Advances in Experimental Medicine and Biology 1326
Cell Biology and Translational Medicine

Kursad Turksen *Editor*

Cell Biology and Translational Medicine, Volume 12

Stem Cells in Development and Disease

Springer

Advances in Experimental Medicine and Biology

Cell Biology and Translational Medicine

Volume 1326

Cell Biology and Translational Medicine aims to publish articles that integrate the current advances in Cell Biology research with the latest developments in Translational Medicine. It is the latest subseries in the highly successful Advances in Experimental Medicine and Biology book series and provides a publication vehicle for articles focusing on new developments, methods and research, as well as opinions and principles. The Series will cover both basic and applied research of the cell and its organelles' structural and functional roles, physiology, signalling, cell stress, cell-cell communications, and its applications to the diagnosis and therapy of disease.

Individual volumes may include topics covering any aspect of life sciences and biomedicine e.g. cell biology, translational medicine, stem cell research, biochemistry, biophysics, regenerative medicine, immunology, molecular biology, and genetics. However, manuscripts will be selected on the basis of their contribution and advancement of our understanding of cell biology and its advancement in translational medicine. Each volume will focus on a specific topic as selected by the Editor. All submitted manuscripts shall be reviewed by the Editor provided they are related to the theme of the volume. Accepted articles will be published online no later than two months following acceptance.

More information about this subseries at http://www.springer.com/series/15838

Kursad Turksen
Editor

Cell Biology and Translational Medicine, Volume 12

Stem Cells in Development and Disease

Springer

Editor
Kursad Turksen *(emeritus)*
Ottawa Hospital Research Institute
Ottawa, ON, Canada

ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISSN 2522-090X ISSN 2522-0918 (electronic)
Cell Biology and Translational Medicine
ISBN 978-3-030-71932-6 ISBN 978-3-030-71933-3 (eBook)
https://doi.org/10.1007/978-3-030-71933-3

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

This next volume in the Cell Biology and Translational Medicine series addresses the topic of stem cells in development and diseases. Amongst specialized topics, there are chapters on the role of stem cells neuronal development and the role of stem cells in diseases including arthritis, aging and cancer.

I remain very grateful to Gonzalo Cordova, associate editor of the series, and acknowledge his continuous support.

I would also like to acknowledge and thank Mariska van der Stigchel, assistant editor, for her outstanding efforts in helping to get this volume to the production stages.

A special thank you goes to Shanthi Ramamoorthy and Rathika Ramkumar for their outstanding efforts in the production of this volume.

Finally, sincere thanks to the contributors not only for their support of the series, but also for their insights and efforts to capture both the advances and the remaining obstacles in their areas of research. I trust readers will find their contributions as interesting and helpful as I have.

Ottawa, ON, Canada — Kursad Turksen

Contents

Adv Exp Med Biol - Cell Biology and Translational Medicine (2021) 12: 1–10
https://doi.org/10.1007/5584_2020_608

Published online: 1 January 2021

Immune Dysregulation and Recurring Mutations in Myelodysplastic Syndromes Pathogenesis

Anacélia Matos, Silvia M. M. Magalhães, and Michael J. Rauh

Abstract

Myelodysplastic syndromes (MDS) are clonal stem cell malignancies characterized by ineffective hematopoiesis leading to peripheral cytopenias and variable risk of progression to acute myeloid leukemia. Inflammation is associated with MDS pathogenesis. Several cytokines, reactive species of oxygen/nitrogen and growth factors are directly or indirectly involved in dysfunction of the MDS bone marrow (BM) microenvironment. Mutations in genes mainly regulating RNA splicing, DNA methylation and chromatin accessibility, transcription factors, signal transduction and the response to DNA damage contribute to ineffective hematopoiesis, genomic instability and MDS development. The inflammation-associated DNA damage in hematopoietic stem cells may also contribute to MDS development and progression with aggressive clinical characteristics. Many studies have aimed at clarifying mechanisms involved in the activity of immature myeloid cells as powerful modulators of the immune response and their correlation with aging, autoimmunity, and development of cancer. In this review, we explore recent advances and accumulating evidence uniting immune dysregulation, inflammaging and recurring mutations in the pathogenesis of MDS.

Keywords

Bone marrow · Hematopoietic stem cells · Inflammation · Mutations · Myelodysplastic syndromes

Authors Silvia M. M. Magalhães and Michael J. Rauh have equally contributed to this chapter.

A. Matos
Cancer Cytogenomic Laboratory, Federal University of Ceará, Fortaleza, Brazil

Post-graduate Program in Medical Science, Federal University of Ceará, Fortaleza, Brazil

S. M. M. Magalhães
Cancer Cytogenomic Laboratory, Federal University of Ceará, Fortaleza, Brazil

Post-graduate Program in Medical Science, Federal University of Ceará, Fortaleza, Brazil

Department of Clinical Medicine, Federal University of Ceará, Fortaleza, Brazil
e-mail: silviamm@ufc.br

M. J. Rauh (✉)
Department of Pathology and Molecular Medicine, Queen's University, Kingston, ON, Canada
e-mail: michael.rauh@queensu.ca; rauhm@queensu.ca

Abbreviations

AML	acute myeloid leukemia
ARG1	arginase 1
ASXL1	Additional Sex Combs Like 1, Transcriptional Regulator
BM	bone marrow

CBL	Casitas B-Lineage Lymphoma Proto-Oncogene
CCUS	clonal cytopenia of undetermined significance
CD	cluster of differentiation
CHIP	clonal hematopoiesis of indeterminate potential
CSF	colony-stimulating factor
DAMP	danger-associated molecular pattern
del	deletion
DNA	deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3A
eMDSC	early MDSC
ETV6	ETS Variant Transcription Factor 6
EZH2	Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit
G	granulocytic
GATA2	GATA Binding Protein 2
GM	granulocyte-monocyte
HLA	human leukocyte antigen
HSPC	hematopoietic stem/progenitor cell
IDH1/2	isocitrate dehydrogenases 1 and 2
IFN-γ	interferon gamma
IL	interleukin
IMC	immature myeloid cell
iNOS	inducible nitric oxide synthase
IPSS	International prognostic scoring system
JAK2	Janus Kinase 2
L-Arg	L-arginine
M	monocytic
MDS	myelodysplastic syndromes
MDSC	myeloid-derived suppressor cell
miR	micro RNA
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NKT	natural killer/T
NO	nitric oxide
NRAS	Neuroblastoma RAS Viral (V-Ras) Oncogene Homolog
PAMP	pathogen-associated molecular pattern
PGE2	prostaglandin E2
PMN	polymorphonuclear
PPM1D	Protein Phosphatase, Mg2+/Mn2+ Dependent 1D
RAD21	RAD21 Cohesin Complex Component
RNA	ribonucleic acid
ROS	reactive oxygen species
RUNX1	RUNX Family Transcription Factor 1
S100A8	S100 Calcium Binding Protein A8
S100A9	S100 Calcium Binding Protein A9
SF3B1	Splicing Factor 3b Subunit 1
SRSF2	Serine And Arginine Rich Splicing Factor 2
STAG2	Stromal Antigen 2
TAM	tumour-associated macrophage
TCR	T-cell receptor
TET2	Ten-Eleven Translocation Methylcytosine Dioxygenase 2
TGF-β	transforming growth factor beta
TIFAB	TRAF-Interacting Protein with Forkhead-Associated Domain, Family Member B
TIRAP	Toll-interleukin-1 receptor domain-containing adaptor protein
TLR	Toll-like receptor
TME	tumour microenvironment
TNF-α	tumour necrosis factor alpha
TP53	Tumour Protein P53
TRAF	tumor necrosis factor receptor-associated factor
Treg	regulatory T-cell
U2AF1	U2 Small Nuclear RNA Auxiliary Factor 1
VEGF	vascular endothelial growth factor
WT1	Wilms tumour 1
ZRSR2	Zinc Finger CCCH-Type, RNA Binding Motif And Serine/Arginine Rich 2

1 Introduction: Immune Dysregulation in MDS

Myelodysplastic syndromes (MDS) constitute a group of diseases which are distinguished by the presence of one or more unexplained peripheral blood cytopenias, dysplastic hematopoietic differentiation, and variable risk to progress to acute myeloid leukemia (AML). The incidence

of these disorders increases with age, with an average of 70 years (Sekeres 2010). Recurrent mutations and haploinsufficiency of particular genes, related epigenetic changes, altered RNA splicing, and disorder in the bone marrow microenvironment all contribute to the disease phenotype (Cazzola 2020).

Inflammation is involved in many disease processes, such as hypertension, cardiovascular disease, rheumatoid arthritis, rheumatoid heart disease, and systemic lupus erythematosus, which are characterized by impairment of immune cell regulatory mechanisms. Several studies have focused on understanding the immunological abnormalities in in MDS (Banerjee et al. 2019; Xin et al. 2019; Corey et al. 2007; Rosenberg and Sinha 2009; Yang et al. 2015).

The pathogenesis of MDS is heterogeneous and includes abnormalities of both innate and adaptive immune systems, as will be described. Understanding how senescence-dependent changes and mutations impact both hematopoietic stem/progenitor cells (HSPC) and the bone marrow microenvironment is essential to understanding the pathogenesis and progression of the disease (Xin et al. 2019; Wang et al. 2018; Glenthoj et al. 2016; Kornblau et al. 2010; Marvel and Gabrilovich 2015).

The innate immune system was traditionally though to dysregulate HSPC proliferation and trigger apoptotic events contributing to the hallmark ineffective hematopoiesis in MDS. The activation of innate immune cells happens through the interaction between pathogen-associated molecular patterns (PAMPs) or host cell-derived danger-associated molecular patterns (DAMPs) with the Toll-like receptors (TLRs) (Kawai and Akira 2010). The TLR signaling pathway results in activation of mitogen-activated protein kinase cascades and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and leads to transcription of pro-inflammatory cytokines such as interleukin-8, which has been described by de Matos et al. (2017). Intrinsic dysregulation of TLR pathways in MDS HSPC results in hyperactive TLR signalling, including a novel inflammatory form of programmed cell death, known as pyroptosis (Basiorka et al. 2016; Barreyro et al. 2018). Moreover, MDS HSPC have a competitive advantage over normal HSPC in the resultant chronic inflammatory environment, which favours their expansion and disease progression (Muto et al. 2020).

Regarding cytokines, the levels of TNF-α, IFN-γ, TGF-β, IL-6 and IL-8 have been observed to be higher in MDS patients, and associated with both dysregulated inflammatory signaling and myeloid differentiation (Yang et al. 2015; Wang et al. 2018; Kornblau et al. 2010; de Matos et al. 2017). According to Xin et al. (2019), who conducted the first meta-analysis focused on inflammatory cytokine levels in MDS patients, there is a close association between immunological microenvironment disorders and the pathogenesis of MDS, with significantly increased TNF-α, IFN-γ, IL-6 and IL-8 in blood and bone marrow of MDS patients (Xin et al. 2019). In addition to the aforementioned intrinsic dysregulation of inflammatory pathways in HPSC, extrinsic influence of these cytokines from chronic infections or host inflammatory disorders may further foster the MDS clonal advantage and disease progression.

MDS-associated mutations and cytogenetic aberrations may also contribute to the inflammatory milieu. In a study made by Kornblau et al. (2010), some cytokines and chemokines were strongly correlated with certain MDS and AML cytogenetic abnormalities, and influenced MDS outcomes beyond the IPSS-calculated risk (Kornblau et al. 2010). The groups of Karsan and Starczynowski have also demonstrated that haploinsufficiency of micro-RNAs (e.g. miR-145 and miR-146a) and genes in del(5q) MDS (e.g. *TIFAB*) contribute to inappropriate TLR activation, including IL-6 production, by affecting expression of signalling mediators, Toll-interleukin-1 receptor domain-containing adaptor protein (TIRAP) and tumor necrosis factor receptor-associated factor-6 (TRAF6) (Starczynowski et al. 2010; Varney et al. 2015). Moreover, recurrent MDS-associated mutations in epigenetic regulators (e.g. *TET2*, *ASXL1*) and components of the spliceosome machinery (e.g. *SF3B1*, *SRSF2*, *U2AF1*) also appear to converge on innate immune pathways, resulting in excessive

inflammasome activation and inflammatory cytokine production, including IL-6 (Basiorka et al. 2016; Smith et al. 2019; Pollyea et al. 2019).

The expression of at least thirty cytokines, chemokines and growth factors in the peripheral blood and bone marrow have been implicated in MDS pathogenesis and clinical outcomes (Banerjee et al. 2019; Xin et al. 2019; Yang et al. 2015; Wang et al. 2018; Glenthoj et al. 2016; Kornblau et al. 2010; Ganan-Gomez et al. 2015). Increased levels of cell death in bone marrow are a hallmark of lower risk disease. On the contrary, in higher risk MDS, with more aggressive clonal expansion, decreased levels of apoptosis are observed (Yang et al. 2015; Glenthoj et al. 2016; Kerbauy and Joachim 2007). Levels of IFN-γ and IL-6 are associated with apoptosis induction in the bone marrow of MDS patients and higher IFN-γ and IL-6 secretion is generally related to lower-risk MDS. In contrast, immunosuppressive cytokines like IL-10 are more strongly secreted in high-risk MDS (Wang et al. 2018). These studies reflect the importance of inflammatory cytokines in dysregulation of the immunological environment in the pathogenesis of MDS. While they show a range in cytokine profiles between different types of MDS and different studies, they importantly also demonstrate convergence in critical innate immune pathways. Increased intramedullary apoptosis and pyroptosis are important contributors to cytopenias in MDS. Additionally, it is very probable that these immunologic aberrations and pressures play a central role in the course of MDS evolution from low- to high-risk MDS or to AML (Banerjee et al. 2019; Glenthoj et al. 2016; Steensma et al. 2015; Valka et al. 2019; Sallman and List 2019).

The role of adaptive immunity in MDS pathogenesis and progression has been considered, although not as extensively as the molecular connections with innate immunity. CD8+ T-cells may become activated and expanded in response to epitopes on MDS stem cells, resulting in suppression of both malignant and normal hematopoiesis, as reviewed by Wang et al. (2018). This is exemplified in lower-risk MDS with trisomy 8, where the Wilms tumor 1 antigen (WT1) is overexpressed by HPSC, WT1 triggers T-cell suppression of hematopoiesis, and this may be ameliorated by T-cell directed immunosuppressive therapy (Sloand et al. 2011). However, as MDS progresses to high-risk and later stages, there is expansion of regulatory T-cell subsets (Treg) and increased expression of inhibitory checkpoint proteins, which likely facilitate evasion of adaptive immunity by mutant MDS clones (see recent reviews (Wang et al. 2018; Barreyro et al. 2018; Sallman and List 2019; Winter et al. 2020)).

Indeed, it is well described that the tumor microenvironment in MDS and other cancers is immunosuppressive, both inhibiting activated immune cells and activating cells with a suppressive phenotype. Multiple cell types contribute to tumor mediated immune suppression, including Treg, type 2 NKT cells, and tumor associated macrophages (TAMs) (Najjar and Finke 2013). More recently, a group of cells named myeloid-derived suppressor cells (MDSCs) has been considered as responsible for suppressing adaptive immunity and mediating pathological effects seen in MDS (Sica and Massarotti 2017; Chen et al. 2013; Kittang et al. 2015; Eksioglu et al. 2017; Sarhan et al. 2018).

2 MDSC: Pathways of Activation and Pathogenesis in MDS

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immune cells that are defined by their myeloid origin. This group of cells strongly expands in pathological situations such as chronic infections and solid tumors, suppressing immune mediated tumor surveillance and T cell polarization (Marvel and Gabrilovich 2015; Gabrilovich et al. 2007). MDSCs also share other non-immunological functions such as promotion of angiogenesis, tumor local invasion and metastases (Filipazzi et al. 2012; Chesney et al. 2017).

Despite the fact that MDSCs are a phenotypically heterogeneous population of myeloid cells at different stages of maturation, one feature common to all MDSCs is that they show remarkable activity to suppress various non-myeloid immune

cells, such as T-cells, B-cells and natural killer (NK) cells (Filipazzi et al. 2012; Salminen et al. 2019a). MDSCs have also been reported to regulate innate immune responses by modulating the cytokine production of macrophages (Raza and Galili 2012; Lopez-Bujanda and Drake 2017). Three main subdivisions of MDSC have been proposed (Bronte et al. 2016): PMN-MDSCs or G-MDSCs (polymorphonuclear or granulocytic) which account for 70–80% of the MDSC population, M-MDSCs (monocytic) which account for 20–30% of MDSCs, and a smaller fraction of early-stage MDSC (eMDSC) (Lopez-Bujanda and Drake 2017; Bronte et al. 2016). There are some phenotypical differences when human and murine markers are compared. In humans, PMN-MDSCs are identified by the $CD11b^{+}CD14^{-}CD15^{+}$ expression pattern or $CD11b^{+}CD14^{-}CD66b^{+}$, while M-MDSC are characterized by $CD11b^{+}CD14^{+}HLA^{low/-}CD15^{-}$ and eMDSC as lineage (CD3/14/15/19/56)-negative/$HLA\text{-}DR^{-}/CD33^{+}$ (Filipazzi et al. 2012; Bronte et al. 2016; Safarzadeh et al. 2019).

Physiologically, MDSCs are present predominantly in the bone marrow (Nadal et al. 2018). In pathological conditions, such as with cancer, chronic inflammation or autoimmunity, a sustained and aberrant differentiation of myeloid cells occurs, leading to MDSC expansion. Inflammatory factors can modulate myeloid cells in the tumor microenvironment, and having them delivered distantly to hematopoietic organs can change normal myelopoiesis and skew the differentiation of myeloid cells in favor of MDSCs (Umansky et al. 2016). The expansion and activation of MDSC are controlled by a complex network of soluble factors like IL-6, IL-10, IL-1β and IFN-γ, granulocyte-macrophage colony stimulating factor (GM-CSF), M-CSF, G-CSF, vascular endothelial growth factor (VEGF) and TLR ligands (Gabrilovich and Nagaraj 2009; Veglia et al. 2018; Kumar et al. 2016a). Under pathological conditions and mediated by these factors, MDSC can be found in higher proportions in the blood circulation and may be also recruited back to the tumor microenvironment (Kumar et al. 2016b). Moreover, a tumor-microenvironment (TME) that is hypoxic, nutrient-deprived and enriched in pro-inflammatory and suppressive cytokines, chemokines, and oxidative agents such as reactive oxygen species (ROS), nitric oxide (NO) and peroxynitrite, further induces the activation of local MDSCs (Tcyganov et al. 2018; Wang et al. 2013; Consonni et al. 2019; Weber et al. 2018).

MDSC are also defined by their functional ability to suppress immune cell responses (Bronte et al. 2016). This is achieved through the expression of many immune suppressive factors as, for instance, arginase (ARG1), NO and ROS. Unrestrained MDSC activation may, in turn cause DNA mutations and genetic instability.

The specific types of MDSCs use different mechanisms of immunosuppression. The essential difference is that the suppressive PMN-MDSCs produce ROS and ARG1, whereas M-MDSCs predominantly express the inducible nitric oxide synthase (iNOS). However, the common and main mechanism of action associated with the immunosuppressive activities of PMN-MDSCs and M-MDSCs is the metabolic conversion of L-arginine (L-Arg) through either iNOS or ARG1. Because both promote the depletion of the amino acid L-Arg and down-regulation of T cell receptor (TCR) ζ-chain expression, this leads to suppression of the cell cycle and T-cell immunosuppression as a result (Consonni et al. 2019).

MDSC expansion therefore inhibits proliferation and antitumor activity of T cells, decreasing cytokine secretion, recruiting regulatory T cells, and consequently, prohibiting natural killer cell (NK cell) activation, thus hampering the host anti-tumor immune response. Furthermore, MDSC also induce Treg differentiation by secreting IL-10 and TGF-β as well as stimulating tumor angiogenesis by secreting VEGF and basic fibroblast growth factor (Gabrilovich and Nagaraj 2009; Gabrilovich et al. 2012; Schmid and Varner 2012).

Clinical and experimental evidence has shown an association between MDSCs and cancer. A significant increase in MDSCs provokes a propitious immune microenvironment associated with high cancer prevalence, poor prognosis and resistance to therapy (Marvel and Gabrilovich 2015; Kirkwood et al. 2018; Lisha et al. 2018). On the

other hand, the impact of age on MDSCs in humans is not well documented. The mechanisms involved in age-related increase of MDSCs seems to be, at least partly, determined by well-known aging-associated processes, including cellular senescence and chronic low-grade inflammation ("inflammaging"), and likely the skewing of hematopoiesis away from the lymphoid toward the myeloid lineage (Kirkwood et al. 2018; Pawelec et al. 2019; Salminen et al. 2019b). However, the recognition of expanded MDSC in MDS may offer a further genetic connection to aging (Chen et al. 2013).

In 2013, it was reported that MDSC were markedly expanded in the blood (Jiang et al. 2013) and bone marrow (Chen et al. 2013) of MDS patients. The latter study also demonstrated a suppressive effect of MDS MDSC on human erythroid and myeloid progenitor cell growth *in vitro*, implicating MDSC in the ineffective hematopoiesis associated with MDS (Chen et al. 2013). Moreover, with murine models, it was demonstrated that MDSC expansion is driven by pro-inflammatory S100A9, signalling through CD33 and mediated by the induction of immunosuppressive IL-10 and TGF-β (Chen et al. 2013). Independent studies subsequently revealed associations between MDSC and Treg expansion and higher MDS clinical risk (Kittang et al. 2015). Novel therapies are now under consideration, directed towards MDSC and with the goal of ameliorating immunosuppression and improving hematopoiesis (Eksioglu et al. 2017; Sarhan et al. 2018). However, the relationship between these immune cell populations, inflammatory signaling and recurring mutations in MDS is not completely understood. The influence of different factors in expansion and activation of MDSCs such as PGE2, VEGF, IL-6, IL-10 and S100A8-A9 has been shown (Rosenberg and Sinha 2009; Marvel and Gabrilovich 2015; Najjar and Finke 2013; Gabrilovich and Nagaraj 2009; Pawelec et al. 2019). Due to the profile of inflammatory cytokine changes with the course of disease, current studies have focused on understanding the abnormalities in immunologic profile in different myeloid disorders, mainly in MDS and AML, and other proposals to deplete the key innate immune cellular effectors, MDSC, are still currently in development (Sallman and List 2019; Pawelec et al. 2019).

3 Considerations for Clonal, Pre-MDS States: CHIP and CCUS

Major breakthroughs in the molecular and genetic basis of MDS have recently been achieved. Genetic lesions, namely recurrent somatic point mutations and/or small insertions/deletions in >40 different genes, have now been associated with MDS pathogenesis (Cazzola 2020; Cull and Rauh 2017; Claus and Lubbert 2003; Issa 2010; Sperling et al. 2017). These mainly occur in genes regulating RNA splicing (e.g., *SF3B1, SRSF2, U2AF1, ZRSR2*), DNA methylation (e.g., *TET2, DNMT3A, IDH1/IDH2*) and chromatin accessibility (e.g., *ASXL1, EZH2, STAG2, RAD21*), transcription factors (e.g. *RUNX1, GATA2, ETV6*), signal transduction (e.g. *CBL, JAK2, NRAS*) and the response to DNA damage (e.g. *TP53, PPM1D*). In the case of *SF3B1*, the presence of mutations is incorporated into the current iteration of the World Health Organization classification of MDS, with a recent proposal to recognized *SF3B1*-mutant MDS as an even more distinct diagnostic entity (Malcovati et al. 2020).

Presently, other MDS-associated mutations are not considered diagnostic of MDS on their own. This is because at least 10–15% of healthy older persons with no hematologic disease acquire somatic mutations that overlap with MDS, drive clonal expansion and, eventually, what is now called clonal hematopoiesis of indeterminate potential (CHIP) (Steensma et al. 2015). Although most individuals who acquire CHIP during aging will never develop MDS, the presence of an MDS-associated somatic mutation, such as in *DNMT3A*, *ASXL1* or *TP53* is a strong predictor of the development of subsequent hematologic malignancy and is associated with worse overall survival (Park et al. 2019; Yoshizato et al. 2015). The presence of a recurrent mutation and otherwise

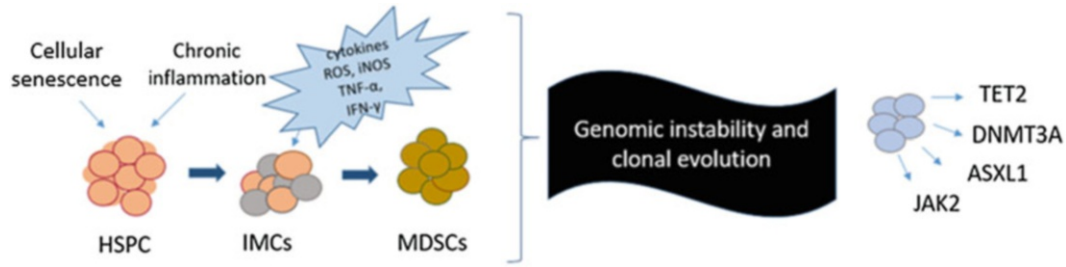


Fig. 1 Some of the main pathways involved in MDS pathogenesis and progression. An aging and inflammatory bone marrow microenvironment induced by cellular senescence and chronic immune stimulation leads to MDSC accumulation and activation. Inflammatory cytokines and soluble factors, such as ROS and iNOS, contribute to increased apoptosis and pyroptosis of HSPCs as well as genomic instability. Somatic mutations contribute to further dysregulation of immune system, suppression of normal hematopoiesis, clonal evolution and susceptibility to leukemic transformation. *HSPC* Hematopoietic stem and progenitor cells, *IMCs* Immature myeloid cells; *MDSCs* Myeloid-derived suppressor cells, *ROS* Reactive oxygen species, *iNOS* Inducible nitric oxide synthase, *TNF-a* Tumor necrosis factor-alpha, *IFN-γ* Interferon gamma, *TET2* Tet methylcytosine dioxygenase 2, *DNMT3A* DNA methyltransferase 3A, *ASXL1* Additional sex combs like 1, *JAK2* Janus Kinase 2

unexplained cytopenias (clonal cytopenias of undetermined significance, CCUS) can be suggestive of progression to MDS, although in this case more specific morphologic criteria are mandatory (Sperling et al. 2017; Cargo et al. 2015; Kwok et al. 2015). A subsequent study with longitudinal follow-up suggests it may be possible to predict patients with CCUS at greatest risk of progression to MDS or other myeloid neoplasm and that, effectively, CCUS patients with particular mutational features may have presumptive evidence of early MDS (Malcovati et al. 2017).

As described, there is a complex process involved in the pathogenesis of MDS, especially mechanisms directly correlated with myeloid-mediated inflammation, the accumulation of genetic damage, immunosuppression and related selective pressures during the evolution of malignant clones (Steensma et al. 2015; Hosono 2019) (Fig. 1). Although we have mainly focused on these aspects in the context of MDS, these processes are likely at play as early as the CHIP phase and into CCUS. For example, common drivers of CHIP and MDS, mutations in *TET2* and *DNMT3A*, have been associated with inflammation and immune cell alterations in humans and murine models, as recently reviewed (King et al. 2020; SanMiguel et al. 2020; Ferrone et al. 2020; Cook et al. 2020). Moreover, spliceosomal mutations (e.g. *SF3B1*, *SRSF2*, *U2AF1*) also appear to converge on innate immune pathways, resulting in excessive inflammasome activation and inflammatory cytokine production, including IL-6 (Smith et al. 2019; Pollyea et al. 2019). Finally, our group has demonstrated increased ARG1 expression in *Tet2*-mutant macrophages (Cull et al. 2017) and in MDS patients with *DNMT3A* and *TET2* mutations (Cull et al. 2018). This could signify a myeloid suppressive phenotype (reminiscent of MDSCs) in response to chronic inflammation but more studies are required to map the relationship between immune dysregulation and mutations in the progression from CHIP to CCUS and MDS. The comprehension of genetic lesions, genomic instability and dysregulated immune response are important to better understand MDS pathogenesis, progression and predictive factors of response to therapy.

4 Conclusions

Though the pathogenesis in MDS is now much more understood, the factors involved in this heterogeneous process are still an attractive and constant focus of research. Clinical and experimental evidence suggest an important link between genetic, epigenetic, and immune systems in the

pathogenesis and progression of MDS. Ongoing studies are looking at more specific molecular and immune pathways and targets with potential clinical significance, notably the role of the inflammatory marrow environment and MDSCs in MDS. Translation from understanding the complex molecular and immunological pathophysiology of MDS to the identification of new targets and novel treatment options are long awaited.

References

Banerjee T, Calvi LM, Becker MW et al (2019) Flaming and fanning: the spectrum of inflammatory influences in myelodysplastic syndromes. Blood Rev 36:57–69

Barreyro L, Chlon TM, Starczynowski DT (2018) Chronic immune response dysregulation in MDS pathogenesis. *Blood* 132(15):1553–1560

Basiorka AA, McGraw KL, Eksioglu EA, Chen X, Johnson J, Zhang L, Zhang Q, Irvine BA, Cluzeau T, Sallman DA, Padron E, Komrokji R, Sokol L, Coll RC, Robertson AA, Cooper MA, Cleveland JL, O'Neill LA, Wei S, List AF (2016) The NLRP3 inflammasome functions as a driver of the myelodysplastic syndrome phenotype. Blood 128(25):2960–2975

Bronte V, Brandau S, Chen SH et al (2016) Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nature 7:12150

Cargo CA, Rowbotham N, Evans PA et al (2015) Targeted sequencing identifies patients with preclinical MDS at high risk of disease progression. Blood 126 (21):2362–2365

Cazzola M (2020) Myelodysplastic syndromes. N Engl J Med 383(14):1358–1374

Chen X, Eksioglu EA, Zhou J, Zhang L, Djeu J, Fortenbery N, Epling-Burnette P, Van Bijen S, Dolstra H, Cannon J, Youn J, Donatelli SS, Qin D, De Witte T, Tao J, Wang H, Cheng P, Gabrilovich DI, List A, Wei S (2013) Induction of myelodysplasia by myeloid-derived suppressor cells. J Clin Invest 123 (11):4596–4611

Chesney JA, Mitchell RA, Yaddanapudi K (2017) Myeloid-derived suppressor cells- a new therapeutic target to overcome resistance to cancer immunotherapy. J Leukoc Biol 102(3):727–740

Claus R, Lubbert M (2003) Epigenetic targets in hematopoietic malignancies. Oncogene 22 (42):6489–6496

Consonni FM, Porta C, Marino A et al (2019) Myeloid-derived suppressor cells: ductile targets in disease. Front Immunol 10:949

Cook EK, Luo M, Rauh MJ (2020) Clonal hematopoiesis and inflammation: partners in leukemogenesis and comorbidity. Exp Hematol 83:85–94

Corey SJ, Minden MD, Barber DL et al (2007) Myelodysplastic syndromes: the complexity of stem-cell diseases. Nat Rev Cancer 7(2):118–129

Cull AH, Rauh MJ (2017) Success in bone marrow failure? Novel therapeutic directions based on the immune environment of myelodysplastic syndromes. J Leukoc Biol 102(2):209–219

Cull AH, Snetsinger B, Buckstein R, Wells RA, Rauh MJ (2017) Tet2 restrains inflammatory gene expression in macrophages. Exp Hematol 55:56–70.e13

Cull AH, Mahendru D, Snetsinger B et al (2018) Overexpression of Arginase 1 is linked to DNMT3A and TET2 mutations in lower-grade myelodysplastic syndromes and chronic myelomonocytic leukemia. Leuk Res 65:5–13

de Matos AG, Ribeiro Junior HL, de Paula BD et al (2017) Interleukin-8 and nuclear factor kappa B are increased and positively correlated in myelodysplastic syndrome. Med Oncol 34(10):168

Eksioglu EA, Chen X, Heider K-H, Rueter B, McGraw KL, Basiorka AA, Wei M, Burnette A, Cheng P, Lancet J, Komrokji R, Djeu J, List A, Wei S (2017) Novel therapeutic approach to improve hematopoiesis in low risk MDS by targeting MDSCs with the Fc-engineered CD33 antibody BI 836858. Leukemia 31(10):2172–2180

Ferrone CK, Blydt-Hansen M, Rauh MJ (2020) Age-associated TET2 mutations: common drivers of myeloid dysfunction, cancer and cardiovascular disease. Int J Mol Sci 21(2):626

Filipazzi P, Huber V, Rivoltini L (2012) Phenotype, function and clinical implications of myeloid-derived suppressor cells in cancer patients. Cancer Immunol Immunother 61(2):255–263

Gabrilovich DI, Nagaraj S (2009) Myeloid-derived-suppressor cells as regulators of the immune system. Nat Rev Immunol 9(3):162–174

Gabrilovich DI, Bronte V, Chen SH et al (2007) The terminology issue for myeloid-derived suppressor cells. Cancer Res 67(1):425–426

Gabrilovich DI, Ostrand-Rosenberg S, Bronte V (2012) Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol 12(4):253–268

Ganan-Gomez I, Wei Y, Starczynowski DT et al (2015) Deregulation of innate immune and inflammatory signaling in myelodysplastic syndromes. Leukemia 29 (7):1458–1469

Glenthoj A, Orskov AD, Hansen JW et al (2016) Immune mechanisms in myelodysplastic syndrome. Int J Mol Sci 17(6):944

Hosono N (2019) Genetic abnormalities and pathophysiology of MDS. Int J Clin Oncol 24(8):885–892

Issa JP (2010) Epigenetic changes in the myelodysplastic syndrome. Hematol Oncol Clin North Am 24 (2):317–330

Jiang HJ, Fu R, Wang HQ, Li LJ, Qu W, Liang Y, Wang GJ, Wang XM, Wu YH, Liu H, Song J, Guan J, Xing LM, Ruan EB, Shao ZH (2013) Increased circulating of myeloid-derived suppressor cells in myelodysplastic syndrome. Chin Med J 126(13):2582–2584

Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. Nat Immunol 11(5):373–384

Kerbauy DB, Joachim H (2007) Apoptosis and anti-apoptotic mechanisms in the progression of MDS. Exp Hematol 35(11):1739–1746

King KY, Huang Y, Nakada D, Goodell MA (2020) Environmental influences on clonal hematopoiesis. Exp Hematol 83:66–73

Kirkwood KL, Zhang L, Thiyagarajan R et al (2018) Myeloid-derived suppressor cells at the intersection of inflammaging and bone fragility. Immunol Investig 47:844–854

Kittang AO, Kordasti S, Sand KE, Constantini B, Kramer AM, Perezabellan P, Seidl T, Rye KP, Hagen KM, Kulasekararaj A, Bruserud O, Mufti GJ (2015) Expansion of myeloid-derived suppressor cells correlates with number of T regulatory cells and disease progression in myelodysplastic syndrome. Onco Targets Ther 5(2):e1062208

Kornblau SM, McCue D, Singh N et al (2010) Recurrent expression signatures of cytokines and chemokines are present and are independently prognostic in acute myelogenous leukemia and myelodysplasia. Blood 116 (20):4251–4261

Kumar V, Patel S, Tcyganov E, Gabrilovich D (2016a) The nature of myeloid-derived suppressor cells in the tumor microenvironment. Trends Immunol 37 (3):208–220

Kumar V, Patel S, Toyganov E, Gabrilovich DI (2016b) The nature of myeloid-derived suppressor cells in the tumor microenvironment. Trends Immunol 37 (3):208–220

Kwok B, Hall JM, Witte S et al (2015) MDS-associated somatic mutations and clonal hematopoiesis are common in idiopathic cytopenias of undetermined significance. Blood 126(21):2355–2361

Lisha A, Mu S, Wang Y et al (2018) Prognostic role of myeloid-derived suppressor cells in cancers: a systematic review and meta-analysis. BMC Cancer 18:1220

Lopez-Bujanda Z, Drake CG (2017) Myeloid-derived cells in prostate cancer progression: phenotype and prospective therapies. J Leukoc Bio 102(2):393–406

Malcovati L, Galli A, Travaglino E, Ambaglio I, Rizzo E, Molteni E, Elena C, Ferretti VV, Catricala S, Bono E, Todisco G, Bianchessi A, Rumi E, Zibellini S, Pietra D, Boveri E, Camaschella C, Toniolo D, Papaemmanuil E, Ogawa S, Cazzola M (2017) Clinical significance of somatic mutation in unexplained blood cytopenia. Blood 129(25):3371–3378

Malcovati L, Stevenson K, Papaemmanuil E, Neuberg D, Bejar R, Boultwood J, Bowen DT, Campbell PJ, Ebert BL, Fenaux P, Haferlach T, Heuser M, Jansen JH, Komrokji RS, Maciejewski JP, Walter MJ, Fontenay M, Garcia-Manero G, Graubert TA, Karsan A, Meggendorfer M, Pellagatti A, Sallman DA, Savona MR, Sekeres MA, Steensma DP, Tauro S, Thol F, Vyas P, Van de Loosdrecht AA, Haase D, Tuchler H, Greenberg PL, Ogawa S, Hellstrom-Lindberg E, Cazzola M (2020) SF3B1-mutant MDS as a distinct disease subtype: a proposal from the International Working Group for the Prognosis of MDS. Blood 136(2):157–170

Marvel D, Gabrilovich DI (2015) Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected. J Clin Invest 125(9):3356–3364

Muto T, Walker CS, Choi K, Hueneman K, Smith MA, Gul Z, Garcia-Manero G, Ma A, Zheng Y, Starczynowski DT (2020) Adaptive response to inflammation contributes to sustained myelopoiesis and confers a competitive advantage in myelodysplastic syndrome HSCs. Nat Immunol 21 (5):535–545

Nadal C, Beguin J, Benchekroun G, Le Roux D (2018) The myeloid derived suppressor cells: Who are they? Can they be used as a diagnostic tool to investigate metastasis in veterinary medicine? Comp Immunol Microbiol Infect Dis 61:5–8

Najjar YG, Finke JH (2013) Clinical perspectives on targeting of myeloid derived suppressor cells in the treatment of cancer. Front Oncol 3:49

Park DS, Akuffo AA, Muench DE et al (2019) Clonal hematopoiesis of indeterminate potential and its impact on patient trajectories after stem cell transplantation. PLoS Comput Biol 15:e1006913

Pawelec G, Verschoor CP, Ostrand-Rosenberg S (2019) Myeloid-derived suppressor cells: not only in tumor immunity. Front Immunol 10:1099

Pollyea DA, Harris C, Rabe JL, Hedin BR, De Arras L, Katz S, Wheeler E, Bejar R, Walter MJ, Jordan CT, Pietras EM, Alper S (2019) Myelodysplastic syndrome-associated spliceosome gene mutations enhance innate immune signaling. Haematologica 104(9):e388–e392

Raza A, Galili N (2012) The genetic basis of phenotypic heterogeneity in myelodysplastic syndromes. Nat Rev Cancer 12(12):849–859

Rosenberg SO, Sinha P (2009) Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol 182(8):4499–4506

Safarzadeh E, Hashemzadeh S, Duijf PHG et al (2019) Circulating myeloid-derived suppressor cells: an independent prognostic factor in patients with breast cancer. J Cell Physiol 234(4):3515–3525

Sallman DA, List A (2019) The central role of inflammatory signaling in the pathogenesis of myelodysplastic syndromes. Blood 133(10):1039–1048

Salminen A, Kaarniranta K, Kauppinen A (2019a) Immunosenescence: the potential role of myeloid-derived suppressor cells (MDSC) in age-related immune deficiency. Cell Mol Life Sci 76 (10):1901–1918

Salminen A, Kauppinen A, Kaarniranta K (2019b) AMPK activation inhibits the functions of myeloid-derived suppressor cells (MDSC): impact on cancer and aging. J Mol Med 97(8):1049–1064

SanMiguel JM, Young K, Trowbridge JJ (2020) Hand in hand: intrinsic and extrinsic drivers of aging and clonal

hematopoiesis. Exp Hematol:S0301-472X(20)30562–2

Sarhan D, Brandt L, Felices M, Guldevall K, Lenvik T, Hinderlie P, Curtsinger J, Warlick E, Spellman SR, Blazar BR, Weisdorf DJ, Cooley S, Vallera DA, Onfelt B, Miller JS (2018) 161533 TriKE stimulates NK-cell function to overcome myeloid-derived suppressor cells in MDS. Blood Adv 2(12):1459–1469

Schmid MC, Varner JA (2012) Myeloid cells in tumor inflammation. Vasc Cell 4(1):14

Sekeres MA (2010) The epidemiology of myelodysplastic syndromes. Hematol Oncol Clin North Am 24(2):287–294

Xin S, Yanhua Z, Li X et al (2019) The inflammatory cytokine profile of myelodysplastic syndromes. A meta-analysis. Medicine 98:22 (e15844

Sica A, Massarotti M (2017) Myeloid suppressor cells in cancer and autoimmunity. J Autoimmun 85:117–125

Sloand EM, Melenhorst JJ, Tucker ZC, Pfannes L, Brenchley JM, Yong A, Visconte V, Wu C, Gostick E, Scheinberg P, Olnes MJ, Douek DC, Price DA, Barrett AJ, Young NS (2011) T-cell immune responses to Wilms tumor 1 protein in myelodysplasia responsive to immunosuppressive therapy. Blood 117(9):2691–2699

Smith MA, Choudhary GS, Pellagatti A, Choi K, Bolanos LC, Bhagat TD, Gordon-Mitchell S, Von Ahrens D, Pradhan K, Steeples V, Kim S, Steidl U, Walter M, Fraser IDC, Kulkarni A, Salomonis N, Komurov K, Boultwood J, Verma A, Starczynowski DT (2019) U2AF1 mutations induce oncogenic IRAK4 isoforms and activate innate immune pathways in myeloid malignancies. Nat Cell Biol 21(5):640–650

Sperling AS, Gibson CJ, Ebert BL (2017) The genetics of myelodysplastic syndrome: from clonal hematopoiesis to secondary leukemia. Nat Rev Cancer 17:5–19

Starczynowski DT, Kuchenbauer F, Argiropoulos B, Sung S, Morin R, Muranyi A, Hirst M, Hogge D, Marra M, Wells RA, Buckstein R, Lam W, Humphries RK, Karsan A (2010) Identification of miR-145 and miR-146a as mediators of the 5q- syndrome phenotype. Nat Med 16(1):49–58

Steensma DP, Bejar R, Jaiswal S, Lindsley RC, Sekeres MA, Hasserjian RP et al (2015) Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. Blood 126(1):9–16

Tcyganov E, Mastio J, Chen E, Gabrilovich DI (2018) Plasticity of myeloid-derived suppressor cells in cancer. Curr Opin Immunol 51:76–82

Umansky V, Blattner C, Gebhardt C et al (2016) The role of myeloid-derived suppressor cells (MDSC) in cancer progression. Vaccines (Basel) 4(4):pii: E36

Valka J, Veselaa J, Votavova H et al (2019) Genetic variant screening of DNA repair genes in myelodysplastic syndrome identifies a novel mutation in the XRCC2 gene. Oncol Res Treat 42:263–268

Varney ME, Niederkorn M, Konno H, Matsumura T, Gohda J, Yoshida N, Akiyama T, Christie S, Fang J, Miller D, Jerez A, Karsan A, Maciejewski JP, Meetei RA, Inoue J, Starczynowski DT (2015) Loss of Tifab, a del(5q) MDS gene, alters hematopoiesis through derepression of toll-like receptor-TRAF6 signaling. J Exp Med 212(11):1967–1985

Veglia F, Perego M, Gabrilovich D (2018) Myeloid-derived suppressor cells coming of age. Nat Immunol 19(2):108–119

Wang L, Chang WY, Wong SC et al (2013) Increased myeloid-derived suppressor cells in gastric Cancer correlate with Cancer stage and plasma S100A8/A9 Proinflammatory proteins. J Immunol 190(2):794–804

Wang C, Yang Y, Gao S et al (2018) Immune dysregulation in myelodysplastic syndrome: clinical features, pathogenesis and therapeutic strategies. Crit Rev Oncol Hematol 122:123–132

Weber R, Fleming V, Hu X et al (2018) Myeloid-derived suppressor cells hinder the anti-cancer activity of immune checkpoint inhibitors. Front Immunol 9:1310

Winter S, Shoaie S, Kordasti S, Platzbecker U (2020) Integrating the "Immunome" in the stratification of myelodysplastic syndromes and future clinical trial design. J Clin Oncol 38(15):1723–1735

Yang L, Qian Y, Eksioglu E et al (2015) The inflammatory microenvironment in MDS. Cell Mol Life Sci 72(10):1959–1966

Yoshizato T, Dumitriu D, Hosokawa K et al (2015) Somatic mutations and clonal hematopoiesis in aplastic anemia. N Engl J Med 373:35–47

Adv Exp Med Biol - Cell Biology and Translational Medicine (2021) 12: 11–37
https://doi.org/10.1007/5584_2020_577

Published online: 11 September 2020

Stem Cell Aging and Regenerative Medicine

Debojyoti De, Parimal Karmakar, and Debalina Bhattacharya

Abstract

Stem cells are a promising source for regenerative medicine to cure a plethora of diseases that are currently treated based on either palliative or symptomatic relief or by preventing their onset and progression. Aging-associated degenerative changes in stem cells, stem cell niches, and signaling pathways bring a step by step decline in the regenerative and functional potential of tissues. Clinical studies and experiments on model organisms have pointed out checkpoints that aging will inevitably impose on stem cell aiming for transplantation and hence questions are raised about the age of the donor. In the following discourse, we review the fundamental molecular pathways that are implicated in stem cell aging and the current progress in tissue engineering and transplantation of each type of stem cells in regenerative medicine. We further focus on the consequences of stem cell aging on their clinical uses and the development of novel strategies to bypass those pitfalls and improve tissue replenishment.

D. De and P. Karmakar
Department of Life science and Biotechnology, Jadavpur University, Kolkata, India

D. Bhattacharya (✉)
Department of Microbiology, Maulana Azad College, Kolkata, India
e-mail: debalina.bhattacharya13@gmail.com; debalina.bhattacharya@maulanaazadcollegekolkata.ac.in

Keywords

Aging · Regeneration · Regenerative medicine · Senescence · Stem cells

Abbreviations

ADSC	Adipose tissue-derived stem cell
ASC	Adult stem cell
BMSC/BMDSC	Bone marrow-derived mesenchymal stem/stromal cell
CSC	Cardiac stem cell
DNMT	DNA methyltransferase
EPC	Endothelial progenitor cell
ESC	Embryonic stem cell
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSC	Germline stem cell
GVHD	Graft versus host disease
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HIF	Hypoxia-Inducible Factor
HSC	Haematopoietic stem cell
ICM	Inner cell mass
iPSC	Induced pluripotent stem cell
ISC	Intestinal stem cell
LDHA	Lactate dehydrogenase A
MDSC	Muscle-derived stem cell
MSC	Mesenchymal stem cell
NAC	N-acetylcysteine

NHEJ	Non-homologous end joining
NPC	Neural precursor cell
NSC	Neural stem cell
PDK	Pyruvate dehydrogenase kinase
PNPase	Polynucleotide Phosphorylase
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
SC	Stem cell
SCNT	Somatic cell nuclear transfer
SGZ	Subgranular zone
SSC	Skin stem cell
SVZ	Subventricular zone
TERT	Telomerase Reverse Transcriptase
UCB	Umbilical cord blood

1 Introduction

Gradual molecular changes in tissues are almost inevitably followed by deterioration in tissue function and an inadequate response to injury. Regenerative reaction to injury and subsequent homeostasis depends on stem cells which possess the ability of self-renewal as well as tissue-specific differentiation (Liu and Rando 2011). Stem cells balance quiescence with proliferation but with age this equilibrium no longer persists leading to loss of regeneration and proliferation, senescence, or even death (Fig. 1). Fundamentally one must figure out the molecular mechanisms behind self-renewal, proliferation, differentiation, quiescence, and stem cell survival to understand their association with age and further ascertain the effectors of stem cell dysfunction (Fig. 2). Besides, such insights will provide information about the potential therapeutic interventions that can either delay or nullify degenerative changes and enhance the scope of regenerative medicine (Sharpless and DePinho 2007). Currently, individuals with diseased organs are treated with organ transplantation, but the availability of donors often does not meet the demand in transplantation, and this is anticipated to worsen with the increase in the aged and diseased population. Although a wide array of stem cells can be used clinically, not all of them are preferred equally in terms of application in regenerative medicine. Some cells regenerate at a high rate *in vivo* but fail to develop *in vitro* whereas some cells do not expand at all (Sharpless and DePinho 2007). Besides developing therapeutics, stem cell research in regenerative medicine can also be utilized in understanding the pathology of diseases by generating innovative tissue and organ models like 'diseases in a dish'. Thus, they can also deliver as clinical instruments for detecting 'factors' of historically incurable diseases and in turn the course of action.

2 Stem Cell Aging

The maintenance of quality and quantity of stem cells are of paramount importance not only for the development but also for proper functioning of organs throughout life. But aging is a great threat to the unusual replicative capacity of stem cells and perhaps this is the major cause of gradual dysfunction of organs with age. Stem cell aging depends on various factors and some of the major factors are described below.

2.1 ROS

ROS levels directly affect stem cells' quiescence, self-renewal, or differentiation through signaling pathways by reacting with various kinases, phosphatases, or transcription factors. In ESCs, intermittent levels of ROS may cause G1 cell cycle arrest, but continuous exposure can lead to apoptosis. ESCs also have a high rate of self-renewal maintained by a controlled ROS production. Similarly, the self-renewal of ASCs is highly dependent on low ROS accumulation and oxidative stress (Kobayashi and Suda 2011; Bigarella et al. 2014; Liang and Ghaffari 2014). This becomes evident when HIFs are genetically impaired. HIFs are part of a transcriptional complex involved in hypoxia-signalling cascade to maintain oxygen homeostasis and help in conserving stem cells' potency. So, when their

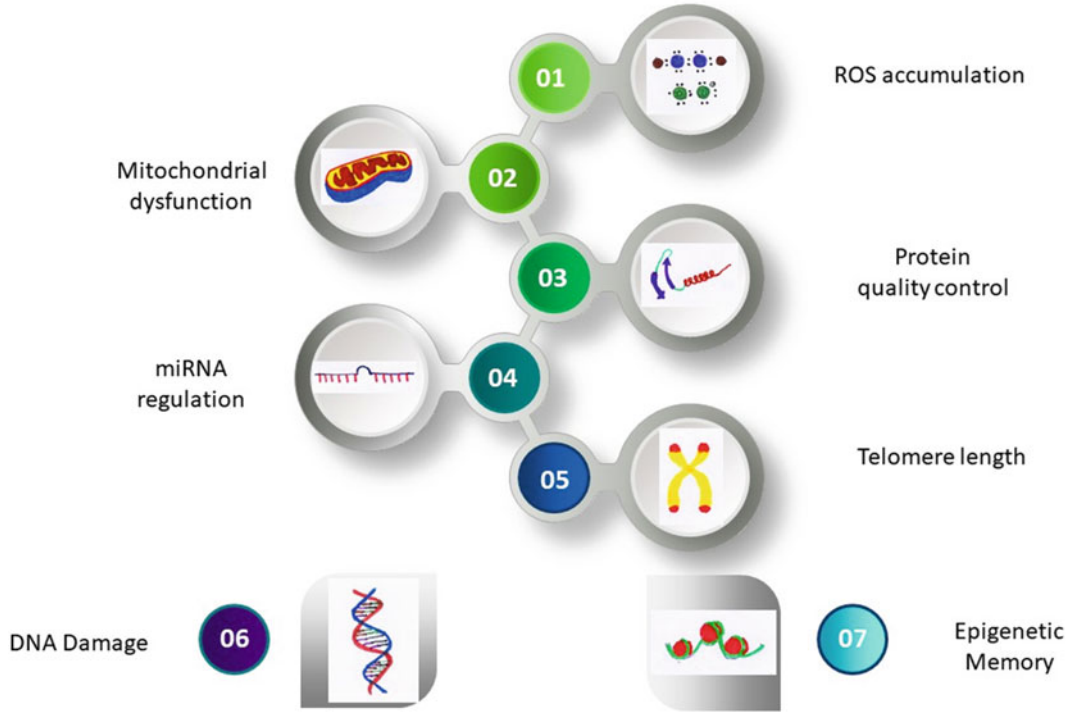


Fig. 1 Multifactorial causes of stem cell aging. Other factors can be changes in RNA splicing and ribosomal machinery, transformed intercellular communication, altered cell polarity, stem-cell niche deterioration, cellular senescence or cell cycle arrest, exhaustion of stem cell pool etc.

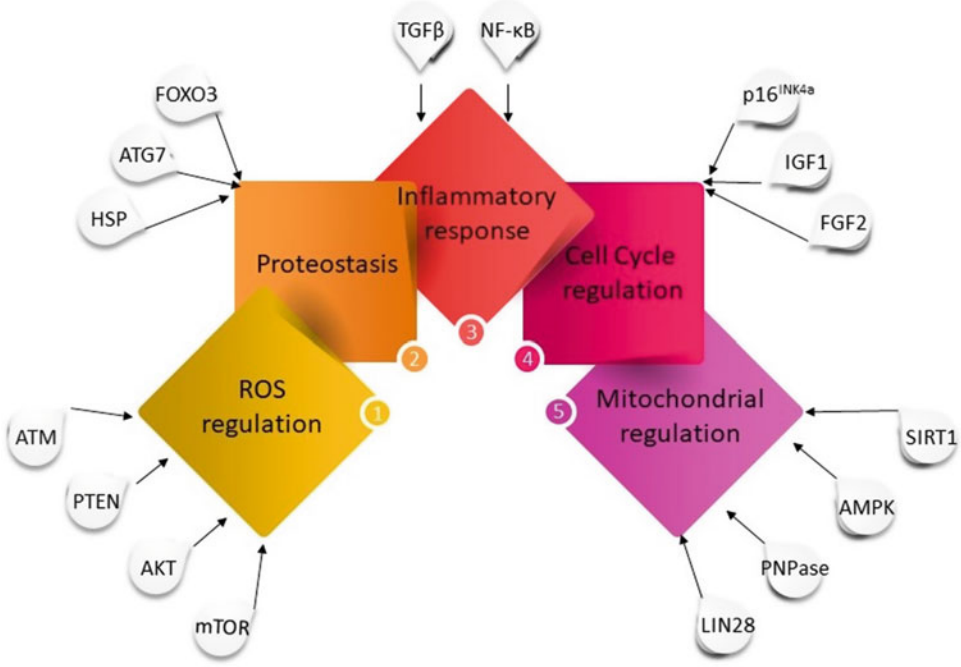


Fig. 2 Common signaling pathways involved in stem cell aging. Although the arrows in the figure are unidirectional, in reality they are all interrelated and interconnected

predetermined actions get compromised oxidative phosphorylation gets activated with higher ROS production, leading to loss of quiescence, self-renewal, and depletion of stem cell pool. To be more precise, MEIS1 gene regulates HIF1α and HIF2α and loss of MEIS1 results in loss of HIF phenotype which can be reversed by ROS scavenging (Kobayashi and Suda 2011; Bigarella et al. 2014). Similarly, glycolytic metabolism is also implicated in stem cell maintenance where loss of crucial glycolytic enzymes like LDHA results in increased ROS production and further defects in HSCs and their progenitors. PDKs serve as checkpoints in the transition between quiescence and proliferation. Genetic ablation of PDKs disrupts this balance and leads to loss of quiescence, increased ROS production and results in HSC pool exhaustion (Bigarella et al. 2014).

Stem cells can also detect health and functioning of mitochondria using ROS as a marker. For example, in mice, deficiencies or mutations in genes such as Lkb1 (Liver kinase B1); (Bid BH3 interacting-domain death agonist); Mortalin; Protein deglycase DJ-1; TSC1 (Tuberous sclerosis 1), which are all responsible for mitochondrial function, lead to increased ROS levels and consequent loss of HSC quiescence (Bigarella et al. 2014).

Heightened ROS levels are found to have resulted from decreased expression of antioxidants like catalase & SOD2 (Superoxide dismutase 2) in NSCs which play a major role in minimising the levels of peroxides and radicals. PTEN (Phosphatase and tensin homolog) AKT (Protein kinase B) mTOR (Mammalian target of rapamycin) signalling pathway also regulates ROS and thereby prevents impairment of HSCs self-renewal and survival. Sirtuins are NAD+ (Nicotinamide adenine dinucleotide) dependent deacetylases that act as redox sensors and maintain mitochondrial metabolism and ROS production during HSC aging and have been implicated in redressing HSC aging through the antioxidant activity of SOD2. p53 (Tumor protein p53), is also a transcriptional regulator of pro-oxidants and antioxidants and its role is noticeable in NSC homeostasis & aging by suppressing ROS related onset of mitophagy (Kobayashi and Suda 2011; Bigarella et al. 2014; Liang and Ghaffari 2014). TERT exercise extranuclear activity by translocating into mitochondria where it protects mtDNA (mitochondrial DNA) from oxidative damage and ROS induced apoptosis and it also slows down senescence in MSCs. On the other hand, oncogene RAS increases mitochondrial ROS in fibroblasts and induces senescence which is believed to withdraw proliferative cells from the cell cycle (Min-Wen et al. 2016). ROS can even affect cell cycle of HSCs within bone marrow. Even during stem cell therapy from aged or differentiated stem cells, ROS is generated in higher quantities and hamper the reprogramming procedure. This implies that there is a need for intervention by hypoxic conditions or ROS scavengers like NAC (Kobayashi and Suda 2011; Liang and Ghaffari 2014).

As ROS influence a plethora of signaling pathways it becomes a real challenge to manipulate stem cell aging by modulating ROS metabolism. Situations can arise whereby manipulating ROS through H_2O_2 signalling pathway stem cells makes them deviate from their predetermined metabolically desired lineages (Bigarella et al. 2014).

2.2 Mitochondria

Mitochondrial bioenergetics is enhanced by an RNA-binding protein called LIN28 via upregulating oxidative phosphorylation enzymes. LIN28 which gets highly expressed in ESCs, gradually declines during differentiation as well as aging whereas let-7 (lethal-7) miRNA which is suppressed by LIN28 in ESCs resurface in senescent and aged tissues. Let-7 is worth mentioning as it blocks survival, dedifferentiation, and regeneration during the aftermath of an injury. The LIN28 let-7 switch regulates mitochondrial metabolism and in turn regeneration and aging (Min-Wen et al. 2016). PNPase is another RNA-binding protein in mitochondria that is responsible for mitochondrial biogenesis via mtRNA (mitochondrial RNA) import and processing. As a matter of fact, PNPase depleted cells or PNPase suppression by an antagonist such

as TCL1 (T-cell leukemia/lymphoma protein 1) protein facilitate more efficient reprogramming of aged somatic cells to produce iPSCs (Min-Wen et al. 2016).

When MDSCs leave the state of quiescence to proliferate, they shift from mitochondrial fatty acid oxidation to glycolysis. To readily activate the genes for proliferation, H4K16 acetylation is increased by a successive decrease in NAD^+ levels and SIRT1 deacetylase activity. In contrast, quiescent MDSCs have SIRT1 (Sirtuin (Silent mating type information regulation 2 homolog)) 1 activated and via transcriptional coactivator PGC1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha), transactivate mitochondrial oxidative phosphorylation related genes and represses glycolytic genes in order to establish mitochondrial fatty acid oxidation. This implies that upregulation of PGC1α can delay aging and is confirmed by the observation in Drosophila NSCs & ISCs where Spargel (PGC1α homolog) regulates mitochondrial biogenesis during differentiation (Min-Wen et al. 2016; Sun et al. 2016).

Dietary restriction (DR) controls and coordinates stem cell aging by regulating mitochondrial homeostasis through signalling pathways like AMPK (5′ adenosine monophosphate-activated protein kinase) or mTOR and can involve in enhancing self-renewal of MDSCs or HSCs. AMPK signalling specifically prevents mitochondrial apoptosis in HSCs, regulates NSCs and, maintains germline stem cells quiescence in *C. elegans*. Upregulation of mTOR results in increased mitochondrial activity and oxidative stress which can drive HSCs out of quiescence and result in depletion of the stem cell pool. This is consolidated by rapamycin inhibition of mTOR which preserves HSCs' self-renewal capability. Furthermore, in ISCs, DR inhibits mTOR signaling in Paneth cells to increase self-renewal and delay degeneration via cADPR (cyclic ADP ribose) mediated Ca^{2+} signalling (Min-Wen et al. 2016; Zhang et al. 2018).

The mtDNA "Mutator" mice suffer from a proofreading impaired mitochondrial DNA polymerase γ (PolG) and thus accumulate mtDNA mutations and age prematurely. They show impaired homeostasis in ESCs, HSCs, NSCs, and ISCs and closely resemble aged hHSCs in self-renewal, age-related anaemia, neurodegeneration, and other senescent phenotypes. ROS levels are not altered significantly in the mutator tissues, still a dysregulated redox balance is indicated as the homeostatic dysfunction in most of the stem cells can be redressed by overexpressing antioxidants. As mentioned previously, induction of PGC1α can also promote mitochondrial biogenesis and revert the skeletal muscle phenotypes but it fails to repair the accumulated mtDNA mutations. Through asymmetric cell divisions stem cells maintain self-renewal by segregating old mitochondria from young, but data from mutator mice showed that once mutagenesis level crosses a threshold limit, the segregation of old from young becomes insufficient and degeneration sets in (Min-Wen et al. 2016; Sun et al. 2016).

2.3 Proteostasis

Stem cell aging gets affected by misfolded, damaged, aggregated, or unnecessary proteins. Even development can be compromised if ESCs accumulate such irregular proteins and pass them to progenitor cells. Naturally, proteostasis or proteome homeostasis must control aging and aging-associated anomalies. Starting from synthesis to the endpoint of degradation, proteome quality in stem cells is regulated by monitoring concentration, localization, folding, and interaction (García-Prat et al. 2017; Lee et al. 2016; Noormohammadi et al. 2017; Vilchez et al. 2014).

With the help of a complex network of chaperones and proteases, ER (endoplasmic reticulum) activates a cellular stress response known as unfolded protein response (UPR^{ER}). UPR^{ER} targets misfolded proteins for ER-associated degradation (ERAD) or cell death programs like autophagy and apoptosis in coordination with mitochondria. Furthermore, in mouse ISCs, UPR^{ER} reverses the loss of stemness caused by Perk–eIF2α induced ER stress. Mediators in UPR^{ER} are found to play diverse roles in stem

cell differentiation. For example, IRE1 (Inositol-requiring enzyme 1) induces higher rates of B cell lymphopoiesis, XBP1 (X-box binding protein 1) positively regulates plasma & osteogenic differentiation, CHOP (CCAAT-enhancer-binding protein homologous protein) advances differentiation of varied cell types like erythrocytes and chondrocytes. Similarly, mitochondria activate UPR^{MT} to maintain mitochondria proteome (García-Prat et al. 2017; Lee et al. 2016; Noormohammadi et al. 2017; Vilchez et al. 2014).

Heat shock proteins (HSP) as molecular chaperones assure correct protein conformational folding and unfolding and ameliorate any proteotoxic stress via another cellular stress response known as heat shock response (HSR). In particular, embryonic stem cells (ESC) have higher levels of HSPs, as observed in murine ESCs (mESC) exhibiting higher levels of HSPA1b (also stands true for hESC), HSPA1a, HSPA9 than the cells which have already differentiated. Interestingly, mESCs differentiate when HSP90 is inhibited which can be partially reversed back by HSP90β. Also, the association of HSP90 with pluripotency maintaining transcription factors such as Oct4 (Octamer-binding transcription factor 4) and Nanog protects them from the ubiquitin-proteasome complex mediated degradation. Other examples of HSPs' role in stem cell metabolism are HSPB5 modulating myogenesis via its regulator MyoD (Myoblast determination protein 1) expression, HSPA8 helping in survival of HSCs by destabilising proapoptotic Bim mRNA, HSP70 promoting erythropoiesis by inhibiting cleavage of transcription factor GATA-1 (GATA-binding factor 1) which is immensely responsible for erythroid lineage differentiation (Lee et al. 2016; Noormohammadi et al. 2017; Vilchez et al. 2014).

When proteins cannot be rescued by UPRs and HSPs, they are degraded through autophagy or Ubiquitin-Proteasome system (UPS). In order to maintain pluripotency hESCs exhibit higher UPS activity and accumulate less damaged proteins than the differentiated somatic cells but whether UPS is enhanced in ASCs remains to be elucidated. ESCs which generally replicate continuously without senescence may be aided by increased proteostasis which helps in ameliorating increased translation errors and maintaining an intact proteome for self-renewal. Degradation of damaged proteins with the initiation of proteasome activator PA28 is one of the first signs of mESCs' differentiation. This proteasome activity is also involved in modulating concentrations of transcription factors required for differentiation or maintaining pluripotency (García-Prat et al. 2017; Noormohammadi et al. 2017; Vilchez et al. 2014).

On early differentiation mESCs as well as hESCs show increased autophagy to prevent damaged proteins from passing on to their differentiated counterparts. This is well observed in foetal and postnatal mHSCs where inhibiting crucial autophagy genes like FIP200 (FAK family kinase-interacting protein of 200 kDa)/Atg7 (Autophagy related 7) hampers proliferation. In a different light, autophagy in ESCs can be viewed as a way to further initiate differentiation by replacing proteins that maintain stemness. Increased ROS levels can also cause protein misfolding as observed when FOXO transcription factors FOXO1 (Forkhead box protein O1), FOXO3, FOXO4 are found deficient in NSCs and HSCs. FOXO transcription factors are responsible for several aspects of stem cell maintenance such as self-renewal, quiescence, stress response and overall survival (García-Prat et al. 2017; Lee et al. 2016; Noormohammadi et al. 2017; Vilchez et al. 2014).

It is a well-known fact that there is always a trade-off going on between reproduction and aging. Resources are preferentially allocated to the germline for proteome maintenance when compared to the somatic tissues and thus eliminating germ cells in *C. elegans* and *D. melanogaster* has proven to be effective in extending their lifespan (Noormohammadi et al. 2017; Vilchez et al. 2014). Dietary restriction (DR) also helps in proteostasis by decreasing protein synthesis which is indicative of more efficient protein folding and degradation. Proteostasis depends on stem cell types as well. Generally, in high turnover cells like epithelia, proteostasis is better maintained in the stem cells

compared to differentiated cells, whereas in neurons that have low or no turnover, proteostasis is better maintained in the differentiated cells (Vilchez et al. 2014).

2.4 DNA Damage

Stem cells are protected populations. Still, they accumulate DNA damages from time to time depending on DNA methylation profile, quiescence, niche, etc. but surprisingly DNA damage is not that strongly connected to most age-associated diseases. Preventing DNA damage at a molecular level is a daunting task as an endogenous DNA repair network is complex and even alleviating just DNA damage will not extend the lifespan of stem cells in every instance. It is more feasible to mitigate the downstream effects of DNA damage, taking into consideration the tissue-specific implications of such damage (Adams et al. 2015; Al zouabi and Bardin 2020; Behrens et al. 2014).

Although HSCs are mostly quiescent, they show a steady accumulation of DNA damage and with age often develop cancer by expressing mutation-driven clonal expansion. Suppressing DNA damage repair pathways via ERCC1 gene of nucleotide excise repair or XRCC (X-ray repair cross-complementing protein 1) gene of NHEJ accelerates aging and aging-associated phenotypes in satellite cells. In support of this, aged satellite cells' functioning has seen to have improved under the influence of antioxidant like Trolox which helps in mitigating DNA damage (Insinga et al. 2014; Kenyon and Gerson 2007; McNeely et al. 2019). Similarly, ISCs which have an excessive need for proliferation are also at a higher risk of cancer from DNA damage followed by mutation-driven clonal expansion. MSCs are also linked to several age-related disorders like osteoporosis but a robust relationship between DNA damage and those disorders is yet to be consolidated. In NSCs, DNA damage leads to proliferation errors such as loss of differentiation towards glial cells like astrocytes and could be linked to age-related disorders like dementia. As a matter of fact, a subset of NSCs is programmed to get exhausted by proliferation to prevent the accumulation of DNA damage (Adams et al. 2015; Al zouabi and Bardin 2020; Behrens et al. 2014; Insinga et al. 2014; Kenyon and Gerson 2007; McNeely et al. 2019). Skin stem cells such as epidermal stem cells (EpSC), hair follicle stem cells (HFSC), or melanocyte stem cells (MeSC) are at a higher risk of DNA damage by external physical and chemical agents as they are located closer to the atmosphere. Interestingly, in EpSCs, DNA replication and proliferation generally occur at night, but with age, this circadian rhythm gets dysregulated which results in proliferation during the day followed by DNA damage via UV & ROS. DNA damage is more pronounced in areas of hair greying & hair loss which are commonly associated with age and can be symptoms of age-related disorders. Like aged HSCs, MeSCs & HFSCs trigger differentiation after significant DNA damage in order to eliminate damaged cells and minimize risks of oncogenic transformation (McNeely et al. 2019). DNA damage in GSCs can either affect the progeny (aneuploidy) or the individual himself (infertility) and even the somatic cells. Spermatogenesis has a higher risk of DNA damage as it requires a perpetual replication process, unlike oocyte proliferation which ceases after a certain period. In mice, due to their highly active telomerase, spermatogonial stem cells' generation potential exceeds even their life span despite accumulating DNA damage. Although oocytes cease proliferation and prevent replication errors, it becomes a challenging task to sustain genomic integrity for such a long time in meiotic prophase I. So, their course of action is to block DNA damage repair and via RNF212 (Ring Finger Protein 212) & HORMAD1 (HORMA domain-containing protein 1) lead damaged cells to double-strand breaks (DSB) induced apoptosis as a quality control mechanism (McNeely et al. 2019).

2.5 Telomere

Consequences of telomere mutations' effects are more pronounced in stem cells like HSCs or ISCs which need to generate a high turnover of cells.

Telomere shortening by proliferative stress or telomere deletion is seen to have reduced self-renewal potential in HSCs. Telomerase activity and the outcome of telomere deficiency like apoptosis, cell cycle arrest is shown to be different between stem cell types and even between their progenies. Telomerase RNA component (TERC) is one of the two core subunits of telomerase and serves as the template for replication. The other one is telomerase reverse transcriptase (TERT) which catalyses the addition of the telomere repeats at the telomere ends (Blasco 2007; Choudhary 2012; Zhang and Ju 2010; Tümpel and Rudolph 2012).

Abnormal telomerase activity results in faulty differentiation in the case of MSCs and inhibition of neurogenesis in NSCs. In ISCs, senescent phenotypes stemming from TERC knockdown can be redressed by deleting EXO1 (Exonuclease 1). EXO1 encodes an exonuclease involved in DNA repair and its deletion inhibits cell cycle arrest and apoptosis and improves stem cell maintenance and lifespan. In contrast deletion of another mismatch repair gene MSH2 (MutS homolog 2) showed a negative effect on the function of HSCs like for instance an increase in microsatellite instability leading to loss in stem cell phenotype (Zhang and Ju 2010; Tümpel and Rudolph 2012; Song et al. 2009).

Telomere dysfunction is also associated with the formation of DNA damage foci which consist of proteins associated with DNA damage and repair such as γH2AX. The foci induce ATM (Ataxiatelangiectasia mutated)/ATR (ATM and RAD3-related) signalling which further triggers cell cycle regulators like p53 & p21 (Cyclin-dependent kinase inhibitor 1) which ultimately leads to senescence. Surprisingly, ATM deletion results in an enhanced aging phenotype. This indicates that ATM is responsible for maintaining telomere function via involvement in DNA damage signals, telomere capping and even telomere-independent functions like maintaining ROS levels which can also lead to aging. p53 being a crucial member of the DNA damage signalling pathway can detect telomere shortening and usually causes apoptosis or senescence in stem cells. p53 inhibition can stall senescence but that occurs only at low levels of telomere dysfunction. At higher levels apoptosis can take place via p53 independent pathways. Interestingly, p21, a downstream target of p53 expresses a dual role in stem cell aging. In general, p21 retains stem cell in a quiescent stage and its knockout can cause a depletion in the stem cell pool. But during telomere dysfunction, upregulation of p21 shows an increased cell cycle arrest or early differentiation, both of which can deplete the stem cell pool. As a matter of fact, p21 deletion in mice shows improved stem cell maintenance and function in telomerase dysfunctional ISCs and HSCs (Zhang and Ju 2010; Tümpel and Rudolph 2012; Song et al. 2009; Ju and Rudolph 2008).

Telomere dysfunction alters both stem cell niche and expression of regulatory cytokines and growth factors. For example, TERC deletion reduces both the quality and quantity of MSC niche stromal cells along with their proliferative capacity. Telomerase dysfunction can also result in an increased level of pro-inflammatory cytokines like elevated G-CSF in the HSC niche cells causing impaired lineage commitment (Song et al. 2009; Ju and Rudolph 2008).

2.6 Epigenetics

DNA methylation via canonical DNMT enzymes DNMT1, DNMT3A and DNMT3B plays a role in stem cell aging by balancing self-renewal and differentiation. In SSCs & NSCs, knocking down DNMT1 reduces self-renewal and abnormal differentiation. In ISCs, DNMT1 knockdown inhibits differentiation, whereas in HSCs, DNMT1 knockdown affects both self-renewal and differentiation. Furthermore, ablating DNMT3A & DNMT3B in HSCs can increase self-renewal but that will automatically result in a loss of differentiation. Stem cell aging is characterized by both DNA hypermethylation as well as hypomethylation. Some methylation patterns are present in all stem cell types while some are specific for cell types as well as certain loci like CpG islands. Stem cells have no direct or significant changes in transcription caused by altered DNA methylation. This could imply that

significant changes, if any, could only be observed in differentiated stem cell progenies because DNA methylation is transmitted downstream. Or it could be possible that alterations are only observed at distal regulation sites like enhancers because till now studies of altered methylation are more or less based only on transcription start sites or within genes (Beerman and Rossi 2015; Brunet and Rando 2017; Krauss and de Haan 2016).

TET (Ten-eleven translocation) are major DNA demethylating enzymes. In NPCs (Neural progenitor cells), knocking down TET1 results in pool exhaustion through the reduction of self-renewal followed by manifestations of accelerated aging-related symptoms like impaired learning and memory. In contrast, TET2 when knocked down in HSCs, results in increased stem cell renewal and manifests a differentiation bias towards myeloid lineage which can indicate aging (Beerman and Rossi 2015).

HATs like CBP (CREB-binding protein), MOZ (Histone acetyltransferase KAT6A), or HAT cofactors like Trrap (Transformation/transcription domain-associated protein), if ablated in HSCs or NPCs, result in a wide range of functional impairment in stem cells such as cell depletion, heightened apoptosis, decreased or faulty proliferation and differentiation. Moreover, several Class I & Class II HDACs if knocked down in ISCs/HSCs/MSCs/NSCs, result in a series of age-related phenotypes like loss of self-renewal via apoptosis, loss of progeny cells, inhibition of proliferation, faulty polarization, etc. In HSCs and satellite cells, Class III HDACs such as SIRT1 ensures adequate self-renewal by maintaining quiescence and preventing premature differentiation whereas in NSCs, proliferation & self-renewal is increased by knocking down SIRT1 (Beerman and Rossi 2015; Yu and Dang 2017; Ren et al. 2017).

Transcriptional state of activeness can be measured by H4K16ac levels and are found to be less in aged HSCs compared to their younger counterparts. H4K16 hypoacetylation can also lead to aging via DNA damage accumulation as it impedes DNA damage response and DNA double-strand breaks repairs. Transcriptional regulation by H3K4 methylation at enhancer regions does not show up in the HSCs but the genes are primed to get expressed in the differentiated progenies. Similarly, in NPCs, H3K4me1 marked loci only put the genes in suspension for a later expression while loci associated with H3K4me1 as well as H3K27ac regulate genes that are actively transcribed. Moreover, some stem cells like HSCs show marked differences in H3K4me pattern between old & young cells. The mono-methylated form of H3K27 is positively correlated with gene expression while the di- and the tri-methylated forms are often negatively correlated. H3K27 is methylated by a polycomb group protein complex called PRC2 (Polycomb repressive complex 2) and the methylation affects a number of stem cell function like self-renewal, differentiation, regenerative potential, maintaining quiescence and stem cell pool but how these methylation changes during aging affect stem cell metabolism is yet to be consolidated. H3K4me3 and H3K27me3 marks can be present together in a locus as bivalent domains and regulate lineage potential and commitment in stem cells. Addition and deletion of bivalent domains in aged stem cells are responsible for dysregulation in stem cell function where repressive H3K27me3 replace H3K4me3 marks. Loss of regeneration potential in aged stem cells can be due to this overexpressing restrictive phenotype as observed in old HSCs and satellite cells (Beerman and Rossi 2015; Brunet and Rando 2017; Krauss and de Haan 2016; Ren et al. 2017).

Epigenetic signalling also involves crosstalk among varied epigenetic modifications because sometimes histone methylation needs to be removed to pave ways for DNA methylation. For example, H3K4me restricts DNMT3A/3B recruitment to promoter regions and inhibit DNA methylation, or in HSCs knocking down H3K4 demethylases Kdm1a/1b (Lysine (K)-specific demethylase 1a/1b) results in loss of DNA methylation. Similarly, during differentiation of ESCs, H3K27me3 regions are substituted by DNA methylation as a way to permanently mark repression of lineage. PRC2 can also inhibit DNA methylation by interacting with TET1 but gets functionally restricted in aged HSCs due to

downregulation of its core factors, which results in further accumulation of DNA methylation (Beerman and Rossi 2015).

3 Stem Cells in Regenerative Medicine

Though the term "regenerative medicine" dated back to a 1992 paper by a US scientist Leland R. Kaiser, it is widely regarded to be coined by another US scientist William Haseltine in 1999 when he described a new frontier in therapeutics that would combine knowledge from tissue engineering, stem cell biology, biomechanics, nanotechnology, etc. It is presently described as a biomedical application to repair or replace damaged cells or tissues when the body fails to do it by itself (Fig. 3). Tissue repair or replacement is a ubiquitous phenomenon of the human body which maintains tissue integrity throughout the physiological events of cellular turnover and external or internal trauma. Cells like neutrophils get replaced within a couple of days whereas cells like cerebral cortex neurons persist throughout life! Whole organ transplantation has certain limitations such as lack of donors, transplantation efficiency, ethico-legal issues which make way for stem cells to be potential alternative sources. Stem cells exhibit potency, are capable of differentiating into all cell types and are involved in physiological regeneration. The subsequent discourse carries on with the attributes of embryonic and adult stem cells and their practice in regenerative medicine (Sampogna et al. 2015).

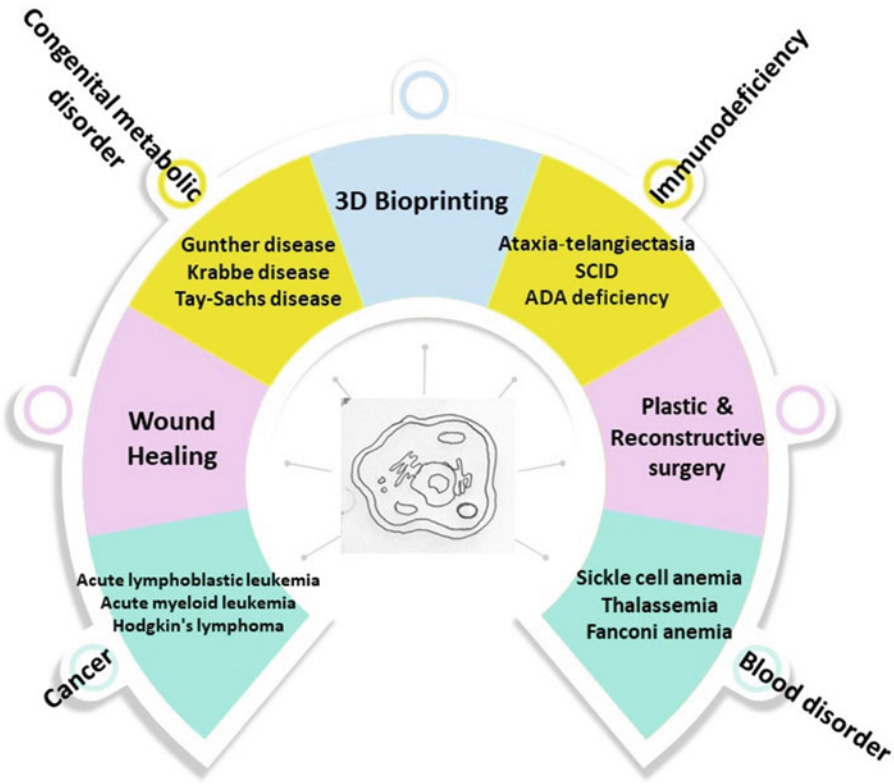


Fig. 3 Potential applications of Stem Cell therapy in regenerative medicine. Currently Hematopoietic stem cell transplantation is the only form that is widely accepted clinically

3.1 Embryonic Stem Cells in Regenerative Medicine

In 1981, Martin Evans & Matthew Kaufmann extracted stem cells from a mouse. After a long journey of 17 years, in 1998, James Thomson from the University of Wisconsin–Madison established the first hESC line (Fig. 4). Nowadays, information on about 400 hESC lines can be retrieved online from NIH Human Embryonic Stem Cell Registry (Bajada et al. 2008).

ESCs are pluripotent cells that can be procured from the ICM of 5/6-day old blastocysts (in embryonic development blastocyst gets implanted in the uterus to evolve into a foetus). The ICM develops into two distinct cell layers, the hypoblast which gets shaped into the yolk sac and the epiblast which is ordered into three distinct layers ectoderm, mesoderm, endoderm to form and fashion all the tissue types. ICM can be isolated from fresh or frozen human embryos by methods like mechanical dissection, laser dissection, immune surgery, micro dissection, laser-assisted biopsy. The isolated pluripotent stem cells are cultured using feeder layers (mouse/human/synthetic or even without feeder layers), scaffold matrices, and other synthetic polymers. Their 'immortality' provides for a stable source of cells for further tissue engineering. To replicate the structurally and functionally well-knit bunch of cells in a human body, we need to grow ESCs in 3D culture conditions because cells in 2D conditions are seen to be readily dying after transplantation. Extracting ESCs and successfully differentiating them into cell types can also unfurl the molecular apparatus of embryogenesis that is involved in defining every cell lineage. For example, hESCs call for a threshold level of pluripotency-associated transcription factors OCT3/4, SOX2 (SRY (sex determining region Y)-box 2), NANOG that help to preserve their potential for self-renewal (Bajada et al. 2008; Riazi et al. 2009; Doğan 2018; Vazin and Freed 2010).

During isolation and culture there are high chances that hESC lines get exposed to pathogenic or immunogenic molecules. This is one of the most crucial criteria that is required to be maintained according to the Current Good Manufacturing Practices (CGMP) guidelines laid out by USFDA in order to utilise the ESC lines for clinical applications.

The isolation of hESCs from IVF blastocysts has been opposed by religion, society, and the government. Blastocyst stage embryos can be considered as sentient human beings which implicates the extraction of cells as immoral as well as unethical and the embryos' chances of implantation also get reduced with extraction of ESCs. Cell lines created and stored have been put under strict laws in some countries and in some of them such research is even entirely outlawed. This further limits the scope of ESCs' application because new hESC lines need to be created as

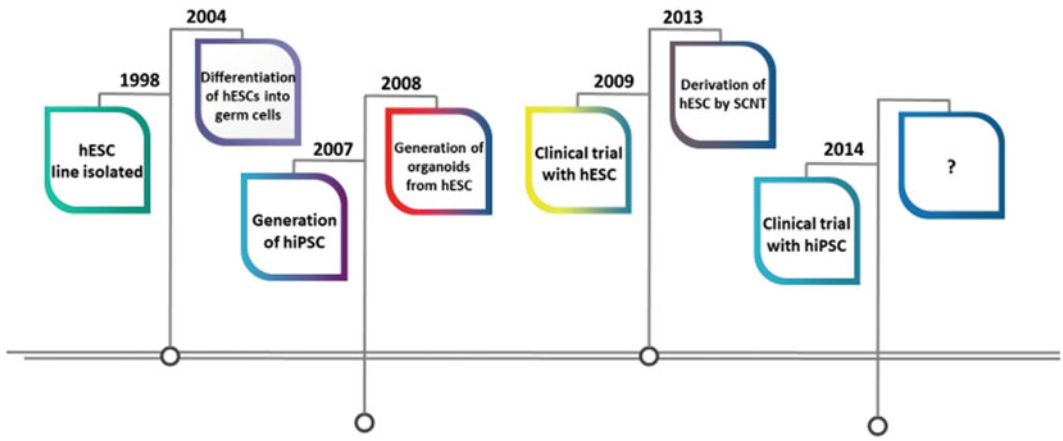


Fig. 4 Timeline of key events in Embryonic Stem Cell research

repeated cell cultures lead to genetic anomalies. There is also an inevitable risk of stem cells dividing out of control and form a teratoma after transplantation. hESC generated grafts' failure of surviving due to allogeneic immune reaction by unmatched histocompatibility antigens can be redressed up to a certain extent by immune suppressors (Bajada et al. 2008; Riazi et al. 2009; Khan et al. 2018).

3.2 Therapeutic Cloning

SCNT for therapeutic cloning can bypass shortcomings of graft rejection, concern over killing a foetus, logistic limitations of collection of ICM from IVF (In vitro fertilization) clinics. The introduction of a nucleus from a donor cell into an enucleated oocyte ensures genetic similarity between the donor and that of the future embryo which indicates that ESC lines can be personalized. Personalized ESC lines avoid allogeneic reactions and immune rejections and can be stored with credentials to be reused in the future. ESC banks can be set up comprising of every HLA (Human leukocyte antigen) allele present in the population for creating an efficient engraftment regime and avoiding transplantation rejections but that will be a humongous task considering the vastness of HLA alleles. Moreover, therapeutic cloning generates only the early developmental stages that are sufficient for efficiently procuring ESCs, unlike reproductive cloning where the embryo persists to develop and grow into a newborn. Nuclear reprogramming can be realised in hybrids between somatic cells & ESCs as indicated by the expression of pluripotency markers like OCT4, NANOG in the somatic cells. Autologous cells can also be generated by nuclear reprogramming utilising oocytes and patient-specific stem cells. Scientists have successfully treated genetic defects using SCNT reprogrammed ESCs in mice and have been trying to emulate the same in human beings (Riazi et al. 2009; Kind and Colman 1999).

The challenges of therapeutic cloning are its little success rate, the scarcity of available volunteers who will donate egg cells, the overall cost, and the safety of the invasive procedure. The low efficiency of SCNT is due to the partial and insufficient reversal of the epigenetic machinery of the nucleus. Consequently, stem cells show higher cloning efficiency than differentiated cells. The fusion between nuclei of an 'old' cell and an enucleated oocyte is primarily implicated in causing developmental anomalies. Alternatives can be hybrid cells established from humans and species like mice which would lessen the involvement of donated oocytes or we can aim for directly converting differentiated cells into pluripotent cells by efficiently expressing specific pluripotency-associated transcription factors. However, the current scenario for nuclear cloning is grim as SCNT research is deemed controversial worldwide even by scientific communities and is banned by law in most countries (Riazi et al. 2009; Kind and Colman 1999).

3.3 Adult Stem Cells in Regenerative Medicine

The ability to self-renew and maintain tissues for a prolonged time demarcates stem cells from progenitor cells. Identifying the unique factors that separate stem cells from other types of cells aided in extracting & proliferating stem cells from physiological locations such as bone marrow and prompted regenerative medicine into the limelight. But at present, a suitable cell transplantation assay required to demarcate stem cells from progenitor cells is presently not well developed for several tissue types and not all tissues even express regenerative potential. Of all the tissues that have shown the prospect of stem cell activity, the one profoundly used in clinical trials and therapy is bone marrow. Multifaceted complex obstacles must be solved in each systematic step of isolation, expansion, proliferation, differentiation, and transplantation. So, it is obvious that primary reports of regeneration from transplanted stem cells were met with either scepticism or enthusiasm. The most crucial factor in regenerative medicine is the source of tissue which can either be from the same person or a different donor or even engineered. Transplantation can

also be troublesome as most of the time cells do not survive due to interaction with a new microenvironment and immune rejection (Riazi et al. 2009; Stocum and Zupanc 2008).

3.4 Hematopoietic Stem Cells

HSCs need to be constantly activated and generate high-yielding haematopoietic progenitor cells to maintain homeostasis. The transplantation model of the immunocompromised mouse has significantly facilitated analysing bone marrow transplantation, isolation & enrichment of HSCs, and examining their homing towards specific tissue regions. Multicolor cell sorting can be utilised to isolate several multilineage progenitors. Unfortunately, many markers that are associated with the purification of mouse HSCs do not express in the human hematopoietic environment. Scientists are still in the process of isolating hHSCs of high purity. Autologous HSC transplantation can be applied for treating non-haematopoietic cancers and allogeneic HSC transplantation in autoimmune disorders as well as blood malignancies. Like any other allogeneic transplantation system, transfer of HSCs via bone marrow transplantation is hindered by a series of limitations such as inflammatory syndromes like GVHD, infections, availability of donors who have their expressed HLA similar to the recipients. Compared to bone marrow transplantation, HSCs from UCB reconstitute and regenerate the hematopoietic system with lesser chances of GVHD. UCB is generally preferred to treat blood disorders in children as adults require a higher effective cell dose which can only be sourced from bone marrow (Riazi et al. 2009).

In the recent past, methodologies are being designed to escalate and expand the effective cell dose from various sources. HSCs can be enriched in a bioreactor with presence of optimal growth factors and constant removal of cells or factors that can induce differentiation. On a molecular level cytokines' expression can be altered or overexpression of transcription factors like HOXB4 (Homeobox protein Hox-B4) that are implicated in expansion of HSCs. Moreover, not only HSCs but differentiated blood lineages like platelet-rich plasma can also be transfused and applied in regenerative medicine. ESCs can be manipulated by lineage-specific markers to differentiate into erythroid or myeloid cell lineages. They can also give rise to such lineages by co-culturing with specific cell lines like OP9 (cell line obtained from newborn mouse skull which enhance haematopoietic development) or utilizing embryoid body (EB). Embryoid bodies are three-dimensional aggregates of pluripotent stem cells procured from regular stem cell sources like blastocyst or via SCNT. However, in contrast to mESCs, adequate generation of HSCs from hESCs are still in process (Müller et al. 2016; Brown and Weissman 2004).

3.5 Neural Stem Cells

Neurodegenerative disorders like Parkinson's or Huntington's result when there is a severe loss of neurons and glia and the resident stem cells fail to yield an adequate regenerative response. Developing adequate therapy is complicated as there is a need for replacement of a vast array of neuronal cells which have either lost their ability to divide postnatally or are damaged or dysfunctional with age or disease. NSCs located in SGZ & SVZ of the brain can be isolated, cultured, and transplanted in mice to produce neural and glial lineages. Primary sources of isolation are aborted foetuses, dead people or NSCs can even be generated from ESCs. Transplantation in spinal cord injuries (SCI) of animal models has shown NSCs successfully engrafting and showing signs of functional recovery but the exact mechanism of regeneration is yet to be consolidated (Riazi et al. 2009; Lindvall et al. 2004; Takahashi 2018; Martino and Pluchino 2006).

Skin-derived precursor cells (SKP) are multipotent cells derived from the neural crest and are located in the dermis. They can differentiate into cells that have markers of Schwann cells (indicating myelination regeneration) or peripheral neurons. Naturally, they are a promising fountainhead of stem cells as they are autologous and obtained with much ease. Many mesenchymal

progenitors from the bone marrow are also derived from neural crest and are similarly reported to be successful in regenerating neurons and glia after transplantation. Such cells when transplanted in SCI and ALS (Amyotrophic lateral sclerosis) patients have shown a positive effect in repair and regeneration. Even cells from non-hematopoietic fraction of UCB expressed stem like characteristics and demonstrated functional recovery in animal models of neurodegeneration. ESCs show an inclination towards differentiating into neurons when treated with retinoic acid. bFGF (Basic fibroblast growth factor) is also a strong mitogen for generating neural precursors. Selectable markers can be expressed under promoters like CNP (2′,3′-cyclic-nucleotide 3′-phosphodiesterase) or Tα1-tubulin which helps in expansion of the generated neuron and glia from ESCs.

NSCs' tropism towards distinct lesions within the brain can be utilised as vehicles for delivering target-specific drugs to induce secretion of growth factors for repair or regeneration or to destroy tumours. Clinically bone marrow mononuclear cell fraction containing BMSCs and GM-CSF are injected to reach the lesions within a therapeutic window following trauma. BMSCs improve axonal regeneration and GM-CSF induces NSCs to proliferate, activates macrophages to clear myelin debris, and impedes apoptosis (Bajada et al. 2008; Riazi et al. 2009).

3.6 Mesenchymal Stem Cells

MSCs are located in several stromal populations and they can differentiate into adipocytes, chondrocytes, osteocytes and myoblasts. The stromal populations also contain several multipotent, heterogenous progenitors. MSCs can be identified and isolated using markers like SSEA-4 (Stage-specific embryonic antigen 4) or Sca-1 (Stem Cell Antigen-1). Besides bone marrow, MSCs can be extracted from UCB, adipose tissue, hair follicles, and even circulating blood. They can be transplanted to redress bone defects with the help of biodegradable scaffolds and in disorders like osteogenesis imperfecta (OI) or osteoporosis. Transplantation of MSCs in OI has shown a positive influence on growth, bone mineral content, and decreases fractures. Even osteoprogenitors present within stromal cells can be adequately engineered for bone regeneration. MSCs are seen to have expressed genes specified for chondrocyte generation like Type II collagen and thereby exhibit chondrogenic potential in regenerating degenerative vertebral discs and nucleus pulposus in an animal model. Unfortunately, the cartilage that forms tends to mineralise. Chondrocytes can also be isolated for cartilage generation, but they are available in an inadequate quantity and moreover, they frequently show loss of function by dedifferentiation when expanded *in vitro* (Riazi et al. 2009; Krampera et al. 2006; Trohatou and Roubelakis 2017; Qu-Petersen et al. 2002).

Satellite cells are multipotent cells located in the muscle fibers and have a positive role in postnatal growth of skeletal muscles along with repair after injury. MDSCs are an additional heterogeneous population of multipotent stem cells located in the skeletal muscles. $CD45^{-}$ subpopulations of MDSCs give rise to myogenic progenitors and even lineages like blood. They have also expressed chondrogenic potential in an animal model. MDSCs may prove to be more effective than satellite cells in muscle regeneration as they can regenerate muscle fibers in higher frequency. Both intravenous and intramuscular routes of transplantation have proven to be effective in treating musculoskeletal disorders (Horwitz et al. 1999; Kuo et al. 2006; Yin et al. 2013).

3.7 Heart

Regeneration of cardiomyocytes is restricted so any considerable loss of cells (during myocardial infarction) cannot be replenished easily and thus requires an extrinsic push for repair and regeneration. CSCs are multipotent cells located in the heart and have exhibited potential to differentiate into smooth muscle cells and cardiomyocytes. They can be extracted based on surface markers such as c-kit or Abcg2 (ATP-binding cassette

super-family G member 2). Other cardiac progenitor cells have also been identified based on presence of receptors like Flk-1 (Fetal Liver Kinase 1) or transcription factors like Islet-1 (Insulin gene enhancer protein).

Cardiomyocytes can be derived from ESCs and transplantation of ESCs in mice hearts can help in restoration of cardiac function but transplanted ESCs show a propensity to differentiate into non-cardiac cells which have become a severe setback for clinical application. Enrichment of cardiomyocyte populations from ESC cultures is dependent on cell lines and the yield can also be increased via expressing antibiotic resistance genes driven by specific promoters such as αMHC (α-myosin heavy chain) or selectable markers like green fluorescence proteins. Myocardial injury or hypertrophy induced by myocardial injury can also be treated by transplanting cells capable of either replacing cardiomyocytes or differentiating into contractile myocytes (Riazi et al. 2009; Beltrami et al. 2003; Urbanek et al. 2005; Boheler et al. 2002).

EPCs, progenitor cells derived from bone marrow and skeletal muscle can also be transplanted to improve heart function after damage or degeneration, but their actions are mainly due to paracrine effects instead of cardiomyocyte generation. For example, EPCs help in neovascularisation and progenitor cells from bone marrow are seen to have affected infarct remodelling in a clinical trial. Interestingly, skeletal muscles can give rise to progenitor cells called skeletal-based precursors of cardiomyocytes (Spoc) which are capable of generating cardiomyocytes. A diversified array of progenitors and stem cells like ESCs, HSCs, MSCs, and EPCs can be used to engineer vascular grafts. BMDSCs are also been used to build heart valves with the help of biodegradable scaffolds. Tissue-engineered heart valves are naturally more stable and durable than the existing mechanical valves though they sometimes suffer limitations due to deformation and calcification. Bone marrow or BMSCs can be transplanted into the coronary arteries or the myocardium to treat ischaemic heart diseases such as myocardial infarction by expanding the resident pool of stem cells and progenitors. They are supposed to be positively modulating angiogenesis, inducing the proliferation of endogenous cardiomyocytes, and preventing their apoptotic death (Müller et al. 2018; Singla et al. 2006; Siepe et al. 2005; Vesely 2005).

3.8 Liver

Generally, after infections or conditions like hepatectomy, the liver shows compensatory growth by hyperplasia via hepatocytes' re-entry into the cell cycle. Even in cases of liver failure due to hepatitis, stem cells and progenitors help in repair and regeneration. On mitogenic stimulation hepatocytes can dedifferentiate into multipotent progenitors or directly transdifferentiate into cholangiocytes to redress bile duct injuries. Hepatic oval cells express markers similar to HSCs such as Sca-1 and c-kit and can give rise to both hepatocytes & cholangiocytes *in vitro*. The oval cells can be isolated using surface marker Thy-1 (Thymocyte differentiation antigen 1) and can be a potential candidate for transplantation and liver regeneration. Human liver stem cells (HLSC) are another distinct variety of progenitors/stem cells that differentiate into cells of hepatic, endothelial and osteogenic lineages. They do not express hepatic oval cell or HSC markers, instead they exhibit markers similar to that of MSCs (Riazi et al. 2009; Shafritz et al. 2006; Nussler et al. 2006; Koenig et al. 2006; Herrera et al. 2006; Adiwinata 2019).

3.9 Pancreas

Pancreatic β cell replacement and/or regeneration are a promising strategy to cure diabetes.

Human pancreatic islets can be isolated and transplanted to manifest a positive outcome but side effects of immunosuppressive drugs, lack of suitable donors, chances of autoimmune diabetes limit their therapeutic applications. The existence of pancreatic stem cells in the ducts or islets has been controversial in the scientific community. Or it may be possible that the resident cells are able to dedifferentiate into progenitor cells that can

further give rise to islets. Pancreatic islet cells have been isolated, made to dedifferentiate, and further induced into cells resembling islets by using peptides like INGAP (Islet Neogenesis Associated Protein) that are associated with neogenesis of islets. Both mESCs & hESCs can be prompted to differentiate into cells that can secrete insulin, but the methods are still in their earlier stages to be applied clinically. Notably, transdifferentiation of hepatocytes, hepatic oval cells, foetal liver cells, and even intestinal cells can result in β cell generation by methods like overexpression of pancreatic transcription factors such as Pdx1 (Pancreas/duodenum homeobox protein 1) (Riazi et al. 2009; Zulewski et al. 2001; Baharvand et al. 2006; Bonner-Weir et al. 2004).

3.10 Lungs

HSCs, MSCs derived from bone marrow or ESCs can be induced to express markers of airway or alveolar epithelial cells *in vitro* but needs to be explored further for clinical applications. Extraction of ESCs and generation of iPSCs from patients with lung diseases (acquired diseases or genetic such as cystic fibrosis) and studied to comprehend the mechanisms behind injury and repair. Stem cells and progenitor cells in the lungs have varied population depending on the region of lungs and can be examined from distinct regions that are damaged from miscellaneous pollutants (Weiss 2014). Club cells can act as stem cells in times of injury to replenish ciliated cells in the trachea, distal airways, and regenerate the bronchiolar epithelium. Bronchioalveolar stem cells (BASCs) are another stem cell population located in lungs and are seen to be proliferating and differentiating into alveolar epithelial cells in alveolar injury models. EPCs can be utilized as biomarkers as they are found to be correlated with clinical variables in various lung diseases. Systemic administration of EPCs has been found to ameliorate lung injuries in several animal models, still the exact mechanism of action is not clear and may be due to synergistic effects of various factors such as paracrine stimulation of resident progenitors or immunomodulation which can vary between diseases. They can also be transfected or transduced to express pro-angiogenic factors or even suicide genes in lung cancer. As of now, transplantation of EPCs has shown functional recovery in patients suffering from pulmonary hypertension. In murine lungs, MSCs have shown to be activating resident airway progenitors and administering MSCs in several lung injury models in animals has been thoroughly successful. Transplantation of MSCs in an infected human lung explant has caused a decrease in inflammation. The mode of action of MSCs is not fully understood and it is believed to be different in each disease. MSCs can have immunomodulatory effects by either leading to secretion of anti-inflammatory molecules such as TSG-6 (Tumor necrosis factor-inducible gene 6 protein) or by interacting with the immune system to minimise inflammation-associated tissue damage in lungs (Weiss 2014).

3.11 Kidney

After Acute Kidney Injury (AKI), BMSCs & HSCs are seen to be migrating to the region of injury for regeneration and thus transplanting BMSCs or HSCs have exhibited improvement in structural and functional recovery after AKI. They can regenerate glomerular & tubular regions by secreting trophic factors and replacing a varied type of resident tissue types such as podocytes and glomerular mesangial cells. The transplanted BMSCs mobilize G-CSF and M-CSF and are proven to be renoprotective in few models of AKI. mESCs have also been shown to differentiate into renal tissues like renal tubule epithelia, tubular cells, podocytes using growth factors like BMP4, BMP7 (Bone morphogenetic protein 7). Amniotic fluid stem cells (AFSCs) can also prove to be a promising source for regenerating renal tissues as they have been observed getting integrated into the murine kidney and proliferate into renal cells. They have also expressed markers of podocytes *in vitro* (Nowacki et al. 2014).

3.12 Urinary Bladder

Stem cell-based bioengineered bladders have been applied in animal models and clinical trials in cases of poorly compliant bladders and in birth defects like myelomeningocele. Autologous bladder cells are cultured and seeded onto biodegradable matrices made from collagen or polyglycolic acid. The engineered bladder construct is then enlarged by cystoplasty. Wrapping the omentum around the construct contributes to its sustainability by aiding in neovascularisation. Although differentiated cells can be used, transplantation of MSCs shows better results by appropriate homing and differentiating into smooth muscle cells, faster repopulation, and proper maintenance of neural function. MSCs are the most sought-after option when autologous bladder cells are insufficient due to conditions like bladder cancer (Bajada et al. 2008; Smolar et al. 2018).

3.13 Dentistry

Dental pulp stem cells (DPSC) can be isolated from dental pulp, expanded *in vitro*, and transplanted to regenerate dental pulp. Stem cells from the apical papilla (SCAP), stem cells from human exfoliated deciduous teeth (SHED) have the similar ability of regeneration, but non-dental stem cells like MSCs derived from human gingiva (GMSC), periodontal ligament stem cells (PDLSC) cannot form similar dentin pulp complex. Composite mixtures of hydroxyapatite/tricalcium phosphate ceramic powder have been commonly used in pulp restoration. For viability, transplanted stem cells require an abundant supply of blood which can be provided by either surgically expanding the root canal space (increases chances of infection) or treating DPSCs with cytokines like G-CSF (enhances both angiogenesis & stem cell proliferation). MSCs (especially PDLSCs or GMSCs) have been demonstrated to regenerate bone & periodontal ligament and prevent inflammation associated damage in periodontal diseases in animal models. Other multipotent cells extracted from regions like dental papilla show differentiation into neurons or hepatocytes, implying application in alternative tissue-specific regenerative therapies (Nakao and Tsuji 2008; Xiao and Nasu 2014).

3.14 Plastic and Reconstructive Surgery

MSCs and other progenitors sourced from BMDSCs have the regeneration potential to develop all craniofacial tissue types. BMDSCs help in augmenting fat grafting and can be applied clinically for treating mandibular defects, mandibular osteoradionecrosis, and fractures. Application of BMDSCs is limited by its low concentration in the bone marrow and its cost per procedure. ADSCs require a less invasive extraction procedure, enrich the fat grafts, and can be clinically used in plastic surgery like facial augmentation by enhancing both osteogenesis & chondrogenesis. They are capable of inducing cytokines and growth factors like PDGF (Platelet-derived growth factor), VEGF (Vascular endothelial growth factor) which aids in cosmetic 'anti-aging' therapies via collagen remodelling. For example, in mice, ADSCs are found to have successfully minimized UVB induced wrinkles. Lipofilling is an already established technique consisting of stem cell-enriched volumetric rejuvenation but this procedure is not stem cell-induced anti-aging per se as transplanted cells fail to prolong 'anti-aging' effects. Similarly, MSCs acting synergistically with hyaluronic acid can improve skin tone by filling in folds in the face. One example of USFDA approved cosmetic surgery is LAVIV, a personalized cell therapy which eliminates smile lines or nasolabial folds utilising autologous fibroblasts (Miller et al. 2016; McArdle et al. 2014).

Growth factors like BMPs bind to MSCs and ADSCs and induce differentiation towards chondrogenic & osteogenic lineage. VEGF is another such growth factor which induces angiogenesis & vasculogenesis and enhances the viability of fat grafts. Some USFDA approved BMPs

such as rhBMP-2, rhBMP-7 can be used clinically like in craniofacial surgery, but further research needs to be done and also there are safety concerns as BMPs are associated with edema or even cancer. Platelet rich plasma (PRP) contains factors like PDGF or TGFβ (Transforming growth factor beta) that are chemotactic for stem cells and positively influence angiogenesis or collagen formation. PRP can be applied in facial plastic surgery like rejuvenation, treating scars and wounds or fat grafting (Miller et al. 2016; Yu 2018).

3.15 Organoids

Organoids are a 3D aggregation of tissues and organs cultured from stem cells or progenitors. They are genetically stable, able to self-renew, differentiate, and mimic morphogenetic features of an equivalent tissue or organ. Specificity in culture conditions is required to maintain both stemness and the environment of the target organ. They help in understanding pathogenesis, how stem cells function, and how they can be manipulated for regenerative therapeutics. Organoids can even be directly involved in regeneration *in vivo* as observed in successful engraftment inside injured intestinal regions. The restriction in growth and differentiation potential by lack of vasculature mediated nutrient support can be solved by connecting *in vivo* derived vasculature. At present, reported stem cell-derived organoids are of stomach, prostate, liver, fallopian tube, and even taste buds (Takata and Eiraku 2017).

4 Stem Cell Aging in Regenerative Medicine

The ramifications of aging shape not only the local milieu but also the systemic environment altogether. Heterochronic parabiosis reveals that the physiological consequences of exchanging cells between subjects of contrasting ages will affect both young and old. Regeneration in the old parabiont gets improved while in the younger individual manifests in a concomitant decline. This alteration in rejuvenation is also extensive instead of being restricted to few types of tissues.

Stem cells act by getting integrated into the patient's system, by locally secreting "factors" or by both from time to time. The relative contributions of cells and factors in modulating the environment are debatable; however the significance of their synergistic outcome is affirmative. Replacing senescent cells or aging-associated factors with factors that aid in rejuvenation is the starting point of stem cell therapy. Know-how related to secreted factors and their respective contributions is restricted as there is a certain degree of ambiguity regarding their identities and activities in each tissue type. They consist of cytokines, growth factors, etc. and constitute signalling pathways that oversee regulation of senescence and biological aging such as Wnt & TGFβ. For example, C1q (Complement component 1q) is an agonist related to Wnt signalling pathway. It is a member of the complement system which gets accumulated in tissues with age and is implicated in decreasing skeletal muscle regeneration. Activin A is another such factor which is a member of TGFβ signalling pathway. It is secreted by senescent cells, causes stem cell dysfunction and thus hampers regeneration. Chemokines such as CCL11 (Chemokine (C-C motif) ligand 1) or MHC (Major histocompatibility complex) components like β2 microglobulin are also implicated in aging and they inhibit regeneration in the nervous system. Cytokines such as IL6 & IL8 (Interleukin 8) are associated with SASP and bind immune response pathways with stem cell aging. Interestingly, acute exposure to SASP factors promotes regeneration responses whereas chronic exposures prompt senescence or worse tumorigenesis. As we can see that immunomodulatory factors can affect regeneration both positively and negatively, therefore altering these pathways to achieve transplantation efficiency can be complicated and is possibly a double-edged sword. The lineage of transplanted HSCs can be traced throughout the whole system instead of only the targeted regions. Similarly, resident cells may integrate into the graft and impact functional outcome.

Nevertheless, the consensus is that secreted factors seem more convenient than stem cells in advancing aging or regeneration. At first, transplanted stem cells go through homing aided by chemokines as a result of signalling by injured tissues, and then the factors take charge by acting locally followed by a systemic cell-cell signalling cascade and/or additional stem cell differentiation and integration. Besides stem cells and secreted factors, the extracellular vesicles (EV) play a crucial role in both local and systemic signalling. EVs are secreted from both stem cells and senescent cells and they contain several proteins, lipids, miRNAs, mRNAs, etc. EVs can increase with senescence and are involved in aging-associated processes such as telomere dysregulation, inflammatory phenotypes, heightened mitogenic signalling, etc. Certain miRNAs that positively modulate stem cell function are found in smaller quantities in EVs from senescent cells (Lau et al. 2019; Conboy et al. 2005).

As mentioned earlier, aged stem cells from a donor and the recipient environment can influence each other and affect survival of the graft with overall outcome of the transplantation procedure. They are also likely to be significant for the systemic rejuvenation or aging of the recipients in age-mismatched transplants. As obvious as it seems, it is worth mentioning that regenerative capacity gets compromised in cases of young recipients and old donors when compared to old recipients and young donors. But regenerative capacity can also be dependent on age of recipients instead of donor age as observed in muscle grafts and transplantation of mammary gland tissues in mice. Regeneration is in fact compromised in older recipients in these cases. Experimental models in mice display the induction of aging and aging-associated phenotypes in young recipients from old donors. Degenerative disorders like osteoarthritis are observed when aged cells are transplanted in the knees of young mice. Even if the percentage of transplanted senescent cells is low in peritoneal cavities of young mice, it is adequate to cause symptoms like aging-associated muscle weakness. Aging-associated modifications of signalling in the stem cell niche microenvironment can also impact transplantation outcome. HSC transplantation in aged patients suffering from leukaemia is associated with increased recurrence of diseases. Not only aging-associated anomalies but most importantly overall lifespan can decline from the impact of donated senescent cells. Although the transplanted cells may remain localized, they can manipulate the recipient cells to express senescence by secreting factors and signal transduction. Besides stem cell activity, aging can also be accelerated from transplantation surgery and immunosuppression. Clinically, the effects of aging in recipients from grafts have not been examined in detail, as the emphasis has more or less revolved around graft survival and functional success. Selective depletion of senescent cells by senolytic agents has been positively correlated with stem cell regeneration and proliferation in HSCs. For example, selective apoptosis of p16Ink4a (Cyclin-dependent kinase inhibitor 2A) positive senescent cells ameliorates degenerative disorders such as pulmonary fibrosis or age-associated osteoporosis. Senolytic drugs exhibit varied specificities against different tissue types and even SASP machinery can be targeted by senomorphic drugs. A substantial advantage of senolytic agents is that they demand infrequent administration as senescent cells need at least a certain window to develop, whereas drugs that target the secreted factors entail regular administration. Senolytic drugs can be administered to the donor, recipient, or the graft itself though side effects like cytopenia may arise in an individual. A wide range of therapeutics are currently in development which specifically target senescent stem cells and are based on viral vectors and nucleic acids (Lau et al. 2019; Conboy et al. 2005).

4.1 Age-Related Clonal Haematopoiesis

Age-related clonal haematopoiesis (ARCH) is a physiological phenomenon where individuals with no previous haematologic malignancies display expansion of specific hematopoietic stem and progenitor cells' (HSPC) clones carrying

repeated disruptive genetic forms. ARCH increases the chances of therapy-related myeloid neoplasms (t-MN) after autologous HSC transplantation and conditions such as cytopenia or leukaemia after allogeneic HSC transplantation. Autologous HSC transplantation is carried out predominantly to treat lymphoma & multiple myeloma. Before HSCs can be isolated, patients need to go through a series of chemotherapy sessions to induce remission. The pre-existing expanded clones which are already expressing the chemoresistant mutations can become dominant post-chemotherapy, increasing the risks of developing t-MN in cancer patients with ARCH. t-MN advances more rapidly than other myeloid malignancies and have lower chances of survival. TP53 mutations which are commonly observed in t-MN are thought to be the major driving force behind ARCH. It was earlier believed that chemotherapy was the reason behind the detected TP53 mutations but later it was found out that not only the mutations manifested in the clones long before the development of t-MN but also got expressed in the clones which were yet to receive chemotherapy. Still, it is worth noting that although chemotherapy is not inducing the TP53 mutations in the HSPCs, it is providing a conducive niche to the already mutated cells. PPM1D (Protein Phosphatase Mg2+/Mn2+ dependent 1D) gene, which regulates p53 is also found to have been mutated in ARCH but unlike p53 their correlation with chemotherapy is still not consolidated. Besides t-MN, therapy-related Myelodysplastic syndrome (t-MDS) patients also express significant levels of TP53 & PPM1D mutations than patients with de novo MDS. All these findings suggest that if it is possible to analyse patient's blood before transplantation and detect significant mutations, it can pave way for predicting transplantation outcomes like relapse and survival rates in patients and further in chalking out treatment regimens. ARCH is found to be positively correlated with a poor survival rate after transplantation and even has implications for haematopoietic recovery. The range of mutations in genes and instances of two or more coexisting mutations in genes related to ARCH can increase in recipients undergoing autologous HSC transplantation and thus occurrences of secondary myeloid neoplasms can also increase. The accumulated mutation load in the HSCs of the ARCH patients is also responsible for the failure of peripheral mobilization of HSCs, thereby generating a need for harvesting cells from bone marrow. We must keep in mind that unlike cancer patients receiving only radiotherapy or chemotherapy, patients who will be having their HSCs harvested after that have an additional burden of regenerating the haematopoietic environment after such intense myelosuppression. Regenerating the hematopoietic system results in replicative stress and may speed up clonal expansion to malignant transformation. This is well observed in the recipients of allogeneic transplantation who often suffer from donor-derived leukaemia, but no signs of leukaemia are manifested in the donors. The malignant transformation not only depends on the mutations of the clones but also on stressors like cytotoxic therapeutic agents because different stressors may favour different clones with separate distinct mutations (Abelson and Wang 2018; Jaiswal et al. 2014).

Donor cell leukaemia (DCL) is a rare phenomenon observed in recipients after HSC transplantation and is reported to be sourced from engrafted donor stem cells. The donors although healthy, carry HSCs with mutations that confer the capability of malignant transformation. The transformation is accelerated after transplantation because the resident bone marrow is already depleted and is under repopulation pressure. This consequently provides the mutated HSCs with a conducive niche microenvironment where there is low competition for growth. Examples of mutated genes that are precursors of malignant clonal expansion or abnormal cytopenia in the HSC recipients are Myelodysplastic syndrome/Acute myeloid leukaemia (MDS/AML) associated genes like DNMT3A or U2AF1 (U2 small nuclear RNA auxiliary factor 1). Interestingly, donors who carry these mutations do not express clinically evident oncogenesis even long after the occurrence of DCL in the transplanted patients. Besides being provided with a less competitive bone marrow niche, the engrafted HSCs

also outcompete their healthy counterparts in the new environment by DNMT3A mutations that bestow on them the power of enhanced self-renewal, survival, and propagation. These HSCs though have an engraftment advantage, fail to recover the hematopoietic system due to abnormal differentiation (Abelson and Wang 2018; Wiseman 2011).

4.2 Impact of Age on T Cell Recovery after Bone Marrow Transplantation

In allogeneic bone marrow transplantation, the primary causes of death are infections, transplantation related toxicity, and GVHD. Immune reconstitution plays a major role in preventing deaths from opportunistic infections and sustaining the grafts after transplantation. The occurrence of both opportunistic infections and GVHD are positively correlated to recipient age as older patients show higher risks of infections from delayed or slower recovery of CD4+ T cells and this delayed immune reaction can be associated with infections. T cell generation from CD34+ HSCs located in bone marrow are also found to have decreased with age when ASCs are compared to ESCs. Another experiment on mice where thymic lobe reconstitution by bone marrow cells was observed, it showed that reconstitution as well as differentiation into various subsets of T cell were similar between mice of different age groups, but reconstitution capacity of older mouse appeared subpar when compared to its younger counterpart. Production of lymphocytes in general gets decreased with age (Azuma et al. 2002; Small et al. 1999). In mouse, CD4+ T cells along with their subpopulation 'naive cells' which exhibit elevated expression of CD45RB surface markers, are found to be generated in lower quantities in more aged specimens. Even the production of cytokines like IL-2 which depends on naive cells, is reduced in the older mice. In bone marrow transplantation procedures where T cells are depleted, older patients suffer from CD4+ T-lymphocytopenia and opportunistic infections occur only in those patients where CD4+ T cell counts are exceptionally low. More importantly, patients with younger donors have lymphocyte responses higher than recipients with older donors. Transplantation in mouse shows that lymphocyte recovery along with CD4+ T cell and CD4+ T naive cell recovery is more pronounced in recipients of HSCs from younger donors. As host defence is dependent on CD4+ T naive cells, faster recovery can reduce the rates of mortality after transplantation. Younger donors also ensure more pronounced recovery of lymphocyte functions like higher proliferative response to mitogens such as concanavalin A (ConA) and pokeweed mitogen (PWM). After transplantation, T cells can generate de novo and is dependent on thymic function which in turn gets affected by age.

Although the reasons behind age-related deterioration of lymphopoiesis and the subsequent immune deficiencies are yet to be elucidated it can be hypothesized that differentiation and lymphocyte generation from T cell progenitors may vary between different age groups (Azuma et al. 2002; Small et al. 1999).

4.3 Age-Related Challenges of Regenerative Therapy by Bone Marrow Derived Mesenchymal Stromal Cells

Bone marrow derived mesenchymal stromal cells (BM-MSC) harvested from old donors exhibit a reduced proliferation capacity *in vitro* and certain reports even point out differences in proliferation capacity between male and female donors. Before transplanting *in vivo*, MSCs need to be cultured and expanded *in vitro*. The cells in culture go through series of replication which lead to shortening of telomeres and developing senescent phenotypes with reduced proliferation. Reduced proliferation is seen to be directly correlated with cell amplification & clonogenic capacity of the BM-MSCs which in turn affect their multipotency. Studies on BM-MSCs revealed that with an increase in donor age, the number of Colony-forming unit fibroblast (CFU-F)

decreases remarkably. Stem cells with surface markers like CD146 are correlated with better proliferative capacity & multipotency. CD146+ BM-MSCs are found more in number when harvested from young donors and this population gets reduced in aged donors (Charif et al. 2017; Stolzing et al. 2008).

Scientists are divided in their opinions about the relationship between donor age and differentiation capability. Some of them strictly believe that there is absolutely no significant relationship whatsoever between donor age or sex and BM-MSCs' osteogenic, adipogenic & chondrogenic differentiation whereas some emphasize on alterations of differentiation phenotypes instead of differentiation capacity. Asserting the opposite, a lot of researchers have found out significant associations between stem cell aging and differentiation capacity but is yet to arrive at a unanimous decision. Although it is commonly believed that osteogenic potential decreases with aged stem cells, the same cannot be said with firmness about adipogenic & chondrogenic potential. These contradictory results stem from the fact that the sources of stem cells were from separate passages which indicate that the experiments may be non-uniform. Aged BM-MSCs from old donors are found to have elevated levels of ROS, NO (Nitric oxide), cell cycle inhibitors like p21, p53, all of which are associated with age-related degeneration, oxidative stress & senescence. Senescence is further elevated by replicative pressure from multiple passages *in vitro*. Even when exposed to oxidizing stress from external sources (H_2O_2/ROS), MSCs from younger donors show more resistance to shortening of telomere length and better proliferative function than MSCs from old donors. Overexpression of ROS inhibitors such as APE1 (Apurinic/apyrimidinic endonuclease 1)/REF-1 (Redox effector factor 1), can mitigate the effects of senescence and facilitate maintenance of MSCs' characteristics for regenerative medicine. Significant differences in telomere length are also evident in MSCs *in vitro* between young and old donors which can be associated with differences in proliferation capacities for regeneration. As telomere shortening is inevitable *in vivo*, we can try to over express non-endogenous telomerase to override the senescent phenotypes and maintain essential and effective proliferation as well as multipotency for regeneration (Charif et al. 2017; Stolzing et al. 2008).

4.4 Aging and Wound Repair by Mesenchymal Stem Cells

Stem cell therapy via systemic or local administration has been an effective tool in healing chronic wounds and even reverses the repercussions of scars or fibrosis. Marked differences are observed *in vivo* when cells from different age groups are transplanted for healing chronic wounds. Most of them are connected to age-related footprints such as proliferation & differentiation potential, expression of senescence-associated molecular markers, telomerase activity and even cell morphology. In general, regenerative capability of MSCs deteriorates with age or multiple subcultures limiting their clinical application in repairing chronic wounds. With prolonged culture, the proliferative ability and the resultant growth rate of MSCs declines, and chances of survival get bleak.

The number of 'young' spindle shaped cells from MSCs sourced from younger donors are more than in aged donors and those cells get gradually lost with increased number of passages. As mentioned previously, there are contradictory reports on alterations of osteogenic, adipogenic, or chondrogenic induction of MSCs with respect to donor age. With increased subcultures the MSCs also fare poorly in their homing ability after transplantation. For quality assurance, aged MSCs can be segregated from their younger counterparts based on senescent markers like senescence-associated β-galactosidase (SA-βgal) activity, senescence-associated heterochromatin foci (SAHFs), senescence-associated DNA damage foci (SDFs). Bone regeneration and homeostasis after transplantation are maintained through actin cytoskeleton dynamics which plays a major role in building and sustaining the three-dimensional matrix. In aging MSCs, this actin cytoskeleton metabolism gets disrupted by a low actin turnover with the consequent upregulation

of transgelin which cross-links actin. This results in low responses of actin to growth factors and other crucial molecules of signalling pathways that are involved in regeneration. In early passage MSCs, the arrangement of mitochondria and their oxygen consumption are markedly different from late passage MSCs which could be indicative of different competence of stem cell differentiation. The more resistant the mitochondria will be against the inevitable ROS damage the better the MSCs will be able to maintain their persistent self-renewal capability. Unfortunately, the antioxidant potential gets reduced with age resulting in accumulated molecular injuries and strained metabolic activities and subsequent dysfunction (Yao et al. 2015; Nuschke 2013).

Whole genome sequencing of *ex vivo* MSC cultures are used to identify the genetic variances between early and late cell passages. In late passages, MSCs exhibit a considerable number of single nucleotide changes (SNC). This can indicate that after transplantation and expansion of MSCs, genetic constitution can get unstable and affect regeneration. In autologous transplantation of diseased patients, the diseased microenvironment can also negatively affect MSCs and impair wound healing. For example, in diabetic patients, the high level of glucose and glycation end products induce apoptosis in MSCs long before differentiation by upregulating pro-apoptotic molecules like BAX (Bcl-2-associated X protein) or FAS and inhibiting anti-apoptotic molecules like BCL-2 (B-cell lymphoma 2). Even circulating cytokines near the wounded regions such as IL-2, IL-7 can lead to apoptosis of transplanted MSCs. Transplanted aged MSCs are often compromised in supporting angiogenesis and sustaining an effective vascular network. They also demonstrate a curtailed immunomodulatory activity resulting in a lowered anti-inflammatory and shielding effect against the wound pathophysiology. Lowering the oxygen concentration from the conventional 21% to 6% in cultures ensures less oxidative stress and has been observed to counteract the effects of aging in MSCs. Culturing MSCs in low temperature utilizes lesser oxygen than normal which in turn reduces the ROS levels, ROS induced damage & apoptosis and stress-induced senescence. Temperature regulation also maintains self-renewal of MSCs by suppressing differentiation via upregulation of p21 & p53 which at controlled lowered temperature can delay cell cycle progression. hTERT vector can be transfected into the cultured MSCs and hTERT can act as a transcriptional modulator and maintain ROS level & oxidative stress. hTERT also helps in maintaining genome stability by intercepting dysregulation of genes that affect ploidy. Transcription factors like NANOG which are associated with maintaining pluripotency can also be transfected in MSCs to reverse the aging-associated deterioration in differentiation potential. Often, MSCs lose their differentiation potential due to culture stress or culture conditions such as the batch of foetal bovine serum (FBS) used. Such undesirable outcomes can be avoided by adding growth factors like FGF2 (Fibroblast growth factor 2) which can aid MSCs in maintaining their differentiation potential. However, addition of FGF2 can lead to alterations in the inherent properties of MSCs. Growth factors can also express therapeutic effects directly on wounds. Addition of natural culture environment (Wharton's jelly extracted from the umbilical cord (UC-MSC)) has been proved to be more effective in maintaining the innate properties of MSCs and significantly suppressing senescence (Yao et al. 2015).

4.5 Age-Related Challenges of Regenerative Therapy by Adipose-Derived Stem Cells

In general, ADSCs are found to be in high density per volume of adipose tissue but the concentration gets altered in diseased conditions like diabetes. Diseased condition acts like a prelude to subsequent aging characteristics like oxidative stress, presence of pro-inflammatory cytokines, and ultimately senescence. Whether yield and expansion of ADSCs are found to be affected by donor's age is debated as conflicting results have been obtained to date. Some reported that the

yield is getting affected by donor age while others showed that the CFUs and the proliferation are getting affected. When compared to BM-MSCs, ADSCs showed higher resistance to senescence with lower expression of biomarkers like senescence-associated β-galactosidase activity. Oncogenic transformation can be associated with aged ADSCs but till now no results have corroborated with that hypothesis. The extent of genetic anomalies due to prolonged culturing is studied thoroughly and the genome is revealed to be more or less stable after multiple passages. Predominantly negligible rates of aneuploidy are observed in the forms of tetrasomy, tetraploidy or trisomy and no gain in proliferative advantage and consequent oncogenic transformation is reported. Oncologic safety is further confirmed *in vivo* in both animal models and clinical trials. For transplantation, selecting cells from early passages as well as cells which have already commenced differentiation can prevent adopting tumoral clones (Dufrane 2017; Kornicka et al. 2015).

The purity of ADSCs is a critical criterion regarding safety as it is frequently observed that non-specific isolation methods result in a heterogeneous cell population where cells that proliferate more rapidly and outgrow others become prevalent after multiple passages. These cells can negatively affect genetic stability in the long run. While some scientists reported that expression of osteogenic proteins such as osteocalcin & RUNX2 (Runt-related transcription factor 2) or alkaline phosphatase is similar between donors of all ages, others found out a weaker osteogenic capability *in vitro* from old ADSCs. To achieve a successful *in vivo* transplantation and bone reconstruction, old ADSCs can be induced to differentiate before transplantation, or the deficiency in growth factor release can be toned down. The tissue-engineered 3D scaffold-free grafts are found not to be affected by donor age in terms of duration of construction, assessed quality of the construct by 3D characterization and tissue remodelling. But the required quantity of adipose tissue for constructing the implant is found to be more for older donors (Dufrane 2017; Kornicka et al. 2015).

4.6 Stem Cell Aging and Derivation of Induced Pluripotent Stem Cells

iPSCs effectively avoid the restrictions and pitfalls of therapeutics generated from ESCs & ASCs such as ethico-legal issues, limitations in the availability of stem cell donors, immune rejection, etc. As evident from the methodology itself, the source or origin of the cells is of chief importance followed by the donor age. Human population is extremely variable in its genetic makeup and this aspect can also affect reprogramming efficiency. Donor age is a key issue indeed as patients who most urgently need regenerative medicine belong to older age groups. First and foremost, one needs to evaluate interrelation between donor cell age and the iPSCs' reprogramming capability & functionality, preservation of epigenetic patterns, tumorigenicity, differentiation potential & phenotypes in the recipients. One must even acknowledge the possibility of reprogramming senescent cells. In previous cloning experiments we have seen that old cells after successful administration suffered from untimely senescence in cloned organisms which can limit their applications for clinical therapeutics. Cells from old donors show poor reprogramming efficiency and tend to retain age-associated DNA methylation patterns, however reprogramming to pluripotency is attainable by targeting mediators & effectors of senescence. For example, effectively expressing pluripotency-associated factors like NANOG or knocking down senescence-associated factors like p21 improves reprogramming efficiency. Even maintaining optimal ROS levels & DNA damage response and optimizing culture conditions improve the quality of aged cells for multiple passages of culture. Cells also vary according to their telomere lengths which are dependent on donor age and it is commonly observed that several iPSC lines have considerably varied telomere lengths. But as of now, telomerase is essentially reactivated in iPSCs to overcome the drawbacks of telomere shortening in aged cells. It has been found out that mitochondria of old cells are more

mutated than their younger counterparts and thus can be a possible target of intervention in order to avoid premature senescence. Epigenetic modifications can be tissue-specific which can hinder differentiation and thereby the generation of the required tissues. However, optimising culture conditions and increasing the number of subcultures of iPSCs can revert the senescence-associated methylation patterns which gradually become comparable to ESCs. The level of DNA mutations and chromosomal aberrations are naturally heightened in older cells and mutations can even form during reprogramming itself. While some scientists believe that the problems can be solved by repeated passaging as iPSCs without these genetic anomalies exhibit a growth advantage, others are of the opinion that repeated passaging will increase the chances of genetic anomalies instead which may lead to oncogenic transformation. This suggests a critical quality check of iPSCs before their clinical applications. Retaining all crucial parameters optimized, the functional potential (as measured by their unlimited proliferation and the ability to regenerate all types of cells) of iPSCs from donors of all age groups, are similar (Strässler et al. 2018; Lo Sardo et al. 2016).

5 Conclusion

Stem cell aging is influenced by the interdependence of so many contrasting cell-intrinsic and cell-extrinsic factors and their crosstalk, that the idea of placing definite weight on any one factor is unfathomable. However, there are specific signals that do reveal themselves more than the others and are implicated to be the most crucial regulators of stem cell aging. Accessibility to an extensive array of stem cells is currently providing a large number of options for clinicians. A universal stem cell-like master key is improbable and hence every disease will require distinctive stem cell type(s). First and foremost, it is of utmost necessity that any stem cell therapy should be deemed harmless. Although unraveling the molecular mechanisms of aging has exposed the constraints of old stem cells in regeneration, data correlating aging of stem cells and their long-term efficacy is conflicting and has not yet been consolidated.

References

Abelson S, Wang J (2018) Age-related clonal hematopoiesis. Curr Opin Hematol 25(6):441–445

Adams P, Jasper H, Rudolph K (2015) Aging-induced stem cell mutations as drivers for disease and cancer. Cell Stem Cell 16(6):601–612

Adiwinata PJ (2019) Exploring the Most promising stem cell therapy in liver failure: a systematic review. Stem Cells Int 2019:1–15

Al zouabi L, Bardin A (2020) Stem cell DNA damage and genome mutation in the context of aging and cancer initiation. Cold Spring Harb Perspect Biol:a036210. https://doi.org/10.1101/cshperspect.a036210

Azuma E, Hirayama M, Yamamoto H, Komada Y (2002) The role of donor age in naive T-cell recovery following allogeneic hematopoietic stem cell transplantation: the younger the better. Leuk Lymphoma 43 (4):735–739

Baharvand H, Jafary H, Massumi M, Ashtiani S (2006) Generation of insulin-secreting cells from human embryonic stem cells. Dev Growth Differ 48 (5):323–332

Bajada S, Mazakova I, Richardson JB, Ashammakhi N (2008) Updates on stem cells and their applications in regenerative medicine. J Tissue Eng Regen Med 2 (4):169–183

Beerman I, Rossi D (2015) Epigenetic control of stem cell potential during homeostasis, aging, and disease. Cell Stem Cell 16(6):613–625

Behrens A, van Deursen J, Rudolph K, Schumacher B (2014) Impact of genomic damage and ageing on stem cell function. Nat Cell Biol 16(3):201–207

Beltrami A, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S et al (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114(6):763–776

Bigarella C, Liang R, Ghaffari S (2014) Stem cells and the impact of ROS signaling. Development 141 (22):4206–4218

Blasco M (2007) Telomere length, stem cells and aging. Nat Chem Biol 3(10):640–649

Boheler KR, Czyz J, Tweedie D, Yang H-T, Anisimov SV, Wobus AM (2002) Differentiation of pluripotent embryonic stem cells into cardiomyocytes. Circ Res 91 (3):189–201

Bonner-Weir S, Toschi E, Inada A, Reitz P, Fonseca S, Aye T et al (2004) The pancreatic ductal epithelium serves as a potential pool of progenitor cells. Pediatr Diabetes 5(s2):16–22

Brown J, Weissman I (2004) Progress and prospects in hematopoietic stem cell expansion and transplantation. Exp Hematol 32(8):693–695

Brunet A, Rando T (2017) Interaction between epigenetic and metabolism in aging stem cells. Curr Opin Cell Biol 45:1–7

Charif N, Li Y, Targa L, Zhang L, Ye J, Li Y et al (2017) Aging of bone marrow mesenchymal stromal/stem cells: implications on autologous regenerative medicine. Biomed Mater Eng 28(s1):S57–S63

Choudhary B (2012) Telomere and telomerase in stem cells relevance in ageing and disease. Front Biosci S4 (1):16–30

Conboy I, Conboy M, Wagers A, Girma E, Weissman I, Rando T (2005) Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 433(7027):760–764

Doğan A (2018) Embryonic stem cells in development and regenerative medicine. Adv Exp Med Biol 1079:1–15

Dufrane D (2017) Impact of age on human adipose stem cells for bone tissue engineering. Cell Transplant 26 (9):1496–1504

García-Prat L, Sousa-Victor P, Muñoz-Cánoves P (2017) Proteostatic and metabolic control of stemness. Cell Stem Cell 20(5):593–608

Herrera M, Bruno S, Buttiglieri S, Tetta C, Gatti S, Deregibus M et al (2006) Isolation and characterization of a stem cell population from adult human liver. Stem Cells 24(12):2840–2850

Horwitz E, Prockop D, Fitzpatrick L, Koo W, Gordon P, Neel M et al (1999) Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 5 (3):309–313

Insinga A, Cicalese A, Pelicci P (2014) DNA damage response in adult stem cells. Blood Cell Mol Dis 52 (4):147–151

Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman P, Mar B et al (2014) Age-related clonal hematopoiesis associated with adverse outcomes. N Engl J Med 371(26):2488–2498

Ju Z, Rudolph L (2008) Telomere dysfunction and stem cell ageing. Biochimie 90(1):24–32

Kenyon J, Gerson S (2007) The role of DNA damage repair in aging of adult stem cells. Nucleic Acids Res 35(22):7557–7565

Khan F, Almohazey D, Alomari M, Almofty S (2018) Isolation, culture, and functional characterization of human embryonic stem cells: current trends and challenges. Stem Cells Int 2018:1–8

Kind A, Colman A (1999) Therapeutic cloning: needs and prospects. Semin Cell Dev Biol 10(3):279–286

Kobayashi C, Suda T (2011) Regulation of reactive oxygen species in stem cells and cancer stem cells. J Cell Physiol 227(2):421–430

Koenig S, Krause P, Drabent B, Schaeffner I, Christ B, Schwartz P et al (2006) The expression of mesenchymal, neural and haematopoietic stem cell markers in adult hepatocytes proliferating *in vitro*. J Hepatol 44 (6):1115–1124

Kornicka K, Marycz K, Tomaszewski K, Marędziak M, Śmieszek A (2015) The effect of age on osteogenic and adipogenic differentiation potential of Human Adipose Derived Stromal Stem Cells (hASCs) and the impact of stress factors in the course of the differentiation process. Oxidative Med Cell Longev 2015:1–20

Krampera M, Pizzolo G, Aprili G, Franchini M (2006) Mesenchymal stem cells for bone, cartilage, tendon and skeletal muscle repair. Bone 39(4):678–683

Krauss S, de Haan G (2016) Epigenetic perturbations in aging stem cells. Mamm Genome 27(7–8):396–406

Kuo C, Li W, Mauck R, Tuan R (2006) Cartilage tissue engineering: its potential and uses. Curr Opin Rheumatol 18(1):64–73

Lau A, Kennedy B, Kirkland J, Tullius S (2019) Mixing old and young: enhancing rejuvenation and accelerating aging. J Clin Investig 129(1):4–11

Lee H, Gutierrez-Garcia R, Vilchez D (2016) Embryonic stem cells: a novel paradigm to study proteostasis? FEBS J 284(3):391–398

Liang R, Ghaffari S (2014) Stem cells, redox signaling, and stem cell aging. Antioxid Redox Signal 20 (12):1902–1916

Lindvall O, Kokaia Z, Martinez-Serrano A (2004) Stem cell therapy for human neurodegenerative disorders–how to make it work. Nat Med 10(S7):S42–S50

Liu L, Rando T (2011) Manifestations and mechanisms of stem cell aging. J Cell Biol 193(2):257–266

Lo Sardo V, Ferguson W, Erikson G, Topol E, Baldwin K, Torkamani A (2016) Influence of donor age on induced pluripotent stem cells. Nat Biotechnol 35(1):69–74

Martino G, Pluchino S (2006) The therapeutic potential of neural stem cells. Nat Rev Neurosci 7(5):395–406

McArdle A, Senarath-Yapa K, Walmsley G, Hu M, Atashroo D, Tevlin R et al (2014) The role of stem cells in aesthetic surgery. Plast Reconstr Surg 134 (2):193–200

McNeely T, Leone M, Yanai H, Beerman I (2019) DNA damage in aging, the stem cell perspective. Hum Genet 139(3):309–331

Miller MQ, Dighe A, Cui Q, Park SS, Christophel JJ (2016) Regenerative medicine in facial plastic and reconstructive surgery. JAMA Facial Plast Surg 18 (5):391

Min-Wen J, Jun-Hao E, Shyh-Chang N (2016) Stem cell mitochondria during aging. Semin Cell Dev Biol 52:110–118

Müller A, Huppertz S, Henschler R (2016) Hematopoietic stem cells in regenerative medicine: astray or on the path? Transfus Med Hemother 43(4):247–254

Müller P, Lemcke H, David R (2018) Stem cell therapy in heart diseases – cell types, mechanisms and improvement strategies. Cell Physiol Biochem 48 (6):2607–2655

Nakao K, Tsuji T (2008) Dental regenerative therapy: stem cell transplantation and bioengineered tooth replacement. Jpn Dent Sci Rev 44(1):70–75

Noormohammadi A, Calculli G, Gutierrez-Garcia R, Khodakarami A, Koyuncu S, Vilchez D (2017) Mechanisms of protein homeostasis (proteostasis) maintain stem cell identity in mammalian pluripotent stem cells. Cell Mol Life Sci 75(2):275–290

Nowacki M, Kloskowski T, Pokrywczyńska M, Nazarewski Ł, Jundziłł A, Pietkun K et al (2014) Is

regenerative medicine a new hope for kidney replacement? J Artif Organs 17(2):123–134
Nuschke A (2013) Activity of mesenchymal stem cells in therapies for chronic skin wound healing. Organogenesis 10(1):29–37
Nussler A, Konig S, Ott M, Sokal E, Christ B, Thasler W et al (2006) Present status and perspectives of cell-based therapies for liver diseases. J Hepatol 45 (1):144–159
Qu-Petersen Z, Deasy B, Jankowski R, Ikezawa M, Cummins J, Pruchnic R et al (2002) Identification of a novel population of muscle stem cells in mice. J Cell Biol 157(5):851–864
Ren R, Ocampo A, Liu G-H, Izpisua Belmonte JC (2017) Regulation of stem cell aging by metabolism and epigenetics. Cell Metab 26(3):460–474
Riazi AM, Kwon SY, Stanford WL (2009) Stem cell sources for regenerative medicine. Methods Mol Biol 482:55–90
Sampogna G, Guraya S, Forgione A (2015) Regenerative medicine: historical roots and potential strategies in modern medicine. J Microsc Ultrastruct 3(3):101–107
Shafritz D, Oertel M, Menthena A, Nierhoff D, Dabeva M (2006) Liver stem cells and prospects for liver reconstitution by transplanted cells. Hepatology 43(S1): S89–S98
Sharpless N, DePinho R (2007) How stem cells age and why this makes us grow old. Nat Rev Mol Cell Biol 8 (9):703–713
Siepe M, Heilmann C, von Samson P, Menasché P, Beyersdorf F (2005) Stem cell research and cell transplantation for myocardial regeneration. Eur J Cardiothorac Surg 28(2):318–324
Singla D, Hacker T, Ma L, Douglas P, Sullivan R, Lyons G et al (2006) Transplantation of embryonic stem cells into the infarcted mouse heart: formation of multiple cell types. J Mol Cell Cardiol 40(1):195–200
Small T, Papadopoulos E, Boulad F, Black P, Castro-Malaspina H, Childs B et al (1999) Comparison of immune reconstitution after unrelated and related T-cell–depleted bone marrow transplantation: effect of patient age and donor leukocyte infusions. Blood 93(2):467–480
Smolar J, Horst M, Sulser T, Eberli D (2018) Bladder regeneration through stem cell therapy. Expert Opin Biol Ther 18(5):525–544
Song Z, Ju Z, Rudolph K (2009) Cell intrinsic and extrinsic mechanisms of stem cell aging depend on telomere status. Exp Gerontol 44(1–2):75–82
Stocum D, Zupanc G (2008) Stretching the limits: stem cells in regeneration science. Dev Dyn 237 (12):3648–3671
Stolzing A, Jones E, McGonagle D, Scutt A (2008) Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. Mech Ageing Dev 129(3):163–173
Strässler E, Aalto-Setälä K, Kiamehr M, Landmesser U, Kränkel N (2018) Age is relative—impact of donor age on induced pluripotent stem cell-derived cell functionality. Front Cardiovasc Med 5:4
Sun N, Youle R, Finkel T (2016) The mitochondrial basis of aging. Mol Cell 61(5):654–666
Takahashi J (2018) Stem cells and regenerative medicine for neural repair. Curr Opin Biotechnol 52:102–108
Takata N, Eiraku M (2017) Stem cells and genome editing: approaches to tissue regeneration and regenerative medicine. J Hum Genet 63(2):165–178
Trohatou O, Roubelakis M (2017) Mesenchymal stem/stromal cells in regenerative medicine: past, present, and future. Cell Reprogram 19(4):217–224
Tümpel S, Rudolph K (2012) The role of telomere shortening in somatic stem cells and tissue aging: lessons from telomerase model systems. Ann N Y Acad Sci 1266(1):28–39
Urbanek K, Torella D, Sheikh F, De Angelis A, Nurzynska D, Silvestri F et al (2005) Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci 102 (24):8692–8697
Vazin T, Freed W (2010) Human embryonic stem cells: derivation, culture, and differentiation: a review. Restor Neurol Neurosci 28(4):589–603
Vesely I (2005) Heart valve tissue engineering. Circ Res 97(8):743–755
Vilchez D, Simic M, Dillin A (2014) Proteostasis and aging of stem cells. Trends Cell Biol 24(3):161–170
Weiss D (2014) Concise review: current status of stem cells and regenerative medicine in lung biology and diseases. Stem Cells 32(1):16–25
Wiseman D (2011) Donor cell leukemia: a review. Biol Blood Marrow Transplant 17(6):771–789
Xiao L, Nasu M (2014) From regenerative dentistry to regenerative medicine: progress, challenges, and potential applications of oral stem cells. Stem Cells Cloning Adv Appl 2014:89
Yao B, Huang S, Gao D, Xie J, Liu N, Fu X (2015) Age-associated changes in regenerative capabilities of mesenchymal stem cell: impact on chronic wounds repair. Int Wound J 13(6):1252–1259
Yin H, Price F, Rudnicki M (2013) Satellite cells and the muscle stem cell niche. Physiol Rev 93(1):23–67
Yu Y (2018) Application of stem cell technology in antiaging and aging-related diseases. Adv Exp Med Biol 1086:255–265
Yu A, Dang W (2017) Regulation of stem cell aging by SIRT1 – linking metabolic signaling to epigenetic modifications. Mol Cell Endocrinol 455:75–82
Zhang J, Ju Z (2010) Telomere, DNA damage, and oxidative stress in stem cell aging. Birth Defects Res C Embryo Today 90(4):297–307
Zhang H, Menzies KJ, Auwerx J (2018) The role of mitochondria in stem cell fate and aging. Development 145(8):dev143420
Zulewski H, Abraham E, Gerlach M, Daniel P, Moritz W, Muller B et al (2001) Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate *Ex Vivo* into pancreatic endocrine, exocrine, and hepatic phenotypes. Diabetes 50(3):521–533

Adv Exp Med Biol - Cell Biology and Translational Medicine (2021) 12: 39–46
https://doi.org/10.1007/5584_2020_606

Published online: 17 December 2020

Potential of Chimeric Antigen Receptor T-Cells in Cancer Therapy

Drashti Desai, R. S. Gaud, and Pravin Shende

Abstract

Novel approaches for targeted delivery like nanoparticles, liposomes, polymer conjugates, etc. with better safety profile needs to be developed for cancer treatment. Chimeric antigen receptors (CAR) with modified thymus cells (T-cells) showed greater potential as a therapy due to its direct effect on immune system responsible for destruction of pathogens and said equivalent to the living drug. On activation of T-cell, it binds to the antigen domains treating refractory or relapsed cancers. The receptors are termed chimeric as it consists of T-cells functioning as well as antigen-binding combined in sole receptor. This therapy showed positive success towards hematological cancers and engineered for specific protein targeting. Though the therapy is associated to several challenges like incompetence towards tumor lysis and cytokine release rate, termination of cytotoxic activity after completion of tumor eradication, etc. The control mechanisms used by CAR T-cells are apoptosis by suicide genes, dual-antigen receptor, ON-switch tumor attack and bispecific molecules as activation switch. In solid tumors, CAR T-cell therapy showed promising signs of efficacy becoming a game-changing cell therapy. CAR T-cells are optimized using different engineering resolutions and lead to broadways for therapy adoption to benefit the cancer patients.

Keywords

Antigen · Domain · Persistence · Receptor · Tumor

Abbreviations

ABD	Antigen-binding domain
ALL	Acute lymphoblastic leukemia
CAR	Chimeric antigen receptors
GVHD	Graft-versus-host disease
ISD	Intracellular signaling domain
TAAs	Tumor-associated antigens
T-cells	Thymus cells
TD	Transmembrane domain

1 Introduction

Cancer is mainly treated with chemo- and radiation- therapies or combinational chemistry for target therapy but it causes cytotoxic effects and the rate of mortality depends on patient-related and technique-related factors like stages of cancer, patient age, patient history, cell engraftment,

D. Desai, R. S. Gaud, and P. Shende (✉)
Shobhaben Pratapbhai Patel School of Pharmacy and Technology Management, SVKM'S NMIMS, Mumbai, India
e-mail: shendepravin94@gmail.com

thymus cell (T-cell) cultivation, etc. To improve such treatment choices, various advance methods are developed like small molecular inhibitors, nanoparticulate-based therapies; laser treatment, monoclonal antibodies, etc. Among these, the first validated T-cell therapy in humans was used as a molecular remission induction technique in donor lymphocyte by infusion for treating myeloid malignancies. Later, it was clinically tested for Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma (Almåsbak et al. 2016; Mandpe et al. 2020; Patil et al. 2020; Shende et al. 2018; Deol and Lum 2010). Normal immune system cells, T-cells, use to fight against infections by killing cancerous cells or by directly attacking on viruses, bacteria and carcinoma using killer T-cells whereas helper T-cells act indirectly by organizing and arranging cancerous cells (Shende and Basarkar 2019). Some cancers evade the immune system by evasion of cytokines, immune suppressive mediators, Tregs, etc. Hence to protect the immunity of an individual, Chimeric Antigen Receptor (CAR) T-cells are prepared by genetic modification from patient's T-cells in the laboratory (Fig. 1). CAR T-cell mainly comprises of an antigen-binding domain (ABD), a transmembrane domain (TD), a hinge and an intracellular signaling domain (ISD) (Rafiq et al. 2020). The target antigen is recognized and redirected to lymphocytes by the CAR extracellular portion known as ABD. It is composed of monoclonal antibodies of variable light and heavy chains coupled with a flexible linker like (Gly4Ser)3 for the development of desirable single chain fragment. This enables the activation of major histocompatibility complex-independent T-cell to target the antigen of cancer cells expressed by the proteins on cell surface and recognize the intracellular tumor-associated antigens (TAAs) (Fig. 2) (Dwivedi et al. 2019; Rafiq et al. 2017; Zhang et al. 2014).

The extracellular antigen-binding domain is connected by the CARS hinge and TD to the ISD by binding moiety, compromising of heavy and light variable fragments joined to a flexible linker. To overcome the steric hindrance, hinge provides a suitable length to reach the antigen for the influence of CAR T-cell cytokine production and for programmed cell death. The CAR hinge domains use sequence of amino acid from IgG1, IgG4, CD8 or CD28 whereas TD is a derivative of type I proteins like CD3ζ, CD8α or CD4 to anchor CAR into the T-cell and affect the stability and CAR functioning (Alabanza et al. 2017; Guedan et al. 2018). An ISD comprises of one or more co-stimulatory domain for immunoreceptor, derived from CD3ζ using tyrosine-based motifs for secretion of cytokines, boosting proliferation and reduction of engrafted T-cells resistance towards immunosuppression. Food & Drug Administration approved CD28 or 4-1BB (CD137)-based stimulatory domains for higher response rates in the patients suffering from cancer.

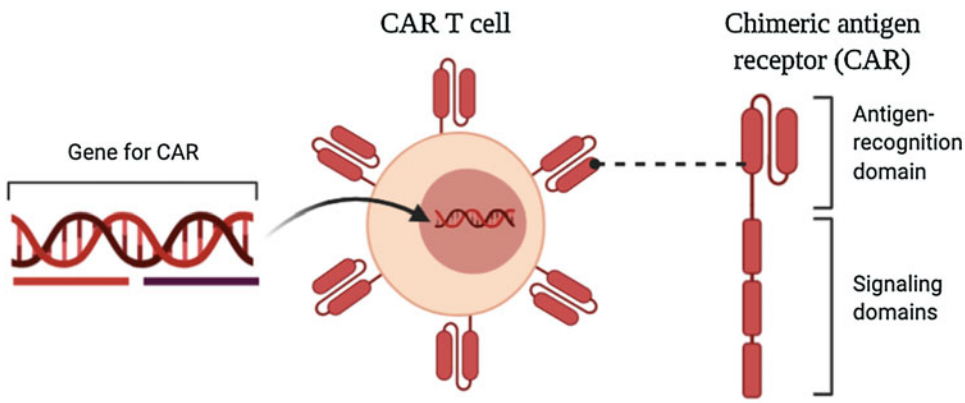


Fig. 1 Structural presentation of CAR

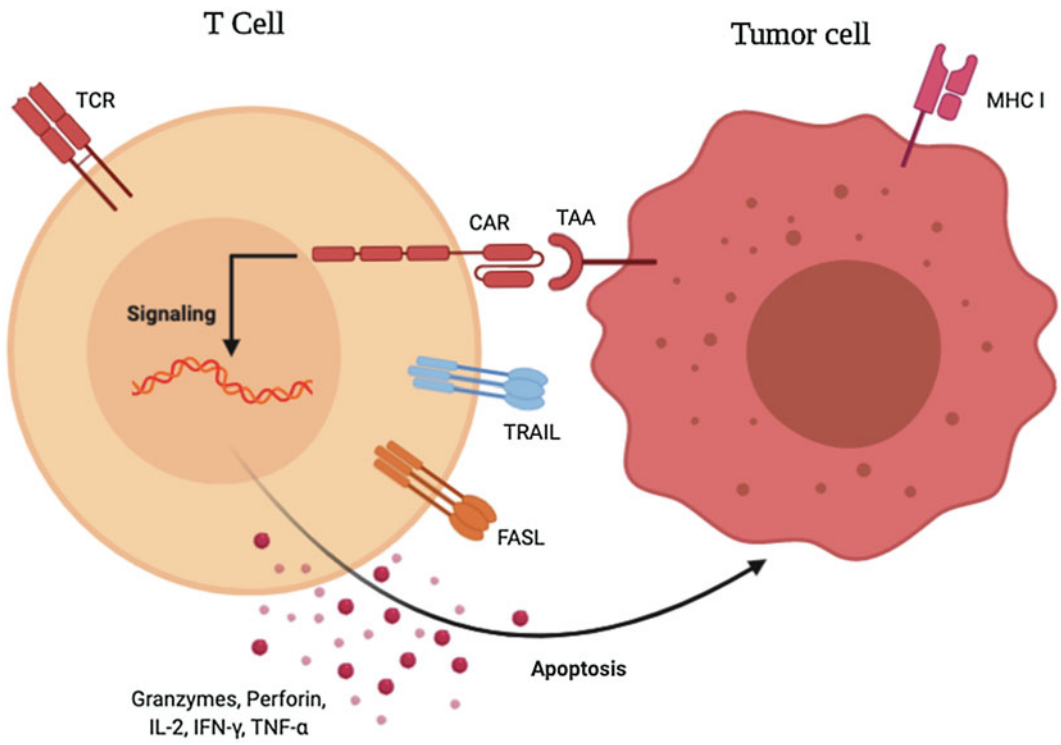


Fig. 2 CAR T-cell interaction with tumor cell

2 CAR T-Cell Derived for Cancer Treatment

In Israel (1989) at Weizmann Institute of Science, Eshhar's group designed first chimeric receptor by modulating extracellular single chain for ovarian cancer using *in vitro* and *in vivo* models. Genetically modified T-cells were developed either via viral-based gene transfer or non-viral methods like DNA-based transposons, CRISPR/Cas9 technology or direct transfer by electroporation of *in-vitro* transcribed-mRNA (Miliotou and Papadopoulou 2018; Gross et al. 1989). The receptor modified T-cells were grown *in-vitro* and administered to the patient to selectively act against oncogenic cells for treating pediatric and adult acute lymphoblastic leukemia (ALL) and large B-cell Non-Hodgkin's lymphoma (Zhang et al. 2017). The elimination through CAR-mediated tumor to execute cytolysis and death receptor signaling uses two pathways redirected by CD4+ and CD8+ T-cells (Chmielewski et al. 2013). The patient may suffer from some side effects like shortness of breath, fever, seizures, joint and muscle aches, low blood pressure, etc. It also causes on-target on-tumor toxicity like release of tumor cell components into normal bloodstream and on-target off-tumor toxicity like inflammation reaction, cognate antigen on healthy tissues, etc. (Abken 2017; Brudno et al. 2016).

There are 3 generations of CARs:

1. **First-generation:** offers fusion proteins containing an extracellular antigen-binding domain (CD3ζ chain) connected to an intracellular signaling domain (T-cell receptor).
2. **Second-generation:** Consists of modified co-stimulatory domain fused to CD3ζ like 4-1BB to enhance the activity and stability.
3. **Third-generation:** Contains various costimulatory domains like CD3ζ, CD27, CD28, OX-40, 4-1BB.
4. **Fourth-generation:** Refers as T-cell redirected for universal cytokine killing with

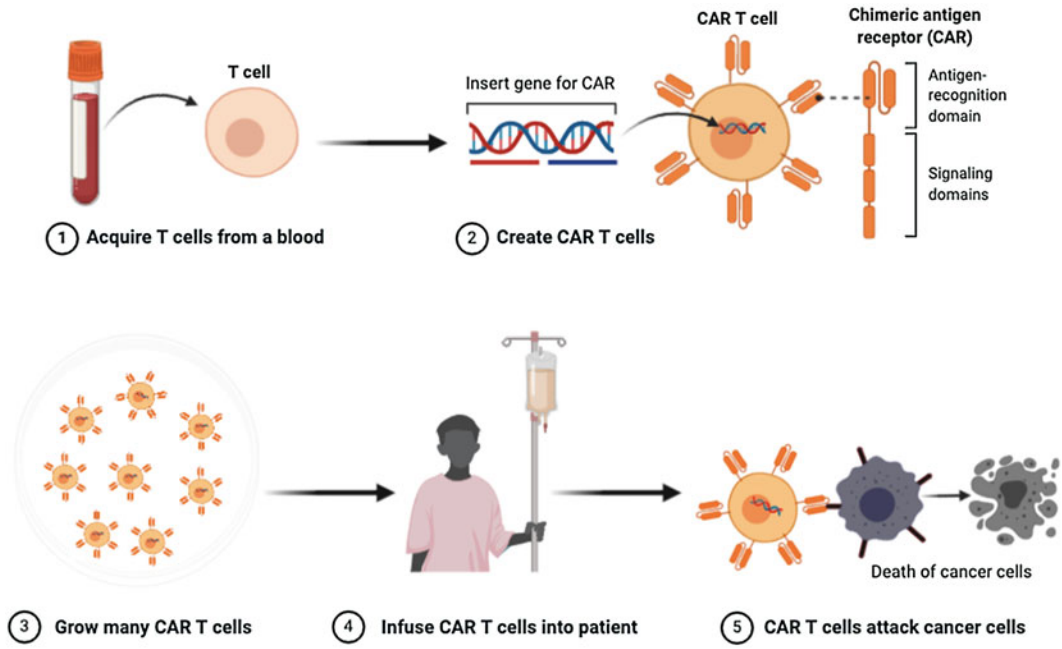


Fig. 3 Schematic presentation of CAR T-cell preparation and action

an ability to induce promotor by utilizing nuclear factor for activated T-cell in relation to IL-12 (Smith et al. 2016).

CAR T-cells can be allogenic i.e. originated from a healthy donor persistent for weeks to month or autologous i.e. originated from any patient persistent for months to year and infused into the patient (Fig. 3). The limitations are comparatively higher cost and restricted dosing depending on the number of cells. The major issues (Depil et al. 2020; Carpenito et al. 2009; June et al. 2018) observed in CAR therapy to restrain the anti-tumor activity are:

1. Graft-versus-host disease (GVHD) occurs during the administration of allogeneic T-cells.
2. Rapid elimination of CAR cells by the host immune system.

GVHD can be avoided by allogeneic CAR T-cells usage derived from a stem cell transplant donor, virus-specific memory T cells or non-αβ T-cells usage or gene editing. Nucleases most prominently like zinc-finger nucleases, transcription activator-like effector nucleases, MegaTALs, etc. are used in precise gene editing (Brudno and Kochenderfer 2016; O'Reilly et al. 2016; Capsomidis et al. 2018; Qasim et al. 2017; Eyquem et al. 2017).

3 Factors Affecting Efficacy and Safety of CAR T-Cell Therapy

Major factors affecting efficacy of CAR T-cell therapy are design of CAR, infused T-cell composition, pre-conditioning regimen and microenvironment. Researchers used combination of first- and second-generations CAR to treat lymphoma patients and showed improvement in persistence of T-cells and CD28 or 4-1BB signaling domains in first-generation whereas in second-generation CAR, rapid expansion and longer persistence observed (Savoldo et al. 2011; Long et al. 2015). Incorporation of CD28-4-1BB or CD28-OX40 to third generation CAR did not show any intense responses in the clinical trials (Karlsson et al. 2015; Till et al. 2012).

Major factors affecting CAR T-cell therapy related to its safety due to threatening cytokine release syndromes and macrophage activation

syndromes for on-target off-tumor toxicity, neurotoxicity and tumor lysis syndrome. This resulted to high circulating levels of several cytokines like interleukin-6 or interferon. Other safety concerns include occurrences of aphasia, tremor and seizures (Maus et al. 2014; Grupp et al. 2013).

4 Overcoming the Toxicities

The two major toxicities associated with activation of T-cell and release of cytokines, and on-target off-tumor effects expressed due to antigen target and CAR cell interaction. In high-volume disease patients, cytokine-related toxicities are attenuated by decreasing the infused cells and associating to CAR designs fundamental modifications like dimerizing agents of CAR sub-unit, signaling downstream inhibitors of the protease inhibitors or CAR for regulation of the CAR protein expression. Complement-dependent or antibody-dependent cellular cytotoxicity is suicide gene system enabling CAR T-cells for elimination via apoptosis induction (Park et al. 2018; Lee et al. 2019; Griffioen et al. 2009). The on-target off-tumor toxicities are overcome by designing TAAs by its multiple functional activation recognition, selectively expressed antigen absence on non-cancerous cells, factor manifestation of tumor cells supplement like recognition of phosphoantigens via γδT cell receptors or through tumor microenvironment like the immunosuppressive cytokine IL-4 (Lamers et al. 2013; Song et al. 2015; Kloss et al. 2013). Alternate strategies are based on conditional expression systems and logic gating, CAR targeting expression of a specific TAA dependent by activation of transgenic receptor structures like synthetic Notch receptors and HIF-CARs intended to express selectively in the hypoxic tumor microenvironment (Fisher and Anderson 2018).

CAR T-cells efficacy is improved by countering antigen escape, increasing CAR T-cell persistence by less-differentiated T-cell subsets usage. The penetration and assembly of CAR T-cells into solid tumors is enriched by stimulating its proficient responses towards tumor-associated chemokines or physical barriers of target tumor microenvironment. CAR T-cells are modified to overcome the tumor microenvironment immunosuppressive factors by evading inhibitory immune checkpoints activity, involving apoptosis or promotion of an inflammatory milieu through cytokines manifestation or any other factors associated to immune-stimulatory like CD40 ligand, antigen-presenting cell, CC-chemokine receptor, cancer-associated fibroblast, etc. (Kuhn et al. 2019; Morgan and Schambach 2018; Zhao et al. 2015; Junttila and de Sauvage 2013; Hardaway et al. 2018).

5 Clinical Trials

FDA approved CAR T-cells therapies include Tisagenlecleucel (KymriahTM), Axicabtagene ciloleucel (YescartaTM) and Tocilizumab (Actemra®). CAR T-cell immunotherapy trials were carried out for various cancers like leukemia, multiple myeloma, lung cancer, cervical cancer, breast cancer, etc. Some of the trials under process are listed in Table 1 (EU Clinical Trials Register; clinical trials.gov).

6 Conclusions

In end-stage patients, clinical trials with CAR therapy showed promising results with 92% full recovery in ALL. To control GVHD, gene editing technologies are strategized to eradicate TCR expression ensuing novel allogeneic CAR T-cells invisible techniques to the host immune system. As it is limited due to therapeutic barriers such as trafficking, CAR T-cell expansion, persistence, etc. within tumors, it emerges as a potential paradigm shift in refractory or relapsed cancers treatment as a gene therapy that further helps to long-term risks characterization related to gene editing in medicine. It also facilitates the resolutions of all the barriers associated to the side effects. The novel designs for CAR T-cells products showed better clinical aids to treat diverse cancer patients.

Table 1 Clinical trials in process related to CAR T-cell treatment in cancer patients

Sr. No.	Medical condition	Objective	Sponsor
1	B-cell Lymphoma	Determination of a safe number of infusions by modified cells (white cells with retrovirus called CD19)	National Cancer Institute (NCI)
2	CD19+ B cell lymphoma or leukemia	Phase II trial of CD 19-targeting CAR T-cells for refractory B-cell melanoma	Uppsala University
3	Multiple myeloma	SLAMF7 CAR-T cells antitumor activity Assess feasibility, safety and of autologous in multiple myeloma	Universitätsklinikum Würzburg
4	Patients with potential malignancy receiving Autolus' CAR T cell therapy	Long-term patients development towards autologous T cells treatment	Autolus Limited
5	CAR T-cell therapy receiving patients	Lentiviral-Based CAR T-Cell Therapy exposed patients follow-up	Novartis Pharma Services AG
6	Acute myeloid leukemia and Multiple myeloma	Multiple myeloma and acute myeloid leukemia exhibiting CD44v6 autologous CAR T-cells showing antitumor activity	MolMed S.p.A.
7	Relapsed and refractory CD19+ leukemia and lymphoma	CD19+ lymphoid relapsed or refractory patients with T-cells transduced by retroviral vector	University Hospital Heidelberg
8	Advanced Lung Cancer	CAR-T cells to treat advanced lung cancer	Sun Yat-sen University Cancer Center Guangzhou
9	Cervical Cancer	Intervention of CAR-T Against Cervical Cancer	Shenzhen Geno-immune Medical Institute
10	Breast Cancer	HER-2-targeting CAR T Cells infusion	Central laboratory in Fuda cancer hospital
11	Ovarian Cancer	MESO-CAR T Cells Therapy for Relapsed and Refractory Ovarian Cancer	Zhejiang University School of Medicine

Conflict of Interest The authors declare there is no conflict of interest.

References

Abken H (2017) Driving CARs on the highway to solid cancer: some considerations on the adoptive therapy with CAR T cells. Hum Gene Ther 28(11):1047–1060

Alabanza L, Pegues M, Geldres C et al (2017) Function of novel anti-CD19 chimeric antigen receptors with human variable regions is affected by hinge and transmembrane domains. Mol Ther 25(11):2452–2465

Almåsbak H, Aarvak T, Vemuri MC (2016) CAR T cell therapy: a game changer in cancer treatment. J Immunol Res 2016:5474602

Brudno JN, Kochenderfer JN (2016) Toxicities of chimeric antigen receptor T cells: recognition and management. Blood 127(26):3321–3330

Brudno JN, Somerville RP, Shi V et al (2016) Allogeneic T cells that express an anti-CD19 chimeric antigen receptor induce remissions of B-cell malignancies that progress after allogeneic hematopoietic stem-cell transplantation without causing graft-versus-host disease. J Clin Oncol 34(10):1112–1121

Capsomidis A, Benthall G, Van Acker HH et al (2018) Chimeric antigen receptor-engineered human gamma delta T cells: enhanced cytotoxicity with retention of cross presentation. Mol Ther 26(2):354–365

Carpenito C, Milone MC, Hassan R et al (2009) Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. Proc Natl Acad Sci U S A 106 (9):3360–3365

Chmielewski M, Hombach AA, Abken H (2013) Antigen-specific T-cell activation independently of the MHC: chimeric antigen receptor-redirected T cells. Front Immunol 4:371

Deol A, Lum LG (2010) Role of donor lymphocyte infusions in relapsed hematological malignancies after stem cell transplantation revisited. Cancer Treat Rev 36(7):528–538

Depil S, Duchateau P, Grupp SA et al (2020) 'Off-the-shelf' allogeneic CAR T cells: development and challenges. Nat Rev Drug Discov 19:185–199

Dwivedi A, Karulkar A, Ghosh S, Rafiq A, Purwar R (2019) Lymphocytes in cellular therapy: functional regulation of CAR T cells. Front Immunol 9:3180

EU Clinical Trials Register. clinicaltrialsregister.eu

Eyquem J, Mansilla-Soto J, Giavridis T et al (2017) Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature 543 (7643):113–117

Fisher J, Anderson J (2018) Engineering approaches in human gamma delta T cells for cancer immunotherapy. Front Immunol 9:1409

Griffioen M, van Egmond EH, Kester MG, Willemze R, Falkenburg JH, Heemskerk MH (2009) Retroviral transfer of human CD20 as a suicide gene for adoptive T-cell therapy. Haematologica 94(9):1316–1320

Gross G, Waks T, Eshhar Z (1989) Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. Proc Natl Acad Sci U S A 86(24):10024–10028

Grupp SA, Kalos M, Barrett D et al (2013) Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med 368(16):1509–1518

Guedan S, Posey AD Jr, Shaw C et al (2018) Enhancing CAR T cell persistence through ICOS and 4-1BB costimulation. JCI Insight 3(1):e96976

Hardaway JC, Prince E, Arepally A, Katz SC (2018) Regional infusion of chimeric antigen receptor T cells to overcome barriers for solid tumor immunotherapy. J Vasc Interv Radiol 29(7):1017–1021.e1

June CH, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC (2018) CAR T cell immunotherapy for human cancer. Science 359(6382):1361–1365

Junttila MR, de Sauvage FJ (2013) Influence of tumour micro-environment heterogeneity on therapeutic response. Nature 501(7467):346–354

Karlsson H, Svensson E, Gigg C et al (2015) Evaluation of intracellular signaling downstream chimeric antigen receptors. PLoS One 10(12):e0144787

Kloss CC, Condomines M, Cartellieri M, Bachmann M, Sadelain M (2013) Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. Nat Biotechnol 31 (1):71–75

Kuhn NF, Purdon TJ, van Leeuwen DG et al (2019) CD40 ligand-modified chimeric antigen receptor T cells enhance antitumor function by eliciting an endogenous antitumor response. Cancer Cell 35(3):473–488.e6

Lamers CH, Sleijfer S, van Steenbergen S et al (2013) Treatment of metastatic renal cell carcinoma with CAIX CAR-engineered T cells: clinical evaluation and management of on-target toxicity. Mol Ther 21 (4):904–912

Lee YG, Chu H, Lu Y et al (2019) Regulation of CAR T cell-mediated cytokine release syndrome-like toxicity using low molecular weight adapters. Nat Commun 10 (1):2681

Long AH, Haso WM, Shern JF et al (2015) 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. Nat Med 21(6):581–590

Mandpe P, Prabhakar B, Shende P (2020) Role of liposomes-based stem cell for multimodal cancer therapy. Stem Cell Rev Rep 16(1):103–117

Maus MV, Grupp SA, Porter DL, June CH (2014) Antibody-modified T cells: CARs take the front seat for hematologic malignancies. Blood 123 (17):2625–2635

Miliotou AN, Papadopoulou LC (2018) CAR T-cell therapy: a new era in cancer immunotherapy. Curr Pharm Biotechnol 19(1):5–18

Morgan MA, Schambach A (2018) Engineering CAR-T cells for improved function against solid tumors. Front Immunol 9:2493

O'Reilly RJ, Prockop S, Hasan AN, Koehne G, Doubrovina E (2016) Virus-specific T-cell banks for 'off the shelf' adoptive therapy of refractory infections. Bone Marrow Transplant 51(9):1163–1172

Park JH, Rivière I, Gonen M et al (2018) Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. N Engl J Med 378(5):449–459

Patil A, Narvenker R, Prabhakar B, Shende P (2020) Strategic consideration for effective chemotherapeutic transportation via transpapillary route in breast cancer. Int J Pharm 586:119563

Qasim W, Zhan H, Samarasinghe S et al (2017) Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. Sci Transl Med 9 (374):eaaj2013

Rafiq S, Purdon TJ, Daniyan AF et al (2017) Optimized T-cell receptor-mimic chimeric antigen receptor T cells directed toward the intracellular Wilms Tumor 1 antigen. Leukemia 31(8):1788–1797

Rafiq S, Hackett CS, Brentjens RJ (2020) Engineering strategies to overcome the current roadblocks in CAR T cell therapy. Nat Rev Clin Oncol 17(3):147–167

Savoldo B, Ramos CA, Liu E et al (2011) CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. J Clin Invest 121(5):1822–1826

Shende P, Basarkar V (2019) Recent trends and advances in microbe-based drug delivery systems. Daru 27 (2):799–809

Shende PK, Desai D, Gaud RS (2018) Role of solid-gas interface of nanobubbles for therapeutic applications. Crit Rev Ther Drug Carrier Syst 35(5):469–494

Smith AJ, Oertle J, Warren D, Prato D (2016) Chimeric antigen receptor (CAR) T cell therapy for malignant cancers: summary and perspective. Jour Cell Immunother 2(2):59–68

Song DG, Ye Q, Poussin M, Liu L, Figini M, Powell DJ Jr (2015) A fully human chimeric antigen receptor with potent activity against cancer cells but reduced risk for off-tumor toxicity. Oncotarget 6(25):21533–21546

Till BG, Jensen MC, Wang J et al (2012) CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB

domains: pilot clinical trial results. Blood 119 (17):3940–3950
Zhang G, Wang L, Cui H et al (2014) Anti-melanoma activity of T cells redirected with a TCR-like chimeric antigen receptor. Sci Rep 4:3571
Zhang C, Liu J, Zhong JF, Zhang X (2017) Engineering CAR-T cells. Biomark Res 5:22
Zhao Z, Condomines M, van der Stegen SJC et al (2015) Structural design of engineered costimulation determines tumor rejection kinetics and persistence of CAR T cells. Cancer Cell 28(4):415–428. http://clinicaltrials.gov/

Adv Exp Med Biol - Cell Biology and Translational Medicine (2021) 12: 47–53
https://doi.org/10.1007/5584_2021_622

Published online: 25 February 2021

Drug Sensitivity and Drug Repurposing Platform for Cancer Precision Medicine

Ekene Emmanuel Nweke and Deepak B. Thimiri Govinda Raj

Abstract

One of the critical Global challenges in achieving the UN Sustainable Development Goals 3 Good Health and Well Being is optimizing drug discovery and translational research for unmet medical need in both communicable and non-communicable diseases. Recently, the WHO reports there has been a shift from communicable diseases to non-communicable diseases with respect to being the leading cause of death globally and particularly in low- and middle-income countries such as South Africa. Hence, there is current drive to establish functional precision medicine program that addresses the unmet medical need using high throughput drug sensitivity and drug repurposing platform. Here, this paper serves as a perspective to showcase the recent development in high throughput drug sensitivity screening platform for the cancer precision medicine. We also elaborate on the benefit and applications of high-throughput drug screening platform for Precision Medicine. From a future perspective, this paper aims to showcase the possibility to integrate existing high-throughput drug sensitivity screening platform with the newly developed platform technologies such as microfluidics based single cell drug screening.

Keywords

Drug repurposing · Drug sensitivity · High throughput drug screening · Precision medicine

1 Introduction

Worldwide, cancer is a leading cause of death and singlehandedly shortens life expectancy in most countries. Due to several factors such as aging, lifestyle and environmental exposures, and socio-economic development, cancer incidence and seem to be increasing, globally. The GLOBOCAN 2018 study across 185 countries estimated 18.1 million and 9.6 million cancer incidence and mortality, respectively (Bray et al. 2018). Recent findings also predict that, in the United States of America, there will be more than 1.8 million new cancer cases diagnosed with an expected death of >600,000 (Siegel et al. 2020). These statistics show a lifetime probability for cancer diagnosis to be 40.1% and

E. E. Nweke
Department of Surgery, Faculty of Health Sciences, University of Witwatersrand, Johannesburg, South Africa

D. B. Thimiri Govinda Raj (✉)
Synthetic Nanobiotechnology and Biomachines Group, ERA Synthetic Biology, Centre for Synthetic Biology and Precision Medicine, Council for Scientific and Industrial Research, Pretoria, South Africa

Biotechnology Innovation Centre, Rhodes University, Grahamstown, South Africa
e-mail: dgovindaraj@csir.co.za

38.7% in men and women, respectively. There has been an observed decline of cancer-related mortality over the years, largely due to behavioral changes like reduction in smoking and recently targeted chemo- and immunotherapies.

Unlike traditional approach to interventions involve the diagnosing and treating of patients based on a standard of care which is a "one-fit-all" model, precision medicine (sometimes referred to as personalized medicine) allows for a more patient-tailored approach (Auffray et al. 2009; Bereczki 2012; Duffy 2016; Erikainen and Chan 2019; Ginsburg and Willard 2009; Gonzalez-Angulo et al. 2010; Tutton 2012; Wallace and Moodie 2014; Hood et al. 2004). Precision medicine utilizes the phenotypic and genotypic characteristics of a patient with the hope to provide a more efficient model for predicting, diagnosing, treating, and monitoring disease. This model, therefore, proposes to provide early accurate diagnosis and more effective treatment strategies (Fierz 2004). Genotypic characteristics include genetic information, and molecular profile of the patient and the phenotype would include clinical and demographic information. The identification of the molecular mechanism of disease in each patient facilitates the use of the appropriate drug for treatment.

This strategy is crucial in oncology where the origin of each cancer is a clonal event evolving into tumor heterogeneity and could be distinct in individual patients. In cancer research, there are several efforts for individualized treatment as a part of a personalized medicine approach (Fig. 1). The most commonly used efforts for individualized treatments are applications through full in-depth integration of clinical and demographic information with omics analyses. Omics analyses include transcriptomics, genomics, metabolomics, metagenomics, and proteomics. Despite several initiatives and generations of large omics datasets, it has been cumbersome to identify the significance of every finding, thereby limiting strategies that benefit patient treatment. Worldwide, there is now also increasing recognition of the requirement for medical researchers and health authorities to develop strategies and platforms aimed at accelerating the precision medicine model. To optimize individual patient benefit, researchers must develop new protocols and combine diagnostic information and the breadth of available therapies (Jarnaess and Taskén 2007). In this review, we discuss how tumour heterogeneity calls for the application of precision medicine. Furthermore, we highlight how proven strategy- drug screening could help circumvent drug resistance and improve accessibility to effective drugs.

2 Tumour Heterogeneity: Origin and Role in Drug Resistance

Tumours have been determined to be heterogeneous. There is a well-known dogma that says "No two cancers are the same". This means that although 2 patients might have the same cancer, the molecular pathogenesis of the disease are different. These differences are exacerbated by genetic and environment factors such as age and exposure to tobacco, thus they can affect disease progression and inform treatment strategy (Alexandrov et al. 2013; Coombs et al. 2017; Jamal-Hanjani et al. 2015; Marusyk et al. 2012; Burrell and Swanton 2014). Similarly, clones and subclones may emerge within a patient's tumour during cancer progression and therapeutic intervention in a phenomenom known as intra tumour heterogeneity (Burrell and Swanton 2014; Merlo et al. 2006). Tumour heterogeneity results in the origination of distinct and diverse cell populations that may exists dependently and/or independently of one another and are able to foster tumour progression and drug resisitance in this manner (Heppner and Miller 1983). Metastatic heterogeneity is also evident, whereby clones in the primary tumours are different from those in the metastatic sites. Inter metastatic heterogeneity involves the process of tumour cells acquiring additional clonal heterogeneity as they migrate and invade other organs. When these cells reach the destined organs they may further evolve in a process known as intra tumour heterogeneity (Jamal-Hanjani et al. 2015; Vogelstein et al. 2013).

Studies have shown that even within an individual patient sample, clonal heterogeneity exists. Factors such as time and the site of sample

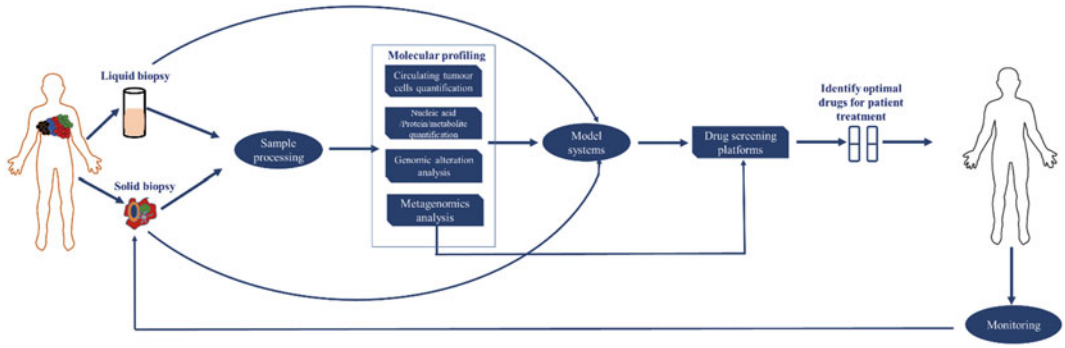


Fig. 1 Precision medicine and the role of drug screening: Biopsy is obtained from a cancer patient and processed before subjected to molecular profiling such as nucleic acid/protein quantification and metagenomics and mutational analysis. An *in vitro* or *in vivo* model system can them be used to screen a panel of drugs and subsequently the optimal drug(s) used to treat the patient. After treatment, patient can be monitored to ensure drug treatment efficacy

collection during clinical progression may influence heterogeneity (Anderson et al. 2011; Landau et al. 2013; Lohr et al. 2014; Snuderl et al. 2011; Gerlinger et al. 2012; Ricketts and Linehan 2014; Bolli et al. 2014; Haffner et al. 2013; Keats et al. 2012; Schuh et al. 2012; Nadeu et al. 2016). Gerlinger et al. performed exome sequencing and chromosomal abnormalities analysis on renal tumours and observed that up to 69% of somatic mutations were absent across tumour regions. Furthermore, using RNA sequencing they determined that the gene expression profiles associated with patient prognosis across these regions (Gerlinger et al. 2012). Another study analysed several cancers and identified subclonal mutations in genes such as *IDH*, *EGFR*, and *KRAS* are crucial to tumour development and progression. These mutations occur at different stages in the tumor evolution and impact therapeutic efficacy (McGranahan and Swanton 2017; McGranahan et al. 2015). A lethal cell clone was determined in prostrate cancer and tracked from its primary to metastatic site. The authors determined that genetic alterations differed as the cancer progresses and suggested the importance of longitudinally monitoring individual patients through tumor progression and treatment. Similarly, clonal mutations in genes such as *NOTCH1* and *P53* were observed in longitudinally collected 48 Chronic Lymphocytic Leukaemia patient samples and predicted overall survival (Nadeu et al. 2016).

Tumour heterogeneity can drive drug resistance. In non-small cell lung cancers patients, EGFR mutations are sensitive to EGFR –specific inhibitors, however it was observed that a secondary T790M mutation was responsible for gefitinib resistance in half of the patients (Kosaka et al. 2006). In gastrointestinal stromal tumour patients, intra- and inter- heterogeneity was demonstrated extensively; specifically the presence of tyrosine kinase inhibitor resistant mutations during treatment (Liegl et al. 2008; Heinrich et al. 2008). Likewise, a study using 23 initial low grade and recurrent brain tumours determined that 50% of mutations found in the former was absent in the latter (Johnson et al. 2014). These studies show and reiterate the role of tumour heterogeneity in drug resistance.

3 Drug Sensitivity and Repurposing in Cancer

High throughput sequencing has highlighted new potentially oncogenic alterations in different cancers both in coding and non-coding areas (Santarpia et al. 2016). For example, genome-wide mutational analysis revealed the localization of mutations in small regions in the genome that

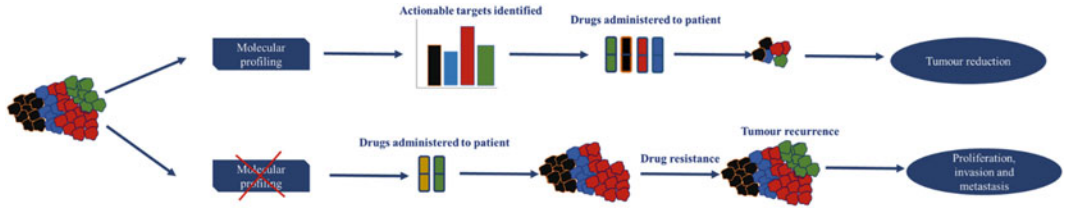


Fig. 2 Drug screening and intra tumour heterogeneity. Drug screening enables the understanding of the molecular events occurring s the tumour evolves and progresses. It allows for the proper identification of actionable targets and the subsequent treatment with the appropriate drugs

were associated with certain cancer types (Alexandrov et al. 2013). In patients with micro and overt metastatic breast cancer, ESR1 mutational analysis revealed tumour evolution and predicted drug resistance (Schiavon et al. 2015).

Integrating tumour molecular profiling in the clinical environment can help circumvent drug resistance associated with tumor heterogeneity. It achieves by unearthing actionable targets, thus providing better understanding of the cancer of individual patients allowing for the administration of effective targeted therapies (Bhutani et al. 2020; Zehir et al. 2017) (Fig. 2).

The development of new drugs for cancer treatment is largely expensive and time consuming. Health economist estimate the financial costs to be about two billion dollars over an approximate 10-year period (Collier 2009). The continuous steady increase in cancer incidence and mortality worldwide hints for an alternate way for the discovery of effective, readily accessible drugs for cancer treatment. Over the years, drug repurposing has increasingly become attractive in this regard. Drug repurposing or repositioning is the process of investigating and identifying novel ways to use already approved drugs for utilization other than its intended use (Ashburn and Thor 2004). For oncology, these drugs may be either cancer or non-cancer approved drugs (Verbaanderd et al. 2017; Pushpakom et al. 2019). During the drug development process, majority of the drugs fail clinical trials due to issues such as safety, formulation and delivery. However, since the drugs intended to be repurposed have already been tested and approved, this makes the drug repurposing process less risky. Furthermore, less financial and resource cost is required in drug repurposing.

Although, drug repurposing is a promising strategy in cancer treatment, there are still several hurdles including legal, financial and ethical, to overcome (Breckenridge and Jacob 2019). Over the years, there has been successes in drug repurposing for cancer treatment. For example, Raloxifene, originally developed for osteoporosis treatment has been repurposed for the treatment of breast cancer. Similarly, the commonly used Aspirin, used in treating pain, is now being utilized in colorectal cancer treatment (Pushpakom et al. 2019). The realization of these successes were brought about by diverse strategies. One of such auspicious strategy is the process of drug screening to observe phenotypic changes. Drug screening would involve the use of highly robust and high throughput platforms. Ultimately, identified drugs to be repurposed post-screening can be one or a combination of drugs; however, an understanding of the biological, physiological and clinical implication of the effect of the drug(s) is required (Boshuizen and Peeper 2020).

4 High Throughput Screening Platform

Here, we focus on origin and current developments high-throughput screening (HTS) platform for medical research and industrial drug discovery pipeline. HTS platform has been one of the main approaches for drug discovery which has been of major interest both in academic

and industrial setting since the last few decade. One of the main purpose for HTS technology is to enable the rapid progress in the drug discovery pipeline for both communicable and non-communicable diseases. Early implementation of high-throughput screening was established first in pharmaceutical companies which enabled progress during early stage drug discovery for the pharmacology research and development. Early evolution of the HTS platform at Pfizer was during the year period 1984–1995 which started with the natural product screening followed by toxicity and drug metabolism screening in 1995–99. During the year 1999, Pfizer fully adapted the integration of HTS platform in the drug discovery studies cycle. Prior to the establishment of HTS platform, the standard approach for drug discovery (biochemical and pharmacological) methods are based on weighed drug compounds and test tube reaction volume of 1 ml (Pereira and Williams 2007). Thus the conventional approach has the limited assay capacity to perform screening upto 50 compounds per week. Hence, conventional approach required 2 years time frame in order to screen approximately 3000 compounds that represent structural diversity for target modulators. Particularly with the progress on recombinant DNA technology enabled access to new therapeutic targets, which results in the need for medium and high-throughput screening to select the structural hits that modulate the new targets. This unmet need for drug discovery set perfect opportunity for HTS to be the go-to platform that can screen more than 100,000 compounds against the therapeutic targets with high speed and accuracy. Some of the comparison between the traditional screening and high throughput screening includes (a) traditional setup is in single tube while high throughput screening is array format 96, 384, 1536 well setup; (b) traditional screening requires large reaction assay volume more than 1 ml, while the HTS assay requires smaller assay volume between 50 and 500 ul; (c) traditional screening requires large amount of compound at the range of 5–10 mg while HTS assay requires only upto 1 ug of compounds; (d) In the traditional screening – components of assay can be added singly while with HTS method, assay components can be added simultaneously; (e) traditional screening mechanical action can be 1:1 while HTS mechanical action would 1:96, 384 and 1536 setup; (f) the compounds can be dissolved in customed solution in traditional screening while compounds are dissolved in dimethyl sulphoxide (DMSO); (g) traditional screening assay are quite laborious and time-consuming while high-throughput screening assay are effective both in terms of speed and efficiency (less than a minute per step for 96, 386, 1536 well); (h) traditional screens can do 20-5- compounds in 2 weeks' time per laboratory while HTS screens can do more than 10,000 screening in a week time per laboratory and finally (i) traditional screening are limited both in numbers and diversity while HTS are unlimited both in screening number and diversity; The origin and evolution of HTS is from 1984 where the natural products screening automation were performed with design capacity at 10,000 assays per week. Then in 1986, HTS concept was introduced to use 30 mM DMSO solutions of synthetic compounds along with 96 well plates (fixed format) with an assay volume upto 100 ul. Yeast ras oncogene was screened with 800–1400 compounds per weeks with manual pattern recognition and photographic records. Similarly, Neurotensin receptor/ligand were screened using dot-blot filtration along with autoradiography and image analysis as a part of HTS therapeutic target. Further in 1987, screening capabilities improved from 1000 to 3000 compounds with introduction of 96-well pipettors and harvesters. HTS screening was implement in applied biotechnology research with improvement of 7200 compounds per weeks with 20 concurrent HTS along with cell-based and biochemical triplicate and RT-PCR multiplex assay to be performed. By 2000, HTS progressed with ADMET and nanotechnology integration with academic universities involvement with national and international research routemap.

With the advancement in HTS technology, several screening platforms were implemented

for the treatment of communicable and non-communicable diseases. Here, we focus on the current and recent development of high-throughput drug sensitivity screening for drug repurposing in precision medicine. Demand for drug sensitivity screening and drug repurposing is mainly due to substantial increased numbers of communicable and non-communicable diseases such as cancer and COVID-19. In addition, several communicable and non-communicable diseases are acquiring treatment resistance and multi-drug resistance thereby there is unmet need to identify effective multi-drug combinations with rapid screening methods like HTS drug sensitivity screening platforms. Further, the HTS drug sensitivity screening platforms enabled drug repurposing to identify effective drug combinations at rapid phase for unmet medical need such during pandemic like COVID19 and unsolvable problem such as cancer and infectious disease.

5 Future Perspective

With the rapid development of Artificial Intelligence and machine learning approaches in precision medicine, there is recent progress in the integration of artificial intelligence and machine learning approaches with high throughput drug sensitivity screening data analysis. Such integrative approach enables rapid advancement in drug discovery and functional precision medicine technologies for unmet medical need in communicable and non-communicable diseases. One of the new initiatives is currently being established at CSIR Synthetic biology and precision medicine centre is to implement integrative functional precision medicine platform for South African Patient cohort with respect to blood cancer and ovarian cancer. In our initiative, we aim to integrate the high throughput drug sensitivity screening along with microfluidics based single-cell drug screening to generate the drug sensitivity screening datasets and implement artificial intelligence approaches to functional precision medicine initiatives in South Africa.

References

Alexandrov LB et al (2013) Signatures of mutational processes in human cancer. Nature 500:415–421

Anderson K et al (2011) Genetic variegation of clonal architecture and propagating cells in leukaemia. Nature 469:356–361

Ashburn TT, Thor KB (2004) Drug repositioning: identifying and developing new uses for existing drugs. Nat Rev Drug Discov 3:673–683

Auffray C, Chen Z, Hood L (2009) Systems medicine: the future of medical genomics and healthcare. Genome Med 1:2

Bereczki D (2012) Personalized medicine: a competitor or an upgrade of evidence-based medicine? Per Med 9:211–221

Bhutani MS, Cazacu IM, Roy-Chowdhuri S, Maitra A, Pishvaian MJ (2020) Upfront molecular profiling of pancreatic cancer patients – an idea whose time has come. Pancreatology 20:391–393

Bolli N et al (2014) Heterogeneity of genomic evolution and mutational profiles in multiple myeloma. Nat Commun 5:2997

Boshuizen J, Peeper DS (2020) Rational Cancer treatment combinations: an urgent clinical need. Mol Cell 78:1002–1018

Bray F et al (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68:394–424

Breckenridge A, Jacob R (2019) Overcoming the legal and regulatory barriers to drug repurposing. Nat Rev Drug Discov 18:1–2

Burrell RA, Swanton C (2014) Tumour heterogeneity and the evolution of polyclonal drug resistance. Mol Oncol 8:1095–1111

Collier R (2009) Drug development cost estimates hard to swallow. CMAJ 180:279–280

Coombs CC et al (2017) Therapy-related clonal Hematopoiesis in patients with non-hematologic cancers is common and associated with adverse clinical outcomes. Cell Stem Cell 21:374–382.e4

Duffy DJ (2016) Problems, challenges and promises: perspectives on precision medicine. Brief Bioinform 17:494–504

Erikainen S, Chan S (2019) Contested futures: envisioning 'Personalized,' 'Stratified,' and 'Precision' medicine. New Genet Soc 38:308–330

Fierz W (2004) Challenge of personalized health care: to what extent is medicine already individualized and what are the future trends? Med Sci Monit 10: RA111–RA123

Gerlinger M et al (2012) Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 366:883–892

Ginsburg GS, Willard HF (2009) Genomic and personalized medicine: foundations and applications. Transl Res 154:277–287

Gonzalez-Angulo AM, Hennessy BTJ, Mills GB (2010) Future of personalized medicine in oncology: a systems biology approach. J Clin Oncol 28:2777–2783

Haffner MC et al (2013) Tracking the clonal origin of lethal prostate cancer. J Clin Invest 123:4918–4922

Heinrich MC et al (2008) Primary and secondary kinase genotypes correlate with the biological and clinical activity of sunitinib in imatinib-resistant gastrointestinal stromal tumor. J Clin Oncol 26:5352–5359

Heppner GH, Miller BE (1983) Tumor heterogeneity: biological implications and therapeutic consequences. Cancer Metastasis Rev 2:5–23

Hood L et al (2004) Predictive and preventative medicine S. Science 306:640

Jamal-Hanjani M, Quezada SA, Larkin J, Swanton C (2015) Translational implications of tumor heterogeneity. Clin Cancer Res 21:1258–1266

Jarnaess E, Taskén K (2007 Nov) Spatiotemporal control of cAMP signalling processes by anchored signalling complexes. Biochem Soc Trans 35(Pt 5):931–937

Johnson BE et al (2014) Mutational analysis reveals the origin and therapy-driven evolution of recurrent glioma. Science 343:189–193

Keats JJ et al (2012) Clonal competition with alternating dominance in multiple myeloma. Blood 120:1067–1076

Kosaka T et al (2006) Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib. Clin Cancer Res 12:5764–5769

Landau DA et al (2013) Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. Cell 152:714–726

Liegl B et al (2008) Heterogeneity of kinase inhibitor resistance mechanisms in GIST. J Pathol 216:64–74

Lohr JG et al (2014) Widespread genetic heterogeneity in multiple myeloma: implications for targeted therapy. Cancer Cell 25:91–101

Marusyk A, Almendro V, Polyak K (2012) Intra-tumour heterogeneity: a looking glass for cancer? Nat Rev Cancer 12:323–334

McGranahan N, Swanton C (2017) Clonal heterogeneity and tumor evolution: past, present, and the future. Cell 168:613–628

McGranahan N et al (2015) Clonal status of actionable driver events and the timing of mutational processes in cancer evolution. Sci Transl Med 7:283ra54

Merlo LMF, Pepper JW, Reid BJ, Maley CC (2006) Cancer as an evolutionary and ecological process. Nat Rev Cancer 6:924–935

Nadeu F et al (2016) Clinical impact of clonal and subclonal TP53, SF3B1, BIRC3, NOTCH1, and ATM mutations in chronic lymphocytic leukemia. Blood 127:2122–2130

Pereira DA, Williams JA (2007) Origin and evolution of high throughput screening. Br J Pharmacol 152:53–61

Pushpakom S et al (2019) Drug repurposing: progress, challenges and recommendations. Nat Rev Drug Discov 18:41–58

Ricketts CJ, Linehan WM (2014) Intratumoral heterogeneity in kidney cancer. Nat Genet 46:214–215

Santarpia L et al (2016) Deciphering and targeting oncogenic mutations and pathways in breast Cancer. Oncologist 21:1063–1078

Schiavon G et al (2015) Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. Sci Transl Med 7:313ra182

Schuh A et al (2012) Monitoring chronic lymphocytic leukemia progression by whole genome sequencing reveals heterogeneous clonal evolution patterns. Blood 120:4191–4196

Siegel RL, Miller KD, Jemal A (2020) Cancer statistics, 2020. CA Cancer J Clin 70:7–30

Snuderl M et al (2011) Mosaic amplification of multiple receptor tyrosine kinase genes in glioblastoma. Cancer Cell 20:810–817

Tutton R (2012) Personalizing medicine: futures present and past. Soc Sci Med 75:1721–1728

Verbaanderd C, Meheus L, Huys I, Pantziarka P (2017) Repurposing drugs in oncology: next steps. Trends Cancer 3:543–546

Vogelstein B et al (2013) Cancer genome landscapes. Science 339:1546–1558

Wallace MP, Moodie EEM (2014) Personalizing medicine: a review of adaptive treatment strategies. Pharmacoepidemiol Drug Saf 23:580–585

Zehir A et al (2017) Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. Nat Med 23:703–713

Adv Exp Med Biol - Cell Biology and Translational Medicine (2021) 12: 55–72
https://doi.org/10.1007/5584_2020_607

Published online: 31 December 2020

Role and Regulation of Lin28 in Progenitor Cells During Central Nervous System Development

Fernando Faunes

Abstract

Lin28 is a highly conserved RNA binding protein that regulates stemness whose molecular role has been widely studied *in vitro*. However, the regulation and the molecular role of Lin28 during the development of the vertebrate central nervous system (CNS) *in vivo* are not completely understood. Here, the expression and the putative role of Lin28 in the development of the mammalian CNS are reviewed in the context of recent results showing the progressive cellular and molecular changes in neural progenitor cells. Downstream genes that may play a role during CNS development and the effect of misregulated expression of Lin28 are discussed. Evidence suggests that Lin28 promotes symmetric divisions over asymmetric divisions, increasing the number of progenitors during early neurogenesis. Future quantitative analysis of Lin28 isoforms levels and stabilities together with single cell transcriptomics data, cell cycle dynamics and cell fate analysis in Lin28 gain- and loss-of-function experiments will provide a better understanding of the molecular role of Lin28 during development.

F. Faunes (✉)
Departamento de Ciencias Biológicas, Facultad de Ciencias de la Vida, Universidad Andres Bello, Viña del Mar, Chile
e-mail: fernando.faunes@unab.cl

Keywords

Central nervous system · Cerebral cortex · Developmental timing · Differentiation · Heterochronic genes · Let-7 · Lin28 · Neural progenitor cells · Proliferation

Abbreviations

AP	Apical progenitor
CNS	Central nervous system
CP	Cortical plate
ESC	Embryonic stem cell
IPC	Intermediate progenitor cell
IZ	Intermediate zone
MZ	Marginal zone
NEC	Neuroepithelium cell
NS	Nervous system
SVZ	Subventricular zone
VZ	Ventricular zone

1 Introduction

Lin28 was one the heterochronic genes identified in analyses of mutants in the nematode *Caenorhabditis elegans* (Ambros and Horvitz 1984). The term "heterochrony" refers to any difference in the timing or duration of a developmental process in an organism relative to other organism (for example, in a mutant relative to the wildtype or in an animal relative to its ancestors).

Heterochronic genes in *C. elegans* control the timing and pattern of cell divisions during larval development (Rougvie 2001). Mutants for these genes show defects in proliferation and differentiation in several tissues because progenitor cells skip, repeat, or delay specific division patterns. For example, *lin28* loss-of-function mutants show precocious differentiation in some lineages because symmetric divisions of progenitor cells do not occur in one of the specific larval stages (Ambros and Horvitz 1984). In contrast, *lin28* gain-of-function induce excess of proliferation due to repetitions of symmetric divisions of progenitor cells (Moss et al. 1997).

Lin28 is highly conserved in animals and two Lin28 orthologues have been identified in vertebrates, Lin28a and Lin28b. Lin28a and Lin28b promote proliferation of stem cells by regulating the expression of genes related to metabolism and cell cycle (Shyh-Chang and Daley 2013; Tsialikas and Romer-Seibert 2015). Accordingly, Lin28a and Lin28b are aberrantly expressed in several tumors (Viswanathan et al. 2009) and enhance the reprogramming of differentiated cells to generate induced pluripotent stem cells (iPSCs) (Yu et al. 2007; Zhang et al. 2016).

Lin28a and Lin28b expression *in vivo* has been studied in different tissues of several vertebrates (Faas et al. 2013; Faunes et al. 2017; Gundermann et al. 2019; La Torre et al. 2013; Moss and Tang 2003; Ouchi et al. 2014; Yang et al. 2015). The general trend is that Lin28a and Lin28b are expressed at higher levels during early stages of embryogenesis or larval development compared to adults, with the highest expression in undifferentiated cells (Moss and Tang 2003; Shyh-Chang and Daley 2013; Yang and Moss 2003). Consistent with the role of Lin28 in mammal progenitor cells and developmental timing in *C. elegans*, gain- and loss-of-function experiments in vertebrates show that Lin28 is involved in growth (Shinoda et al. 2013; Zhu et al. 2010), metabolism (Zhu et al. 2011), regeneration (Shyh-Chang et al. 2013), puberty (Zhu et al. 2010) and metamorphosis (Faunes et al. 2017; Gonzalez-Itier et al. 2018).

Lin28a and Lin28b are RNA binding proteins that regulate the translation of several mRNAs and the biogenesis of specific microRNAs (Shyh-Chang and Daley 2013; Tsialikas and Romer-Seibert 2015). Lin28a and Lin28b regulate the levels of proteins involved in cell cycle, cell growth, protein synthesis and metabolism by direct binding to mRNAs or indirectly through inhibition of the biogenesis of the micro-RNA *let-7*. In addition, Lin28a controls transcription by binding to promoters (Zeng et al. 2016). Therefore, Lin28a and Lin28b are master regulators of gene expression. Although several target genes have been identified in stem cells *in vitro*, the repertoire of genes controlled by Lin28a and Lin28b during embryogenesis is still incomplete.

In this work, recent evidence about the role of Lin28a and Lin28b *in vivo* is reviewed, specifically, in the development of the vertebrate central nervous system (CNS). The focus is on the cerebral cortex of mice because several studies have characterized the types of cell divisions (symmetric versus asymmetric divisions), the cell cycle length, and the cell fate of the progeny of neural progenitors during embryogenesis. In addition, single cells analyses of neural progenitors have revealed how gene expression changes during neurogenesis. These works are reviewed to discuss the expression of Lin28 and the effect of gain- and loss-of-function experiments on the CNS.

2 Overview of the CNS Development in Mice

Nervous system (NS) originates from the definitive ectoderm, which is committed into neuroectoderm around embryonic day 7.5 (E7.5) by the underlying notochord. Rapid cell divisions thicken the neuroectoderm (neural plate), which then begins to fold to form the neural tube. The neural tube is closed around E9/E10. The most anterior (rostral) part of the neural tube -the forebrain- is divided into telencephalon (from which the cerebral cortex is derived) and diencephalon.

Neurogenesis in vertebrates has been extensively reviewed (Cardenas and Borrell 2020; Florio and Huttner 2014; Paridaen and Huttner 2014). Here, only some of the processes that occur in mice with a focus on the cerebral cortex development are summarized. Neuroepithelium cells (NECs) expressing Sox1, Sox2, Sox3, and Lin28 undergo symmetric amplifying divisions that expand the number of NECs (Fig. 1a). Around E10, some of these NECs are converted into radial glia cells (RGCs) and express Pax6, Nestin and Vimentin. This population of NECs and RGCs is heterogenous and contains cells with different potential. Some of these NECs and RGCs self-renew (i.e. one daughter cell is a new NEC or RGC and the other daughter cell is a different cell type) and can generate neurons, astrocytes, and oligodendrocytes and, therefore, they are neural stem cells. However, other cells can generate only neurons and, therefore, they are neural progenitors and have a more limited proliferation capacity. At present, there are no expression markers to clearly distinguish between neural stem cells (that can generate all the cell types in the NS) and neural progenitor cells (that can generate only one cell type). In this review, the term "neural progenitor cells" is used, but it is important to consider that some of these cells may have a greater potential. NECs and RGCs are also known as apical progenitors (APs) and have apical and basal processes in contact with the ventricle (apical side) and the pia (basal side) of the neuroepithelium, respectively (Fig. 1a).

After the expansion of progenitors through symmetric divisions at the beginning of neurogenesis, RGCs switch from symmetric to asymmetric divisions. Studies using ^{3}H and BrdU incorporation have estimated that 11 cell cycles occur between E11 and E17 with an increase in the cell cycle length from 8 h at E11 to 18 h at E16-17 and an increase in the proportion of asymmetric divisions in the neuroepithelium (Takahashi et al. 1995, 1996) (Fig. 1a, b).

Different types of asymmetric divisions have been described: (1) RGCs can self-renew into one RGC daughter cell and one neuron (direct neurogenesis); (2) RGCs can self-renew into one RGC daughter cell and one intermediate progenitor cell (IPC) that later divides into two neurons (indirect neurogenesis); and (3) RGCs can divide into two neurons (Florio and Huttner 2014). The contribution of these types of divisions to the final set of cells of the NS is not completely clear and may vary in time and regions, but it is thought that direct neurogenesis occurs early, and indirect neurogenesis predominates at later stages. During indirect neurogenesis, IPCs switch from Pax6 to Tbr2/Eomes expression and migrate to the basal side of the VZ, forming the sub-ventricular zone (SVZ). IPCs are considered one of the two types of basal progenitors (the other type, the basal glia radial bRG is highly abundant in primates and is not further discussed here (Cardenas and Borrell 2020; Florio and Huttner 2014)). In the SVZ, IPCs lose the expression of markers of RGCs and most of the IPCs divide once, generating two neurons.

Recent *in vivo* single cell analysis using a pulse-label method in VZ-born cells at different times show that APs change their identity from E12 to E15 (Telley et al. 2019). Gene expression in APs change from "internally driven" at E12 to a more "environmentally driven" at E15 as revealed for transcriptomic analyses. Intrinsic genetic programs -including cell cycle regulation- are predominant in early APs and environment sensing programs -including ion transport-related processes- predominate at later stages. This temporal change in APs is correlated with an increase in the duration of the cell cycle, consistent with a previous work (Takahashi et al. 1995). Cell cycle length has been proposed to be important for differentiation and this aspect is later discussed considering the role of Lin28 on proliferation. Importantly, the transcriptional program underlying differentiation is conserved among temporally different APs, suggesting that the diversity of cell types of the progeny is mainly generated from the temporal progression of molecular programs in APs. Interestingly, the transcriptional changes from early APs to late APs occur independently of cell division, as observed after simultaneous blocking of cell division and differentiation of E11–12 APs by overexpressing the cell cycle inhibitor p18 and the Notch-intracellular domain

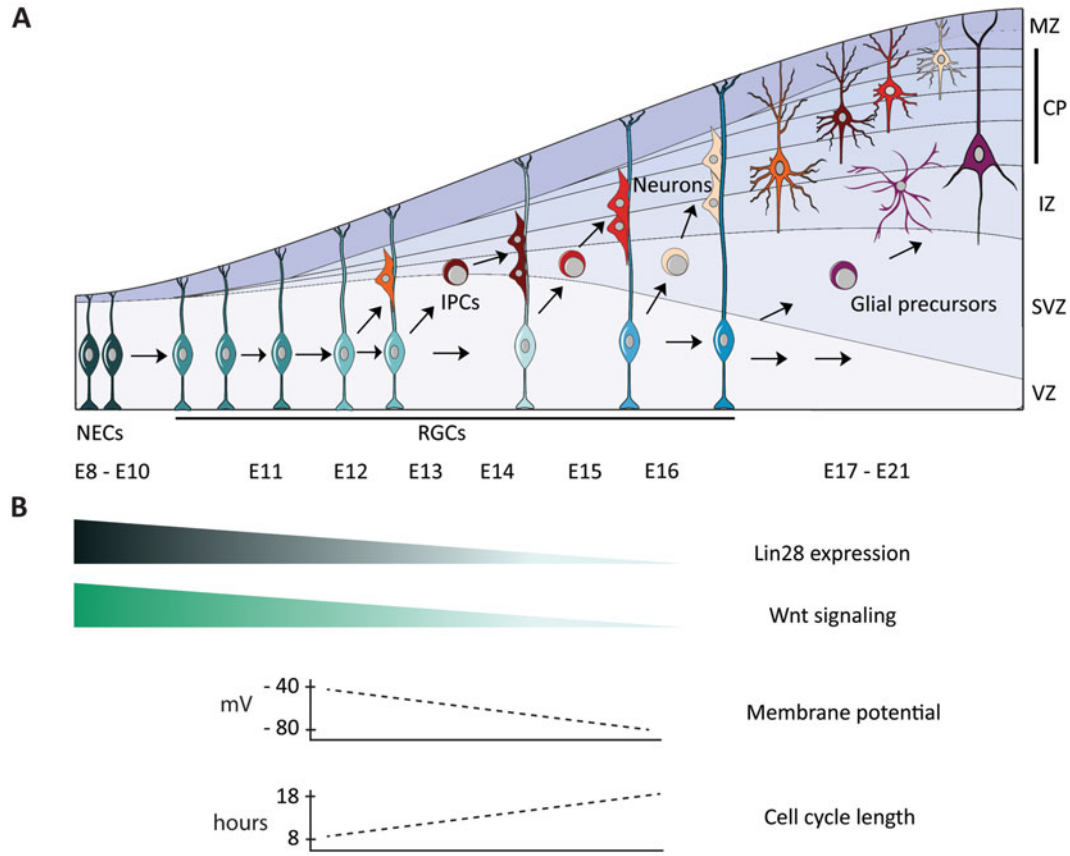


Fig. 1 Overview of cerebral cortex development. (**a**) Scheme of neurogenesis in mice showing the types of divisions of neural progenitors and the progeny during embryogenesis (E8 to E21, embryonic days are approximate). Neuroepithelial cell (NECs) and radial glial cells (RGCs) undergo symmetric divisions that expand the pool of progenitors at the beginning of neurogenesis (dark green). Asymmetric divisions of RGCs (light green) can generate a neuron (direct neurogenesis) or an intermediate progenitor cell (IPC), which divides into two neurons (indirect neurogenesis). Glial progenitors (blue) are not shown as generated from neurogenic RGCs to indicate that the population of progenitors is heterogenous. Different layers of the epithelium are shown at approximate times of generation. VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; MZ, marginal zone. (**b**) Cellular changes during neurogenesis. Lin28 levels and Wnt/β-catenin signaling decreases in progenitor cells during neurogenesis. Membrane potential and cell cycle length are only shown in the temporal window described in the text

(NICD), respectively (Okamoto et al. 2016). These results show that the cell division program can be decoupled from the genetic network that regulates AP identity.

These gene expression transitions in APs are also linked to membrane properties. Membrane potential increases from E12.5 (around −40 mV) to E15.5 (around −80 mV), as measured in cortical slices using patch clamp (Vitali et al. 2018). Strikingly, by modifying the membrane potential specifically in APs through *in utero* electroporation of a potassium channel to hyperpolarize and electroporation of a DREADD (Designer Receptor Exclusively Activated by Designer Drug) to restrict hyperpolarization, the fate of the progeny is altered. At E12.5, low membrane potential (−40 mV) promotes direct neurogenesis and the generation of early fate neurons. Progressive hyperpolarization represses Wnt/β-catenin signaling and promotes indirect neurogenesis and the

birth of late fate neurons. Hyperpolarization may also regulate the ability of APs to respond to extracellular signals, consistent with the evidence of the predominant extracellular control at later stages (Telley et al. 2019). How hyperpolarization, Wnt/β-catenin signaling, and AP identity are linked at the molecular levels requires further investigation.

After E15, RGCs produce GFAP+ astrocytes and O4+ oligodendrocytes (gliogenesis). As Lin28 is not expressed in neural progenitors at these later stages, gliogenesis is not described here. In conclusion, cell proliferation, expression of self-renewal and differentiation factors, signaling pathways and membrane potential must be tightly coordinated in time to generate the high cell diversity during CNS development.

3 Expression of Lin28a and Lin28b during CNS Development in Mice

3.1 Endogenous Expression During Embryogenesis and Post-natal Development

To understand the role of Lin28 in the CNS, the endogenous expression of Lin28a and Lin28b during neurogenesis is first described. The focus is on Lin28a and Lin28b expression during mice development. Different splice isoforms, post-transcriptional and post-translational mechanisms described for Lin28 in other biological contexts are discussed.

During mice embryogenesis, Lin28a protein is detected in embryonic and extraembryonic ectoderm and endoderm at E6.5 (Yang and Moss 2003). Lin28a is expressed in several tissues derived from the three germ layers at E8.5 but then the expression is mainly restricted to some epithelia, including neuroepithelia (Yang and Moss 2003). Between E9.5 and E12, *in situ* hybridization and immunofluorescence show that Lin28a is detected in the neural tube (Balzer et al. 2010), mainly in the ventricular zone (VZ) of the developing cerebral cortex (Yang et al. 2015). During these stages, Lin28a colocalizes with Sox2+ and Nestin+ cells and with the most apical subset of Pax6+ cells, indicating that Lin28 is mainly expressed in APs (Balzer et al. 2010; Herrlinger et al. 2019; Yang et al. 2015). Interestingly, at E11.5, Lin28a expression is higher in the forebrain compared to midbrain and hindbrain, indicating that expression may be spatially controlled (Herrlinger et al. 2019). In contrast to Lin28a, spatial expression of Lin28b has not been characterized in detail.

Western blots in developing cerebral cortex extracts show that Lin28a and Lin28b protein levels decrease from E10.5 to birth (Yang et al. 2015). High levels of Lin28a protein are observed at E9.5 compared to E12.5 and no Lin28a expression is detected after E16.5. Similarly, Lin28b levels are higher at E9.5 compared to E18.5. These results are consistent with the progressive increase of differentiated cells compared to undifferentiated cells. Interestingly, Lin28a has been detected by western blot in the cerebral cortex at post-natal day 1 and 3 (P1 and P3) and not detected at later stages P7 to P42 (Nathan et al. 2020). Different antibodies and procedures used by these studies can explain this post-natal detection, but it is also probable that different regions of the cortex may have different levels of Lin28a. Notably, Lin28a expression colocalizes at P1 and P3 with the terminal differentiation marker Neu by immunofluorescence, indicating that Lin28a expression is compatible with the expression of neuronal markers (Nathan et al. 2020).

Different protein isoforms have been described for Lin28a in mouse hippocampal cells (Amen et al. 2017) and for Lin28b in human hepatocarcinoma cells (Guo et al. 2006) and brain tadpoles in *Xenopus* (Faunes et al. 2017) (Fig. 2a, b). Importantly, the expression of the isoforms correlates with the differentiation of cells. The most characterized Lin28a isoform (25 kDa) is typically detected in undifferentiated cells but a different specific band around 37 kDa is observed in differentiated cells (Amen et al. 2017). Similarly, full length Lin28b (around 35 kDa), which is associated with undifferentiated cells, promotes proliferation and inhibits the biogenesis of *let-7* (Guo et al. 2006; Mizuno et al. 2018). In contrast, a short form of Lin28b

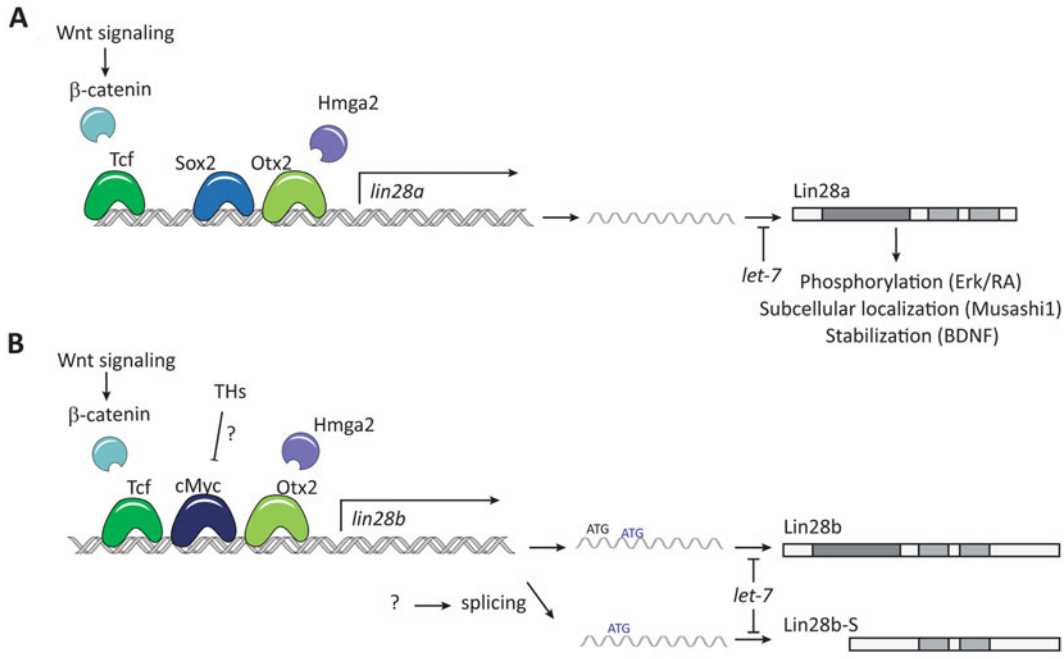


Fig. 2 Control of Lin28 expression and activity. Regulation of Lin28a (**a**) and Lin28b (**b**) at the transcriptional, post-transcriptional and post-translational levels. In the case of Lin28b, splicing generates two RNAs which are translated from different start codons

(Lin28b-S, around 22 kDa), which is detected in differentiated cells, is not able to promote proliferation or to regulate *let-7* biogenesis (Guo et al. 2006; Mizuno et al. 2018). Lin28b-S is generated by alternative splicing and starts with an open reading frame with a different start codon keeping the same downstream protein sequence (Fig. 2b, black "ATG" and blue "ATG"). These results indicate that alternative splicing controls the balance of isoforms during differentiation and that these isoforms may play different roles in progenitor and differentiated cells. In addition to alternative splicing, different *lin28b* transcripts can be generated from alternative promoter usage in hepatocarcinoma and medulloblastoma cells (Guo et al. 2018; Hovestadt et al. 2014). How different promoters of Lin28 are used and how splicing is regulated during embryogenesis has not been studied. These works suggest that proper levels of Lin28 isoforms may be crucial to control proliferation and differentiation. Thus, if Lin28a detected by immunofluorescence at post-natal stages corresponds to the 25 kDa or the 37 KDa isoform or both is unknown. The use of antibodies to detect specific segments of each isoform by immunofluorescence would complement the study of the differential expression of isoforms. In addition, it would be interesting to determine the stability and the molecular targets of each isoform of Lin28a and Lin28b in neural progenitor cells and neurons.

In the retina, another structure of the CNS, both Lin28a and Lin28b RNAs decrease from E12 to P3 (Xia et al. 2018). However, Lin28a shows a small but significant increase in adults compared to E18. A similar pattern is observed for Lin28b protein and no expression is detected at P1 and P3 (La Torre et al. 2013). Interestingly, Lin28a is also detected in neurons Neu+, similar to the cerebral cortex (Nathan et al. 2020). In summary, Lin28a and Lin28b are highly expressed in neural progenitors at early stages of embryogenesis, their expression decreases during development of the CNS, low levels are detected

after birth (including expression in neurons) and no expression is observed after P7.

3.2 Regulation of Lin28a and Lin28b in Neural Stem and Progenitor Cells

The decrease of Lin28a and Lin28b levels during development has been described in several tissues. However, the specific transcription factors and signaling pathways that regulate their expression *in vivo* have not been characterized.

Some factors that directly regulate Lin28 expression *in vitro* have been identified and are described here to compare with the expression and role of Lin28 *in vivo*. In mouse embryonic stem cells (ESCs), Lin28 expression is controlled by the core pluripotency network, including the transcription factors Oct4 and Nanog, which directly bind to Lin28 promoter (Marson et al. 2008). ESCs exist in functionally distinct states: ground, naïve and primed pluripotency, which are maintained in specific culture conditions, exhibit distinct properties, and represent different stages of their counterparts *in vivo* (Nichols and Smith 2009; Ying et al. 2008). Lin28 increases in the presence of serum (naïve state) and in the presence of FGF/activin (primed state) compared to ground state (Ghimire et al. 2018; Kalkan et al. 2017; Kumar et al. 2014; Marks et al. 2012; Parisi et al. 2017). This increase has been interpreted as a reinforcement of self-renewal under differentiation stimuli (serum or FGF/activin). However, it is also probable that this increase in Lin28 expression is required to change cell cycle parameters and accelerate proliferation as a first step in the differentiation program. Accordingly, Lin28a and Lin28b double KO (*lin28a*$^{-/-}$;*lin28b*$^{-/-}$) cells maintain a ground state morphology in the presence of FGF/Activin (Zhang et al. 2016) and high expression of *nanog* (Li et al. 2017), suggesting that the increase of Lin28 may be involved in the exit of ground pluripotency of ESCs *in vitro*. Consistent with this idea, Lin28 is expressed in proliferating cells and not in the basal stem cells in the adult intestine (Yang and Moss 2003). Furthermore, Lin28a expression is undetectable in muscle satellite stem cells, but it is expressed during myoblast differentiation *in vitro* (Polesskaya et al. 2007). Finally, Lin28a and Lin28b increase during reprogramming of fibroblasts to transit to a state similar to primed and naive states and then decrease at the ground state (Zhang et al. 2016). Altogether, these results indicate that Lin28a and Lin28b promote cell proliferation during commitment of ESCs, regeneration in adult tissues and reprogramming *in vitro*. Similarly, Lin28 may accelerate cell division *in vivo* to increase the number of neural progenitor cells, as discussed below.

The transcription factors Otx2 and HMGA2 also bind to promoters and increase the expression of Lin28a and Lin28b during the transition to the primed state (Parisi et al. 2017). In human neural stem cells, Sox2 binds to and controls the activation of the *lin28a* promoter (Cimadamore et al. 2013). In neuroblastoma cell lines and other human and mouse tumors, cMyc directly regulates Lin28b expression (Beckers et al. 2015; Chang et al. 2009). Otx2, HMGA2, Sox2 and cMyc are expressed during CNS development and, therefore, they may control the expression of Lin28a and Lin28b *in vivo* (Fig. 2).

The interplay of these transcription factors with signaling pathways in the control of Lin28 *in vivo* has not been studied. In adult mammalian retina, Wnt/β-catenin pathway regulates Lin28a and Lin28b expression after injury (Yao et al. 2016). In *Xenopus*, Lin28a and Lin28b are downregulated after FGF inhibition during early development (Faas et al. 2013). During *Xenopus* metamorphosis, Lin28b decreases in the brain while thyroid hormones (THs) increase (Faunes et al. 2017). However, if THs directly inhibit Lin28b expression has not been studied. Considering the important role of THs in nervous system development and the increase of THs that occur during late embryogenesis and the peri-natal period in mice (Barez-Lopez and Guadano-Ferraz 2017), it would be interesting to determine if THs regulate the expression of Lin28. THs directly inhibit the transcription of cMyc in neuroblastoma cells (Perez-Juste et al. 2000) and, therefore, THs may indirectly decrease the levels of Lin28 through downregulation of cMyc. In summary,

Wnt/β-catenin, FGF and THs may control the transcription of Lin28 genes, but further research is required to determine if these signaling pathways directly regulate Lin28 expression during neurogenesis.

Lin28 translation is repressed by the miRNA *let-7* in hepatocellular carcinoma and P19 cells (Guo et al. 2006; Wu and Belasco 2005). Consistent with this mutual inhibition between Lin28 and *let-7* in cell lines, *let-7* expression is detected in APs and increases during the development of the CNS in mice (Fairchild et al. 2019; Zhao et al. 2010) and in the brain and spinal cord during *Xenopus* metamorphosis (Faunes et al. 2017). Interestingly, in the neuroblast Ad12 HER10 cell line, the levels of *let-7* oscillate during the cell cycle with higher levels in G2/M compared to G1 (Fairchild et al. 2019). At E13.5, *let-7* expression is mainly detected at the basal side of the VZ and lower expression is detected at the apical side, indicating that *let-7* levels are dynamically controlled during the cell cycle, consistent with the interkinetic nuclear migration of neural progenitors in the VZ. However, this dynamic expression in the VZ is no longer observed at later stages and *let-7* is expressed at higher levels (Fairchild et al. 2019).

In addition to transcriptional and post-transcriptional regulation, Lin28 is regulated at the post-translational level (Fig. 2a). The brain-derived neurotrophic factor (BDNF) increases Lin28a expression through protein stabilization in primary murine hippocampal neurons prepared from P0 mice (Amen et al. 2017). This work suggests that Lin28a half-life is low or that Lin28a is synthesized at low levels at post-natal stages but that Lin28 levels can be modulated by extracellular signals. In P19 cells, Lin28a is phosphorylated at the serine 200 and cells expressing a Lin28a-S200A mutant form (phospho-deficient) show higher proliferation and reduced differentiation upon retinoic acid treatment, indicating that phosphorylation of Lin28a promotes differentiation (Liu et al. 2017). Furthermore, the cellular localization of Lin28 may also be modulated by other proteins such as Musashi1 in differentiation of ESCs (Kawahara et al. 2011). Interestingly, before E13.5, Lin28 localizes to the nucleolus and cytoplasm in the cerebral cortex and, after E13.5, the nucleolar localization is not detected (Herrlinger et al. 2019). These results indicate that Lin28a level and function are modulated by several post-translational mechanisms. It would be interesting to study how phosphorylation and subcellular localization together with transcriptional and splicing regulation are coordinated during neurogenesis *in vivo*. Detailed immunofluorescence analysis of Lin28 isoforms with specific antibodies and gene expression studies in specific cell populations isolated at different time points would give us insights into these mechanisms.

In summary, the complex regulation of splicing, translation, and post-translational modifications of Lin28 suggests that the balance of Lin28 (compared to other regulators such as *let-7*) is crucial for normal development. This balance may regulate the dynamics and type of cell division and the levels of regulators of cell cycle and factors that control commitment and differentiation.

4 Role of Lin28 in Proliferation and Cell Fate Decisions in the CNS

Several approaches have been used to study the role of Lin28a and Lin28b during CNS development. In this section, gain- and loss-of-function experiments are reviewed with emphasis on the developmental time when Lin28a or Lin28b are affected, the strategy used, and the read-out analyzed.

By using the Cre-lox system activated under the control of *nestin* promoter (i.e. active in AP cells), Lin28a was continuously expressed in the progeny of Nestin+ cells (Yang et al. 2015). In these *lin28a Tg; Nestin-Cre* animals, brain size and the cerebral cortex thickness increase at E18.5 compared to controls, consistent with an increase of proliferation detected at E15.5. This overexpression of Lin28a increases the number of Pax6+ cells and decreases the number of Tbr2+ cells compared to controls, indicating an imbalance between APs (Pax6+) and IPCs (Tbr2+). Interestingly, this phenotype is identical to the

obtained after the overexpression of a stabilized form of β-catenin under the control of an enhancer of *nestin* (Wrobel et al. 2007), suggesting that Lin28a may be one the downstream genes of Wnt/β-catenin in APs, similar to the control in the mammalian adult retina, as mentioned above (Yao et al. 2016).

Overexpression of Lin28a has also been done by *in utero* electroporation at E14.5. In this case, plasmids carrying Lin28a-GFP under the control of the constitutive promoter CAG were injected in the right ventricle and cortical cells were harvested 2 days after electroporation (at E16.5) and cultured *in vitro* (Bhuiyan et al. 2013). At day *in vitro* (DIV) 0, the number of proliferating cells (Ki67+) is higher in Lin28a overexpressing cells compared to controls. In addition, Lin28a overexpression prevents apoptosis after 13 DIV. It is important to mention that in these conditions, Lin28a-GFP colocalizes with the neuronal marker Neu+, indicating that Lin28a overexpression is also compatible with the expression of neuronal markers *in vitro*. Consistent with this effect, overexpression of Lin28a in Sox2-knockdown neural stem cells rescues the defects in proliferation and the expression of the neuronal differentiation, Tuj1 (Cimadamore et al. 2013). Interestingly, Lin28 overexpression does not rescue the expression of the neuronal marker MAP2, suggesting that some functions of Sox2 are independent of Lin28a or, alternatively, overexpression of Lin28a prevents full terminal differentiation. Accordingly, morphology and action potential analysis of neurons of post-natal animals that overexpress Lin28a from E14.5 are altered compared to controls, indicating that continuous presence of Lin28a affects terminal differentiation (Jang et al. 2019). Considering the changes in membrane potential during development, it would be interesting to study the molecular link between Lin28 and its targets and membrane potential in progenitors. Furthermore, Lin28a overexpressing animals show memory deficits determined in water maze tests (Jang et al. 2019). These results indicate that it is important to consider functional analyses in addition to the study of expression of progenitor and neuronal markers.

Altogether these experiments show that Lin28 induces neurogenesis *in vivo* probably by expansion of early APs when Lin28a and Lin28b are expressed at higher levels. In P19 cells that stably overexpress Lin28a or Lin28b, RA-induced differentiation towards neurons is preferred over glia, based on Tuj1 and GFAP expression, respectively (Balzer et al. 2010). Although a direct neurogenesis-inducer role of Lin28a or Lin28b cannot be ruled-out, another possibility is that Lin28a and Lin28b control cell cycle parameters to favor rapid divisions like those seen in early APs that produce neurons (see below).

In contrast to the effect on proliferation induced by overexpression of Lin28a during embryonic neurogenesis, Lin28a overexpression during postnatal stages by electroporation of plasmids into the lateral ventricle at day P0 reduced the number of neurons in the olfactory bulb and has no effect on proliferation studied by the Ki67 marker 3 days after electroporation (Romer-Seibert et al. 2019). In this experimental setup, Lin28a overexpression decreases the number of Sox2+ cells in the SVZ and increases the number of Doublecortin (DCX) + cells, suggesting that Lin28a is converting APs into neuroblasts and decreasing the pool of progenitors. The reason why Lin28a in these conditions has no effect on proliferation is not clear. One possibility is that APs at postnatal stages are intrinsically different to APs present at early stages of embryogenesis. In addition, the extracellular environment is different and antiproliferative signals present at post-natal stages probably prevent amplifying divisions of APs. Interestingly, despite the increase of DCX+ cells, the final number of neurons and astrocytes in the olfactory bulb in Lin28a-overexpressing animals is reduced compared to controls and the proportion of neuronal subtypes is altered. If this fate depends on cell cycle length and if Lin28a plays a role in this parameter is unknown. Lin28a overexpression may also affect migration, similar to the effect of gain-of-function of Wnt3 (Wrobel et al. 2007).

The absence of effect of Lin28 overexpression on proliferation in post-natal or adult stages has also been described in other biological contexts. In the retina, transfection of Lin28 at E16 increases the number of Brn3+ cells (early fate)

compared to controls but the transfection of Lin28 at P1 has no effect on the number of Brn3 + cells. Similarly, Lin28b upregulation has no effect in proliferation, ganglion size, and *let-7* expression during early postnatal development (Hennchen et al. 2015). Consistent with a possible anti-proliferative environment at post-natal and adult stages, damage in the nervous system induces the expression of Lin28 to promote regeneration or repair in some contexts (see below). These experiments suggest that Lin28 accelerates proliferation when extracellular proliferative signals are present (or anti-proliferative signals are reduced), but Lin28 is not sufficient to induce proliferation. This ability of Lin28 to promote rapid cell divisions is thought to be important for reprogramming of fibroblast to iPSCs (Hanna et al. 2009; Zhang et al. 2016). Altogether, these results indicate that the role of Lin28 *in vivo* is incompletely understood and future studies on the differences among embryonic and post-natal progenitors, the effect on proliferation, cell fate decision, migration, maturation and neuronal function may reveal new aspects of the function of Lin28.

Consistent with the effect of gain-of-function of Lin28 on early neurogenesis, Lin28a/b double knockout (dKO) mice show reduced proliferation in the neuroepithelium at E9.5 and in the VZ of the cerebral cortex at E11.5 compared to wild-type animals (Herrlinger et al. 2019). Importantly, the number of Tuj1+ cells is higher in dKO animals compared to controls, suggesting precocious differentiation, and indicating that Lin28a or Lin28b are not required to initiate the neuronal differentiation program. dKO animals present neural tube closure defects and die before birth. In contrast to dKO animals, no defects are observed in single Lin28b KO mutants. In Lin28a KO mutants, brain and other organs are smaller compared to controls at P1 (Yang et al. 2015). In Lin28a KO animals, proliferation in the cerebral cortex is reduced compared to controls at E15.5. Interestingly, the number of Pax6+ cells and Tbr2+ cells is significantly lower in Lin28a KO animals compared to controls at E17.5 and P1 in the cerebral cortex but no difference is detected at E15.5. These defects are more severe when one allele of Lin28b is lost in Lin28a KO mutants (*lin28a*$^{-/-}$; *lin28b*$^{+/-}$), suggesting that Lin28a and Lin28b play overlapping roles in CNS development. Thus, Lin28a and Lin28b are required for the maintenance and expansion of APs during the development of the CNS (Herrlinger et al. 2019; Yang et al. 2015).

In summary, Lin28a overexpression during embryogenesis increases the proliferation of APs but does not prevent the expression of neuronal markers. However, terminal differentiation seems to be affected by the continuous presence of Lin28a. Considering the normal morphology of animals that overexpress Lin28a, these results show that the differentiation program starts normally and overcomes the ectopic expression of Lin28a, probably due to post-translational modifications that inhibit the function of Lin28a. Consistently, Lin28a and Lin28b are required for normal proliferation during development, as shown by single Lin28a KO and dKO mutants. In the absence of Lin28a and Lin28b, neuronal differentiation occurs preciously but the neural tube is not closed, and animals die during embryogenesis probably due to defects in several tissues.

5 Molecular Mechanisms Underlying the Role of Lin28 in the CNS

Lin28a and Lin28b regulate the translation of mRNAs in the cytosol and inhibit the processing of the *let-7* precursor in the nucleus and cytosol (Tsialikas and Romer-Seibert 2015). Several genes are known to be directly regulated by Lin28 in stem cells, tumors and cell lines *in vitro*, including *cyclins*, *cyclin-dependent kinases* (*cdks*), *insulin growth factor 2* (*igf2*), ribosomal proteins, glycolytic enzymes and mitochondrial enzymes (Balzeau et al. 2017; Hafner et al. 2013; Peng et al. 2011; Polesskaya et al. 2007; Shyh-Chang et al. 2013; Xu et al. 2009; Zhu et al. 2011). In addition, by inhibiting the biogenesis of *let-7*, Lin28 indirectly controls the levels of the *let-7* target genes *Myc*, *Ras*, *Cyclin D1*, *cyclin D2*, *Hmga2*, among others (Shyh-Chang and Daley 2013). Therefore, the module

Lin28/*let-7* is a global regulator of genes related to metabolism, protein synthesis and cell cycle and the balance between Lin28 and *let-7* is crucial for proliferation and differentiation in progenitor cells. In this section, genes and processes regulated by Lin28 in the CNS and neural stem cell lines are briefly described.

In the neural stem cell line NE-4C (established from cerebral vesicle of E9 mice), Lin28a binds mRNAs of *Imp1* (*IGF2 mRNA-binding protein 1*, also known as *Igf2bp1*), *IGF2* and *Hmga2* (*high-mobility group AT-hook 2*) (Yang et al. 2015). Accordingly, Lin28a overexpression up-regulates IGF2 and HMGA2 in culture neurons after *in utero* electroporation (Bhuiyan et al. 2013; Jang et al. 2019) and knockdown using RNAi for Lin28 decrease the levels of the receptor of IGF1 (IGF1R) and HMGA2 (Jang et al. 2019). *In vivo*, IGF1R and HMGA2 protein levels decrease in progenitor cells isolated from cerebral cortex of $lin28a^{-/-};lin28b^{+/-}$ animals compared to wild-type animals (Yang et al. 2015). This effect of Lin28 on Imp1, IGF2, IGF1R indicates that Lin28 regulates IGF2-mTOR signaling. Consistently, the activation of this pathway induced by IGF2 is decreased in cerebral cortex dissected at E12.5 of Lin28a mutant animals compared to wild-type animals (Yang et al. 2015). In addition, phosphorylation of the S6 ribosome protein, a readout of Igf2-mTOR signaling, is reduced in progenitor cells of Lin28a KO animals compared to controls at P1. This reduction in Igf2-mTOR signaling is also observed in the brain of $lin28a^{-/-}b^{+/-}$ animals at E14.5 (Yang et al. 2015). These results confirm that mTOR signaling is directly regulated by Lin28 in the CNS.

HMGA2 is also directly regulated by Lin28. HMGA2 is a chromatin-associated protein that controls transcription and is widely expressed in undifferentiated cells. HMGA2 is enriched in early neural progenitors (Telley et al. 2019) and promotes self-renewal by decreasing the levels of the tumor suppressors $p16^{Ink4a}$ and $p19^{Arf}$ (Nishino et al. 2008). Therefore, in addition to directly promote cell proliferation through regulation of cyclins, Lin28 favors proliferation through regulation of HMGA2 and the levels of tumor suppressors. However, it has also been described that Lin28a represses the translation of HMGA2 in primed ESCs and HMGA2 cooperates with Otx2 to increase the expression of Lin28 (Parisi et al. 2017). Considering that both Lin28 and HMGA2 are targets of *let-7*, this evidence indicates that the levels of Lin28, HMGA2 and *let-7* are tightly balanced during proliferation and differentiation in the CNS.

Consistent with the proliferation induced by Lin28 overexpression, mis-expression of *let-7* reduces proliferation of neural stem cells *in vitro* (Cimadamore et al. 2013). Overexpression of *let-7* decreases the number of cells in the S phase in Ad12 HER10 cells, in cortical stem cells isolated from E14.5 brain and in primary cultures of E11.5 cortex and lengthens the cell cycle in Ad12 HER10 cells (Fairchild et al. 2019). Accordingly, knockdown of *let-7* with antagomirR shortens the cell cycle in these cultures, indicating that *let-7* controls proliferation and cell cycle exit in neural progenitors. These results indicate that Lin28 and *let-7* form a regulatory loop that mutually controls their levels and the levels of their target genes and, consequently, the dynamic of the cell cycle. It would be interesting to determine if the levels of Lin28 and HMGA2 change during the cell cycle.

Lin28a modulates translation both positively and negatively (Cho et al. 2012). To determine which of these activities is predominant during the development of the CNS, a genetic analysis using a mouse containing a hypomorphic allele of the ribosomal protein L24 ($Rpl24^{Bst/+}$) was performed. $Rpl24^{Bst/+}$ mice show reduced global protein synthesis. Whereas $Lin28a^{-/-}$ and $Rpl24^{Bst/+}$ animals do not show neural closure defects at E11.5, $lin28a^{-/-};Rpl24^{Bst/+}$ animals present open neural tubes, similar to Lin28 dKO embryos (Herrlinger et al. 2019), suggesting that the predominant activity of Lin28 during CNS development is to promote translation. Consistent with this role, $Rpl24^{Bst/+}$ rescues the increase in brain size and the defect in the ratio of Pax6+/Tbr2+ cells observed in animals that overexpress Lin28a. Altogether, these results indicate that Lin28a mainly promotes translation in neural progenitors.

Transcriptome analysis between E11.5 neuroepithelium from wild-type and Lin28 dKO

animals show that only 15 genes decrease and only 19 increase in mutants. In contrast, the analysis of mRNAs associated with polysomes show that 368 genes decrease, and 187 genes increase in the mutants compared to wild-type animals (Herrlinger et al. 2019). Gene Ontology (GO) analysis indicated that categories related to ribosome biogenesis and protein synthesis decreased and categories related to neurotransmitter complexes and the post-synapse increased in mutants. All these results are consistent with the role of Lin28 in promoting translation of genes associated to protein synthesis and a role in blocking translation of some genes associated to differentiation during CNS development.

The cellular and molecular effects of gain- and loss-of-functions experiments described here indicate that the role of Lin28 in CNS is directly linked to cell division and global protein synthesis (Fig. 3a). Lin28 may contribute to the control the dynamics of the cell cycle of early APs (when is highly expressed during neurogenesis), promoting symmetric over asymmetric cell divisions. Consequently, overexpression of Lin28 alters the timing of differentiation without preventing the continuous activation of commitment and differentiation factors (Fig. 3b). This alteration in timing has also been observed for overexpression of cdk4/cyclin D1 (Lange et al. 2009) and loss of p27 (Durand et al. 1998).

Based on estimations of cell cycle length and type of divisions (Takahashi et al. 1995, 1996), it can be proposed that Lin28 is part of a module that determines a threshold, which must be reached to trigger the transition to symmetric to asymmetric divisions (Fig. 3b). This threshold probably decreases during neurogenesis as Lin28 levels- or activity- decreases. Increasing the levels of Lin28 in APs (for example, using the *Nestin* promoter) would increase the threshold and may induce a couple of one or two rounds of symmetric divisions instead of asymmetric divisions between E11 and E14 increasing the number of progenitor cells. If commitment and differentiation factors levels or activity continuously increase, consistent with the idea that transcriptional programs are independent of cell cycle (Okamoto et al. 2016), this threshold is reached, but a couple of divisions after the normal timing (Fig. 3b). After this threshold is reached, an asymmetric division is produced (to generate a neuron in direct neurogenesis or an IPC in indirect neurogenesis) and the excess of Lin28 would not affect the program of expression of neuronal markers, consistent with increase in brain size and the higher proportion of Pax6+ cells over Tbr2+ cells observed in animals that overexpress Lin28 (Yang et al. 2015). This proposal is consistent with the delay observed in other biological contexts after Lin28 overexpression, probably due to the effect on crucial tissues (Faunes et al. 2017; Moss et al. 1997; Zhu et al. 2010).

This idea of Lin28 being part of a module that determines a threshold for asymmetric divisions in APs is consistent with the "cell cycle length hypothesis", which proposes that a certain period of time is required for the neurogenic factors to trigger differentiation (Calegari and Huttner 2003; Götz and Huttner 2005). In this cell cycle length hypothesis, lengthening the cell cycle, mainly in G1, favors the exit of the cell cycle because there is enough time for differentiation factors to act and the threshold can be reached. Shorter cell cycles prevent that the threshold is reached causing progenitor cells to proliferate. Under this scenario, Lin28 overexpression may favor short cell cycles, preventing reaching of the threshold at the normal timing (consistent with the opposite effect of *let-7* overexpression). However, in this case, it is also necessary that commitment or differentiation factors continuously increase even after cell divisions, so the threshold be reached, but at later divisions compared to the normal development. This idea is also consistent with the decreased proliferation and precocious differentiation observed in Lin28 KO animals. In this case, the threshold to trigger asymmetric division is set below the normal level and the number of progenitor cells is low to allow the normal closure of the neural tube and differentiation begins earlier. How this threshold works at the molecular level, how is linked to cell cycle dynamics, signaling and metabolic pathways and how the rate of these processes is affected in the absence or excess of Lin28 is unknown. Future quantitative measurements of Lin28 levels in

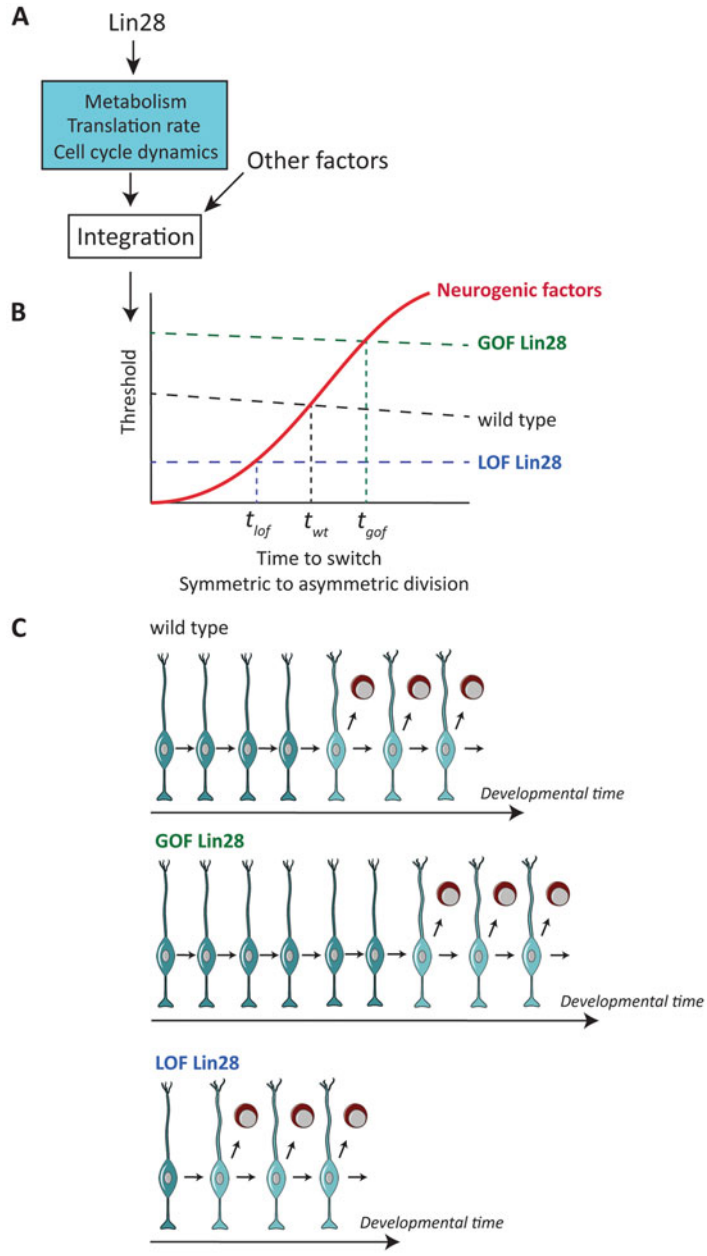


Fig. 3 Model of action of Lin28 in neural progenitors. (**a**) Lin28 regulates the levels of metabolic enzymes, proteins involved in translation and regulators of the cell cycle that must be are coordinated with other factors to set a threshold that switch from symmetric to asymmetric division in a single progenitor cell. (**b**) The genetic network that controls the expression of commitment and neurogenic factors works independently of cell cycle and Lin28, and the level of these factors continuously increases. When the threshold is reached, the progenitor cell will divide asymmetrically to generate a neuron or an intermediate progenitor. In this scheme, the wild type threshold is proposed to decrease during development as Lin28 decreases. However, it is also possible that other levels keep the threshold at the same level during embryogenesis. In gain-of-function of Lin28 (GOF Lin28, green), the threshold is higher than the normal level, so the switch occurs later. In loss-of-function of Lin28 (LOF Lin28, blue), the threshold is lower than the normal level, so the switch occurs earlier. (**c**) The consequence of these changes is an excess of progenitor cells in GOF Lin28 and precocious differentiation with lower number of progenitor cells in LOF Lin28 relative to the wild type

single cells and during cell cycle together with studies on cell cycle length, division patterns and the fate of the progeny after Lin28 overexpression with different promoters or during specific temporal windows would be useful to address these questions.

6 Reactivation of Lin28 in NS Regeneration and Misregulation in Diseases

Lin28 expression is not detected in most adult tissues (Yang and Moss 2003). As mentioned above, Lin28a is not expressed in intestinal basal stem cells but is expressed in proliferating cells (transit amplifying cells). Lin28 is also expressed in skeletal muscle and *in vitro* studies in myoblasts have shown that Lin28a is required for muscle differentiation (Polesskaya et al. 2007). This evidence suggests that Lin28 expression is required in tissues undergoing normal proliferation and repair, such as the intestine and the muscle.

Lin28a also increases after damage in the NS. In the peripheral NS of mice, Lin28a and Lin28b increase in the L4/5 dorsal root ganglia (DRG) after sciatic nerve axotomy (Wang et al. 2018). Importantly, overexpression of Lin28a specifically in the L4/5 DRG and ubiquitous and inducible overexpression of Lin28b promote axon regeneration in this experimental model. Consistent with a role of Lin28 in axon regeneration, simultaneous knock-down of Lin28a and Lin28b decreases axon regeneration compared to controls (Wang et al. 2018).

Overexpression of Lin28a or Lin28b also promotes regeneration in the CNS after optic nerve crush (Nathan et al. 2020; Wang et al. 2018), retinal injury (Yao et al. 2016; Zhang et al. 2019) and spinal cord injury (Nathan et al. 2020). In the retina, Lin28 promotes proliferation of Müller glia (Yao et al. 2016). This role of Lin28 in the regeneration of the retina has been also described in zebrafish (Gorsuch et al. 2017; Mitra et al. 2019; Ramachandran et al. 2010). Altogether, all these works indicate that Lin28 is re-activated after damage and promotes regeneration in the mammalian NS.

In contrast to the regulated re-activation of Lin28 under repair and regeneration, Lin28 is aberrantly upregulated in cancer and other diseases (Balzeau et al. 2017; Thornton and Gregory 2012; Viswanathan et al. 2009). Specifically in the NS, misregulated expression of Lin28 is observed in neuroblastoma (Beckers et al. 2015; Hennchen et al. 2015; Molenaar et al. 2012) and medulloblastoma (Hovestadt et al. 2014). In addition to tumors, increased expression of Lin28 is detected in Rett Syndrome due to downregulation of MECP2 (Kim et al. 2019). In contrast, conditional knockout of Lin28a in mice results in degeneration of midbrain-type dopamine neurons (Chang et al. 2019). These works indicate that the regulation of Lin28 levels is critical for normal homeostasis of the NS in adults. Understanding the mechanisms underlying the control of expression and activity of Lin28 and its downstream genes may provide strategies to restore normal levels in these contexts.

7 Perspectives

The detailed analysis of the endogenous expression and the role of Lin28 *in vivo* are still incomplete. The balance of Lin28 with commitment and differentiation factors seems to be crucial for the decision between proliferate and differentiate, as described in other contexts (Radzisheuskaya et al. 2013; Sansom et al. 2009). Different splice isoforms of Lin28 and post-translational regulation add new layers of complexity to this process. Progressive changes in intrinsic molecular programs in progenitors must be coordinated with Lin28-regulated genes and with the factors that control the duration of cell cycle, which it has been proposed to regulate these decisions. To determine the dynamics of these changes during the cell cycle and neurogenesis through single cell analyses, real time reporters and pulse-label approaches together with a detailed quantitative

analysis of different isoforms of Lin28 will give us new insights into the cellular and molecular role of Lin28 during embryogenesis.

Acknowledgements I thank Dr. Carlos Oliva and Dr. Gonzalo Cordova for critical reading of the manuscript. Images were adapted from Illustration Toolkits Biology and Molecular Cell Biology by Motifolio.

References

Ambros V, Horvitz HR (1984) Heterochronic mutants of the nematode Caenorhabditis elegans. Science 226:409–416

Amen AM, Ruiz-Garzon CR, Shi J, Subramanian M, Pham DL, Meffert MK (2017) A rapid induction mechanism for Lin28a in trophic responses. Mol Cell 65 (490–503):e497

Balzeau J, Menezes MR, Cao S, Hagan JP (2017) The LIN28/let-7 pathway in Cancer. Front Genet 8:31

Balzer E, Heine C, Jiang Q, Lee VM, Moss EG (2010) LIN28 alters cell fate succession and acts independently of the let-7 microRNA during neurogliogenesis in vitro. Development 137:891–900

Barez-Lopez S, Guadano-Ferraz A (2017) Thyroid hormone availability and action during brain development in rodents. Front Cell Neurosci 11:240

Beckers A, Van Peer G, Carter DR, Gartlgruber M, Herrmann C, Agarwal S, Helsmoortel HH, Althoff K, Molenaar JJ, Cheung BB, Schulte JH, Benoit Y, Shohet JM, Westermann F, Marshall GM, Vandesompele J, De Preter K, Speleman F (2015) MYCN-driven regulatory mechanisms controlling LIN28B in neuroblastoma. Cancer Lett 366:123–132

Bhuiyan MI, Lee JH, Kim SY, Cho KO (2013) Expression of exogenous LIN28 contributes to proliferation and survival of mouse primary cortical neurons in vitro. Neuroscience 248:448–458

Calegari F, Huttner WB (2003) An inhibition of cyclin-dependent kinases that lengthens, but does not arrest, neuroepithelial cell cycle induces premature neurogenesis. J Cell Sci 116:4947–4955

Cardenas A, Borrell V (2020) Molecular and cellular evolution of corticogenesis in amniotes. Cell Mol Life Sci 77:1435–1460

Chang TC, Zeitels LR, Hwang HW, Chivukula RR, Wentzel EA, Dews M, Jung J, Gao P, Dang CV, Beer MA, Thomas-Tikhonenko A, Mendell JT (2009) Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation. Proc Natl Acad Sci U S A 106:3384–3389

Chang MY, Oh B, Choi JE, Sulistio YA, Woo HJ, Jo A, Kim J, Kim EH, Kim SW, Hwang J, Park J, Song JJ, Kwon OC, Henry Kim H, Kim YH, Ko JY, Heo JY, Lee MJ, Lee M, Choi M, Chung SJ, Lee HS, Lee SH (2019) LIN28A loss of function is associated with Parkinson's disease pathogenesis. EMBO J 38: e101196

Cho J, Chang H, Kwon SC, Kim B, Kim Y, Choe J, Ha M, Kim YK, Kim VN (2012) LIN28A is a suppressor of ER-associated translation in embryonic stem cells. Cell 151:765–777

Cimadamore F, Amador-arjona A, Chen C, Huang C-T, Terskikh AV (2013) SOX2–LIN28/let-7 pathway regulates proliferation and neurogenesis in neural precursors. Proceedings of the National Academy of Sciences 2013

Durand B, Fero ML, Roberts JM, Raff MC (1998) p27Kip1 alters the response of cells to mitogen and is part of a cell-intrinsic timer that arrests the cell cycle and initiates differentiation. Curr Biol 8:431–440

Faas, L., Warrander, F.C., Maguire, R., Ramsbottom, S. A., Quinn, D., Genever, P., Isaacs, H.V., 2013. Lin28 proteins are required for germ layer specification in Xenopus. Development (Cambridge, England) 140, 976–986

Fairchild CLA, Cheema SK, Wong J, Hino K, Simo S, La Torre A (2019) Let-7 regulates cell cycle dynamics in the developing cerebral cortex and retina. Sci Rep 9:15336

Faunes F, Gundermann DG, Munoz R, Bruno R, Larrain J (2017) The heterochronic gene Lin28 regulates amphibian metamorphosis through disturbance of thyroid hormone function. Dev Biol 425:142–151

Florio M, Huttner WB (2014) Neural progenitors, neurogenesis and the evolution of the neocortex. Development 141:2182–2194

Ghimire S, Van der Jeught M, Neupane J, Roost MS, Anckaert J, Popovic M, Van Nieuwerburgh F, Mestdagh P, Vandesompele J, Deforce D, Menten B, Chuva de Sousa Lopes S, De Sutter P, Heindryckx B (2018) Comparative analysis of naive, primed and ground state pluripotency in mouse embryonic stem cells originating from the same genetic background. Sci Rep 8:5884

Gonzalez-Itier S, Contreras EG, Larrain J, Glavic A, Faunes F (2018) A role for Lin-28 in growth and metamorphosis in Drosophila melanogaster. Mech Dev 154:107–115

Gorsuch RA, Lahne M, Yarka CE, Petravick ME, Li J, Hyde DR (2017) Sox2 regulates Muller glia reprogramming and proliferation in the regenerating zebrafish retina via Lin28 and Ascl1a. Exp Eye Res 161:174–192

Götz M, Huttner WB (2005) The cell biology of neurogenesis. Nat Rev Mol Cell Biol 6:777–788

Gundermann DG, Martinez J, De Kervor G, Gonzalez-Pinto K, Larrain J, Faunes F (2019) Overexpression of Lin28a delays Xenopus metamorphosis and down-regulates albumin independently of its translational regulation domain. Dev Dynamics 248:969–978

Guo Y, Chen Y, Ito H, Watanabe A, Ge X, Kodama T, Aburatani H (2006) Identification and characterization of lin-28 homolog B (LIN28B) in human hepatocellular carcinoma. Gene 384:51–61

Guo W, Hu Z, Bao Y, Li Y, Li S, Zheng Q, Lyu D, Chen D, Yu T, Li Y, Zhu X, Ding J, Zhao Y, He X, Huang S (2018) A LIN28B tumor-specific transcript in cancer. Cell Rep 22:2016–2025

Hafner M, Max KE, Bandaru P, Morozov P, Gerstberger S, Brown M, Molina H, Tuschl T (2013) Identification of mRNAs bound and regulated by human LIN28 proteins and molecular requirements for RNA recognition. RNA 19:613–626

Hanna J, Saha K, Pando B, van Zon J, Lengner CJ, Creyghton MP, van Oudenaarden A, Jaenisch R (2009) Direct cell reprogramming is a stochastic process amenable to acceleration. Nature 462:595–601

Hennchen M, Stubbusch J, Abarchan-El Makhfi I, Kramer M, Deller T, Pierre-Eugene C, Janoueix-Lerosey I, Delattre O, Ernsberger U, Schulte JB, Rohrer H (2015) Lin28B and Let-7 in the control of sympathetic neurogenesis and neuroblastoma development. J Neurosci 35:16531–16544

Herrlinger S, Shao Q, Yang M, Chang Q, Liu Y, Pan X, Yin H, Xie LW, Chen JF (2019) Lin28-mediated temporal promotion of protein synthesis is crucial for neural progenitor cell maintenance and brain development in mice. Development 146

Hovestadt V, Jones DT, Picelli S, Wang W, Kool M, Northcott PA, Sultan M, Stachurski K, Ryzhova M, Warnatz HJ, Ralser M, Brun S, Bunt J, Jager N, Kleinheinz K, Erkek S, Weber UD, Bartholomae CC, von Kalle C, Lawerenz C, Eils J, Koster J, Versteeg R, Milde T, Witt O, Schmidt S, Wolf S, Pietsch T, Rutkowski S, Scheurlen W, Taylor MD, Brors B, Felsberg J, Reifenberger G, Borkhardt A, Lehrach H, Wechsler-Reya RJ, Eils R, Yaspo ML, Landgraf P, Korshunov A, Zapatka M, Radlwimmer B, Pfister SM, Lichter P (2014) Decoding the regulatory landscape of medulloblastoma using DNA methylation sequencing. Nature 510:537–541

Jang HJ, Kim JY, Kim SY, Cho KO (2019) Persistent Lin28 expression impairs neurite outgrowth and cognitive function in the developing mouse neocortex. Mol Neurobiol 56:3780–3795

Kalkan T, Olova N, Roode M, Mulas C, Lee HJ, Nett I, Marks H, Walker R, Stunnenberg HG, Lilley KS, Nichols J, Reik W, Bertone P, Smith A (2017) Tracking the embryonic stem cell transition from ground state pluripotency. Development 144:1221–1234

Kawahara H, Okada Y, Imai T, Iwanami A, Mischel PS, Okano H (2011) Musashi1 cooperates in abnormal cell lineage protein 28 (Lin28)-mediated let-7 family microRNA biogenesis in early neural differentiation. J Biol Chem 286:16121–16130

Kim JJ, Savas JN, Miller MT, Hu X, Carromeu C, Lavallee-Adam M, Freitas BCG, Muotri AR, Yates JR 3rd, Ghosh A (2019) Proteomic analyses reveal misregulation of LIN28 expression and delayed timing of glial differentiation in human iPS cells with MECP2 loss-of-function. PLoS One 14:e0212553

Kumar RM, Cahan P, Shalek AK, Satija R, DaleyKeyser A, Li H, Zhang J, Pardee K, Gennert D, Trombetta JJ, Ferrante TC, Regev A, Daley GQ, Collins JJ (2014) Deconstructing transcriptional heterogeneity in pluripotent stem cells. Nature 516:56–61

La Torre A, Georgi S, Reh TA (2013) Conserved microRNA pathway regulates developmental timing of retinal neurogenesis. Proc Natl Acad Sci U S A 110:E2362–E2370

Lange C, Huttner WB, Calegari F (2009) Cdk4/cyclinD1 overexpression in neural stem cells shortens G1, delays neurogenesis, and promotes the generation and expansion of basal progenitors. Cell Stem Cell 5:320–331

Li MA, Amaral PP, Cheung P, Bergmann JH, Kinoshita M, Kalkan T, Ralser M, Robson S, von Meyenn F, Paramor M, Yang F, Chen C, Nichols J, Spector DL, Kouzarides T, He L, Smith A (2017) A lncRNA fine tunes the dynamics of a cell state transition involving Lin28, let-7 and de novo DNA methylation. elife 6

Liu X, Chen M, Li L, Gong L, Zhou H, Gao D (2017) Extracellular signal-regulated kinases (ERKs) phosphorylate Lin28a protein to modulate P19 cell proliferation and differentiation. J Biol Chem 292:3970–3976

Marks H, Kalkan T, Menafra R, Denissov S, Jones K, Hofemeister H, Nichols J, Kranz A, Stewart AF, Smith A, Stunnenberg HG (2012) The transcriptional and epigenomic foundations of ground state pluripotency. Cell 149:590–604

Marson A, Foreman R, Chevalier B, Bilodeau S, Kahn M, Young Ra, Jaenisch R (2008) Wnt signaling promotes reprogramming of somatic cells to pluripotency. Cell Stem Cell 3:132–135

Mitra S, Sharma P, Kaur S, Khursheed MA, Gupta S, Chaudhary M, Kurup AJ, Ramachandran R (2019) Dual regulation of lin28a by Myc is necessary during zebrafish retina regeneration. J Cell Biol 218:489–507

Mizuno R, Chatterji P, Andres S, Hamilton K, Simon L, Foley SW, Jeganathan A, Gregory BD, Madison B, Rustgi AK (2018) Differential regulation of LET-7 by LIN28B isoform-specific functions. Mol Cancer Res: MCR 16:403–416

Molenaar JJ, Domingo-Fernandez R, Ebus ME, Lindner S, Koster J, Drabek K, Mestdagh P, van Sluis P, Valentijn LJ, van Nes J, Broekmans M, Haneveld F, Volckmann R, Bray I, Heukamp L, Sprussel A, Thor T, Kieckbusch K, Klein-Hitpass L, Fischer M, Vandesompele J, Schramm A, van Noesel MM, Varesio L, Speleman F, Eggert A, Stallings RL, Caron HN, Versteeg R, Schulte JH (2012) LIN28B induces neuroblastoma and enhances MYCN levels via let-7 suppression. Nat Genet 44:1199–1206

Moss EG, Tang L (2003) Conservation of the heterochronic regulator Lin-28, its developmental expression and microRNA complementary sites. Dev Biol 258:432–442

Moss EG, Lee RC, Ambros V (1997) The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. Cell 88:637–646

Nathan FM, Ohtake Y, Wang S, Jiang X, Sami A, Guo H, Zhou FQ, Li S (2020) Upregulating Lin28a promotes axon regeneration in adult mice with optic nerve and spinal cord injury. Mol Ther 28:1902–1917

Nichols J, Smith A (2009) Naive and primed pluripotent states. Cell Stem Cell 4:487–492

Nishino J, Kim I, Chada K, Morrison SJ (2008) Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf expression. Cell 135:227–239

Okamoto M, Miyata T, Konno D, Ueda HR, Kasukawa T, Hashimoto M, Matsuzaki F, Kawaguchi A (2016) Cell-cycle-independent transitions in temporal identity of mammalian neural progenitor cells. Nat Commun 7:11349

Ouchi Y, Yamamoto J, Iwamoto T (2014) The heterochronic genes lin-28a and lin-28b play an essential and evolutionarily conserved role in early zebrafish development. PLoS One 9:e88086

Paridaen JT, Huttner WB (2014) Neurogenesis during development of the vertebrate central nervous system. EMBO Rep 15:351–364

Parisi S, Passaro F, Russo L, Musto A, Navarra A, Romano S, Petrosino G, Russo T (2017) Lin28 is induced in primed embryonic stem cells and regulates let-7-independent events. FASEB J 31:1046–1058

Peng S, Chen LL, Lei XX, Yang L, Lin H, Carmichael GG, Huang Y (2011) Genome-wide studies reveal that Lin28 enhances the translation of genes important for growth and survival of human embryonic stem cells. Stem Cells 29:496–504

Perez-Juste G, Garcia-Silva S, Aranda A (2000) An element in the region responsible for premature termination of transcription mediates repression of c-myc gene expression by thyroid hormone in neuroblastoma cells. J Biol Chem 275:1307–1314

Polesskaya A, Cuvellier S, Naguibneva I, Duquet A, Moss EG, Harel-Bellan A (2007) Lin-28 binds IGF-2 mRNA and participates in skeletal myogenesis by increasing translation efficiency. Genes Dev 21:1125–1138

Radzisheuskaya A, Chia Gle B, dos Santos RL, Theunissen TW, Castro LF, Nichols J, Silva JC (2013) A defined Oct4 level governs cell state transitions of pluripotency entry and differentiation into all embryonic lineages. Nat Cell Biol 15:579–590

Ramachandran R, Fausett BV, Goldman D (2010) Ascl1a regulates Muller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. Nat Cell Biol 12:1101–1107

Romer-Seibert JS, Hartman NW, Moss EG (2019) The RNA-binding protein LIN28 controls progenitor and neuronal cell fate during postnatal neurogenesis. FASEB J 33:3291–3303

Rougvie AE (2001) Control of developmental timing in animals. Nat Rev Genet 2:690–701

Sansom SN, Griffiths DS, Faedo A, Kleinjan DJ, Ruan Y, Smith J, van Heyningen V, Rubenstein JL, Livesey FJ (2009) The level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. PLoS Genet 5:e1000511

Shinoda G, Shyh-Chang N, Soysa TY, Zhu H, Seligson MT, Shah SP, Abo-Sido N, Yabuuchi A, Hagan JP, Gregory RI, Asara JM, Cantley LC, Moss EG, Daley GQ (2013) Fetal deficiency of lin28 programs life-long aberrations in growth and glucose metabolism. Stem Cells 31:1563–1573

Shyh-Chang N, Daley GQ (2013) Lin28: primal regulator of growth and metabolism in stem cells. Cell Stem Cell 12:395–406

Shyh-Chang N, Zhu H, Yvanka de Soysa T, Shinoda G, Seligson MT, Tsanov KM, Nguyen L, Asara JM, Cantley LC, Daley GQ (2013) Lin28 enhances tissue repair by reprogramming cellular metabolism. Cell 155:778–792

Takahashi T, Nowakowski RS, Caviness VS Jr (1995) The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. J Neurosci 15:6046–6057

Takahashi T, Nowakowski RS, Caviness VS Jr (1996) The leaving or Q fraction of the murine cerebral proliferative epithelium: a general model of neocortical neuronogenesis. J Neurosci 16:6183–6196

Telley L, Agirman G, Prados J, Amberg N, Fievre S, Oberst P, Bartolini G, Vitali I, Cadilhac C, Hippenmeyer S, Nguyen L, Dayer A, Jabaudon D (2019) Temporal patterning of apical progenitors and their daughter neurons in the developing neocortex. Science 364:eaav2522

Thornton JE, Gregory RI (2012) How does Lin28 let-7 control development and disease? Trends Cell Biol 22:474–482

Tsialikas J, Romer-Seibert J (2015) LIN28: roles and regulation in development and beyond. Development 142:2397–2404

Viswanathan SR, Powers JT, Einhorn W, Hoshida Y, Ng TL, Toffanin S, O'Sullivan M, Lu J, Phillips LA, Lockhart VL, Shah SP, Tanwar PS, Mermel CH, Beroukhim R, Azam M, Teixeira J, Meyerson M, Hughes TP, Llovet JM, Radich J, Mullighan CG, Golub TR, Sorensen PH, Daley GQ (2009) Lin28 promotes transformation and is associated with advanced human malignancies. Nat Genet 41:843–848

Vitali I, Fievre S, Telley L, Oberst P, Bariselli S, Frangeul L, Baumann N, McMahon JJ, Klingler E, Bocchi R, Kiss JZ, Bellone C, Silver DL, Jabaudon D (2018) Progenitor hyperpolarization regulates the sequential generation of neuronal subtypes in the developing neocortex. Cell 174(1264–1276):e1215

Wang XW, Li Q, Liu CM, Hall PA, Jiang JJ, Katchis CD, Kang S, Dong BC, Li S, Zhou FQ (2018) Lin28 signaling supports mammalian PNS and CNS axon regeneration. Cell Rep 24(2540–2552):e2546

Wrobel CN, Mutch CA, Swaminathan S, Taketo MM, Chenn A (2007) Persistent expression of stabilized beta-catenin delays maturation of radial glial cells into intermediate progenitors. Dev Biol 309:285–297

Wu L, Belasco JG (2005) Micro-RNA regulation of the mammalian lin-28 gene during neuronal differentiation of embryonal carcinoma cells. Mol Cell Biol 25:9198–9208

Xia X, Teotia P, Ahmad I (2018) Lin28a regulates neurogliogenesis in mammalian retina through the Igf signaling. Dev Biol 440:113–128

Xu B, Zhang K, Huang Y (2009) Lin28 modulates cell growth and associates with a subset of cell cycle regulator mRNAs in mouse embryonic stem cells. RNA 15:357–361

Yang DH, Moss EG (2003) Temporally regulated expression of Lin-28 in diverse tissues of the developing mouse. Gene Expr Patterns 3:719–726

Yang M, Yang SL, Herrlinger S, Liang C, Dzieciatkowska M, Hansen KC, Desai R, Nagy A, Niswander L, Moss EG, Chen JF (2015) Lin28 promotes the proliferative capacity of neural progenitor cells in brain development. Development 142:1616–1627

Yao K, Qiu S, Tian L, Snider WD, Flannery JG, Schaffer DV, Chen B (2016) Wnt regulates proliferation and neurogenic potential of Muller glial cells via a Lin28/let-7 miRNA-dependent pathway in adult mammalian retinas. Cell Rep 17:165–178

Ying Q-L, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A (2008) The ground state of embryonic stem cell self-renewal. Nature 453:519–523

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920

Zeng Y, Yao B, Shin J, Lin L, Kim N, Song Q, Liu S, Su Y, Guo JU, Huang L, Wan J, Wu H, Qian J, Cheng X, Zhu H, Ming GL, Jin P, Song H (2016) Lin28A binds active promoters and recruits Tet1 to regulate gene expression. Mol Cell

Zhang J, Ratanasirintrawoot S, Chandrasekaran S, Wu Z, Ficarro SB, Yu C, Ross CA, Cacchiarelli D, Xia Q, Seligson M, Shinoda G, Xie W, Cahan P, Wang L, Ng SC, Tintara S, Trapnell C, Onder T, Loh YH, Mikkelsen T, Sliz P, Teitell MA, Asara JM, Marto JA, Li H, Collins JJ, Daley GQ (2016) LIN28 regulates stem cell metabolism and conversion to primed pluripotency. Cell Stem Cell 19:66–80

Zhang Y, Williams PR, Jacobi A, Wang C, Goel A, Hirano AA, Brecha NC, Kerschensteiner D, He Z (2019) Elevating growth factor responsiveness and axon regeneration by modulating presynaptic inputs. Neuron 103(39–51):e35

Zhao C, Sun G, Li S, Lang M-F, Yang S, Li W, Shi Y (2010) MicroRNA let-7b regulates neural stem cell proliferation and differentiation by targeting nuclear receptor TLX signaling. Proc Natl Acad Sci U S A 107:1876–1881

Zhu H, Shah S, Shyh-Chang N, Shinoda G, Einhorn WS, Viswanathan SR, Takeuchi A, Grasemann C, Rinn JL, Lopez MF, Hirschhorn JN, Palmert MR, Daley GQ (2010) Lin28a transgenic mice manifest size and puberty phenotypes identified in human genetic association studies. Nat Genet 42:626–630

Zhu H, Shyh-Chang N, Segrè AV, Shinoda G, Shah SP, Einhorn WS, Takeuchi A, Engreitz JM, Hagan JP, Kharas MG, Urbach A, Thornton JE, Triboulet R, Gregory RI, Altshuler D, Daley GQ (2011) The Lin28/let-7 axis regulates glucose metabolism. Cell 147:81–94

Adv Exp Med Biol - Cell Biology and Translational Medicine (2021) 12: 73–93
https://doi.org/10.1007/5584_2021_625

Published online: 25 February 2021

Cartilage Repair by Mesenchymal Stem Cell-Derived Exosomes: Preclinical and Clinical Trial Update and Perspectives

Leila Taghiyar, Shahrbano Jahangir, Mojtaba Khozaei Ravari, Mohammad Amin Shamekhi, and Mohamadreza Baghaban Eslaminejad

Abstract

Osteoarthritis (OA) and other degenerative joint diseases are characterized by articular cartilage destruction, synovial inflammation, sclerosis of subchondral bone, and loss of extracellular matrix (ECM). Worldwide, these diseases are major causes of disability. Cell therapies have been considered to be the best therapeutic strategies for long-term treatment of articular cartilage diseases. It has been suggested that the mechanism of stem cell-based therapy is related to paracrine secretion of extracellular vesicles (EVs), which are recognized as the main secretion factors of stem cells. EVs, and in particular the subclass exosomes (Exos), are novel therapeutic approaches for treatment of cartilage lesions and OA. The results of recent studies have shown that EVs isolated from mesenchymal stem cells (MSCs) could inhibit OA progression. EVs isolated from various stem cell sources, such as MSCs, may contribute to tissue regeneration of the limbs, skin, heart, and other tissues. Here, we summarize recent findings of preclinical and clinical studies on different MSC-derived EVs and their effectiveness as a treatment for damaged cartilage. The Exos isolation techniques in OA treatment are also highlighted.

Keywords

Cartilage · Exosomes · Extracellular vesicles · Mesenchymal stem cells · Regeneration

Abbreviations

ACI	Autologous chondrocyte implantation
ADMSCs	Adipose-derived MSCs
AFM	Atomic force microscopy
DLS	Dynamic light scattering
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assays

Shahrbano Jahangir and Mojtaba Khozaei Ravari contributed equally with all other contributors.

L. Taghiyar, S. Jahangir, M. Khozaei Ravari, and M. B. Eslaminejad (✉)
Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
e-mail: eslami@royaninstitute.org

M. A. Shamekhi (✉)
Department of Polymer Engineering, Islamic Azad University, Sarvestan, Iran
e-mail: mshamekhi@gmail.com

EVs	Extracellular vesicles
GFP	Green fluorescent protein
GMP	Good manufacturing practice
HA	Hyaluronic acid
IPFP	Infrapatellar fat pad
iPSCs	Induced pluripotent stem cells
MACI	Matrix-induced autologous chondrocyte implantation
MMP	Matrix metalloproteinase
MSCs	Mesenchymal stem cell
MVs	Microvesicles
NPCs	Nucleus pulposus cells
NTA	Nanoparticle tracking analysis
OA	Osteoarthritis
PCs	Progenitor cells
PRP	Platelet-rich plasma
SEM	Scanning electron microscopy
STR	Short tandem repeat
TEM	Transmission electron microscopy
TFF	Tangential flow filtration

1 Introduction

Articular cartilage possesses a decreased natural ability to repair itself after an injury. Once a defect develops, the area underneath the subchondral bone becomes involved and results in an osteochondral defect, which is recognized as osteoarthritis (OA) (Brittberg et al. 2016). An estimated 250 million people currently suffer from cartilage defects, and there is a predicted six–sevenfold increase in OA in the next decade (Mora et al. 2018). Currently, no gold standard clinical treatment exists for articular cartilage defects. Traditional and investigational new drugs for cartilage lesions or OA disease focus on rescue from pain and inflammation, but they lack the capacity to regenerate damaged cartilage (Zhang et al. 2016a). Current surgery based-therapeutic approaches include microfracture and osteochondral grafts; however, they result in the formation of a fibrocartilage type tissue (Medvedeva et al. 2018). Recent cell-based therapeutic approaches, such as chondrocyte or stem cell therapy, are powerful strategies for cartilage tissue regrowth. Long-term analysis of clinical trials for OA treatment by autologous chondrocyte implantation (ACI) indicated that more than 90% of the patients continued to have good function five years post-implantation (Mithoefer et al. 2012). Concerns regarding autologous cell transplantation include loss of chondrocyte phenotype, limited donor availability, and fibrocartilage tissue development (Mithoefer et al. 2012).

Injection of undifferentiated cells, including stem cells, increases the risk for cell migration toward an incorrect site, and they may differentiate into ectopic tissue (Herberts et al. 2011). The use of modified or allogenic cells can induce rejection of the implanted cells by increasing autoimmune reactions and may increase the risk of cancer or other side effects of the cell therapy after cartilage repair (Kim and Cho 2015).

An abundance of evidence has shown that cell-free approaches are the most recent techniques for treatment of cartilage lesions. For instance, extracellular vesicles (EVs) and soluble factors released by mesenchymal stem cells (MSCs) are responsible for the therapeutic effectiveness of stem cells (Gimona et al. 2017). EVs that originate from the endosome (30–150 nm in diameter) contain a membrane that encapsulates mRNAs, microRNAs, proteins, and liposomes. These factors have potential use in development of drug delivery therapeutic biomarkers (Li et al. 2019). Recently, EVs such as Exos have emerged as potent cell-free transfer tools because of their elevated physicochemical strength and biocompatibility.

Three major EV classes secreted by cells include apoptotic bodies, microvesicles (MVs), and Exos. It has been assumed that these vesicle populations are homogeneous in size and density; however, the subtypes are heterogeneous in nature (Kim et al. 2013). Recent evidence confirmed that MSCs release distinct-sized EV sub-populations that have different biophysical, proteomic, and RNA repertoires (Willms et al. 2016). MSCs can secrete many diverse subtypes of vesicles That are composed of various RNA, miRNA, DNA, and proteins (Zhang et al. 2018a).

Risks for embolism or tumorigenesis are not associated with Exos therapies compared to modified cell therapies, and this has made Exos

a promising tool for regenerative medicine. Furthermore, their low toxicity and the ability to cross the blood-brain barrier makes them far preferable to other delivery procedures such as microcarriers and synthetic drug carriers (Jiang and Gao 2017).

Although the results of several preclinical studies showed a robust ability of EVs-MSCs as treatments for various diseases, safety considerations in clinical trials should still be addressed. In addition, the standard expansion of large scale GMP-grade Exos-based pharmaceuticals are main current challenges that should be resolved (Navabi et al. 2005). In the MSC culture settings, Exos are harvested according to procedures that need standardization, however, it is expected that mutagenicity and oncogenicity concerns of Exos-based clinical trials will be less when compared to live cell MSC trials.

The purpose of the present review is to summarize the studies with Exos, modified Exos, or those derived from manipulated cells as natural nanocarrier treatments of cartilage lesions or OA. We also discuss different isolation methods used to harvest EVs.

2 Articular Cartilage Structure, Injuries, and Repair

2.1 Articular Cartilage Structure and Function

Articular cartilage is a highly dense tissue characterized by a smooth, lubricated surface (Eckstein et al. 1996). The main role of articular cartilage is to support the surrounding soft tissues, absorb shock, lubricate for the joints and facilitate bone movements, mechanical load-bearing, and protect the subchondral bones from frictional shear forces (Luo et al. 2017). This crucial function is related to the composition of the extracellular matrix (ECM), particularly the arrangement and orientation of the collagen fibers and its connection to the ECM macromolecules (glycosaminoglycan [GAGs] and glycoproteins) (Shen 2005). Remarkably, articular cartilage is an avascular, aneural, alymphatic, and hypocellular tissue that receives its nutrients by a double diffusion barrier via the synovial fluid and subchondral bone (Kwan et al. 1991). Articular cartilage varies in thickness from 1 to 7 mm in humans, depending on the location of the joint. Chondrocytes are one type of specialized cartilage cell that constitutes only 1–5% of the articular cartilage volume; thus, they have no cell-to-cell interactions, and are similar to osteocytes that reside in bone tissue (Kozhemyakina et al. 2015). Chondrocytes are highly specialized cells that are responsible for the synthesis, maintenance, and turnover of the specialized matrix infrastructure that is rich in GAGs and proteoglycans. Proteoglycans and their associated GAGs have important functional roles in tissue remodeling, and they maintain the fluid, uptake of proteins, intracellular signaling, cell migration, and the electrolyte balance in articular cartilage. Despite its high specification, articular cartilage is a thin layer that has a low potential for self-regeneration. Over the past three decades, regeneration of articular cartilage and the underlying mechanisms of cartilage restoration have been primary challenges in clinical and experimental settings. However, current approaches have not shown acceptable outcomes for restoration of articular cartilage function. Nevertheless, it is important to understand current therapeutic strategies and their impacts (Luo et al. 2017; Shen 2005; Kwan et al. 1991; Ryan et al. 2009).

2.2 Articular Cartilage Injuries and their Repair

Chondral lesions are attributed to several factors and their symptoms include swelling, localized pain, and locking. Local cartilage lesions are classified in stages according to the severity of the lesions (Zhang et al. 2018b; Kwon et al. 2019). Chondral damages include clefts, fissures, chondral flaps or tears, and loss of part of the articular cartilage and are triggered by acute or repetitive trauma (Zhang et al. 2018b). In osteochondral lesions, injuries extend into the subchondral bone and cause hemorrhaging and fibrin clot formation. Inflammatory responses

may also occur at the injured site in this condition (Buckwalter 2002).

Chondral and osteochondral damages are the most common types of joint injuries and they are graded according to the depth of the lesion. In addition to the size of the lesion, the patient's age is also an important aspect in non-healing cartilage (Lespasio et al. 2017).

Failure to seek initial treatment along with progressive cartilage degeneration followed by excessive focal stresses on the joints can lead to OA. On the other hand, chondral injuries are progressive and mainly occur because of traumatic or abnormal loading on the joints. In this progressive event, chondrocytes throughout the cartilage thickness undergo apoptosis or necrosis and subsequent damage to the ECM. These crucial conditions lead to secondary hypoxic damage that develops as a result of activation of a cascade of inflammatory factors, tissue necrosis, scar repair, and remodeling. When the full-thickness lesions occur, destruction progresses to the subchondral bone and there is an influx of blood cells that include MSCs and medullary bone elements into the lesion site from medullary bone marrow. Thereafter, the full-thickness defect is filled by a fibrocartilage-like tissue with type I collagen fibers secreted by the MSCs that penetrated into the injured site. However, biochemical, biomechanical, and load-bearing properties of alternative tissues are inferior compared to hyaline cartilage and are not appropriate for articular cartilage (Bhosale and Richardson 2008).

The presence of blood vessels is essential for tissue repairs; hyaline cartilage lack blood vessels and, thus, fails to appropriately rebuild or restore the damaged tissue. Strategies for repairing cartilage defects are categorized into two completely different approaches - traditional surgery and novel tissue engineering/cell-based methods.

2.2.1 Traditional Treatment of Cartilage Defects

(a) Microfracture (bone marrow stimulation)

In 1984, Steadman introduced the microfracture technique as the primary surgery method for hyaline cartilage restoration. This method involves orderly removal of all the calcified cartilage covering followed by the generation of small fractures in the underlying bone in order to release bone marrow clots into the site of the cartilage defect, which would induce hyaline-like tissue formation (Xu et al. 2015). Significant improvements were reported in more than 80% of patients after microfracture surgery when compared with the pre-operative condition. Long-term follow-up indicated the formation of fibrous tissue, which primarily consisted of type I collagen, at the defect site. Although this method was reported to be effective by most studies, inconsistency and variable results, along with degradation of newly formed tissue have been reported (Shen 2005; Kwan et al. 1991). Asik et al. used the microfracture technique for cartilage repair in 90 patients who had focal full-thickness articular cartilage defects. The patients reported considerable pain relief and better cartilage performance. Furthermore, a correlation existed between functional performance and prognostic parameters such as age, size of the defect, and body mass index (Asik et al. 2008). Mithoefer et al. stated that knee function was good to excellent for 67% and poor for 8% of 48 study patients with articular cartilage lesions during a short follow-up of 2 years (Mithoefer et al. 2005).

It is believed that the quality and quantity of bone marrow-derived stem cells and patient's age play a critical role in the microfracture efficacy (Kozhemyakina et al. 2015). The new tissue forms nearly 8 weeks after surgery; thus, the postoperative recovery seems to be effective (Chu et al. 2018).

(b) Arthroscopic debridement and drilling of osteochondral lesions

Debridement of joints was first carried out by Pridi in 1959 on an experimental rabbit model. Magnusson established this procedure more than 60 years ago to treat human knee injuries (Hubbard 1996). Debridement is an arthroscopic surgery in human and veterinary medicine where small holes are generated in the subchondral bone and unstable cartilage and necrotic bone are removed with a curette to the border of healthy

tissue. This stimulation causes the release of surface proteoglycans, which can encourage later adhesion of reparative cells from the synovial membrane. Microfracture is often used for full-thickness lesions in joints, whereas drilling is performed for the initial stage of joint lesions where damaged cartilage is less than or equal to 10 mm in diameter. On the other hand, debridement is indicated in cases of necrotic bone, and the overlying cartilage is intact or compromised (Logli et al. 2019; Bexkens et al. 2017). Improvement was observed in 74% of 78 patients after a 1-year follow-up.

(c) Mosaicplasty

Osteochondral autograft transfer mosaicplasty is a common surgical procedure for osteochondral lesions that was first described in 1993 by Matsusue (Matsusue et al. 1993). In this method, cylindrical osteochondral plugs from low-bearing areas of the articular cartilage are grafted into the cartilage defect. Notwithstanding the suitable outcomes, inadequate donor tissue is the main problem of autologous osteochondral grafts. Moreover, the replaced fragments may not integrate with the native hyaline cartilage and, in some cases, may cause cyst formation due to diffusion of the synovial fluid inside the joints (Hangody et al. 1998; Hangody and Fules 2003; Smith et al. 2005).

(d) Soft tissue grafts

In 1990, Homminga engrafted autologous perichondrium as a biomembrane in another regenerative method to repair cartilage lesions (Homminga et al. 1990). The periosteum, which also has both osteogenic and chondrogenic capabilities due to progenitor cells (PCs) that reside on the cambium layer, is another alternative. PCs are maintained within the periosteum and are recruited in response to injury. Therefore, these biomembranes are ideal biological tools for repair of cartilage lesions (Bouwmeester et al. 1997; Duchamp de Lageneste et al. 2018). Accordingly, transplantation of periosteum and perichondrium flaps have been widely used in full-thickness defects of articular cartilage in animal models and in human clinical trials (Bouwmeester et al. 1999; Carranza-Bencano et al. 1999).

(e) Osteotomy

An osteotomy is a controlled surgical break of bone that allows for bone realignment. It is performed to correct primary knee deformities and as a treatment for knee OA. Smith, in 1958, first used this technique to realign the knee joint of early or medial unicompartmental OA (Smith et al. 2005). Tibial and femoral osteotomies are two types of surgical procedures. The osteotomy is a corrective surgical procedure where cutting and realignment of the bone distributes the joint loading and prevents pressure on the cartilage surface. This procedure may reduce pain, enhance function, postpone knee deterioration, and delay the need for a partial or total knee replacement surgery (Brouwer et al. 2014; Schultz and Gobel 1999).

2.2.2 Novel Tissue Engineering and Cell-Based Methods

(a) Engineered cartilage tissue

In the last few years, tissue engineering has been developed as an alternative to traditional procedures. Accordingly, supportive scaffolds that carry cells and guide matrix production are well-known as promising tools for cartilage regeneration (Nam et al. 2018). Scaffold-based techniques is a cutting-edge technology that uses a three-dimensional (3D) structured material to rebuild new tissue that has a high degree of similarity in architecture and function to the native cell environment. Furthermore, they must allow for successful infiltration, and stimulate cellular differentiation and proliferation via providing suitable bioactive molecules. Although there are numerous scaffolds from different origins (synthetics or natural), alignments and structures, an ideal scaffold should have the capability to induce chondrogenesis and ECM formation, it should be biocompatible, biodegradable and absorbable, and non-immunogenic with appropriate mechanical properties comparable to native cartilage (Nam et al. 2018). Moreover, the surface topography, elasticity, mechanical, and

biochemical properties of a scaffold play a principal role in cell behavior. Recently, the use of 3D bio-printed scaffolds enabled fabrication of customized structures specific for individual defect sites (Medvedeva et al. 2018). Although this method is a high-resolution strategy to fabricate scaffolds, it is relatively expensive.

The choice of a cell source has enormous impact on the success of cartilage restoration and is one of the foremost challenges related to populating scaffolds. Autologous cell sources such as chondrocytes may avoid immune response, but other crucial factors for choosing the proper cell source include accessibility, reproducibility, responsiveness to growth factors, and not tumorigenic. In order to overcome these limitations, MSCs are considered to be an alternative allogenic cell source for cartilage repair because they lack the limitations associated with chondrocytes. In addition, anti-inflammatory and immunomodulatory properties of MSCs facilitate tissue wound repair and chondrogenesis without the need to suppress inflammation (Solheim et al. 2016).

The use of autologous chondrocyte spheroids (chondrospheres) is a novel scaffold-free approach to regenerate lesions. Its advantages include not interrupting cell-cell interactions and high integration potential with adjacent tissue. In a study of a minipig model, chondrocyte spheroids were well-integrated with the host tissue (Meyer et al. 2012). An ongoing randomized phase III clinical study (CO.Don® AG) is based on chondrospheres and the results are not yet available (Fickert et al. 2012).

In this section, clinical interventions for articular cartilage regeneration were briefly discussed in addition to some emerging technologies that are promising for cartilage rehabilitation. Yet, a well-characterized technology should be developed to address the appropriate bioactivity, integrity, biomechanical, and biological properties of the articular cartilage tissue. In the next section, we discuss in detail the emerging technology of cell therapy for cartilage regeneration and focus on its challenges and potentials.

(b) Cell-based therapies in cartilage regeneration

An emerging technology for articular cartilage regeneration is cell therapy based on autologous or allogenic cells, differentiated or stem cells (Wang et al. 2017a). Stem cells are multipotent cells found in various tissues that have tremendous potential for self-renewal and differentiation.

The ACI procedure is a form of tissue engineering as a treatment for deep focal chondral defects. ACI is the first application of cell therapy for cartilage regeneration, which was developed by Peterson in 1987. A cartilage biopsy is surgically collected from a low-weight-bearing area and chondrocytes are released from the ECM following enzymatic treatment. They undergo large scale expansion *in vitro* for implantation into the chondral defects (Brittberg et al. 1996). The cartilage defect is covered by a membrane once the chondrocytes are implanted into the defect site. Clinical outcomes have shown that ACI is an effective therapy for large cartilage defects (>4 cm^2) (Zhang et al. 2018a; Buckwalter 2002). The longest follow-up investigation showed overall improvement in knee functions in 84% of the patients.

Potential immune issues are avoided with ACI because the patient's own cells are used (Bhosale and Richardson 2008; Xu et al. 2015). Matrix-induced autologous chondrocyte implantation (MACI) is a refined version of ACI in which isolated autologous chondrocytes are cultured on type I or III collagen membranes (Shen 2005). Despite promising results, ACI and MACI have donor limitations and complications that include donor site morbidity and graft failure. Chondrocyte hypertrophy in response to *in vitro* expansion is another challenge. Limitations such as the need for an additional operation and dedifferentiation potential during *in vitro* cultivation should be addressed (Fisher et al. 2017).

Recently, various commercial chondrocyte-based samples prepared under good manufacturing practice (GMP) conditions are on the market and in different clinical trial phases. However, once the specialized cells are implanted, immunosuppressive agents must be administered to prevent graft rejection (Ebrahimi et al. 2014). Some studies have used ACI for cartilage disorders treatments (Peterson et al. 2003; Brittberg et al. 1994; Steinwachs 2009).

In parallel, nasal septum chondrocytes are an alternative terminally differentiated cell for cartilage regeneration. An ongoing study by the University Hospital of Basel is using nasal chondrocytes (Nose2Knee), and has completed a phase I clinical trial after successful outcomes on the safety and feasibility of this procedure (Onuora 2016).

MSCs have been considered as an alternative allogenic cell source for cartilage repair because they do not have the limitations associated with chondrocyte. MSCs are multipotent stromal cells found in various tissues and organs such as the bone marrow, umbilical cord blood, adipose tissue, and synovial fluid. The chondrogenic potential of MSCs depends on the tissue source. Yoshimura et al. conducted a comparison study between rat MSCs derived from bone marrow, synovium, periosteum, adipose tissue, and muscle (Yoshimura et al. 2007). It has been reported that the synovium-derived MSCs have a 100-fold higher colony number per nucleated cells than bone marrow-derived MSCs (BmMSCs). Moreover, synovium-derived MSCs have the highest potential for proliferation and chondrogenesis. An advantage of MSCs over terminally differentiated chondrocytes is their easier *in vitro* expansion (Nam et al. 2018).

Currently, articular cartilage regeneration by transplantation of autologous MSCs is a widely used procedure (Negoro et al. 2018). Intra-articular administration of MSCs is a minimally invasive method in articular cartilage regeneration due to the presence of synovial fluid with less tissue damage (Nasiri et al. 2019). However, it is important to investigate the fate and homing of the cells. A research group in Germany established a reliable tracking method of genetically labeled MSCs in distant organs of rat models after injection into articular knee (Zwolanek et al. 2017; Satue et al. 2019). Although a few MSCs were spotted in the lungs of one rat 1 day after the injection, there was no other evidence of donor cells observed in the distant organs during a 6-month observation period. The injected MSCs improved cartilage regeneration and supported the safety and efficacy of an intra-articular injection of MSCs. In a completed phase I-II clinical trial, 15 patients with chronic OA each received a single injection of intra-articular autologous BmMSCs. The patients were observed for 12 months after the injection (Soler et al. 2016). Both the regenerative and anti-inflammatory results supported the feasibility and safety of this procedure. Re-Join is another MSC therapy for OA that is based on autologous adipose-derived MSCs (ADMSCs). The results of a phase II clinical trial (Lu et al. 2019) that enrolled 26 patients who received Re-Join injections showed significant improvements in terms of joint function and cartilage regeneration after 12 months of follow-up.

A study of dose selection of ADSC injections explored the impact of cell dosage on cartilage regeneration in 18 patients with severe knee OA (Pers et al. 2016). The phase I clinical trial outcomes showed no major adverse effect and patients had significantly improved pain levels and cartilage function at the low-dose MSC injection (cells) after 6 months of follow-up. A similar, recent study of 50 selected patients who had knee OA assessed three doses of intra-articular autologous MSC injections and compared them with platelet-rich plasma (PRP) injections. After 12 months of follow-up, patients who received the PRP injections reported no significant improvement, whereas radiological and arthroscopic examinations showed improved hyaline cartilage regeneration with the mid-dose MSC (5×10^7 cells) injection (Filardo et al. 2015). Ozeki et al. investigated the effect of single-dose or multiple-dose injections of synovial MSCs on rat OA models (Ozeki et al. 2016). It was concluded that intra-articular injected green fluorescent protein (GFP)-labeled MSCs mostly migrated into the synovium while maintaining their undifferentiated state. These MSCs expressed anti-inflammatory and chondroprotective proteins TSG-6, PRG4, and BMPs that hindered OA progression. The number of MSCs in the synovium decreased over time; thus, a weekly injection of cells for up to 12 weeks maintained the long-term effects of the procedure. In addition to autologous MSC therapy, allogeneic treatments have been conducted in animals with promising outcomes. In a recent

study, canine models were subjected to an intra-articular injection of either hyaluronic acid (HA) (2 mL, 1%) or allogeneic BmMSCs (1×10^7cells) in conjunction with HA (2 mL, 1%) (Li et al. 2018). After 28 weeks, the animals were sacrificed and examined in terms of cartilage regeneration or emerging adverse effects. According to histological staining and immunohistochemistry, allogeneic MSCs plus HA resulted in more cartilaginous tissue than HA alone. In a similar study, OA rabbits received intra-articular allogeneic BmMSCs (1×10^6) in combination with HA (0.4 mL, 1%) (Chiang et al. 2016). It was concluded that histological scores and inhibition of OA were significantly higher in the animals injected with allogeneic MSCs plus HA. Stempeucel® is a biologic product based on allogeneic pooled human BmMSCs for OA treatment. In a clinical study, 60 patients with knee OA received an intra-articular injection of either 25, 50, 75, or 150×10^6 cells of Stempeucel® in combination with HA (2 mL, 1%) (Gupta et al. 2016). Adverse events of pain and swelling were observed at the higher doses of MSCs (above 50×10^6 cells). Although Stempeucel® was safe at the lowest dose and an improving trend was observed for cartilage repair and pain relief, the MRI score revealed no significant improvements compared with the placebo (PLASMA-LYTE) group. This finding suggests that additional, thorough investigations are essential. Another promising approach for cartilage regeneration via cell therapy is the combined injection of MSCs and chondrocytes. A phase I/II clinical trial on IMPACT by University Medical Center Utrecht was based on the intra-articular injection of autologous chondrocytes (10–20%) in combination with allogeneic MSCs (80–90%) (de Windt et al. 2017). After 12 months' follow-up, there were no adverse effects observed in the patients. MRI scans indicated that the defects were filled in patients with cartilage tissue, and tissue biopsies showed elevated levels of proteoglycans and collagen type II. Short tandem repeat (STR) analysis revealed that after 12 months, the biopsy tissues had only autologous DNA and no allogeneic DNA was identified. No significant difference was observed in 10% or 20% of the chondrocytes. Thus, it can be concluded that transplantation of allogeneic or autologous MSCs for articular cartilage repair is effective in terms of pain relief and short-term tissue restoration. However, long-term assessment is crucial to confirm the safety and efficacy of the underlying procedure.

Many strategies have been proposed to efficiently induce chondrogenic differentiation of iPSCs through formation of embryoid bodies (Umeda et al. 2012; Lee et al. 2015), differentiation into intermediate MSCs (Nejadnik et al. 2015; Chijimatsu et al. 2017), co-culture with primary chondrocytes (Wei et al. 2012; Qu et al. 2013), or the use of growth factors (Cheng et al. 2014; Saito et al. 2015); however, no solid, reproducible protocol has been developed. Although iPSCs are superior in proliferation rate and chondrogenic potential compared to MSCs, other limitations restrict their use in therapeutic applications (Ko et al. 2014). Autologous iPSC therapy is very expensive and allogeneic therapy encounters safety and immunological issues (Lo Monaco et al. 2018). Major challenges in the chondrogenic differentiation of iPSCs include obtaining a purified and homogeneous population of cells and the risk of tumorigenesis. Kotaka et al. provided a strategy for iPSCs delivery to the defect site that used magnetic-labeled cells. Briefly, human fetal lung cell-derived iPSCs were labeled with iron nanoparticles and purified by an external magnetic field (Kotaka et al. 2017). Then, 18 nude rats with patellar defects were treated with a suspension of magnetic-labeled iPSCs in 3% atelocollagen at 10^7 cells/ml. At 8 weeks after the transplantation, all of the defects were covered by a smooth surface hyaline-like cartilage and no tumors were observed. However, a follow-up study of more than 8 weeks would be needed to prove the safety of this procedure. Saito et al. established a 2 week chondrogenic differentiation protocol of human neonatal dermal fibroblast-derived iPSCs and cultured the differentiated cells on a permeable membrane for 1 week *in vitro* (Saito et al. 2015). The membranes were subsequently transplanted into full-thickness femoral condyle defects in 36 mice. After 8 or 16 weeks, the femurs were collected

and examined for chondrogenic differentiation and tumorigenesis. After 8 weeks no tumor was seen, but after 16 weeks, an immature teratoma was observed in one mouse, which indicated that increasing the follow-up duration might show an increase in the risk of tumorigenesis. Hence, there is an emphasis on the significance of avoiding tumorigenesis in clinical applications of iPSCs.

Despite the diverse cell therapy strategies for cartilage regeneration, there is no universally approved, applicable protocol that fully restores tissue structure and function. The use of terminally differentiated chondrocytes or iPSCs as promising cell sources for cartilage repair necessitate additional research in cell fate determination to prevent dedifferentiation or tumor formation. Among many cell therapies, injections of allogeneic MSCs has achieved the most reliable outcomes in animal and preclinical studies because of its immunomodulatory effects, chondrogenic potential, and paracrine properties. Although the risk of tumorigenesis and rejection of MSCs has not been solved. It is inferred that paracrine cues like TGF-β superfamily growth factors play significant roles in the mechanism of action of MSCs in cartilage regeneration (Bobick et al. 2009). Moreover, MSC-derived EVs (EV-MSCs) induce the formation of a cartilaginous matrix (Zhang et al. 2016b). Therapeutic evidence shows that MSCs-secreted EVs and soluble factors are effective. Therefore, cell-free therapy using EV-MSCs might constitute an alternative point of view for researchers (Kotaka et al. 2017). In the next section, we provide a detailed description of the role of Exos in cartilage repair.

Although, in many preclinical experiments or ongoing clinical trials, MSC therapy appears to be a promising strategy to treat cartilage lesions because of their immunomodulatory and paracrine properties, the risks of tumorigenesis and rejection have not been determined. Scientists are optimistic about the results obtained from EV-MSCs therapy for cartilage defects from OA or rheumatoid arthritis (RA); however, the putative therapeutic effects and mechanism of EV-MSCs on inflammation-induced alignment remains unknown.

3 Exosomes (Exos) as a Promising Substitute for Cell Therapy

Recently, the therapeutic effects of MSCs have been attributed to the paracrine secretion of trophic factors such as EVs. EV-mediated tissue regeneration, as a novel cell-free therapeutic approach, has generated renewed optimism for tissue repair. EV therapy may overcome the complications related to stem cell therapy (Musial-Wysocka et al. 2019; Lukomska et al. 2019). The results of numerous studies have suggested that EVs are the most important mediator of cellular information exchange, which are present in the MSCs secretome (Nooshabadi et al. 2018). Despite therapeutic effects of EV-MSCs in facilitating tissue repair in liver disease (La Greca et al. 2018; Di Rocco et al. 2016), cancer (Ren 2019), myocardial infraction (Bang and Kim 2019; Wang et al. 2008; Muller et al. 2018; Ong and Wu 2015), and Alzheimer's disease (AD) (Iranifar et al. 2019), the mechanism of action and effect of EVs on cartilage regeneration has not been fully investigated.

Exos are nanometer-sized vesicles of about 30–100 nm that are enclosed in a bilayer membrane and are secreted by various cell types. EVs contain an active cargo comprised of proteins, mRNA and a wide range of mRNAs and metabolites that could regulate inflammatory responses, angiogenesis, and immune-modulation (Nooshabadi et al. 2018). To date, different types of vesicles found in cells, including micro-vesicle bodies (VBs), Exos, and apoptotic bodies are categorized according to their morphology, size, biogenesis, potential release pathways, and content. Many cell types are known to secrete EVs and these include immune cells such as macrophages, mast cells, B and T lymphocytes, dendritic cells, all types of stem cells (adult, embryonic, and cord blood), and chondrocytes. Unlike cells, EVs do not elicit

acute immune rejection, and they can be produced at a large scale and stored until needed (Lu et al. 2017).

However, biodistribution and *in vivo* tracking techniques should be investigated in order to increase therapeutic efficacy and avoid the possible off-target effects of EVs (Mitchell et al. 2019). The different therapeutic effects of EVs derived from various cell sources are strictly related to the parental cell origin. On the other hand, the therapeutic effects of EVs depend on their content and include inflammatory mediators, tropic factors, signaling molecules, and nucleic acids. EVs derived from cultured cells are functionally and therapeutically dissimilar to *in vivo* derived EVs because of signals received from the microenvironment to the parental cells. Furthermore, mimicking the *in vivo* condition with specific mediators may improve therapeutic outcomes (Seo et al. 2019). Despite *advances in transgenic cell therapy,* the use of genetically modified cells is limited regenerative medicine because *gene therapy* is principally a viral vector-based treatment.

The results of numerous studies have shown that MSC-derived conditional medium has positive effects on various diseases such as myocardial infarction, renal diseases, and complete hepatic destruction (Qin et al. 1996; Rota et al. 2019; Nicolas et al. 2016). Thereafter, for the first time, EVs isolated from cardiac PCs (CPCs) hold great cardiac regeneration potential and can be an alternative to stem cell therapies (Galieva et al. 2019; Rovira et al. 2017; Luo et al. 2018). The therapeutic potential of EVs has been shown in the repair of intervertebral disc degeneration. Exos derived from BmMSCs and nucleus pulposus cells (NPCs) have been functionally evaluated. Exos-NPC stimulated BmMSC migrated and differentiated to a nucleus pulposus phenotype after they were taken up by cells (Jin et al. 2018). ADSC vesicles and soluble proteins stimulate skeletal muscle regeneration (Watson et al. 2016). EV-MSCs could also act as immunomodulatory mediators of immune related diseases to prevent the difficulties associated with traditional cell therapies (Haraszti et al. 2018). EVs have been successfully applied for nerve disorders where preclinical studies have shown promising results for diseases such as AD (Reza-Zaldivar et al. 2018), Parkinson's disease (PD) (Yu et al. 2020), amyotrophic lateral sclerosis (ALS) (Ferrara et al. 2018), multiple sclerosis (MS) (Blonda et al. 2018), stroke and neurotrauma (Colao et al. 2018).

EVs have been used for acute and chronic renal injuries and ureteral strictures (Yan et al. 2018; Li et al. 2017). In a preclinical study on rats with acute liver failure, the EVs released from human ADSCs (hADMSCs) had an enhanced survival rate in the experimental group compared to the control group (Greening et al. 2015).

Despite the therapeutic abilities of EVs, numerous issues remain unsolved such as the lack of distinct manufacturing processes and contaminating endogenous exosomes (Exos) of the serum. These issues necessitate large scale processing of the medium.

Recently, bioreactor cultures are appropriate alternatives for large scale production of EVs for clinical applications. Hollow fiber bioreactors have been used to produce EVs and the results showed that the bioreactor culture yielded ~40-fold more EV per mL of conditioned medium as compared to a conventional T flask cell culture (Watson et al. 2016). The 3D cell cultures based on microcarriers are widely used to grow adherent cells. Tangential flow filtration (TFF) is a method for concentrating proteins or viruses from large quantities of cell culture media. Microcarrier-based 3D culture and TFF allow for scalable production of biologically active Exos from MSCs (Haraszti et al. 2018; Corso et al. 2017; Huang and He 2017). Herein, we explain the current protocols for EVs isolation and characterization.

3.1 Isolation and Characterization of Exosomes (Exos)

Recent Exos isolation methods have been reported that use conditioned media of cultured cells with biological body fluids such as blood and plasma (Properzi et al. 2013). The functional properties, biodistribution, and membrane

integrity of Exos is mainly related to the isolation approach. Exos biomarkers are often used as diagnostic and prognostic tools for different cancers; however, Exos fractions might become contaminated by other co-isolated membranous vesicles and lipoproteins. Therefore, the collected conditioned medium should be carefully inspected to ensure that the isolated vesicles are produced by the cells of interest. For instance, culture medium supplemented with fetal bovine serum (FBS) might contain an abundance of Exos. In order to overcome this problem, one should either use another supplementary ingredient such as bovine serum albumin (BSA) or the FBS should be centrifuged at a high speed before use (Mithoefer et al. 2005).

Current isolation techniques depend on size differences between EVs or specific surface markers. Methodologies that include ultracentrifugation, density-gradient centrifugation, ultrafiltration, precipitation, immunoisolation, and chromatography have been used to isolate Exos (Lim et al. 2019) and will be explained in detail.

Ultracentrifugation is the most commonly used approach for isolation and refining of Exos. The particles form pellets after centrifuging at different speeds. A low g-force centrifugation (e.g., 500 for 5–10 min) is performed for separation of intact cells and cellular debris followed by high g-forces (e.g., 100,000 g for 1–2 h) to isolate the Exos. The ultracentrifugation procedure depends on the g-force, rotor type, clearing factor (k-factor for a rotor describes its pelleting efficiency), and viscosity of the solution (Chu et al. 2018).

Precipitation is another widely used method to detect lipid vesicles that involves polymer solutions. The polymer solution is prepared at an optimized salt concentration and low temperature to reduce vesicle solubility. Subsequently, after low speed centrifugation, the pellet is resuspended in phosphate-buffered saline (PBS) or another appropriate solvent for analysis. This procedure may be contaminated by proteins such as albumin and immunoglobulin (Lim et al. 2019).

Ultrafiltration is one of the size-based Exos isolation techniques. A semi-permeable membrane is used for isolation purposes depending on the particle size and different molecular weights. For instance, hollow fiber bioreactor filtration is an appropriate, practical method for Exos isolation which follows this mechanism (Rim and Kim 2016).

Density-gradient ultracentrifugation is a powerful isolation method utilized to separate and isolate different sub-cellular components by a linear sucrose gradient (e.g., sucrose, Ficoll). The centrifugation takes place at ~100,000 × g for ~16 h. The Exos are then located in the density region between 1.10 and 1.18 g mL^{-1} and the proteins are pelleted at the bottom of the tube. Size-exclusion chromatography (SEC) is a chromatographic technique that acts according to molecular size and can be used to isolate Exos from proteins.

In SEC, larger particles (e.g., Exos) elute faster with the mobile phase and small analytes (e.g., proteins) remain in the stationary phase. Consequently, the Exos can be separated by retrieving the eluted fraction at a definite time (Contreras-Naranjo et al. 2017; Chiriaco et al. 2018). In addition to size-based approaches, other techniques use immunoaffinity-based approaches to isolate Exos. Exos contain numerous specific membrane proteins such as CD63, CD81, CD82, CD9, Alix, annexin, EpCAM, and Rab5 on their surfaces that can be coupled with their corresponding antibodies. Predominantly, the antibodies could be fixed on different types of materials such as magnetic beads, chromatography matrices, plates, and microfluidic devices. Magnetic beads and magnetic nanowires are the most common matrices used in flow cytometry cell sorting. However, this technique is not feasible for isolation of Exos from large quantities of biological samples (Liu and Su 2019).

Most importantly, characterization of Exos is another challenge in exosome mediated therapies. Current methods used to characterize Exos are based on the analysis of specific Exos parameters, which include size, surface markers, protein analysis, and nucleic acid content. Although techniques like transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM) are widely

used for morphological investigation of individual Exos, other approaches are needed to determine their concentration and size range distribution. Nanoparticle analysis apparatuses like dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) have been used to quantify the distribution of Exos, while NTA is more applicable for Exos concentration.

Traditional immunoassays that include enzyme-linked immunosorbent assays (ELISA), Western blot, total protein analysis (bicinchoninic acid [BCA], Bradford assays), and flow cytometry are also used to characterize the surface marker and protein content of Exos. Other novel nano-based techniques such as resistive pulse sensing (RPS), surface plasmon resonance (SPR)-based nanosensors, and nano-deterministic lateral displacement (DLD) have been used to isolate and detect Exos (Rim and Kim 2016). TEM is the best technique for determining particle size distribution and shape. TEM images of EVs are normally seen as round and saucer/cup shaped particles (Rim and Kim 2016). The same mechanism is true for SEM, except the electron beam is reflected from the sample. DLS is the standard technique used to measure size concentration of nanoparticles such as Exos by light scattered from particles under Brownian motion in a liquid suspension (Hubbard 1996).

NTA is a novel method that estimates particle sizes that range from 10 to 1000 nm based on the Brownian motion of nanoparticle in liquids (Hangody and Fules 2003). Various Exos surface marker proteins are characterized by flow cytometry, although Western Blotting is a more common approach for surface marker protein analysis. RPS has a detection limit of 100 nm–100 μm to measure particle size and concentration. The principle is based on the difference in electrical resistance of two nanoparticle filled and non-filled cells in a weak electrolyte. The two cells are connected with a nano constriction (Rim and Kim 2016; Szatanek et al. 2017). SPR is based on incident light stimulation of oscillating electrons at the boundary between positive and negative permittivity material. This technique has been optimized into a nano-based device, the nano-plasmonic exosome (nPLEX), in order to characterize Exos. Extraordinary optical transmission (EOT) in periodic nanoholes is the basis of nPLEX. The mechanism is based on a spectral change during the binding of an Exos to nanoholes coated with affinity ligands for various Exos protein markers in the nanopore optical transmittance. Finally, nano-DLD is a continuous process used in microfluidic devices that use pillar array gradients with a critical cutoff diameter defined in their geometry. Therefore, DLD is used to isolate or detect parasites, bacteria, Exos, blood cells, and circulating tumor cells in the blood. (Rim and Kim 2016; Rana et al. 2018; Smith et al. 2018). Thorough characterization of EVs is the first major step to identify and preserve therapeutic components. The long-term safety of paracrine secretomes need more investigations. Next, we intend to focus on particular applications of Exos as drugs or drug delivery systems in articular cartilage, OA and RA in clinical settings (Fig. 1).

3.2 Clinical Applications of Stem Cell-Derived Exosomes (Exos) in Cartilage Defects

The present studies related to cartilage and/or OA repair using EVs are limited to experimental animal models of inflammation and OA. EVs have been shown to reduce inflammation and enhance hyaline-like cartilage formation *in vitro* and *in vivo*. For instance, Zhang et al. considered the effect of weekly intra-articular injections of human embryonic MSC-derived Exos in rat models with osteochondral defects. The results showed enhanced appearance and histological scores compared with PBS-treated defects. Interestingly, complete restoration of cartilage and subchondral bone was observed in the Exos-treated defects after 12 weeks (Zhang et al. 2016b). A related study found that Exos derived from embryonic MSCs have successful therapeutic effects on OA by balancing the synthesis and degradation of ECM cartilage (Wang et al. 2017b). According to the majority of scientists, the therapeutic effects of MSCs are largely dependent upon the form of secretory vesicles

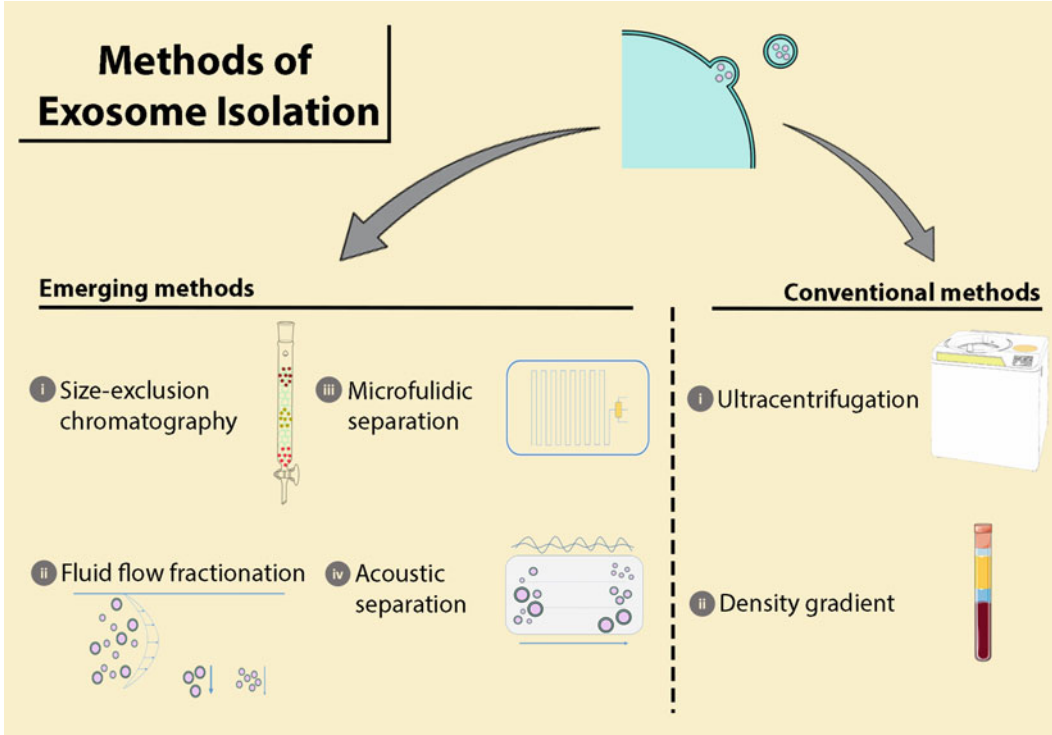


Fig. 1 Current methods for exosome (Exos) isolation. Emerging methods that consist of chromatography, microfluidic separation, fluid flow fraction, and acoustic separation. Conventional methods consist of ultracentrifugation and density gradient

(Manferdini et al. 2013). Cosenza et al. assessed the function of Exos or microparticles (MPs) in OA. They found that MPs and Exos had similar *in vitro* chondroprotective and anti-inflammatory activities, and protected mice from OA progression *in vivo*. Their data indicated that the key therapeutic effects of BmMSCs were addressed by either Exos or MPs. (Cosenza et al. 2017). Vonk et al. demonstrated that Exos harvested from BmMSCs restored OA cartilage by reducing inflammatory responses and stimulating osteoarthritic chondrocytes to secret ECM (Vonk et al. 2018).

Although promising results were reported in small animals, to date, few studies have examined the effects of EVs in large animals and in clinical settings. However, with longer follow-up periods need to confirm the presence of repaired cartilage or a reduction in OA progression. In addition, it has been shown that Exos have biological functions like the cells from which they are derived and there are no unwanted effects like immunogenicity or tumorigenesis with the use of Exos (Xin et al. 2014; Burger et al. 2015). For instance, MSCs isolated from synovial fluid can potentially repair cartilage; however, the use of these cells have limitations such as immunogenicity. In this regard, Tao et al. compared the therapeutic effect of two types of Exos released from synovial derived MSCs (SMSC-Exos) and SMSCs that overexpress miR-140-5p (SMSC-140-Exos) in a rat OA model (Tao et al. 2017). MiR-140-5p plays an important role in MSC chondrogenic differentiation as well as cartilage homeostasis and development (Miyaki et al. 2009). *In vitro* and *in vivo* evaluations have shown that SMSC-140 can enhance the *in vitro* proliferation and migration of articular chondrocytes. Furthermore, relative to SMSC-Exos, SMSC-140-Exos substantially prevented

OA in an OA rat model (Tao et al. 2017). Thus, Exos from gene-manipulated cells show remarkable therapeutic ability for use in clinical settings.

Chondrocytes are the only resident cells in cartilage tissue; evidence has shown that apoptosis of chondrocytes can be a major cause for initiation and progression of OA. Qi et al. have reported that MSC-Exos can inhibit chondrocyte apoptosis and improve their viability under inflammatory conditions (Qi et al. 2019). Therefore, mounting evidence suggests that MSC-Exos could be a beneficial, effective tool in a novel cell-free approach for OA treatment.

Some scientist believe that EVs derived from specific tissues can imitate the niche or microenvironment of the cells by stimulating the tissue-inductive mediators due to the presence of tissue-related factors (mRNA and proteins) that play an important role in local induction of tissue regeneration (Becerra et al. 2011). For instance, chondrocytes are the main cells in cartilage tissue that maintain the cartilage microstructure (Leyh et al. 2014; Zhao et al. 2017; Ahmed et al. 2007). The effect of chondrocyte-derived Exos (CC-Exos), as a stimulator of chondrogenesis in subcutaneous environments, was investigated by Chen et al. for successful ectopic cartilage regeneration compared to Exos derived from BmMSCs (BmMSC-Exos) (Chen et al. 2018). The cartilage generated in the presence of CC-Exos was associated with minimal hypertrophy and angiogenesis, whereas hypertrophy was evident in the presence of BMSC-Exos. They concluded that CC-Exos could imitate the chondrogenesis niche in the subcutaneous environment.

The results of studies indicated the important role of MSC paracrine factors in tissue regeneration, and researchers reported that the condition media of ADSCs exhibited anti-inflammatory properties in OA chondrocytes (Ratajczak et al. 2014; Platas et al. 2013). Tofiño-Vian et al. investigated the chondroprotective function of EVs isolated from hADSCs on OA chondrocytes. MVs and Exos reduced the levels of inflammatory factors such as TNF-a, IL-6, PGE2, and NO in OA chondrocytes stimulated by IL-1β. In OA chondrocytes, EVs reduced the release of matrix metalloproteinase (MMP) activity and MMP-13 expression, but significantly enhanced anti-inflammatory cytokine IL-10 and collagen II expressions (Tofino-Vian et al. 2018).

Despite numerous therapeutic properties of Exos as alternative cell-free therapies for cartilage regeneration, the method of its administration is the main challenge for EVs. Injections are the most common administration route for EVs; however, this is not effective for treatment of cartilage disorders because of rapid leakage from the defect site. On the other hand, constant supervision of EVs at the injury site is a promising method for cartilage repair. Loading Exos into the hydrogel is an appropriate technique to stabilize them into the defect site. Exos have been encapsulated into the photo-induced imine crosslinking (PIC) hydrogel glue in an attempt to prepare an Exos-complex hydrogel tissue patch. The researchers demonstrated that this tissue patch could be easily integrated with the native cartilage tissue and the Exos were effectively maintained at the defect site. This tissue patch, as a novel cell-free material, has been proposed for the comprehensive repair of tissues and organs (Liu et al. 2017).

Under normal conditions, chondrocytes have a dynamic balance of anabolic and catabolic activity that depends on glycolysis activity and is required to provide basic energy (Mobasheri et al. 2017). In OA, chondrocytes lose their metabolic flexibility properties, which results in decreased cellular mitochondrial biogenesis and increased mitochondrial DNA damage (Mobasheri et al. 2017; Luo et al. 2015). Currently, evidence suggests that Exos play an important role in intercellular mitochondrial communication. Exos contents may include the mitochondrial genome or the entire mitochondria (Singh et al. 2017). Chen et al. investigated the effect of MSC-derived Exos on mitochondrial homeostasis. They fabricated a 3D printed scaffold made of ECM, GelMA, and Exos. They found that this construct promoted chondrocyte migration into the defect site and sustainably released Exos. The study results indicated that damages were caused by mitochondrial dysfunction; oxidative stress in the degraded cartilage could be recovered by MSC-Exos through mitochondrial related proteins (Chen et al. 2019).

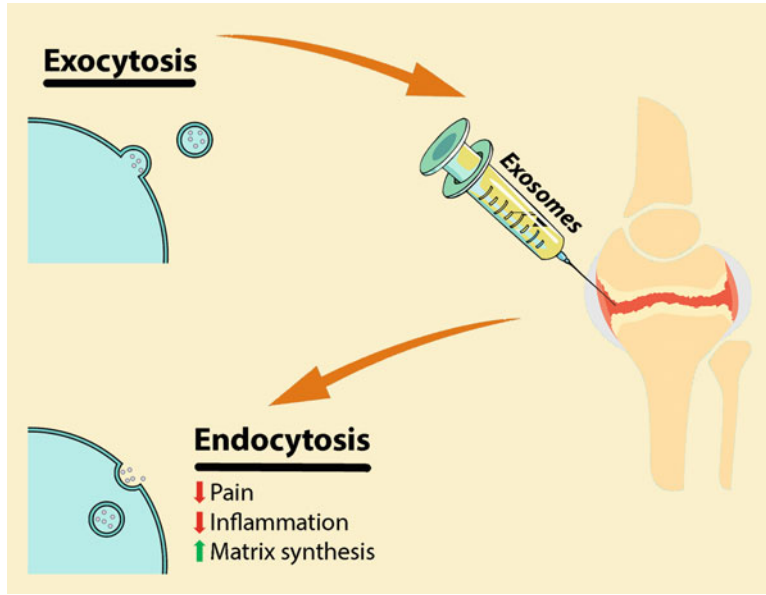


Fig. 2 Cell-free therapeutic approaches for rheumatoid arthritis (RA). Exosome (Exos) isolation from mesenchymal stem cells (MSCs). Injection into the synovial space and endocytosis of Exos by cells

In another study, Wu et al. demonstrated that infrapatellar fat pad (IPFP) MSC-derived Exos (MSCIPFP-Exos) could inhibit the apoptosis of cartilage, balance the anabolic and catabolic processes, and protect cartilage from OA. They recommended that this mechanism could be correlated with the miR100-5p-mediated inhibition of the mTOR-autophagy pathway (Wu et al. 2019). Taken together, Exos could be considered as a new treatment for cartilage injuries (Fig. 2).

3.3 Limitations, Future Trends, and Concluding Remarks

Recently, cell-free regenerative medicine, which is based on the unique ability of EVs derived from stem cells, is a promising new candidate therapy (Pang et al. 2020). Although numerous studies have demonstrated the tremendous ability of EVs isolated from stem cells to improve treatments of various diseases (Reza-Zaldivar et al. 2018; Yu et al. 2020; Ferrara et al. 2018), the use of EVs in cartilage regeneration and OA pathogenesis is still in its early stage. Consequently, EV therapy for large animal models is essential before clinical trials can be conducted (Cheng and Schorey 2013; Yang et al. 2017; Toghraie et al. 2011). Due to the complexity of the cartilage structure, regeneration of focal defects more than 3 cm must be treated with a combination of EVs and other appropriate matrices under dynamic conditions (Brittberg et al. 2003). Therefore, researchers should address the following: how to use EVs; determine the biological properties of different types of EVs; the optimal dose of EVs in relation to different sizes of cartilage lesions; stability of EVs at the defect site; and determine the role of EVs in homeostasis and pathogenesis of joints.

A major challenge for the combination of novel biomaterials and EVs is to discover the optimal EV dose. Further experimentations should be designed in order for these stem cell-derived EVs to become available for clinical settings. Although phenomenal progress has been made in understanding the Exos cargo's biological properties, future studies must also concentrate on the challenges of obtaining regulatory approval and their future translation into clinical platforms.

In summary, the most important challenges of clinical applications for EVs to be recognized are the pharmacodynamics and biological distribution of the injected Exos. Although homing of the Exos to soft organs such as the lungs, liver

Table 1 Osteoarthritis (OA) and arthritis treated by exosomes (Exos) in animal models of disease

Disease model	MSC-product nomenclature	Isolation method	Dose assessment	Dose	References
Critical-sized osteochondral defects	hESC-MSC-derived Exos	Tangential flow filtration (TFF)	Protein assay	100 mg	Zhang et al. (2016b)
OA induced in male C57BL/6 mice	MSCIPFP-Exos	Exoquick ™(EQ) reagent kit (SBI) and ultrafiltration	Nanosight LM10 instrument	10^{10} particles/ml	Wu et al. (2019)
OA model induced in male Sprague-Dawley rats	SMSCs-Exos	DLS	Protein isolation kit	10^{11} Exos particles/ml	Tao et al. (2017)
Collagenase-induced OA	Exos	Centrifugation	Protein	250 ng/5 ml	Cosenza et al. (2017)
Thickness cylindrical Osteochondral defect (rabbit)	hiPSC-MSC-derived Exos	Ultracentrifugation	Qnano platform (TRPS)	2.45×10^{12} in 200 ml of prepared Exos-hydrogel	Liu et al. (2017)
OA model induced in C57BL/6 J mice	ESC-MSC-derived Exos	Ultracentrifugation	Protein	5 μl exos	Wang et al. (2017b)
Subcutaneous non-chondrogenic sites	Cartilage cells and BmMSC-exos	Ultracentrifugation (ultra-clear tube)	Protein	6 μg Exos per 20 ml injection	Chen et al. (2018)

OA Osteoarthritis, *MSCs* Mesenchymal stem cells, *Exos* Exosomes, *BmMSC-Exos* Bone marrow MSC-derived Exos, *MSCIPFP-Exos* Infrapatellar fat pad MSC-derived Exos

or spleen have been reported a few minutes after the injection, more thorough investigations of the pharmacokinetics, metabolism, and biological dosage should be conducted for safety. These investigations will take time before Exos can be used in clinical applications (Table 1).

References

Ahmed N et al (2007) Soluble signalling factors derived from differentiated cartilage tissue affect chondrogenic differentiation of rat adult marrow stromal cells. Cell Physiol Biochem 20(5):665–678

Asik M et al (2008) The microfracture technique for the treatment of full-thickness articular cartilage lesions of the knee: midterm results. Arthroscopy 24 (11):1214–1220

Bang OY, Kim EH (2019) Mesenchymal stem cell-derived extracellular vesicle therapy for stroke: challenges and progress. Front Neurol 10:211

Becerra J et al (2011) The stem cell niche should be a key issue for cell therapy in regenerative medicine. Stem Cell Rev Rep 7(2):248–255

Bexkens R et al (2017) Clinical outcome after arthroscopic debridement and microfracture for osteochondritis dissecans of the capitellum. Am J Sports Med 45 (10):2312–2318

Bhosale AM, Richardson JB (2008) Articular cartilage: structure, injuries and review of management. Br Med Bull 87:77–95

Blonda M et al (2018) New insights into immune cell-derived extracellular vesicles in multiple sclerosis. Front Neurol 9:604

Bobick BE et al (2009) Regulation of the chondrogenic phenotype in culture. Birth Defects Res C Embryo Today 87(4):351–371

Bouwmeester SJ et al (1997) Long-term results of rib perichondrial grafts for repair of cartilage defects in the human knee. Int Orthop 21(5):313–317

Bouwmeester P et al (1999) Histological and biochemical evaluation of perichondrial transplants in human articular cartilage defects. J Orthop Res 17(6):843–849

Brittberg M et al (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 331(14):889–895

Brittberg M et al (1996) Rabbit articular cartilage defects treated with autologous cultured chondrocytes. Clin Orthop Relat Res 326:270–283

Brittberg M et al (2003) Articular cartilage engineering with autologous chondrocyte transplantation. A review of recent developments. J Bone Joint Surg Am 85A (Suppl 3):109–115

Brittberg M et al (2016) Cartilage repair in the degenerative ageing knee. Acta Orthop 87(Sup 363):26–38

Brouwer RW et al (2014) Osteotomy for treating knee osteoarthritis. Cochrane Database Syst Rev 12: CD004019

Buckwalter JA (2002) Articular cartilage injuries. Clin Orthop Relat Res 402:21–37

Burger D et al (2015) Human endothelial colony-forming cells protect against acute kidney injury: role of exosomes. Am J Pathol 185(8):2309–2323

Carranza-Bencano A et al (1999) Comparative study of the reconstruction of articular cartilage defects with free costal perichondrial grafts and free tibial periosteal grafts: an experimental study on rabbits. Calcif Tissue Int 65(5):402–407

Chen Y et al (2018) Exosomes derived from mature chondrocytes facilitate subcutaneous stable ectopic chondrogenesis of cartilage progenitor cells. Stem Cell Res Ther 9(1):318

Chen P et al (2019) Desktop-stereolithography 3D printing of a radially oriented extracellular matrix/mesenchymal stem cell exosome bioink for osteochondral defect regeneration. Theranostics 9(9):2439–2459

Cheng Y, Schorey JS (2013) Exosomes carrying mycobacterial antigens can protect mice against Mycobacterium tuberculosis infection. Eur J Immunol 43 (12):3279–3290

Cheng A et al (2014) Cartilage repair using human embryonic stem cell-derived chondroprogenitors. Stem Cells Transl Med 3(11):1287–1294

Chiang ER et al (2016) Allogeneic mesenchymal stem cells in combination with hyaluronic acid for the treatment of osteoarthritis in rabbits. PLoS One 11(2): e0149835

Chijimatsu R et al (2017) Characterization of mesenchymal stem cell-like cells derived from human iPSCs via neural crest development and their application for osteochondral repair. Stem Cells Int 2017:1960965

Chiriaco MS et al (2018) Lab-on-chip for exosomes and microvesicles detection and characterization. Sensors (Basel) 18(10):3175

Chu CR et al (2018) Minimally manipulated bone marrow concentrate compared with microfracture treatment of full-thickness chondral defects: a one-year study in an equine model. J Bone Joint Surg Am 100(2):138–146

Colao IL et al (2018) Manufacturing exosomes: a promising therapeutic platform. Trends Mol Med 24 (3):242–256

Contreras-Naranjo JC, Wu HJ, Ugaz VM (2017) Microfluidics for exosome isolation and analysis: enabling liquid biopsy for personalized medicine. Lab Chip 17(21):3558–3577

Corso G et al (2017) Reproducible and scalable purification of extracellular vesicles using combined bind-elute and size exclusion chromatography. Sci Rep 7 (1):11561

Cosenza S et al (2017) Mesenchymal stem cells derived exosomes and microparticles protect cartilage and bone from degradation in osteoarthritis. Sci Rep 7(1):16214

de Windt TS et al (2017) Allogeneic mesenchymal stem cells stimulate cartilage regeneration and are safe for single-stage cartilage repair in humans upon mixture with recycled autologous chondrons. Stem Cells 35 (1):256–264

Di Rocco G, Baldari S, Toietta G (2016) Towards therapeutic delivery of extracellular vesicles: strategies for in vivo tracking and biodistribution analysis. Stem Cells Int 2016:5029619

Duchamp de Lageneste O et al (2018) Periosteum contains skeletal stem cells with high bone regenerative potential controlled by Periostin. Nat Commun 9(1):773

Ebrahimi A, Hosseini SA, Rahim F (2014) Immunosuppressive therapy in allograft transplantation: from novel insights and strategies to tolerance and challenges. Cent Eur J Immunol 39(3):400–409

Eckstein F et al (1996) Determination of knee joint cartilage thickness using three-dimensional magnetic resonance chondro-crassometry (3D MR-CCM). Magn Reson Med 36(2):256–265

Ferrara D et al (2018) Role of extracellular vesicles in amyotrophic lateral sclerosis. Front Neurosci 12:574

Fickert S et al (2012) One-year clinical and radiological results of a prospective, investigator-initiated trial examining a novel, purely autologous 3-dimensional autologous chondrocyte transplantation product in the knee. Cartilage 3(1):27–42

Filardo G et al (2015) Platelet-rich plasma: why intra-articular? A systematic review of preclinical studies and clinical evidence on PRP for joint degeneration. Knee Surg Sports Traumatol Arthrosc 23 (9):2459–2474

Fisher JN et al (2017) The application of stem cells from different tissues to cartilage repair. Stem Cells Int 2017:2761678

Galieva LR et al (2019) Therapeutic potential of extracellular vesicles for the treatment of nerve disorders. Front Neurosci 13:163

Gimona M et al (2017) Manufacturing of human extracellular vesicle-based therapeutics for clinical use. Int J Mol Sci 18(6):1190

Greening DW et al (2015) A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. Methods Mol Biol 1295:179–209

Gupta PK et al (2016) Efficacy and safety of adult human bone marrow-derived, cultured, pooled, allogeneic mesenchymal stromal cells (Stempeucel(R)): preclinical and clinical trial in osteoarthritis of the knee joint. Arthritis Res Ther 18(1):301

Hangody L, Fules P (2003) Autologous osteochondral mosaicplasty for the treatment of full-thickness defects of weight-bearing joints: ten years of experimental and clinical experience. J Bone Joint Surg Am 85-A(Suppl 2):25–32

Hangody L et al (1998) Mosaicplasty for the treatment of articular cartilage defects: application in clinical practice. Orthopedics 21(7):751–756

Haraszti RA et al (2018) Exosomes produced from 3D cultures of MSCs by tangential flow filtration show higher yield and improved activity. Mol Ther 26 (12):2838–2847

Herberts CA, Kwa MS, Hermsen HP (2011) Risk factors in the development of stem cell therapy. J Transl Med 9:29

Homminga GN et al (1990) Perichondral grafting for cartilage lesions of the knee. J Bone Joint Surg Br 72 (6):1003–1007

Huang T, He J (2017) Characterization of extracellular vesicles by size-exclusion high-performance liquid chromatography (HPLC). Methods Mol Biol 1660:191–199

Hubbard MJ (1996) Articular debridement versus washout for degeneration of the medial femoral condyle. A five-year study. J Bone Joint Surg Br 78(2):217–219

Iranifar E et al (2019) Exosomes and microRNAs: new potential therapeutic candidates in Alzheimer disease therapy. J Cell Physiol 234(3):2296–2305

Jiang XC, Gao JQ (2017) Exosomes as novel bio-carriers for gene and drug delivery. Int J Pharm 521 (1–2):167–175

Jin Y et al (2018) Extracellular vesicles secreted by human adipose-derived stem cells (hASCs) improve survival rate of rats with acute liver failure by releasing lncRNA H19. EBioMedicine 34:231–242

Kim N, Cho SG (2015) New strategies for overcoming limitations of mesenchymal stem cell-based immune modulation. Int J Stem Cells 8(1):54–68

Kim DK et al (2013) EVpedia: an integrated database of high-throughput data for systemic analyses of extracellular vesicles. J Extracell Vesicles 2013:2

Ko JY et al (2014) In vitro chondrogenesis and in vivo repair of osteochondral defect with human induced pluripotent stem cells. Biomaterials 35(11):3571–3581

Kotaka S et al (2017) Magnetic targeted delivery of induced pluripotent stem cells promotes articular cartilage repair. Stem Cells Int 2017:9514719

Kozhemyakina E, Lassar AB, Zelzer E (2015) A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation. Development 142(5):817–831

Kwan AP et al (1991) Macromolecular organization of chicken type X collagen in vitro. J Cell Biol 114 (3):597–604

Kwon H et al (2019) Surgical and tissue engineering strategies for articular cartilage and meniscus repair. Nat Rev Rheumatol 15(9):550–570

La Greca A et al (2018) Extracellular vesicles from pluripotent stem cell-derived mesenchymal stem cells acquire a stromal modulatory proteomic pattern during differentiation. Exp Mol Med 50(9):119

Lee J et al (2015) Early induction of a prechondrogenic population allows efficient generation of stable chondrocytes from human induced pluripotent stem cells. FASEB J 29(8):3399–3410

Lespasio MJ et al (2017) Knee osteoarthritis: a primer. Perm J 21:16–183

Leyh M et al (2014) Osteoarthritic cartilage explants affect extracellular matrix production and composition in cocultured bone marrow-derived mesenchymal stem cells and articular chondrocytes. Stem Cell Res Ther 5(3):77

Li P et al (2017) Progress in exosome isolation techniques. Theranostics 7(3):789–804

Li L et al (2018) Mesenchymal stem cells in combination with hyaluronic acid for articular cartilage defects. Sci Rep 8(1):9900

Li X et al (2019) Challenges and opportunities in exosome research-perspectives from biology, engineering, and cancer therapy. APL Bioeng 3(1):011503

Lim J et al (2019) Direct isolation and characterization of circulating exosomes from biological samples using magnetic nanowires. J Nanobiotechnol 17(1):1

Liu C, Su C (2019) Design strategies and application progress of therapeutic exosomes. Theranostics 9 (4):1015–1028

Liu X et al (2017) Integration of stem cell-derived exosomes with in situ hydrogel glue as a promising tissue patch for articular cartilage regeneration. Nanoscale 9(13):4430–4438

Lo Monaco M et al (2018) Stem cells for cartilage repair: preclinical studies and insights in translational animal models and outcome measures. Stem Cells Int 2018:9079538

Logli AL et al (2019) Osteochondritis dissecans lesions of the capitellum in overhead athletes: a review of current evidence and proposed treatment algorithm. Curr Rev Musculoskelet Med 12(1):1–12

Lu K et al (2017) Exosomes as potential alternatives to stem cell therapy for intervertebral disc degeneration: in-vitro study on exosomes in interaction of nucleus pulposus cells and bone marrow mesenchymal stem cells. Stem Cell Res Ther 8(1):108

Lu L et al (2019) Treatment of knee osteoarthritis with intra-articular injection of autologous adipose-derived mesenchymal progenitor cells: a prospective, randomized, double-blind, active-controlled, phase IIb clinical trial. Stem Cell Res Ther 10(1):143

Lukomska B et al (2019) Challenges and controversies in human mesenchymal stem cell therapy. Stem Cells Int 2019:9628536

Luo Z et al (2015) Mechano growth factor (MGF) and transforming growth factor (TGF)-beta3 functionalized silk scaffolds enhance articular hyaline cartilage regeneration in rabbit model. Biomaterials 52:463–475

Luo Y et al (2017) The minor collagens in articular cartilage. Protein Cell 8(8):560–572

Luo J et al (2018) Bone marrow mesenchymal stem cells reduce ureteral stricture formation in a rat model via the paracrine effect of extracellular vesicles. J Cell Mol Med 22(9):4449–4459

Manferdini C et al (2013) Adipose-derived mesenchymal stem cells exert antiinflammatory effects on chondrocytes and synoviocytes from osteoarthritis patients through prostaglandin E2. Arthritis Rheum 65(5):1271–1281

Matsusue Y, Yamamuro T, Hama H (1993) Arthroscopic multiple osteochondral transplantation to the chondral defect in the knee associated with anterior cruciate ligament disruption. Arthroscopy 9(3):318–321

Medvedeva EV et al (2018) Repair of damaged articular cartilage: current approaches and future directions. Int J Mol Sci 19(8):2366

Meyer U et al (2012) Cartilage defect regeneration by ex vivo engineered autologous microtissue – preliminary results. In Vivo 26(2):251–257

Mitchell R et al (2019) Secretome of adipose-derived mesenchymal stem cells promotes skeletal muscle regeneration through synergistic action of extracellular vesicle cargo and soluble proteins. Stem Cell Res Ther 10(1):116

Mithoefer K et al (2005) The microfracture technique for the treatment of articular cartilage lesions in the knee. A prospective cohort study. J Bone Joint Surg Am 87 (9):1911–1920

Mithoefer K et al (2012) Evolution and current role of autologous chondrocyte implantation for treatment of articular cartilage defects in the football (soccer) player. Cartilage 3(1 Suppl):31S–36S

Miyaki S et al (2009) MicroRNA-140 is expressed in differentiated human articular chondrocytes and modulates interleukin-1 responses. Arthritis Rheum 60(9):2723–2730

Mobasheri A et al (2017) The role of metabolism in the pathogenesis of osteoarthritis. Nat Rev Rheumatol 13 (5):302–311

Mora JC, Przkora R, Cruz-Almeida Y (2018) Knee osteoarthritis: pathophysiology and current treatment modalities. J Pain Res 11:2189–2196

Muller P, Lemcke H, David R (2018) Stem cell therapy in heart diseases – cell types, mechanisms and improvement strategies. Cell Physiol Biochem 48 (6):2607–2655

Musial-Wysocka A, Kot M, Majka M (2019) The pros and cons of mesenchymal stem cell-based therapies. Cell Transplant 28(7):801–812

Nam Y et al (2018) Current therapeutic strategies for stem cell-based cartilage regeneration. Stem Cells Int 2018:8490489

Nasiri N et al (2019) Targeted cell delivery for articular cartilage regeneration and osteoarthritis treatment. Drug Discov Today 24(11):2212–2224

Navabi H et al (2005) Preparation of human ovarian cancer ascites-derived exosomes for a clinical trial. Blood Cells Mol Dis 35(2):149–152

Negoro T et al (2018) Trends in clinical trials for articular cartilage repair by cell therapy. NPJ Regen Med 3:17

Nejadnik H et al (2015) Improved approach for chondrogenic differentiation of human induced pluripotent stem cells. Stem Cell Rev Rep 11(2):242–253

Nicolas C et al (2016) Stem cell therapies for treatment of liver disease. Biomedicine 4(1):2

Nooshabadi VT et al (2018) The extracellular vesicles-derived from mesenchymal stromal cells: a new therapeutic option in regenerative medicine. J Cell Biochem 119(10):8048–8073

Ong SG, Wu JC (2015) Exosomes as potential alternatives to stem cell therapy in mediating cardiac regeneration. Circ Res 117(1):7–9

Onuora S (2016) Regenerative medicine: a nose for cartilage repair. Nat Rev Rheumatol 12(12):691

Ozeki N et al (2016) Not single but periodic injections of synovial mesenchymal stem cells maintain viable cells in knees and inhibit osteoarthritis progression in rats. Osteoarthr Cartil 24(6):1061–1070

Pang B et al (2020) Extracellular vesicles: the next generation of biomarkers for liquid biopsy-based prostate cancer diagnosis. Theranostics 10(5):2309–2326

Pers YM et al (2016) Adipose mesenchymal stromal cell-based therapy for severe osteoarthritis of the knee: a phase i dose-escalation trial. Stem Cells Transl Med 5 (7):847–856

Peterson L et al (2003) Treatment of osteochondritis dissecans of the knee with autologous chondrocyte transplantation: results at two to ten years. J Bone Joint Surg Am 85A(Suppl 2):17–24

Platas J et al (2013) Conditioned media from adipose-tissue-derived mesenchymal stem cells downregulate degradative mediators induced by interleukin-1beta in osteoarthritic chondrocytes. Mediat Inflamm 2013:357014

Properzi F, Logozzi M, Fais S (2013) Exosomes: the future of biomarkers in medicine. Biomark Med 7 (5):769–778

Qi H et al (2019) Exosomes derived from mesenchymal stem cells inhibit mitochondrial dysfunction-induced apoptosis of chondrocytes via p38, ERK, and Akt pathways. In Vitro Cell Dev Biol Anim 55 (3):203–210

Qin Set al (1996) Folk medicine of the Qiang nationality. Zhongguo Zhong Yao Za Zhi 21(8):453–456, 509

Qu C et al (2013) Chondrogenic differentiation of human pluripotent stem cells in chondrocyte co-culture. Int J Biochem Cell Biol 45(8):1802–1812

Rana A, Zhang Y, Esfandiari L (2018) Advancements in microfluidic technologies for isolation and early detection of circulating cancer-related biomarkers. Analyst 143(13):2971–2991

Ratajczak MZ et al (2014) New advances in stem cell research: practical implications for regenerative medicine. Pol Arch Med Wewn 124(7–8):417–426

Ren K (2019) Exosomes in perspective: a potential surrogate for stem cell therapy. Odontology 107(3):271–284

Reza-Zaldivar EE et al (2018) Potential effects of MSC-derived exosomes in neuroplasticity in Alzheimer's disease. Front Cell Neurosci 12:317

Rim KT, Kim SJ (2016) Quantitative analysis of exosomes from murine lung cancer cells by flow cytometry. J Cancer Prev 21(3):194–200

Rota C, Morigi M, Imberti B (2019) Stem cell therapies in kidney diseases: progress and challenges. Int J Mol Sci 20(11):2790

Rovira J et al (2017) Therapeutic application of extracellular vesicles in acute and chronic renal injury. Nefrologia 37(2):126–137

Ryan JA et al (2009) Mechanical compression of articular cartilage induces chondrocyte proliferation and inhibits proteoglycan synthesis by activation of the ERK pathway: implications for tissue engineering and regenerative medicine. J Tissue Eng Regen Med 3(2):107–116

Saito T et al (2015) Hyaline cartilage formation and tumorigenesis of implanted tissues derived from human induced pluripotent stem cells. Biomed Res 36(3):179–186

Satue M et al (2019) Intra-articularly injected mesenchymal stem cells promote cartilage regeneration, but do not permanently engraft in distant organs. Sci Rep 9 (1):10153

Schultz W, Gobel D (1999) Articular cartilage regeneration of the knee joint after proximal tibial valgus osteotomy: a prospective study of different intra- and extra-articular operative techniques. Knee Surg Sports Traumatol Arthrosc 7(1):29–36

Seo Y, Kim HS, Hong IS (2019) Stem cell-derived extracellular vesicles as immunomodulatory therapeutics. Stem Cells Int 2019:5126156

Shen G (2005) The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. Orthod Craniofac Res 8(1):11–17

Singh B, Modica-Napolitano JS, Singh KK (2017) Defining the momiome: promiscuous information transfer by mobile mitochondria and the mitochondrial genome. Semin Cancer Biol 47:1–17

Smith GD, Knutsen G, Richardson JB (2005) A clinical review of cartilage repair techniques. J Bone Joint Surg Br 87(4):445–449

Smith JT et al (2018) Integrated nanoscale deterministic lateral displacement arrays for separation of extracellular vesicles from clinically-relevant volumes of biological samples. Lab Chip 18(24):3913–3925

Soler R et al (2016) Final results of a phase I-II trial using ex vivo expanded autologous mesenchymal stromal cells for the treatment of osteoarthritis of the knee confirming safety and suggesting cartilage regeneration. Knee 23(4):647–654

Solheim E et al (2016) Results at 10-14 years after microfracture treatment of articular cartilage defects in the knee. Knee Surg Sports Traumatol Arthrosc 24 (5):1587–1593

Steinwachs M (2009) New technique for cell-seeded collagen-matrix-supported autologous chondrocyte transplantation. Arthroscopy 25(2):208–211

Szatanek R et al (2017) The methods of choice for extracellular vesicles (EVs) characterization. Int J Mol Sci 18(6):1153

Tao SC et al (2017) Exosomes derived from miR-140-5p-overexpressing human synovial mesenchymal stem cells enhance cartilage tissue regeneration and prevent osteoarthritis of the knee in a rat model. Theranostics 7(1):180–195

Tofino-Vian M et al (2018) Microvesicles from human adipose tissue-derived mesenchymal stem cells as a new protective strategy in osteoarthritic chondrocytes. Cell Physiol Biochem 47(1):11–25

Toghraie FS et al (2011) Treatment of osteoarthritis with infrapatellar fat pad derived mesenchymal stem cells in rabbit. Knee 18(2):71–75

Umeda K et al (2012) Human chondrogenic paraxial mesoderm, directed specification and prospective isolation from pluripotent stem cells. Sci Rep 2:455

Vonk LA et al (2018) Mesenchymal stromal/stem cell-derived extracellular vesicles promote human cartilage regeneration in vitro. Theranostics 8(4):906–920

Wang CH, Cherng WJ, Verma S (2008) Drawbacks to stem cell therapy in cardiovascular diseases. Futur Cardiol 4(4):399–408

Wang M et al (2017a) Advances and prospects in stem cells for cartilage regeneration. Stem Cells Int 2017:4130607

Wang Y et al (2017b) Exosomes from embryonic mesenchymal stem cells alleviate osteoarthritis through balancing synthesis and degradation of cartilage extracellular matrix. Stem Cell Res Ther 8(1):189

Watson DC et al (2016) Efficient production and enhanced tumor delivery of engineered extracellular vesicles. Biomaterials 105:195–205

Wei Y et al (2012) Chondrogenic differentiation of induced pluripotent stem cells from osteoarthritic chondrocytes in alginate matrix. Eur Cell Mater 23:1–12

Willms E et al (2016) Cells release subpopulations of exosomes with distinct molecular and biological properties. Sci Rep 6:22519

Wu J et al (2019) miR-100-5p-abundant exosomes derived from infrapatellar fat pad MSCs protect articular cartilage and ameliorate gait abnormalities via inhibition of mTOR in osteoarthritis. Biomaterials 206:87–100

Xin H, Li Y, Chopp M (2014) Exosomes/miRNAs as mediating cell-based therapy of stroke. Front Cell Neurosci 8:377

Xu X et al (2015) Full-thickness cartilage defects are repaired via a microfracture technique and intraarticular injection of the small-molecule compound kartogenin. Arthritis Res Ther 17:20

Yan IK et al (2018) Use of a hollow fiber bioreactor to collect extracellular vesicles from cells in culture. Methods Mol Biol 1740:35–41

Yang VK et al (2017) Circulating exosome microRNA associated with heart failure secondary to myxomatous mitral valve disease in a naturally occurring canine model. J Extracell Vesicles 6(1):1350088

Yoshimura H et al (2007) Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. Cell Tissue Res 327(3):449–462

Yu H et al (2020) Potential roles of exosomes in Parkinson's disease: from pathogenesis, diagnosis, and treatment to prognosis. Front Cell Dev Biol 8:86

Zhang W et al (2016a) Current research on pharmacologic and regenerative therapies for osteoarthritis. Bone Res 4:15040

Zhang S et al (2016b) Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration. Osteoarthr Cartil 24(12):2135–2140

Zhang L et al (2018a) Exosomal miRNA profiling to identify nanoparticle phagocytic mechanisms. Small 14(15):e1704008

Zhang Y et al (2018b) Repair of full-thickness articular cartilage defect using stem cell-encapsulated thermogel. Mater Sci Eng C Mater Biol Appl 88:79–87

Zhao X et al (2017) Chondrogenesis by bone marrow-derived mesenchymal stem cells grown in chondrocyte-conditioned medium for auricular reconstruction. J Tissue Eng Regen Med 11(10):2763–2773

Zwolanek D et al (2017) Tracking mesenchymal stem cell contributions to regeneration in an immunocompetent cartilage regeneration model. JCI Insight 2(20):e87322

Adv Exp Med Biol - Cell Biology and Translational Medicine (2021) 12: 95–109
https://doi.org/10.1007/5584_2020_603

Published online: 27 December 2020

Kaempferol Induces Cell Death and Sensitizes Human Head and Neck Squamous Cell Carcinoma Cell Lines to Cisplatin

Mabel Catalán, Catalina Rodríguez, Ivonne Olmedo, Javiera Carrasco-Rojas, Diego Rojas, Alfredo Molina-Berríos, Mario Díaz-Dosque, and José A. Jara

Abstract

Cisplatin is a first-line chemotherapeutic drug commonly used to treat patients with head and neck cancer; nevertheless, cisplatin resistance poses a main challenge for its clinical efficacy. Recent studies have shown that kaempferol, a natural flavonoid found in various plants and foods, has an anticancer effect. The following study evaluated the cytotoxic effects of kaempferol on head and neck tumor cells and their mechanism of action, evaluating the effects on proliferation, the oxygen consumption rate, transmembrane potential, tumor cell migration and induction of apoptosis. Moreover, we determined the effects of a combination of kaempferol and cisplatin on head and neck tumor cells. We found that kaempferol inhibited the oxygen consumption rate and decreased the intracellular ATP content in tumor cells. This novel mechanism may inhibit the migratory capacity and promote antiproliferative effects and apoptosis of tumor cells. Additionally, our in vitro data indicated that kaempferol may sensitize head and neck tumor cells to the effects of cisplatin. These effects provide new evidence for the use of a combination of kaempferol and cisplatin *in vivo* and their future applications in head and neck cancer therapy.

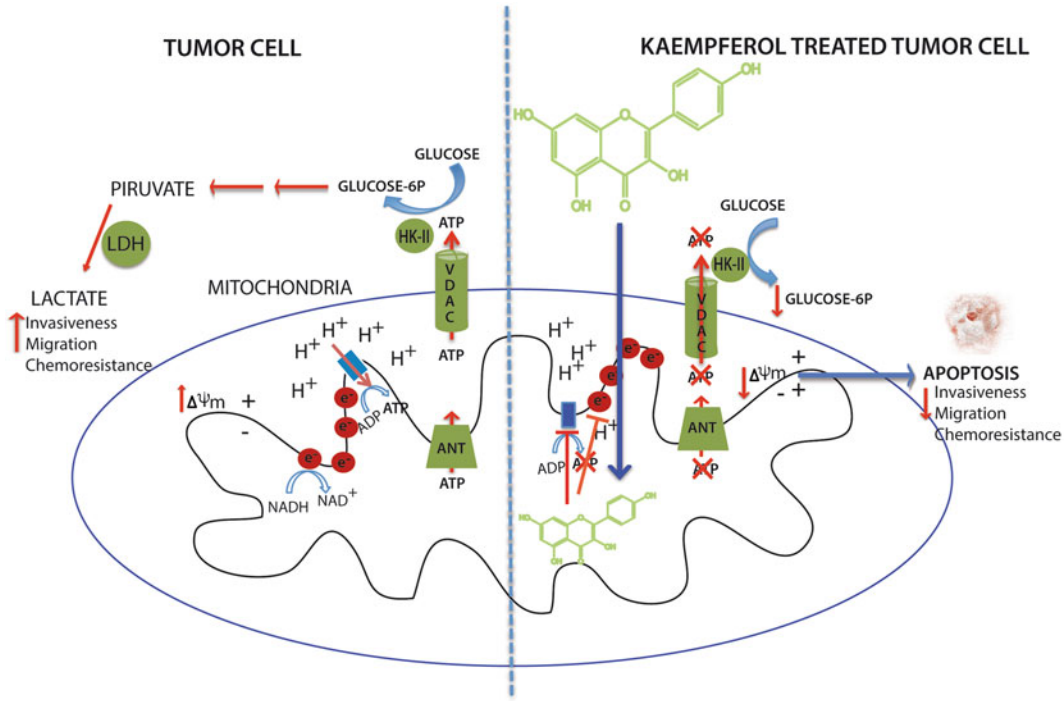
Mabel Catalán and Catalina Rodríguez contributed equally with all other contributors.

M. Catalán and D. Rojas
Clinical and Molecular Pharmacology Program, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, Universidad de Chile, Santiago, Chile

C. Rodríguez, J. Carrasco-Rojas, A. Molina-Berríos, M. Díaz-Dosque, and J. A. Jara (✉)
Institute for Research in Dental Sciences (ICOD), Faculty of Dentistry, Universidad de Chile, Santiago, Chile
e-mail: jsandovalj@u.uchile.cl

I. Olmedo
Physiopathology Program, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, Universidad de Chile, Santiago, Chile

Graphical Abstract



Keywords

3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one · Cisplatin and sensitization · Kaempferol · Mitochondria · Oral Cancer

Abbreviations

AKT	Protein kinase B
AV	Annexin V
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CoQ	Coenzyme Q
CSC	Cancer stem cells
DOK	Dysplastic oral keratinocytes
EGFR	epidermal growth factor receptor
ETC	Electron transport chain
HNSCC	Head and neck squamous cell carcinomas
IC_{50}	Concentration necessary to achieve 50% viability inhibition.
MMP-2	Metalloproteinase-2
MMP-9	Metalloproteinase-9
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OCR	Oxygen consume rate
OxPhos	Oxidative phosphorylation
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinases
PTEN	Phosphatase and tensin homolog protein
TMRME	Tetra methyl rhodamine methyl ester
VEGF	Vascular endothelial growth factor
ΔΨm	Mitochondrial transmembrane potential

1 Introduction

Head and neck squamous cell carcinomas (HNSCC) comprise cancers of the oral cavity, pharynx and larynx. Moreover, squamous cell carcinomas account for more than 90% of all cases of this type of cancer and contribute to the

global cancer burden. The prognosis of this highly malignant disease is still poor, with 5-year survival rates between approximately 50 and 60%, even with combined treatment involving surgery, radiotherapy, chemotherapy, or new monoclonal antibody drugs (Budach and Tinhofer 2019).

Additionally, more than 50% of all patients with HNSCC are diagnosed initially at locally advanced stages, such as stages III or IV, involving regional node or distant metastases, which contribute to their poor prognosis. Despite significant developments in drugs established for other cancers, historically, there have been few novel drugs available for HNSCC. Indeed, drugs older as 5-fluorouracil, cisplatin and docetaxel are still frequently used in chemotherapy treatments, despite adverse effects and a poor efficacy in these types of cancers. Although several chemotherapeutic drugs have been tested during recent decades, the results have been disappointing, and overall survival for patients progressing after failure of platinum-based chemotherapy remains unacceptably poor. It is necessary to search for new cytotoxic molecules that can exert antitumor effects alone or in combination with classic or new antitumor drugs and result in decreases in the doses and subsequently the adverse effects related to antineoplastic drug use (Muratori et al. 2019). Among diverse antitumor drugs, cisplatin is a drug widely used in head and neck cancer treatment to date, but its clinical efficacy is often impaired due to tumor resistance. Nevertheless, this decrease in the efficacy may be improve with the combination of cisplatin with other agents. These adverse events severely affect the quality of life of patients (Ojha et al. 2016; Shahid et al. 2018; Sun et al. 2019). Accordingly, it is necessary to search for new treatment alternatives to improve the efficacy, decrease the doses needed for cisplatin and consequently decrease the toxic effects observed.

Phytochemical molecules are natural plant-derived compounds that have been used for treating various diseases, including various types of tumors. These molecules are widely available and well tolerated with respect to synthetic drugs. Furthermore, in recent years, many studies have shown that numerous phytochemicals are capable of sensitizing tumor cells to antitumor drugs, reversing tumor drug resistance and decreasing toxic effects in various malignancies (Ng et al. 2018).

Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4 one) is a flavonoid that is found in a wide variety of vegetables and fruits, such as broccoli, tomatoes, onions, red fruits, grapes and tea (Chen and Chen 2013; Georgiev et al. 2014). This polyphenol has long been used in traditional medicine to treat several diseases, and it also has antioxidant, inflammatory and antineoplastic effects (Wang et al. 2018). Furthermore, it has been demonstrated that kaempferol can induce diverse mechanisms to disturb the survival or regulation of some cancer cells. For example, this compound inhibited cell growth and migration of pancreatic tumor cells through the inhibition of epidermal growth factor receptor (EGFR) pathways *in vitro* (Lee and Kim 2016).

Moreover, previous studies have reported that some flavonoids at high concentrations may induce the inhibition of the oxygen transport chain in rat hepatocytes (Dorta et al. 2005), and it was shown that genistein and quercetin, other flavonoids, modulate the activity of the mitochondrial transition pore (Ortega and Garcia 2009). Flavonoids present a high structural homology with the quinone moiety of CoQ (Coenzyme Q); thus, the inhibition of complex I of the electron transport chain would be induced by the competition between kaempferol and CoQ by the binding site for CoQ in complex I (Lagoa et al. 2011). Not all tumor cells show principally glycolytic metabolism due to tumor heterogeneity and the different populations of tumor cells in the tumor mass. Additionally, it is currently known that the phosphorylation of glucose to glucose 6-phosphate in glycolytic tumor cells is mediated by hexokinase II, which is highly dependent on recently synthetized ATP from mitochondrial oxidative phosphorylation (OxPhos) (Li et al. 2015; Roberts and Miyamoto 2015). Furthermore, a recent study showed that in hypoxic tumor zones, the development of tumor stem cells is favored; the cells obtain energy through ATP

from OxPhos, despite having concentrations of oxygen lower than 5%; show high resistance to antitumor drugs and have a high capacity for repopulating tumors after treatments (Peiris-Pages et al. 2016). Consequently, some authors have suggested a strategy of inducing mitochondrial dysfunction through phytochemical molecules to improve the antitumor efficacy of or decrease the adverse effects induced by classic antitumor drugs. Thus, natural flavonoids such as apigenin were shown to induce sensitization to cisplatin in prostate CSC (Cancer Stem Cells) (Erdogan et al. 2017). Furthermore, morin, a natural flavonoid and a known inhibitor of NF-κB, can sensitize ovarian cancer cells to cisplatin by decreasing the expression of galectin-3, which is an antiapoptotic protein regulated by the NF-κB transcription factor (Bieg et al. 2018).

Accordingly, in this manuscript, the effects of kaempferol on oral squamous cell carcinoma alone or in combination with cisplatin were evaluated. Moreover, the effects of kaempferol on mitochondrial function and the sensitization of head and neck tumor cells treated with kaempferol to cisplatin were assessed.

2 Results

2.1 Cytotoxic and Antiproliferative Effects of Kaempferol on Head and Neck Tumor Cells

To evaluate the effects of kaempferol in head and neck tumor cells viability and determine the IC_{50} values we realized MTT cell viability test. The results showed that kaempferol (Fig. 1a) has a cytotoxic effect on oral cancer cell lines such as tongue cell carcinoma, which are cisplatin resistant (Cal-27 cell line), and laryngeal squamous cell carcinoma cell lines (HEp-2), which are cisplatin naïve cells (Fig. 1). Furthermore, the effect was dependent on the time of incubation and the concentration of kaempferol, with lower efficacy at 48 h than at 72 h in both tumor cell lines (Figs. 1b and c). However, kaempferol did not affect the dysplastic cell line DOK, a nontumor cell line used as a control to screen the selectivity of kaempferol on nontumor cells (Fig. 1d). These cytotoxic effects are less potent than those of cisplatin because the IC_{50} values at 72 h were 120 μM and 104 μM for the Cal-27 and HEp-2 cell lines, respectively, in contrast to the IC_{50} values of cisplatin, which was 40 μM and > 300 μM at 72 h for the Cal-27 and HEp-2 cells, respectively (Table 1). Additionally, we assessed the antiproliferative effects of kaempferol through a colony formation assay. The results showed that kaempferol induced a concentration-dependent inhibition of proliferation in both tumor cell lines at 72 h (Fig. 1e and f).

2.2 Effects of Kaempferol on Mitochondrial Function

To evaluate the effects of kaempferol on mitochondrial function, we determined the oxygen consumption of head and neck carcinoma cells. Figure 2a shows the representative traces for the oxygraphy realized in tumor cells. Figure 2a shows a decrease in the oxygen consumption rate (OCR) in the presence of diverse concentrations of kaempferol on Cal-27 tumor cells, but this diminution was not concentration dependent in either tumor cell line (Fig. 2b and c). This since despite to observe a decrease of oxygen consume rate at 70, 140 and 280 μM respect to control (determined through one-way ANOVA, $p < 0,05$) in both cell lines. However, there was no statistically significant difference between the different concentrations of kaempferol evaluated (determined through one-way ANOVA, $p > 0,05$) (Fig. 2b and c).

2.3 Effects of Kaempferol on Mitochondrial Transmembrane Potential and Intracellular ATP Content

To evaluate the possible effects of kaempferol in tumor cells mitochondria, we determine the effects of diverse concentration of Kaempferol on two pivotal mitochondrial parameters as mitochondrial transmembrane potential and

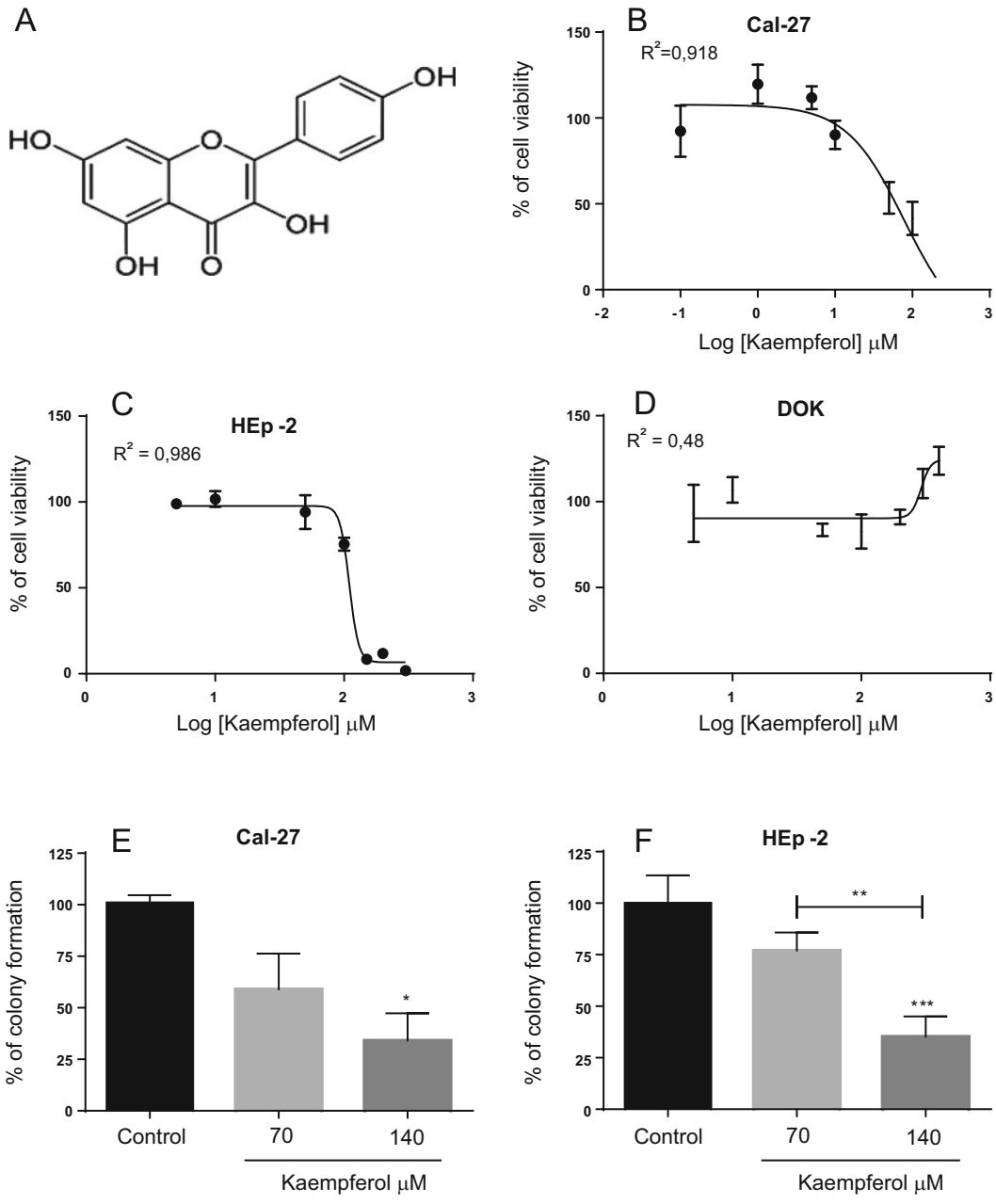


Fig. 1 Cell viability and colony formation of head and neck cancer cell lines after exposure to kaempferol
The MTT assay was performed at incubation times of 72 h. (**a**) Chemical structure of kaempferol. (**b**) Cal-27, (**c**) HEp-2 and (**d**) DOK cell viability curves. Both are sigmoidal dose-response curves (variable slope), providing a graphic representation of the cytotoxicity of kaempferol. (**e**) Cal-27 and F) Hep-2 cell colonies after kaempferol treatment. The values represent the mean of at least three independent experiments ± SD. Each assay was performed in triplicate

Table 1 Cytotoxicity and selectivity of Kaempferol in the Head and Neck cancer cell line

	Kaempferol 48 h	Kaempferol 72 h	Cisplatin 48 h	Cisplatin 72 h
CAL-27	143 μM ± 39	120 μM ± 44	47 μM ± 5	40 μM ± 1
HEp-2	111 μM ± 3	104 μM ± 5	>300 μM	>300 μM
DOK	>300 μM	>300 μM	>500 μM	>500 μM

IC_{50} = concentration necessary to achieve 50% toxicity in the cell culture after 48 or 72 h of treatment. Values were calculated from the respective sigmoidal dose-response curves, representing the mean ± SD values of at least three independent experiments. Each assay was performed in triplicate

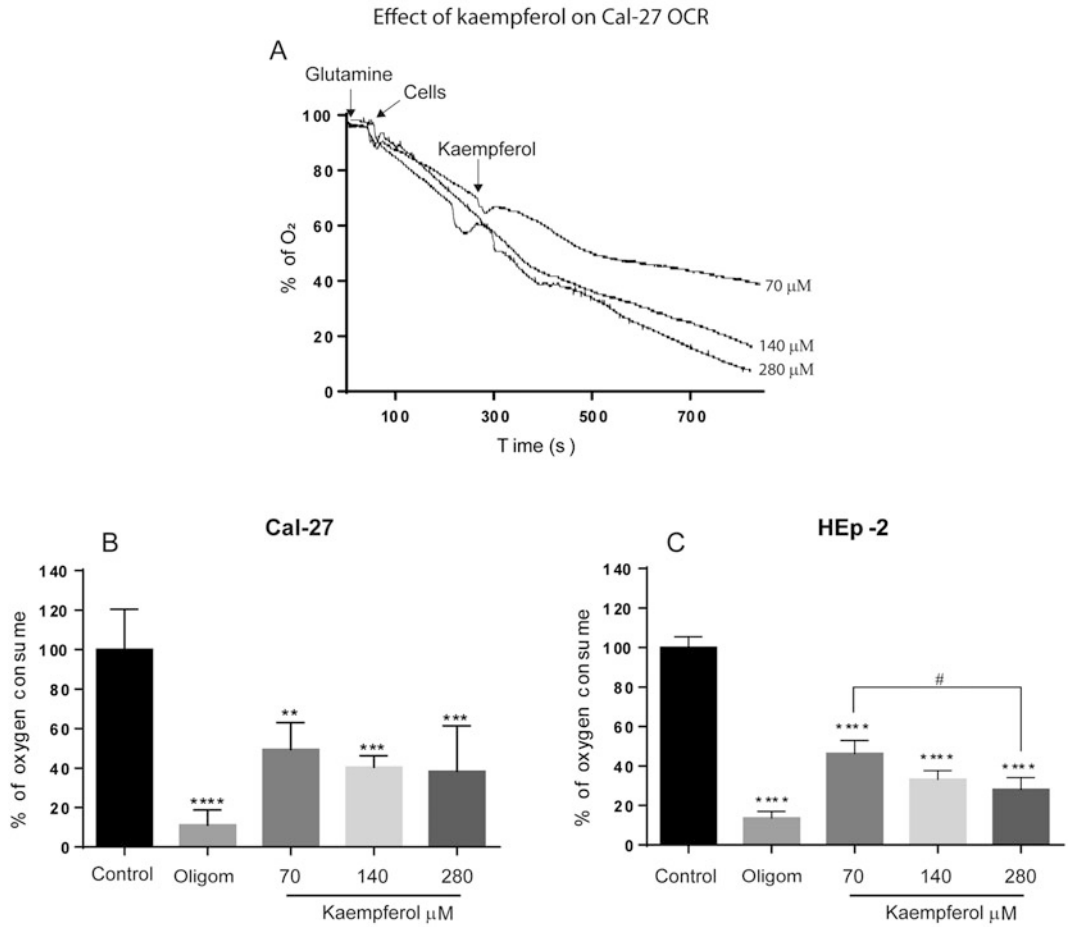


Fig. 2 The effect of kaempferol on cell respiration in head and neck cancer cell lines

(**a**) Schematic graphic of cell respiration and procedures. The graph represents oxygen consumption versus time of Cal-27 cells exposed to different concentrations of Kaempferol. Cells were incubated initially with Glutamine, which is used by tricarboxylic acid cycle (TCA) as a substrate for activate the electron transport chain (ETC) of mitochondria. (**b**) The OCRs of Cal-27 and (**c**) HEp-2 cells after exposure to different concentrations of kaempferol. The OCR in the presence of the experimental compounds was normalized to the control OCR. Oligomycin was used as a positive control. The data shown represent the mean ± SD values of at least five independent experiments. Significant differences between the experimental conditions vs. the control conditions are indicated by asterisks (one-way ANOVA, Bonferroni post hoc test); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

intracellular ATP content. In Fig. 3, the results showed that kaempferol may induce a decrease in mitochondrial transmembrane potential (ΔΨm) but only in the Cal-27 (Fig. 3a) cell line and at a high concentration under the assessed conditions. In the HEp-2 cell line, the effect was not statistically significant at all concentrations assessed (Fig. 3b). However, kaempferol induced a decrease in the intracellular ATP content at almost all concentrations assessed in both Cal-27 and HEp-2 tumor cells (Fig. 3c and d).

2.4 Effects of Kaempferol on Tumor Cell Migration

Figure 4 shows the effects of kaempferol on some proteins pivotal for tumor cell migration. In Fig. 4a and b, the results show that kaempferol may induce a decrease in the activation of the metalloproteinases 2 and 9 (MMP-2, MMP-9) and vascular endothelial growth factor (VEGF) proteins in Cal-27 and HEp-2 tumor cells, respectively (Fig. 4a and b). Moreover, in Fig. 4c,

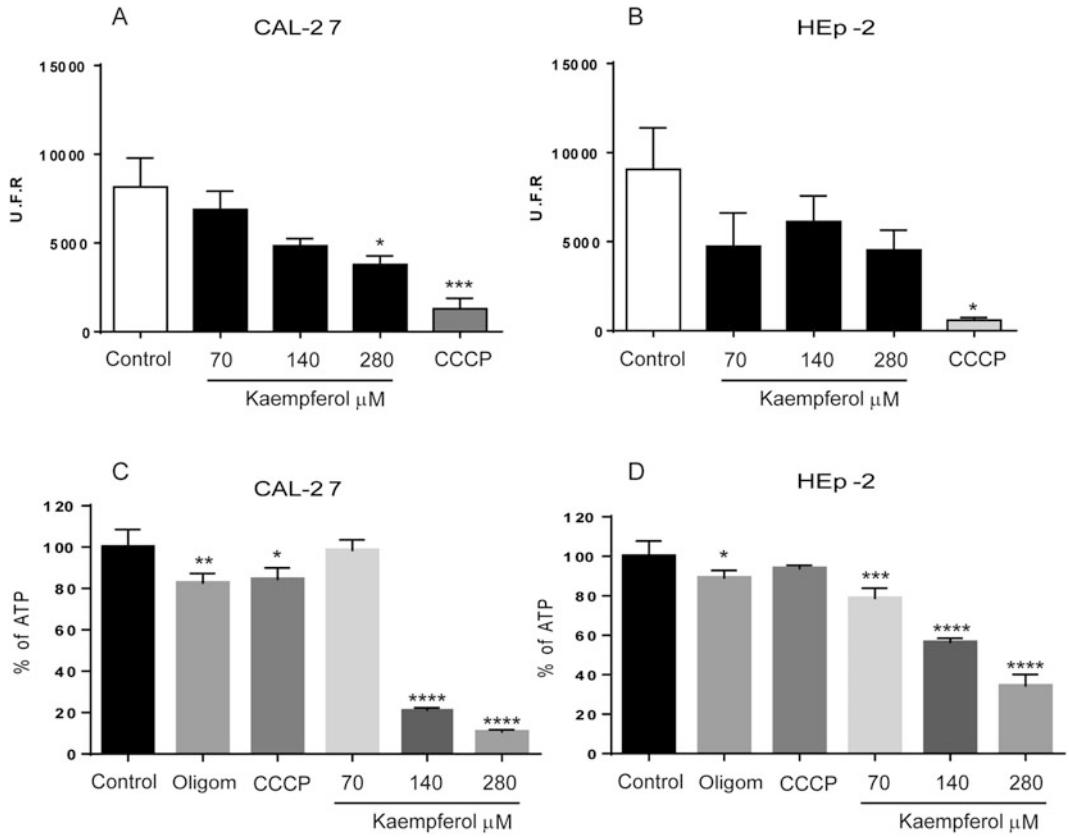


Fig. 3 Evaluation of metabolic alterations produced by kaempferol in head and neck cancer cell lines
The effects of various concentrations of kaempferol on ΔΨm were measured using a TMRM probe sensitive to mitochondrial transmembrane potential after 30 min of incubation with the compounds in (**a**) Cal-27 and (**b**) HEp-2 cells. The data showed the dissipation of intensity of the TMRM probe, a signal of mitochondrial potential loss. The data were normalized to the control values. The ΔΨm values shown represent the mean of four independent experiments ± SD. In addition, the effects of various concentrations of kaempferol after 4 h of incubation on intracellular ATP levels were measured by CellTiter-Glo Luminescent Assays of (**c**) Cal-27 and (**d**) HEp-2 cells. The values show the average ATP levels for each condition and the comparison with the control. CCCP, a classic uncoupler of electron transport chain (ETC), was used as a positive control. Significant differences between the experimental conditions and the control conditions are indicated by asterisks (one-way ANOVA, Bonferroni post hoc test); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

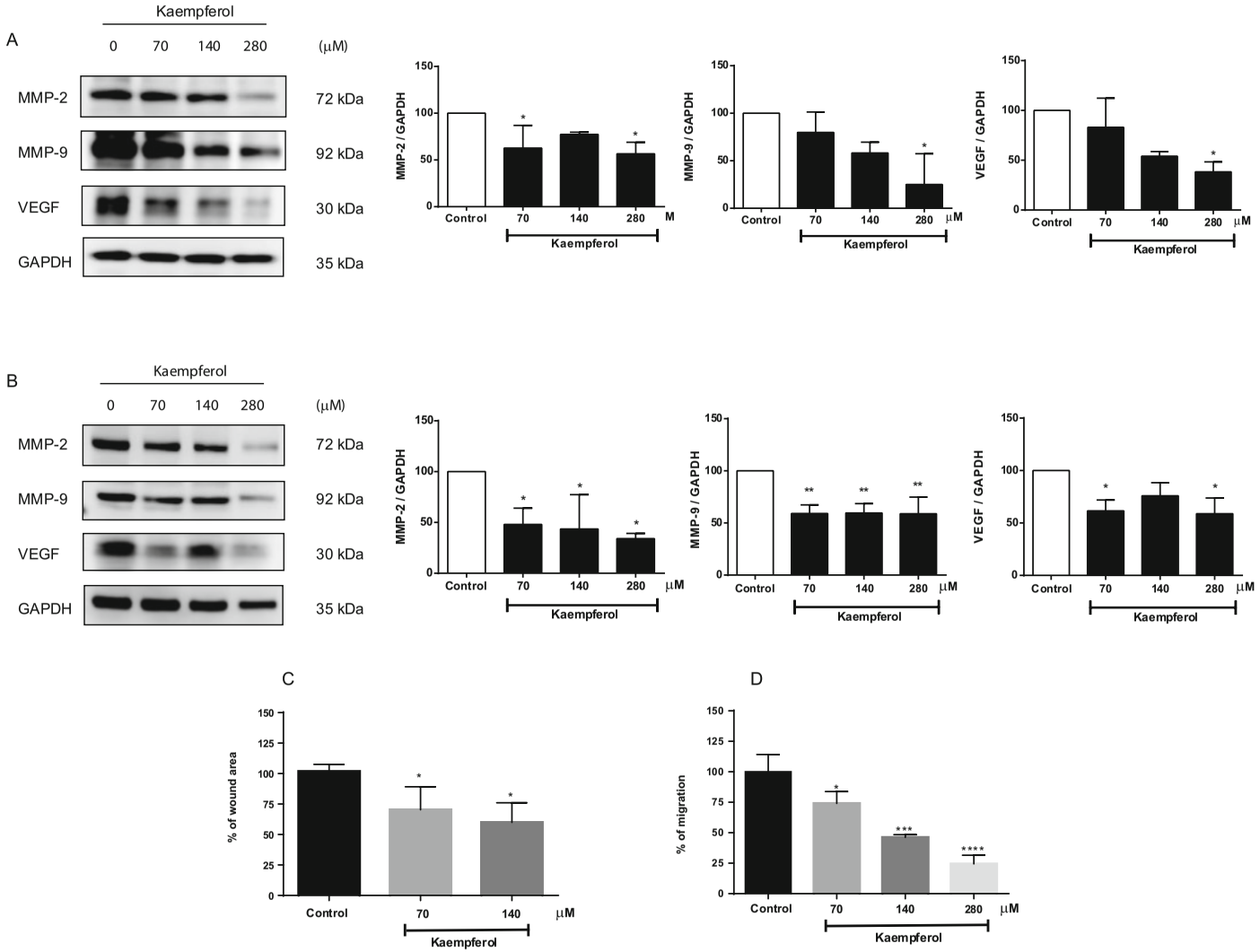


Fig. 4 Evaluation of antimigratory effect produced by kaempferol in head and neck cancer cell lines

The protein levels of metalloproteinase 2 (MMP2), metalloproteinase 9 (MMP9) and vascular endothelial growth factor (VEGF) were evaluated by western blot. Cells were stimulated for 24 h with kaempferol at various concentrations for (**a**) Cal-27 and (**b**) HEp-2 cells. In addition, in the Cal-27 cell line, cell migration was evaluated by (**c**) scratch would and (**d**) Transwell assays. Values shown represent the mean of five independent experiments. Significant differences between the experimental conditions and the control conditions are indicated by asterisks (one-way ANOVA, Bonferroni post hoc test); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

kaempferol decreased the wound area on Cal-27 cells, and we observed the same effects on HEp-2 tumor cells (data not shown). Finally, in Fig. 4d, the results show that kaempferol may induce a significant diminution of cell migration evaluated through Transwell assays.

2.5 Effects of Kaempferol on Induction of Apoptosis

To determine the cell death type induced by kaempferol in head and neck tumor cells, we assessed through flow cytometry the annexin V (AV) and propidium iodide (PI) fluorescence, which permit to evaluate apoptosis and necrosis, respectively. In Fig. 5a and b, dot plots representative of the effects of kaempferol on Cal-27 and HEp-2 cells are shown. The quadrants in the lower and upper right show the cells positive for AV, an apoptotic marker. In the upper left quadrant are cells positive for propidium iodide, a necrosis marker. The quantification shown in graphs 4C and 4D indicates that kaempferol may induce apoptosis in both tumor cells in a nonconcentration-dependent manner.

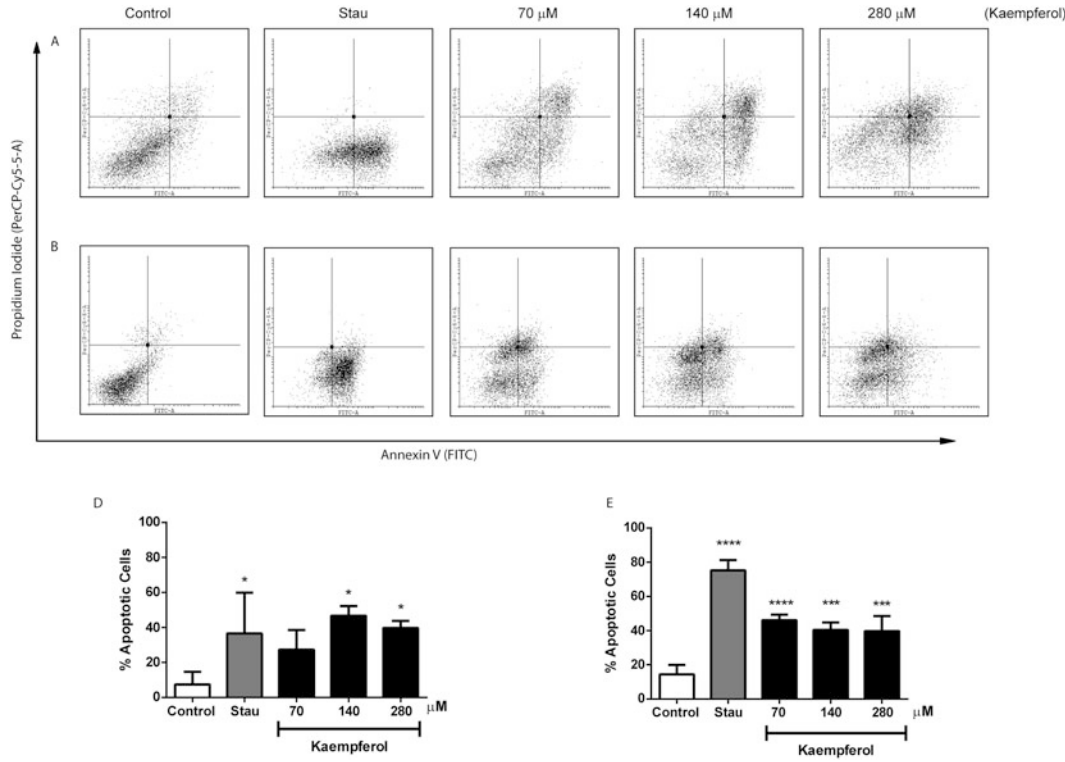


Fig. 5 Apoptosis evaluation of Kaempferol in head and neck cancer cell lines

Apoptosis levels induced by the compounds after 48 h of incubation were assessed in Cal-27 and HEp-2 cell lines. Cells were exposed to Annexin V (AV)-FITC probe, that detects phosphatidylserine exposed to the extracellular space as an early apoptosis event. In addition, cells were exposed to propidium iodide (PI)-PE probe, that enter to the cell when membrane permeability is disrupt, and intercales in the DNA. Representative scatterplots are shown for (**a**) Cal-27 and (**b**) HEp-2 cells, where left lower quadrant is AV-/PI- cells which are living cells; right lower is AV+/PI- which early apoptosis; left upper quadrant is AV-/PI+ which is necrotic cells; and right upper quadrant is AV+/PI+ for late apoptosis. Apoptosis measurements are graphically represented for each compound in (**c**) Cal-27 and (**d**) HEp-2 cells. Assessments were performed using Cyflogic software (version 1.2.1). Staurosporine (Stau; 10 μM) was used as a positive apoptosis-induction control. Apoptosis was assessed by quantifying Annexin V-FITC-positive cells using flow cytometry (FACSCanto, BD Biosciences). The data shown represent the mean ± SD of four independent experiments. Significant differences between the experimental conditions and the control are indicated by asterisks (one-way ANOVA, Bonferroni's post hoc test); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

2.6 Effects of Kaempferol in Combination with Cisplatin

Figure 6 shows that kaempferol sensitized tumor cells to the effects of cisplatin, a common drug used in the treatment of head and neck cancer. Moreover, kaempferol at lower concentrations than their IC_{50} values (140 μM) improved the effects of cisplatin, which we did not observe using cisplatin alone, for example, at concentrations of 25, 50 and 100 μM cisplatin.

3 Discussion

Previous studies of different kinds of polyphenols have shown that these molecules have cytotoxic effects on tumor cells and antineoplastic effects. In this work, kaempferol showed cytotoxic (Fig. 1) and antiproliferative effects, the latter evaluated through colony formation assays (Fig. 1e and f); furthermore, kaempferol had antimigratory effects on head and neck tumor cells. These results are concordant with reports describing similar properties in other types of cancers (Batra and Sharma 2013; Greenwell and Rahman 2015; Abdal Dayem et al. 2016).

With respect to the IC_{50} values obtained in the cell viability experiments for the head and neck cancer cell lines Cal-27 and HEp-2, as shown in Table 1, the values at 48 and 72 h were greater than 100 μM. These values are in accordance with the IC_{50} values for other polyphenols described in other tumor cells and cancers or above those observed in other cancers (Yi et al. 2016; Han et al. 2018). These values in general are higher than the IC_{50} values for drugs used in clinical practice for each tumor, but polyphenols have very high therapeutic windows. Furthermore, the viability assays showed selective cytotoxic effects of kaempferol on tumor cell lines and nonsignificant cytotoxic effects on nontumor cells, such as the DOK cell line, a dysplastic tongue cell line (Fig. 1d). Moreover, a higher

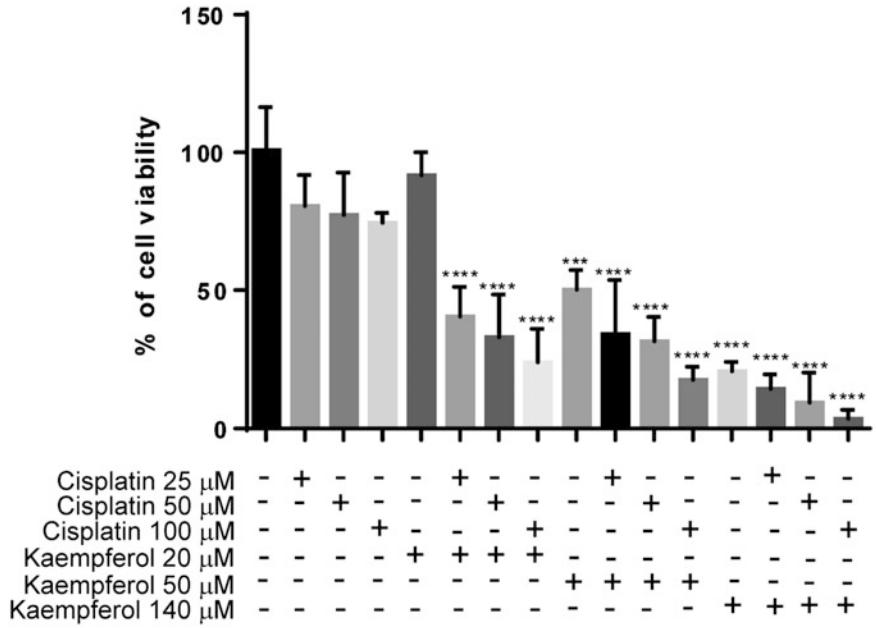


Fig. 6 The effect in cell viability of Kaempferol in combination with Cisplatin in Cal-27 cell line
Cal-27 cells were incubated for 48 h with kaempferol and cisplatin at different concentrations, and cell viability was evaluated by MTT assays. The data shown represent the mean ± SD of four independent experiments. Significant differences between the experimental conditions and the control are indicated by asterisks (one-way ANOVA, Bonferroni's post hoc test); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

concentration of kaempferol improved the viability of dysplastic cells.

Mitochondria are the respiratory and energetic centers of cells where multiple signal transduction pathways converge, leading to dysfunction of those organelles and apoptotic and/or necrotic cell death. Consequently, mitochondria-targeted anticancer drugs are referred to as mitocans with different molecular modes of action (Neuzil et al. 2013). In this context, polyphenols from plants and their synthetic or semisynthetic derivatives exhibit pleiotropic biological activities, including modes of action of diverse mitocans. Some of them, such as genistein, theaflavin and their gallate conjugates, and EGCG, have already been tested as class V mitocans or electron redox chain-targeting agents (Salvi et al. 2002; Li et al. 2012; Valenti et al. 2013). Based on this background, we evaluated the effects of kaempferol on the OCR as an additional mechanism that may partially explain the mode of action in head and neck tumor cells. Our results suggest that although kaempferol induces a decrease in the OCR in a concentration-independent manner, it does not induce a relevant decrease in the mitochondrial transmembrane potential but induces a significant decrease in the intracellular ATP content without loss of cell viability (Fig. 3). In this study, we did not identify the specific site of inhibition in the electron transport chain (ETC), which represents a weakness in our work. However, another author has reported that kaempferol in isolated mitochondria of rat brain induced an inhibition of complex I of the ETC (Lagoa et al. 2011).

In the context of diabetic retinopathy, 25 μM kaempferol inhibited the proliferation and migration of human retinal endothelial cells (HRECs), as shown by CCK-8, transwell, scratch wound, and tube formation assays (Xu et al. 2017). Additionally, a mild effect on cell viability but a significant reduction in VEGF gene expression were observed in OVCAR-3 and A2780/CP70 ovarian tumor cells (Luo et al. 2009). Moreover, in SSC-4 tongue tumor cells, it has been shown that kaempferol induces an antimetastatic effect associated with reduced expression of the MMP-2 and TIMP-2 mRNA and protein levels. Kaempferol also produced an inhibitory effect on the phosphorylation of ERK1/2 (Lin et al. 2013).

Accordingly, our data showed that kaempferol inhibited the migration of head and neck tumor cells by inhibiting VEGF expression and the activation of MMP-2 and MMP-9 metalloproteinases that degrade extracellular matrix and permit the migration and invasion of tumor cells.

However, kaempferol inhibited proliferation and induced apoptosis and autophagy in human lung cancer A549 cells. Additionally, kaempferol might exert these effects through upregulation of miR-340, along with upregulation of PTEN and inactivation of the PI3K/AKT pathway (Han et al. 2018). Moreover, other authors have described induction of apoptosis in MCF-7 breast cancer cells (Yi et al. 2016). Once again, our results are in accordance with these previous studies, but the Cal-27 cell line showed slight resistance to apoptosis compared with the HEp-2 cell line. Moreover, in both tumor cell lines, the effects were not observed in a concentration-dependent manner, which suggests that kaempferol may induce a cell death mechanism different from necrosis, which was low in our assays (data not shown).

Cisplatin-based chemotherapy regimens are used as adjuvant and palliative treatments and play a pivotal role against head and neck cancer, especially for cancers with aggressive characteristics. However, cisplatin resistance is a key limitation for its clinical efficacy (Suzuki et al. 2011; Pendleton and Grandis 2013; Yang et al. 2019). Therefore, combined treatment with other sensitizing agents, for which some polyphenols have been proposed as supportive therapy for other types of cancer cells (Garg et al. 2005) is an effective strategy to overcome cisplatin resistance. Herein, we provide experimental evidence that the addition of kaempferol to cisplatin cytotoxic effects may improve the therapeutic potential for human head and neck cancer.

In addition, we found that kaempferol may induce inhibition of the mitochondrial oxygen consumption rate, which leads to a decrease in intracellular ATP content. This novel mechanism may partially explain the inhibition of tumor cell migration and induction of apoptosis observed in

our assays. In this sense, kaempferol mitochondrial effects are in agreement with the findings related to other polyphenols such as quercetin and resveratrol, both also described as potential anticancer agents to sensitize classical drugs used as a gold standard treatment for many types of cancer (Gupta et al. 2011; Vafadar et al. 2020).

Our data revealed a novel function of kaempferol and enhanced the value of kaempferol as an anticancer and sensitizing agent in head and neck cancer, which is a group of cancers with poor therapeutic alternatives and high therapy failure rates.

4 Materials and Methods

4.1 Chemical and Reagents

DMEM culture medium, carbonyl cyanide m-cholorophenyl hydrazone (CCCP), Glutamine, Glucose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), Fetal Bovine Serum (FBS), staurosporine, trypan blue 0.4% solution, were purchased from Sigma Chemical Co. (St. Louis, MO). Kaemperol was purchased from Sigma-Aldrich. Cisplatin was purchased from Santa Cruz Biotechnology. Penicillin/streptomycin, trypsin, were purchased from Hyclone Laboratories (South Logan, UT). All other reagents were purchased from Sigma (Sigma-Aldrich, USA).

4.2 Cell Lines and Cell Culture

The human oral squamous cell lines, Cal 27 (ATCC® CRL-2095™) was acquired from American Type Culture Collection (ATCC). The laryngeal cell line HEp-2 was acquired from Instituto de Salud pública de Chile. DOK, oral dysplastic keratinocites were acquires from The European Collection of Authenticated Cell Cultures (ECACC). Cal-27 and HEp-2 cell lines were grown in DMEM supplemented with 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin in a humidified 5% CO_2 at 37 °C. DOK cells were grown in DMEM supplemented with 400 ng/mL hydrocortisone plus 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin in a humidified 5% CO_2 at 37 °C. For passages, cells were detached by using trypsin-EDTA and seeded in culture plates for different experiments.

4.3 Cell Viability Assay

Cells were seeded into a 96-well plate (1×10^4 cell per well). After 24 h, kaempferol compounds were added at increasing concentrations into the wells and incubated for 48 and 72 h, in culture DMEM medium supplemented with 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin in a humidified 5% CO_2 at 37 °C. After this time, the cells were washed twice with PBS (phosphate buffer saline) 1X, and then 100 μL of 0.5 mg/mL MTT solution was added to each well. After 2 h of incubation, MTT was removed and crystals of formazan were dissolved in 40 μL of DMSO. Absorbance was measured at 570 nm with a microplate ELISA reader (Infinite F50®Tecan Group Ltd., Swiss).

4.4 Colony Assay

For this assay 2×10^3 cells were cultured in a 6 well plate. Then, the cells were incubated during 24 h at 37 °C and 5% of CO_2. Later the cells were treated for 24 h with different concentrations of kaempferol. Then, the culture medium was changed for culture medium without compound, and then the cells were incubated during 72 h. Finally, crystal violet 0.5% in methanol was applied to the cells for 30 min. The number of colonies was photographed and determined by using Image J software program (NIH, Bethesda, MD, USA).

4.5 Oxygen Consumption Assay

The cell respiration rates were measured polarographically (Frey et al. 2007). Tumor cells were

cultured at 80% of confluency and then trypsinized and counted. 5×10^6 cells were used for experimental measurement. 0.6 mL of cell suspension in PBS 1X (pH 7.4) at 25 °C was added to the electrode chamber with L-glutamine (8.3 mM). The respiration rate which accounts for the OxPhos system was firstly inhibited by 2.5 μg/mL oligomycin. Then, the full uncoupled respiration rates were assessed adding 0.133 μM of CCCP (Carbonyl cyanide m-cholorophenyl hydrazone), which is used as control. The effects of oxygen consumption rates (OCR) provoked by each compound under study were measured by adding increasing concentrations of each of them (70, 140 and 280 μM). The results were compared respect to the OCR for CCCP.

4.6 Mitochondrial Transmembrane Potential (ΔΨm)

Changes in transmembrane potential (ΔΨm) were determined using tetra methyl rhodamine methyl ester (TMRME) as a probe. Cells were seeded into 24-well plates (1×10^5 cells per well) for 24 h and then incubated with increasing concentrations of kaempferol (70, 140 and 280 μM) for 30 min. Passed 15 min with kaempferol cells was exposed to 200 nM TMRM probe. Then, cells were washed with PBS 1X, detached and suspended in cold PBS for analysis using Flow Cytometry FACS (FACSAria® III, BD Biosciences) at a wavelength of 540Ex/595Em nm.

4.7 Intracellular ATP Levels

The tumor cells ATP levels were measured using the CellTitle-Glo Luminescent Assay (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 1×10^4 cells per well were seeded into 96-wells plate, and were cultured during 24 h. Each well was incubated with kaempferol at different concentrations for 4 h. Then, 100 μL of the cell suspension were transferred to an opaque 96-well plate and incubated at room temperature for 10 min in the dark. Then, the luminescence was captured using Thermo-Scientific Varioskan Flash spectral scanning reader. As a control assay for cell viability and cell membrane integrity for the measurement of intracellular ATP content, PI incorporation was assessed through flow cytometry (FACS Canto, BD Biosciences, San Jose, CA) according to described previously (Jara et al. 2014).

4.8 Apoptosis Induction

The induction of apoptosis by kaempferol was determined through flow cytometry using Annexin V and PI probes (Annexin V-FITC Apoptosis Detection Kit, Abcam), according to the manufacturer's instructions (FACS Canto, BD Biosciences). Briefly, 1×10^5 cells/mL were incubated with kaempferol for 48 h at 37 °C and 5% CO_2. The cells were suspended in 500 μL of 1X Annexin V-binding buffer, and then were added 5 μL both annexin V and PI to the samples and then we incubated the cells for 5 min at room temperature. The samples were measured at Ex/Em = 488/530 nm for Annexin V-FITC and Ex/Em = 488/575 nm for PI and 10,000 events were recorded. The results were analyzed using the Cyflogic program (non-commercial version, CyFlo Ltd.).

4.9 Scratch Assays

1×10^5 cells were seeded in a 24 well plate. These cells were incubated during 24 h in DMEM supplemented with 10% FBS. Later, the cells were washed with PBS 1X and a wound was made with a 200 μL micropipette tip. Then, cells were washed with PBS and incubated with different concentrations of kaempferol in culture medium without FBS with 5-bromodeooxyuridine (BrdU) 50 μM (to inhibit the cells proliferation). As a positive control was utilized, DMEM supplemented with 10% of FBS. Each sample was photographed at 72 h, respectively. The samples were compared with their own wound area, which was registered at the

start of the assay, using Image J software (NIH, Bethesda, MD, USA).

4.10 Tumor Cells Migration Assay

The Transwell chambers with 8 μm pores were obtained from Corning (Corning, NY, USA). Cells were harvested for the migration assay, resuspended in DMEM without FBS, at a concentration of 1×10^5 cells in 100 μL. The cells were seeded into Transwell 24 well-plate upper chamber and different concentrations of the each compound were added. The lower chamber were filled with 600 μL of DMEM supplemented with 10% FBS. Cells were then incubated for 16 h at 37 °C and 5% of CO_2. At the end of the assay, cells that migrated into the reverse side of the Transwell membrane were fixed with methanol, stained with violet crystal 0.5%, and then photographed under a light microscope. The number of migratory cells was measured using Image J software (NIH, Bethesda, MD, USA).

4.11 Activation of Metalloproteinases and VEGF by Western Blot Analysis

Cells were treated with kaempferol for 48 h and then washed in cold PBS 1X and lysed with 40 × −diluted RIPA buffer (50 mM Tris-HCl; 150 mM NaCl; 0.1% sodium dodecyl sulfate) containing a proteinase and phosphatase inhibitor cocktail (Cell Signaling Technologies, Boston, MA). A 40 μg sample of each protein was separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the resolved proteins were transferred to a nitrocellulose membrane (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 at room temperature for 1 h and then incubated with the primary antibody for MMP-9 (dilution 1:1000), MMP-2 (dilution 1:1000), VEGF (dilution 1:1000), or β-actin (dilution 1:1000) at 4 °C overnight. Membranes were washed, next incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature (dilution 1:5000). After washing, the membranes were exposed to an enhanced chemiluminescent reagent (EZ-ECL). A ChemiDoc MP System was used for Western blotting. Digital blots were quantified using ImageJ software.

Acknowledgements Fondecyt iniciación Grant N°11180533 (JAJ), PRI-ODO 18/003 (JAJ), Fondecyt iniciación Grant N°11160281 (MC), Fondecyt iniciación Grant N°11170962 (IOA).

Conflict of Interest The authors declare they have no conflict of interests.

References

Abdal Dayem A, Choi HY, Yang GM, Kim K, Saha SK, Cho SG (2016) The anti-cancer effect of polyphenols against breast cancer and cancer stem cells: molecular mechanisms. Nutrients 8(9)

Batra P, Sharma AK (2013) Anti-cancer potential of flavonoids: recent trends and future perspectives. 3 Biotech 3(6):439–459

Bieg D, Sypniewski D, Nowak E, Bednarek I (2018) Morin decreases galectin-3 expression and sensitizes ovarian cancer cells to cisplatin. Arch Gynecol Obstet 298(6):1181–1194

Budach V, Tinhofer I (2019) Novel prognostic clinical factors and biomarkers for outcome prediction in head and neck cancer: a systematic review. Lancet Oncol 20(6):e313–e326

Chen AY, Chen YC (2013) A review of the dietary flavonoid, kaempferol on human health and cancer chemoprevention. Food Chem 138(4):2099–2107

Dorta DJ, Pigoso AA, Mingatto FE, Rodrigues T, Prado IM, Helena AF, Uyemura SA, Santos AC, Curti C (2005) The interaction of flavonoids with mitochondria: effects on energetic processes. Chem Biol Interact 152(2–3):67–78

Erdogan S, Turkekul K, Serttas R, Erdogan Z (2017) The natural flavonoid apigenin sensitizes human CD44(+) prostate cancer stem cells to cisplatin therapy. Biomed Pharmacother 88:210–217

Frey C, Pavani M, Cordano G, Munoz S, Rivera E, Medina J, Morello A, Diego Maya J, Ferreira J (2007) Comparative cytotoxicity of alkyl gallates on mouse tumor cell lines and isolated rat hepatocytes. Comp Biochem Physiol A Mol Integr Physiol 146 (4):520–527

Garg AK, Buchholz TA, Aggarwal BB (2005) Chemosensitization and radiosensitization of tumors by plant polyphenols. Antioxid Redox Signal 7 (11–12):1630–1647

Georgiev V, Ananga A, Tsolova V (2014) Recent advances and uses of grape flavonoids as nutraceuticals. Nutrients 6(1):391–415

Greenwell M, Rahman PK (2015) Medicinal plants: their use in anticancer treatment. Int J Pharm Sci Res 6 (10):4103–4112

Gupta SC, Kannappan R, Reuter S, Kim JH, Aggarwal BB (2011) Chemosensitization of tumors by resveratrol. Ann N Y Acad Sci 1215:150–160

Han X, Liu CF, Gao N, Zhao J, Xu J (2018) Kaempferol suppresses proliferation but increases apoptosis and autophagy by up-regulating microRNA-340 in human lung cancer cells. Biomed Pharmacother 108:809–816

Jara JA, Castro-Castillo V, Saavedra-Olavarria J, Peredo L, Pavanni M, Jana F, Letelier ME, Parra E, Becker MI, Morello A, Kemmerling U, Maya JD, Ferreira J (2014) Antiproliferative and uncoupling effects of delocalized, lipophilic, cationic gallic acid derivatives on cancer cell lines. Validation in vivo in singenic mice. J Med Chem 57(6):2440–2454

Lagoa R, Graziani I, Lopez-Sanchez C, Garcia-Martinez V, Gutierrez-Merino C (2011) Complex I and cytochrome c are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria. Biochim Biophys Acta 1807(12):1562–1572

Lee J, Kim JH (2016) Kaempferol inhibits pancreatic cancer cell growth and migration through the blockade of EGFR-related pathway in vitro. PLoS One 11(5): e0155264

Li B, Vik SB, Tu Y (2012) Theaflavins inhibit the ATP synthase and the respiratory chain without increasing superoxide production. J Nutr Biochem 23(8):953–960

Li X, Lu Y, Lu H, Luo J, Hong Y, Fan Z (2015) AMPK-mediated energy homeostasis and associated metabolic effects on cancer cell response and resistance to cetuximab. Oncotarget 6(13):11507–11518

Lin CW, Chen PN, Chen MK, Yang WE, Tang CH, Yang SF, Hsieh YS (2013) Kaempferol reduces matrix metalloproteinase-2 expression by down-regulating ERK1/2 and the activator protein-1 signaling pathways in oral cancer cells. PLoS One 8(11):e80883

Luo H, Rankin GO, Liu L, Daddysman MK, Jiang BH, Chen YC (2009) Kaempferol inhibits angiogenesis and VEGF expression through both HIF dependent and independent pathways in human ovarian cancer cells. Nutr Cancer 61(4):554–563

Muratori L, La Salvia A, Sperone P, Di Maio M (2019) Target therapies in recurrent or metastatic head and neck cancer: state of the art and novel perspectives. A systematic review. Crit Rev Oncol Hematol 139:41–52

Neuzil J, Dong LF, Rohlena J, Truksa J, Ralph SJ (2013) Classification of mitocans, anti-cancer drugs acting on mitochondria. Mitochondrion 13(3):199–208

Ng CY, Yen H, Hsiao HY, Su SC (2018) Phytochemicals in skin cancer prevention and treatment: an updated review. Int J Mol Sci 19(4)

Ojha S, Venkataraman B, Kurdi A, Mahgoub E, Sadek B, Rajesh M (2016) Plant-derived agents for counteracting cisplatin-induced nephrotoxicity. Oxidative Med Cell Longev 2016:4320374

Ortega R, Garcia N (2009) The flavonoid quercetin induces changes in mitochondrial permeability by inhibiting adenine nucleotide translocase. J Bioenerg Biomembr 41(1):41–47

Peiris-Pages M, Martinez-Outschoorn UE, Pestell RG, Sotgia F, Lisanti MP (2016) Cancer stem cell metabolism. Breast Cancer Res 18(1):55

Pendleton KP, Grandis JR (2013) Cisplatin-based chemotherapy options for recurrent and/or metastatic squamous cell Cancer of the head and neck. Clin Med Insights Ther 2013(5)

Roberts DJ, Miyamoto S (2015) Hexokinase II integrates energy metabolism and cellular protection: Akting on mitochondria and TORCing to autophagy. Cell Death Differ 22(2):248–257

Salvi M, Brunati AM, Clari G, Toninello A (2002) Interaction of genistein with the mitochondrial electron transport chain results in opening of the membrane transition pore. Biochim Biophys Acta 1556 (2–3):187–196

Shahid F, Farooqui Z, Khan F (2018) Cisplatin-induced gastrointestinal toxicity: an update on possible mechanisms and on available gastroprotective strategies. Eur J Pharmacol 827:49–57

Sun CY, Zhang QY, Zheng GJ, Feng B (2019) Phytochemicals: current strategy to sensitize cancer cells to cisplatin. Biomed Pharmacother 110:518–527

Suzuki M, Ishikawa H, Tanaka A, Mataga I (2011) Heterogeneity of anticancer drug sensitivity in squamous cell carcinoma of the tongue. Hum Cell 24(1):21–29

Vafadar A, Shabaninejad Z, Movahedpour A, Fallahi F, Taghavipour M, Ghasemi Y, Akbari M, Shafiee A, Hajighadimi S, Moradizarmehri S, Razi E, Savardashtaki A, Mirzaei H (2020) Quercetin and cancer: new insights into its therapeutic effects on ovarian cancer cells. Cell Biosci 10:32

Valenti D, de Bari L, Manente GA, Rossi L, Mutti L, Moro L, Vacca RA (2013) Negative modulation of mitochondrial oxidative phosphorylation by epigallocatechin-3 gallate leads to growth arrest and apoptosis in human malignant pleural mesothelioma cells. Biochim Biophys Acta 1832(12):2085–2096

Wang J, Fang X, Ge L, Cao F, Zhao L, Wang Z, Xiao W (2018) Antitumor, antioxidant and anti-inflammatory activities of kaempferol and its corresponding glycosides and the enzymatic preparation of kaempferol. PLoS One 13(5):e0197563

Xu XH, Zhao C, Peng Q, Xie P, Liu QH (2017) Kaempferol inhibited VEGF and PGF expression and in vitro angiogenesis of HRECs under diabetic-like environment. Braz J Med Biol Res 50(3):e5396

Yang Z, Liao J, Carter-Cooper BA, Lapidus RG, Cullen KJ, Dan H (2019) Regulation of cisplatin-resistant head and neck squamous cell carcinoma by the SRC/ETS-1 signaling pathway. BMC Cancer 19(1):485

Yi X, Zuo J, Tan C, Xian S, Luo C, Chen S, Yu L, Luo Y (2016) Kaempferol, a flavonoid compound from Gynura Medica induced apoptosis and growth inhibition in Mcf-7 breast cancer cell. Afr J Tradit Complement Altern Med 13(4):210–215

Adv Exp Med Biol - Cell Biology and Translational Medicine (2021) 12: 111–137
https://doi.org/10.1007/5584_2020_612

Published online: 1 January 2021

A Comprehensive Approach to Urticaria: From Clinical Presentation to Modern Biological Treatments Through Pathogenesis

Marco Folci, Giacomo Ramponi, and Enrico Brunetta

Abstract

Urticaria is characterized by the cutaneous presence of wheals (hives), angioedema or both. Acute and chronic urticaria are distinguished based on a duration of less or more than 6 weeks. Chronic urticaria can be further classified into a spontaneous form and several inducible types triggered by specific external stimuli. Lifetime prevalence of urticaria may be up to 20%, with the acute form being way more common than the chronic one. Exacerbating factors (e.g. infections, drugs, food) and immune system alterations have been investigated as main triggers of mast cell activation, which in turn leads to increased vascular permeability and extravasation of inflammatory cells. While diagnostic workup is focused upon history taking, several emerging biomarkers correlate with severity and/or prognosis of the disease and can be necessary to differentiate chronic spontaneous urticaria from other disorders, such as vasculitis and autoinflammatory diseases. Treatment of acute urticaria is based upon H1 antihistamines and short courses of steroids. While H1 antihistamines are also used in chronic spontaneous urticaria, omalizumab is the standard of care in patients who are unresponsive to these. Recently, several new drugs have entered clinical trials to offer a therapeutic possibility for patients unresponsive to omalizumab. Numerous target molecules, such as mediators of mast cells activation, are under investigation. Amongst these, new anti-IgE therapies and possibly IL-5 pathway blockade seem to have reached enough data to move to advanced clinical trials.

Keywords

Acute urticaria · Angioedema · Biologics · Biomarkers · Chronic spontaneous urticaria · Ligelizumab · Mast cells · Omalizumab · Urticaria · Vasculitis

M. Folci (✉) and E. Brunetta
Internal medicine, division of Clinical Immunology, Humanitas Clinical and Research Center – IRCCS, Milan, Italy

Department of Biomedical Sciences, Humanitas University, Milan, Italy
e-mail: marco.folci@humanitas.it; mdfolci@gmail.com

G. Ramponi
Internal medicine, division of Clinical Immunology, Humanitas Clinical and Research Center – IRCCS, Milan, Italy

Abbreviations

AAC	area above the curve
ACEi	Angiotensin converting enzyme inhibitors
ANA	anti-nuclear antibodies
AOSD	Adult onset Still's disease
ASST	autologous serum skin test
AU	Acute urticaria
BHRA	basophil histamine release assay
BTK	Bruton's tyrosine kinase
C5aR	Complement 5a receptor
CAPS	Cryopirin-associated periodic syndromes
CIndU	Chronic Inducible Urticaria
COX-1	cyclooxygenase 1
CRP	c-reactive protein
CRTH2	chemoattractant receptor homologous molecule expressed on the Th2 cell
CSU	Chronic Spontaneous Urticaria
CU	Chronic urticaria
CU-Q20L	Chronic Urticaria Quality of Life
DARPins	designed ankyrin repeat proteins
ESR	erythrocyte sedimentation rate
F1 + 2	Prothrombin fragment 1 + 2
FcεRI	high-affinity IgE receptor
FcεRIα	high-affinity IgE receptor alpha chain
HLA	Human Leukocyte Antigen
HUV	hypocomplementemic urticarial vasculitis
IFNγ	interferon gamma
IL-1	interleukin 1
IL-18	interleukin 18
IL-24	interleukin 24
IL-25	interleukin 25
IL-33	interleukin 33
IL-4	interleukin 4
IL-5	interleukin 5
IL-6	interleukin 6
Il-6sR	interleukin 6 soluble receptor
InH-AAE	idiopathic nonhistaminergic acquired angioedema
ITAMs	immunoreceptor tyrosine-based activation motifs
LCN2	serum Lipocalin-2
MC_T	tryptase-positive chymase-negative mast cells
MC_{TC}	tryptase-positive chymase-positive mast cells
MMP-9	matrix metalloproteinase-9
MPV	mean platelet volume
NGF	nerve growth factor
NSAID	non-steroidal anti-inflammatory drug
PG	Prostaglandins.
PGD2	prostaglandin D2
sgp130	soluble glycoprotein 130
Siglec	Sialic acid-binding immunoglobulin-like lectin
SYK	spleen tyrosine kinase
Th1	T helper 1
Th2	T helper 2
TPO	thyroid peroxidase
TSH	thyroid stimulating hormone
TSLP	thymic stromal lymphopoietin
UAS7	weekly urticaria activity score
USS	Urticaria Severity Score (USS)
UV	urticarial vasculitis

1 Definition

Urticaria is a common clinical condition characterized by the cutaneous appearance of wheals (hives), angioedema or both (Zuberbier et al. 2018).

Urticarial wheals are defined by three features: a central swelling of variable dimensions surrounded by an erythematous border; an intense sensation of itching or burning; a short-lived appearance with the skin returning to normal in less than 24 h (Zuberbier et al. 2018). Wheals are mainly due to vasodilation in superficial dermal causing swelling of tissues, local oedema and concurrent sensory neural activation which is associated to pruritus (Antia et al. 2018).

Angioedema is characterized by sudden swelling of the lower dermis and subcutis or mucous membranes (with occasional erythema), sporadic presence of pain and a slower resolution in comparison to wheals (up to 72 h) (Zuberbier et al. 2018). Angioedema is also caused by

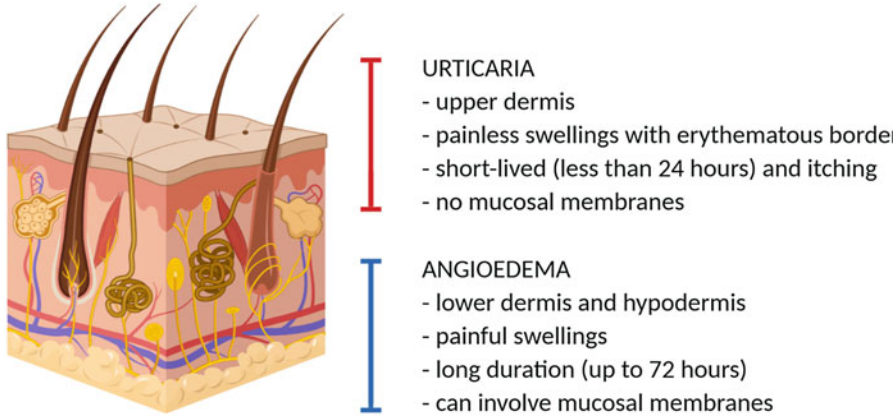


Fig. 1 Features of Urticaria and Angioedema Urticaria is characterized by painless erythematous and itching wheals which involve the upper dermis and are short lived. Angioedema presents as painful swellings with minimal or no skin alteration and long duration. It can involve also mucosal membranes

vasodilation, plasma extravasation and neural stimulation, but contrary to urticaria these phenomena occur in deeper layers of the skin (reticular dermis and subcutaneous tissue) (Antia et al. 2018) (Fig. 1).

2 Classification

Urticaria can have a wide spectrum of presentations and subtypes, which can typically coexist in the same patient. Acute urticaria (AU) is distinguished from chronic urticaria (CU) based upon its duration, with a defining period of less than 6 weeks (compared to a duration longer than 6 weeks for CU, Table 1A). In acute cases of urticaria, anaphylaxis should be excluded by evaluating for signs of respiratory, gastrointestinal, neurologic upset or hemodynamic instability (Antia et al. 2018). The presence of a clear external trigger (e.g. insect sting, food ingestion) or a suggestive past medical history should equally be red flags alarming the physician to consider urticaria as a possible feature of anaphylaxis (Estelle Simons Ledit 2013).

While most cases of acute urticaria remain idiopathic after history taking, the most common associated factors are respiratory infections (40% of cases), drug reactions (9,2%) and food reactions (0.9%) (Antia et al. 2018; Zuberbier et al. 1996).

Chronic urticaria can be divided into chronic inducible urticaria (CIndU) and chronic spontaneous urticaria (CSU). The subdivision is based upon the clinical reproducibility of wheals following exposure to a direct stimulus (e.g. cold, pressure, water, etc.) or absence of such trigger (Zuberbier et al. 2018). While CSU is considered as a single clinical entity, although multiple pathogenetic subsets may be present, CIndU is further split into several conditions based in the specific trigger mechanisms as presented in Table 1B. Behind the appearance of a minor disorder, chronic urticaria carries an important burden of disease for affected patients and their family members (Zuberbier et al. 2018). A reduction in objective well-being and subjective health has been observed in patients suffering from chronic spontaneous urticaria, especially when the condition is refractory to treatment or just partially responsive (Maurer et al. 2017a). Along with an impaired quality of life, chronic spontaneous urticaria is also associated with a considerable impairment in work productivity, which can be a result of the bothersome itching sensation and aesthetic disfigurement (in severe cases) (Maurer et al. 2017b; Maurer et al. 2016).

Rarely, urticaria can be the initial presentation of a systemic autoimmune, hematologic, autoinflammatory or neoplastic disorder (Zuberbier et al. 2018). Therefore, the presence

Table 1A The current classification distinguishes urticaria based upon its duration (acute when lasting less than 6 weeks, chronic when lasting for a longer time). Chronic urticaria is further subdivided into inducible and spontaneous forms. Patients with CSU have wheals that appear spontaneously, with no external trigger needed. On the contrary, patients with CIndU suffer from wheals only when exposed to specific physical triggers

Duration	Subtype	Description
Acute <6 weeks		
Chronic >6 weeks	*CSU*	Unknown trigger
	Inducible	Known trigger

Table 1B Classification of chronic inducible urticaria. Patients with CIndU suffer from wheals only when exposed to specific physical triggers. Sometimes, CIndU and CSU may coexist in the same patient

Subtype	Description	Test
Symptomatic dermographism	Urticarial lesions in response to pressure, rubbing or scratching (human contact or clothes) (Dice 2004). Wheals appear immediately after contact and remit within less than an hour	Fric test: Plastic tool used to evaluate the severity of dermographism reproducibly (Mlynek et al. 2013)
Acquired cold urticaria	Wheals after exposure to cold fluids, solids or gaseous substances	The reaction can be elicited by applying the cold provocation test (Zuberbier et al. 2018)
Heat urticaria	Hot objects induce the formation of wheals when brought in contact with the skin. Some familial cases have also been described (Radonjic-Hoesli et al. 2018)	A heat provocation test shall be performed for confirmation (Zuberbier et al. 2018)
Delayed pressure urticaria	Formation of skin lesions several hours after exposure to perpendicular pressure. It often coexists with CSU and other forms of physical urticaria (Radonjic-Hoesli et al. 2018)	Pressure test to verify and quantify angioedema (Zuberbier et al. 2018)
Solar urticaria	Exposure to sunlight, especially UV-A light. This condition is very rare (Radonjic-Hoesli et al. 2018)	The patient may be tested with light of different wavelengths (Zuberbier et al. 2018).
Cholinergic urticaria	It precipitates after an immediate increase in body temperature (e.g. hot baths, physical exercise, emotional stress, hot food) (Abajian et al. 2014)	Provocation testing (Zuberbier et al. 2018)
Vibratory angioedema	Angioedema occurs after local exposure of the skin to vibrations. Interestingly, a genetic mutation (ADGRE2) has been observed in the autosomal dominant variant of the disease (Boyden et al. 2016)	Testing with vibration (e.g. mixer) (Zuberbier et al. 2018)
Contact Urticaria	When the patient experiences contact with the eliciting substance, wheals appear which persist for a few hours. Contact urticaria may sometimes be an occupational disease (Williams et al. 2008)	Provocation testing with suspected substance (Zuberbier et al. 2018)
Aquagenic Urticaria	Rare form which occurs after contact with water, including body fluids such as sweat and tears	Diagnosis can be obtained by performing a water challenge test (Rothbaum and McGee 2016)

of atypical associated features should prompt the clinician towards a more extensive diagnostic approach.

3 Epidemiology and Genetics

Surveys about the lifetime prevalence of any form of urticaria yielded results ranging from less than 1% to more than 20%, depending on the age of patients, the methods of the study and its location (Antia et al. 2018; Weller et al. 2010). Both AU and CU have been more commonly observed in females, with an apparent ratio of 2 females for each male (Antia et al. 2018; Sánchez-Borges et al. 2015a; Amsler et al. 2014; Lapi et al. 2016; Kalogeromitros et al. 2011; Magen et al. 2013). Apparently, the gender gap in prevalence

seems to narrow among the elderly, the children and when considering some forms of CIndU (Cassano et al. 2016; Ban et al. 2014).

AU lifetime prevalence was reported in Europe with estimations going from 12% to 24%, qualifying therefore as a very common condition (Konstantinou et al. 2011; Pogorelov et al. 2016). As only a minority of patients with AU progress to the chronic form of disease, the prevalence of the latter condition is clearly lower. A study from the US estimated the 1-year prevalence of CU to be as low as 0.08%, while data from Europe reported a prevalence for the same period ranging between 0.38% and 0.8% (Lapi et al. 2016; Pogorelov et al. 2016; Zazzali et al. 2012).

In Spain, chronic urticaria was observed more commonly in patients between 25 and 55 years old (Gaig et al. 2004). In contrast, a recent Italian study observed a higher incidence of CSU among patients younger than 20 years and those older than 50 years old (Kalogeromitros et al. 2011). Other studies actually observed a lower incidence of all forms of urticaria in children, with the prevalence of any type of urticaria ranging between 3.4% and 5.4% (Antia et al. 2018; Dilek et al. 2016; Kjaer et al. 2008).

Urticaria is clearly a disorder with no Mendelian inheritance. Nevertheless, several HLA class II alleles were associated with CSU according to the results of a study conducted in 1999 on British patients (O'Donnell et al. 1999). A subsequent Italian study observed a higher prevalence of the disease among family members of patients, suggesting a familial inheritance. Later, additional HLA alleles (class I and class II) were also associated with the disease (Asero 2002; Coban et al. 2008).

4 Pathogenesis

Disclosing the pathophysiology of urticaria appears to be the principal need to bring successful treatment in a clinical setting. Nowadays, it is widely accepted the thesis involving mast cells as the principal mediators of damage. The wheal is microscopically characterized by a pro-inflammatory microenvironment generated by the degranulation of activated mast cells which determine the release of a great amount of soluble molecules such as histamine, leukotrienes, prostaglandins (PGs) and others cytokines.

This pathological niche presents high amount of mediators that have the ability to shape the infiltrate enhancing the activity of the homed cells leading to the perpetration of the damage. The analysis of soluble proteins from lesional skin suggests a mixed Th1/Th2 response by the presence of high amount of interleukin IL-4, IL-5, IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) as well as interferon-gamma (IFNγ) (Caproni et al. 2005; ELIAS et al. 1986; Kay et al. 2015).

These inflammatory factors determine the presence of a perivascular aggregation of immune cells mainly composed by mast cells, lymphocytes, monocytes, neutrophils, eosinophils (Vonakis and Saini 2008) and basophils (Ying et al. 2002); moreover, markers of vascular leakage and angiogenesis were found to be highly expressed on the surface of endothelium of the affected vessels (Kay et al. 2014).

4.1 Acute Urticaria

Considering the acute form of urticaria, the pathophysiology has been less extensively studied with respect to the abnormalities occurring in the chronic condition. Despite its higher frequency in population, AU presents a short duration of disease as well as effective treatments. Precipitating factors have been described in less than 50% of cases. The most common one is the presence of a concomitant viral infection, followed by drugs, food and insect sting reactions (Zuberbier et al. 1996; Kulthanan et al. 2008). Along with viruses, several reports described association with bacterial infections such as cystitis and tonsillitis (Wedi et al. 2009), instead, consumption of raw fish and exposure to Anisakis simplex nematodes, was highlighted only in some studies (Wedi et al. 2009; Del Pozo et al. 1997).

Scientific evidence identifies the same pathological alterations in both forms. An abnormal

release of histamine, platelet-activating factor and cytokines by activated mast cells leads to vasodilation, increased vascular permeability and stimulation of sensory nerve endings in the affected skin.[1] Furthermore, mediators released from mast cells may act as chemo-attractants for cells of the immune system, such as eosinophils, neutrophils and lymphocytes. As a consequence, the affected dermis becomes inflamed (Radonjic-Hoesli et al. 2018).

4.2 Chronic Urticaria

The chronic form, defined as a spontaneous or induced occurrence of wheals, is considered to be caused by a persistent derangement in functioning of mast cells. The inappropriate and uncontrolled degranulation of those determines the clinical manifestation with the appearance of the short-lived typical lesions.

Even if the pathogenesis of CIndU is clearly dependent upon definite extrinsic factors which activate and enhance mast cells degranulation (Tables 1A and 1B), the pathogenesis of the spontaneous form remains elusive. Because of the peculiar features and not well understood role of mast cells in the human organism, several elements have been analysed tempting to define cellular and molecular pathways responsible of CSU. Recently, the alteration in mast cells activation has been mostly attributed to complex mechanisms of autoimmunity involving various component of the immune system besides to complement cascade and coagulation.

4.2.1 Cellular Alterations

Within skin lesions, a mixed perivascular pro-inflammatory infiltrate can be observed. Attracted by chemokines which are mainly released by mast cells, eosinophils, monocytes, basophils and T helper cells penetrate the skin layers occupying the perivascular space. Interestingly, a peculiar mixed Th1 and Th2 responses seems to be present, with a concurrent increase of both spectra main mediators (Radonjic-Hoesli et al. 2018; Kay et al. 2015; Ying et al. 2002).

Strong evidence in support of the autoimmune pathogenesis of CSU is certainly the documented presence of autoreactive CD4+ T cells targeting FcεRIα in subjects with the disease. In a recent study, these lymphocytes were detected in more than a quarter of investigated patients, while they were completely absent in controls. They were associated with a Th1 phenotype, with secretion of IFN-γ (Auyeung et al. 2016).

Mast Cells

Mast cells are derived from bone marrow CD34+, CD117+ (Kit), CD13+ pluripotent progenitor cells that mature under the local environment of the tissues into which they migrate (Kirshenbaum and Metcalfe 2006; Bandara et al. 2015). Studies focusing on the content of their granules led to identify two different subtypes which present different interaction with the other cells of immune system as well as a dissimilar localization in the human body. In particular, mast cells which are tryptase-positive but chymase-negative (MC_T) (Irani and Schwartz 1989) which contain complete scroll, are located at mucosal tissues, such as the intestine, lung and nose, are T-lymphocyte dependent and are increased in number in allergic disease (Irani and Schwartz 1989; Otsuka et al. 1995). The other subtype is defined to be constituted by both tryptase and chymase-positive granules (MC_{TC}) which appear to form grating or lattice structure (Irani and Schwartz 1989). They are found primarily in the skin and gastrointestinal submucosa and are independent of lymphocytes (Smith et al. 1995).

The role of mast cells in CSU is consensually acknowledged. However, it's still controversial whether they are increased in numbers in patients with the disease. While some studies supported this hypothesis, others found no association (Saini and Kaplan 2018). Only a few studies that analysed the presence of mast cells in the lesional skin distinguished between MC_T and MC_{TC}. One of these, in particular, highlighted how there is no difference between the number of MC_{TC} between CSU patients and heathy ones; however, the presence of the subtype MC_T varies significantly (Bradding et al. 1995).

Evidence for mast cell degranulation come firstly from the measurement of total serum tryptase levels, which appear to be slightly elevated in subjects with CSU compared to both healthy and atopic subjects even if still within the normal range (Ferrer et al. 2010). According to some evidence, a functional defect in these cells activation may lead to increased histamine release in CSU (Bédard et al. 1986; Jacques et al. 1992). This alternative hypothesis attributes the chronic evolution of acute urticaria to a defect in mast cells intracellular signalling, rather than autoimmune disturbances (Bracken et al. 2019). Findings in support of this assumption rely on release tests which demonstrate, by the use of 48/80-induced histamine responses via skin chambers, how mast cells coming from CSU patients appear to be more prone to degranulate (Bédard et al. 1986; Jacques et al. 1992).

The molecular basis of the altered response appears to lies in the structure of FcεR1, which contains immunoreceptor tyrosine-based activation motifs (ITAMs). After phosphorylation, ITAMs allow the activation of spleen tyrosine kinase (SYK) and downstream pathways which eventually elicit the degranulation of mast cells (Bracken et al. 2019). Mast cells from CSU patients, when cultured in vitro, were observed to release significantly higher levels of histamine than their counterparts from healthy controls.

Activated mast cells were shown to express significantly higher levels of SYK during the same experiment (Saini et al. 2009). Interestingly, a subsequent study did not found SYK to be upregulated from anti-FcεRI autoantibodies. This implies that the upregulation of SYK observed in a subset of CSU patients depends upon causes other than autoimmunity or, more accurately, other than autoantibodies (MacGlashan 2019).

The presence of activated mast cells has profound effects on local microenvironment. Some recent evidence points the attention on the ability of these cells to produce high amount of neuropeptides, in particular NGF (Aloe et al. 1994; Peters et al. 2011), which is markedly elevated in subject affected by allergic diseases and CSU (Bonini et al. 1996). NGF is a member of neurotrophin, has pivotal roles for the development and survival of nociceptive neurons but also is involved in chronic inflammation maintaining and promoting the proliferation and activity of lymphocytes, neutrophils, eosinophils and mast cells (Minnone et al. 2017).

In CSU has been demonstrated how NGF is able to act stimulate the proliferation of mast cells besides act as a chemotactic factor. This evidence could be at the base for a pathological loop in which the activated mast cells through the stimulation of sensory nerves could enhance the immune infiltrate supported by inflamed nerves (Fig. 2) (Minnone et al. 2017).

Basophils

Basophils are circulating granulocytes which can express IgE receptors, release histamine and produce several cytokines, such as IL-4, IL-13 and others, in response to IgE receptor activation (Saini and Kaplan 2018; Schroeder 2009; Raap et al. 2017). Blood basophils in patients with CSU were characterized by multiple abnormalities. Along with a reduction in their number (basopenia), reduced histamine release in response to anti-IgE (basophils hyporesponsiveness) and basophil infiltration of skin lesions have been observed (Vonakis and Saini 2008; Saini and Kaplan 2018; Rorsman 1962). Several alterations in the function and number of basophils have been observed in CSU patients suggesting a recruiting from the bloodstream into urticarial skin lesions (Grattan et al. 2003).

Based on the response to IgE-receptor-mediated histamine degranulation, it is possible to identify two different subsets of basophils in CSU patients which are confirmed by a study monitoring CD63 induction after IgE-receptor activation (Rauber et al. 2017). Improvements in both basopenia and basophil IgE-receptor abnormalities are seen in natural remission of CSU and point to basophils as contributor to disease (Eckman et al. 2008). Even if the pathways involved in the processes which recruit basophils to skin lesions are not yet elucidated, some evidence focused the attention on the prostaglandin D2 (PGD2) pathway and the chemoattractant receptor homologous molecule

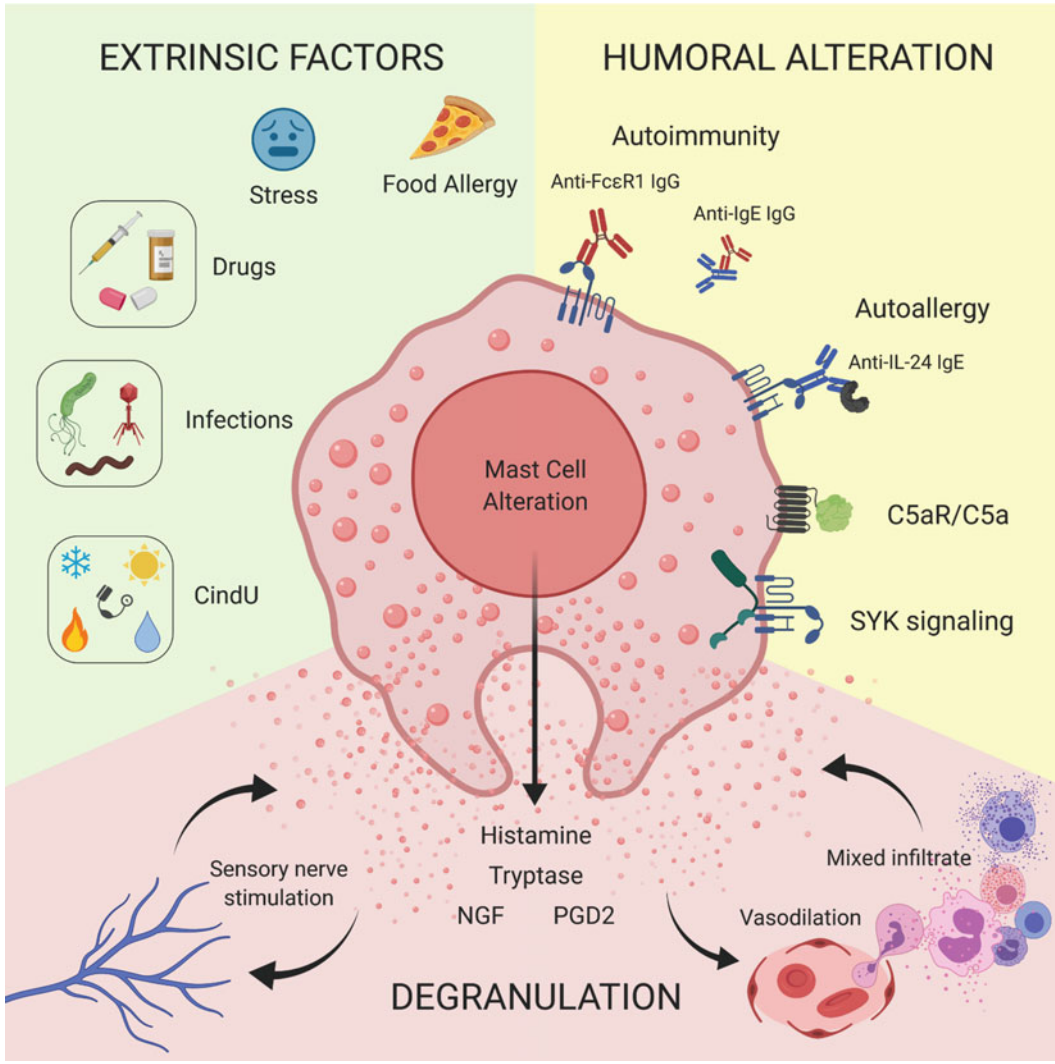


Fig. 2 Possible mechanisms of mast cell activation in urticaria

Extrinsic factors (green section). Several extrinsic elements can exacerbate urticaria via mast cells activation. Infections and drugs are identified as one of the most frequent precipitating factors. Some evidence demonstrate how emotional stress has been strictly linked to a worsening of skin symptoms instead, foods and food additives have been implicated mainly as a consequence of food allergy. CindU are well-known examples of triggered urticaria

Humoral alteration (yellows section). The main pathogenic mechanisms actually identified and under validation are: Autoimmunity**.** IgG autoantibodies (IgG) target IgE-receptors (FcεR1) on mast cells (MC). This leads to dimerization of receptors and mast cell activation, with consequent degranulation (DMC). Concurrently, formation of complement fragments (C5a) and binding to C5a receptor (C5aR) on mast cells enhances the process. Autoallergy**.** IgE antibodies (IgE) targeting human proteins such as interleukin 24 (IL-24) are present in serum. These bind to FcεR1 via their Fc region, leading to allergic reactions against "autoallergens". Intracellular signalling defects**.** An abnormal activation of spleen tyrosine kinase (SYK) in mast cells of patients with chronic urticaria leads to increased intracellular signalling and consequent degranulation without external triggers

Degranulation (red section). Mast cells degranulation could be facilitated by intrinsic cell defects which diminishing the activation threshold lead to an enhanced exocytosis. The release of histamine, tryptase, PGD2 and NGF determine an increase in vascular permeability causing the extravasation of several inflammatory cells. Perivascular mixed infiltrate interacts with tissue mast cells generating a pathological loop which worsen and maintain a pro-urticaria microenvironment. The same auto stimulatory condition could be occurring with free nerve endings by secretion of NGF

expressed on the Th2 cell (CRTH2) (Oliver et al. 2016).

Moreover, data from omalizumab phase III trials in CSU shows a correlation between the clinical response and that improvement in basopenia (Saini et al. 2017).

Recent finding observed increased IL-31 in skin as well as serum levels of CSU patient (Raap et al. 2010). This molecule is considered to be the major player inducing pruritus in skin diseases such as atopic dermatitis but also determine a powerful chemotactic action on basophils recruitment. This indicates that IL-31 plays a role in the orchestration and accumulation of basophils in inflammatory skin diseases; furthermore, it has been widely demonstrated how basophils are the main cellular sources of IL-31 in skin lesions of CSU patients (Raap et al. 2017). The inhibition of this pathological loop could be thus of therapeutic interest in controlling itch-related symptoms as well as inducing the remission of disease (Fig. 2).

4.2.2 Humoral Alterations

The initial study which provided strength to this hypothesis was the development and testing of the autologous serum skin test (ASST) (Grattan et al. 1986). More than three decades ago, few patients with chronic spontaneous urticaria were drawn blood; serum was separated from it and reinjected intradermally with the result to develop wheals when exposed (Grattan et al. 1986). Clearly, this seemed to imply the presence of an intrinsic serum factor triggering urticaria; however, this finding was later doubted when evidence came out that a substantial percentage of healthy controls also developed positive ASST reactions (Taskapan et al. 2008).

Thyroid autoimmunity, especially Hashimoto's thyroiditis, has also been associated with CSU for a long time (Levy et al. 2003; Nuzzo et al. 2011). This seemed to provide evidence of a common autoimmune mechanism or predisposition. According to recent evidence, which emerged from a European multicentric study (the PURIST study) (Schoepke et al. 2019), a subset of patients with purely autoimmune pathogenesis may exist, although its relative weight appears minor. Defined three criteria as a red-flag of derangements in immune function, only 8% of the patients studied fulfilled all the conditions, even if this group had higher activity of the disease (UAS 7), lower IgE levels and higher anti-TPO IgG levels.

Besides the association with thyroid autoimmunity, a high frequency of antinuclear antibodies (ANA) has also been observed among these patients (Viswanathan et al. 2012). However, their meaning in CSU is poorly understood and, as ANA are well known to occur in a considerable fraction of the healthy population, they bear no practical use in CSU (Viswanathan et al. 2012).

On the contrary, antibodies specific to CSU and relevant to its pathogenesis are those targeting FcεR1. These were described for the first time in a group of 26 patients with CSU. It was hypothesized that they could act similarly to anti TSHr-antibodies in Graves disease, leading to the activation, rather than the neutralization, of their target (Hide et al. 1993). Specifically, these IgG antibodies bind to FcεRI on mast cells, leading to their activation and degranulation. This mechanism alone, due to activating autoantibodies, seemed to explain up to one quarter of cases of CSU (as anti- FcεR1 IgGs are prevalent in about 25% of patients) (Niimi et al. 1996).

Fascinatingly, in a small subset of patients, anti-IgE autoantibodies were also observed (Niimi et al. 1996). This may sound puzzling to those who are well acquainted with the therapeutic effectiveness of anti-IgE monoclonal antibodies (eg. Omalizumab) in CSU. However, a recent study conducted on asthma patients demonstrated that naturally occurring anti-IgE IgGs are quite frequent and not necessarily inhibitory (Chan et al. 2014). Some of these actually lead to activation of basophils, a finding which may also be coherent with their presence in CSU patients.

Along with humoral autoimmunity, a role for humoral "autoallergy" has been postulated in CSU. Autoallergy refers to the presence of IgE antibodies against human proteins, which would be capable of inducing mast cells degranulation without extrinsic triggers. The first autoallergen to be identified in CSU was thyroid peroxidase (TPO). Anti-TPO IgEs were shown to be

significantly more prevalent in patients than in controls (Altrichter et al. 2011). Subsequently, interleukin 24 (IL-24) was identified as a more sensitive and specific autoallergen for CSU (Schmetzer et al. 2018). Anti-IL-24 IgEs were additionally shown to be able to bind mast cells and lead to histamine release, which is a needed condition to prove their pathogenicity (Schmetzer et al. 2018).

Despite that, some difficulties arise when trying to develop a coherent model of pathogenesis for autoallergens and their antibodies. In fact, TPO is not present in the skin and IL-24, which is present in the epidermis, is not known to be found in the dermis as well (Poindexter et al. 2009). As the epidermis is normal in CSU, a definite role of autoallergy can't be ultimately proven (Fig. 2) (Kaplan 2019).

Complement

Whereas autoantibodies constitute the humoral arm of the adaptive immune system, the complement system provides innate immunity with an equally effective means of molecular defence. Unfortunately, when complement derails from its normal function, it may compound damage in a dysregulated immune system.

Soon after the discovery of autoantibodies targeting IgE receptors, it was found that a subset of these was able to activate mast cells only *in vivo*. This was hypothesized to be due to the exclusive *in vivo* presence of complement and its fragments, which could bind to antibodies and complement receptors (C5aR.) on mast cells (Fiebiger et al. 1995).

Purified C5a fragments were later tested for their effects upon histamine release in the presence of anti-IgE receptor IgGs. C5a was associated with a significant increase in histamine release from mast cells, when compared to complement-depleted serum (Ferrer et al. 1999). It was therefore suggested that IgGs cross binding FcεRI on mast cells could have a stronger degranulating effect in the presence of higher amounts of activated complement fragments (Fig. 2) (Kikuchi and Kaplan 2002).

Coagulation

Although the coagulation cascade is not directly linked with the immune system, its relevance in the pathogenesis of CSU has been supported by several studies (Asero et al. 2007). After initial findings of an increased activation of the coagulation, use of anticoagulant drugs was considered as a valid adjuvant in the treatment for CSU (although no increase in the risk of thrombosis was observed in these patients) (Cugno et al. 2010).

The increased activation of fibrin and other coagulation factors may be the result of vascular damage taking place in the affected skin. The same group of researchers also showed a prompt decrease in levels of D-Dimer (a degradation product of fibrin) in patients with a clinical response after being treated with either cyclosporine or omalizumab (Asero 2015; Asero et al. 2017).

4.2.3 Extrinsic Precipitating Factors

As it is the case for AU, the chronic spontaneous form has also been associated with several extrinsic factors. Among these, infections of viral, parasitic, bacterial or fungal aetiology have all been implicated in causing chronic spontaneous urticaria or its exacerbations (Antia et al. 2018). Helicobacter pylori was long believed to play a causative role in a significant subset of patients.

A study highlighted how the eradication of the bacterium had provided significantly beneficial effects to patients with chronic spontaneous urticaria, with a complete or partial remission of symptoms (Di Campli et al. 1998). Unfortunately, results were not replicated by subsequent studies, which didn't found any association with H. pylori, nor any improvement of CSU eradication therapy (Curth et al. 2015; Kohli et al. 2018).

Several foods and food additives have been implicated and extensively studied in the pathogenesis of chronic spontaneous urticaria (Antia et al. 2018). Patients may sometimes indicate certain foods as the cause of their symptoms and consequently develop avoidance mechanisms when choosing what to eat. However, studies

which investigated the presence of urticarial symptoms after oral food challenge only observed them in a small minority of patients with such claims (Hsu and Li 2012; Chung et al. 2016).

Drugs can exacerbate urticaria through different mechanisms. ACE inhibitors are well known to cause angioedema by causing an increase in the amounts of bradykinin (Scalese and Reinaker 2016). Nonsteroidal anti-inflammatory drugs (NSAIDs) may also induce urticaria, either through an allergic (IgE-mediated) reaction or through the inhibition of COX-1 and increased production of leukotrienes (Antia et al. 2018).

Emotional stress has also been associated with chronic spontaneous urticaria. Patients suffering from the disorder suffer from higher rates of depression, anxiety and somatoform disorders with respect to the general population. However, it's unclear whether this is a consequence of coping with the disorder or a possible predisposing factor (Fig. 2) (Uguz et al. 2008).

5 Diagnosis

According to the latest guidelines AU is to be considered as a self-limiting disorder, which requires no investigation unless a couple of specific features are present. Namely, these are the presence of a type I food allergy (IgE-mediated) or of another eliciting factor such as NSAIDs (or other drugs) (Zuberbier et al. 2018). In these cases, the patient should be counselled towards allergy tests and avoidance of re-exposure to proven precipitating factors.

In CU, the workup is more extensive. The most relevant component of the investigation is the history taking, which, if conducted properly, usually spares the physician and the patients time-consuming, costly and often improper laboratory testing.

5.1 Diagnostic Workup

A summary of the crucial questions in history taking and their diagnostic role has been included in Table 2, still based upon the latest international recommendations (Zuberbier et al. 2018).

After conducting history taking, physical examination should be performed. Along with a general survey of the patient, attention should be focused on features of the skin lesions (when present at the time of the visit). Eventually, each form of CIndU should be confirmed with the appropriate testing (see Tables 1A and 1B).

CSU wheals may have variable appearance, but they should always blanch with pressure and leave no trace after 24 h. In patients with typical features of CSU, lab testing should be restricted to a differential blood count, ESR and CRP. In patients with atypical features (e.g. wheals not resolving within 24 h, presence of angioedema only) or other associated symptoms, a more extensive workup may be appropriate. Testing for Helicobacter pylori, functional autoantibodies presence (e.g. ASST), thyroid autoimmunity, allergy tests, vasculitis (e.g. skin biopsy) or other systemic disorders may be warranted depending upon the history (Zuberbier et al. 2018).

5.2 Biomarkers of Diagnosis and Severity

Biomarkers are clinical features (clinical biomarkers) or molecules present in serum (molecular biomarkers) which are able to offer some diagnostic, therapeutic or prognostic information. They clearly depend upon the disease pathogenesis and are one of the most important fields of clinical research in many conditions, including CSU. Clinical biomarkers for diagnosis, stratification of disease and treatment response have been observed in CSU. These include age and gender (Folci et al. 2018). Being female has been associated with a more prolonged clinical course and a worse quality of life, while an association of older age and longer duration of disease has also been established (Gregoriou et al. 2009; Hiragun et al. 2013).

The presence of angioedema is particularly taken in consideration because of a strict correlation to a less favorable prognosis (Toubi et al.

Table 2 History taking in chronic urticaria. A list of items which should be assessed when first visiting a patient with urticaria and/or angioedema

Anamnestic item	Diagnostic relevance
Time of onset	Crucial to classify urticaria as acute or chronic (>6 weeks)
Shape, size, frequency, and duration of wheals	Although wheals may have variable appearance and size, they last characteristically less than 24 h in CSU. When lasting longer than 24 h, the suspicion of an urticarial vasculitis should arise
Presence of angioedema	When angioedema is the main complaint the clinician should suspect acquired angioedema (testing of C4, C1-inhibitor levels and function) and hereditary angioedema (gene mutation analysis)
Associated symptoms (such as arthralgias, bone pain, fever, abdominal pains)	This item is fundamental to decide when to extend the laboratory testing in the suspicion of a systemic disorder, with urticaria as its presenting feature
Family and personal history of wheals and angioedema	A positive family history for angioedema is quite suggestive of hereditary angioedema (Tosi 1998; Pappalardo et al. 2000)
Induction by external physical agents or physical exercise	The patient should be asked whether he can "make his wheals come". In this case, a form of CIndU is likely present and the specific trigger should be investigated. In some patients, CSU and CIndU coexist, so that they are not exclusive categories
Association with certain foods or drugs (e.g. NSAIDs, ACEi.)	This is fundamental to rule out the presence of a pharmacological trigger
Allergies, autoimmune disorders, gastrointestinal or other disease	This item allows a better understanding of the overall patient's health status and may be helpful in the setting of suspected differential diagnosis
Previous treatment and responsiveness	It's fundamental to ask about previous treatment in order to implement correctly the stepwise approach which is adopted in CSU
Previous diagnostic testing	This is often useful to avoid repeating laboratory testing

2004; Champion et al. 1969). Exacerbations occurring with the use of aspirin or non-steroidal anti-inflammatory drugs (NSAID) have been widely described as being related to a more severe and chronic disease (Sánchez-Borges et al. 2015b; Shin et al. 2015).

For what concerns molecular biomarkers, a considerable number is present, even if no one of them has yet gained the clinical practice. C reactive protein (CRP) is certainly one of the most important and reproducible. High CRP levels were demonstrated to be associated with higher activity of disease and quality of life impairment (Kolkhir et al. 2017; Kolkhir et al. 2018). Another molecule with marked inflammatory activity, interleukin 6 (IL-6), was also found to be increased (Kasperska-Zajac et al. 2015). Furthermore, Vitamin D, well-known because of its crucial role in bone metabolism, less because of its immunomodulatory activity, has been implicated in the pathogenesis of chronic urticaria. Apparently, depletion in Vitamin D is associated with an increased disease severity. As a consequence, it may be an effective marker with potential therapeutic implications (Vitamin D supplementation) (Piemonti et al. 2000; Holick and Vitamin 2007; Woo et al. 2015).

As it was previously described, D-Dimer levels were long thought to be an effective marker of response to treatment, especially when high before administration (Takeda et al. 2011). However, recent evidence contradicts this view and actually confirms the validity of D-dimer only as a marker of CSU severity.

In order to evaluate patients who are less likely to respond to Omalizumab promptly, due to the presence of anti-IgE receptor IgGs, the basophil histamine release assay (BHRA) may be used. BHRA evaluates the presence of IgGs targeting FcεRI in serum. In patients who test positive, omalizumab will take time to be effective. This is due to the need for all FcεRI on mast cells to be uncovered from IgE (which occurs immediately) and slowly decay (over several weeks) (Grattan et al. 1986; Folci et al. 2018; Metz et al. 2017; Saini and MacGlashan 2012).

Table 3 Biomarkers of diagnosis, prognosis and severity

Biomarker		Diagnosis	Prognosis and severity	
			Better	*Worse*
Clinical	Age	–	Young pts	Older pts
	Gender	–	Male	Female
	Duration of CSU	–	Less than 1 yr	Longer than 1 yr
	Angioedema	–	Absent	Present
	NSAID exacerbation	–	Not associated	Associated
Molecular	Anti-IL-24 IgE	Positive association	–	–
	IL-6	–	–	High levels
	IL-6 sR	–	–	High levels
	Sgp130	–	–	High levels
	IL-18	–	–	High levels
	MMP-9	–	–	High levels
	CRP	–	–	High levels
	D-dimer	–	–	High levels
	F1 + 2	–	–	High levels
	Vitamin D	–	–	Low levels
	MPV	–	–	High values
	Basophils count	–	Normal number	Low number
	CD203c-basophils	–	–	Increased

MMP-9 Metalloproteinase-9, *CRP* C reactive protein, *F1 + 2* Prothrombin fragment 1 + 2, *Il-6sR* Interleukin 6 soluble receptor, *sgp130* Soluble glycoprotein 130, *mpv* mean platelet volume, *ESR* Erythrocyte sedimentation rate, *CRP* c-reactive protein

Other biomarkers, such as interleukin 18 (IL-18) and matrix metalloproteinase-9 (MMP-9), have also been considered. IL-18 may be involved in the recruitment of eosinophils in skin affected from urticaria, while MMP-9 may be involved in the migration of immune cells by remodelling tissue extracellular matrix (Novick et al. 2001; Wang et al. 2001; Ram et al. 2006; Song et al. 2013; Belleguic et al. 2002). However, results are far from being conclusive and they will need validation from other groups.

Eventually, IL-24 should be mentioned once again. If a standardized assay (e.g. ELISA testing) for the quantification of anti-IL-24 IgE were developed, it would appear more feasible to start considering autoallergy in the clinical setting. Also in this case, although results are still preliminary, they seem to be quite promising (Table 3).

5.3 Differential Diagnosis

A broad spectrum of disorders is included in the differential diagnosis of chronic urticaria, with several of these conditions being rare but severe systemic disorders (Radonjic-Hoesli et al. 2018). The clinician should be particularly careful when urticaria is associated with other signs and symptoms or presents with atypical features (Radonjic-Hoesli et al. 2018). When wheals last for longer than 24 h, lesions are burning more than itching and don't disappear after digital pressure or the patient suffers from a systemic autoimmune disorder (such as systemic lupus erythematosus or primary Sjogren's syndrome), urticarial vasculitis (UV) may be present. Several other autoimmune, autoinflammatory and hematologic disorders may likewise present with urticaria (a summary of the most relevant conditions with their distinctive features has been included in Table 4).

6 Treatment

While treatment of acute urticaria has remained roughly the same in the last decades, the approach to management of chronic urticaria has been

Table 4 Main differential diagnoses of chronic urticaria

Diagnosis	Peculiar features
Urticarial vasculitis (UV)	The patient may present with wheals lasting longer than 1 day and cutaneous purpura. UV is a small-vessel vasculitis characterized by inflammatory injury of the capillaries of the dermis and the postcapillary venules (Jachiet et al. 2015). In order to reach the diagnosis, a skin biopsy is needed. UV can be further split into a normocomplementemic and a hypocomplementic (HUV, defined by low C1q levels, normal C1-inhibitor, frequent presence of anti-C1q antibodies) subtype. While normocomplementemic UV is usually idiopathic, HUV is usually associated with SLE or SS (Jachiet et al. 2015)
Bullous pemphigoid	In this systemic autoimmune disease, autoantibodies target hemidesmosomes and cause subepidermal blistering and skin detachment (Feliciani et al. 2015). However, the characteristic bullae do not appear in a minority of patients (20%), so that an urticarial rash and intense itching may be the only presentation (Bech et al. 2018)
Cryopyrin-associated periodic syndromes (CAPS)	Urticaria is almost invariably present in CAPS (consisting of familial Cold autoinflammatory Syndrome, Muckle-Wells Syndrome or neonatal Onset Multisystem Inflammatory Disease) together with recurrent fever bouts, arthralgias, eye inflammation and other symptoms which may be exacerbated by exposure to cold (Kuemmerle-Deschner et al. 2017). The disorder is mediated by an increased secretion of interleukin 1 (IL-1), due to a genetic mutation, and can be treated effectively with IL-1 blockade (e.g. anakinra, canakinumab)
Adult-onset Still's disease (AOSD)	AOSD is defined by recurrent bouts of fever, a typical salmon-coloured evanescent rash, a neutrophilic leukocytosis and arthralgias. Additionally, lymphadenopathy or splenomegaly, along with liver dysfunction, may be present (Yamaguchi et al. 1992). Urticaria has been described as a presenting feature of AOSD (Yamamoto 2012)
Schnitzler's syndrome	It's defined by the presence of fever, arthralgia, lymphadenopathies and urticaria in the presence of a monoclonal gammopathy (Lipsker et al. 2001). The disease has been grouped within autoinflammatory syndromes due to the pathogenic role of IL-1 and other cytokines (the chemoattractant CCL2). Besides, in some cases, it has been successfully managed with IL-1 blockade (de Koning et al. 2007; Krause et al. 2019)
Urticaria pigmentosa	This is a form of cutaneous mastocytosis which appears as a maculopapular rash of tan to brown colour which usually spares the palms and soles. Required investigations include serum tryptase and skin biopsy, which shows the presence of mast cell aggregates and increased number of mast cells in cutaneous mastocytosis (Macri and Cook 2019)
Breast, prostate and colon cancer	Some case reports have been published in which CSU was a paraneoplastic phenomenon in the setting of solid cancer (breast, prostate and colorectal cancer) (O'Donnell and Havyer 2014; Kasi et al. 2016; Baroni et al. 2012; Santiago-Vázquez et al. 2019)

revolutionized by the development of biologic drugs. Omalizumab is now the recommended drug in patients with CSU refractory to antihistamines treatment. Soon enough, a new generation of biologic and target drugs promises to further reduce the fraction of patients who does not respond fully to available therapies.

6.1 Acute Urticaria

Emergency physicians and general practitioners are often involved in the management of acute urticaria. Being described as a self-limiting condition, its treatment was not discussed by the most recent guidelines on urticaria (nor the previous ones), where it was just suggested that possible provoking factors should be avoided and symptomatic relief should be warranted (Zuberbier et al. 2018; Zuberbier et al. 2014). Previous literature on this topic is equally sparse. A short course of prednisone was proven to be effective in obtaining a quicker response in one trial, while in a nationwide survey conducted in the US, physicians reported the use of histamine (H1) antagonists and prednisone in affected children (Pollack and Romano 1995; Beno et al. 2007). In a recent review written by a worldwide expert on allergy and urticaria, it's suggested that H1 antihistamines are given up to four times a day, with an additional short course of steroids when severity requires it (eg. 40 mg of prednisone for 3 days, tapered by 5 mg/day or stopped without tapering) (Kaplan 2019). Second generation

H1 antihistamines (eg. cetirizine, levocetirizine, loratadine) are recommended over first generation ones due to improved safety profile, especially a lower incidence of drowsiness (which can still be present, especially when more than one tablet is taken daily) (Snidvongs et al. 2017).

6.2 Chronic Urticaria

The treatment of chronic urticaria depends on the presence of specific trigger factors, as it occurs in CIndU, or the absence of such, as in CSU. Importantly, the physician should acknowledge the difficulties experienced by the patient coping with a long-lasting disorder with no curative treatment. In order to provide the most effective solution without exposing patients to unnecessary risks, a stepwise approach should be followed. Corticosteroids should generally be withdrawn from therapy due to their profile of severe side effects in chronic use.

6.2.1 Chronic Inducible Urticaria

The management of CIndU clearly involves the avoidance of physical stimuli, although this is rarely possible. While it may be feasible to substitute latex products with other equivalent ones in the case of a contact urticaria, it may be more complex to avoid sunlight, exposure to cold or to water. In the case of delayed pressure urticaria, it's important to make the patient aware that by using wider handles for heavy bags the surface in contact with the skin is increased and, as a consequence, the pressure exerted is reduced (Zuberbier et al. 2018). Second generation H1 antihistamines have been successfully used in acquired cold urticaria, where four daily dosage (i.e. 20 mg desloratadine daily) was associated with improved clinical course with respect to lower dosages and placebo (Siebenhaar et al. 2009).

In those forms of inducible urticaria which are responsive to antihistamines, these are clearly the indicated therapy as long as needed. However, delayed pressure urticaria is unresponsive to these (Kaplan 2019). For these and other patients refractory to antihistamines, omalizumab has proven effective and safe in several studies (Metz et al. 2014; Quintero et al. 2017; Maurer et al. 2018). For the few patients who don't respond to omalizumab, no effective pharmacological treatment is available. Patients would clearly respond to steroids but the side effects of a prolonged treatment would almost certainly offset the benefits (Kaplan 2019).

6.2.2 Chronic Spontaneous Urticaria

Management of CSU is a careful process characterized by an add-on strategy to achieve the best control of disease, if not a complete remission.

Identification and Avoidance of Aggravating Factors

When reported in the course of history taking, drugs suspected of exacerbating CSU should be withdrawn from therapy and substituted with others, if possible. Other factors which have been associated with CSU are infections, such as H. pylori colonization. As previously described, the association between H. pylori and CSU has not been proven conclusively. However, when colonization is found, eradication therapy should be pursued, also because of the association of H. pylori with several other disorders (e.g. gastric cancer) (Zuberbier et al. 2018; Curth et al. 2015; Kohli et al. 2018). Reduction in emotional stress and avoidance of certain foods may also provide some degree of benefit, although this is questionable and not strongly supported by available research.

Pharmacological Management: A Stepwise Approach

The pharmacological management of CSU begins, as with all types of urticaria, with second generation, H1 antihistaminergic drugs. These are preferred over first generation H1 antihistamines because of their lower propensity to cause drowsiness. According to the most recent guidelines on treatment, these drugs are roughly equivalent, and patients may choose any of them (e.g. cetirizine, levocetirizine, desloratadine, ebastine, fexofenadine). However, the use of multiple antihistamines at the same time is not

recommended (Zuberbier et al. 2018). In many patients, standard dosage is not effective and it may need to be escalated up to four times in order to warrant remission. Although this approach is also supported by urticaria guidelines, it may pose the patient at a higher risk of sleepiness and is not necessarily compliant to all countries prescribing regulation.

When high doses of regularly administered antihistamines are not effective, omalizumab should be prescribed as an add-on to therapy. Omalizumab is a humanized monoclonal IgG immunoglobulin which targets IgE and prevents their binding to FcεRI (Tonacci et al. 2017). With time, uncovered FcεRI on basophils and mast cells undergo a process of slow decay and reduction in number (Saini and MacGlashan 2012). Omalizumab was administered for the first time in patients with CSU in a small study carried out in 2008 (Kaplan et al. 2008). The effectiveness and safety of the drug was later confirmed by one phase two and three phase three trials (Saini et al. 2011; Maurer et al. 2013; Kaplan et al. 2013; Saini et al. 2015).

These trials brought evidence that doses of 150 mg and 300 mg of omalizumab monthly were both effective, although the 300 mg dose was clearly more effective than the lower one. About 40% of patients reached complete remission and more than half (around 60%) obtained partial remission (calculated with UAS7 score lower than 6) (Kaplan 2019). It also emerged how patients often relapsed when the drug was discontinued, which didn't seem to affect the course of the disease in the long term. Interestingly, omalizumab also proved to be effective in patients suffering from idiopathic nonhistaminergic acquired angioedema (InH-AAE) in a preliminary case series. This led the investigators to hypothesize that InH-AAE may actually be an IgE-mediated disease, sharing the pathogenesis with CSU (Brunetta et al. 2018).

In patients who are refractory to omalizumab, along with reconsideration of alternative diagnoses from CSU, it is recommended that ciclosporin A is added to treatment. Ciclosporin is an immunosuppressant drug whose main mechanism is the inhibition of calcineurin in T cells. However, it has long been known to impede histamine release in mast cells and basophils (Cirillo et al. 1990; Stellato et al. 1992). Ciclosporin has been proven to be an effective drug for the treatment of CSU by two randomized controlled clinical trials, however, with low number of patients treated, and with short periods of treatment and observation after the interruption of therapy (Grattan et al. 2000; Vena et al. 2006).

Moreover, its use is limited by the severe side effects of hypertension and renal injury, which are dose-dependent and require monthly controls (Kulthanan et al. 2018). A daily ciclosporin dose of 3–3.5 mg/kg has obtained a success rate of 70–80%, while higher doses (4–4.5 mg/kg) are usually avoided because of higher risks of toxicity (Kaplan 2019). A recent metanalysis established the efficacy of cyclosporin with respect to placebo along with its mixed safety profile, with the common occurrence of side effects requiring interruption of treatment or reduction of the dosage (Kulthanan et al. 2018).

In patients in whom antihistamines, omalizumab and cyclosporin failed, a number of other medications have been used with variable success. These include leukotriene receptor antagonists, mycophenolate mofetil, methotrexate and hydroxicloroquine (Rutkowski and Grattan 2017). Guidelines do not discard completely this off-label approaches, which may be useful in individual and well-selected patients, but they instruct the physician to adopt a certain degree of caution due to the very low level of evidence which supports them (Zuberbier et al. 2018).

Biomarkers of Response to Treatment

Nowadays predicting the efficacy of a therapeutical scheme before prescribing the drug, seems pivotal to reach remission, improve the health of the patients and avoid waste. Several studies tried to identify and cluster groups of patients by clinical efficacy to a defined drug; nonetheless, foreseeing the response remains an unmet need in CSU treatment. In the table below are listed the main biomarkers described by the recent evidence (Table 5).

Table 5 Biomarkers with a potential prediction on drug treatment

Biomarker	Antihistamines	Omalizumab	Cyclosporine
Resistance			
Clinical	Hispanic	None	None
	Atopic asthma		
	Rhinosinusitis		
	Hypertension		
	Thyroid diseases		
	High UAS7		
	Longer duration of wheals		
Molecular	↑ C5a	BHRA +	None
	↑ IL-6	ASST +	
	↑ D-dimer	Low IgE levels	
	ASST +	CD203c up-regulation basophils	
Response			
Clinical	None	None	Short duration of CSU
			High initial severity
Molecular	↑ LCN2	BHRA -	BHRA +
	↑ Clusterin	ASST -	D-dimer
		Lack of CD203c basophils	
		High IgE levels	
		High FCεRI	
Increased relapse			
Clinical	None	High UAS7 at baseline	None
		Low UAS7 AAC	
Molecular	None	None	None

LCN2 Serum Lipocalin-2, *BHRA* Basophil histamine release assay, *ASST* Autologous serum skin test, *AAC* Area above the curve

6.3 Investigational Therapies

While existing pharmacological measures are effective in the majority of patients, they do not offer a long-lasting remission of symptoms after discontinuation of treatment. Moreover, they are not focused upon pathogenetic subsets within the disorder, but rather tackle CSU in a standardized manner.

On the contrary, the emerging approach of tailored treatment aims at providing the appropriate drug for each patient. This has become feasible due to an improved knowledge of CSU pathogenesis and biomarkers of disease. Currently, several drugs are under clinical and preclinical development for chronic urticaria.

6.3.1 Anti-IgE Humanized Monoclonal Antibodies

Of the drugs currently under investigation, the following two are certainly those with the greatest expectations in CSU. These are the anti-IgE humanized monoclonal antibodies quilizumab and ligelizumab.

Quilizumab targets membrane bound IgEs on IgE-switched B lymphocytes and plasma cells. It was found to reduce levels of total and specific IgEs in serum in patients with asthma and CSU (Harris et al. 2016a). However, in two randomized trials conducted in asthma and CSU, it was unable to provide any clinical benefit compared respectively to standard therapy and placebo (Harris et al. 2016a, b).

Ligelizumab mechanism of action is instead closer to omalizumab, as it equally binds soluble IgEs (Kocatürk et al. 2017). However, ligelizumab appears to be six to nine-fold more powerful than omalizumab. Furthermore, it shows a more prolonged suppression of IgE levels in serum (Kocatürk et al. 2017). This may be due to a different functional profile of the two drugs (Gasser et al. 2020). Indeed, not only does ligelizumab neutralize serum IgEs with increased

affinity, but it also appears to inhibit Ige production (Gasser et al. 2020). According to a recent study, ligelizumab may be able to downregulate IgE production by binding the CD23:IgE complex on the surface of B-cells, a feature which has not been shown with omalizumab (Gasser et al. 2020).

In a phase IIb randomized controlled trial which was recently published, 382 patients were either administered omalizumab, ligelizumab or placebo with varying doses and the response on weekly urticaria activity (with a focus on complete control of hives) was compared (Maurer et al. 2019). The primary endpoint of the study (complete control of hives) was assessed after 12 weeks of treatment. The study successfully determined a dose-response curve for ligelizumab and a statistically significant improvement in symptoms control with respect to omalizumab (complete hives response in 51% of patients treated with 72 mg ligelizumab with respect to 26% of patients who received 300 mg omalizumab) (Maurer et al. 2019). As the authors of the trial observed, the low percentage of patients who responded to omalizumab is puzzling and inconsistent with previous literature (Maurer et al. 2019). This has been interpreted as a consequence of patients selections criteria. Dose-limiting side effects were not observed, with generally no patients reporting anaphylaxis (Maurer et al. 2019).

6.3.2 Blockade of IL-1

Due to the efficacy of IL-1 blockade in autoinflammatory syndromes such as CAPS and Schnitzler's syndrome, along with the evidence of effect in some patients with physical urticarias, anti-IL-1 drugs have also been investigated in CSU (Bodar et al. 2009; Krause et al. 2012; Lenormand and Lipsker 2012). A phase II trial was conducted on the efficacy of canakinumab compared to placebo by the University of Zurich but results haven't been published yet (although the study was scheduled to end several years ago) (Fig. 3).

6.3.3 DARPins

A different and novel group of drugs is that of designed ankyrin repeat proteins (DARPins). These are small molecules, engineered to mimic antibodies binding capabilities, which can be administered orally (Kocatürk and Zuberbier 2018). Two of these were able to inhibit the binding of IgEs to their mast cells receptors and consequently block their activation (Kim et al. 2012). Unfortunately, they haven't reached yet a clinical stage of experimentation (Fig. 3).

6.3.4 SYK Inhibitors

In mast cells, spleen tyrosine kinase (SYK) plays an important role in intracellular signalling. It leads to a cascade of events ultimately causing release of histamine, prostaglandins and other mediators. The development of a topical formulation of a SYK inhibitor (GSK2646264) is currently being studied clinically in healthy controls and patients with CSU (Fig. 3) (Ramirez Molina et al. 2019; Barker et al. 2018).

6.3.5 BTK Inhibitors

A mechanistically similar target of future therapies may be Bruton's tyrosine kinase. Involved in B cell signalling and relevant to the production of IgEs, the inhibition of BTK yielded encouraging results in a study investigating the efficacy of oral ibrutinib (a BTK inhibitor) on mast cells and basophils activation (Dispenza et al. 2018; Herman et al. 2018). A phase II trial is now being conducted on patients with CSU for a different oral BTK inhibitor, fenebrutinib, in order to understand its safety and efficacy (Fig. 3).

6.3.6 Anti-Prostaglandin

CRTH2, a PGD2 receptor, was observed to be over-expressed by eosinophils in CSU patients (Yahara et al. 2010). An oral antagonist of CRTH2, AZD1981, was therefore studied in patients with CSU and H1-antihistamines refractoriness (Oliver et al. 2019). Interestingly, the short trial (4 weeks, placebo-controlled) showed benefit especially in terms of reduced itching, rather than hives extension. The drug was well tolerated and, at least in some aspects, more effective than placebo. Although not yet investigated in CSU, fevipiprant, a drug acting similarly to AZD1981 (a CRTH2 antagonist), has been

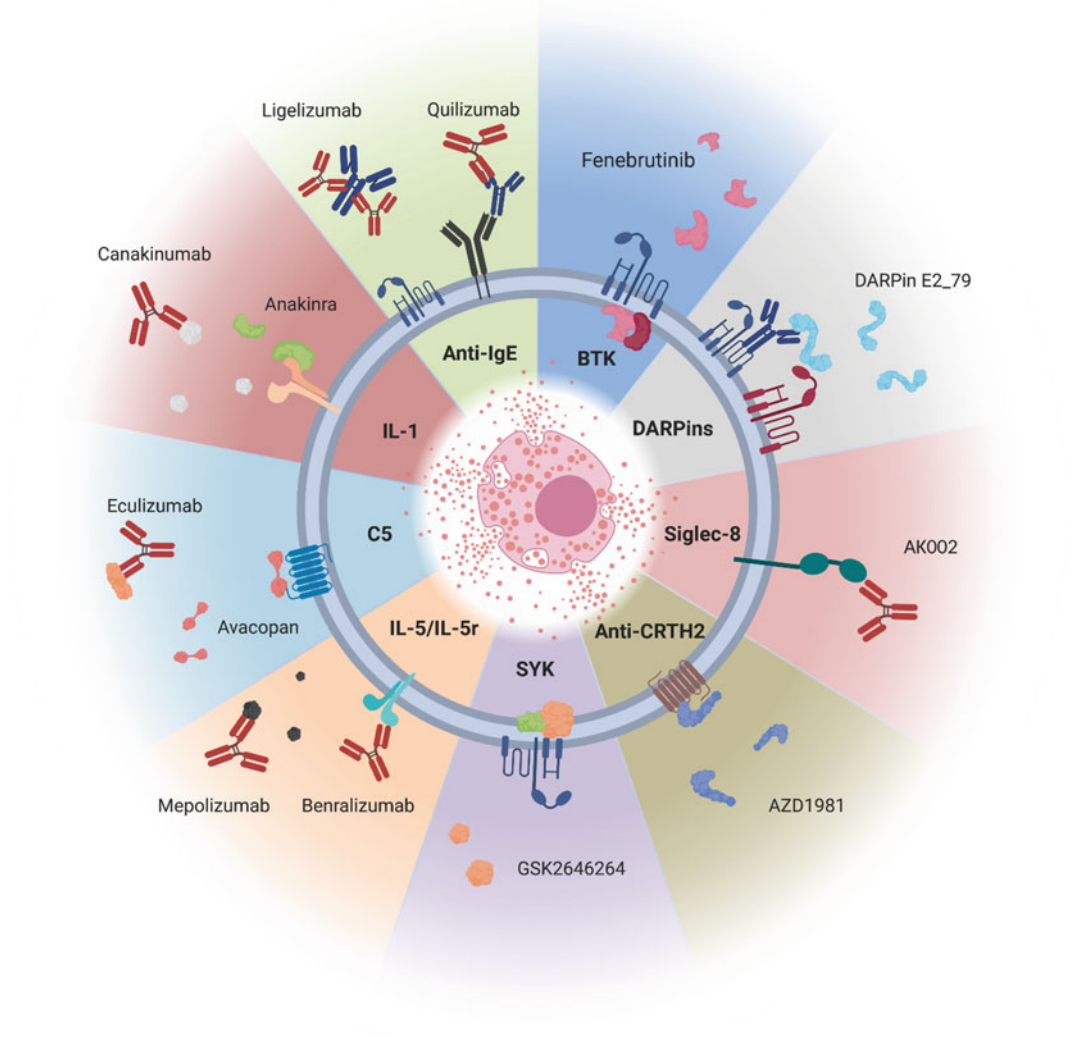


Fig. 3 Investigational therapies in CSU
Each segment represents a distinct pathway involved in the activation of mast cells (centre). Molecular mechanisms are represented on the outer part of the figure, as well as drugs studied to block them (Grey circle: cellular membrane; BTK: Bruton's tyrosine kinase; SYK: spleen tyrosine kinase)

extensively studied in asthma with important preliminary success, especially in severe eosinophilic asthma (Fig. 3) (White et al. 2018).

6.3.7 Anti-Complement Fragments

As described by several studies, complement binding plays a crucial role in enhancing the activating effects of IgG autoantibodies targeting mast cells IgE receptors (Fiebiger et al. 1995). In particular, C5 fragments have been postulated to bind mast cells C5a receptors leading to their activation (Kikuchi and Kaplan 2002). Eculizumab is a monoclonal antibody targeting C5, which has proven effective in paroxysmal nocturnal hemoglobinuria and atypical hemolytic-uremic syndrome. It would be of

great interest to evaluate the safety and efficacy of eculizumab in CSU (Kocatürk et al. 2017). Besides, a molecule targeting C5a receptor was recently developed and tested with preliminary success in patients with granulomatosis with polyangiitis (previously known as Wegener's granulomatosis, a vasculitis part of the group of ANCA-associated vasculitides) (Jayne et al. 2017; Tesar and Hruskova 2018). This molecule, whose name is avacopan, should equally be studied in CSU, where it may provide an additional clinical benefit in patients refractory to other treatments (Fig. 3).

6.3.8 Anti-IL-5 and IL-5 Receptors

Eventually, monoclonal antibodies targeting interleukin 5 and its receptor (respectively mepolizumab and benralizumab) may be effective in CSU, at least according to those who place eosinophils at the centre of urticarial pathogenesis (Kocatürk et al. 2017). This theory has gained popularity after a case was reported in which mepolizumab caused complete remission of CSU refractory to other treatments in a patient with concurrent severe uncontrolled asthma (Magerl et al. 2018). Currently, a phase I study of mepolizumab is undergoing in patients with CSU (Fig. 3) (Kocatürk and Zuberbier 2018). Recently, a non-randomized trial on 12 patients was published which evaluated the response to benralizumab in patients unresponsive to antihistamines (Bernstein et al. 2020). Patients were first administered placebo and subsequently three monthly doses of benralizumab (30 mg). The primary endpoint was the change in UAS7 after 20 weeks, which was found to be highly statistically significant (−15.7 points, 95% CI: −6.6 to −24.8, $p < 0.001$) (Bernstein et al. 2020). However, due to the small size of the patient group and the absence of randomization or even a control group treated with standard of care (omalizumab), it is difficult to infer any reliable information on benralizumab efficacy from this preliminary data.

6.3.9 Anti Sialic Acid-Binding Immunoglobulin-like Lectins (Siglecs)

The expression of Sialic acid-binding immunoglobulin-like lectins (Siglecs), a family receptors member of type I lectin, on human leukocytes is well demonstrated. The function of Siglecs in the human immune system is various, with inhibitory and activatory function depending on the cells expressing them (Varchetta et al. 2012). In particular, Siglec-8 is selectively expressed on human eosinophils, basophils and mast cells (Crocker et al. 2007). Its activation brings eosinophils to apoptosis and mast cells to inhibition of response (Nutku et al. 2003). For this reason, an anti Siglec-8 receptor was synthetized, AK002. Treatment of healthy subjects showed a rapidly depletion in blood eosinophils even after the single dose (Rasmussen et al. 2018). An open-label, phase 2a, pilot study showed how almost 92% of CSU patients naïve to omalizumab and 36% omalizumab-refractory obtained a complete remission (Fig. 3).

7 Prognosis

More than 80% of cases of acute urticaria don't progress beyond 6 weeks (Antia et al. 2018; Zuberbier et al. 1996; Aoki et al. 1994). In a study conducted on children, the rate of acute urticaria becoming recurrent or turning into chronic urticaria was estimated at about 30% (Mortureux et al. 1998). For what concerns the duration of chronic urticaria, different studies have provided variable results. A rate of 3-year remission of 32%, a 1-year remission rate of 47% and a 55% 1-year remission rate were all described by different authors (Champion et al. 1969; Quaranta et al. 1989; Kozel et al. 2001). 5-year remission rates have been observed to be 29% in adults and 67.7% in children (van der Valk et al. 2002; Chansakulporn et al. 2014).

In order to assess severity of disease and quality of life in chronic spontaneous urticaria, several tools have been developed. Among these, Chronic Urticaria Quality of Life Questionnaire (CU-Q20L) focuses on health-related quality of life, instead, the Urticaria Severity Score (USS) evaluates quality of life and need for medications. The most used in the clinical setting is the urticaria activity score-7 (UAS-7), which evaluates severity of the disease (Antia et al. 2018) by the calculation of a weekly score based upon daily itching (0–3 points) and the number of wheals (0–3 points) (Mlynek et al. 2013; Mathias et al. 2010). When the total weekly score sums up to less than 7, disease is well controlled. With higher scores, disease is progressively less controlled.

Studies performed on patients with CSU, in order to assess their quality of life and satisfaction with treatment, have invariably brought forward worrying results. From an online survey conducted in Germany, it emerged that approximately half of the more than 17,000 participants felt their symptoms were uncontrolled (Maurer et al. 2016). A subsequent observational study, conducted on patients with CSU who had persistence of symptoms despite treatment, demonstrated that more than half of the patients had moderate-to-severe disease activity based upon the UAS-7 (Maurer et al. 2017b). Unfortunately, according to another recent study, it appears that a large percentage of patients is undertreated due to lack of adherence to guidelines. In a cohort of patients with CSU refractory to antihistamines, only a minority was informed about the possibility of omalizumab treatment (Maurer et al. 2017a).

Eventually, it should be kept in mind how CSU, although not a life-threatening condition, is a chronic disorder carrying an important burden on patients health and quality of life. While treatment is not always effective, it may still elicit adverse reactions. The ineffectiveness of treatment may be due to the standardized "one-size-fits-all" stepwise approach which is adopted in CSU. Indeed, the possibility of adopting customized therapies for individual patients is not presently available in CSU.

However, the implementation of a laboratory diagnostics (a set of molecular biomarkers) capable of distinguishing subsets of patients with different mechanisms of disease is the contemporary goal of the most respected investigators. Concurrently, an impressive number of novel medications are being tested for future use in CSU. Some of these, due to their specific targets, may only be used in specific cases. All things considered, it seems to be a matter of time before the revolution of precision medicine fully takes place in the setting of urticaria.

Acknowledgments Pictures edited with BioRender (www.BioRender.com).

References

Abajian M, Schoepke N, Altrichter S, Zuberbier T, Maurer M (2014) Physical Urticarias and cholinergic Urticaria. Immunol Allergy Clin N Am 34:73–88

Aloe L, Skaper SD, Leon A, Levi-Montalcini R (1994) Nerve growth factor and autoimmune diseases. Autoimmunity 19:141–150

Altrichter S et al (2011) IgE mediated autoallergy against thyroid peroxidase – a novel Pathomechanism of chronic spontaneous Urticaria? PLoS One 6:e14794

Amsler E, Soria A, Vial-Dupuy A (2014) What do we learn from a cohort of 219 French patients with chronic urticaria? Eur J Dermatol 24:700–701

Antia C, Baquerizo K, Korman A, Bernstein JA, Alikhan A (2018) Urticaria: a comprehensive review. J Am Acad Dermatol 79:599–614

Aoki T, Kojima M, Horiko T (1994) Acute Urticaria: history and natural course of 50 cases. J Dermatol 21:73–77

Asero R (2002) Chronic idiopathic urticaria: a family study. Ann Allergy Asthma Immunol 89:195–196

Asero R (2015) Plasma D-dimer levels and clinical response to ciclosporin in severe chronic spontaneous urticaria. J Allergy Clin Immunol 135:1401–1403

Asero R et al (2007) Activation of the tissue factor pathway of blood coagulation in patients with chronic urticaria. J Allergy Clin Immunol 119:705–710

Asero R, Marzano AV, Ferrucci S, Cugno M (2017) D-dimer plasma levels parallel the clinical response to Omalizumab in patients with severe chronic spontaneous Urticaria. Int Arch Allergy Immunol 172:40–44

Auyeung P, Mittag D, Hodgkin PD, Harrison LC (2016) Autoreactive T cells in chronic spontaneous urticaria target the IgE Fc receptor Iα subunit. J Allergy Clin Immunol 138:761–768.e4

Ban G-Y et al (2014) Clinical features of elderly chronic urticaria. Korean J Intern Med 29:800

Bandara G, Metcalfe DD, Kirshenbaum AS (2015) Growth of human mast cells from bone marrow and peripheral blood-derived CD34(+) pluripotent hematopoietic cells. Methods Mol Biol 1220:155–162

Barker MD et al (2018) Discovery of potent and selective Spleen Tyrosine Kinase inhibitors for the topical treatment of inflammatory skin disease. Bioorg Med Chem Lett 28:3458–3462

Baroni A, Faccenda F, Russo T, Teresa R, Piccolo V (2012) Figurate paraneoplastic urticaria and prostate cancer. Ann Dermatol 24:366–367

Bech R, Kibsgaard L, Vestergaard C (2018) Comorbidities and treatment strategies in bullous pemphigoid: an appraisal of the existing Litterature. Front Med 5:238

Bédard PM, Brunet C, Pelletier G, Hébert J (1986) Increased compound 48/80 induced local histamine release from nonlesional skin of patients with chronic urticaria. J Allergy Clin Immunol 78:1121–1125

Belleguic C et al (2002) Increased release of matrix metalloproteinase-9 in the plasma of acute severe asthmatic patients. Clin Exp Allergy 32:217–223

Beno SM, Nadel FM, Alessandrini EA (2007) A Survey of Emergency Department Management of Acute Urticaria in Children. Pediatr Emerg Care 23:862–868

Bernstein JA et al (2020) Benralizumab for chronic spontaneous Urticaria. N Engl J Med 383:1389–1391

Bodar EJ, Simon A, de Visser M, van der Meer JWM (2009) Complete remission of severe idiopathic cold urticaria on interleukin-1 receptor antagonist (anakinra). Neth J Med 67:302–305

Bonini S et al (1996) Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. Proc Natl Acad Sci 93:10955–10960

Boyden SE et al (2016) Vibratory Urticaria Associated with a Missense Variant in ADGRE2. N Engl J Med 374:656–663

Bracken SJ, Abraham S, MacLeod AS (2019) Autoimmune theories of chronic spontaneous Urticaria. Front Immunol 10:627

Bradding P, Okayama Y, Howarth PH, Church MK, Holgate ST (1995) Heterogeneity of human mast cells based on cytokine content. J Immunol 155:297–307

Brunetta E et al (2018) Omalizumab for idiopathic nonhistaminergic angioedema: evidence for efficacy in 2 patients. Case Rep Immunol 2018:8067610

Caproni M et al (2005) Chronic idiopathic urticaria: infiltrating cells and related cytokines in autologous serum-induced wheals. Clin Immunol 114:284–292

Cassano N, Colombo D, Bellia G, Zagni E, Vena GA (2016) Gender-related differences in chronic urticaria. G Ital Dermatol Venereol 151:544–552

Champion RH, Roberts SO, Carpenter RG, Roger JH (1969) Urticaria and angio-oedema. A review of 554 patients. Br J Dermatol 81:588–597

Chan Y-C et al (2014) 'Auto-anti-IgE': naturally occurring IgG anti-IgE antibodies may inhibit allergen-induced basophil activation. J Allergy Clin Immunol 134:1394–1401.e4

Chansakulporn S et al (2014) The natural history of chronic urticaria in childhood: a prospective study. J Am Acad Dermatol 71:663–668

Chung BY, Cho YS, Kim HO, Park CW (2016) Food allergy in Korean patients with chronic Urticaria. Ann Dermatol 28:562–568

Cirillo R et al (1990) Cyclosporin A rapidly inhibits mediator release from human basophils presumably by interacting with cyclophilin. J Immunol 144:3891–3897

Coban M et al (2008) HLA class I and class II genotyping in patients with chronic Urticaria. Int Arch Allergy Immunol 147:135–139

Crocker PR, Paulson JC, Varki A (2007) Siglecs and their roles in the immune system. Nat Rev Immunol 7:255–266

Cugno M, Marzano AV, Asero R, Tedeschi A (2010) Activation of blood coagulation in chronic urticaria: pathophysiological and clinical implications. Intern Emerg Med 5:97–101

Curth H-M et al (2015) Effects of helicobacter pylori eradication in chronic spontaneous Urticaria: results from a retrospective cohort study. Am J Clin Dermatol 16:553–558

de Koning HD, Bodar EJ, van der Meer JWM, Simon A (2007) Schnitzler syndrome: beyond the case reports: review and follow-up of 94 patients with an emphasis on prognosis and treatment. Semin Arthritis Rheum 37:137–148

Del Pozo MD et al (1997) Anisakis simplex, a relevant etiologic factor in acute urticaria. Allergy 52:576–579

Di Campli C et al (1998) Beneficial effects of helicobacter pylori eradication on idiopathic chronic urticaria. Dig Dis Sci 43:1226–1229

Dice J (2004) Physical urticaria. Immunol Allergy Clin N Am 24:225–246

Dilek F et al (2016) Plasma levels of matrix Metalloproteinase-9 in children with chronic spontaneous Urticaria. Allergy Asthma Immunol Res 8:522

Dispenza MC, Pongracic JA, Singh AM, Bochner BS (2018) Short-term ibrutinib therapy suppresses skin test responses and eliminates IgE-mediated basophil activation in adults with peanut or tree nut allergy. J Allergy Clin Immunol 141:1914–1916.e7

Eckman JA, Hamilton RG, Gober LM, Sterba PM, Saini SS (2008) Basophil phenotypes in chronic idiopathic Urticaria in relation to disease activity and autoantibodies. J Invest Dermatol 128:1956–1963

Elias J, Boss E, Kaplan A (1986) Studies of the cellular infiltrate of chronic idiopathic urticaria: prominence of T-lymphocytes, monocytes, and mast cells. J Allergy Clin Immunol 78:914–918

Estelle Simons Ledit RF Ardusso Vesselin Dimov Motohiro Ebisawa Yehia M El-Gamal Richard F Lockey Mario Sanchez-Borges Gian Enrico Senna Aziz Sheikh Bernard Y Thong Margitta Worm FR, Rosario D, Estelle Simons FR (2013) World allergy organization anaphylaxis guidelines: 2013 update of

the evidence base. Int Arch Allergy Immunol 162:193–204
Feliciani C et al (2015) Management of bullous pemphigoid: the European dermatology forum consensus in collaboration with the European academy of dermatology and venereology. Br J Dermatol 172:867–877
Ferrer M, Nakazawa K, Kaplan AP (1999) Complement dependence of histamine release in chronic urticaria☆☆☆. J Allergy Clin Immunol 104:169–172
Ferrer M et al (2010) Serum total tryptase levels are increased in patients with active chronic urticaria. Clin Exp Allergy 40:1760–1766
Fiebiger E et al (1995) Serum IgG autoantibodies directed against the alpha chain of Fc epsilon RI: a selective marker and pathogenetic factor for a distinct subset of chronic urticaria patients? J Clin Invest 96:2606–2612
Folci M, Heffler E, Canonica GW, Furlan R, Brunetta E (2018) Cutting edge: biomarkers for chronic spontaneous Urticaria. J Immunol Res 2018:5615109
Gaig P, Olona M, Muñoz Lejarazu D, Caballero MT, Domínguez FJ, Echechipia S, García Abujeta JL, Gonzalo MA, Lleonart R, Martínez Cócera C, Rodríguez A, Ferrer M (2004) Epidemiology of urticaria in Spain. J Investig Allergol Clin Immunol 14:214–220
Gasser P et al (2020) The mechanistic and functional profile of the therapeutic anti-IgE antibody ligelizumab differs from omalizumab. Nat Commun 11:1–14
Grattan CE, Wallington TB, Warin RP, Kennedy CT, Bradfield JW (1986) A serological mediator in chronic idiopathic urticaria–a clinical, immunological and histological evaluation. Br J Dermatol 114:583–590
Grattan CEH et al (2000) Randomized double-blind study of cyclosporin in chronic 'idiopathic' urticaria. Br J Dermatol 143:365–372
Grattan CEH, Dawn G, Gibbs S, Francis DM (2003) Blood basophil numbers in chronic ordinary urticaria and healthy controls: diurnal variation, influence of loratadine and prednisolone and relationship to disease activity. Clin Exp Allergy 33:337–341
Gregoriou S et al (2009) Etiologic aspects and prognostic factors of patients with chronic urticaria: nonrandomized, prospective, descriptive study. J Cutan Med Surg 13:198–203
Harris JM et al (2016a) A randomized trial of the efficacy and safety of quilizumab in adults with inadequately controlled allergic asthma. Respir Res 17:29
Harris JM et al (2016b) A randomized trial of quilizumab in adults with refractory chronic spontaneous urticaria. J Allergy Clin Immunol 138:1730–1732
Herman AE et al (2018) Safety, pharmacokinetics, and pharmacodynamics in healthy volunteers treated with GDC-0853, a selective reversible Bruton's tyrosine kinase inhibitor. Clin Pharmacol Ther 103:1020–1028
Hide M et al (1993) Autoantibodies against the high-affinity IgE receptor as a cause of histamine release in chronic Urticaria. N Engl J Med 328:1599–1604
Hiragun M et al (2013) Prognosis of chronic spontaneous urticaria in 117 patients not controlled by a standard dose of antihistamine. Allergy 68:229–235
Holick M, Vitamin F (2007) D deficiency. N Engl J Med 357:266–281
Hsu M-L, Li L-F (2012) Prevalence of food avoidance and food allergy in Chinese patients with chronic urticaria. Br J Dermatol 166:747–752
Irani AM, Schwartz LB (1989) Mast cell heterogeneity. Clin Exp Allergy 19:143–155
Jachiet M et al (2015) The clinical Spectrum and therapeutic Management of Hypocomplementemic Urticarial Vasculitis: data from a French Nationwide study of fifty-seven patients. Arthritis Rheumatol 67:527–534
Jacques P, Lavoie A, Bédard PM, Brunet C, Hébert J (1992) Chronic idiopathic urticaria: profiles of skin mast cell histamine release during active disease and remission. J Allergy Clin Immunol 89:1139–1143
Jayne DRW et al (2017) Randomized trial of C5a receptor inhibitor Avacopan in ANCA-associated Vasculitis. J Am Soc Nephrol 28:2756–2767
Kalogeromitros D et al (2011) Can internet surveys help us understanding allergic disorders? – results from a large survey in urticaria in Greece. J Eur Acad Dermatol Venereol 25:532–537
Kaplan AP (2019) Treatment of urticaria: a clinical and mechanistic approach. Curr Opin Allergy Clin Immunol 19:387–392
Kaplan AP, Joseph K, Maykut RJ, Geba GP, Zeldin RK (2008) Treatment of chronic autoimmune urticaria with omalizumab. J Allergy Clin Immunol 122:569–573
Kaplan A et al (2013) Omalizumab in patients with symptomatic chronic idiopathic/spontaneous urticaria despite standard combination therapy. J Allergy Clin Immunol 132:101–109
Kasi PM, Hieken TJ, Haddad TC (2016) Unilateral arm Urticaria presenting as a paraneoplastic manifestation of Metachronous bilateral breast Cancer. Case Rep Oncol 9:33–38
Kasperska-Zajac A, Grzanka A, Damasiewicz-Bodzek A (2015) IL-6 transsignaling in patients with chronic spontaneous Urticaria. PLoS One 10:e0145751
Kay AB et al (2014) Elevations in vascular markers and eosinophils in chronic spontaneous urticarial weals with low-level persistence in uninvolved skin. Br J Dermatol 171:505–511
Kay AB, Clark P, Maurer M, Ying S (2015) Elevations in T-helper-2-initiating cytokines (interleukin-33, interleukin-25 and thymic stromal lymphopoietin) in lesional skin from chronic spontaneous ('idiopathic') urticaria. Br J Dermatol 172:1294–1302
Kikuchi Y, Kaplan AP (2002) A role for C5a in augmenting IgG-dependent histamine release from basophils in chronic urticaria. J Allergy Clin Immunol 109:114–118
Kim B et al (2012) Accelerated disassembly of IgE-receptor complexes by a disruptive macromolecular inhibitor. Nature 491:613–617

Kirshenbaum AS, Metcalfe DD (2006) Growth of human mast cells from bone marrow and peripheral blood-derived CD34+ pluripotent progenitor cells. Methods Mol Biol 315:105–112

Kjaer HF, Eller E, Høst A, Andersen KE, Bindslev-Jensen C (2008) The prevalence of allergic diseases in an unselected group of 6-year-old children. The DARC birth cohort study. Pediatr Allergy Immunol 19:737–745

Kocatürk E, Zuberbier T (2018) New biologics in the treatment of urticaria. Curr Opin Allergy Clin Immunol 18:425–431

Kocatürk E, Maurer M, Metz M, Grattan C (2017) Looking forward to new targeted treatments for chronic spontaneous urticaria. Clin Transl Allergy 7:1

Kohli S et al (2018) Clinicoepidemiologic features of chronic Urticaria in patients with versus without subclinical *Helicobacter pylori* infection: a cross-sectional study of 150 patients. Int Arch Allergy Immunol 175:114–120

Kolkhir P, André F, Church MK, Maurer M, Metz M (2017) Potential blood biomarkers in chronic spontaneous urticaria. Clin Exp Allergy 47:19–36

Kolkhir P, Altrichter S, Hawro T, Maurer M (2018) C-reactive protein is linked to disease activity, impact, and response to treatment in patients with chronic spontaneous urticaria. Allergy 73:940–948

Konstantinou GN et al (2011) Childhood acute urticaria in northern and southern Europe shows a similar epidemiological pattern and significant meteorological influences. Pediatr Allergy Immunol 22:36–42

Kozel MMA, Mekkes JR, Bossuyt PMM, Bos JD (2001) Natural course of physical and chronic urticaria and angioedema in 220 patients. J Am Acad Dermatol 45:387–391

Krause K, Metz M, Makris M, Zuberbier T, Maurer M (2012) The role of interleukin-1 in allergy-related disorders. Curr Opin Allergy Clin Immunol 12:477–484

Krause K et al (2019) Association of CCL2 with systemic inflammation in Schnitzler syndrome. Br J Dermatol 180:859–868

Kuemmerle-Deschner JB et al (2017) Diagnostic criteria for cryopyrin-associated periodic syndrome (CAPS). Ann Rheum Dis 76:942–947

Kulthanan K, Chiawsirikajorn Y, Jiamton S (2008) Acute urticaria: etiologies, clinical course and quality of life. Asian Pac J Allergy Immunol 26:1–10

Kulthanan K et al (2018) Cyclosporine for chronic spontaneous Urticaria: a meta-analysis and systematic review. J Allergy Clin Immunol Pract 6:586–599

Lapi F et al (2016) Epidemiology of chronic spontaneous urticaria: results from a nationwide, population-based study in Italy. Br J Dermatol 174:996–1004

Lenormand C, Lipsker D (2012) Efficiency of Interleukin-1 blockade in refractory delayed-pressure Urticaria. Ann Intern Med 157:599

Levy Y, Segal N, Weintrob N, Danon YL (2003) Chronic urticaria: association with thyroid autoimmunity. Arch Dis Child 88:517–519

Lipsker D et al (2001) The Schnitzler syndrome. Four new cases and review of the literature. Medicine (Baltimore) 80:37–44

MacGlashan D (2019) Autoantibodies to IgE and FcεRI and the natural variability of spleen tyrosine kinase expression in basophils. J Allergy Clin Immunol 143:1100–1107.e11

Macri A, Cook C (2019) Urticaria Pigmentosa (cutaneous Mastocytosis). StatPearls. StatPearls Publishing

Magen E, Mishal J, Schlesinger M (2013) Clinical and laboratory features of chronic idiopathic urticaria in the elderly. Int J Dermatol 52:1387–1391

Magerl M et al (2018) Benefit of mepolizumab treatment in a patient with chronic spontaneous urticaria. J Dtsch Dermatol Ges 16:477–478

Mathias SD et al (2010) Development of a daily diary for patients with chronic idiopathic urticaria. Ann Allergy Asthma Immunol 105:142–148

Maurer M et al (2013) Omalizumab for the treatment of chronic idiopathic or spontaneous Urticaria. N Engl J Med 368:2340–2341

Maurer M et al (2016) ATTENTUS, a German online survey of patients with chronic urticaria highlighting the burden of disease, unmet needs and real-life clinical practice. Br J Dermatol 174:892–894

Maurer M et al (2017a) H1-antihistamine-refractory chronic spontaneous urticaria: it's worse than we thought – first results of the multicenter real-life AWARE study. Clin Exp Allergy 47:684–692

Maurer M et al (2017b) The burden of chronic spontaneous urticaria is substantial: real-world evidence from ASSURE-CSU. Allergy 72:2005–2016

Maurer M et al (2018) Omalizumab treatment in patients with chronic inducible urticaria: a systematic review of published evidence. J Allergy Clin Immunol 141:638–649

Maurer M et al (2019) Ligelizumab for chronic spontaneous Urticaria. N Engl J Med 381:1321–1332

Metz M, Ohanyan T, Church MK, Maurer M (2014) Omalizumab is an effective and rapidly acting therapy in difficult-to-treat chronic urticaria: a retrospective clinical analysis. J Dermatol Sci 73:57–62

Metz M et al (2017) Clinical efficacy of omalizumab in chronic spontaneous urticaria is associated with a reduction of FcεRI-positive cells in the skin. Theranostics 7:1266–1276

Minnone G, De Benedetti F, Bracci-Laudiero L (2017) NGF and its receptors in the regulation of inflammatory response. Int J Mol Sci 18:1028

Mlynek A et al (2013) A novel, simple, validated and reproducible instrument for assessing provocation threshold levels in patients with symptomatic dermographism. Clin Exp Dermatol 38:360–366

Mortureux P et al (1998) Acute Urticaria in infancy and early childhood. Arch Dermatol 134:319

Niimi N et al (1996) Dermal mast cell activation by autoantibodies against the high affinity IgE receptor in chronic urticaria. J Invest Dermatol 106:1001–1006

Novick D et al (2001) A novel IL-18BP ELISA shows elevated serum IL-18BP in sepsis and extensive decrease of free IL-18. Cytokine 14:334–342

Nutku E, Aizawa H, Hudson SA, Bochner BS (2003) Ligation of Siglec-8: a selective mechanism for induction of human eosinophil apoptosis. Blood 101:5014–5020

Nuzzo V, Tauchmanova L, Colasanti P, Zuccoli A, Colao A (2011) Idiopathic chronic urticaria and thyroid autoimmunity: experience of a single center. Dermatoendocrinol 3:255–258

O'Donnell E, Havyer R (2014) Breast malignancy masquerading under the cloak of acute urticaria. BMJ Case Rep 2014:bcr2013202494

O'Donnell BF et al (1999) Human leucocyte antigen class II associations in chronic idiopathic urticaria. Br J Dermatol 140:853–858

Oliver ET, Sterba PM, Devine K, Vonakis BM, Saini SS (2016) Altered expression of chemoattractant receptor–homologous molecule expressed on T H 2 cells on blood basophils and eosinophils in patients with chronic spontaneous urticaria. J Allergy Clin Immunol 137:304–306.e1

Oliver ET et al (2019) Effects of an Oral CRTh2 antagonist (AZD1981) on eosinophil activity and symptoms in chronic spontaneous Urticaria. Int Arch Allergy Immunol 179:21–30

Otsuka H, Inaba M, Fujikura T, Kunitomo M (1995) Histochemical and functional characteristics of metachromatic cells in the nasal epithelium in allergic rhinitis: studies of nasal scrapings and their dispersed cells. J Allergy Clin Immunol 96:528–536

Pappalardo E et al (2000) Frequent de novo mutations and exon deletions in the C1inhibitor gene of patients with angioedema. J Allergy Clin Immunol 106:1147–1154

Peters EMJ et al (2011) Nerve growth factor partially recovers inflamed skin from stress-induced worsening in allergic inflammation. J Invest Dermatol 131:735–743

Piemonti L et al (2000) Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells. J Immunol 164:4443–4451

Pogorelov D, Olisova O, Nekrasova T, Kolkhir P (2016) Chronische spontane Urtikaria in Verbindung mit einer primär billiären Cholangitis: ein Fallbericht und Literaturüberblick. J Dtsch Dermatol Ges 14:1134–1136

Poindexter NJ et al (2009) IL-24 is expressed during wound repair and inhibits TGFα-induced migration and proliferation of keratinocytes. Exp Dermatol 19:714–722

Pollack CV, Romano TJ (1995) Outpatient management of Acute Urticaria: the role of prednisone. Ann Emerg Med 26:547–551

Quaranta JH et al (1989) The natural history and response to therapy of chronic urticaria and angioedema. Ann Allergy 62:421–424

Quintero OP, Arrondo AP, Veleiro B (2017) Rapid response to omalizumab in 3 cases of delayed pressure urticaria. J Allergy Clin Immunol Pract 5:179–180

Raap U et al (2010) Increased levels of serum IL-31 in chronic spontaneous urticaria. Exp Dermatol 19:464–466

Raap U et al (2017) Human basophils are a source of – and are differentially activated by – IL-31. Clin Exp Allergy 47:499–508

Radonjic-Hoesli S et al (2018) Urticaria and angioedema: an update on classification and pathogenesis. Clin Rev Allergy Immunol 54:88–101

Ram M, Sherer Y, Shoenfeld Y (2006) Matrix Metalloproteinase-9 and autoimmune diseases. J Clin Immunol 26:299–307

Ramirez Molina C et al (2019) GSK2646264, a spleen tyrosine kinase inhibitor, attenuates the release of histamine in ex vivo human skin. Br J Pharmacol 176:1135–1142

Rasmussen HS, Chang AT, Tomasevic N, Bebbington C (2018) A randomized, double-blind, placebo-controlled, ascending dose phase 1 study of AK002, a novel Siglec-8 selective monoclonal antibody, in healthy subjects. J Allergy Clin Immunol 141:AB403

Rauber MM, Pickert J, Holiangu L, Möbs C, Pfützner W (2017) Functional and phenotypic analysis of basophils allows determining distinct subtypes in patients with chronic urticaria. Allergy 72:1904–1911

Rorsman H (1962) Basophilic leucopenia in different forms of urticaria. Acta Allergol 17:168–184

Rothbaum R, McGee J (2016) Aquagenic urticaria: diagnostic and management challenges. J Asthma Allergy 9:209–213

Rutkowski K, Grattan CEH (2017) How to manage chronic urticaria 'beyond' guidelines: a practical algorithm. Clin Exp Allergy 47:710–718

Saini SS, Kaplan AP (2018) Chronic spontaneous Urticaria: the Devil's itch. J Allergy Clin Immunol Pract 6:1097–1106

Saini SS, MacGlashan DW (2012) Assessing basophil functional measures during monoclonal anti-IgE therapy. J Immunol Methods 383:60–64

Saini SS et al (2009) Cultured peripheral blood mast cells from chronic idiopathic urticaria patients spontaneously degranulate upon IgE sensitization: relationship to expression of Syk and SHIP-2. Clin Immunol 132:342–348

Saini S et al (2011) A randomized, placebo-controlled, dose-ranging study of single-dose omalizumab in patients with H1-antihistamine-refractory chronic idiopathic urticaria. J Allergy Clin Immunol 128:567–573.e1

Saini SS et al (2015) Efficacy and safety of Omalizumab in patients with chronic idiopathic/spontaneous Urticaria who remain symptomatic on H 1 antihistamines: a randomized, placebo-controlled study. J Invest Dermatol 135:67–75

Saini SS et al (2017) Effect of Omalizumab on blood basophil counts in patients with chronic idiopathic/spontaneous Urticaria. J Invest Dermatol 137:958–961

Sánchez-Borges M, Capriles-Hulett A, Caballero-Fonseca F (2015a) Demographic and clinical profiles in patients with acute urticaria. Allergol Immunopathol (Madr) 43:409–415

Sánchez-Borges M, Caballero-Fonseca F, Capriles-Hulett-A, González-Aveledo L (2015b) Aspirin-exacerbated cutaneous disease (AECD) is a distinct subphenotype of chronic spontaneous urticaria. J Eur Acad Dermatol Venereol 29:698–701

Santiago-Vázquez M, Barrera-Llaurador J, Carrasquillo OY, Sánchez S (2019) Chronic spontaneous urticaria associated with colon adenocarcinoma: a paraneoplastic manifestation? A case report and review of literature. JAAD Case Rep 5:101–103

Scalese MJ, Reinaker TS (2016) Pharmacologic management of angioedema induced by angiotensin-converting enzyme inhibitors. Am J Health Syst Pharm 73:873–879

Schmetzer O et al (2018) IL-24 is a common and specific autoantigen of IgE in patients with chronic spontaneous urticaria. J Allergy Clin Immunol 142:876–882

Schoepke N et al (2019) Biomarkers and clinical characteristics of autoimmune chronic spontaneous urticaria (aiCSU): Results of the PURIST Study. Allergy 74(12):2427–2436

Schroeder JT (2009) Basophils beyond effector cells of allergic inflammation. Adv Immunol 101:123–161

Shin YS, Suh D-H, Yang E-M, Ye Y-M, Park H-S (2015) Serum specific IgE to thyroid peroxidase activates basophils in aspirin intolerant Urticaria. J Korean Med Sci 30:705–709

Siebenhaar F, Degener F, Zuberbier T, Martus P, Maurer M (2009) High-dose desloratadine decreases wheal volume and improves cold provocation thresholds compared with standard-dose treatment in patients with acquired cold urticaria: a randomized, placebo-controlled, crossover study. J Allergy Clin Immunol 123:672–679

Smith C, Kepley C, Schwartz L, Lee T (1995) Mast cell number and phenotype in chronic idiopathic urticaria. J Allergy Clin Immunol 96:360–364

Snidvongs K, Seresirikachorn K, Khattiyawittayakun L, Chitsuthipakorn W (2017) Sedative effects of Levocetirizine: a systematic review and meta-analysis of randomized controlled studies. Drugs 77:175–186

Song J, Wu C, Zhang X, Sorokin LM (2013) In vivo processing of CXCL5 (LIX) by matrix metalloproteinase (MMP)-2 and MMP-9 promotes early neutrophil recruitment in IL-1 -induced peritonitis. J Immunol 190:401–410

Stellato C et al (1992) Anti-inflammatory effect of Cyclosporin A on human skin mast cells. J Invest Dermatol 98:800–804

Takeda T et al (2011) Increase of coagulation potential in chronic spontaneous urticaria. Allergy 66:428–433

Taskapan O, Kutlu A, Karabudak O (2008) Evaluation of autologous serum skin test results in patients with chronic idiopathic urticaria, allergic/non-allergic asthma or rhinitis and healthy people. Clin Exp Dermatol 33:754–758

Tesar V, Hruskova Z (2018) Avacopan in the treatment of ANCA-associated vasculitis. Expert Opin Investig Drugs 27:491–496

Tonacci A, Billeci L, Pioggia G, Navarra M, Gangemi S (2017) Omalizumab for the treatment of chronic idiopathic Urticaria: systematic review of the literature. Pharmacotherapy 37:464–480

Tosi M (1998) Molecular genetics of C1 inhibitor. Immunobiology 199:358–365

Toubi E et al (2004) Clinical and laboratory parameters in predicting chronic urticaria duration: a prospective study of 139 patients. Allergy 59:869–873

Uguz F, Engin B, Yilmaz E (2008) Axis I and Axis II diagnoses in patients with chronic idiopathic urticaria. J Psychosom Res 64:225–229

van der Valk PGMGM, Moret G, Kiemeney LALMALM (2002) The natural history of chronic urticaria and angioedema in patients visiting a tertiary referral centre. Br J Dermatol 146:110–113

Varchetta S et al (2012) Engagement of Siglec-7 receptor induces a pro-inflammatory response selectively in monocytes. PLoS One 7:e45821

Vena GA, Cassano N, Colombo D, Peruzzi E, Pigatto P (2006) Cyclosporine in chronic idiopathic urticaria: a double-blind, randomized, placebo-controlled trial. J Am Acad Dermatol 55:705–709

Viswanathan RK, Biagtan MJ, Mathur SK (2012) The role of autoimmune testing in chronic idiopathic urticaria. Ann Allergy Asthma Immunol 108:337–341.e1

Vonakis BM, Saini SS (2008) New concepts in chronic urticaria. Curr Opin Immunol 20:709–716

Wang W et al (2001) Interleukin-18 enhances the production of interleukin-8 by eosinophils. Eur J Immunol 31:1010–1016

Wedi B, Raap U, Wieczorek D, Kapp A (2009) Urticaria and infections. Allergy Asthma Clin Immunol 5:10

Weller K et al (2010) Chronische Urtikaria. Hautarzt 61:750–757

White C, Wright A, Brightling C (2018) Fevipiprant in the treatment of asthma. Expert Opin Investig Drugs 27:199–207

Williams JDL et al (2008) Occupational contact urticaria: Australian data. Br J Dermatol 159:125–131

Woo YR, Jung KE, Koo DW, Lee JS (2015) Vitamin D as a marker for disease severity in chronic Urticaria and its possible role in pathogenesis. Ann Dermatol 27:423–430

Yahara H, Satoh T, Miyagishi C, Yokozeki H (2010) Increased expression of CRTH2 on eosinophils in allergic skin diseases. J Eur Acad Dermatol Venereol 24:75–76

Yamaguchi M et al (1992) Preliminary criteria for classification of adult Still's disease. J Rheumatol 19:424–430

Yamamoto T (2012) Cutaneous manifestations associated with adult-onset Still's disease: important diagnostic values. Rheumatol Int 32:2233–2237

Ying S, Kikuchi Y, Meng Q, Kay AB, Kaplan AP (2002) TH1/TH2 cytokines and inflammatory cells in skin biopsy specimens from patients with chronic idiopathic urticaria: comparison with the allergen-induced late-phase cutaneous reaction. J Allergy Clin Immunol 109:694–700

Zazzali JL, Broder MS, Chang E, Chiu MW, Hogan DJ (2012) Cost, utilization, and patterns of medication use associated with chronic idiopathic urticaria. Ann Allergy Asthma Immunol 108:98–102

Zuberbier T, Iffländer J, Semmler C, Henz BM (1996) Acute urticaria: clinical aspects and therapeutic responsiveness. Acta Derm Venereol 76:295–297

Zuberbier T et al (2014) The EAACI/GA2/LEN/EDF/WAO Guideline for the definition, classification, diagnosis, and management of urticaria: the 2013 revision and update. Allergy 69:868–887

Zuberbier T et al (2018) The EAACI/GA^{2}LEN/EDF/WAO guideline for the definition, classification, diagnosis and management of urticaria. Allergy 73:1393–1414

Adv Exp Med Biol - Cell Biology and Translational Medicine (2021) 12: 139–157
https://doi.org/10.1007/5584_2021_619

Published online: 22 February 2021

The Potentials and Pitfalls of Using Adult Stem Cells in Cancer Treatment

Mrinal K. Das, Taral R. Lunavat, Hrvoje Miletic, and Jubayer A. Hossain

Abstract

Stem cells play a pivotal role in the developmental stages of an organism and in adulthood as well. Therefore, it is not surprising that stem cells constitute a focus of extensive research. Indeed, several decades of stem cell research have tremendously increased our knowledge on the mechanistic understandings of stem cell biology. Interestingly, revealing the fundamental principles of stem cell biology has also fostered its application for therapeutic purposes. Many of the attributes that the stem cells possess, some of which are unique, allow multifaceted exploitation of stem cells in the treatment of various diseases. Cancer, the leading cause of mortality worldwide, is one of the disease groups that has been benefited by the potentials of therapeutic applications of the stem cells. While the *modi operandi* of how stem cells contribute to cancer treatment are many-sided, two major principles can be conceived. One mode involves harnessing the regenerative power of the stem cells to promote the generation of blood-forming cells in cancer patients after cytotoxic regimens. A totally different kind of utility of stem cells has been exercised in another mode where the stem cells can potentially deliver a plethora of anti-cancer therapeutics in a tumor-specific manner. While both these approaches can improve the treatment of cancer patients, there exist several issues that warrant further research. This review summarizes the basic principles of the utility of the stem cells in cancer treatment along with the current trends and pinpoints the major obstacles to focus on in the future for further improvement.

Keywords

Cancer · Extracellular vesicles · Gene therapy · Regenerative medicine · Stem cells · Virotherapy

M. K. Das (✉)
Department of Molecular Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway
e-mail: m.k.das@medisin.uio.no

T. R. Lunavat
Department of Biomedicine, University of Bergen, Bergen, Norway

H. Miletic and J. A. Hossain (✉)
Department of Biomedicine, University of Bergen, Bergen, Norway

Department of Pathology, Haukeland University Hospital, Bergen, Norway
e-mail: jhossain@mail.com

Abbreviation

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
bCD	Bacterial cytosine deaminase
BM	Bone marrow

CAR	Coxsackievirus–adenovirus receptor
CNS	Central nervous system
EGF	Epidermal growth factor
EPGT	Enzyme/prodrug gene therapy
ESCs	Embryonic stem cells
EVs	Extracellular vesicles
GVHD	Graft-versus-host disease
HGF	Hepatocyte growth factor
HSCs	Hematopoietic stem cells
HSV-1	Herpes simplex virus type 1
HSV-TK	Herpes simplex virus thymidine kinase gene
iPSCs	Induced pluripotent stem cells
MSCs	Mesenchymal stem cells
MVBs	Multi-vesicular bodies
NSCs	Neural stem cells
OVs	Oncolytic viruses
PTX	Paclitaxel
SSCs	Somatic stem cells
VEGF	Vascular endothelial growth factor

1 Introduction

Cancer is the leading cause of death worldwide (Siegel et al. 2016). Despite enormous improvements in diagnostics and therapeutics, millions of patients die of cancer each year. Surgery still remains the first-line treatment option for direct removal of most solid tumors. Radio- and chemotherapy, in addition, have evolved as standard treatment regimens for a wide range of tumors. Radiotherapy is used to kill tumor cells by damaging their DNA. Highly cytotoxic chemotherapy helps to slow down or stop tumor growth. In the pursuit of novel treatment regimens, molecularly targeted therapies emerged as promising candidates and have been hailed as the future of cancer treatment in the last two decades. However, the encouraging preclinical results have mostly been unmet in large-scale clinical trials (Maeda et al. 2018).

Metastatic cancer cells are almost impossible to eradicate completely by using the current regimens, and recurrence in these cases is extremely likely. Therefore, there is an impetus of developing new, effective therapies, that will exert low or no toxicity in normal cells. Recently, gene-, immuno-, and virotherapy have emerged as important therapeutic options for various cancer types with improved clinical outcomes. Harnessing the therapeutic potential of a whole new class of delivery vehicles such as extracellular vesicles (EVs), has also been of interest in recent years. The functional significance of these aforementioned novel therapies largely depends on efficient delivery tools or vectors. Because of possessing distinctive properties, such as migration towards cancer cells, secretion of bioactive factors, and escaping clearance by immunosuppression, stem cells appear to be very effective cellular vehicles and thus stem cell-based therapies are promising strategies to treat cancer. Numerous stem cell-based therapies have been under investigation in preclinical trials, and they have shown great promises for cancer treatment (Gomes et al. 2017). In addition, post-treatment recovery, especially after cytotoxic chemo-radiotherapeutic regimens, is an inherent component of modern cancer treatment. Stem cell treatment, in this context, also plays a very important role by promoting hematopoietic regeneration. Thus, the stem cells can be exploited in a multifaceted way in cancer treatment. Indeed, numerous studies over a long period of time have shown the multifaceted potential of stem cells in the context of cancer treatment (Fig. 1). Nevertheless, there remain scientific concerns regarding the use of stem cell therapies, which strongly recommends pursuing further studies to validate preclinical findings. This review summarizes current trends and future anticipations in the field of stem cell research in relation to cancer treatment.

2 Stem Cells: Overview and Classification

We have been perplexed for centuries to comprehend how the fertilized egg develops into the many different cells and tissues that structure the organs of a mature individual. Leonardo da Vinci's fifteenth-century drawings of the idealized human anatomy 'Vitruvian Man'

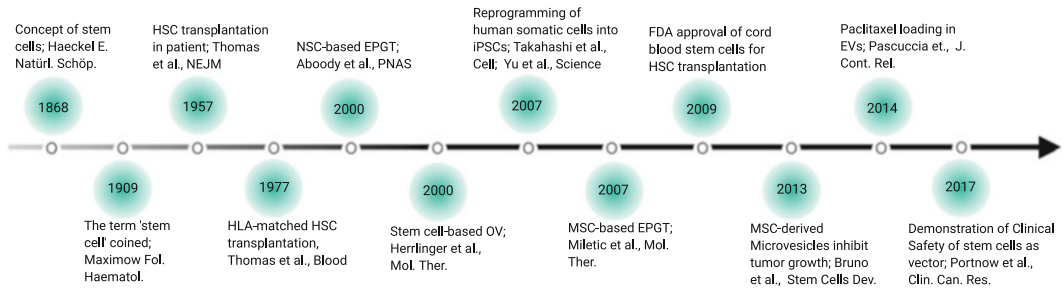


Fig. 1 Timeline showing selected milestones in exploiting stem cells for cancer treatment

provided a portrayal of anatomic development. However, it was the twentieth century, when mankind discovered that genetic information, encoded in the DNA of all cells is the key to understand development. The fate of individual cells and eventually the shape and function of various tissues and organs of the individual are dictated by different genes and signal circuitries originated thereof. In this developmental journey, various stem cells play a key role. Stem cell is the collective term that refers to a certain type of undifferentiated cells that have the ability to self-renew and to stay in an undifferentiated state, or when stimulated by signals in an appropriate environment, are capable of differentiating into many different cell lineages to produce numerous cell types.

Stem cells can be divided into two main categories – embryonic stem cells (ESCs) and somatic stem cells (SSCs). SSCs correspond to adult stem cells, which are generally multipotent and can differentiate into any cell type with a specific lineage, including neural stem cells (NSCs), mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), and others.

2.1 Human Embryonic Stem Cells (hESCs)

About 40 years ago, the first isolation of embryonic stem cells from a mouse opened new horizons towards producing all kinds of differentiated cell types in vitro (Evans et al. 1981; Martin 1981). In due course, the first derivation of hESC was reported in 1998, and since then, the potential of hESCs for cellular therapy has become a subject of great interest for both the scientists and the general public alike (Thomson et al. 1998).

In humans, the living organism from the time of implantation in the uterus until the end of the second month of gestation is defined as the embryo and hESCs are derived from surplus human embryos generated by *in vitro* fertilization (Edwards 2001). As pluripotent cells, ESCs can differentiate into any cell types except those in the placenta (Lin et al. 2013) and are therefore used as gold standards in the evaluation of all pluripotent cells cultured *in vitro*. However, the use of ESCs in scientific studies and human clinical trials is limited due to ethical considerations. In this context, the recent discovery of induced pluripotent stem cells (iPSCs) has largely replaced the need for ESCs.

2.2 Somatic Stem Cells

SSCs are intermittent, quiescent cells with a restricted self-renewal and differentiation ability. Isolation of different precursor cells from adult tissues indicates that all tissues may have their own niche of stem cells. These adult stem cells are in charge of regenerating cells that expire within a given organ, either due to physiological or pathological processes (Chagastelles et al. 2011). In the following section, we will discuss the adult stem cell types which are often employed in cancer treatment.

2.2.1 Haematopoietic Stem Cells

HSCs, found in the bone marrow (BM), are multipotent stem cells that can generate the entire blood compartment, including those of myeloid and lymphoid lineages. In 1963, HSCs were first described in mice in the early 1960s as bone marrow cells that could reconstitute the hematopoietic system after secondary transplantation (Becker et al. 1963; Siminovitch et al. 1963). HSC transplantation is the only available treatment for hematopoietic regeneration to repair irradiation damage (Weissman 2000). The clinical use of HSC transfer for a number of malignant and nonmalignant conditions is life-saving (Thomas et al. 1957; Goldman et al. 1986; Kersey et al. 1987; Walters et al. 1996; Appelbaum 2007; Dohner et al. 2015; Chabannon et al. 2018).

2.2.2 Mesenchymal Stem Cells

MSCs are non-hematopoietic multipotent stem cells. They reside in virtually every tissue and particularly the BM and adipose tissues. Approximately 40 years ago, Friedenstein and coworkers discovered these cells in mice as BM cells, which were capable of generating bone tissue (Friedenstein et al. 1970; Friedenstein 1976). Up till now, MSCs have been successfully derived from various organs including brain, liver, lung, kidney, muscle, thymus, pancreas, skin, bone marrow adipose tissue, fetal tissues, and umbilical cord (Murphy et al. 2013). Multipotent MSCs have also been differentiated into adipocytes, myocytes, osteocytes, and chondrocytes (Minguell et al. 2001; Zuk et al. 2001; Nombela-Arrieta et al. 2011). MSCs in the human body are known to be mainly involved in immunosuppression, in response to injury and inflammation, and in the stimulation of angiogenesis. These properties make them attractive candidates as vehicles for use in a variety of cancer therapies. Various preclinical trials also indicate the potentials of MSCs in cancer treatment (Gomes et al. 2017).

2.2.3 Neural Stem Cells

NSCs refer to the progenitor cells of central nervous system (CNS). With self-renewal capacity, NSCs have the potential to differentiate into three major types of CNS cells: (i) neurons; (ii) astrocytes; and (iii) oligodendrocytes (Brustle et al. 1997). So far, fetal, neonatal or postnatal tissues have been used as sources for the derivation of NSCs. Over the past 20 years, the potential of NSCs has been investigated for treating neurodegenerative diseases and it has been speculated that the multipotent nature of NSCs may contribute to formulating a novel cell replacement platform for such diseases. Various attributes of NSCs, similar to MSCs, make them a suitable candidate as vehicles for various cancer therapies. In recent years, both *in vitro* and *in vivo* studies have shown a unique migratory capacity of NSCs throughout the brain (Aboody et al. 2000; Benedetti et al. 2000).

2.3 Induced Pluripotent Stem Cells

iPSCs are an engineered type of ESC-like stem cells which are generated by reprogramming differentiated somatic cells. In 2006, Takahashi and Yamanaka conveyed a groundbreaking discovery to the scientific world that transduction of mouse fibroblasts with Oct 3/4, Sox2, Klf4, and c-Myc could reprogram terminally differentiated somatic cells into a pluripotent state, similar to ESCs (Takahashi and Yamanaka 2006). They coined the term induced pluripotent stem cells to designate this new type of cells. Subsequently, Takahashi and Yu reported the generation of human iPSCs in 2007 (Takahashi et al. 2007; Yu et al. 2007).

The discovery of iPSCs established the fact that the fate of a cell could be transformed. Furthermore, it opened up a new avenue for cell therapy with the possibility of creating cell banks from a patient's skin for autologous transplantation. Since patient-derived iPSCs can evade ethical concerns and government restrictions surrounding the use of embryonic stem cells, an enormous focus on iPSC technology has prevailed rapid progress in recent years. In addition, due to the possibility of autologous use, iPSC-based therapy may avert the risk of graft-versus-host disease (GVHD), often observed in allogeneic stem cell transplants where the stem

cells are collected from either a matched related or unrelated donor (Singh et al. 2016). So far, iPSCs have been generated from a variety of different cell types, indicating that the majority of somatic cells can be reprogrammed to pluripotency (Raab et al. 2011).

3 Therapeutic Application of Stem Cells in Cancer Treatment

Unique attributes of stem cells make them potential biologics in cancer treatment. Indeed, stem cells harnessed from various sources have been used in multiple stages of cancer treatment both in the laboratory and the clinic. While the principle of using stem cells and the functional significances thereof in cancer treatment is many-sided, the *modi operandi* can be conceptually divided into two major categories: (a) as a vehicle for various anti-cancer therapeutics and (b) as a regenerative regimen to promote regeneration of hematopoietic cells during cancer treatment.

3.1 Application of Stem Cells as Carriers of Therapeutic Payload

Stem cells exhibit inherent tropism towards neoplastic lesions– a property which makes these cells ideal carriers for gene- and virotherapy treatments designed for solid cancers. Extensive research has been conducted on this front and stem cells have emerged as a prominent cellular vehicle for anticancer treatment. In this context, stem cells have been exploited to deliver enzymes for enzyme/prodrug gene therapy (EPGT), immunostimulatory genes for immunotherapy, oncolytic viruses (OVs) and stem cell-derived extracellular vesicles (Evs) loaded with therapeutic molecules. Stem cells function as efficient carriers of these types of payloads and owing to their tumor-homing ability the stem cells are adept at delivering therapeutics specifically into tumor tissue.

3.1.1 Mechanisms for the Tumor Tropism of Stem Cells

The tumor-tropic nature of stem cells originates from their inherent regenerative and reparative roles. To bring about cellular homeostasis following a pathological condition, stem cells must sense certain signals from the pathological tissues. Such signaling patterns resemble various pro-tumorigenic signals such as angiogenesis, hypoxia, inflammatory signals etc. As a result, stem cells become naturally equipped with cellular machineries to respond to the various factors abundant in the tumor tissues and subsequently colonize the solid tumor bed. Stem cell factor-1, monocyte chemoattractant protein-1 and stromal cell-derived factor-1, secreted by the tumor cells (and/or the stromal cells), have been identified to be important in this context (Erlandsson et al. 2004; Imitola et al. 2004; Widera et al. 2004). Various growth factors have also been shown to be central mediators in this process. For example, hepatocyte growth factor (HGF) (Kendall et al. 2008), vascular endothelial growth factor (VEGF) (Schmidt et al. 2005) and epidermal growth factor (EGF) (Boockvar et al. 2003) have been reported to be potent chemoattractants for stem cells. Pro-inflammatory cytokines (such as TNF-α and IL-1β) induced by tumor-associated immune cells also play crucial roles in the migration and differentiation of stem cells within the tumor (Uchibori et al. 2013; Sullivan et al. 2014).

3.1.2 Stem Cells as Carriers in Gene Therapy

EPGT, also known as suicide gene therapy or gene-directed enzyme prodrug therapy, is one of the most prominent gene therapies for cancer treatment (Malekshah et al. 2016; Hossain et al. 2019a, b, 2020a, b). EPGT can be conceptualized as a two-step system where the first step involves the delivery of a foreign gene (called 'suicide gene') into the tumor cells or in the tumor tissue and the second step is associated with administration of an innocuous prodrug that is catalyzed into a toxic drug by the action of the delivered foreign gene. The resultant toxic drug induces tumor cell

death and results in tumor elimination. Stem cells can be used as a vehicle for the suicide gene (Fig. 2). Initially, viral vectors were developed for delivering the suicide gene into the tumor cells which then undergo cell death due to the accumulation of the toxic drug following the action of the suicide gene. The stem cells, however, cannot deliver the suicide gene directly into the tumor cells and the functionality of stem cell-directed EPGT depends on a different mechanism which is known as 'bystander effect'. Bystander effect is a phenomenon whereby the metabolized prodrug can disperse from a donor cell (i.e. the stem cells) to the recipient cells (i.e. the tumor cells) and exerts cytotoxicity in the recipient cells even though the tumor cells themselves are devoid of the suicide gene *per se* (Hossain et al. 2020a, b). The transfer of metabolized prodrug can occur by both cell-contact-dependent (such as via gap junctions) and independent (such as via EVs) mechanisms (Mooney et al. 2017). Bystander effect is a hallmark of EPGT and plays a pivotal role in the success of EPGT regardless of the vehicular choice. In the stem cell-mediated EPGT system, bystander effect represents the sole mechanism behind tumor cell death. There exist various types of EPGT systems (with different combinations of suicide genes and prodrugs) and each system may rely on different mechanisms of bystander effect (Hossain et al. 2020a, b). However, the most prominent mechanisms have been reported to be either direct diffusion through gap junctions or transfer through soluble extracellular vehicles.

Aboody et al. first reported the potential use of stem cells as a vector for EPGT (Aboody et al. 2000). The authors used a multipotent and immortalized neural progenitor cell line (C17.2) which had been derived from the external germinal layer of neonatal mouse cerebellum. The authors further engineered the C17.2 cell line by introducing the bacterial cytosine deaminase (bCD) gene in the genome of C17.2. bCD is one of the most widely used suicide genes. The bCD protein deaminates 5-fluorocytosine into 5-fluorouracil (5FU) which is a pyrimidine analog and can therefore inhibit nucleic acid synthesis causing cell cycle arrest and apoptosis. By engrafting the engineered C17.2 cells in the brain of mice with glioma-burden, the authors showed that NSCs could colonize the experimental tumor bed and killed tumor cells (Aboody et al. 2000). Interestingly the NSCs were able to migrate in a tumor-specific manner, both ipsilaterally and contralaterally in the mouse brain. Human NSCs also show similar migratory capacity. HB1.F3 is a widely used immortalized human NSC line which was originally derived from human fetal brain and has been utilized by independent research groups to deliver the suicide gene bCD for the treatment of medulloblastoma (Kim et al. 2006; Shimato et al. 2007), glioblastoma (Lee et al. 2014), brain metastases of lung cancer (Wang et al. 2012) and breast cancer (Joo et al. 2009). The functionality of NSCs in this context was further improved by introducing additional suicide genes. Wang et al. engineered a human fetal brain-derived NSC line with a suicide gene known as herpes simplex virus thymidine kinase gene (HSV-TK) in addition to bCD (Wang et al. 2012). HSV-TK is, so far, the most widely used suicide gene which converts its prodrug ganciclovir or acyclovir into toxic phosphorylated forms that promote apoptosis in cancer cells (Hossain et al. 2019a, b). The resultant dual-suicide gene carrying NSC line resulted in superior therapeutic efficacy compared to only bCD-carrying NSCs in brain tumor metastasis models.

As aforementioned, stem cell-mediated EPGT solely depends on the bystander effect and thus a higher bystander effect can further improve the therapeutic efficacy. To enhance the bystander effect, various strategies have been developed including the generation of a secretory suicide gene. The prime example is the recombinant human carboxylesterase (hCE1m6) enzyme that can catalyze the prodrug CPT-11 (irinotecan) to a potent topoisomerase I inhibitor and kill the tumor cells (Metz et al. 2013). While the bCD killing radius has been estimated to be around 25 μm, the secretable hCE1m6 can attain two-fold greater killing radius (Barish et al. 2017; Mooney et al. 2017).

Apart from NSCs, MSCs have also been shown to be an efficient vehicle for EPGT.

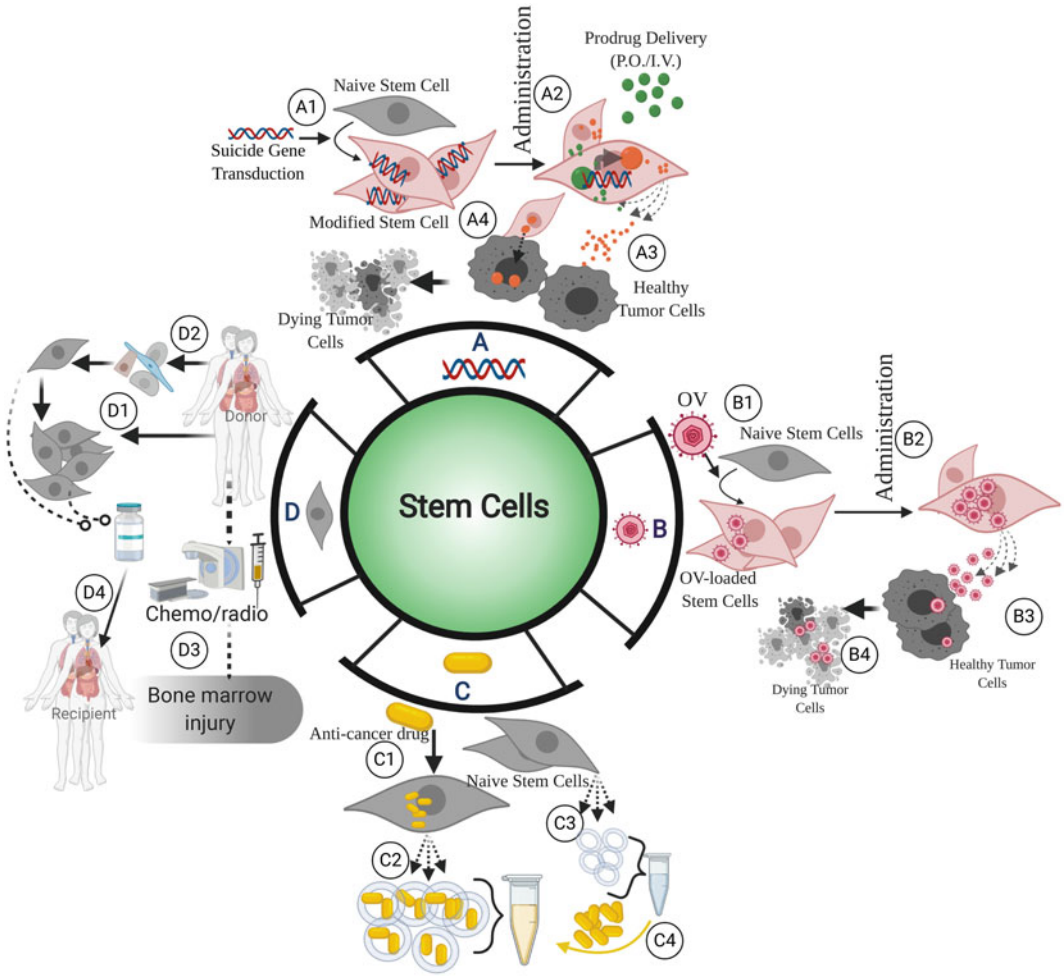


Fig. 2 The figure depicts the major *modi operandi* of stem cells in cancer treatment. Section A-C involves the mechanism of stem cell-based anti-cancer therapy and section D portrays the simplified principle and mechanism of using stem cells during or after chemo-radiotherapy. (A1-A2) Naïve stem cells collected from various sources are modified to express the 'suicide gene' which is then administered to the patients either locally or systemically. The administered 'armed stem cells' subsequently enter the tumor bed, metabolize the non-toxic prodrug (green), which is administered I.V. or P.O., into toxic products (orange). The metabolized toxic drug then enters the tumor cells by either soluble metabolites (A3) or direct cell-to-cell contact (A4). The transfer of this metabolite results in tumor cell death in the final stage. (B1) Oncolytic viruses are loaded in the stem cells which are then administered to the patients (B2). OV-armed stem cells subsequently migrate to the tumor bed, amplify the viral replication and thus maximize infection of tumor cells (B3). Ideally, oncolysis of tumor cells also releases more viruses in the tumor bed. Section C depicts the use of stem cell-derived EVs in cancer treatments. Various types of anti-cancer agents can be loaded into stem cell-derived EVs. However, the figure illustrates only the anti-cancer drug (such as paclitaxel). In the endogenous loading strategy (C1), drug-treated stem cells secrete EVs loaded with the drug which are then collected and purified. The exogenous method (C3) involves harnessing of EVs from naïve stem cells which are then loaded with the drug by various strategies such as electroporation, saponin, dialysis and extrusion (C4)

A different kind of role that is played by stem cells in cancer treatment is regeneration of the hematopoietic system following chemo-radiotherapeutic regimen. In this context, stem cells from bone marrow are first isolated from the patients (donor) prior to the cytotoxic regimen (D1). Although still experimental, this system can be substituted by collecting a wide variety of differentiated cells which can then be reprogrammed to iPSCs (D2). Following cytotoxic regimen which leads to severe loss of hematopoietic cells (D3), the patients (recipient) then are infused with the stem cells (D4) which then home to bone marrow and promote recovery of blood-forming cells. The recipients can be the same patient as donor (autologous transplantation) or different (allogenic transplantation)

Miletic et al. first reported use of MSC-based EPGT system for the treatment of glioblastoma (Miletic et al. 2007). The MSCs were derived from murine bone marrow and engineered with HSV-TK. Upon administration of the prodrug ganciclovir, HSV-TK-mediated catalysis yielded the toxic drug which was transferred to the neighboring tumor cells by gap junctions (Miletic et al. 2007; Matuskova et al. 2010). Recently it has been shown that the mRNA of HSV-K can also be dispersed via exosomes and results in killing of neighboring tumor cells (Pastorakova et al. 2020). The efficacy of MSC-based EPGT has been independently reported by several research groups with both HSV-TK and bCD for the treatment of different cancer types (Chang et al. 2010; Altaner et al. 2014; Zhang et al. 2014). MSCs obtained from sources other than bone marrow, such as from adipose tissue, have also been exploited successfully in EPGT systems (Matuskova et al. 2010).

3.1.3 Delivery of OVs by Stem Cells

OVs have recently emerged as potential therapeutic modality (Harrington et al. 2019). The *modus operandi* of OVs involves selective (or preferred) viral replication in tumor cells and consequent oncolysis. A variety of OVs has been developed over the last two decades targeting multiple cancer types. The most widely used method of OV-treatment is to inject naked OVs intratumorally or systemically. However, this methodology poses a few challenges such as (I) recognition of the OVs by the host immune system leading to clearance of the viral particles (II) limited spreading potential as viral distribution in the tumor tissue is a passive process (III) insufficient viral load and (IV) hepatic sequestration of OVs during systemic administration. Adopting a 'Trojan Horse' approach, by using cell-based carriers, can mend these shortcomings. In this approach, the cellular vehicle is first loaded (mostly infected however surface attachment is also an alternative) with the OVs before implantation or systemic injection of the vector cells. The cellular vehicle then not only functions as a carrier but also as a factory ceaselessly producing OVs for a certain period of time (Fig. 2).

The use of virus-carrying cells was historically adopted when fibroblasts were used to deliver retroviral vectors for brain tumor gene therapy as it showed greater efficacy than the direct injection of the retroviral vectors (Short et al. 1990). Likewise, human teratocarcinoma cells were also used to deliver OVs (Coukos et al. 1999). However, the use of fibroblasts or cancer cells is not optimal as these cells do not necessarily offer tumor-selective migration. In addition, administration of tumor cells may not be appropriate from a clinical perspective. On the other hand, stem cells, owing to their beneficial capabilities as mentioned in the previous sections, offer to be the best cell-based carriers available right now. Herrlinger et al. first reported the use of murine NSC C17.2 to deliver a conditionally replicating OV (Herrlinger et al. 2000). By using a rRp450-mutant herpes simplex virus type 1 (HSV-1), the authors showed that this strategy resulted in better penetration compared to direct injection. Similarly, MSCs were also shown to be capable of delivering conditionally replicating adenoviruses to glioma cells (Sonabend et al. 2008) and resulted in better therapeutic efficiency in breast and lung tumor cells (Hakkarainen et al. 2007). A number of different stem cell-based OVs have been developed for the treatment of different cancer types over the past two decades. Some notable examples are MSC-based delivery of Newcastle disease virus (Kazimirsky et al. 2016) and adipose-derived stem cell-based delivery of Myxoma virus (Josiah et al. 2010) for glioblastoma treatment, NSC-based delivery of chimeric Orthopoxvirus for the treatment of metastatic ovarian cancers (Hammad et al. 2020) and MSC-based delivery of HSV-1 virus for the treatment of brain metastatic melanomas (Du et al. 2017) among others.

3.1.4 Delivery of Therapeutic EVs by Stem Cells

Extracellular vesicles (EVs) including apoptotic bodies, microvesicles and exosomes, are a group of heterogeneous membrane lipid vesicles that are released by many cell types including stem cells, in an evolutionarily conserved manner from prokaryotes to eukaryotes (Chatterjee et al.

1967; Beveridge 1999; Ellis et al. 2010). A few decades back, EVs were considered as inert cellular debris but now they are known to be an important means of intercellular communications (Chargaff et al. 1946; Wolf 1967; Gyorgy et al. 2011). Their intrinsic ability to shuttle different molecules between cells and alter the recipient phenotype has led to an increased amount of studies defining their role in progression as well as using them as vehicles for therapeutic delivery (Valadi et al. 2007; Skog et al. 2008; Alvarez-Erviti et al. 2011).

3.1.4.1 Types of EVs

It is challenging to classify EVs into their respective subtypes due to their overlapping sizes and markers as well as their heterogeneous nature. Nevertheless, based on studies from different researchers, they have been classified by their sizes with exosomes having a size range of 30–150 nm, while microvesicles are 100–1000 nm, and apoptotic bodies are 500–5000 nm in size (Crescitelli et al. 2013; Lunavat et al. 2015). Exosomes are small lipid vesicles derived from multi-vesicular bodies (MVBs) fused with plasma membrane for secretion into extracellular space making them capable of passing through the blood brain barrier (van Niel et al. 2018), which is an important aspect for treating CNS-tumors. Microvesicles, usually referred to as ectosomes or microparticles, are on the other hand often budded into the extracellular space from the plasma membrane (Thery et al. 2009). Apoptotic bodies are considered much larger vesicles that are released as apoptotic blebs in the extracellular milieu when the cells are programmed for apoptosis (Kerr et al. 1972; Elmore 2007). Even though the nomenclature is well-defined for EV subtypes, the detailed molecular machinery for EV biogenesis is still not resolved completely. In the following sections, we will use the ISEV (International Society for Extracellular Vesicles) generic term for EV-related designations (Lotvall et al. 2014; Thery et al. 2018).

3.1.4.2 Stem Cell-Derived EVs in Cancer Treatment

Stem cell-released EVs can contain transcription factors, growth factors, signaling proteins, ECM proteins, metabolic enzymes, DNA and RNA binding proteins, as well as mitochondrial and genomic DNA and RNA subtypes (Mead et al. 2017; Sansone et al. 2017; Sharma 2018; Thery et al. 2018; Toh et al. 2018). While some studies have shown that the stem cell-derived EVs could promote tumor progression *in vitro* and *in vivo* (Zhu et al. 2012; Vallabhaneni et al. 2015), various other studies have reported opposite results showing that stem cell-derived EVs possess potent anti-cancer properties. An early report by Bruno et al. demonstrated that EVs derived from MSCs inhibited progression of established tumors (Bruno et al. 2013). Anti-tumour activity of EVs derived from naïve stem cells have been reported by independent researchers afterwards (Fonsato et al. 2012; Lee et al. 2013; Hao et al. 2019; Lopatina et al. 2019; Brossa et al. 2020; He et al. 2020). Although EVs derived from naïve stem cells can exert anti-cancer effects, tremendous variations are present in different types of stem cells. The need for standardization and cancer-specific customizations has led to tailored stem cells that secrete EVs with desired anti-cancer agents (Moore et al. 2017; Ma et al. 2018; Vakhshiteh et al. 2019; Yokoi et al. 2019). This method is known as endogenous loading techniques where the therapeutic molecule/drug is encapsulated in EVs upon biogenesis. Another method of loading drugs/therapeutic molecules into EVs is by exogenous loading whereby the natural EVs are harnessed and impregnated with desired anti-cancer agents by using various biophysical techniques.

3.1.4.3 Nucleic Acid and Protein Loading in EVs Derived from Stem Cells

Nearly 15 years ago, Valadi et al. first showed the presence of miRNAs and mRNAs in exosomes which could be shuttled between cells thus altering the phenotype (Valadi et al. 2007). Based on this

study, there has been a huge development within the EV community and further challenges to engineer the EVs with RNA/DNA loading capacity.

As an example, Munoz and colleagues reported endogenous loading of nucleic acids in MSCs (Munoz et al. 2013). They transfected MSCs with anti-miR-9-Cy5 oligonucleotide and harnessed the microvesicles which could enter into glioblastoma cells rendering them more sensitive to chemotherapy. In line, MSCs engineered with miR-146b secreted exosomes that could inhibit the growth of primary brain tumors in xenograft models (Katakowski et al. 2013).

Another mechanism to enrich stem cell-derived EVs is to alter the endogenous RNA sorting machinery. There is evidence that the RNA profile of EVs depends solely on the parental cell types (Mateescu et al. 2017). These interpretations could possibly suggest that distinct RNAs can be sorted in EVs by a novel mechanism to deliver specific therapeutic RNAs by applying genetic engineering. Until now, there have been several reported RNA sorting mechanisms including the RISC (RNA induced silencing complex), miRNA-mRNA ratio, the ceramide pathways, ribonucleoprotein interaction with specific RNA sorting sequence motif and non-template terminal nucleotide additions (Yanez-Mo et al. 2015; Vasconcelos et al. 2019). Endogenous loading of proteins into stem cell-derived EVs has also been pursued successfully. Yuan Z and colleagues showed that EVs isolated from MSCs overexpressing TRAIL induced apoptosis in several cancer cell lines in a dose-dependent manner (Yuan et al. 2017). Although the utilization of recombinant TRAIL (rTRAIL) in clinical trials have failed to demonstrate increased therapeutic efficacy (Micheau et al. 2013), this strategy provides an important proof-of-principle and encourages development of similar strategies.

Exogenous loading of RNA has also been studied in stem cell-derived EVs. Electroporation is a widely used method for this purpose. Electroporation of LNA (locked nucleic acid)-modified anti-miR-142-3p oligonucleotides into MSC-derived EVs were shown to suppress breast cancer cells' growth in vitro and in vivo (Naseri et al. 2018).

3.1.4.4 Drug Loading in Stem Cell-Derived EVs

Similar to nucleic acids and proteins, particular anti-cancer drugs can also be loaded into EVs to treat cancer (Fig. 2). Endogenous loading of EVs obtained from MSCs treated with paclitaxel (PTX) have been shown to reduce breast cancer growth *in vivo* (Kalimuthu et al. 2018). In line, PTX-loaded EVs were highly potent in pancreatic cancer cells suggesting that MSCs can be used as loading factories for producing EV therapeutics agents (Pascucci et al. 2014). Similarly, another anti-cancer drug, taxol, was delivered by using exosomes obtained from MSCs which were pre-incubated with the drug. The taxol-loaded exosomes could inhibit the growth of a number of cancer cells such as lung, ovarian and breast cancer (Melzer et al. 2019). Moreover, these exosomes significantly reduced the extent of organ metastases observed in lung, liver, spleen, and kidney by 50% (Melzer et al. 2019).

Fuhrmann G and colleagues demonstrated the use of EVs from different cell sources (endothelial cancer and stem cells) for exogenous loading of porphyrins (Fuhrmann et al. 2015). EVs were encapsulated with passive and active methods such as electroporation, saponin, dialysis and extrusion. Passive loading of hydrophobic compounds in EVs significantly enhanced the cellular uptake and photodynamic effect of porphyrins *in vitro* when compared with free or liposome-encapsulated drug. On the other hand, active loading of EVs with the saponin-assisted method in particular allowed over 11 fold drug loading of hydrophilic porphyrins, showing induced stronger phototoxic effect than free drug in a cancer cell model (Fuhrmann et al. 2015). However, the major drawback of exogenous loading is that only a small amount of drugs/agents can be loaded and have less efficiency and larger inconsistency compared to other studies (Sutaria et al. 2017; Antimisiaris et al. 2018).

3.1.5 Delivery Route

For any biologics, delivery route is an important issue. For stem cell-based anti-cancer treatment, no single delivery route is perfect. In fact, the choice of delivery route will largely depend on the tumor type (and as a corollary, location) and

also the nature of the payload being delivered. The two most common delivery routes are local administration and systemic administration. While a systemic approach is suitable for multiple administrations and non-invasive, the efficacy of this method is likely to be limited for many solid tumors. For example, systemically delivered NSCs have been shown to be able to home into intracerebral glioma cells, albeit with lower efficiency compared to locally administered NSCs. (Aboody et al. 2000). Thus, local administration in this context may provide enhanced potency but might be restricted to a single administration. One open question pertinent to the local administration is whether to perform it intratumorally or into the resection cavity. The answer, however, may depend on specific tumor types. An emerging delivery route for the treatment of CNS-tumors is the intranasal delivery system (Balyasnikova et al. 2014; Li et al. 2015).

3.1.6 Clinical Scenarios of Stem Cell-Based Anti-cancer Treatment Regimens

Promising preclinical results have spurred the process of clinical translation of stem cell-mediated anti-cancer therapeutics. At least four clinical trials have been undertaken so far among which one was suspended due to non-clinical reasons (NCT02192359). The first-in-human trial conducted involved local administration of bCD-carrying NSCs for the treatment of glioblastoma (NCT01172964). This pilot study, conducted in 15 patients, showed the feasibility of using stem cells for EPGT without any major side effects and the NSCs were able to migrate to distant tumor foci (Portnow et al. 2017). The successful completion of this trial warranted further clinical studies and a phase I trial with 18 patients was started in 2017 (NCT02015819). The first-in-human clinical trial involving stem cell-mediated delivery of OVs was started in 2017 for the treatment of glioblastoma (NCT03072134). This study has been completed, however the results are yet to be published. No clinical trial has so far been pursued to test the efficacy of stem cell-derived EVs for cancer therapy, although few clinical trials have been undertaken or already completed for other types of diseases such as acute ischemic stroke, periodontitis, and treatment of acute respiratory distress syndrome (ARDS) (e.g. NCT04356300, NCT04270006 etc.).

3.1.7 Challenges of Using Stem Cells as Delivery Vehicles for Therapeutic Payloads

3.1.7.1 Longevity

A major drawback of using stem cells as vectors for therapeutic payloads is the poor post-transplantation survival of the genetically modified stem cells— possibly accounting for about 90% atrocity in the first few days (Mooney et al. 2018a, b). This issue has prompted researchers to utilize additional means to enhance survival of the stem cells. Kauer et al. reported the use of a synthetic extracellular matrix to extend the survival of stem cells after implantation in vivo (Kauer et al. 2011). Mooney et al. in this context took a different approach and exploited the functionality of antiapoptotic protein bcl2 (Mooney et al. 2018a, b). The authors showed that transient overexpression of Bcl-2 by the stem cells increases their retention following transplantation and results in improved therapeutic efficacy in a glioblastoma model. This modification can result in the survival of stem cells up to a few weeks. However, it is not clear if this time period would be sufficient for particular treatments. For example, in the context of EPGT, short-term suicide gene activity is a limiting factor for successful treatment of glioblastoma (Hossain et al. 2019a, b, 2020a, b).

3.1.7.2 Immunosuppression

Another common challenge of stem cell-based delivery system is the immunosuppressive nature of the stem cells. The immunosuppressive nature of stem cells actually serves as a double-edged sword. Minimal or low immunogenicity, reflected by low or undetectable MHC expression, enables the stem cells to evade the immune system in the tumor bed of the allogenic host for sufficient period of time (Aboody et al. 2008; Hossain et al. 2020a, b). This property indeed makes the

stem cells ideal carriers in the first place. However the immunosuppressive signature of these cells can harm overall therapy as the activation of an anti-tumor immune response is important for a successful anti-cancer therapy in most, if not all, cancers (Hossain et al. 2020a, b).

3.1.7.3 Safety Issues

Arguably, the biggest safety concern is the possibility of secondary tumorigenicity. The stem cells can theoretically transform and create neoplastic lesions. However this phenomenon has not been observed in preclinical models with NSCs (Mooney et al. 2017) and in line the first-in-human trial also showed that the NSCs were non-tumorigenic (Portnow et al. 2017; Mooney et al. 2018a, b). However, from a theoretical perspective, different types of stem cells in different tumor niches can behave differently and thus the possibility of secondary tumorigenesis cannot be excluded completely. Nevertheless, safety can be ensured by introducing a suicide gene into the stem cells (for EPGT systems which will mandatorily be present) for the delivery of OVs or EVs which can be subject to elimination by prodrug administration in case the issue of secondary tumorigenesis practically arises.

3.1.7.4 Compatibility

When it comes to the delivery of OVs, two critical challenges emerge. The first one is the amenability of the stem cells towards viral loading. The loading of the OVs will depend on the expression of the relevant receptors on the stem cell surface and this stipulation may be a rate-limiting step. For instance, the expression of coxsackievirus–adenovirus receptor (CAR), which is one of the most important receptors for most serotypes of adenoviruses is relatively low on MSCs and thus the serotype 5 adenovirus loading on MSCs is challenging (Hakkarainen et al. 2007). While the use of high viral doses can circumvent this problem to some extent, a more sophisticated solution is to engineer the viral tropism. Redirecting the viral entry mechanisms has been extensively studied for oncolytic adenoviruses and various alternatives such as CAR-independent entry, αvβ integrin- or HSPG-targeted entry have been successfully adopted (Hakkarainen et al. 2007; Hammer et al. 2015; Kim et al. 2015). The second challenge encountered in stem cell-mediated delivery of OVs presents a *catch-22* situation. While the OVs must be efficient self-replicators and potent killers for proper functionality, it is simultaneously imperative to safeguard the stem cells from the OVs to prevent self-lysis for at least a sufficient period of time. One way to deal with this problem is to temporarily rein in cell division of the stem cells. Since a majority of OVs rely on regular proliferation of the tumor cells to selectively replicate in those cells, temporary and reversible suppression of proliferation in the stem cells can enable them to avoid self-atrocity and after a certain period of implantation when the stem cells are ready to deliver the OVs this restriction can be taken off (Herrlinger et al. 2000). Another way of circumventing this issue is to use tumor-specific promoter (such as survivin) so that the OVs replicate more efficiently after delivery into the tumor cells (Ahmed et al. 2011; Mooney et al. 2018a, b) or using antioxidant drugs (Kim et al. 2013).

4 Application of Stem Cells in Regenerative Medicine

Regenerative medicine appears to be one of the emerging branches of therapeutics, that develops methods for the functional restoration of a specific tissue and/or organ of the patients suffering from acute injuries or chronic disease conditions, in the context where patients' own regenerative responses do not avail (Mason et al. 2008). Therefore, access to unlimited numbers of specific cell types has been a long-standing aspiration in regenerative medicine. Stem cells' self-renewal capacity and a great potential to differentiate into other types of cells render them as frontiers of regenerative medicine. Successful differentiation of embryonic or adult stem cells into functional progeny followed by their transplantation or *in vivo* stimulation of endogenous adult stem cells have led to the development of novel stem cell-based therapeutic approaches for regenerative medicine.

Metastatic patients often receive very high doses of chemotherapy and/or radiotherapy, which eventually results in severe death of blood-forming cells and leukocytes (Copelan 2006). To counteract the loss, the patients are treated with the intravenous infusion of autologous or allogeneic HSCs as regenerative medicine. During the last two decades, the use of HSC transplant has increased globally and evolved technologically. According to WHO, a total of 1,298,897 HSC transplant activities were reported worldwide from 1957 to 2016 (Niederwieser et al. 2016).

4.1 Homing to Bone Marrow

Regeneration of blood-forming cells via HSC transplant depends on the migration of the systemically administered cells into the bone marrow through a distinct process. By this process, called homing, the HSCs rapidly migrate into a specific stem cell niche in the BM to form the desired blood cells. HSC homing process mainly depends on the active interaction between the chemokine SDF-1 and its receptor CXCR4 (Lapidot et al. 2005). However, some studies demonstrated that HSC homing to the BM was independent of the SDF-1–CXCR4 interaction (Brenner et al. 2004; Kahn et al. 2004) and in line Adamiak et al. showed that phosphosphingolipid sphingosine-1-phosphate was a more potent chemotactic factor for HSCs than SDF-1 (Adamiak et al. 2015). Other potential factors involved in the homing of HSCs to the BM are extracellular ATP or UTP (Rossi et al. 2007), and Ca2+ and H+ ions (Adams 2006; Okajima 2013). Furthermore, while HSCs migrate through blood vessels, they interact with endothelial cells via LFA-1, VLA-4/5, CD44, and MMP-2/9 (Lapidot et al. 2005).

4.2 *Satus quo* of Stem Cell-Based Regenerative Regimen in Cancer Treatment

4.2.1 HSC-Based Regenerative Regimen

Nowadays, HSC transplantation is primarily used to treat multiple myeloma, leukemia, and lymphomas, followed by high-dose radiotherapy or chemotherapy (Copelan 2006). Sources of HSCs for transplantation include a matched related sibling donor or a matched unrelated volunteer donor. In allogeneic HSC transplant, use of alternative donor has also become optional, which includes mismatched unrelated donor, haploidentical related donor, and umbilical cord blood transplant. In 2009, FDA approved only cord blood cell treatment for HSC transplant under authoritative guidance. However, in the beginning, HSC transplant was very challenging due to the deadly reaction of GVHD. In 1957, Edward D Thomas and his co-workers pioneered the use of first HSC transplant in cancer patient (Thomas et al. 1957). In the study, they treated the patients with infusion of marrow from a normal donor, followed by chemoradiotherapy. Out of these six patients, only two engrafted, and eventually, all died within 100 days of transplantation. Since very little was known about the histocompatibility antigens at that time, there was no matching screening between the donors and the recipients prior to transplantation. Therefore, many researchers abandoned the field after repeated failures. Nevertheless, Edward D Thomas kept his hope in the potential of HSC transplantation. In 1969, he pursued a clinical trial program in Seattle for allogeneic HSC transplantation, where they tested the human leukocyte antigen for matching between patients and donors. In 1977, his group reported 100 transplantations, followed by chemotherapy and radiation therapy in 54 patients with acute myeloid leukemia (AML) and in 46 patients with acute lymphoblastic leukemia (ALL). Although only 13 patients survived without disease 1–4.5 years after HSC transplant, it was the first clinical proof of success in HSC transplant therapy (Thomas et al. 1977). Subsequently, Thomas and his team showed that allogeneic HSC transplant resulted in a cure rate of 50% for AML patients in first remission (Thomas et al. 1979). Currently, HSC transplantation procedure is also intensively studied in clinical trials, together with chemotherapy or immunotherapy, to treat other kinds of cancer, such as brain tumors (NCT00528437), neuroblastoma, sarcomas (NCT01807468), and breast cancer

(NCT00003927). Nevertheless, using allogeneic sources of HSCs is yet challenging due to GVHD occurrence, which is often treated with immunosuppressive drugs with less effectiveness and serious side effects (Casper et al. 2010).

4.2.2 MSC-Based Regenerative Regimen

In addition to HSC-transplantation, other types of stem cells are also used in this context. MSC-based infusion has been found to be beneficial in enhancing the overall outcome of the treatment by supporting HSCs to keep their undifferentiated and proliferative state (Le Blanc and Ringden 2007; Sacchetti et al. 2007; Mendez-Ferrer et al. 2010). Furthermore, MSCs with immunomodulatory effects could effectively reduce strong immune responses in patients with refractory GVHD. MSCs are also found to facilitate the recovery of injured organs and could enable body tolerance to high-dose chemotherapy that is to improve tumor-killing effects (Lee 2011).

4.2.3 iPSC-Based Regenerative Regimen

In the context of regenerative medicine, the advent of iPSCs also opened up a new avenue to treat cancer patients after chemoradiotherapy. Patient-derived healthy iPSCs can theoretically be employed to regenerate tumor- or treatment-injured tissues. Due to the pluripotent nature of iPSCs, almost all tissues can be obtained for the therapeutic applications. Nevertheless, regenerative therapy mediated by human iPSCs needs vigorous *in vivo* engraftment of iPSC-derived tissues. Currently, only a few types of human iPSC-derived cells such as hepatocytes have been successfully engrafted in animal models (Batlle et al. 1963).

4.3 Challenges Associated with Stem Cell-Based Regenerative Regimen

Despite the intensive use of HSC transplantation with improved technology, the major limitation of HSC transplant lies with the high rate of mortality and morbidity. The main reasons for transplant-related mortality are GVHD and infections. Since the discovery of iPSCs, researchers held a great hope that autologous iPSCs would be a likely solution of GVHD complications (Nishikawa et al. 2008; Zhao et al. 2008). However, few recent studies investigated iPSC immunogenicity in autologous settings and raised questions about this assumption. Araki et al. reported that autologous iPSC-derived teratomas were rejected by immune-competent mice indicating that autologous transplantation models can still induce an immune response (Araki et al. 2013). de Almeida also identified the immunogenic properties of the autologous iPSCs (de Almeida et al. 2014), opposing the concept of immune-privileged iPSCs. In the study, they found that undifferentiated autologous iPSCs stimulated an immune response with increased lymphocytic infiltration and elevated granzyme-B, IFN-γ, while autologous iPSC-derived endothelial cells were accepted by immune mechanisms similar to self-tolerance, suggesting that terminal differentiation of iPSCs with high purity is an inherent factor in successful autologous HSC transplantations. Teratoma formation after transplantation also poses as a matter of concern for the therapeutic application, which may also be prevented by reducing the number of undifferentiated cells within the graft (Menard et al. 2005). In that regard, well-known cell surface markers can be a useful tool to sort out the high yield of differentiated cells (Keirstead et al. 2005).

5 Concluding Remarks

Stem cell research has been extensively performed in the last few decades which has not only provided us with fundamental understanding on stem cells' significance in homeostasis and pathology, but also presented unique opportunities of exploiting the stem cells in the context of cancer treatment. While the application of stem cells in this context is multifaceted, two major categories can be defined: as a vector for various anti-cancer therapeutics and as regenerative biologics during or following cytotoxic cancer treatment. The regenerative applications of

stem cells during cytotoxic treatment is currently being used in the clinic mostly for hematologic/lymphoid cancers such as leukemia, multiple myeloma, and lymphomas. Use of stem cells in anti-cancer treatment is currently being tested in two clinical trials. Although both approaches stipulate distinct features for a successful application, there are many common challenges that require further studies for improvement.

One of the biggest questions of using stem cells is whether to use autologous or allogenic source. Autologous sources offer some beneficial features such as avoiding risks of graft-versus-host disease and avoiding premature immune-mediated clearance. However, the benefits are associated with some challenges as well. For example, limited supply and difficulty in standardization create hurdles for clinical use. Perhaps the biggest challenge is the heterogeneic nature of stem cells from different patients which can potentially make it difficult (or even impossible) to tailor the stem cells for the intended purpose. This issue is more pertinent to stem cell-based anti-cancer treatment strategies such as carrying suicide gene or virus or loading with certain anti-cancer therapeutics. Therefore, unfortunately, the question of which source to use is not unequivocally resolved. The allogenic source is, so far, most popular for the anti-cancer use of stem cells. However, both autologous and allogenic sources seem to be used for regenerative application (Henig et al. 2014).

Regardless of the source, another important and common challenge is 'longevity'. To extend the post-transplantation life of stem cells immunosuppressive regimens are widely used. However, various lines of evidence suggest that an immunosuppressive regimen can sabotage the efficacy of total anti-cancer regimen. Thus, more research should be directed in aiming at extending the longevity of the stem cells following transplantation without dampening the immune system—which could result in more efficacious and cost-effective options for the treatment of cancer patients in future.

References

Aboody KS et al (2000) Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. Proc Natl Acad Sci U S A 97(23):12846–12851

Aboody KS et al (2008) Stem and progenitor cell-mediated tumor selective gene therapy. Gene Ther 15 (10):739–752

Adams GB (2006) Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. Nature 439(7076):599–603

Adamiak M et al (2015) Evidence for the involvement of sphingosine-1-phosphate in the homing and engraftment of hematopoietic stem cells to bone marrow. Oncotarget 6(22):18819–18828

Ahmed AU et al (2011) Neural stem cell-based cell carriers enhance therapeutic efficacy of an oncolytic adenovirus in an orthotopic mouse model of human glioblastoma. Mol Ther 19(9):1714–1726

Altaner C et al (2014) Complete regression of glioblastoma by mesenchymal stem cells mediated prodrug gene therapy simulating clinical therapeutic scenario. Int J Cancer 134(6):1458–1465

Alvarez-Erviti L et al (2011) Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol 29(4):341–345

Antimisiaris SG et al (2018) Exosomes and exosome-inspired vesicles for targeted drug delivery. Pharmaceutics 10(4):218

Appelbaum FR (2007) Hematopoietic cell transplantation from unrelated donors for treatment of patients with acute myeloid leukemia in first complete remission. Best Pract Res Clin Haematol 20(1):67–75

Araki R et al (2013) Negligible immunogenicity of terminally differentiated cells derived from or embryonic stem cells. Nature 494(7435):100–104

Balyasnikova IV et al (2014) Intranasal delivery of mesenchymal stem cells significantly extends survival of irradiated mice with experimental brain tumors. Mol Ther 22(1):140–148

Barish ME et al (2017) Human neural stem cell biodistribution and predicted tumor coverage by a diffusible therapeutic in a mouse glioma model. Stem Cells Transl Med 6(6):1522–1532

Batlle E et al (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature 197:452–454

Becker AJ et al (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature 197:452–454

Benedetti S et al (2000) Gene therapy of experimental brain tumors using neural progenitor cells. Nat Med 6 (4):447–450

Beveridge TJ (1999) Structures of gram-negative cell walls and their derived membrane vesicles. J Bacteriol 181(16):4725–4733

Boockvar JA et al (2003) Constitutive EGFR signaling confers a motile phenotype to neural stem cells. Mol Cell Neurosci 24(4):1116–1130

Brenner S et al (2004) CXCR4-transgene expression significantly improves marrow engraftment of cultured hematopoietic stem cells. Stem Cells 22(7):1128–1133

Brossa A et al (2020) Extracellular vesicles from human liver stem cells inhibit renal cancer stem cell-derived tumor growth in vitro and in vivo. Int J Cancer 147 (6):1694–1706

Bruno S et al (2013) Microvesicles derived from human bone marrow mesenchymal stem cells inhibit tumor growth. Stem Cells Dev 22(5):758–771

Brustle O et al (1997) In vitro-generated neural precursors participate in mammalian brain development. Proc Natl Acad Sci U S A 94(26):14809–14814

Casper J et al (2010) Allogeneic hematopoietic stem-cell transplantation in patients with hematologic malignancies after dose-escalated treosulfan/fludarabine conditioning. J Clin Oncol 28(20):3344–3351

Chabannon C et al (2018) Hematopoietic stem cell transplantation in its 60s: a platform for cellular therapies. Sci Transl Med 10(436):eaap9630

Chagastelles PC et al (2011) Biology of stem cells: an overview. Kidney Int Suppl 1(3):63–67

Chang DY et al (2010) The growth of brain tumors can be suppressed by multiple transplantation of mesenchymal stem cells expressing cytosine deaminase. Int J Cancer 127(8):1975–1983

Chargaff E et al (1946) The biological significance of the thromboplastic protein of blood. J Biol Chem 166 (1):189–197

Chatterjee SN et al (1967) Electron microscopic observations on the excretion of cell-wall material by Vibrio cholerae. J Gen Microbiol 49(1):1–11

Copelan EA (2006) Hematopoietic stem-cell transplantation. N Engl J Med 354(17):1813–1826

Coukos G et al (1999) Use of carrier cells to deliver a replication-selective herpes simplex virus-1 mutant for the intraperitoneal therapy of epithelial ovarian cancer. Clin Cancer Res 5(6):1523–1537

Crescitelli R et al (2013) Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. J Extracell Vesicles 2:20677

de Almeida PE et al (2014) Transplanted terminally differentiated induced pluripotent stem cells are accepted by immune mechanisms similar to self-tolerance. Nat Commun 5:3903

Dohner H et al (2015) Acute Myeloid Leukemia. N Engl J Med 373(12):1136–1152

Du W et al (2017) Stem cell-released oncolytic herpes simplex virus has therapeutic efficacy in brain metastatic melanomas. Proc Natl Acad Sci U S A 114(30): E6157–E6165

Edwards RG (2001) IVF and the history of stem cells. Nature 413(6854):349–351

Ellis TN et al (2010) Virulence and immunomodulatory roles of bacterial outer membrane vesicles. Microbiol Mol Biol Rev 74(1):81–94

Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35(4):495–516

Erlandsson A et al (2004) Stem cell factor is a chemoattractant and a survival factor for CNS stem cells. Exp Cell Res 301(2):201–210

Evans MJ et al (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292 (5819):154–156

Fonsato V et al (2012) Human liver stem cell-derived microvesicles inhibit hepatoma growth in SCID mice by delivering antitumor microRNAs. Stem Cells 30 (9):1985–1998

Friedenstein AJ (1976) Precursor cells of mechanocytes. Int Rev Cytol 47:327–359

Friedenstein AJ et al (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3 (4):393–403

Fuhrmann G et al (2015) Active loading into extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins. J Control Release 205:35–44

Goldman JM et al (1986) Bone marrow transplantation for patients with chronic myeloid leukemia. N Engl J Med 314(4):202–207

Gomes JPA et al (2017) Deepening a simple question: can MSCs be used to treat cancer? Anticancer Res 37 (9):4747–4758

Gyorgy B et al (2011) Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci 68(16):2667–2688

Hakkarainen T et al (2007) Human mesenchymal stem cells lack tumor tropism but enhance the antitumor activity of oncolytic adenoviruses in orthotopic lung and breast tumors. Hum Gene Ther 18(7):627–641

Hammad M et al (2020) Neural stem cells improve the delivery of oncolytic chimeric Orthopoxvirus in a metastatic ovarian cancer model. Mol Ther Oncolytics 18:326–334

Hammer K et al (2015) Engineered adenoviruses combine enhanced oncolysis with improved virus production by mesenchymal stromal carrier cells. Int J Cancer 137 (4):978–990

Hao SC et al (2019) hUC-MSCs secreted exosomes inhibit the glioma cell progression through PTENP1/miR-10a-5p/PTEN pathway. Eur Rev Med Pharmacol Sci 23(22):10013–10023

Harrington K et al (2019) Optimizing oncolytic virotherapy in cancer treatment. Nat Rev Drug Discov 18(9):689–706

He Z et al (2020) Human umbilical cord mesenchymal stem cells-derived exosomes deliver microRNA-375 to downregulate ENAH and thus retard esophageal squamous cell carcinoma progression. J Exp Clin Cancer Res 39(1):140

Henig I et al (2014) Hematopoietic stem cell transplantation-50 years of evolution and future perspectives. Rambam Maimonides Med J 5(4):e0028

Herrlinger U et al (2000) Neural precursor cells for delivery of replication-conditional HSV-1 vectors to intracerebral gliomas. Mol Ther 1(4):347–357

Hossain JA et al (2019a) Long-term treatment with valganciclovir improves lentiviral suicide gene therapy of glioblastoma. Neuro-Oncology 21(7):890–900
Hossain JA et al (2019b) Cancer suicide gene therapy with TK.007. Methods Mol Biol 1895:11–26
Hossain JA et al (2020a) Suicide gene therapy for the treatment of high-grade glioma: past lessons, present trends, and future prospects. Neurooncol Adv 2(1): vdaa013
Hossain JA et al (2020b) Letter regarding "Extensive brainstem infiltration, not mass effect, is a common feature of end-stage cerebral glioblastomas". Neuro-Oncology 22(12):1882–1883
Imitola J et al (2004) Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. Proc Natl Acad Sci U S A 101(52):18117–18122
Joo KM et al (2009) Human neural stem cells can target and deliver therapeutic genes to breast cancer brain metastases. Mol Ther 17(3):570–575
Josiah DT et al (2010) Adipose-derived stem cells as therapeutic delivery vehicles of an oncolytic virus for glioblastoma. Mol Ther 18(2):377–385
Kahn J et al (2004) Overexpression of CXCR4 on human CD34+ progenitors increases their proliferation, migration, and NOD/SCID repopulation. Blood 103 (8):2942–2949
Kalimuthu S et al (2018) A new approach for loading anticancer drugs into mesenchymal stem cell-derived exosome mimetics for cancer therapy. Front Pharmacol 9:1116
Katakowski M et al (2013) Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. Cancer Lett 335(1):201–204
Kauer TM et al (2011) Encapsulated therapeutic stem cells implanted in the tumor resection cavity induce cell death in gliomas. Nat Neurosci 15(2):197–204
Kazimirsky G et al (2016) Mesenchymal stem cells enhance the oncolytic effect of Newcastle disease virus in glioma cells and glioma stem cells via the secretion of TRAIL. Stem Cell Res Ther 7(1):149
Keirstead HS et al (2005) Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J Neurosci 25(19):4694–4705
Kendall SE et al (2008) Neural stem cell targeting of glioma is dependent on phosphoinositide 3-kinase signaling. Stem Cells 26(6):1575–1586
Kerr JF et al (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26(4):239–257
Kersey JH et al (1987) Comparison of autologous and allogeneic bone marrow transplantation for treatment of high-risk refractory acute lymphoblastic leukemia. N Engl J Med 317(8):461–467
Kim SK et al (2006) Human neural stem cells target experimental intracranial medulloblastoma and deliver a therapeutic gene leading to tumor regression. Clin Cancer Res 12(18):5550–5556
Kim CK et al (2013) N-acetylcysteine amide augments the therapeutic effect of neural stem cell-based antiglioma oncolytic virotherapy. Mol Ther 21(11):2063–2073
Kim J et al (2015) Stem cell-based cell carrier for targeted oncolytic Virotherapy: translational opportunity and open questions. Viruses 7(12):6200–6217
Lapidot T et al (2005) How do stem cells find their way home? Blood 106(6):1901–1910
Le Blanc K, Ringden O (2007) Immunomodulation by mesenchymal stem cells and clinical experience. J Intern Med 262(5):509–525
Lee RH (2011) Therapeutic factors secreted by mesenchymal stromal cells and tissue repair. J Cell Biochem 112 (11):3073–3078
Lee JK et al (2013) Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. PLoS One 8 (12):e84256
Lee JY et al (2014) Double suicide gene therapy using human neural stem cells against glioblastoma: double safety measures. J Neuro-Oncol 116(1):49–57
Li YH et al (2015) Intranasal delivery of stem cells as therapy for central nervous system disease. Exp Mol Pathol 98(2):145–151
Lin HT et al (2013) Stem cell therapy: an exercise in patience and prudence. Philos Trans R Soc Lond Ser B Biol Sci 368(1609):20110334
Lopatina T et al (2019) Extracellular vesicles from human liver stem cells inhibit tumor angiogenesis. Int J Cancer 144(2):322–333
Lotvall J et al (2014) Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J Extracell Vesicles 3:26913
Lunavat TR et al (2015) Small RNA deep sequencing discriminates subsets of extracellular vesicles released by melanoma cells–evidence of unique microRNA cargos. RNA Biol 12(8):810–823
Ma T et al (2018) MicroRNA-132, delivered by mesenchymal stem cell-derived exosomes, promote angiogenesis in myocardial infarction. Stem Cells Int 2018:3290372
Maeda H et al (2018) Analyses of repeated failures in cancer therapy for solid tumors: poor tumor-selective drug delivery, low therapeutic efficacy and unsustainable costs. Clin Transl Med 7(1):11
Malekshah OM et al (2016) Enzyme/prodrug systems for cancer gene therapy. Curr Pharmacol Rep 2 (6):299–308
Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78(12):7634–7638
Mason C et al (2008) A brief definition of regenerative medicine. Regen Med 3(1):1–5
Mateescu B et al (2017) Obstacles and opportunities in the functional analysis of extracellular vesicle RNA – an

ISEV position paper. J Extracell Vesicles 6 (1):1286095
Matuskova M et al (2010) HSV-tk expressing mesenchymal stem cells exert bystander effect on human glioblastoma cells. Cancer Lett 290(1):58–67
Mead B et al (2017) Bone marrow-derived mesenchymal stem cells-derived exosomes promote survival of retinal ganglion cells through miRNA-dependent mechanisms. Stem Cells Transl Med 6(4):1273–1285
Melzer C et al (2019) Taxol-loaded MSC-derived exosomes provide a therapeutic vehicle to target metastatic breast cancer and other carcinoma cells. Cancers (Basel) 11(6):798
Menard C et al (2005) Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium: a preclinical study. Lancet 366 (9490):1005–1012
Mendez-Ferrer S et al (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466(7308):829–834
Metz MZ et al (2013) Neural stem cell-mediated delivery of irinotecan-activating carboxylesterases to glioma: implications for clinical use. Stem Cells Transl Med 2 (12):983–992
Micheau O et al (2013) Death receptors as targets in cancer. Br J Pharmacol 169(8):1723–1744
Miletic H et al (2007) Bystander killing of malignant glioma by bone marrow-derived tumor-infiltrating progenitor cells expressing a suicide gene. Mol Ther 15 (7):1373–1381
Minguell JJ et al (2001) Mesenchymal stem cells. Exp Biol Med (Maywood) 226(6):507–520
Mooney R et al (2017) Cell-mediated enzyme prodrug cancer therapies. Adv Drug Deliv Rev 118:35–51
Mooney R et al (2018a) Concise review: neural stem cell-mediated targeted cancer therapies. Stem Cells Transl Med 7(10):740–747
Mooney R et al (2018b) Bcl-2 overexpression improves survival and efficacy of neural stem cell-mediated enzyme prodrug therapy. Stem Cells Int 2018:7047496
Moore C et al (2017) The emerging role of exosome and microvesicle- (EMV-) based cancer therapeutics and immunotherapy. Int J Cancer 141(3):428–436
Munoz JL et al (2013) Delivery of functional anti-miR-9 by mesenchymal stem cell-derived exosomes to glioblastoma multiforme cells conferred chemosensitivity. Mol Ther Nucleic Acids 2:e126
Murphy MB et al (2013) Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. Exp Mol Med 45:e54
Naseri Z et al (2018) Exosome-mediated delivery of functionally active miRNA-142-3p inhibitor reduces tumorigenicity of breast cancer in vitro and in vivo. Int J Nanomedicine 13:7727–7747
Nishikawa S et al (2008) The promise of human induced pluripotent stem cells for research and therapy. Nat Rev Mol Cell Biol 9(9):725–729
Niederwieser D et al (2016) Hematopoietic stem cell transplantation activity worldwide in 2012 and a SWOT analysis of the Worldwide Network for Blood and Marrow Transplantation Group including the global survey. Bone Marrow Transplant 51(6):778–785
Nombela-Arrieta C et al (2011) The elusive nature and function of mesenchymal stem cells. Nat Rev Mol Cell Biol 12(2):126–131
Okajima F (2013) Regulation of inflammation by extracellular acidification and proton-sensing GPCRs. Cell Signal 25(11):2263–2271
Pascucci L et al (2014) Paclitaxel is incorporated by mesenchymal stromal cells and released in exosomes that inhibit in vitro tumor growth: a new approach for drug delivery. J Control Release 192:262–270
Pastorakova A et al (2020) Suicide gene therapy mediated with exosomes produced by mesenchymal stem/stromal cells stably transduced with HSV thymidine kinase. Cancers (Basel) 12(5):1096
Portnow J et al (2017) Neural stem cell-based anticancer gene therapy: a first-in-human study in recurrent high-grade glioma patients. Clin Cancer Res 23 (12):2951–2960
Raab S et al (2011) A Comparative View on Human Somatic Cell Sources for iPSC Generation. Stem Cells Int. 2014; 2014:768391
Rossi L et al (2007) The extracellular nucleotide UTP is a potent inducer of hematopoietic stem cell migration. Blood 109(2):533–542
Sacchetti B et al (2007) Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 131(2):324–336
Sansone P et al (2017) Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer. Proc Natl Acad Sci U S A 114(43):E9066–E9075
Schmidt NO et al (2005) Brain tumor tropism of transplanted human neural stem cells is induced by vascular endothelial growth factor. Neoplasia 7 (6):623–629
Sharma A (2018) Role of stem cell derived exosomes in tumor biology. Int J Cancer 142(6):1086–1092
Shimato S et al (2007) Human neural stem cells target and deliver therapeutic gene to experimental leptomeningeal medulloblastoma. Gene Ther 14 (15):1132–1142
Short MP et al (1990) Gene delivery to glioma cells in rat brain by grafting of a retrovirus packaging cell line. J Neurosci Res 27(3):427–439
Siegel RL et al (2016) Cancer statistics, 2016. CA Cancer J Clin 66(1):7–30
Siminovitch L et al (1963) The distribution of colony-forming cells among spleen colonies. J Cell Comp Physiol 62:327–336
Singh AK et al (2016) Fatal GvHD induced by PD-1 inhibitor pembrolizumab in a patient with Hodgkin's lymphoma. Bone Marrow Transplant 51(9):1268–1270
Skog J et al (2008) Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and

provide diagnostic biomarkers. Nat Cell Biol 10 (12):1470–1476
Sonabend AM et al (2008) Mesenchymal stem cells effectively deliver an oncolytic adenovirus to intracranial glioma. Stem Cells 26(3):831–841
Sullivan CB et al (2014) TNFalpha and IL-1beta influence the differentiation and migration of murine MSCs independently of the NF-kappaB pathway. Stem Cell Res Ther 5(4):104
Sutaria DS et al (2017) Achieving the promise of therapeutic extracellular vesicles: the devil is in details of therapeutic loading. Pharm Res 34(5):1053–1066
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
Takahashi K et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872
Thery C et al (2009) Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 9(8):581–593
Thery C et al (2018) Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles 7(1):1535750
Thomas ED et al (1957) Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. N Engl J Med 257(11):491–496
Thomas ED et al (1977) One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow. Blood 49(4):511–533
Thomson JA et al (1998) Embryonic stem cell lines derived from human blastocysts. Science 282 (5391):1145–1147
Toh WS et al (2018) MSC exosome works through a protein-based mechanism of action. Biochem Soc Trans 46(4):843–853
Uchibori R et al (2013) NF-kappaB activity regulates mesenchymal stem cell accumulation at tumor sites. Cancer Res 73(1):364–372
Vakhshiteh F et al (2019) Mesenchymal stem cell exosomes: a two-edged sword in cancer therapy. Int J Nanomedicine 14:2847–2859
Valadi H et al (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9 (6):654–659
Vallabhaneni KC et al (2015) Extracellular vesicles from bone marrow mesenchymal stem/stromal cells transport tumor regulatory microRNA, proteins, and metabolites. Oncotarget 6(7):4953–4967
van Niel G et al (2018) Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol 19 (4):213–228
Vasconcelos MH et al (2019) Extracellular vesicles as a novel source of biomarkers in liquid biopsies for monitoring cancer progression and drug resistance. Drug Resist Updat 47:100647
Walters MC et al (1996) Bone marrow transplantation for sickle cell disease. N Engl J Med 335(6):369–376
Wang C et al (2012) Neural stem cell-based dual suicide gene delivery for metastatic brain tumors. Cancer Gene Ther 19(11):796–801
Weissman IL (2000) Stem cells: units of development, units of regeneration, and units in evolution. Cell 100 (1):157–168
Widera D et al (2004) MCP-1 induces migration of adult neural stem cells. Eur J Cell Biol 83(8):381–387
Wolf P (1967) The nature and significance of platelet products in human plasma. Br J Haematol 13 (3):269–288
Yanez-Mo M et al (2015) Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 4:27066
Yokoi A et al (2019) Mechanisms of nuclear content loading to exosomes. Sci Adv 5(11):eaax8849
Yu JMA et al (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318 (5858):1917–1920
Yuan Z et al (2017) TRAIL delivery by MSC-derived extracellular vesicles is an effective anticancer therapy. J Extracell Vesicles 6(1):1265291
Zhang TY et al (2014) Gene recombinant bone marrow mesenchymal stem cells as a tumor-targeted suicide gene delivery vehicle in pulmonary metastasis therapy using non-viral transfection. Nanomedicine 10 (1):257–267
Zhao R et al (2008) From fibroblasts to iPS cells: induced pluripotency by defined factors. J Cell Biochem 105 (4):949–955
Zhu W et al (2012) Exosomes derived from human bone marrow mesenchymal stem cells promote tumor growth in vivo. Cancer Lett 315(1):28–37
Zuk PA et al (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7(2):211–228

Adv Exp Med Biol - Cell Biology and Translational Medicine (2021) 12: 159–186
https://doi.org/10.1007/5584_2020_581

Published online: 15 September 2020

The Outcome of Stem Cell-Based Therapies on the Immune Responses in Rheumatoid Arthritis

Peyvand Parhizkar Roudsari, Sepideh Alavi-Moghadam, Mostafa Rezaei-Tavirani, Parisa Goodarzi, Akram Tayanloo-Beik, Forough Azam Sayahpour, Bagher Larijani, and Babak Arjmand

Abstract

Rheumatoid arthritis as a common autoimmune inflammatory disorder with unknown etiology can affect 0.5–1% of adults in developed countries. It involves more than just the patient's joints and can be accompanied by several comorbidities and affect cardiovascular, pulmonary, and some other systems of the human body. Although cytokine-mediated pathways are mentioned to have a central role in RA pathogenesis, adaptive and innate immune systems and intracellular signaling pathways all have important roles in this process. Non-steroidal anti-inflammatory drugs, glucocorticoids, conventional disease-modifying anti-rheumatic drugs, and biological agents are some mentioned

P. P. Roudsari and A. Tayanloo-Beik
Metabolomics and Genomics Research Center, Endocrinology and Metabolism Molecular Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran
e-mail: Peyvand.parhizkar@yahoo.com; a.tayanloo@gmail.com

S. Alavi-Moghadam
Cell Therapy and Regenerative Medicine Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran
e-mail: sepidalavi@gmail.com

M. Rezaei-Tavirani
Proteomics Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
e-mail: Tavirany@yahoo.com

P. Goodarzi
Brain and Spinal Cord Injury Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran
e-mail: pr_goodarzi@yahoo.com

F. A. Sayahpour
Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
e-mail: fa_rosacea@yahoo.com

B. Larijani
Endocrinology and Metabolism Research Center, Endocrinology and Metabolism Clinical Sciences Institute, Tehran University of Medical sciences, Tehran, Iran
e-mail: emrc@tums.ac.ir

B. Arjmand (✉)
Metabolomics and Genomics Research Center, Endocrinology and Metabolism Molecular Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran

Cell Therapy and Regenerative Medicine Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran
e-mail: barjmand@sina.tums.ac.ir

medications used for RA. They are accompanied by some adverse effects and treatment failures which elucidates the needing for novel and more powerful therapeutic approaches. Stem cell-based therapies and their beneficial effects on therapeutic processes of different diseases have been founded so far. They can be an alternative and promising therapeutic approach for RA, too; due to their effects on immune responses of the disease. This review, besides some explanations about RA characteristics, addresses the outcome of the stem cell-based therapies including mesenchymal stem cell transplantation and hematopoietic stem cell transplantation for RA and explains their effects on the disease improvement.

Keywords

Arthritis · Autoimmune diseases · Hematopoietic stem cells · Mesenchymal stem cells · Rheumatoid · Stem cell transplantation

Abbreviations

ACPA	Anti–Citrullinated Protein Antibody
Ad-MSCs	Adipose-Derived Mesenchymal Stem Cells
ADSCs	Adipose-Derived Stem Cells
AHSCT	Autologous Hematopoietic Stem Cell Transplantation
Allo-HSCT	Allogeneic Hematopoietic Stem Cell Transplantation
AP-1	Activator Protein 1
APTC	Antiplatelet Trialists Collaboration
BAFF	B-cell Activating Factor
BM	Bone Marrow
BM-MSCs	Bone Marrow-MSCs
CCR7	CC-Chemokine Receptor type 7
CD40L	CD40 Ligand
CFA	Complete Freund's Adjuvant
CIA	Collagen-Induced Arthritis
COX	Cyclooxygenase
CXCR5	Chemokine C-X-C Motif Receptor5
CYC	Cyclophosphamide
DAS28	Disease Activity Score 28
DMARDs	Disease-Modifying Anti-Rheumatic Drugs
ESR	Erythrocyte Sedimentation Rate
FLSs	Fibroblast-Like Synoviocytes
Foxp3	Forkhead box protein P3
G-CSF	Granulocyte-Colony Stimulating Factor
GI	Gastrointestinal
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GvHD	Graft-versus-Host Disease
hAdMSCs	human Adipose-derived Mesenchymal Stem Cells
haplo-SCT	haplo-identical SCT
HDC	High-Dose Chemotherapy
HLA	Human Leukocyte Antigen
HSCs	Hematopoietic Stem Cells
HSCT	Hematopoietic Stem Cell Transplantation
hUCMSCs	Human UC-MSCs
ICOS	Inducible Costimulatory Molecule
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
ILD	Interstitial Lung Disease
iNOS	inducible Nitric Oxide Synthase
JAK	Janus Kinase
JIA	Juvenile Idiopathic Arthritis
M-CSF	Macrophage-Colony Stimulating Factor
MHC	Major Histocompatibility Complex
MMPs	Matrix Metalloproteinases
MPCs	Mesenchymal Precursor Cells
MS	Multiple Sclerosis
MSCs	Mesenchymal Stem Cells
MSCT	MSC Transplantation
MTX	Methotrexate
NF-Kb	Nuclear Factor Kappa B
NK	Natural Killer
NLRs	NOD-Like Receptors
NOD	Non-Obese Diabetic
NOD	Nucleotide-Binding Oligomerization Domain
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs

PBSCs	peripheral blood stem cells
PBSCT	Peripheral Blood Stem Cell Transplantation
PD-1	Programmed Cell Death Protein 1
PGE2	Prostaglandin E2
PI 3-kinase	Phosphatidylinositol 3-Kinase
PPI	Proton Pump Inhibitor
PTCL	Peripheral T Cell Lymphoma
RA	Rheumatoid Arthritis
RANKL	Receptor Activator of Nuclear Factor κB Ligand
RF	Rheumatoid Factor
rhTNFR: Fc	recombinant human TNF Receptor: Fc Fusion Protein
RORγt	RAR-related Orphan Receptor Gamma
SCT	Stem Cell Transplantation
SDF-1	Stromal Cell-Derived Factor-1
SLE	Systemic Lupus Erythematosus
SS	Sjögren's Syndrome
T1D	Type 1 Diabetes
Tfh	T Follicular helper
TGFβ	Transforming Growth Factor β
Th	T helper
TLRs	Toll-Like Receptors
TNF	Tumor Necrosis Factor
Treg	T regulatory cells
UCB	Umbilical Cord Blood
UC-MSCs	Umbilical Cord MSCs
VAS	Visual Analog Scale

1 Introduction

Rheumatoid arthritis (RA) is a common systemic autoimmune disease with unknown specific etiology in which chronic, symmetric, and progressive inflammatory polyarthritis are mentioned as its characteristics (Zerbini et al. 2017; Aletaha and Smolen 2018). Cytokine-mediated pathways have the main role in the pathogenesis of RA in its immune responses (McInnes et al. 2016) along with innate, adaptive, and stromal responses (McInnes and Schett 2011). Although the primary cause that initiates the destructive processes of RA is unknown, it has been founded that genetic and lifestyle factors can alter the occurrence risk. Immune system dysfunction, genetic predisposition (human leukocyte antigen (HLA)-DR4 was founded in two-thirds of patients), hormones, age, viruses, smoking, and patient environments are mentioned as some of those risk factors (Swann 2011). RA which affects 0.5–1% of adults in developed countries (Lazzerini et al. 2017) can lead to socioeconomic costs, progressive disability, and even death (McInnes and Schett 2011). In addition to the synovial inflammation and bone destruction, it is also accompanied by some systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders (McInnes and Schett 2011; Aletaha and Smolen 2018). It is mentioned that RA is not a curable disease, but therapeutic tools can be helpful to achieve improved clinical symptoms (Swann 2011; Aletaha and Smolen 2018). Nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, conventional disease-modifying anti-rheumatic drugs (DMARDs), and biologic agents are the medications can be taken for it (Littlejohn and Monrad 2018). Renal, hepatic, cardiovascular, and gastrointestinal (GI) system can be affected by NSAIDs' adverse reactions (Crofford 2013). Moreover, GI side effects, hepatotoxicity, pulmonary toxicity, hematologic toxicity, and carcinogenicity are some of the possible side effects of methotrexate (MTX) as an important conventional DMARD which are accompanied by several side-effects and declining efficacy (Weaver 2004; Wang et al. 2018). On the other hand, biological agents may also lead to reactivation of tuberculosis, psoriasiform skin changes, exacerbation of demyelinating diseases and nonmelanoma skin cancer as some of their adverse effects (Aletaha and Smolen 2018). Thus, these drugs are in association with adverse effects and treatment failures which develop the needing for more effective therapeutic tools.Hereupon, stem cell-based therapies are introduced as alternative treatment approaches for RA (Jung 2018). Stem cell therapies can be utilized widely in the fields of immunotherapy and gene therapy (Brignier and Gewirtz 2010; Arjmand et al. 2017) and their ability in the treatment of various diseases such as obesity (Payab et al. 2018),

epilepsy disorder (Goodarzi et al. 2014), Alzheimer's Disease (Larijani et al. 2012; Goodarzi et al. 2019c), Parkinson's disease (Larijani et al. 2012; Goodarzi et al. 2015; Larijani et al. 2019a), Autism spectrum disorders (Larijani et al. 2020), diabetes (Rahim et al. 2018; Arjmand et al. 2019; Larijani et al. 2019b), stroke (Aghayan et al. 2014), multiple sclerosis (MS), and graft-versus-host disease (GvHD) have been also discussed (Larijani et al. 2012). With the aim of seeking new treatment approaches for RA, many preclinical studies and clinical trials have been carried out by the means of mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) as representative adult stem cells (Jung 2018). MSCs are multipotent cells with a great potential ability of suppressive activity for inhibiting pro-inflammatory cells from both the innate and adaptive immune system. Thus, hypo-immunogenicity, immune modulation effect, and capability for tissue regeneration make MSCs potent to be a promising therapeutic tool for RA treatment (Jung 2018; Goodarzi et al. 2019a; Luque-Campos et al. 2019; Aghayan et al. 2020). On the other hand, 'immune reset' is mentioned to be the base concept for HSCs treatment strategy in which autologous transplantation has more commonly used for the clinical trials (because of bone marrow (BM) failure and GvHD which may be occurred along with allogeneic transplantation) (Jung 2018). In current review, the authors are trying to introduce RA through the characteristics of symptoms, causes, immunopathogenesis mechanisms, and current treatment options. Additionally, they are also tried to discuss the importance of stem cell-based therapies and explaining the effects of stem cells on the immune system of RA subjects.

2 Rheumatoid Arthritis

RA as a common inflammatory arthropathy can affect 1% of the world's population (Firestein and McInnes 2017; Bergot et al. 2020). It occurs three times more in women than men (Swann 2011). Although the peak incidence of RA is in the sixth decade, it can affect the population at any age (Aletaha and Smolen 2018). Inflammatory changes of the joints' synovial tissue, cartilage, bone, and also extra-articular sites are its characteristics (Scherer et al. 2020). Additionally, chronic inflammation of the synovial membrane can lead to articular cartilage and juxta-articular bone damage (Aletaha and Smolen 2018). According to different evidence and researches, chronic inflammation and articular destruction could be propagated by immune-mediated etiology in association with stromal tissue dysregulation (Firestein and McInnes 2017). Herein, RA can cause severe joint damage and disability. There is no known-cause for RA; however, there is a correlation between RA and genetic/environmental factors (Aletaha and Smolen 2018). Smoking, obesity, and periodontal disease, the gut microbiome, and infections are some of this known risk factors for RA development (Littlejohn and Monrad 2018). On the other hand, Lyme disease, psoriatic arthritis, tophaceous gout, and osteoarthritis are some of RA's differential diagnoses (Cush et al. 2010). Although RA is in association with socioeconomic costs, progressive disability, systemic complications, and even early death, more knowledge about the immune mechanisms of RA has provided the new therapeutic agents which can lead to reduced mortality rate (McInnes and Schett 2011).

2.1 Symptoms and Causes

RA is associated with synovial inflammation and hyperplasia (swelling), because of the leukocyte infiltration to the synovial compartment that causes the synovitis. Furthermore, bone destruction and deformity also occur (McInnes and Schett 2011). Joint inflammation, central sensitization, and structural joint damage as predominant problems can lead to the occurrence of pain in RA subjects. Moreover, they can affect RA individual's psychosocial conditions (Walsh and McWilliams 2012). Rheumatoid nodules and serum rheumatoid factor are in association with chronic deforming arthritis and severe systemic manifestations of the disease (Askari et al. 1974).

Morning stiffness is another symptom of RA that reduces the ability of patients for work and it is related to early retirement (Mattila et al. 2014). RA as a polyarticular symmetric disease is primarily initiated in the wrists and metacarpophalangeal, metatarsophalangeal, and proximal interphalangeal joints with morning joint stiffness more than 30 min (Fig. 1) (Aletaha and Smolen 2018). Fatigue is also commonly founded in RA patients (Matcham et al. 2015).

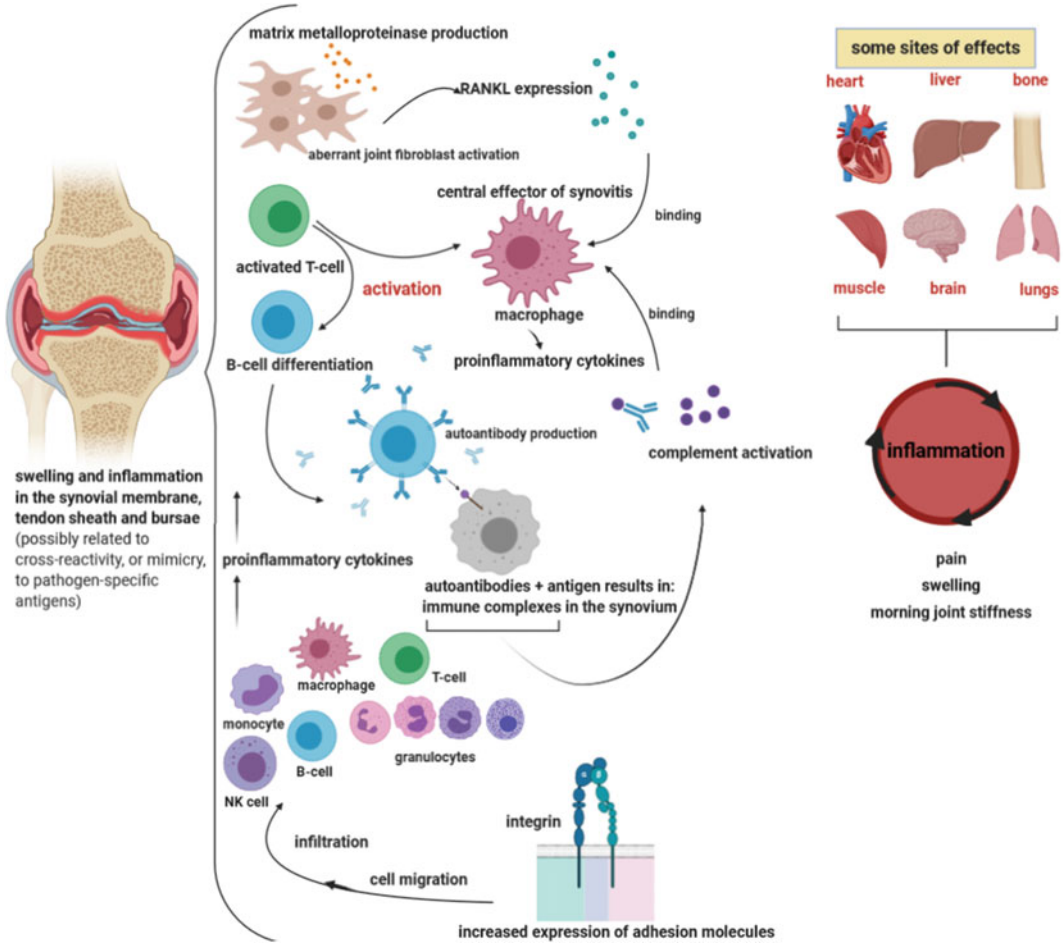


Fig. 1 Pathogenic Processes in Rheumatoid Arthritis and Some Affected Sites. Swelling and inflammation in the synovial membrane, tendon sheath, and bursae are some aspects of RA pathogenesis (possibly related to cross-reactivity, or mimicry, to pathogen-specific antigens (Bergot et al. 2020)) (McInnes and Schett 2011). Increased expression of adhesion molecules such as integrins can control the cell migration into the synovium and lead to synovitis due to leukocyte infiltration (by granulocytes, monocytes/macrophages, NK cells, B cells, and T cells) in the synovial compartment that causes the pro-inflammatory cytokines production (Mellado et al. 2015). Activated and differentiated T cells can activate macrophages and B cells leading to the production of autoantibodies from plasma cells. Then immune complexes (autoantibodies-autoantigen binding forms) lead to higher complement activation and they cause pro-inflammatory cytokines secretion by binding to macrophages. Fibroblasts that express RANKL, can activate macrophages, too (Aletaha and Smolen 2018). They also lead to MMPs release which have roles in generating osteoclasts (Hirohata et al. 2001; Aletaha and Smolen 2018). This process results in inflammation in RA (swelling (McInnes and Schett 2011), pain (Walsh and McWilliams 2012) and morning stiffness are some of its symptoms (Mattila et al. 2014)) and can also affect other sites such as heart, brain, liver, lungs, muscle, and bone (McInnes and Schett 2011). *NK cell* Natural killer cell, *RANKL* receptor activator of nuclear factor κB ligand

Sleep disturbances (such as sleep fragmentation) are reported in patients which it might have effects on symptoms of mood and pain (Irwin et al. 2012). RA may be accompanied by multiple comorbidities. Hereupon, cardiovascular disease is the primary cause of death in RA patients (Aletaha and Smolen 2018) and they are at higher risk for cardiovascular death, ischemic heart disease, and heart failure. Increasing evidence of inflammation and an increased proportion of unstable plaques have been also founded in RA patients (Gabriel 2008). On the other hand, brain, liver, lungs, exocrine glands (such as secondary Sjögren's syndrome (SS)), muscles and bones (osteoporosis) can be infected by inflammation in RA patients, too (Fig. 1). RA may be along with a higher risk for lymphoma, too (McInnes and Schett 2011). Vasculitis, Felty's syndrome, glomerulonephritis, pericarditis, pleuritis, scleritis, and interstitial lung disease (ILD) along with morbidity and premature death are mentioned to be some severe extra-articular manifestations that may develop in RA individuals (Farquhar et al. 2019). The initial trigging factors of RA have not been founded, but genetic and lifestyle risk factors have important effects, and interaction of factors may lead to the disease (Swann 2011). Accordingly, it has been founded that there is an important association with HLA–DRB1 locus in patients who are positive for rheumatoid factor (RF) or an autoantibody named anti-citrullinated protein antibody (ACPA). Alleles with common amino acid motif (QKRAA) in the HLA-DRB1 region which is termed shared epitope, provide this particular susceptibility. Predisposing T-cell repertoire selection, antigen presentation, or alteration in peptide affinity, and molecular mimicry of the shared epitope by microbial proteins are some possible explanations for the link presents between rheumatoid arthritis and the shared epitope (McInnes and Schett 2011). On the other hand, epigenetic modifications have some roles in promoting inflammatory responses, too (Aletaha and Smolen 2018). An immune system dysfunction is also attributed to the disease and over activation of the 'T' cells and the 'B' cells has been founded to have roles in the inflammation processes of RA. Hormones are also mentioned factors. Accordingly, decreased oestrogen and progesterone levels following childbirth may lead to RA. Further, the below-normal level of cortisol is mentioned in the RA patients. The role of viruses such as *Proteus mirabilis* for triggering RA and making malfunctions in the immune system is discussable, too (Swann 2011). Smoking is a major and preventable risk factor for RA. The risk due to smoking can be affected by genotype in addition to the amount of smoking; therefore, smoking cessation is mentioned to be an important step in RA management (Källberg et al. 2011; Harris et al. 2016).The increasing rate of RA occurrence in certain countries also indicates the role of diet and lifestyle in triggering RA (Swann 2011). Herein, in a study, diet has been shown to have an effect on approximately one-quarter of RA subjects with longstanding disease and a potential link between sugar consumption and inflammation in RA patients has been founded (Tedeschi et al. 2017). In another study, the influence of weather conditions especially daily mean temperature on RA symptoms has been also demonstrated (Abasolo et al. 2013). Among these mentioned factors, immune responses of the disease and involved cytokines (which have a great effect on RA mechanisms) are going to be explained further.

2.2 Mechanisms of the Disease

As mentioned, RA initially lead to inflammation in the synovial membrane (Synovium is a delicate structure which produces synovial fluid and can protect the articular). It also can affect tendon sheath and the sacs of fluid (bursae) (Fig. 1). In RA as an autoimmune disease, the immune system treats healthy tissues as same as invading pathogens leads to inflammation and cytokine production (which are normal parts of healing in the presence of a foreign body, infection or a virus). Excess synovial fluid and thus swelling are caused by the attraction of the immune cells done by cytokines (Swann 2011). In some cases, this autoimmunity might be related to cross-

reactivity, or mimicry, to pathogen-specific antigens (Bergot et al. 2020). Therefore, some cellular and molecular processes play important roles in the clinical disease expression and infiltration of the synovial membrane (with T cells, B cells, and monocytes), activation of endothelial cells, and neovascularization are some other hallmarks of RA synovitis. Moreover, hyperplastic synovial lining layer is formed by the expansion of synovial fibroblast-like and macrophage-like cells which causes bony erosions and cartilage degradation. Pro-inflammatory cytokines, including tumor necrosis factor (TNF) and interleukin (IL)-6, induce receptor activator of nuclear factor κB ligand (RANKL), prostaglandins, and matrix metalloproteinases (MMPs) are mediators of the signs and symptoms which have roles in generating osteoclasts within the synovial membrane followed by bony damage (Aletaha and Smolen 2018).

2.2.1 Immune Responses of the Disease and Involved Cytokines

Adaptive immune pathways, activation of the innate immune system, cytokines and intracellular signaling pathways all have important roles in the pathogenesis of RA. RA is also in association with aberrant joint fibroblast activation which is connected to joint destruction (McInnes and Schett 2011; El-Jawhari et al. 2014). Cytokines (IL-12, 15, 18, and 23), HLA class II, and costimulatory molecules are expressed by numerous myeloid cells and plasmacytoid dendritic cells which can be founded in the synovium of RA cases. Mentioned cytokines are important for T-cell activation and antigen presentation (McInnes and Schett 2011). T lymphocytes are important factors in the immunopathogenesis of RA. T helper (Th) and T regulatory cells (Tregs) have a key role in the synovial membrane to form and maintain chronic inflammatory lesions (Warrington et al. 2001). On the other hand, increased expression of adhesion molecules such as selectins and their ligands, integrins, adhesion molecules, and chemokines and their receptors in the endothelium, can control the cell migration into the synovium and lead to synovitis (due to leukocyte infiltration of the synovial compartment). Cellular infiltration of granulocytes, monocytes/macrophages, natural killer (NK) cells, B cells, and especially CD4+ and CD8+ T cells causes the production of chemokines and pro-inflammatory cytokines in large amounts (Fig. 1). Involvement of Th1, Th17, Th22, and Treg cells in RA has been clearly founded. Th17 cells express cytokines including IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-22 with effects on synovial inflammation because of their neutrophil activation role (Mellado et al. 2015). It has been demonstrated that Th17/Treg imbalance is closely related to the severity of RA (Ma et al. 2019). T follicular helper (Tfh) cells which provide a novel Th lineage and offer a help for antigen-specific B cells (for supporting B cells), are also increased and combined with autoantibodies in patients with RA. Positive expression of chemokine C-X-C motif receptor 5 (CXCR5), inducible costimulatory molecule (ICOS), programmed cell death protein 1 (PD)-1, CD40 ligand (CD40L), and secretion of interleukin IL-21 along with the decreased expression of CC-chemokine receptor type 7 (CCR7) are mentioned to be some characteristics of RA (Liu et al. 2015). The importance of TNF-α has been also founded in RA pathogenesis. TNF-α plays an important role by activating cytokine and chemokine expression. Protection of synovial fibroblasts, promotion of angiogenesis, suppression of Treg cells, and induction of pain are also mentioned to be done by it (McInnes and Schett 2011). Besides, bone marrow derived CD34+ cells from RA patients have shown abnormal responses to TNF-α following by their differentiation into fibroblast-like cells and producing MMP-1 (a feature that is unique to type B synoviocytes) (Hirohata et al. 2001). Moreover, the ability of BM CD34+ cells for supporting B cells survival, sustaining their responses, and also their contribution to the RA pathogenesis has been also discussed (Hirohata et al. 2000). Activated and differentiated T cells release lymphokines that activate macrophages and provide help for B cells via the production of autoantibodies by their differentiation into plasma cells. Autoantibodies-autoantigen binding forms immune complexes in the synovium which elicit other B cells generate rheumatoid factor (anti-

immunoglobulin G (IgG) antibody). These enlarged complexes can lead to higher complement activation. Furthermore, they can also bind to macrophages and cause the secretion of pro-inflammatory cytokines and other mediators such as TNF and IL-6 (activators of leukocytes and osteoclasts and are involved in B-lymphocyte differentiation (McInnes and Schett 2011)) (Aletaha and Smolen 2018). On the other hand, fibroblasts with expressing RANKL can activate macrophages and may also lead to resorbing bone from the synovial, exostal site (Fig. 1) (Aletaha and Smolen 2018). In addition to cognate interactions with T cells and immune complexes, toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) cytokines, lipoprotein particles, and liver X–receptor agonists have effects on activation of macrophages (McInnes and Schett 2011). Monocytes/macrophages are other important sources of pro-inflammatory cytokines and chemokines such as TNFα, IL-6 and IL-1, and MMPs (Mellado et al. 2015). Generally, macrophages (central effectors of synovitis) beside mast cells (producers of vasoactive amines, cytokines, chemokines) and NK cells (as innate effector cells), are founded in the synovial membrane. Macrophages act by the means of releasing the cytokines, reactive oxygen intermediates, and nitrogen intermediates. Production of prostanoids, matrix degrading enzymes, phagocytosis, and antigen presentation are other parts of their roles. (McInnes and Schett 2011). Interleukin-4, 13, 15 (in early rheumatoid arthritis) and IL-1 family cytokines including interleukin-1α, 1β, 18, and 33 (with their effects on promoting the activation of leukocytes, endothelial cells, chondrocytes, and osteoclasts), have some roles in the immunopathogenesis of RA. IL-21 (activator of Th17 and B-cell subset), IL-32 (activator of cytokine production), IL-7 (with its effects on activation of T-cell, NK cell, and blocking apoptosis) are other involved cytokines in the pathogenesis of RA (McInnes and Schett 2011). IL-19 has anti-inflammatory roles in arthritis, while IL-20 and IL-24 are in a correlation with bone degradation. IL-22 has been shown to have a relation with bone erosion and IL26 induces pro-inflammatory cytokines in RA pathogenesis (Kragstrup et al. 2018). Moreover, serum IL-2 level may also be elevated in certain RA conditions (Tebib et al. 1991). Altogether, cytokines play an important role in the pathogenesis of RA and their contribution to the induction and maintenance of inflammation may help to achieve therapeutic targets (Noack and Miossec 2017). Herein, pathogenic processes in rheumatoid arthritis and some affected sites are showed in Fig. 1.

3 Treatment Approaches for Rheumatoid Arthritis

Since RA and osteoarthritis are two common forms of arthritis with different treatments and outcomes, primary care approaches should distinguish the clinical presentation of RA from osteoarthritis and other causes of joint pain. Early recognition is also important to initiate the treatment earlier. This can lead to better long-term outcomes with lower disease activity or complete remission. Moreover, early treatment may lead to preventing irreversible structural damage and chronic functional impairment. NSAIDs and glucocorticoids are anti-inflammatory agents which are important in the first steps of managing pain and inflammation. Additionally, DMARDs and biologic agents are also other medications that could be taken (their classifications and adverse effects are concluded in Fig. 2) (Littlejohn and Monrad 2018). Moreover, the advantages of stem cell transplantation for immunity reeducation via using autologous and allogeneic MSCs in RA have been also discussed (To and Khan 2019). Autologous and allogeneic forms of hematopoietic stem cell transplantation (HSCT) in patients with RA have been also continued to be investigated in RA patients (Moore et al. 2001, 2003). Regular exercise, physical therapy, dietary changes, physiotherapists, occupational therapists, and even surgery are considered to be other treatment approaches for RA (Swann 2011). Although RA is incurable, modern therapeutic approaches can help to achieve excellent control of the disease, relieve pain, and prevent joint damage from

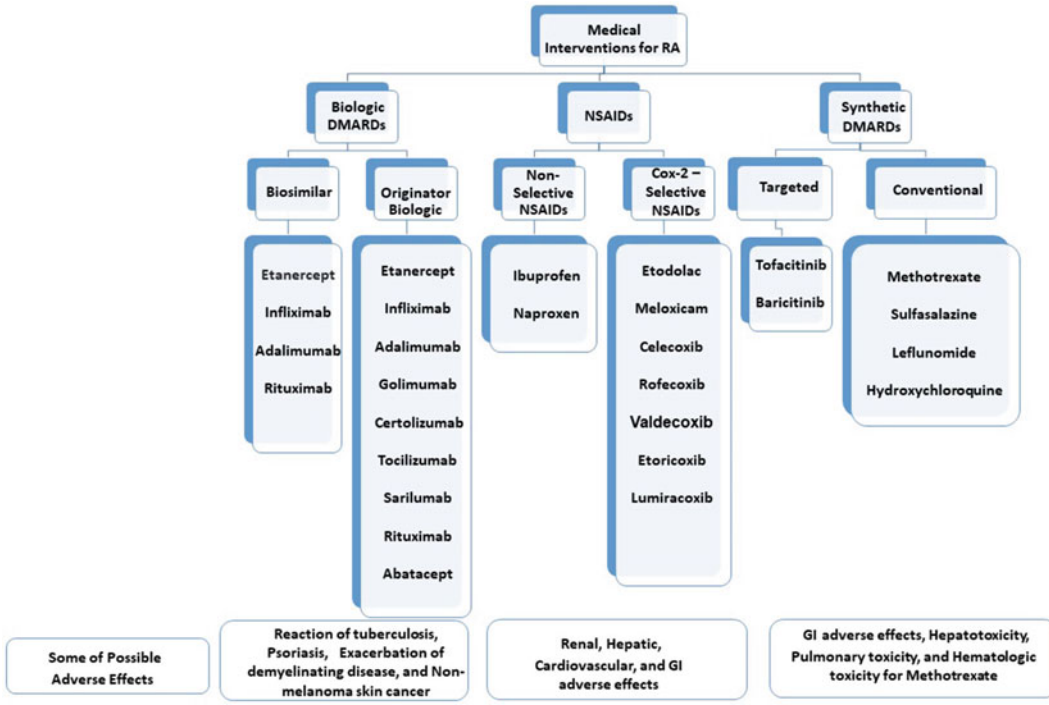


Fig. 2 Some medical interventions for RA and their adverse effects. Although RA is an incurable disease, therapeutic approaches can be beneficial for relieving pain and preventing joint damage from progressing (Swann 2011; Aletaha and Smolen 2018). NSAIDs and glucocorticoids can be used at the first steps (Littlejohn and Monrad 2018);renal, hepatic, cardiovascular and GI events are some possible adverse effects of NSAIDs (Crofford 2013). However, COX-2–selective type of NSAIDs can reduce the risk of ulcer complications comparing to non-selective NSAIDs (Chan et al. 2002). Synthetic DMARDs (including targeted and conventional drugs) and biologic DMARDs (including originator biologic and biosimilar drugs) are other medications that can be used for RA (Aletaha and Smolen 2018). GI side effects, hepatotoxicity, pulmonary toxicity, hematologic toxicity, and carcinogenicity are some of the possible side effects of methotrexate (as a conventional DMARD) (Wang et al. 2018). Reactivation of tuberculosis, psoriasiform skin changes, exacerbation of demyelinating diseases, and nonmelanoma skin cancer can be mentioned as adverse effects for biologic agents (Aletaha and Smolen 2018). *RA* Rheumatoid arthritis, *NSAIDs* Nonsteroidal anti-inflammatory drugs, *GI* Gastrointestinal, *COX* Cyclo-oxygenase, *DMARDs* Disease-modifying anti-rheumatic drugs

progressing (Swann 2011; Aletaha and Smolen 2018).

3.1 Non-steroidal Anti-inflammatory Drugs

NSAIDs are therapeutic agents that target cyclo-oxygenase (COX) 1 and 2 isozymes. They are used as first-line therapy in rheumatic joint disorders (Bickham et al. 2016). As it was mentioned, pain is described to be an important cause of reduced health-related quality of life in rheumatic diseases like RA. Herein, NSAIDs are anti-inflammatory and analgesic agents used for symptomatic therapy (relieving the pain, swelling, and stiffness of arthritis). They act by the means of inhibiting the biosynthesis of prostaglandins at the level of the COX enzyme which is responsible for many of their adverse effects, too (Crofford 2013). Altering the blood pressure, developing hyporeninemic hypoaldosteronism, idiosyncratic reaction accompanied by massive proteinuria, and acute interstitial nephritis could be mentioned as some of their renal adverse effects. Elevations of one or more liver tests can also occur; hepatic adverse events especially could be accompanied by diclofenac and sulindac. Additionally, the increased relative risk of cardiovascular hazards like myocardial

infarction is another possible adverse effect of NSAIDs (Crofford 2013). NSAIDs may increase the risk for GI complications, too; thus, co-prescribed therapy with a gastroprotective agent (such as a proton pump inhibitor (PPI)) may have beneficial effects in these patients. Patient adherence to this kind of therapy is an important challenge in clinical practice (Henriksson et al. 2013). In addition, using NSAIDs that are selective for COX-2 (instead of those conventional ones), can reduce the risk of ulcer complications. Herein, COX-2–selective NSAIDs celecoxib and rofecoxib have been evidenced to be effective anti-inflammatory agents with minimal gastric effects. In a study, it has been demonstrated that among patients who have a recent history of ulcer bleeding, celecoxib, acts as effective as treatment with diclofenac plus omeprazole (with respect to the prevention of recurrent bleeding); however, renal adverse events were common among susceptible individuals had taken celecoxib or diclofenac plus omeprazole (Chan et al. 2002). It has been also known that at moderate doses, celecoxib was non-inferior to ibuprofen or naproxen with regard to the primary Antiplatelet Trialists Collaboration (APTC) criteria of cardiovascular outcome. The definition of the criteria contains death from cardiovascular causes, including hemorrhagic death, nonfatal myocardial infarction, or nonfatal stroke (Nissen et al. 2016). In other study, it has been concluded that the COX-2 selective NSAIDs including etodolac, meloxicam, celecoxib, rofecoxib, valdecoxib, etoricoxib, and lumiracoxib were similar to non-selective NSAIDs (including ibuprofen and naproxen) in their effects on symptomatic relief of RA. Additionally, the amount of evidence for protective effects of COX-2 selective NSAIDs against serious GI events significantly varied among individual drugs (Chen et al. 2008). Despite all this, NSAIDs and pain medications mainly work on improving symptoms. They are not able to prevent damage progression and irreversible disability; thus, they are only usable as adjunctive, symptomatic therapy or during the short phase before an established diagnosis has been achieved (Aletaha and Smolen 2018).

3.2 Conventional Disease-Modifying Anti-rheumatic Drugs

Oral DMARDs remain the cornerstone of treatment strategies for RA. They are still used as initial treatment for the disease. It has been founded that more aggressive and earlier therapy with nonbiologic and biologic DMARDs can lead to reduced symptoms and slower progression of the disease. It can be mentioned that adherence to this kind of therapy and following the clinicians' prescription are important issues. Nonadherence may cause worse outcomes such as radiological damage, loss of function, and a lower quality of life (Xia et al. 2016). It has been founded that tapering and stopping DMARDs can be a beneficial approach for a subset of patients with RA who have entered clinical remission; although complete withdrawal of DMARDs only can be accessed when full remission happens (Schett et al. 2016). DMARDs are classified as synthetic and biologic agents. Synthetic DMARDs consist of targeted and conventional drugs (such as MTX, sulfasalazine, leflunomide, hydroxychloroquine) (Aletaha and Smolen 2018). Among non-biologic DMARDs, MTX is the most effective, the most tolerable, and the safest. Its efficacy could be compared with biologic agents according to parallel clinical trials of DMARD-naïve patients. Herein, about two-thirds of patients with RA in usual care show adequate responses to MTX monotherapy or its combinations with other non-biologic DMARDs. Weekly low-dose MTX with co-administration of folic acid as the "anchor drug" for RA has been strongly supported; because of its efficacy, effectiveness, tolerability, and safety (Pincus and Castrejón 2013). MTX is taken with daily folic acid or weekly folinic acid. The aim of that is to prevent potential side effects that are attributed to its folate antagonism. MTX is a structural analog of folic acid and acts on impairing DNA synthesis, it affects several and various enzymes and causes enhanced adenosis release. This process leads to reduced inflammatory responses and lymphocyte proliferation. Monotherapy with a DMARD (commonly MTX) forms the first step of the treatment. However, if it

does not leads to adequate disease control, hydroxychloroquine (an antimalarial agent with anti-inflammatory and immunomodulatory properties) and sulfasalazine (that has salicylate and sulfa antibiotic components) can be used in addition to MTX as triple therapy (Littlejohn and Monrad 2018). Combination of MTX 17.5 mg/week, sulfasalazine 500 mg bid, and hydroxychloroquine 200 mg bid has been shown to have both significantly improved statistical and clinical effects in multiple clinical variables; for instance in patients who have not adequate responses to MTX alone and also in those who have shown suboptimal responses to the combination of sulfasalazine-hydroxychloroquine (O'Dell et al. 1996). It has been concluded that triple therapy (MTX + sulfasalazine + hydroxychloroquine) is non-inferior to etanercept (a biologic DMARD (Aletaha and Smolen 2018)) plus MTX in RA patients with active disease despite MTX therapy (O'Dell et al. 2013). Despite all this, MTX might be in association with different side effects from mild to severe forms; GI side effects, hepatotoxicity, pulmonary toxicity, hematologic toxicity, and carcinogenicity are some of the possible side effects. This indicates the importance of long-term monitoring, appropriate dose administration, and also a careful pretreatment inspection (Wang et al. 2018). Taken together, side-effects and declining efficacy limit conventional DMARDs' ability in treatment durability over the long term (Weaver 2004).

3.3 Biological Agents

When two or more conventional synthetic DMARDs do not lead to adequate responses, it means that they are not likely to achieve the therapeutic target. The presence of autoantibodies, early joint damage, and high disease activity are the markers for poor prognostic conditions that are in association with the rapid progression of the disease. This condition may be slow down if biologic DMARDs or targeted synthetic DMARDs (Janus kinase (JAK) inhibitors such as tofacitinib and baricitinib) are added. Biologic DMARDs contain originator biologic (including etanercept, infliximab, adalimumab, golimumab, certolizumab, tocilizumab, sarilumab, rituximab, and abatacept) and biosimilar (including etanercept, infliximab, adalimumab, and rituximab) DMARDs (Aletaha and Smolen 2018). In the presence of incomplete or adverse responses to conventional DMARDs, TNF inhibitors are in the first-line of biologic therapy. They block the interaction of TNF-α with its receptor. TNF-α has important roles in the immunopathogenesis of RA through its effects on stimulating the production of other pro-inflammatory cytokines and the expression of endothelial cell adhesion molecules. Production of metalloproteinases and stimulation of osteoclasts are also other of its actions. Thus, using these agents may lead to the improvement of joint inflammation and radiographic damage (Littlejohn and Monrad 2018). Infliximab, etanercept, adalimumab, golimumab, and certolizumab are TNF-α antagonists which have been used widely for RA treatment. Some adverse events such as infections and injection-site reaction can occur because of them (these events are mild or moderate and with a low rate of incidence mostly); certain patients do not show responses to anti-TNF-α therapies and some patients may need to shift to another anti-TNF-α agent (do not continue the first one) (Ma and Xu 2013). Tocilizumab is a humanized and monoclonal anti-human IL-6 receptor antibody which acts on blocking IL-6 signaling. It is mentioned to be a beneficial agent when MTX or TNF inhibitor does not lead to effective responses. The effective and successful use of tocilizumab in RA treatment, followed by the development of other antibodies with biological targets of IL-6 pathway including sarilumab (which is anti-IL-6 receptor antibody) and sirukumab (as an anti-IL-6 antibody) (Lee and Seo 2018). Reactivation of tuberculosis, neutropenia, and hyperlipidemia are some of the adverse events of tocilizumab and sarilumab (Aletaha and Smolen 2018). In patients who have persistent active RA, etanercept (a soluble TNF receptor) in combination with MTX has been shown well safety, tolerability, and additional benefit compared to using MTX alone (Weinblatt et al. 1999). In those patients (with persistently active RA) repeated doses of infliximab combined to MTX may also lead to

beneficial effects and slow down the progression of damage (Lipsky et al. 2000). Besides, a single course of two infusions of rituximab (chimeric anti-CD20 monoclonal antibody) in combination with cyclophosphamide or MTX or either alone has been also shown to be effective for improving disease symptoms (Edwards et al. 2004). In addition, adalimumab is a fully human monoclonal tumor necrosis factor α antibody and in combination with MTX therapy can lead to more beneficial effects in reducing signs and symptoms compared to MTX plus placebo (Weinblatt et al. 2003). Despite all these mentioned beneficial effects, an association between ILD and several of the newer biologic agents has been also founded which recalls clinicians' awareness (Hadjinicolaou et al. 2011). Wide range of different skin lesions (such as Chronic inflammatory or infectious) can be also seen during anti-TNF-α therapy which indicates the need for long-term observations (Lee et al. 2007). Therefore, it has been mentioned that the possible risks and benefits should be considered carefully, due to the ability of biological agents for increasing the risk of adverse effects which are higher compared to synthetic ones (Lampropoulos et al. 2015).

4 An Introduction to Stem Cell Medicine

Stem cells are presented as the cells which are able to self-renew and differentiate into various cell types under correct conditions. A wide and different range of cell types can emerge, depending on the type of stem cell and they can be restricted to a few lineages, too. Stem cells have great therapeutic potential in regenerative medicine; thus, they have been widely researched (Biehl and Russell 2009; O'Connor 2017; Goodarzi et al. 2018). Blood diseases (such as thalassemia, multiple myeloma, and cycle cell anemia), heart disease, bone disease, renal disease, and respiratory diseases are some of the disorders which can benefit from stem cell-based approaches. There are evidence that the immunomodulatory and differentiation capacity of stem cells can be beneficial for controlling immune-based diseases (Larijani et al. 2012). Regenerative potential effects of stem cells (such as reconstituting muscle, cartilage, or bone) on RA have been also investigated (Jorgensen et al. 2001). The effects of stem cell therapies on this disease are going to be explained more in the next parts.

4.1 Sources of Stem Cells

Stem cells can be respectively classified into 4 categories by a hierarchal system and their differentiation potency including totipotent, pluripotent, multipotent, and unipotent stem cells. They can also be classified into categories depending on cell origin including, induced pluripotent, fatal, adult and embryonic stem cells (Brignier and Gewirtz 2010). Induced pluripotent stem cells are generated directly from somatic cells with defined factors and they can propagate indefinitely (Zhao et al. 2011; Joseph et al. 2020). Fetal stem cells can be defined as multipotent cells with less differentiation ability than embryonic stem cells (Goodarzi et al. 2019b) (which differentiate into all of the somatic cells in an embryo). The inner cell mass of the human blastocyst is the derivation source of embryonic stem cells (O'Connor 2017). The researches about pluripotent stem cells have been come from embryos mainly and thus result in the name of embryonic stem cells. Pluripotent stem cells can become all cell types of the body by the means of differentiation into multipotent stem cells (Biehl and Russell 2009). Despite the pluripotency potential of these cells, there are some moral ethical and legal issues about their use for clinical application (Tuan et al. 2002). As was mentioned, through subsequent divisions, the differentiation ability of cells will be restricted to multiple lineages and then they will be called multipotent with the capability of forming a limited number of cell types which is the characteristic of adult stem cells (or somatic stem cells/nonembryonic stem cells) (Brignier and Gewirtz 2010). Thus, adult stem cells are multipotent cells with the main role of maintaining hemostasis. They can be founded in different tissues and organs and can give rise to

cell types of one particular tissue. The great potential therapeutic effects of these cells (because of their functional and structural regenerative capabilities) have been founded (Gurusamy et al. 2018; Caglayan et al. 2019); they can be easily isolated, expanded, and transplanted back that makes them have no chance of immune rejection with lack of ethical or legal issues. Adult stem cells are harvested from bone marrow typically and mainly include two types of cells: MSCs and HSCs (Vishwakarma et al. 2015). Besides, the existence of adult stem cells in mammary tissue with the capacity to differentiate into ductal, alveolar, and myoepithelial cells has been demonstrated (Woodward et al. 2005). Small populations of adult stem cells are responsible for the epithelial lining of the intestine for efficient renewal and repairing, too (Barker 2014). The presence of neural stem cells in the adult brain (Johansson et al. 1999) and the existence of a multipotent stem-like cell in the olfactory mucosa have been also known (Murrell et al. 2005). On the other hand, skin epithelial stem cells and corneal stem cells can be also mentioned as adult stem cells that are of great interest in clinical application (Kiatpongsan et al. 2006). MSCs have the potential ability to differentiate into chondrocytes, osteoblasts, adipocytes, fibroblasts, marrow stroma, and other tissues of mesenchymal origin. Bone marrow aspiration is considered to provide the most accessible and enriched source of MSCs. Besides, trabecular bone, dermis, adipose tissue, periosteum, pericyte, and blood are the other source tissues of adult MSCs in where they can reside and process the regeneration ability (Tuan et al. 2002). In addition, fibroblast-like synoviocytes (FLSs) are resident mesenchymal cells of synovial joints (with an important role in RA pathogenesis) (Mor et al. 2005; Noss and Brenner 2008; El-Jawhari et al. 2014). Umbilical cord (A postnatal organ discarded after birth) could be also mentioned as another source of MSCs. Umbilical cord MSCs (UC-MSCs) indicate such an immunoregulatory capacity makes them permissive for allogeneic transplantation (Liu et al. 2010). On the other hand, HSCs with their ability of self-renewal and differentiation into all blood lineages have roles in the lifelong production of blood cells (Mikkola and Orkin 2006). They are mostly isolated from bone marrow and some of them (which have roles in homeostasis) circulate in the blood. Herein, peripheral blood and umbilical cord blood (UCB) are other sources. HSCs should mobilize from bone marrow to the circulation to provide the ability for isolating them from peripheral blood. Administration of hematopoietic cytokines such as granulocyte-colony stimulating factor (G-CSF) is needed for this isolation. Advantages of using HSC for treatment a range of hematologic diseases (especially cancer) and also the disorders with different origins such as liver, heart, and brain have been discussed so far (O'Connor 2017). The therapeutic effects of stem cell-based therapies on different disorders are going to be discussed in the next part.

4.2 The Therapeutic Effects of Stem Cell-Based Strategies on Some Disorders with Immune Pathways

Transplantation of HSCs in addition to its effects on the treatment of different lethal diseases (such as leukemia and lymphoma) can be applied for severe autoimmune diseases (Sykes and Nikolic 2005). Since 1995, autologous hematopoietic stem cell transplantation (AHSCT) has been utilized as a treatment approach for MS. The high degree of safety and its effects on inducing long term remissions for MS patients have been demonstrated (Ge et al. 2019). On the other hand, MSC by its effects on releasing cytokines (those act synergistically) like IL6, IL-10, transforming growth factor β (TGFβ), prostaglandin E2 (PGE2), and HLA-G has been shown to have important influences on modulating the immune system (Kartiko et al. 2017). Therefore, it has been founded to be an ideal candidate for the treatment of MS, too; because of this high immunomodulatory effects and regenerative capabilities (Clark et al. 2019). Increased numbers of Tregs, decreased demyelination and inflammation, and modified microglia cells can

be mentioned as the effects of MSCs. Suppressed pathogenic effector CD4+ T and modulated effector CD8+ T cell subsets have been also seen in the animal studies of MS (Zhou et al. 2019). On the other hand, both HSCT and mesenchymal stem cell transplantation (MSCT) (with widely different safety and tolerability profiles) have beneficial effects on the treatment of systemic lupus erythematosus (SLE), too. The process was initiated with an interest in high-dose immunosuppression supported by HSCT and followed by researches in the MSCT field (Leung et al. 2017). Reestablishing the osteoblastic niche, recovering forkhead box protein P3 (Foxp3+ cells), and downregulating Th17 levels are some of the effects of allogenic MSCT on SLE treatment (Sun et al. 2009). Two other autoimmune diseases including type 1 diabetes (T1D) (defined as the destruction of the insulin-secreting beta islet cells) and SS (affects salivary glands and lachrymal glands) have been shown to may be affected by stem cell transplantation. (Faustman and Davis 2010). Intrapancreatic MSCT leads to an elevated level of serum insulin and C-peptide, and decreasing blood glucose. Moreover, AHSCT has been shown to be in association with decreased expansion/function of Th1 and Th17 (inhibition of T-cell proliferation and pro-inflammatory cytokine production) in T1D (Ye et al. 2017). On the other hand, according to the study, MSCs extract and MSCs therapies may lead to lower lymphocytic infiltration and anti-SS-A antibodies, less B

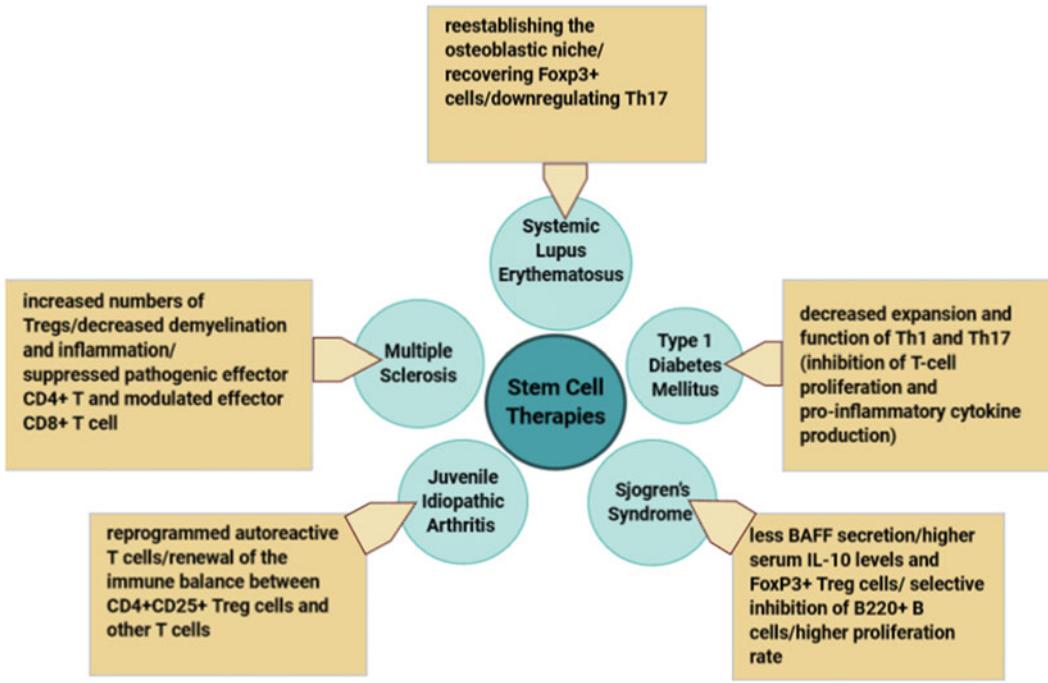


Fig. 3 Stem Cell-Based Therapies Effects on Autoimmune Diseases. The outcome of stem cell-based therapies on the immune responses of some autoimmune diseases has been mentioned including increased numbers of Tregs, decreased demyelination and inflammation, suppressed pathogenic effector CD4+ T and modulated effector CD8 + T cell subsets in Multiple Sclerosis (Zhou et al. 2019), reestablishing the osteoblastic niche, recovering Foxp3+ cells and downregulating Th17 in Systemic Lupus Erythematous (Sun et al. 2009), decreased expansion and function of Th1 and Th17 (inhibition of T-cell proliferation and pro-inflammatory cytokine production) in Type 1 Diabetes Mellitus (Ye et al. 2017), lower lymphocytic infiltration and anti-SS-A antibodies, less BAFF secretion, higher serum IL-10 levels and FoxP3+ Treg cells, selective inhibition of B220+ B cells and higher proliferation rate in Sjogren's Syndrome (Abughanam et al. 2019), and reprogrammed autoreactive T cells, renewal of the immune balance between CD4 + CD25+ regulatory T cells and other T cells in Juvenile Idiopathic arthritis (Wulffraat et al. 2006). *Treg* T regulatory cells; *Foxp3* Forkhead box protein P3, *Th* T helper, *BAFF* B-cell activating factor, *IL* Interleukin

cell activating factor (BAFF) secretion, higher serum IL-10 levels and FoxP3+ Treg cells, selective inhibition of B220+ B cells, and higher proliferation rate of the glands in non-obese diabetic (NOD) mice with SS-like disease (Abughanam et al. 2019). Juvenile idiopathic arthritis (JIA) patients may also benefit from stem cell-based approaches. Herein, allogeneic hematopoietic stem cell transplantation (allo-HSCT) is mentioned to be a potentially curative immunomodulatory strategy for them (Silva et al. 2018). In addition, the use of autologous stem cell transplantation may lead to reprogrammed autoreactive T cells; besides, it can be effective for the renewal of the immune balance between CD4 + CD25+ Treg cells and other T cells in these patients (Wulffraat et al. 2006). The outcome of stem cell-based therapies on the immune responses of these autoimmune diseases has been mentioned in Fig. 3. The potential therapeutic effects of stem cell-based therapies on RA have been also demonstrated (Sykes and Nikolic 2005; Ra et al. 2011; Jung 2018) that are going to be discussed further in the next part.

5 Stem Cell-Based Therapies and the Immune Responses of Rheumatoid Arthritis

Adverse effects and treatment failures associated with current RA treatments (such as biologics and targeted anti-rheumatic drugs) limit their abilities; thus, stem cell-based therapies using adult stem cells have been suggested as an alternative therapeutic approach and MSCs/HSCs are now underway (Jung 2018). Immune responses (such as B and T cells functions) have an important role in the pathogenesis of autoimmune diseases; moreover, as it was mentioned aberrant joint fibroblast activation is also related to joint destruction in RA. MSC-based therapies initially had focused on utilizing their multi-lineage differentiation capabilities to regenerate bone and cartilage, but the emergence of their ability in immunomodulation processes has been also provided a potential therapeutic approach to correct the breakdown of immune tolerance in RA (El-Jawhari et al. 2014). The effect of MSC administration on anti-inflammatory, immune modulation, and immune tolerance induction has been founded. These effects can lead to improved immune function and clinical symptoms (Karamini et al. 2020). Additionally, HSC therapy based on the concept of 'immune reset' provides other safe treatment in which pathologic immune cells are replaced by healthy ones. The immunosuppressive effect of HSCs by inducing anergy and the proliferation of regulatory cells have been also mentioned (Jung 2018). These findings are going to be explained more precisely in the next parts.

5.1 Mesenchymal Stem Cell Transplantation

As the bone marrow was the original discovered site of MSCs, bone marrow-MSCs (BM-MSCs) have been widely studied which have great immunomodulatory effects. Some of them act on T cell-mediated immune responses; such as inhibiting CD4+ T cell proliferation and IL-2, TNF-α production by T cells. Differentiating classic $CD4^{+}CD25^{hi}$ $FOXP3^{+}$ Tregs and maintaining their inhibitory function are also other MSC's effects on T cells that are dependent on interferon (IFN)-γ. Cell-to-cell contact between MSC and T cells may also lead to the suppressed function of T cells. The secretion of IDO which results in the inhibition of cell proliferation (by destructing the synthesis of different cellular proteins) can be mentioned as an immunoregulatory mechanism of MSCs. Additionally, the production of inducible nitric oxide synthase (iNOS) leading to the inhibited proliferation and the secretory/cytolytic functions of T cells are other immunoregulatory mechanisms associated with MSCs. MSC's acts on inhibiting B cell function and differentiation have been also demonstrated; B cells in co-culture with MSC (with IFN-γ dependency) may cause reduced expression of the chemokine receptors including CXCR4, CXCR5, and CCR7 on B cells that lead to the suppressed chemotaxis of B cells. On the other hand, BM-MSCs inhibit the production of dendritic cells from monocytes. Their functions

can also lead to promoted production of IL-10 and reduced expression of HLA-DR, CD80, and CD86 co-stimulatory molecules (on antigen presenting cells) (El-Jawhari et al. 2014; Karamini et al. 2020). Low expression levels of major histocompatibility complex -I (MHC-I) and no expression of MHC-II and costimulatory molecules make MSCs have low immunogenicity (Ra et al. 2011). In addition, IL-1 is significantly downregulated after the injection of MSCs. Increased levels of TGF-β and decreased serum IL-1β and IL-6 were also founded following MSCs therapy. It can be also mentioned that TLRs which are expressed on MSCs surface, affect MSCs to direct into whether inflammatory or anti-inflammatory actions. Stimulated TLR-4 causes the production of IL-6, IL-8, and TGF-β as pro-inflammatory cytokines that result in MSC1 phenotype development; while TLR-3 has roles in stimulation of immunosuppressive MSC2 cells and therefore in IDO secretion (Abdelmawgoud and Saleh 2018). IL-6 and macrophage-colony stimulating factor (M-CSF) are secreted by MSCs leading to the suppressed functions of T and B lymphocytes and dendritic cells. These characteristics make MSCs have the ability to be used in the treatment of RA and other several autoimmune diseases. This ability of MSCs for releasing anti-inflammatory and anti-apoptotic molecules may also lead to protect damaged tissues (Ra et al. 2011). It has been stated that biomarkers such as IFN-levels can be helpful for predicting the responses to MSC therapy (Karamini et al. 2020). The promising results in the treatment of induced RA in rats have been achieved with BM-MSCs utilizing that is needed to be investigated in humans (El Qashty et al. 2018). BM-MSC can inhibit the generation of pro-inflammatory cytokines produced by Th cells and T cytotoxic cells. It can cause increased mRNA expression of anti-inflammatory molecules that suggests their potential therapeutic aspect (Pedrosa et al. 2015). Despite their potentials, subsets of subchondral BM-MSCs may have abnormal behaviors playing roles in the induction of synovitis and enthesitis (Berthelot et al. 2019). On the other hand, it has been demonstrated that FLSs are not only space-filling but also have a direct influence on the cartilage destruction and inducing inflammation and autoimmunity (Mor et al. 2005; El-Jawhari et al. 2014). These pro-inflammatory cells could provide valuable treatment for RA and their combination with MSCs therapies should be considered to develop novel MSC-based strategies (El-Jawhari et al. 2014). Hereupon, in physiological states, MSCs are believed to have roles in the maintenance and repairing joint tissues; but in RA condition, inflammatory milieu makes this function be repressed. In this state, MSCs will have roles in the progression of joint damage instead. FLSs are also a part of the stroma (of the synovial membrane) in which RA condition makes them have roles in causing damage to articular cartilage and bone. Indeed, RA FLSs have an ability to augment B cells and increase their function and survival; although, in normal circumstances, FLSs obstruct T cell proliferation and inhibit differentiation of monocytes into dendritic cells (De Bari 2015; Karamini et al. 2020). RA FLS becomes metabolically active cells in which glucose metabolism is increased. Hypoxia and inflammatory mediators in FLS will lead to chronic glucose metabolic changes, activation of many signaling pathways, and activation of essential glycolytic enzymes (have important roles in FLSs function). These functions make them be a potential therapeutic target for RA (de Oliveira et al. 2019). In addition, it has been demonstrated that UC-MSCs can provide an opportunity to suppress T cell activation and induce Tregs generation by their inhibitory effects on the proliferation, invasive behavior, and inflammatory responses of FLSs. however, UC-MSCs therapy has been also shown the amelioration of collagen-induced arthritis (CIA) *in vivo* which exhibits their potential therapeutic ability for RA (Liu et al. 2010). In a study on CIA rats, human UC-MSCs (hUCMSCs) have shown the ability for inhibiting splenic T cells proliferation, serum IL-17 expression, and promoting TGF-β expression in the serum. HUCMSCs therapy also led to downregulated RAR-related orphan receptor gamma (RORγt) mRNA and protein expression, decreased Th17 cell ratio, and induced T cells apoptosis. Upregulated Foxp3

mRNA and protein expression, and increased Treg cell ratio in the spleen were also shown. HUCMSCs can also downregulate the expression of joint RORγt and Foxp3 which are markers of Th17 and Treg cells. Altogether, these functions indicate the immunoregulatory role of hUCMSCs in T lymphocyte functions and inflammatory factor pathways which results in improved arthritis and inhibited synovial hyperplasia in CIA rats. It can also lead to delayed radiological progression and reduced swelling degree of hindfoot and arthritis index (Ma et al. 2019). The results of the other study about the effects of UC-MSC and the biologic treatments (such as an anti-TNF antibody, recombinant human TNF receptor:Fc fusion protein (rhTNFR:Fc) and anti-CD20 antibody) on ameliorating synovial inflammation and joint damage have been shown comparable findings in decreasing the severity of arthritis and histological scores (Sun et al. 2017). In a study of intravenous infusion of UC-MSCs (single-dose regime), it has been also shown that UC-MSC therapy in RA patients provides safe and effective clinical therapy with rapid responses and without any serious side-effects up to 8 months after therapy (Wang et al. 2013; Clark et al. 2019; To and Khan 2019). In addition, the results of a 3-year cohort study of UC-MSC therapy combined with a personalized low dose DMARD have been shown stabled serological indexes, improved clinical symptoms, and low activity level of RA which provides a higher quality of life for patients (Clark et al. 2019). Besides, allogenic derived MSCT (from the umbilical cord of healthy donors) for three patients showed effective and safe approach and results in decreased erythrocyte sedimentation rate (ESR), lower disease activity, and disappeared bone pain that in two of them were combined with reduced blood Treg cells and increased Th17 (Wang et al. 2011). Also, allogeneic MSCs transplantation can lead to decreased 28-joint count disease activity score (DAS28), and pain visual analog scale (VAS) score leading to remission symptoms. The ability of UC-MSCs to inhibit Tfh cell differentiation through IDO production in response to IFN-γ has been also founded that indicates the good responses of patients with high IFN-γ levels to MSCT (Liu et al. 2015). Adipose tissue is another attractive source of MSCs used for autologous stem cell therapy. Systemic infusion of human adipose-derived mesenchymal stem cells (hAdMSCs) can downregulate Th1-driven autoimmune and inflammatory responses. It can also decrease the incidence and severity of experimental arthritis (induced by CIA in vivo), and induce IL-10 in lymph nodes and joints. Therefore, it results in a lower incidence and severity of experimental arthritis. De novo generation of antigen-specific CD4 + CD25 + FoxP3+ Treg cells are also promoted by hAdMSCs (Ra et al. 2011). Herein, a study of intravenous and intra-articular infusion of adipose tissue MSCs was carried out in 2011 with the aim of finding out the adipose-derived mesenchymal stem cells (Ad-MSCs) therapies' effects on RA patients. Liposuction was done and Ad-MSCs were obtained. One of the patients, received two separate IV injections of 300×10^6 Ad-MSC, other received an initial dose of 800×10^6 Ad-MSCs (Once 200×10^6 (i.-v.) + 100×10^6 (intraarticular), Once 350×106 (i.v.) + 150×10^6 (intraarticular)). Four separate IV injections of 200×10^6 Ad-MSCs in intervals of 1 month were received to the last patient. The results showed the clinical improvements in all three patients regardless of the dosing regimen (Ra et al. 2011; To and Khan 2019). Moreover, reduced levels of L-6, IL-17, TNF-α, and IFN-γ as pro-inflammatory cytokines has been founded by hAdMSCs (in another study of administering hAdMSCs in an animal model of experimental arthritis). (Kim et al. 2014; Baharlou et al. 2017). In another study, locally administered adipose-derived stem cells (ADSCs) in RA have been explored by the injection of single-cell ADSCs and three dimensionally-cultured ADSC spheroids intra-articularly into the knees of RA model mice. Synovial inflammation was improved and the regeneration of articular cartilage occurred. The suppressed function of activated inflammatory cells and macrophage function, downregulated rate of inflammatory cytokines, and upregulated TSG-6 and TGFβ1 significantly indicated the effectiveness of intra-articular administration of ADSC single cells and

spheroids. Therefore, they may provide a novel and beneficial localized strategy for RA patients (Ueyama et al. 2020). Additionally, the ability of Ad-MSCs (in suppressing the production of pro-inflammatory cytokines and inducing the production of TGF-β by lymphocytes) in combination with conventional treatment can even lead to better responses to treatment (Baharlou et al. 2017). Besides autologous forms, intravenous injection of allogeneic adipose tissue MSCs in RA patients has been also carried out. It also showed beneficial effects that appear to cease at 3 months following treatment indicates the needing for repeated administrations (Álvaro-Gracia et al. 2017; To and Khan 2019).

5.2 Hematopoietic Stem Cell Transplantation

As was mentioned, 'Immune reset' is the main aim of HSCT. Besides, there are also some immunosuppressive effects for it; direct contact with cytotoxic T cells may induce anergy because of MHC class I and class II expression but not the B7 family of co-stimulatory molecules. Additionally, inducing proliferation of peripheral Foxp3+ Treg cells is another pathway done because of their ability to activate notch signaling by direct intracellular contact and secrete soluble factors (such as GM-CSF). Herein, HSCs can be recognized by their cell surface markers including CD34, in addition to CD59, CD90, and CD117 in humans (Jung 2018). HSCT can be done by the products from BM, peripheral blood, and UCB (Hatzimichael and Tuthill 2010; Arbeláez-Cortés et al. 2013). Autologous transplantation, syngeneic transplantation (with minimal risk of GvHD), and allogeneic transplantation (graft free of autoreactive cells + increased risk of complications) are three types of HSCT. AHSCT is mentioned to be more frequent than other types. Herein, peripheral blood stem cells (PBSCs) are the main source (bone marrow is the other one), because of their easy procurement and also faster and more stable engraftment. Additionally, stem cell collection and transplantation phase (including conditioning regimen, aplastic phase, and engraftment) are included in this HSCT procedure (Arbeláez-Cortés et al. 2013). In this process, the anterior or posterior iliac crest is the usual site for BM collection. For peripheral blood (because there are not enough stem cells), medications (such as G-CSF) are used for mobilization and collection. G-CSF can enhance neutrophils to release serine proteases; thus, results in a breaking of vascular adhesion molecules leading to the HSCs release from the bone marrow (Arbeláez-Cortés et al. 2013; Khaddour and Mewawalla 2019). G-CSF was founded to be able to activate neutrophil elastase to induce stromal cell-derived factor-1 (SDF-1) degradation. Thus, it can promote the peripheral movements of HSCs. SDF-1 is a secreted chemokine by stromal cells in the BM and their CXCR4 is expressed by HSCs. Signaling between them was shown to have important roles in stem cell quiescence, proliferation, retention within niches, and migration to the outside (Jung 2018). SDF-1 has been founded to be overproduced in RA and its expression may be upregulated by IL-17 in RA FLS (mediated by phosphatidylinositol 3-kinase (PI 3-kinase), nuclear factor kappa B (NF-Kb), and activator protein 1 (AP-1)) (Kim et al. 2007). HSCs are able to go towards inflamed joints overexpressing SDF-1 when they are grafted. The migration of HSCs to the peripheral blood towards damaged tissues with secreted SDF-1 (by damaged tissues) have effects on the local infiltration of stem cells (Jung 2018). Furthermore, PBSCs can be prepared by the administration of chemotherapy. Herein, cyclophosphamide (CYC) is a mobilization chemotherapy drug used in autoimmune diseases. Conditioning regimen (high or intermediate immunosuppressive therapy with the aim of ablation of the immune system), aplastic phase, and engraftment are involved in the next steps of AHSCT in autoimmune diseases (Arbeláez-Cortés et al. 2013). Using HSCT can lead to regenerate a new, antigen-naïve, and self-tolerant immune system. It can result in bypassed triggering factors of autoimmune responses. The conditioning regimen which consists of high-dose immunosuppression (with the aim of ablating immune system) may lead to a prolonged anti-inflammatory effect. Elimination of the bulk of

autoreactive cells and de novo reconstitution of the immune system by HSC (autoimmunity reset) is the second possible explanation for rationale stated for using HSCT. It has been mentioned that both B and T cells have significant roles in this process; the effects of HSCT on the fate of Tregs cells and TH17 cells (as well as other immune system components) have been also demonstrated and founded to be important for RA autoimmunity pathogenesis (Nikolov and Pavletic 2008). In a study, it was reported that injection of UCB stem cells (MSCs ad HSCs) into complete Freund's adjuvant (CFA) rats, resulted in significantly decreased arthritis severity and disappeared paw swelling after 21–34 days of therapy; considerable downregulated levels of TNF-α, IL-1 and IFN-γ, and upregulated levels of IL-10 were also found after HSCs and MSCs injection (Abdelmawgoud and Saleh 2018). In 1999, a study has been demonstrated the safety and effectiveness of AHSCT in inducing major clinical response. It was shown to be successful for maintaining considerable responses for two patients (among four of them) at 9 months and 20 months post-treatment. As a result, the needing for more aggressive T cell depletion of the autograft, using a myeloablative regimen, or using an allograft was mentioned to may be necessary for decreased relapse rates (Burt et al. 1999a). Later, in another study, high-dose chemotherapy (HDC) followed by AHSCT led to temporarily increased health status and functionality of the patients (Teng et al. 2005). Several other AHSCTs have been done with the sources of PBSC and BM that showed feasible and safe treatment steps in all patients (with no unexpected major toxicity or treatment-related mortality). However, most of the patients experienced a recurrence of disease activity within 2 years (Verburg et al. 2001). Survived autoreactive T cells in inflamed synovia and pannus (where lymphoablative treatment does not have sufficient effects) might be the cause for early relapses in RA. It suggests that eradication of those intra-articular T-cell clones by the means of myeloablative conditioning treatment can lead to a longer remission period (Zeher et al. 2017). About the immune responses of HSCT, it was also founded that IgG and several T-cell markers, such as CD27 and CD45RO in the synovium, have a higher expression rate before autologous hematopoietic stem cell transplantation (SCT) in patients who had favorable responses to it. It was shown that infiltration (with lymphocytes and plasma cells) before SCT (high dose cyclophosphamide and CD34 selected autologous stem cell transplantation) was almost absent after therapy. It should be mentioned that severely diminished cellular infiltration appeared 3 months post-transplantation. It has been founded that survival of T or B cells, synoviocytes or macrophages (despite high dose chemotherapy), reinfusion of autoaggressive lymphocytes (despite purging of the graft), reactivation of autoaggressive lymphocytes (caused by new autoantigens), dysregulation (because of intrinsic stem cell defects) or a combination of those factors above can be accompanied by reappearance of the autoimmune disease after autologous SCT (Toes et al. 2002). Furthermore, the seronegative disease has founded to be more responsive to the process (Snowden et al. 2012). In another report from a young patient with a long history of RA, who underwent haplo-identical SCT (haplo)-SCT for treatment of peripheral T cell lymphoma (PTCL), as the result, both RA and PTCL had complete remission after almost 3 years of follow-up. Intensive immunosuppressive regimen prior to haplo-SCT and post-transplant high dose cyclophosphamide were concluded to may be helpful for preventing immune memory transferring to the donor T-cells (and the recurrence of RA). It is done by the means of destructing all antigen-experienced and immunocompetent cells of the recipient's bone marrow. The inability of donor immunocompetent cells to be activated by a putative RA antigen was another possible explanation. Contribution of the shared epitope to the disease recurrence has been also founded. Herein, haplo-SCT (according to its context) comparing to HLA-identical allo-SCT or autologous SCT may be less considered to recognize and respond to the antigen through clonal expansion. The excellent response of this case, provide potential evidence of using haplo-SCT for treating severe and/or refractory RA. This also indicates that the

disease is driven by a T-cell dependent mechanism (Atoui et al. 2019).

6 Conclusion, Limitations and Future Perspectives

MSCs have a great ability in differentiating into different lineages. Moreover, their immune-suppressive properties and their effects on dendritic cells, T cells, and B cells have been widely researched. It has been known that they provide promising cell therapy for autoimmune diseases such as RA (Liu et al. 2015). Important roles of MSCs in the inflammatory processes of RA pathogenesis have been known and their transplantation has been shown to be effective and safe; however, technological advancements (like those in tissue engineering) and new strategies (for delivering MSCs) are still needed for better responses. Discovering the correct target audience, with a responsive phenotype (for MSCT) is another issue we face in this field. There are wide ranges of phenotypical variations of RA's effects on different patients (including joint involvement patterns or major organs involvement) which creates a great challenge in clinical trials to organize control groups. This may also be responsible for less reliable outcome measurements (based on disease improvement criteria). On the other hand, many clinical trials of MSCT have not shown significant benefits in refractory RA which makes the other challenge that MSCT may only be effective for a certain number of disease phenotypes. Besides, another challenge is to know whether MSC therapies can be beneficial for patients who are not refractory to conventional treatments or not. Because now the candidates for MSC-based therapies usually are chosen from those who are refractory to DMARDs and biological treatments. Thus, now, scientists are in the early stage of conducting clinical trials which are used restrictedly for those failed to achieve remission with other treatment strategies. Permanent tolerance of the immune system is an important issue, too; because the current data has shown the short-term effects (Karamini et al. 2020). Additionally, MSC source discrepancies, make it difficult to compare outcomes. Further, adjustment of treatment regimens and dosing of administration are needed to be more investigated in further studies (To and Khan 2019). Herein, administration dosage and implementing the re-administration period should be considered carefully to achieve the safety goals as well as appropriate patient profile and imaging data that should be prepared for better assessments. As was mentioned, there are variable clinical efficacy outcomes which demand more knowledge about the biomarkers, too to be able for predicting these different RA responses (Karamini et al. 2020). The dependence of MSC effects on the disease etiology and inflammation degree makes it important to have more information about interactions between MSCs and pathogenic immune cells (such as memory T cells) too (Luque-Campos et al. 2019). Thus, allogeneic mesenchymal precursor cells (MPCs) therapy, was shown to have encouraging results and may provide a promising therapeutic approach in biologic-refractory RA patients; however, there are some challenges needed to be assessed more (Kafaja et al. 2017). About different sources of MSCs, it could be mentioned that Ad-MSCs may offer a more reliable source than BM-MSCs (because of some controversy effects) (Karamini et al. 2020). Culture-expanded autologous Ad-MSCs have shown clinical benefits in RA and also in a wide spectrum of autoimmune diseases independent of patient's age; however, it needs a sufficient number of patients and more investigations to be confirmed about therapeutic efficacy and long term benefits (Ra et al. 2011). Local transplantation of adipose-derived stem cells has also shown a significant therapeutic effect in a mouse model of RA and provides a promising therapeutic strategy for the treatment of RA; although there were some limitations. For instance, molecular mechanisms of ADSCs effects had not been characterized. Also, using mouse ADSCs instead of human and slight differences between RA severity in the mouse model were the two other limitations (Ueyama et al. 2020). Also, intravenous administration of expanded allogeneic Ad-MSCs in refractory RA is another promising

and innovative therapeutic approach that needs further investigations about the duration of the therapeutic effects, the optimal dosing strategy, and the most suitable patient profile. The superiority of allogeneic cells instead of autologous (because of their more availability, less complicated processes, and shorter time for cell expansion) have been also provided in the study (Álvaro-Gracia et al. 2017). Taken together, immunomodulatory aspects of Ad-MSCs which have important roles in RA pathogenesis, make them have great therapeutic advantages (Baharlou et al. 2017); although in a study, the paradoxical effect of them, suggests that disease phase can affect the mechanisms of the action and attention should be paid for safe and effective use of MSCs immunosuppressive effects (Kim et al. 2014). On the other hand, intravenous infusion of hUCB-MSCs has been also shown to be an effective therapeutic option for refractory RA patients or for those who are intolerant of MTX (Liu et al. 2010; Park et al. 2018). It has shown long-term safety and efficacy with maintained effects. Stabled clinical outcomes for 3 years has been also seen that lead to a higher quality of life; however, it still needs a larger a multiple-center/controlled study (Clark et al. 2019). In addition, the MSC therapy should consider targeting the inflammatory factors within the synovium and mesenchymal stromal cells which can provide new chances for newer RA therapeutic strategies (Karamini et al. 2020). Stimulation of the inflammation and tissue damage are mentioned to be the abilities of FLS which make them beneficial targets for the treatment of inflammatory arthritis; although it needs more understandings to develop new agents for RA treatment (Noss and Brenner 2008). On the other hand, another study has demonstrated that MSCs therapy can be superior to HSC and MTX treatment; because of their effectiveness to decrease RA inflammation (Abdelmawgoud and Saleh 2018). The field of HSC therapy has had a progression, too. It provides relatively safe and efficacious treatment which 'immune reset' is its main concept (Jung 2018). Ablation of a self-reactive immune system and resetting auto-tolerance by regeneration of HSCs make AHSCT be an efficacious therapeutic strategy for autoimmune diseases (Álvaro-Gracia et al. 2017). Infusion of hematopoietic progenitor cells (CD34 cells) with the ability for expansion and differentiation into T and B cells, macrophages, and monocytes can provide the possibility of reconstitution new hematopoietic and immune system (Burt et al. 1999b). It should be mentioned that the conditioning regimen affects the responses of the transplantation widely (Jung 2018). HSCT can cause improvements in the clinical symptoms; such as resolved morning stiffness and disappeared rheumatoid nodules. Normalized sedimentation rate and disappeared rheumatoid factor are other clinical benefits of the therapy; Although remission has not been founded to be permanent and relapse within 2 years is a common occurrence (Burt et al. 2001). Autologous type of transplantation is more widely used in clinical trials because the allogeneic form is accompanied by potential adverse effects such as BM failure and GvHD (Jung 2018). Lymphocyte depletion (CD34 + enrichment) of an allo-graft can lead to decreased GVHD risk, but it can also increase the risk of graft failure. The fully matched donor is needed for allogeneic stem cell transplantation to provide safe and lower intensity conditioning. Besides, the intensity of the conditioning regimen (which is immunosuppression) has an inverse correlation with relapse occurrence after AHSCT (Burt et al. 2001). Hereupon, unrelated UCB has a lower risk of GVHD and syngeneic transplantation is also mentioned to have minimal risk of it (Álvaro-Gracia et al. 2017). Rapid collection and administration, and less frequency of infections are mentioned to be other advantages of cord blood transplantation (it could be also stated that peripheral blood stem cell transplantation (PBSCT) provides a more rapid engraftment rate than bone marrow) (Khaddour and Mewawalla 2019). The safety and feasibility of AHSCT following high-dose chemotherapy (that can provide long-term improvement of disease activity) have been founded in the patients who had not successful responses to conventional DMARDs and TNF blocking agents. It could also increase the functionality and health status of patients with severe/refractory RA. Despite all

Table 1 Stem cell-based trials for RA (https://clinicaltrials.gov/)

Study title	Study start date	Intervention/treatment	Phase	Status	Identifier number
The safety and effects of mesenchymal stem cell (MSCs) in the treatment of rheumatoid arthritis	December 2017	Biological: UC-MSCs	Not applicable	Recruiting	NCT03798028
Transplantation of autologous bone marrow derived stem cells in patients with rheumatoid arthritis	November2016	Biological: stem cell transplantation	Phase 1	Active, not recruiting	NCT03067870
Evaluation of stem cell therapy effects on the immune response in rheumatoid arthritis patients	June 20, 2016	Biological: autologous mesenchymal stem cells	Phase 1	Completed	NCT03333681
Umbilical cord tissue-derived mesenchymal stem cells for rheumatoid arthritis	October 2013	Biological: umbilical cord mesenchymal stem cells	Phase 1 Phase 2	Active, not recruiting	NCT01985464
Safety and efficacy study of umbilical cord-derived mesenchymal *stem cells* for *rheumatoid arthritis*	April 2013	Biological: Umbilical Cord-Derived Mesenchymal Stem Cells (UC-MSCs) Drug: Rheumatoid Arthritis with Disease-Modifying Drugs (DMARDs) Biological: UC-MSC + DMARDS	Phase 1 Phase 2	Unknown	NCT01547091
Transplantation of bone marrow derived mesenchymal *stem cells* in affected knee osteoarthritis by *rheumatoid arthritis*	October 2009	Biological: mesenchymal cell transplantation biological: placebo	Phase 2 Phase 3	Completed	NCT01873625
Rheumatoid arthritis: tolerance induction by mixed chimerism	September 2002	Biological: hematopoietic stem cell transplantation drug: fludarabine (and 5 other drugs)	Phase 1	Terminated	NCT00282412
Stem cell support in patients with rheumatoid arthritis	June 1997	Biological: immune ablation and hematopoietic stem cell transplant	Phase 1	Terminated	NCT00278551

this, risk/benefit assessments should be done more precisely. In addition, more prospective, randomized controlled trials are needed to be conducted with a long term follow up (Verburg et al. 2001; Álvaro-Gracia et al. 2017). The role of post-transplant immunosuppression, the timing of HSCT, and constituents of the conditioning regimen are some other issues we face in this kind of therapy (Van Laar and Tyndall 2006). Here on Table 1, there are some of the stem cell-based trials carried out with the aim of RA treatment:

Declarations Funding: There is no financial support for this work.

Conflicts of interest/Competing interests: There is no Conflicts of/Competing interest.

Availability of data and material: Not Applicable.

Code availability: Not Applicable.

Authors' contributions: Peyvand Parhizkar Roudsari wrote the first draft. Parisa Goodarzi , Akram Tayanloo-Beik, and Forough Azam Sayahpour helped to study and gather information, Mostafa Rezaei-Tavirani and Sepideh

Alavi-Moghadam extensively edited the manuscript. Bagher Larijani participated in a critical review. Babak Arjmand helped supervise the project and gave final approval of the version to be published.

Compliance with Ethical Standards

Funding: We did not receive any funding from any institution.

Conflict of Interest: There is no conflict of interest.

Ethical Approval: This article does not contain any studies with animals and human participants performed by any of the authors.

References

Abasolo L, Tobías A, Leon L, Carmona L, Fernandez-Rueda JL, Rodriguez AB, Fernandez-Gutierrez B, Jover JA (2013) Weather conditions may worsen symptoms in rheumatoid arthritis patients: the possible effect of temperature. Reumatología Clínica (English Edition) 9(4):226–228

Abdelmawgoud H, Saleh A (2018) Anti-inflammatory and antioxidant effects of mesenchymal and hematopoietic stem cells in a rheumatoid arthritis rat model. Adv Clin Exp Med 27(7):873–880

Abughanam G, Elkashty OA, Liu Y, Bakkar MO, Tran SD (2019) Mesenchymal stem cells extract (MSCsE)-based therapy alleviates xerostomia and Keratoconjunctivitis sicca in Sjogren's syndrome-like disease. Int J Mol Sci 20(19):4750

Aghayan HR, Soleimani M, Goodarzi P, Norouzi-Javidan-A, Emami-Razavi SH, Larijani B, Arjmand B (2014) Magnetic resonance imaging of transplanted stem cell fate in stroke. J Res Med Sci 19(5):465–471

Aghayan HR, Payab M, Mohamadi-Jahani F, Aghayan SS, Larijani B, Arjmand B (2020) GMP-compliant production of human placenta-derived mesenchymal stem cells. In: Methods in molecular biology. Springer, New York

Aletaha D, Smolen JS (2018) Diagnosis and management of rheumatoid arthritis: a review. JAMA 320 (13):1360–1372

Álvaro-Gracia JM, Jover JA, García-Vicuña R, Carreño L, Alonso A, Marsal S, Blanco F, Martínez-Taboada VM, Taylor P, Martín-Martín C (2017) Intravenous administration of expanded allogeneic adipose-derived mesenchymal stem cells in refractory rheumatoid arthritis (Cx611): results of a multicentre, dose escalation, randomised, single-blind, placebo-controlled phase Ib/IIa clinical trial. Ann Rheum Dis 76(1):196–202

Arbeláez-Cortés Á, Bejarano-Pineda L, Toro-Gutiérrez CE (2013) Autologous peripheral hematopoietic stem cell transplantation. Autoimmunity: from Bench to Bedside [Internet]. El Rosario University Press

Arjmand B, Goodarzi P, Mohamadi-Jahani F, Falahzadeh K, Larijani B (2017) Personalized regenerative medicine. Acta Med Iran 55(3):144–149

Arjmand B, Goodarzi P, Aghayan HR, Payab M, Rahim F, Alavi-Moghadam S, Mohamadi-Jahani F, Larijani B (2019) Co-transplantation of human fetal mesenchymal and hematopoietic stem cells in type 1 diabetic mice model. Front Endocrinol (Lausanne) 10:761

Askari A, Moskowitz RW, Goldberg VM (1974) Subcutaneous rheumatoid nodules and serum rheumatoid factor without arthritis. JAMA 229(3):319–320

Atoui A, El Labban M, Ghaoui N, El Darsa H, El Cheikh J, Bazarbachi A (2019) Complete remission of seronegative rheumatoid arthritis following haplo-identical stem cell transplantation for peripheral T-cell lymphoma. Bone Marrow Transplant 55:979–981

Baharlou R, Ahmadi-Vasmehjani A, Faraji F, Atashzar MR, Khoubyari M, Ahi S, Erfanian S, Navabi S-S (2017) Human adipose tissue-derived mesenchymal stem cells in rheumatoid arthritis: regulatory effects on peripheral blood mononuclear cells activation. Int Immunopharmacol 47:59–69

Barker N (2014) Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. Nat Rev Mol Cell Biol 15(1):19–33

Bergot A-S, Giri R, Thomas R (2020) The microbiome and rheumatoid arthritis. Best Pract Res Clin Rheumatol:101497. https://doi.org/10.1016/j.berh.2020.101497

Berthelot J-M, Le Goff B, Maugars Y (2019) Bone marrow mesenchymal stem cells in rheumatoid arthritis, spondyloarthritis, and ankylosing spondylitis: problems rather than solutions? Arthritis Res Ther 21 (1):239

Bickham K, Kivitz AJ, Mehta A, Frontera N, Shah S, Stryszak P, Popmihajlov Z, Peloso PM (2016) Evaluation of two doses of etoricoxib, a COX-2 selective non-steroidal anti-inflammatory drug (NSAID), in the treatment of Rheumatoid Arthritis in a double-blind, randomized controlled trial. BMC Musculoskelet Disord 17(1):331

Biehl JK, Russell B (2009) Introduction to stem cell therapy. J Cardiovasc Nurs 24(2):98

Brignier AC, Gewirtz AM (2010) Embryonic and adult stem cell therapy. J Allergy Clin Immunol 125(2): S336–S344

Burt RK, Georganas C, Schroeder J, Traynor A, Stefka J, Schuening F, Graziano F, Mineishi S, Brush M, Fishman M (1999a) Autologous hematopoietic stem cell transplantation in refractory rheumatoid arthritis: sustained response in two of four patients. Arthritis Rheum 42(11):2281–2285

Burt RK, Traynor A, Burns W (1999b) Hematopoietic stem cell transplantation of multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus. In: Advances in allogeneic hematopoietic stem cell transplantation. Springer, Boston, pp 157–184

Burt RK, Barr W, Oyama Y, Traynor A, Slavin S (2001) Future strategies in hematopoietic stem cell

transplantation for rheumatoid arthritis. J Rheumatol Suppl 64:42–48
Caglayan S, Ahrens TD, Cieślar-Pobuda A, Staerk J (2019) Chapter 3 Modern ways of obtaining stem cells. In: Stem cells and biomaterials for regenerative medicine. Elsevier, pp 17–36
Chan FK, Hung LC, Suen BY, Wu JC, Lee KC, Leung VK, Hui AJ, To KF, Leung WK, Wong VW (2002) Celecoxib versus diclofenac and omeprazole in reducing the risk of recurrent ulcer bleeding in patients with arthritis. N Engl J Med 347(26):2104–2110
Chen Y, Jobanputra P, Barton P, Bryan S, Fry-Smith A, Harris G, Taylor R (2008) Cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs (etodolac, meloxicam, celecoxib, rofecoxib, etoricoxib, valdecoxib and lumiracoxib) for osteoarthritis and rheumatoid arthritis: a systematic review and economic evaluation. NIHR Health Technology Assessment programme: Executive Summaries, NIHR Journals Library
Clark K, Zhang S, Barthe S, Kumar P, Pivetti C, Kreutzberg N, Reed C, Wang Y, Paxton Z, Farmer D (2019) Placental mesenchymal stem cell-derived extracellular vesicles promote myelin regeneration in an animal model of multiple sclerosis. Cell 8(12):1497
Crofford LJ (2013) Use of NSAIDs in treating patients with arthritis. Arthritis Res Ther 15(3):S2
Cush JJ, Kavanaugh A, Weinblatt ME (2010) Rheumatoid arthritis: early diagnosis and treatment. Professional Communications, West Islip
De Bari C (2015) Are mesenchymal stem cells in rheumatoid arthritis the good or bad guys? Arthritis Res Ther 17(1):113
de Oliveira PG, Farinon M, Sanchez-Lopez E, Miyamoto S, Guma M (2019) Fibroblast-like Synoviocytes glucose metabolism as a therapeutic target in rheumatoid arthritis. Front Immunol 10:1743
Edwards JC, Szczepański L, Szechiński J, Filipowicz-Sosnowska A, Emery P, Close DR, Stevens RM, Shaw T (2004) Efficacy of B-cell–targeted therapy with rituximab in patients with rheumatoid arthritis. N Engl J Med 350(25):2572–2581
El Qashty RMN, Mohamed NN, Radwan LRS, Ibrahim FMM (2018) Effect of bone marrow mesenchymal stem cells on healing of temporomandibular joints in rats with induced rheumatoid arthritis. Eur J Oral Sci 126(4):272–281
El-Jawhari J, El-Sherbiny Y, Jones E, McGonagle D (2014) Mesenchymal stem cells, autoimmunity and rheumatoid arthritis. QJM Int J Med 107(7):505–514
Farquhar H, Vassallo R, Edwards AL, Matteson EL (2019) Pulmonary complications of rheumatoid arthritis. In: Seminars in respiratory and critical care medicine. Thieme Medical Publishers, New York, NY
Faustman DL, Davis M (2010) Stem cells in the spleen: therapeutic potential for Sjogren's syndrome, type I diabetes, and other disorders. Int J Biochem Cell Biol 42(10):1576–1579
Firestein GS, McInnes IB (2017) Immunopathogenesis of rheumatoid arthritis. Immunity 46(2):183–196
Gabriel SE (2008) Cardiovascular morbidity and mortality in rheumatoid arthritis. Am J Med 121(10):S9–S14
Ge F, Lin H, Li Z, Chang T (2019) Efficacy and safety of autologous hematopoietic stem-cell transplantation in multiple sclerosis: a systematic review and meta-analysis. Neurol Sci 40(3):479–487
Goodarzi P, Aghayan HR, Soleimani M, Norouzi-Javidan-A, Mohamadi-Jahani F, Jahangiri S, Emami-Razavi SH, Larijani B, Arjmand B (2014) Stem cell therapy for treatment of epilepsy. Acta Med Iran 52 (9):651–655
Goodarzi P, Aghayan HR, Larijani B, Soleimani M, Dehpour AR, Sahebjam M, Ghaderi F, Arjmand B (2015) Stem cell-based approach for the treatment of Parkinson's disease. Med J Islam Repub Iran 29:168
Goodarzi P, Larijani B, Alavi-Moghadam S, Tayanloo-Beik A, Mohamadi-Jahani F, Ranjbaran N, Payab M, Falahzadeh K, Mousavi M, Arjmand B (2018) Mesenchymal stem cells-derived exosomes for wound regeneration. Adv Exp Med Biol 1119:119–131
Goodarzi P, Alavi-Moghadam S, Payab M, Larijani B, Rahim F, Gilany K, Bana N, Tayanloo-Beik A, Foroughi Heravani N, Hadavandkhani M, Arjmand B (2019a) Metabolomics analysis of mesenchymal stem cells. Int J Mol Cell Med 8(Suppl1):30–40
Goodarzi P, Falahzadeh K, Aghayan H, Payab M, Larijani B, Alavi-Moghadam S, Tayanloo-Beik A, Adibi H, Gilany K, Arjmand B (2019b) Therapeutic abortion and ectopic pregnancy: alternative sources for fetal stem cell research and therapy in Iran as an Islamic country. Cell Tissue Bank 20(1):11–24
Goodarzi P, Payab M, Alavi-Moghadam S, Larijani B, Rahim F, Bana N, Sarvari M, Adibi H, Foroughi Heravani N, Hadavandkhani M, Arjmand B (2019c) Development and validation of Alzheimer's disease animal model for the purpose of regenerative medicine. Cell Tissue Bank 20(2):141–151
Gurusamy N, Alsayari A, Rajasingh S, Rajasingh J (2018) Adult stem cells for regenerative therapy. Prog Mol Biol Transl Sci (Elsevier) 160:1–22
Hadjinicolaou AV, Nisar MK, Bhagat S, Parfrey H, Chilvers ER, Östör AJ (2011) Non-infectious pulmonary complications of newer biological agents for rheumatic diseases—a systematic literature review. Rheumatology 50(12):2297–2305
Harris HE, Tweedie F, White M, Samson K (2016) How to motivate patients with rheumatoid arthritis to quit smoking. J Rheumatol 43(4):691–698
Hatzimichael E, Tuthill M (2010) Hematopoietic stem cell transplantation. Stem Cells Cloning Adv Appl 3:105
Henriksson K, Jörgensen L, From J, Stratelis G (2013) FRI0539 Patient reported adherence to co-prescribed ppi gastro protection treatment in osteoarthritis, rheumatoid arthritis or ankylosing spondylitis patients using nsaid. Ann Rheum Dis 72(Suppl 3):A557–A557
Hirohata S, Yanagida T, Nakamura H, Yoshino S, Tomita T, Ochi T (2000) Bone marrow CD34+

progenitor cells from rheumatoid arthritis patients support spontaneous transformation of peripheral blood B cells from healthy individuals. Rheumatol Int 19 (4):153–159

Hirohata S, Yanagida T, Nagai T, Sawada T, Nakamura H, Yoshino S i, Tomita T, Ochi T (2001) Induction of fibroblast-like cells from CD34+ progenitor cells of the bone marrow in rheumatoid arthritis. J Leukoc Biol 70 (3):413–421

Irwin MR, Olmstead R, Carrillo C, Sadeghi N, FitzGerald JD, Ranganath VK, Nicassio PM (2012) Sleep loss exacerbates fatigue, depression, and pain in rheumatoid arthritis. Sleep 35(4):537–543

Johansson CB, Svensson M, Wallstedt L, Janson AM, Frisén J (1999) Neural stem cells in the adult human brain. Exp Cell Res 253(2):733–736

Jorgensen C, Noel D, Apparailly F, Sany J (2001) Stem cells for repair of cartilage and bone: the next challenge in osteoarthritis and rheumatoid arthritis. Ann Rheum Dis 60(4):305–309

Joseph R, Wang Y, Zhong L, Palecek SP, Nagy K, Agha-Mohammadi S (2020) Advances in induced pluripotent stem cell. In: Advances in induced pluripotent stem cell, p 185

Jung SY (2018) Adult stem cell treatment for rheumatoid arthritis. J Rheum Dis 25(3):158–168

Kafaja S, Segal K, Skerrett D, Itescu S, Furst D (2017) FRI0220 Allogeneic mesenchymal precursor cells (MPCS): a novel approach to treating biologic refractory rheumatoid arthritis. Ann Rheum Dis 76(Suppl 2):566–567

Källberg H, Ding B, Padyukov L, Bengtsson C, Rönnelid J, Klareskog L, Alfredsson L, E. S. Group (2011) Smoking is a major preventable risk factor for rheumatoid arthritis: estimations of risks after various exposures to cigarette smoke. Ann Rheum Dis 70 (3):508–511

Karamini A, Bakopoulou A, Andreadis D, Gkiouras K, Kritis A (2020) Therapeutic potential of mesenchymal stromal stem cells in rheumatoid arthritis: a systematic review of in vivo studies. Stem Cell Rev 16:276–287

Kartiko BH, Siswanto FM, Purwata TE (2017) Mesenchymal stem cell (MSC) as a potential cell therapy for immune related disease. Bali Med J 6(1):38–43

Khaddour K, Mewawalla P (2019) Hematopoietic stem cell transplantation. StatPearls [internet]. StatPearls Publishing

Kiatpongsan S, Tannirandorn Y, Virutamasen P (2006) Introduction to stem cell medicine. J Med Assoc Thail 89(1):111

Kim KW, Cho ML, Kim HR, Ju JH, Park MK, Oh HJ, Kim JS, Park SH, Lee SH, Kim HY (2007) Up-regulation of stromal cell–derived factor 1 (CXCL12) production in rheumatoid synovial fibroblasts through interactions with T lymphocytes: role of interleukin-17 and CD40L–CD40 interaction. Arthritis Rheum 56(4):1076–1086

Kim J-H, Lee YT, Oh K, Cho J, Lee D-S, Hwang Y-i (2014) Paradoxical effects of human adipose tissue-derived mesenchymal stem cells on progression of experimental arthritis in SKG mice. Cell Immunol 292(1–2):94–101

Kragstrup TW, Andersen T, Heftdal LD, Hvid M, Gerwien J, Sivakumar P, Taylor PC, Senolt L, Deleuran B (2018) The IL-20 cytokine family in rheumatoid arthritis and spondyloarthritis. Front Immunol 9:2226

Lampropoulos C, Orfanos P, Bournia V, Karatsourakis T, Mavragani C, Pikazis D, Manoussakis M, Tzioufas A, Moutsopoulos H, Vlachoyiannopoulos P (2015) Adverse events and infections in patients with rheumatoid arthritis treated with conventional drugs or biologic agents: a real world study. Clin Exp Rheumatol 33(2):216–224

Larijani B, NASLI EE, Amini P, Nikbin B, Alimoghaddam K, Amiri S, Malekzadeh R, Mojahed YN, Ghodsi M, Dowlati Y (2012) Stem cell therapy in treatment of different diseases. Acta Med Iran 50 (2):79–96

Larijani B, Goodarzi P, Payab M, Alavi-Moghadam S, Rahim F, Bana N, Abedi M, Arabi M, Adibi H, Gilany K, Arjmand B (2019a) Metabolomics and cell therapy in diabetes mellitus. Int J Mol Cell Med 8 (Suppl1):41–48

Larijani B, Goodarzi P, Payab M, Tayanloo-Beik A, Sarvari M, Gholami M, Gilany K, Nasli-Esfahani E, Yarahmadi M, Ghaderi F, Arjmand B (2019b) The design and application of an appropriate Parkinson's disease animal model in regenerative medicine. Adv Exp Med Biol 50:79–96

Larijani B, Foroughi Heravani N, Alavi-Moghadam S, Goodarzi P, Rezaei-Tavirani M, Payab M, Gholami M, Razi F, Arjmand B (2020) Cell therapy targets for autism Spectrum disorders: hopes, challenges and future directions. Adv Exp Med Biol

Lazzerini PE, Capecchi PL, Laghi-Pasini F (2017) Systemic inflammation and arrhythmic risk: lessons from rheumatoid arthritis. Eur Heart J 38(22):1717–1727

Lee YH, Seo YH (2018) SAT0167 Comparison of the efficacy and tolerability of tocilizumab, sarilumab, and sirukumab in patients with active rheumatoid arthritis: a bayesian network meta-analysis of randomized controlled trials. Ann Rheum Dis 77 (Suppl 2):944–944

Lee H-H, Song I-H, Friedrich M, Gauliard A, Detert J, Röwert J, Audring H, Kary S, Burmester G-R, Sterry W, Worm M (2007) Cutaneous side-effects in patients with rheumatic diseases during application of tumour necrosis factor-α antagonists. Br J Dermatol 156(3):486–491

Leung PSC, Shuai Z, Liu B, Shu SA, Sun L (2017) Stem cell therapy in the treatment of rheumatic diseases and application in the treatment of systemic lupus erythematosus. In: Mina-Osorio P (ed) Next-generation therapies and technologies for immune-mediated inflammatory diseases. Springer International Publishing, Cham, pp 167–198

Lipsky PE, van der Heijde DM, Clair EWS, Furst DE, Breedveld FC, Kalden JR, Smolen JS, Weisman M, Emery P, Feldmann M (2000) Infliximab and methotrexate in the treatment of rheumatoid arthritis. N Engl J Med 343(22):1594–1602

Littlejohn EA, Monrad SU (2018) Early diagnosis and treatment of rheumatoid arthritis. Prim Care 45 (2):237–255

Liu Y, Mu R, Wang S, Long L, Liu X, Li R, Sun J, Guo J, Zhang X, Guo J, Yu P, Li C, Liu X, Huang Z, Wang D, Li H, Gu Z, Liu B, Li Z (2010) Therapeutic potential of human umbilical cord mesenchymal stem cells in the treatment of rheumatoid arthritis. Arthritis Res Ther 12 (6):R210

Liu R, Li X, Zhang Z, Zhou M, Sun Y, Su D, Feng X, Gao X, Shi S, Chen W, Sun L (2015) Allogeneic mesenchymal stem cells inhibited T follicular helper cell generation in rheumatoid arthritis. Sci Rep 5 (1):12777

Luque-Campos N, Contreras-López RA, Paredes-Martínez MJ, Torres MJ, Bahraoui S, Wei M, Espinoza F, Djouad F, Elizondo-Vega RJ, Luz-Crawford P (2019) Mesenchymal stem cells improve rheumatoid arthritis progression by controlling memory T cell response. Front Immunol 10:8459281

Ma X, Xu S (2013) TNF inhibitor therapy for rheumatoid arthritis. Biomed Rep 1(2):177–184

Ma D, Xu K, Zhang G, Liu Y, Gao J, Tian M, Wei C, Li J, Zhang L (2019) Immunomodulatory effect of human umbilical cord mesenchymal stem cells on T lymphocytes in rheumatoid arthritis. Int Immunopharmacol 74:105687

Matcham F, Ali S, Hotopf M, Chalder T (2015) Psychological correlates of fatigue in rheumatoid arthritis: a systematic review. Clin Psychol Rev 39:16–29

Mattila K, Buttgereit F, Tuominen R (2014) Impact of morning stiffness on working behaviour and performance in people with rheumatoid arthritis. Rheumatol Int 34(12):1751–1758

McInnes IB, Schett G (2011) The pathogenesis of rheumatoid arthritis. N Engl J Med 365(23):2205–2219

McInnes IB, Buckley CD, Isaacs JD (2016) Cytokines in rheumatoid arthritis—shaping the immunological landscape. Nat Rev Rheumatol 12(1):63

Mellado M, Martínez-Muñoz L, Cascio G, Lucas P, Pablos JL, Rodríguez-Frade JM (2015) T cell migration in rheumatoid arthritis. Front Immunol 6:384

Mikkola HK, Orkin SH (2006) The journey of developing hematopoietic stem cells. Development 133 (19):3733–3744

Moore J, Milliken S, David JB, Brooks P (2001) Peripheral blood stem cell transplantation for rheumatoid arthritis–Australian experience. J Rheumatol 64:8–12

Moore J, Snowden J, Pavletic S, Barr W, Burt R (2003) Hematopoietic stem cell transplantation for severe rheumatoid arthritis. Bone Marrow Transplant 32(1): S53–S56

Mor A, Abramson SB, Pillinger MH (2005) The fibroblast-like synovial cell in rheumatoid arthritis: a key player in inflammation and joint destruction. Clin Immunol 115(2):118–128

Murrell W, Féron F, Wetzig A, Cameron N, Splatt K, Bellette B, Bianco J, Perry C, Lee G, Mackay-Sim A (2005) Multipotent stem cells from adult olfactory mucosa. Dev Dyn 233(2):496–515

Nikolov NP, Pavletic SZ (2008) Technology insight: hematopoietic stem cell transplantation for systemic rheumatic disease. Nat Clin Pract Rheumatol 4 (4):184–191

Nissen SE, Yeomans ND, Solomon DH, Lüscher TF, Libby P, Husni ME, Graham DY, Borer JS, Wisniewski LM, Wolski KE (2016) Cardiovascular safety of celecoxib, naproxen, or ibuprofen for arthritis. N Engl J Med 375:2519–2529

Noack M, Miossec P (2017) Selected cytokine pathways in rheumatoid arthritis. Semin Immunopathol 39 (4):365–383

Noss EH, Brenner MB (2008) The role and therapeutic implications of fibroblast-like synoviocytes in inflammation and cartilage erosion in rheumatoid arthritis. Immunol Rev 223(1):252–270

O'Connor D (2017) Introduction to gene and stem-cell therapy. In: Molecular and cellular therapies for motor neuron diseases. Elsevier, San Diego, pp 141–165

O'Dell JR, Haire C, Erikson N, Drymalski W, Palmer W, Maloley P, Klassen LW, Wees S, Moore GF (1996) Efficacy of triple DMARD therapy in patients with RA with suboptimal response to methotrexate. J Rheumatol Suppl 44:72–74

O'Dell JR, Mikuls TR, Taylor TH, Ahluwalia V, Brophy M, Warren SR, Lew RA, Cannella AC, Kunkel G, Phibbs CS (2013) Therapies for active rheumatoid arthritis after methotrexate failure. N Engl J Med 369(4):307–318

Park EH, Lim H s, Lee S, Roh K, Seo KW, Kang KS, Shin K (2018) Intravenous infusion of umbilical cord blood-derived mesenchymal stem cells in rheumatoid arthritis: a phase Ia clinical trial. Stem Cells Transl Med 7 (9):636–642

Payab M, Goodarzi P, Foroughi Heravani N, Hadavandkhani M, Zarei Z, Falahzadeh K, Larijani B, Rahim F, Arjmand B (2018) Stem cell and obesity: current state and future perspective. Adv Exp Med Biol 1089:1–22

Pedrosa M, Duarte C, Laranjeira P, Gomes J, Ribeiro T, Santos F, Antunes B, Pedreiro S, Henriques A, Martinho A, Fardilha M, Trindade H, da Silva J, Paiva A (2015) A1.10 Human bone marrow-derived mesenchymal stromal cells strongly inhibit cytokine production by naive, memory and effector $CD4^+$ and $CD8^+$ T cells from rheumatoid arthritis patients, independently of disease activity status. Ann Rheum Dis 74 (Suppl 1):A4–A5

Pincus T, Castrejón I (2013) Update on methotrexate as the anchor drug for rheumatoid arthritis. Bull NYU Hosp Jt Dis 71(suppl 1):S9

Ra JC, Kang SK, Shin IS, Park HG, Joo SA, Kim JG, Kang B-C, Lee YS, Nakama K, Piao M, Sohl B, Kurtz A (2011) Stem cell treatment for patients with autoimmune disease by systemic infusion of culture-expanded autologous adipose tissue derived mesenchymal stem cells. J Transl Med 9(1):181

Rahim F, Arjmand B, Shirbandi K, Payab M, Larijani B (2018) Stem cell therapy for patients with diabetes: a systematic review and meta-analysis of metabolomics-based risks and benefits. Stem Cell Investig 5:40

Scherer HU, Häupl T, Burmester GR (2020) The etiology of rheumatoid arthritis. J Autoimmun 110:102400

Schett G, Emery P, Tanaka Y, Burmester G, Pisetsky DS, Naredo E, Fautrel B, van Vollenhoven R (2016) Tapering biologic and conventional DMARD therapy in rheumatoid arthritis: current evidence and future directions. Ann Rheum Dis 75(8):1428–1437

Silva JMF, Ladomenou F, Carpenter B, Chandra S, Sedlacek P, Formankova R, Grandage V, Friswell M, Cant AJ, Nademi Z (2018) Allogeneic hematopoietic stem cell transplantation for severe, refractory juvenile idiopathic arthritis. Blood Adv 2(7):777–786

Snowden JA, Saccardi R, Allez M, Ardizzone S, Arnold R, Cervera R, Denton C, Hawkey C, Labopin M, Mancardi G, Martin R, Moore JJ, Passweg J, Peters C, Rabusin M, Rovira M, van Laar JM, Farge D, E. A. D. W. P. on behalf of the and P. Paediatric Diseases Working (2012) Haematopoietic SCT in severe autoimmune diseases: updated guidelines of the European Group for Blood and Marrow Transplantation. Bone Marrow Transplant 47 (6):770–790

Sun L, Akiyama K, Zhang H, Yamaza T, Hou Y, Zhao S, Xu T, Le A, Shi S (2009) Mesenchymal stem cell transplantation reverses multiorgan dysfunction in systemic lupus erythematosus mice and humans. Stem Cells 27(6):1421–1432

Sun Y, Kong W, Huang S, Shi B, Zhang H, Chen W, Zhang H, Zhao C, Tang X, Yao G (2017) Comparable therapeutic potential of umbilical cord mesenchymal stem cells in collagen-induced arthritis to TNF inhibitor or anti-CD20 treatment. Clin Exp Rheumatol 35 (2):288–295

Swann J (2011) Understanding rheumatoid arthritis: causes and treatment. Brit J Healthc Assist 5(1):15–17

Sykes M, Nikolic B (2005) Treatment of severe autoimmune disease by stem-cell transplantation. Nature 435 (7042):620–627

Tebib JG, Boughaba H, Letroublon MC, Bienvenu J, Noel E, Armanet P, Colson F, Roullet A, Bouvier M (1991) Serum IL-2 level in rheumatoid arthritis: correlation with joint destruction and disease progression. Eur Cytokine Netw 2(4):239–243

Tedeschi SK, Frits M, Cui J, Zhang ZZ, Mahmoud T, Iannaccone C, Lin TC, Yoshida K, Weinblatt ME, Shadick NA (2017) Diet and rheumatoid arthritis symptoms: survey results from a rheumatoid arthritis registry. Arthritis Care Res 69(12):1920–1925

Teng Y, Verburg R, Sont J, Van den Hout W, Breedveld F, Van Laar J (2005) Long-term followup of health status in patients with severe rheumatoid arthritis after high-dose chemotherapy followed by autologous hematopoietic stem cell transplantation. Arthritis Rheum 52(8):2272–2276

To K, Khan W (2019) Mesenchymal stem cell transplantation in rheumatoid arthritis. In: Stem cell transplantation for autoimmune diseases and inflammation. Springer, Cham, pp 63–74

Toes RE, Fibbe WE, Breedveld FC, van Laar JM (2002) High dose chemotherapy and autologous hematopoietic stem cell transplantation for rheumatoid arthritis: a review. Hum Immunol 63(8):627–637

Tuan RS, Boland G, Tuli R (2002) Adult mesenchymal stem cells and cell-based tissue engineering. Arthritis Res Ther 5(1):32

Ueyama H, Okano T, Orita K, Mamoto K, Sobajima S, Iwaguro H, Nakamura H (2020) Local transplantation of adipose-derived stem cells has a significant therapeutic effect in a mouse model of rheumatoid arthritis. Sci Rep 10(1):3076

Van Laar J, Tyndall A (2006) Adult stem cells in the treatment of autoimmune diseases. Rheumatology 45 (10):1187–1193

Verburg RJ, Kruize AA, van den Hoogen FH, Fibbe WE, Petersen EJ, Preijers F, Sont JK, Barge RM, Bijlsma JW, van de Putte LB (2001) High-dose chemotherapy and autologous hematopoietic stem cell transplantation in patients with rheumatoid arthritis: results of an open study to assess feasibility, safety, and efficacy. Arthritis Rheum 44(4):754–760

Vishwakarma A, Sharpe P, Shi S, Ramalingam M (2015) Chapter 1 An introduction to stem cell biology and tissue engineering. In: Stem cell biology and tissue engineering in dental sciences. Elsevier, pp 1–13

Walsh DA, McWilliams DF (2012) Pain in rheumatoid arthritis. Curr Pain Headache Rep 16(6):509–517

Wang Q, Li X-F, Ma L-H, Li J-X, Chen J-W, Li F, Xue L-J, Bai J (2011) Allogenic derived mesenchymal stem cells transplantation for 4 patients with rheumatoid arthritis [J]. Chin J Allergy Clin Immunol 3

Wang L, Wang L, Cong X, Liu G, Zhou J, Bai B, Li Y, Bai W, Li M, Ji H (2013) Human umbilical cord mesenchymal stem cell therapy for patients with active rheumatoid arthritis: safety and efficacy. Stem Cells Dev 22(24):3192–3202

Wang W, Zhou H, Liu L (2018) Side effects of methotrexate therapy for rheumatoid arthritis: a systematic review. Eur J Med Chem 158:502–516

Warrington KJ, Takemura S, Goronzy JJ, Weyand CM (2001) CD4+, CD28− T cells in rheumatoid arthritis patients combine features of the innate and adaptive immune systems. Arthritis Rheum 44(1):13–20

Weaver A (2004) The impact of new biologicals in the treatment of rheumatoid arthritis. Rheumatology 43 (suppl_3):iii17–iii23

Weinblatt ME, Kremer JM, Bankhurst AD, Bulpitt KJ, Fleischmann RM, Fox RI, Jackson CG, Lange M, Burge DJ (1999) A trial of etanercept, a recombinant tumor necrosis factor receptor: Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. N Engl J Med 340(4):253–259

Weinblatt ME, Keystone EC, Furst DE, Moreland LW, Weisman MH, Birbara CA, Teoh LA, Fischkoff SA, Chartash EK (2003) Adalimumab, a fully human anti–tumor necrosis factor α monoclonal antibody, for the treatment of rheumatoid arthritis in patients taking concomitant methotrexate: the ARMADA trial. Arthritis Rheum 48(1):35–45

Woodward WA, Chen MS, Behbod F, Rosen JM (2005) On mammary stem cells. J Cell Sci 118 (16):3585–3594

Wulffraat NM, De Kleer IM, Prakken B (2006) Refractory juvenile idiopathic arthritis: using autologous stem cell transplantation as a treatment strategy. Expert Rev Mol Med 8(26):1–11

Xia Y, Yin R, Fu T, Zhang L, Zhang Q, Guo G, Li L, Gu Z (2016) Treatment adherence to disease-modifying antirheumatic drugs in Chinese patients with rheumatoid arthritis. Patient Prefer Adherence 10:735

Ye L, Li L, Wan B, Yang M, Hong J, Gu W, Wang W, Ning G (2017) Immune response after autologous hematopoietic stem cell transplantation in type 1 diabetes mellitus. Stem Cell Res Ther 8(1):90

Zeher M, Papp G, Nakken B, Szodoray P (2017) Hematopoietic stem cell transplantation in autoimmune disorders: from immune-regulatory processes to clinical implications. Autoimmun Rev 16(8):817–825

Zerbini CAF, Clark P, Mendez-Sanchez L, Pereira RMR, Messina OD, Uña CR, Adachi JD, Lems WF, Cooper C, Lane NE, I. O. F. C. I. on behalf of the and G. Bone Structure Working (2017) Biologic therapies and bone loss in rheumatoid arthritis. Osteoporos Int 28(2):429–446

Zhao T, Zhang Z-N, Rong Z, Xu Y (2011) Immunogenicity of induced pluripotent stem cells. Nature 474 (7350):212–215

Zhou Y, Zhang X, Xue H, Liu L, Zhu J, Jin T (2019) Autologous mesenchymal stem cell transplantation in multiple sclerosis: a meta-analysis. Stem Cells Int 2019:8536785

Adv Exp Med Biol - Cell Biology and Translational Medicine (2021) 12: 187–188
https://doi.org/10.1007/978-3-030-71933-3

Index