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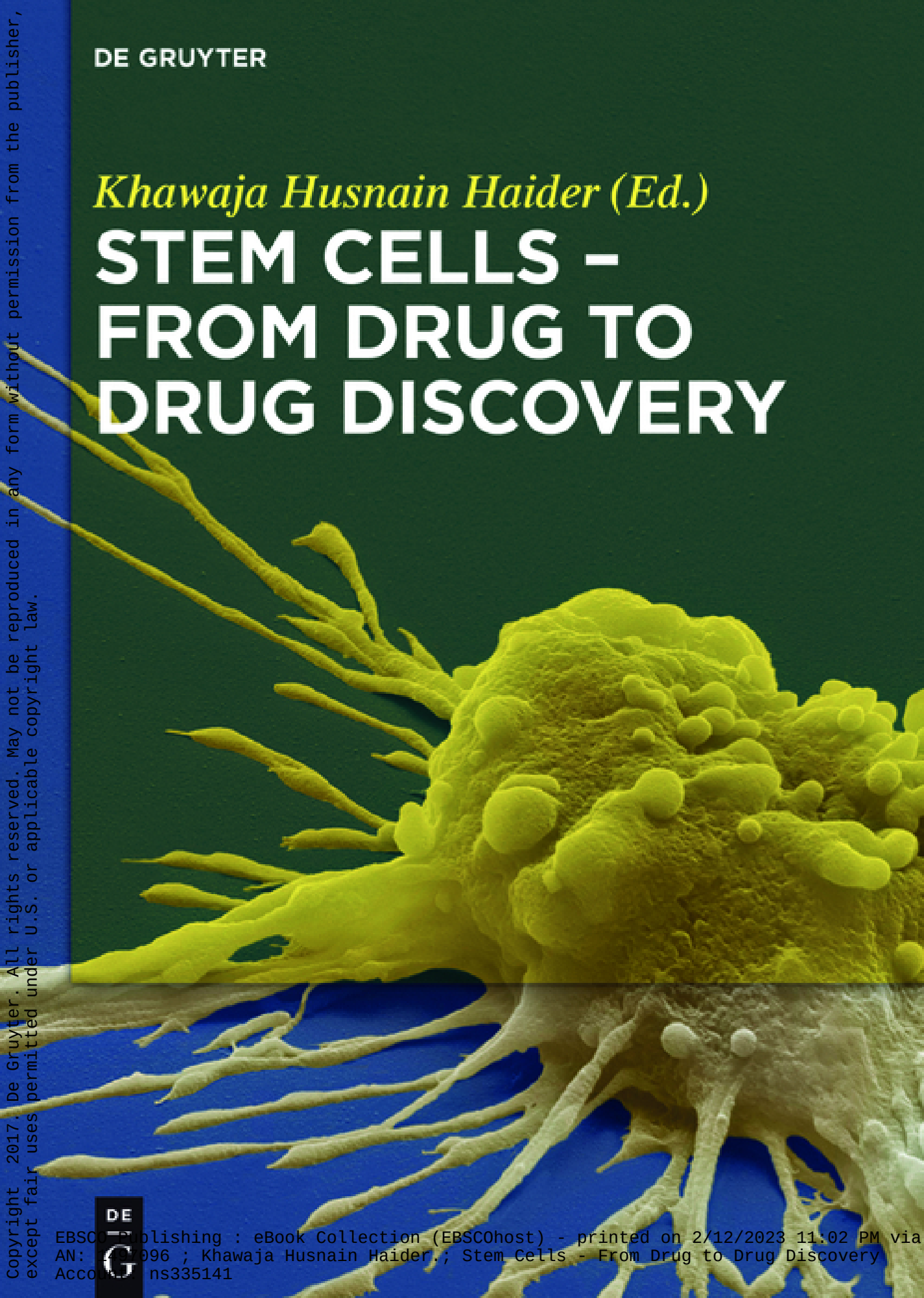
Khawaja Husnain Haider (Ed.)

STEM CELLS – FROM DRUG TO DRUG DISCOVERY



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EBSCO Publishing : eBook Collection (EBSCOhost) - printed on 2/12/2023 11:02 PM via
AN: [illegible]096 ; Khawaja Husnain Haider.; Stem Cells - From Drug to Drug Discovery
Acco[illegible] ns335141

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Stem Cells – From Drug to Drug Discovery

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ISBN 978-3-11-049628-4
e-ISBN (PDF) 978-3-11-049376-4
e-ISBN (EPUB) 978-3-11-049355-9

Library of Congress Cataloging-in-Publication data
A CIP catalog record for this book has been applied for at the Library of Congress.

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available in the Internet at http://dnb.dnb.de.

© 2017 Walter de Gruyter GmbH, Berlin/Boston
Cover image: STEVE GSCHMEISSNER/Brand X Pictures/Getty Images
Typesetting: Compuscript Ltd., Shannon, Ireland
Printing and binding: CPI books GmbH, Leck
♾ Printed on acid-free paper
Printed in Germany

www.degruyter.com

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Preface

Divergent from the contemporary progress in pharmacological interventions that can merely improve the quality of life of the patients, cell replacement therapy epitomizes a shift from conventional symptomatic treatment strategy to addressing the root cause of the disease process. Stem cells offer hope to patients suffering from diseases such as Alzheimer's, diabetes, myocardial infarction, etc., which have always been considered incurable. On the same note, stem cells provide excellent *in vitro* disease models for drug development. Sterling efforts of researchers around the globe during the last two decades have culminated in an exponential increase in understanding the nature and functioning of stem cells, nevertheless the routine clinical use of stem-cell-based therapeutic intervention still seems a distant goal. Given the clinical significance of the field of stem cells, this book is a compilation of the bench experience of experts from various research labs involved in this cutting edge area of research. The scope of the book spans over a wide range of topics from adult stem cells to pluripotent stem cells in regenerative medicine, either alone or as part of the combinatorial therapeutic approach as "drugs" besides their application as tools (disease model) during drug development. An important feature of the book is that it is an assortment of contributions from seasoned researchers to the young faculty from various prestigious institutions around the globe, sharing their first-hand research knowledge/data from the bench-side. Chapter 1 encompasses the novel application of pluripotent stem cells as a promising *in vitro* cellular platform for development of novel proangiogenic and antiangiogenic drug candidates. Given their ability of self-renewal, pluripotency and proficiency to recapitulate the embryological, physiological and pathological states of angiogenesis, the chapter highlights the benefits of using human pluripotent stem cell-derived vascular cells for drug discovery. Additionally, the authors elaborate on the methods developed in their research laboratory for differentiation of human embryonic stem cells to vascular cells and various co-culture models for drug screening studies in the pursuit to develop clinically useful drug compounds. Chapter 2 addresses the contentious issue of limited differentiation capacity of multipotent stem cells to adopt cardiac phenotype. While describing a detailed account of the issue, the authors introduce a list of synthetic and naturally occurring compounds with a focus on the role of small molecules in promoting differentiation of multipotent stem cells into morphofunctionally competent cardiac-like cells. On the same note, Chapter 3 provides an account of the findings relevant to the use of different miRNA clusters in the induction, maintenance and directed endothelial differentiation of pluripotent stem cells. The chapter is important on account of the emerging role of miRNAs in controlling the gene expression profile of a particular cell type in determining its differentiation fate in general and vascular development and regeneration in particular. Chapter 4 offers an update on different approaches for the cellular repair of damages and diseases of skin and cartilage and includes a review of stem cell sources and clinical perspectives on stem-cell-based therapeutic strategies.

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Unlike their multi- and pluripotent counterparts, satellite cells constitute a pool of resident stem cells of skeletal muscle on par with resident stem cells in other organs and tissues. They have been extensively studied in regenerative medicine and as carriers of transgene *in vivo* for stem-cell-based gene therapy. Chapter 5 discusses at length the biology of satellite cells, their characterization in experimental studies and successful reprogramming to pluripotency for application in regenerative medicine and drug development. Chapter 6 is an overview of the application of nano-vehicles of less than 100 nm size for genetic engineering of stem cells with a focus on the key issues and future directions in nanoparticle-based gene transfer. The authors provide an eloquent description of the advancements in the field including the beacon-like and missile-like modifications that are being used for tracking the fate of transplanted cells in real-time. Neural stem cells were first introduced in the early 90s and since then, remain the most well-studied cells in the field. Chapter 7 provides an overiew of the advancements in the field of neural stem cells and their clinical applications. The emerging paradigm shift in understanding the mechanism of stem-cell-based therapeutic benefits from differentiation capacity to paracrine activity of the transplanted cells has paved the way for new strategies to exploit their advantages from a clinical perspective. Chapter 8 summarizes the paracrine activity associated mechanisms of improved prognosis during cell-based therapy based on the available data with a special focus on the cell types which, after extensive characterization *in vitro* and during the pre-clinical studies, have progressed to clinical evaluation in human patients. The target audience of this book includes students studying regenerative medicine, cellular pharmacology, scientists involved in stem cell research in general and those involved in specific areas of adult stem cells as well as pluripotent stem cells, researchers in drug development interested in using stem cells as *ex-vivo* models for drug testing.

Last of all, while thanking my co-authors for their contributions, I take the opportunity to briefly share my personal experience of working with stem cells for nearly two decades. Handling these tiny little cells, I have learnt much more than their use in regenerative medicine. They have been my mentors who taught me the importance of team-work. They have taught me the significance of commitment and dedication and more than that, they have shown me the importance of truthfulness and resilience to stand for truth in life with a small wish “only if truth has a tongue”.

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List of abbreviations

3D= three dimensional
5-aza= 5-azacytidine
AC-dECM= acellular porcine chondrocyte-derived ECM
AD= Alzheimer's disease
ADSCs= adipose-derived MSCs
AGO= argonaute
AscI1= achaete-scute homolog 1
BDNF= brain-derived neurotrophic factor
bFGF= basic fibroblast growth factor
BLBP= brain lipid binding protein
BM= bone marrow
BM-MSCs= bone-marrow-derived mesenchymal stem cells
BMP-2= bone morphogenic protein-2
BMP-4= bone morphogenetic protein-4
bPEI= branched polyethyleneimine
CAD= coronary artery disease
CAM= chorioallontoic membrane
CDCs= cardiosphere-derived cells
CME= clathrin-mediated endocytosis
CSCs= cardiac stem cells
CST= cystathionine-y-lyase
cTn-I= cardiac troponin I
cTn-T= cardiac troponin T
CvME= caveolae-mediated endocytosis
CX43= connexin-43
CXCR4= CX chemokine receptor 4
DMD= Duchenne muscular dystrophy
DMSO= dimethylsulfoxide
DNMTs= NA methyltransferases
EBs= embryoid bodies
ECM= extracellular matrix
ECs= endothelial cells
ECVAM= European Center for the Validation of Alternative Methods
EGF= epidermal growth factor
ELISA= enzyme-linked immunosorbent assay
EPC= endothelial progenitor cells
EPO= erythropoietin
EPOR= erythropoietin receptor
ERK= extracellular signal related kinases
ESCs= embryonic stem cells
FACS= flow activated cell sorter
FGF= fibroblast growth factor
GAGs= glycosaminoglycans
G-CSF= granulocyte colony-stimulating factor
GFAP= glial-fibrillary acidic protein
GLAST= glutamate aspartate transporter
GM-CSF= granulocyte-macrophage colony-stimulating factor

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GMP= good manufacturing practice
GNDF= glial cell-derived neurotropic factor
GRPs= glial-restricted progenitor cells
GSK-3β= glycogen synthase kinase-3 beta
GT= gelatin/β-tricalcium phosphate
HA= hyaluronic acid
hAHFSCs= human amniotic fluid stem cells
HA-MSCs= human amniotic membrane-derived stem cells
HDAC9= histone deacetylase 9
HDACs= histone deacetylases
HGF= hepatocyte growth factor
HMG-CoA= 3-hydroxy-3-methylglutaryl coenzyme-A
HMONs= hybrid mesoporous organosilica nanoparticles
HOXA5= homeobox A5
hPAMAM= hyperbranched poly(amidoamine)
ICAM-1= intercellular adhesion molecule-1
ICCVAM= Interagency Coordinating Committee on the Validation of Alternative Methods
IFN= interferon
IGF= insulin-like growth factor
IL= interleukin
IPCs= intermediate progenitor cells
iPSCs= induced pluripotent stem cells
JACVAM= Japanese Center for the Validation of Alternative Methods
LAD= left anterior descending
LV= left ventricle
MBD= methyl cytosine binding domain
MCP-1= monocyte chemoattractant protein-1
MCs= mural cells
MEF2c= myocyte enhancer factor-2c
MEFs= mouse embryonic fibroblasts
MET= mesenchymal-to-epithelial
MHC= myosin heavy chain
miR/miRNA= micro RNA
MMP-2= matrix metalloproteinase-2
MPC= muscle precursor cells
MRFs= myogenic regulatory factors
MSCs= mesenchymal stem cells
Myf5= myogenic factor 5
MyoD= myogenic differentiation
NBMPR= nitrobenzylmercaptopurine riboside
NCAM= neural cell adhesion molecule
NCSCs= neural crest stem cells
NEPs= neuro-epithelial cells
NeuN= neural nuclear antigen
NFT= neurofibrillary tangles
NGF= nerve growth factor
NLC= nuclear localization signal
NPC= nuclear pore complexes
NPY= neuropeptide Y
NRPs= neuron-restricted progenitor cells
NRSF= neuron-restrictive silencer factor

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NSC= neural stem cells
NSE= non-specific enolase
nt.= nucleotide
OA= osteoarthritis
OB= olfactory bulb
OPMD= oculopharyngeal muscular dystrophy
Pax7= paired box transcription factor 7
PD= Parkinson's disease
PDGF= platelet-derived growth factor
PECAM-1= platelet endothelial adhesion molecule-1
PEDF= pigment epithelium derived factor
PEG= polyethylene glycol
PEI= polyethyleneimine
PEMS= placenta-derived extracellular matrix sponges
PHD-2= propyl-hydroxylase domain 2
PlGF= placenta-derived growth factor
PNS= peripheral nervous system
pri-miRs= primary micro RNA
PRP= platelet-rich plasma
PSCs= pluripotent stem cells
PTEN= phosphatase and tensin homolog
RA= rheumatoid arthritis
REST= repressor element1-silencing transcription
RG= radial glia
RISC= RNA-induced silencing complex
RITS= RNA-induced transcriptional silencing
ROS= reactive oxygen species
RSM= rostral migratory stream
SCI= spinal cord injury
SDF= stem-cell-derived factor
SF-MSCs= synovial fluid MSCs
SGZ= subgranular zone
SIP= sphingosine-1-phosphate
SkMs= skeletal myoblasts
SMCs= smooth muscle cells
SNAP= S-nitroso-N-acetyl-D,L-penicillamine
Sox2= SRY (sex determining region Y)-box 2
SPARC= secreted protein acidic and rich in cysteine
SSD= silver sulfadiazine
SVZ= subventricular zone
Tcf4= transcription factor 4
TGF-β= transforming growth factor-β
TIMPS= tissue inhibitor of metalloproteinase
TNF-α= tumor necrosis factor-α
tPA= tissue plasminogen activator
Trib3= tribbles homologue 3
VEGF= vascular endothelial growth factor
VSELs= very small embryonic epiblast-like stem cells
vSMCs= vascular smooth muscle cells
WJ-MSCs= Wharton's jelly MSCs

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Sriram Gopu and Cao Tong

1 Human pluripotent stem-cell-derived vascular cells: *in vitro* model for angiogenesis and drug discovery

Abstract: Angiogenesis is the process of formation of new blood vessels in development, health and diseased states. It is an intricate process involving a multitude of overlapping phases that encompass cell-cell and cell-matrix interactions between the vascular cells (in particular, endothelial cells and vascular smooth muscle cells/pericytes) and the surrounding extracellular matrix. Assays to investigate angiogenesis are used in the screening of novel proangiogenic and antiangiogenic drug candidates that have potential in the treatment of ischemic diseases, cancers, a range of vascular disorders and for therapeutic vascularization in regenerative medicine. Most assays rely on *in vitro* models of angiogenesis using monolayer cultures of primary and immortalized ECs and *in vitro* animal models. Pluripotent stem cells (PSCs) like human embryonic stem cells (ESCs) due to their ability to self-renew, differentiate to any cell type in the body, and recapitulate the embryological, physiological, and pathological states of angiogenesis, provide a promising *in vitro* cellular platform for development of novel proangiogenic and antiangiogenic drug candidates. This chapter highlights the benefits of using human PSC-derived vascular cells for drug discovery, the methods developed in our laboratory for differentiation of human ESCs to vascular cells and various co-culture models for drug screening studies. Further, we will discuss some of the obstacles and challenges that need to be addressed for stem-cell-based approaches to be used as an effective tool in the pursuit to develop clinically successful drug compounds.

1.1 Introduction

With the tremendous amount of investment and growth in automation and high-throughput screening, increasingly large number of compound libraries are synthesized and screened for angiogenesis promoters and inhibitors. However, the discovery of drugs targeting angiogenesis is hampered by the wide gap between lead compound validation and its clinical success. One of the primary reasons for this inefficiency in translating lead compounds into the clinic is the unpredictability of the currently used *in vitro* angiogenesis assays and the complexity of *in vivo* microenvironment. The vascular system is composed of a complex and heterogeneous three-dimensional (3D) organization of endothelial cells (ECs) and mural cells (that include pericytes, vascular smooth muscle cells (vSMCs), and fibroblasts) within the surrounding extracellular matrix (ECM). To compound this complexity, both ECs and mural cells are

DOI 10.1515/9783110493764-001

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heterogeneous populations with differences in gene expression and functional phenotype among different vessels (arteries, veins, arterioles, venules, capillaries and lymphatics) and among different tissues and organs [1–5]. On the contrary, most high-throughput screening assays utilize monolayer cultures of primary or immortalized ECs without the 3D microenvironment and their interaction with surrounding mural cells and ECM, a highly reductionist approach wherein the crucial elements of drug-biology interaction are lost [6]. Furthermore, the lead compounds are validated and optimized in similar simplified *in vitro* systems and animal models. Based on the regulatory requirements, lead compounds are validated on at least two animal models (one rodent and one non-rodent species). Numerous data have confirmed the limited predictive value of animal models (especially rodent assays) in the human context due to fundamental differences in the physiology between humans and animals [7, 8].

These shortcomings eventually lead to clinical failures and dramatic increases in the costs of the drug discovery process that are recognized by the pharmaceutical industry, yet few solutions are currently available. This demand for better *in vitro* angiogenesis models has triggered researchers within academia and pharmaceutical industry to develop physiologically more relevant *in vitro* human models for screening and validation. From this perspective, this book chapter highlights the promise of human pluripotent stem cells (PSCs) and their vascular progenies for developing angiogenesis assays and their use in drug screening. Further, we will discuss some of the obstacles and challenges that need to be addressed for stem-cell-based approaches for use as an effective tool in pursuit to develop clinically successful drug compounds.

1.2 Formation of blood vessels

Neoangiogenesis is the formation of new blood vessels during embryonic development and postnatal life that can occur through two distinct processes, namely vasculogenesis and angiogenesis. Vasculogenesis is the process of formation of primitive plexus of vessels *de novo* from differentiation of vascular progenitors [9, 10]. Vasculogenesis predominantly occurs during the early stages of embryonic development. In the embryo, vascular progenitors derived from lateral plate mesoderm give rise to ECs that form the primitive blood vessels. Mounting evidence from the published data suggests that vasculogenesis occurs in the postnatal life as well through the endothelial progenitors in the bone marrow and circulating angiogenic cells.

Angiogenesis, however, is the formation of new blood vessels from a pre-existing vascular network. This process is involved in the development of vasculature in the embryo, growing tissues, tissue repair, uterine endometrium and in diseased states including cancer [11]. Angiogenesis is a complex morphogenetic process that

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implicates both cellular and extracellular components and includes the following events:

1. Activation of resting ECs of the target (preexisting) vessel to a migratory EC phenotype.
2. Degradation of ECM surrounding the target blood vessel.
3. Migration of activated ECs through the basement membrane into the surrounding stromal tissues.
4. Proliferation of the activated ECs to form solid sprouts or vascular cords directed towards the angiogenic source.
5. Migration and proliferation of mural cells (pericytes and/or vascular smooth muscle cells) along the perimeter of the vascular cords. Mural cells are contractile and supportive cells that are associated with all vascular channels and play a role in vascular maturation, vascular remodeling and maintaining the vessel tone [12]. These mural cells are mostly found in the larger vessels (arteries and veins) and "are known as vSMCs, whereas the ones present in association with the smaller channels (arterioles, capillaries, venules) are referred to as pericytes [13].
6. Lumenization of the vascular cords resulting in patent blood vessels.
7. Remodeling and maturation of the newly formed blood vessels through the recruitment of mural cells. Formation of mature and functional microvascular network relies on the interaction between ECs and mural cells [14, 15]. During early embryogenesis and throughout the postnatal life, newly formed endothelial vessels recruit mural cells resulting in a complex network of arteries, arterioles, capillaries, venules and veins [9, 10]. Without the mural cells, neo-endothelial structures undergo regression thus suggeting the crucial role of mural cells in providing structural and functional support to the nascent endothelial vessels [11, 15].

1.3 Current models to assess angiogenesis

Insufficient vascularization contributes to the pathogenesis of ischemic disorders such as myocardial infarction, peripheral vascular disease and stroke. However, abnormal or excessive vascularization can contribute to the pathogenesis of cancer, diabetic retinopathy, macular degeneration, psoriasis, vascular malformations and other diseases [11]. Therapeutic strategies using proangiogenic and antiangiogenic agents that enhance or decrease the growth of blood vessels, respectively, could be used as therapeutic targets to treat these disorders. Various *in vitro* assays are currently used to assess proangiogenic and antiangiogenic activities that encompass the key events in angiogenesis, i.e. matrix degradation, migration, proliferation and morphogenesis (Tab. 1.3.1).

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Tab. 1.3.1: *In vitro* assays to assess vasculogenesis and angiogenesis.

	Angiogenic event assayed	Cell types	ECM
Monolayer assays			
– Zymogen assay	Matrix degradation	ECs	–
– Transwell/Boyden chamber assay	Migration	ECs (± MCs)	–
– Scratch wound assay	Migration	ECs (± MCs)	–
– Thymidine/BrdU incorporation	Proliferation	ECs	–
– MTT assay	Proliferation	ECs	–
– Annexin V/TUNEL assays	Proliferation (apoptosis)	ECs	–
– Matrigel assay (Seed On)	Morphogenesis	ECs (± MCs)	+
– Transendothelial electrical resistance (TEER) assay	Vascular permeability	ECs	–
– Transendothelial vascular permeability assay	Vascular permeability	ECs	–
3D assays			
– Collagen/Matrigel encapsulation assay	Morphogenesis, EC-Mural cell interaction	ECs (± MCs)	+
– Collagen sandwich assay	Morphogenesis, EC-Mural cell interaction	ECs (± MCs)	+
– Spheroid assay (Matrix embedded)	Morphogenesis, EC-Mural cell interaction	ECs (± MCs)	+
– Collagen/Matrigel invasion assay	Matrix degradation, migration, morphogenesis, EC-Mural cell interaction	ECs (± MCs)	+
3D-organ culture assays			
– Aortic ring assay	Matrix degradation, proliferation, migration, morphogenesis	ECs + MCs	+
– Chick aortic arch assay	Matrix degradation, proliferation, migration, morphogenesis	ECs + MCs	+
– Mouse metatarsal assay	Matrix degradation, proliferation, migration, morphogenesis	ECs + MCs	+

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Tab. 1.3.1 (continued)

	Angiogenic event assayed	Cell types	ECM
Stem-cell-based models			
– 2D co-culture model (ECs seeded on lawn of mural cells)	Vasculogenesis and angiogenesis (proliferation, morphogenesis), EC-Mural cell interaction	Stem cell-derived ECs and MCs	±
– Embryoid body model	Vasculogenesis and angiogenesis (matrix degradation, proliferation, migration, morphogenesis)	Stem cell-derived ECs and/or MCs	+
– 3D vascular spheroid model (floating/ embedded in ECM)	Morphogenesis, EC-Mural cell interaction, migration, matrix degradation	Stem cell-derived ECs and MCs	±
– 3D vascular organoid model (*in vitro* vascularized tissue equivalents)	Vasculogenesis and angiogenesis (matrix degradation, proliferation, migration, morphogenesis), EC-MC interaction, vascular permeability,	Stem cell-derived ECs and MCs	+

ECs = endothelial cells, ECM = extracellular matix, MCs = mural cells

1.3.1 *In vitro* assays

1.3.1.1 *In vitro* cell-based assays

Matrix degradation

Sprouting of new vessels requires degradation and proteolysis of basement membrane and connective tissue surrounding the parent blood vessel. Matrix degradation involves a family of proteases that includes matrix metalloproteinases (MMPs), other metalloproteinases, cathepsins, serine proteases and aminopeptidases. Activity of MMPs can be assessed using zymogen and matrix invasion assays. Zymogen assays are relatively inexpensive and provide the identities and relative levels of MMPs present in supernatants and lysates from endothelial cultures [16]. However, they do not allow differentiation of active and inactive MMPs or quantification of their relative expression levels.

Endothelial migration

Matrix degradation is followed by migration of ECs into the surrounding tissue either through chemokinesis (random cellular movements) or chemotaxis (directed

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migration towards a stimulatory agent). Assays that could be used to investigate EC migration include scratch wound, transwell and under-agarose assays. In the scratch wound assays, a wound is introduced on a confluent layer of ECs and the amount of coverage of the wound is quantified over time. With the advent of automated imaging and image analysis tools, scratch wound assays are relatively simple and fast assays amenable for high-throughput screening. One of the limitations of this assay includes the difficulty in creating uniform wound size resulting in intra- and inter-experiment variability. Recently, specialized culture inserts are commercially available that could provide wounds of defined size and margins. Though scratch wound assays measure cell motility, distinction between chemokinesis or chemotaxis is not possible. In the transwell assay (also known as Boyden chamber assay), the migration of ECs across a porous membrane (pore size ≥3 μm) in response to a potential angiogenic agent is quantified [17]. Advantages of transwell assays include their high degree of sensitivity and reproducibility relative to other migration assays. Since the concentrations of angiogenic agent across the porous membrane would normalize quickly, the migration of ECs is more likely due to chemokinesis rather than chemotaxis. Migration assays that allow assessment of chemotaxis are under-agarose assay and chemotaxis chamber slides. In the under-agarose assay, the migration of ECs across a 2 mm thick agarose gel in the direction of a potential migratory factor is assessed in relation to the distance of migration on the opposite direction [18]. It is inexpensive and allows clear distinction between chemokinesis and chemotaxis, but is less sensitive than transwell assays, difficult to quantify and not amenable for high-throughput screening. Chemotaxis chamber slides (ibidi GmbH, Germany) are specialized slides that allow the quantification of ECs migration. These chamber slides contain a strip where ECs are cultured and two chambers on either side of the culture strip (one chamber for chemoattractant and the other for plain media). Live cell imaging and automated image analysis of ECs migration across the culture area allows differentiation and quantification of the amount of ECs migration due to chemokinesis and chemotaxis.

Endothelial proliferation and apoptosis

ECs migration is followed by endothelial proliferation and increased survival that provides the cells needed for new vessel formation. Effect of potential proangiogenic or antiangiogenic agents on ECs proliferation can be assessed by quantification of cell count, DNA synthesis (thymidine incorporation assays) and metabolic activity (MTT/MTS assay). Similarly, assays used for assessment of ECs apoptosis include TUNEL, caspase and annexin V assays.

Assays for vascular morphogenesis

Neoangiogenesis involves the assembly and reorganization of ECs into cords and lumenized vessels. This stage of vascular morphogenesis can be assayed using ECs plated on, in or between ECM substrates like Matrigel or collagen type-I. The most

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commonly used assay for endothelial morphogenesis is the Matrigel tube formation assay [19]. In this assay, ECs are plated on Matrigel (an ECM isolated from Engelbreth-Holm-Swarm mouse sarcoma cells) and the formation of a network of branching structures is imaged typically over a period of 6–24 hours. Various parameters like length and branching points of capillary-like structures per unit area are quantified. This assay is easy to set up, rapid, amenable for imaging and high-throughput assessment. However, the Matrigel is extremely rich in angiogenic growth factors and hence, formation of capillary-like structures is not induced above baseline (positive control like VEGF) [20]. Growth-factor reduced preparations of Matrigel allow the assessment above baseline, but are not completely free of endogenous growth factors. Second, most of the endothelial structures on Matrigel resemble cords or cell process and do not contain a lumen. Further, other cell types including fibroblasts, mesenchymal stem cells and cancer cells also have the ability to form network-like structures on Matrigel. Hence, this assay is most suitable for assaying antiangiogenic agents.

An alternative assay for vascular morphogenesis is to suspend or encapsulate ECs with or without mural cells in collagen gels and assess the formation of capillary-like structures [21]. In contrast to plating ECs on Matrigel, encapsulation within ECM gels allows the formation of capillary-like structures with lumen and maintenance over several days. This assay allows the assessment of both angiogenic and antiangiogenic agents. However, quantification of the endothelial structures in thick gels requires imaging at multiple planes using histological sections, confocal imaging or two-photon microscopy. Another alternative *in vitro* assay to produce endothelial tubes is the collagen sandwich assay [22]. In this assay, ECs are plated at sub-confluence onto a layer of collagen gel. After the ECs adhere to the collagen gel, they are covered with a second layer of collagen. The capillary-like structures formed in this assay can be maintained for several days similar to the encapsulation method. Though time consuming, the formation of capillary-like structures primarily in two dimensions at the junction of the collagen gels enables imaging and easier quantification. As exogenous growth factors are needed for the formation of extensive network of endothelial structures, this assay is well suited for assessment of proangiogenic agents.

Sprouting angiogenesis assays

Angiogenesis *in vivo* involves sprouting from a confluent layer of ECs in pre-existing vessels. Several assays have been developed to model sprouting angiogenesis using confluent ECs monolayers. Sprouting angiogenesis can be assayed by inducing ECs grown to confluence on collagen or fibrin gels to invade the matrix and form lumen-containing endothelial structures. Alternatively, sprouting angiogenesis can be modelled using endothelial spheroids or ECs grown to confluence on microbeads which are then embedded within the collagen or fibrin gels. Since, the lumen-containing sprouts form in 3D, assessment of endothelial sprouting requires histological sectioning or confocal microscopy. Recently, Zeitlin et al. developed a novel sprouting angiogenesis assay termed Responsive Angiogenic Implanted Network (RAIN)-Droplet

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model of angiogenesis [23]. In this model, ECs were allowed to self-assemble into toroids (doughnut-shaped) within a hydrogel droplet and then plated onto a collagen gel. This model allowed the formation of radial sprouts of endothelial structures in the horizontal plane allowing easy visualization and quantification.

Considerations on *in vitro* assays

During the process of angiogenesis, the resting phenotype of ECs changes to an activated, migratory state and then back to resting state. In the activated state, the ECs move from a monolayer to a 3D macromolecular environment. Secondly, the interaction between ECs and mural cells play a crucial role in the formation of mature and functional blood vessels. Furthermore, interaction between ECs and surrounding ECM actively regulates the behavior of ECs through various mechanisms that include the provision of substratum for attachment and migration of ECs, acting as reservoir of soluble angiogenic factors, influence on the gene expression patterns, and provision of directional and mechanical cues for blood vessel development. In this regard, it should be noted that the response of ECs grown on monolayer cultures would be different from those grown in 3D macromolecular matrices and in co-culture microenvironments. Hence, the observations from assays based on ECs monolayer cultures must be interpreted with caution. *In vivo* models and to certain extent, 3D *in vitro* assays allow dynamic interaction between multiple cell types and ECM. Monolayer *in vitro* assays allow assessment of isolated events in angiogenesis, while 3D *in vitro* and *in vivo* assays allow investigation of angiogenesis as a whole. *In vitro* assays are less expensive, allow genetic manipulations, can be adapted for high-throughput screening, and automated analysis. Compared to monolayer culture assays, 3D *in vitro* assays have the potential to investigate the effect of diffusion gradients of factors that have stimulatory and inhibitory effects on angiogenesis.

However, it is important to note the need for *in vitro* models that mimic the complex *in vivo* physiology and yet be cost-efficient, amenable for high-throughput screening and disease modelling. An ideal *in vitro* angiogenesis model should have the following characteristics: (1) be amenable for quantitative analysis of the structure of the new vasculature that includes vessel length, surface area, volume, number of vessels, branch-points and other fractal dimensions of the vascular network; (2) provide a quantitative degree of functional characteristics of the new vasculature that includes ECs migration rate, proliferation rate, canalization rate and vascular permeability; (3) provide the release and spatiotemporal concentration distribution of stimulatory/inhibitory factor(s) for generating dose-response curves; (4) provide distinction between newly formed and existing vasculature; (5) amenable for long-term non-invasive monitoring, and (6) cost-effective, easy to use, reliable, and reproducible [24].

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1.3.1.2 Organ culture assays

The cell-culture-based assays previously described are primarily based on monoculture of ECs. In contrast, organ culture assays permit the assessment of angiogenesis in a microenvironment composed of multiple cell lineages. In addition to modeling various stages of angiogenesis, organ culture assays also assess the recruitment of pericytes to new vessels. All the organ culture assays are all similar in that the segments of specific tissues are cultured *ex vivo* in a 3D matrix, and the vessel outgrowths observed over a period of 1–2 weeks. In general, the *ex vivo* explant culture systems are considered to mimic *in vivo* angiogenesis because they include mural cells and a supporting matrix. Additionally, the phenotype of ECs is not altered by *in vitro* subculture.

Aortic ring assay

Aortic ring assay is an organ culture method wherein the isolated rat or mouse thoracic aorta is cut into segments (after removal of adventitia) and embedded into Matrigel or collagen gels [6, 25, 26]. Over the next 12 weeks, the aortic explants are monitored for endothelial outgrowths (in the presence or absence of test agents). Length and abundance of vessel-like outgrowths are quantified using phase contrast or immunohistological methods. This assay allows evaluation of both proangiogenic and antiangiogenic agents. However, this assay system is associated with various disadvantages. Variability in amount of residual adventitia and thickness of each segment influences the amount of vessel outgrowth. Further, differences in the age and strain of the mice or rat also lead to variability in angiogenic responses. Finally, sprouting angiogenesis *in vivo* is primarily a microvascular event and not from major vessels like the aorta.

Chick aortic arch assay is a modification of the aortic ring assay in which segments of embryonic aortic arch are cultured *ex vivo* [27]. In spite of their origin from large vessels, embryonic ECs from embryonic aortic arch are similar to microvascular ECs. However, as these cells are derived from developing embryos, they have inherently proliferative nature and hence, do not model the quiescent state of ECs. This assay also carries the advantage of being rapid and ability to perform under serum-free conditions.

Mouse metatarsal assay

Mouse metatarsal assay is an *ex vivo* organ culture assay in which vessel outgrowths arise from smaller vessels within 17-day-old fetal mouse metatarsals [28, 26]. Vessel outgrowths from the microvascular ECs within the perichondrium are robust within 7–10 days and can be used to assess proangiogenic and antiangiogenic agents. The endothelial outgrowth from bone microvasculature mimics the typical angiogenesis observed *in vivo* compared to that from large vessels like aorta. However, it is time consuming to isolate the metatarsal bone and is associated with variability depending on the amount of surrounding tissues and degree of handling. Similar to the

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chick aortic arch assay, the metatarsals are embryonic in origin and hence, do not represent the quiescent state of micro vessels.

A major drawback of all the organ culture assays is the use of non-human tissues and hence raises concerns over their translation as preclinical screening assays. To overcome the species-specificity related concerns, explant models based on human placental or umbilical blood vessels have been proposed. However, these are large vessels and are not truly representative of *in vivo* angiogenesis.

1.3.2 *In vivo* models of angiogenesis

Generally, combination of several *in vitro* and *ex vivo* assays described in the previous sections are used to assess proangiogenic and antiangiogenic agents. However, the results must be interpreted with caution and drawing comparisons between different assays are often difficult due to differences in origin of ECs, passage numbers, matrix and media compositions used. Secondly, the *in vitro* conditions do not mimic the complex microenvironment observed *in vivo*. Hence, *in vivo* evaluation of the agents is a crucial step in the drug development process. Various *in vivo* assays and disease models have been developed and are used to assess proangiogenic and antiangiogenic potential of compounds. These include chick chorioallantoic membrane (CAM) assay, Matrigel plug assay, mouse ischemic retinal angiogenesis assay, corneal angiogenesis assay, incorporation into retinal vasculature of diabetic rats, tumor xenograft models, mouse hind-limb ischemia models, myocardial ischemia models, stroke models and vascularization in dermal wounds [6, 26, 29, 30].

1.3.2.1 Chick CAM assay

The chick CAM assay is a relatively simple, physiological system for *in vivo* assessment of angiogenesis [31, 30]. The assay is performed by placing the test substance on the CAM, through a carefully cut window. Angiogenesis is assessed by removal of CAM around the placement of test substance and counting number of vessels and branch points under a dissecting microscope. Advantages of the CAM assay include the simplicity, cost-effective, ready availability of experimental material, accessibility for serial or multiple applications, suitability for large-scale screening and maintenance of the test substances in circulation for extended periods (due to lack of excretion). However, there are various limitations: CAM is natively well-vascularized, and hence, difficult to distinguish between the existing and the new capillaries. Chemical or physical irritation from the shell dust generated during the creation of window in the shell can trigger an inflammatory response, which in turn can induce angiogenesis, and thus hinder the specificity of the assay. Finally, the assay is carried out on chick cells, which may limit the translation to humans.

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1.3.2.2 Zebrafish models

Zebrafish embryos offer the ability to study early vascular development and angiogenesis due to their optical transparency and development outside the mother. [31, 30]. The embryos are relatively economical to generate and maintain, with hundreds of embryos produced every week. This also enables large-scale screening. Further, the duration of experiments are relatively short and require minute amounts of the test substance per experiment. The method of administration of test substances does not require much technical expertise. Depending on the physicchemical characteristics of the test substances, they can be simply added to the water or injected into the yolk sac of the developing embryos. Furthermore, it is possible to generate knockdown, overexpression or transgenic zebrafish models for assessment of gene function and visualization. Visualization of developing blood vessels is relatively simple due to their transparency and a number of techniques have been employed including direct observation under low-power microscope, dye injection, alkaline phosphatase staining, confocal micro-angiography and more recently, use of transgenic zebrafish expressing green fluorescent protein under the control of promoters specific to vascular development.

1.3.2.3 Corneal angiogenesis assay

This assay is one of the best *in vivo* assays as the cornea is the only avascular transparent tissue in the body, and any vessels developing into the corneal stroma is newly formed, readily visible and quantifiable [26, 32]. Briefly, a pocket is made in the cornea (mouse, rat or rabbit), and test substance or tumor tissues are placed into the pocket. Alternatively, the test substances can be administered orally or systemically. The degree of ingrowth of new vessels from limbal vasculature into the corneal stroma is quantified. This assay is advantageous in terms of lack of existing background vasculature and ability to monitor over a period of time. Though the assay is reliable and quantifiable, it is expensive, time intensive and technically demanding. As the cornea is natively avascular, the relevance of the assay is questionable.

1.3.2.4 Matrigel plug and sponge implantation assays

In the Matrigel plug assay, Matrigel mixed with test substances or cells is injected into mouse subcutaneous tissue, where it solidifies to form a plug [6, 26, 33]. Another modification of the assay is to encapsulate the Matrigel in a plexiglass chamber or flexible plastic tubing before subcutaneous implantation. As mentioned earlier, Matrigel is extremely rich in angiogenic factors and hence, use of growth-factor-reduced Matrigel is commonly used. The angiogenic response in Matrigel plug is dependent on the age of the mice and site of injection [33]. Young mice (<6months) are less angioresponsive as compared to the older mice (1–2 years). The ventral surface of the animal offers higher angiogenic response compared to dorsal surface.

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Alternatively, a variety of sponges and polymer matrices of defined dimensions impregnated with test substances have been used [26]. The Matrigel plug or sponges are harvested after 1–3 weeks and examined histologically for invasion of blood vessels. Since the Matrigel plug or sponge implant is avascular upon implantation, presence of any blood vessel is an indication of neoangiogenesis. Fluorescence labelled dextran or other dyes are also used to assess the blood flow non-invasively and in real-time. Sponge implants are generally more time-consuming than Matrigel plug assays. Sponge implants also elicit a foreign body reaction resulting in encapsulation in a fibrovascular tissue. The formation of fibrovascular tissue allows assessment of neovascularization, wound healing, collagen deposition and evolution of granulation tissue. Some of these responses can be due to the sponge material itself, so the results must be interpreted with caution.

1.3.2.5 Mouse ischemic retinal angiogenesis assay

In this model of angiogenesis, the unilateral retinal ischemia is induced by increasing the hydrostatic pressure in the anterior chamber of one of the eye [34]. After around 2 hours, the intra-ocular pressure is normalized resulting in reperfusion injury. The other eye serves as a control. This model of retinal ischemia-reperfusion injury results in ischemia due to vaso-obliteration, damage to the retinal endothelium and generation of acellular capillaries [35]. Angiogenic potential of cells or drugs is assessed after either intravitreal or systemic administration. The retina of the eye is isolated and imaged under confocal microscope for evidence of repair and angiogenesis. This model of angiogenesis is specifically suited to study retinal angiogenesis; however, it has several limitations. These include the complexity and timing of the surgical procedure, higher costs, and animal testing regulations which precludes its use for large-scale screening applications.

1.3.2.6 Mouse hind-limb ischemia model

Unilateral hind-limb ischemia is induced by ligating the proximal end of femoral artery and distal portion of saphenous artery [34, 36]. This model results in unilateral reduction of blood flow to the hind-limb, while the other hind-limb serves as a control. Reparative angiogenesis and restoration of blood flow to the affected hind-limb is assessed by laser-Doppler perfusion imaging and histological analysis. This mouse model is commonly used as a preclinical model of peripheral arterial occlusive disease [36]. Interspecies and inter-strain variations in hind-limb ischemia and blood flow recovery need to be considered before choosing the appropriate mouse model. Secondly, vessel location, degree of collateral branching and their occlusion contribute to degree of ischemia after ligation.

1.3.2.7 Myocardial infarction model

Myocardial infarction in rodent models is based on either permanent coronary artery occlusion or temporary surgical occlusion followed by reperfusion [34, 37]. The second

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approach mimics the clinical setting of ischemia-reperfusion injury. These models of myocardial infarction are currently used for evaluation cardiovascular drugs and stem-cell-based regenerative approaches. The progress of cardiac remodeling is generally monitored using non-invasive and invasive techniques of assessment of cardiac function that include echocardiography and left ventricular catheterization. This is followed by postmortem histological analysis.

1.3.2.8 Consideration on *in vivo* models

No single *in vivo* model of angiogenesis can completely elucidate the entire process of angiogenesis. This inability can be attributed to inter-species differences, heterogeneity in the microenvironment of vasculature among different tissues and organs, differences between embryonic and adult tissues, the mode of administration of test substances, and methods of evaluating the individual steps of angiogenesis. In many of the *in vivo* assays, it is difficult to assess and quantify angiogenesis. More importantly, all the *in vivo* animal models provide significant information on angiogenesis but it is unlikely that efficacy and relevance are completely predictable and translatable to humans.

1.4 Promise of stem-cell-based angiogenesis models

Currently, ECs and mural cells are primarily obtained from umbilical, adipose and dermal sources. Reliance on these primary sources is associated with inadequate availability of donor tissues and limited expansion *in vitro*. These limitations of the primary cells are compounded by the high variability between different batches of cells and their donors. Evidence of the existence of vascular progenitors during the postnatal life has provided the opportunity to obtain provasculogenic and proangiogenic cells present in bone marrow, peripheral blood, cord blood, blood vessel wall and adipose tissues [38]. These adult stem cell sources are promising alternative to primary cells; however the abundance, proliferative potential and trans-differentiation capabilities towards vascular cells are limited.

The discovery of human embryonic stem cells (ESCs) [39] and human induced pluripotent stem cells (iPSCs) [40, 41] capable of unlimited proliferation and differentiation into all cell types of the three germ layers in the human body has generated enormous interest in the field of developmental biology, drug discovery and regenerative medicine [42]. Owing to their pluripotent status, these stem cells could be utilized to generate unlimited numbers of identical, well-defined, genetically characterized and morphofunctionally competent ECs and mural cells that could be used for drug discovery process. Ability to direct differentiation of these human PSCs towards vascular lineage provides a valuable model to generate primitive vascular progenitors capable of differentiating to ECs and mural cells similar to embryologic process of

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vasculogenesis [38]. Additionally, these human PSCs could be coaxed into distinct anatomic and embryologic phenotypes. We and others have recently, demonstrated the ability to obtain arterial, venous and lymphatic ECs [43, 44]. Similarly, we and others have demonstrated the ability to obtain pericytes/vSMCs from distinct embryological origins namely lateral plate mesoderm, paraxial mesoderm and neural crest [3, 45]. Furthermore, human ESCs and human iPSCs generated from embryos or individuals bearing genetic mutations, defects and diseases could be a valuable tool for disease modelling and toxicological studies.

Recent progress in 3D culture systems and biomaterial fabrication techniques has led to the development of physiologically complex *in vitro* tissue equivalents or organoids. These organoids are a promising *in vitro* tool to screen pharmaceutical products and assess toxicity with relevance to humans. The need to develop physiologically relevant 3D models has led to tremendous increase in the interests and efforts to develop multicellular spheroid models, [46–48] organoid models, [49–54] 3D bio-printing [55–57] and miniaturized organ-on-chip systems [58–61]. Stem-cell-based models have provided increased understanding of the key signaling events required for induction and formation of multicellular organs as evidenced by the increasing reports of organoid models using human ESCs and human iPSCs [49, 51–53]. Through recapitulating the complexity of the 3D cellular and extracellular microenvironment, and rudiments of function of their *in vivo* equivalents, these stem-cell-based organoid models have the potential to be translated from academic proof-of-concepts to valuable tools in the drug discovery process.

Taken together, ECs and pericytes/vSMCs derived from human PSCs have many potential applications in the pharmaceutical industry including screening and safety pharmacology. These cells would serve as an inexhaustible, reproducible and human *in vitro* angiogenesis model system.

1.5 Differentiation of human PSCs into vascular lineages

The use of human PSC-derived vascular cells for drug development and pharmacological safety requires the establishment of reliable and efficient protocols to differentiate human PSCs to vascular cells. Previous studies have demonstrated that human ESCs and iPSCs can be directed towards ECs and pericytes/vSMCs. Various strategies of directed differentiation towards ECs and pericytes/vSMCs have been outlined in Fig. 1.5.1.

The protocols involve one or combination of following methods: (1) embryoid body (EB)-based differentiation (2) co-culture over murine/human stromal cells like OP9, mouse embryonic fibroblasts, M2-10B4, human foreskin fibroblasts, (3) culture of human ESCs or iPSCs as monolayers over ECM proteins like Matrigel, and collagen IV, and/or (4) growth factor, cytokines or small molecule mediated differentiation in either serum containing or serum-free conditions [62, 38, 63].

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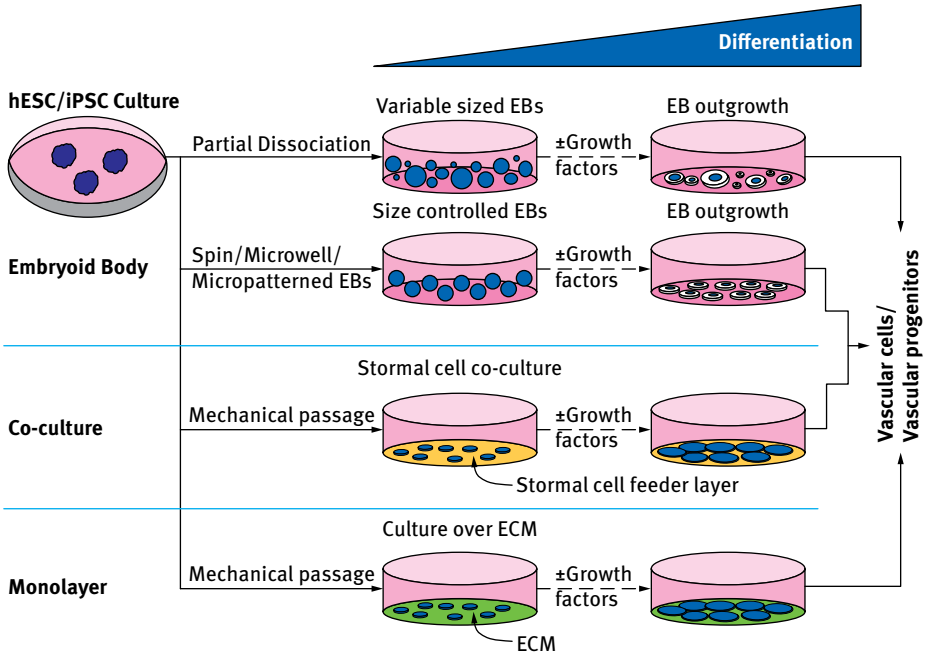

Fig. 1.5.1: The schematic illustrates the three major approaches for directing the differentiation of human PSCs towards vascular lineage: EBs, co-culture and monolayer-based methods. EB-based approach range from a simple partial dissociation of PSC colonies followed by suspension culture that yields EBs of varying sizes to methods that could precisely control the size of the EBs (Spin-, microwell- and micropatterned-EBs). The EBs could be spontaneous differentiated or differentiated under the influence of various growth factor combinations to yield vascular cells or vascular progenitors. Alternatively the human PSCs could be differentiated over stromal cell feeder layers or extracellular matrices to direct the differentiation towards vascular lineage.

1.5.1 Embryoid body-mediated differentiation

When colonies of human PSCs are suspended in hanging drop cultures or in ultra-low attachment plates, they form cell aggregates/spheroids with mesodermal cells sandwiched between ectoderm-like cells in the core, and endodermal cells on the outer layer of the spheroids [63]. These cell spheroids exhibit similarity to early post-implantation embryos and hence are termed as EBs. The cells in the EBs generally differentiate to progenies of all the three germ layers [64]. The pioneering study of EB differentiation of human ESCs to ECs reported isolation of $CD31^{+}$ cells after 13 days of differentiation; and demonstrated the expression of EC markers (CD34, CD31, VE-Cadherin, VEGFR2) and formation of tube-like structures over Matrigel [65]. Following the pioneering work, several studies have demonstrated that human ESCs undergo spontaneous differentiation through EB formation towards endothelial progenitors, ECs, hematopoietic cells and vSMCs [66–68, 35, 69–71]. The simultaneous differentiation of hematopoietic cells, ECs, vSMCs and the formation of vessel-like

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structures within the EBs suggests that EBs produce factors necessary for differentiation of all the components of the vascular system and mimics the events of vasculogenesis occurring in the developing embryo. However, these protocols suffer from low differentiation efficiency (<10%) and lack reproducibility due to various reasons including the heterogeneity of EB size, un-optimized microenvironment of differentiation within the EBs, and the presence of undefined components like serum.

Towards addressing the abovementioned limitations of the EB-based differentiation strategy, various studies have demonstrated enhanced efficiency of differentiation through induction of vascular signaling pathways using growth factors, cytokines and/or small molecules, [72, 73] transduction with angiogenic factors, [69] and elimination of serum in the differentiation milieu, [74] and/or generation of EBs of uniform and defined sizes [75–78]. These studies on optimization of EBs differentiation protocols provide evidence that human PSCs could be directed towards vascular lineages *in vitro*. Though the 3D structure of the EBs offer an advantage over monolayer culture system, the heterogeneous cell populations within EBs prohibit their utility in large-scale industrial and clinical applications.

1.5.2 Co-culture mediated differentiation

Another approach for differentiation of human PSCs to ECs and mural cells is to differentiate them on a layer of appropriate feeder cells that provide direct cell-cell contacts, growth factors, cytokines, and ECM. Murine feeder cells commonly used for vascular induction include stromal cells i.e. OP9, S17, MS-5, and M2-10B4, [79–85] mouse embryonic fibroblasts [86, 87] or mouse ECs [88]. The use of OP9 stromal cell co-cultures for hematopoietic and endothelial differentiation have been extensively characterized by Vodyanik *et al* [79, 83, 84]. In order to avoid the use of murine feeders and to optimize serum-free culture conditions, human foreskin derived fibroblasts (Hs27) were used as feeders in a serum-free medium and the role of BMP4 in induction of vascular progenitors ($CD34^+CD31^+$ cells) from human ESCs was demonstrated [89]. BMP4 supplementation during early stages of culture promotes the induction of vascular progenitors while inclusion of TGFβ suppressed the provascular induction potential of BMP4. While the optimization of co-culture strategy eliminates the need for EB-based differentiation, it only enables the production of less than 20% vascular progenitors. Additionally, the presence of feeders is associated with the inclusion of undefined factors, batch to batch variation and restricts the ability to tune the culture milieu.

1.5.3 Directed vascular differentiation with specific factors and matrix components

Differentiation protocols involving EBs and their co-culture based methods have provided valuable insights in understanding the early stages of vascular commitment of human PSCs and the role of various signaling molecules involved therein. However,

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significant scientific obstacles warrant resolution in terms of developing efficient differentiation strategies and removal of animal-derived products in the culture milieu for their utilization in large-scale applications. Feeder-free monolayer differentiation is an attractive alternative to other differentiation strategies. In this differentiation strategy, monolayer of human PSCs lack the complex diffusion barriers present in EBs and the feeder cells present in co-culture model. Hence, theoretically it provides a platform to precisely control the application of growth factors and other relevant interventions. Furthermore, human PSCs grown over feeder-free culture systems could be directly differentiated without additional steps including re-plating and EB formation.

Collagen IV is one of the predominant matrix proteins present in the vascular basement membrane. Hence, the use of collagen IV and Matrigel for vascular differentiation has been extensively studied. Monolayer differentiation over collagen IV was initially studied using mouse ESCs [90–92]. A differentiation protocol using collagen IV as substrate for differentiation of human ESCs was first reported wherein the human ESCs were directed to ECs in the presence of VEGF and to vSMCs in the presence of PDGFβ [93]. Likewise, a serum-free monolayer differentiation system was reported using collagen IV and a cocktail of growth factors for directed differentiation of human ESCs to $CD31^+VECadherin^+$ ECs phenotype with an efficiency of ~50% after 6 days of differentiation [94].

Using Matrigel as substrate in serum-free conditions, a recent report indicates the differentiation of human ESCs and iPSCs to $CD34^+$ cells (~13–20%) by combined regulation of MEK/ERK and BMP4 signaling pathways [95]. Moreover, the study demonstrated the role of MEK/ERK and BMP4 signaling in mesodermal induction; and bFGF and VEGF in induction of mesodermal cells to $CD34^+$ cells capable of tri-lineage differentiation to ECs, vSMCs and hematopoietic cells. A study using fibronectin as substrate reported the differentiation of human ESCs to $VE\text{-}Cadherin^+CD31^+$ ECs (~80%) after 21 days of differentiation under serum-free culture conditions [96]. The same group subsequently modified their protocol with the inclusion of good manufacturing practice (GMP)-grade components in the differentiation system and obtained vascular cells under GMP-compliant conditions [97]. A recent report using mouse ESCs verified the role of various ECM substrates including gelatin, fibronectin, laminin, collagen I and collagen IV in endothelial differentiation using serum-free conditions [98]. Their results indicate that the rate of endothelial induction was maximized on fibronectin while culture over gelatin, laminin and collagen I favored induction towards vSMCs. Moreover, collagen IV was an optimal substrate for co-differentiation of ECs and vSMCs [98].

We have recently reported the commitment of human ESCs (grown on Matrigel) to lateral plate mesoderm ($VEGFR2^+CD34^+$ cells) and paraxial mesoderm ($PDGFR\beta^+CD34^-$ cells) derivatives that could be subsequently directed to ECs and vSMCs, respectively, using chemically defined medium and sequential modulation of Wnt/β-catenin, BMP4 and VEGF signaling pathways [45]. The differentiation was induced in Stemdiff APEL medium (human albumin, polyvinyl alcohol, essential lipids) which is chemically defined, xeno-free medium commercially available from StemCell Technologies. Using a similar differentiation strategy, recent reports have demonstrated efficient differentiation of human ESCs and human iPSCs to functional ECs [99, 101]. Recently, we

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further modified our earlier protocol using human plasma fibronectin as substrate and stage-specific addition of small molecules and growth factors including CHIR99021, bFGF, BMP4 and VEGF to direct the sequential differentiation of human ESCs to primitive streak, early mesoderm, lateral plate mesoderm, endothelial progenitors and finally ECs [44]. This lineage-specific differentiation strategy resulted in a significantly enhanced efficiency of 90–95% of human ESCs committed towards endothelial lineage. These data was evidenced by the proportion of cells that expressed early endothelial cell markers, i.e. VEGFR2, CD34 and CD31. The human ESC-derived endothelial progenitors were successfully differentiated to morphofunctionally competent ECs under serum-free conditions with the inclusion of bFGF, EGF and VEGF as shown in Fig. 1.5.2.

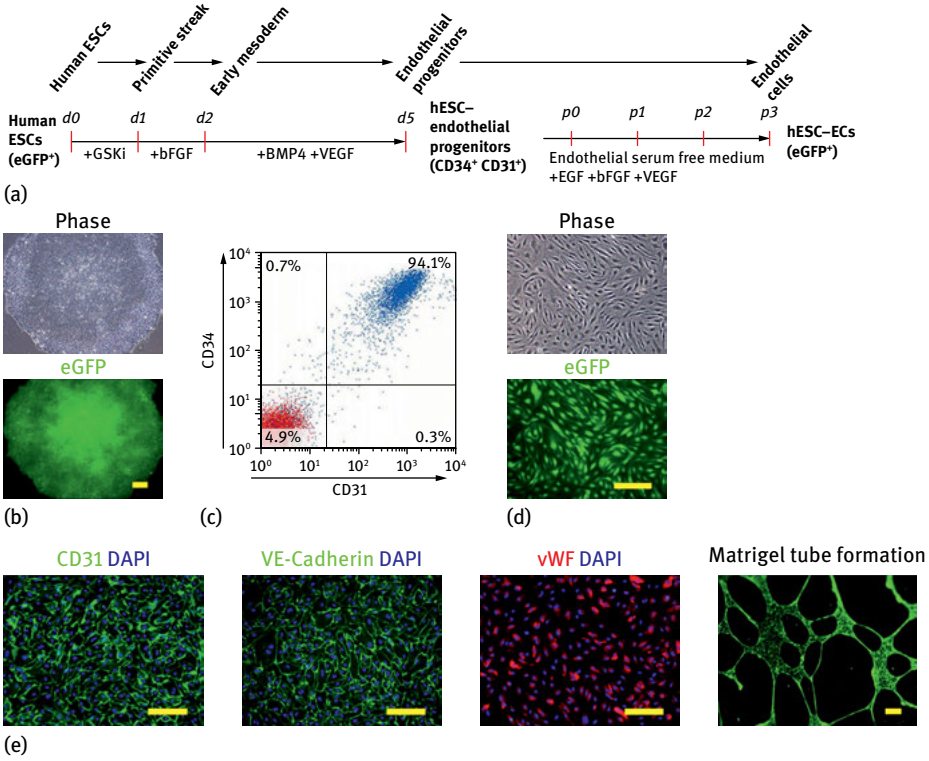

Fig. 1.5.2: Endothelial differentiation of human ESCs. **(a)** The schematic timeline shows the differentiation of human ESC-ECs through an intermediate stage of human ESC-endothelial progenitors. Induction of differentiation of eGFP-expressing human ESCs **(b)** using sequential treatment with CHIR99021, bFGF, BMP4 and VEGF under feeder-free and serum-free conditions results in sequential emergence of primitive streak, early mesoderm and endothelial progenitors. **(c)** This differentiation protocol results in robust commitment to CD34 and CD31 expressing endothelial progenitors. **(d)** These endothelial progenitors upon further differentiation yield eGFP-expressing human ESC-ECs. **(e)** These human ESC-ECs are characterized by the expression of CD31, vascular endothelial-cadherin (VE-Cadherin), von Willebrand factor (vWF) and formation of cord-like structures on Matrigel. Scale bar: 200 µm.

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vSMCs/pericytes are heterogeneous group of cells derived from diverse embryological origins that include lateral plate mesoderm, paraxial mesoderm and neural crest [102]. Various approaches have been reported to induce *in-vitro* differentiation of human PSCs towards vSMC/ pericyte lineage [62]. Most of these protocols rely on the use of serum and do not involve lineage-specific differentiation strategies. vSMCs are derived through a human PSC-derived mesenchymal stem cell intermediates [103, 104]. Recently, lineage-specific differentiation of human PSCs to vSMCs through lateral plate mesoderm, paraxial mesoderm and neural crest progenitors has been reported [3]. Similarly, we have recently demonstrated the differentiation of human

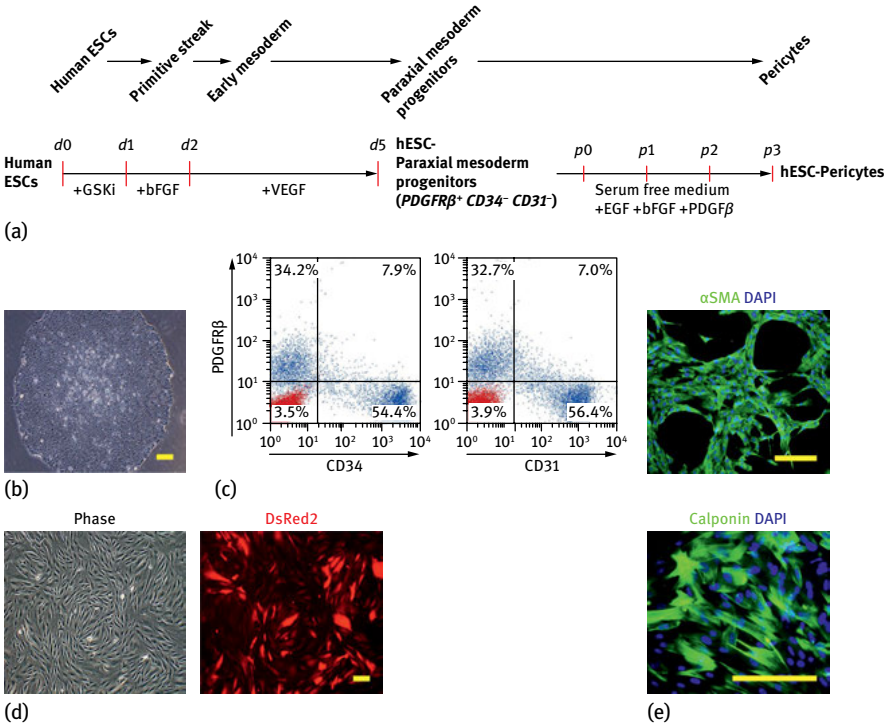

Fig. 1.5.3: Differentiation of human ESCs to pericyte-like cells. **(a)** The schematic timeline shows the differentiation of human ESCs to pericyte-like cells through an intermediate stage of human ESC-paraxial mesoderm progenitors. Induction of differentiation of human ESCs **(b)** using sequential treatment with CHIR99021, bFGF and VEGF under feeder-free and serum-free conditions results in sequential emergence of primitive streak, early mesoderm and paraxial mesoderm progenitors. **(c)** This differentiation results in robust commitment to paraxial mesoderm progenitors ($PDGFR\beta^+CD34^-CD31^-$ cells) and endothelial progenitors ($PDGFR\beta^-CD34^+CD31^+$ cells). **(d)** Differentiation of PDGFRβ-expressing paraxial mesoderm progenitors in the presence of epidermal growth factor (EGF), bFGF and platelet-derived growth factor-β (PDGFβ) yields human ESC-pericytes. **(e)** These human ESC-pericytes are labelled with DsRed2 and are characterized by the expression of **(e)** α-smooth muscle actin (αSMA) and calponin. Scale bar: 200 µm.

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ESCs to vSMCs through paraxial mesoderm intermediates under feeder-free, chemically defined conditions using sequential modulation of Wnt/β-catenin, BMP4 and VEGF signaling pathways [45]. Using an identical lineage-specific differentiation strategy, human PSCs could be differentiated to pericyte-like cells [100, 101]. Similarly, we have developed and optimized a feeder-free and serum-free protocol to differentiate human ESCs to pericytes through stage-specific addition of CHIR99021, bFGF, VEGF and PDGFβ that sequentially directs the differentiation of human ESCs to pericytes through intermediate stages reminiscent of primitive streak, early mesoderm, and paraxial mesoderm progenitors [105]. The differentiation to pericyte-like cells is characterized by the expression of pericyte-associated surface markers (PDGFRβ, NG2, CD73, CD90) and cytoskeletal markers (αSMA, calponin, SM22) (Fig. 1.5.3).

Hence, the monolayer culture conditions for differentiation over various ECM substrates and the use of specific growth factors and/or small molecules provide a platform for directing an efficient differentiation of human PSCs towards vascular lineages. This strategy also provides a platform amenable to better control over the differentiation process through elimination of undefined animal-derived components in the culture milieu. The increased differentiation efficiency and xeno-free system permits their utility in large-scale industrial and clinical applications.

1.6 Building blood vessels *in vitro* – PSC models of angiogenesis

Essentially, the PSC-derived vascular cells could be utilized as an alternative source of vascular cells to perform almost all the *in vitro* assays that are conventionally performed using primary or immortalized cells. It is well known that heterogeneity among ECs and mural cells (arterial, venous, microvascular or lymphatic) is associated with variability in angiogenesis assays. Similar variability can be expected between vascular cells derived from different PSCs and differentiation protocols. Nevertheless, stem-cell-based models open opportunities to derive tissue and lineage specific vascular cells that can model inherent heterogeneity among these cell types. Improvisations in the differentiation efficiency and standardization of differentiation protocols would enable reducing the cell-type-dependent variables. In the subsequent sections, various co-culture models of angiogenesis using PSC-derived vascular cells are described.

1.6.1 2D co-culture angiogenesis assay

ECs have an inherent capability to self-assemble upon 2D direct co-culture or 3D culture within proangiogenic biomaterials. The development of mature and functional vasculature depends not only on the ECs but also requires interaction with mural

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cells (vSMCs/pericytes) [14, 15, 106]. Additionally, they require the ECM to adhere, migrate and assemble into anastomosing network of vascular channels. A confluent monolayer culture of vSMCs/pericytes provides the microenvironment needed for cell-cell interaction between ECs and mural cells, and also acts as a source of native cell-derived ECM.

The 2D co-culture assay is based on the previously published methodology in which human umbilical vein ECs (HUVECs) are co-cultured over pericytes/vSMCs [107–109]. This assay has been validated previously by Evensen and colleagues for high-throughput screening of antiangiogenic molecules. Exploiting their ability to efficiently differentiate human ESCs to ECs and pericytes, we have developed a 2D co-culture based angiogenesis assay (unpublished data). Further, we used enhanced green fluorescent protein (eGFP) expressing human ESCs to derive the ECs, which allows real-time imaging and quantification of the endothelial network formation. Human ESC-derived ECs (human ESC-ECs) were seeded on top of a confluent monolayer of human ESC-derived pericytes (human ESC-pericytes). After the initial seeding, human ESC-ECs form islands/nests on top of the human ESC-pericyte lawn. Upon further culture, the islands of human ESC-ECs form interconnecting networks of endothelial sheets. These endothelial sheets reorganize into thin, anastomosing cords of ECs (Fig. 1.6.1).

The parameters of endothelial networks like cord length and branch points are dependent on the addition of exogenous factors like VEGF, TGFβ1. A similar co-culture strategy has been recently published to establish the *in vitro* functionality of human PSC-derived ECs and pericytes [101].

The process of formation of endothelial islands that subsequently remodel to form vessel-like structures with the support from surrounding pericytes recapitulates the essential steps of primary vascular plexus formation. This model of 2D co-culture angiogenesis provides a platform for analysis of blood vessel

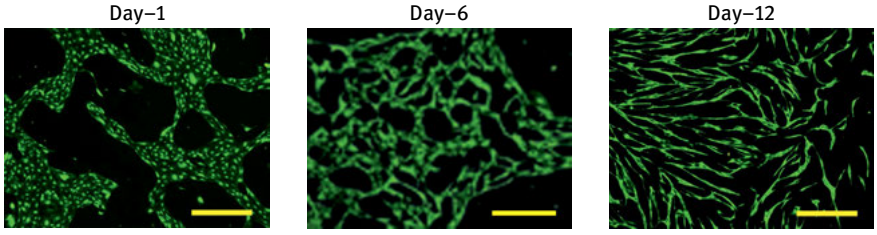

Fig. 1.6.1: 2D co-culture angiogenesis model. The photomicrographs show the morphological changes of eGFP-expressing human ESC-ECs (green) upon co-culture over a confluent layer of human ESC-pericytes. The human ESC-ECs form islands or nests (day 1) that undergo morphological change to form interconnecting networks of endothelial sheets (day 6) and further reorganize into thin, anastomosing cords of ECs (day 12).

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formation in monolayer cultures and relies on the paracrine factors and direct cell-cell interactions between human ESC-ECs and human ESC-pericytes. Hence, this assay can be used to investigate EC-pericyte interactions and its role in EC remodeling. The 2D co-culture angiogenesis assay could be used as high-throughput screening model for the discovery of novel proangiogenic and antiangiogenic compounds.

1.6.2 Embryoid body-based 3D angiogenesis models

Isolation of primary ECs, generation of immortalized and/or genetically modified EC lines and establishment of serum and serum-free culture conditions are valuable achievements in the field of vascular biology. However, these cultures do not provide the 3D microenvironment necessary for the interactions between ECs, adjacent mural cells and the surrounding ECM that are crucial for the regulation of angiogenesis. Furthermore, the mature ECs are not amenable to study basic mechanisms related to vasculogenesis. On the contrary, given their capacity for differentiation to virtually all cell types in the human body, human PSCs provide a valuable *in vitro* model for investigating embryonic vascular development.

Suspension culture of colonies of human PSCs as hanging drop cultures or within ultra-low attachment plates, result in formation of cell aggregates/spheroids known as EBs. The differentiating EBs provide a simulated *in vivo* microenvironment for *de novo* vascular development along with continuous interaction with surrounding non-ECs and other tissues. The various events associated with vascular development within differentiating mouse and human EBs have been extensively studied and reported [110–113]. Vascular development within differentiating EBs could be studied in various ways that include *de novo* vascular development within floating EBs and sprouting angiogenesis from EBs seeded onto adherent culture or encapsulated within 3D Matrigel, collagen or fibrin gels [110]. As the differentiation proceeds, the onset of vasculogenesis is associated with the development of hemangioblasts (a common precursor for hematopoietic and endothelial lineages) which expresses T-cell acute leukemia-1/stem cell leukemia (TAL/SCL), VEGFR2, and brachyury [112]. Upon further differentiation, these hemangioblasts commit towards either hematopoietic or endothelial lineage. The commitment of endothelial cell lineage is associated with the expression of endothelial markers VEGFR2, CD31, CD34, vascular endothelial-cadherin (VE-CAD), Tie-1 and Tie-2 [111,112]. As the differentiation proceeds, the endothelial progenitors differentiate towards ECs and result in the formation of primary vascular plexus. Encapsulation of these differentiating EBs within Matrigel/collagen gels is associated with sprouting angiogenesis into body of the matrix; and this process can be regulated

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by the addition of exogenous angiogenesis stimulants or inhibitors. In parallel to the differentiation of ECs, development of pericytes/vSMCs also occurs. The vascular sprouts protruding from the central core of the EBs, sprouts and invades the surrounding matrix, branches and forms occasional networks [110]. The endothelial sprouts are surrounded by perivascular cells that express NG2 and α-smooth muscle actin similar to the pericytes observed *in vivo*. Upon further culture, the endothelial cell sprouts develop further with the formation of vascular lumen and network [110].

The process of vascular development within differentiating EBs described above has high striking similarities to that observed in developing embryos. Hence, these EB-based vascular models are valuable platform to study human embryonic vascular development *in vitro*. Considering the ethical and practical issues related to access to human embryos, human PSC-derived EBs are an excellent alternative *in vitro* human platform to study the proangiogenic and antiangiogenic drug candidates from a developmental perspective which otherwise is limited to animal models. Further, special ultra-low attachment plates with microwells (Aggrewell™ plates, StemCell Technologies) are available commercially. These plates can be used to generate EBs of defined size by controlling the number of undifferentiated cells seeded and the process of forced aggregation. This provides a platform for large-scale production of EBs that could be used to high-throughput analysis of vascular development and drug discovery.

1.6.3 3D vascular spheroidal co-culture model

3D spheroid culture models are widely used in the field of cancer biology and ESC differentiation. Based on the principle of formation of cellular spheroids upon suspension culture, a 3D vascular spheroidal co-culture model using human ESC-ECs and human ESC-pericytes has been developed (unpublished data). The 3D vascular spheroidal co-culture assay has previously been established using primary ECs and vSMCs seeded within a methylcellulose-based gel or low attachment plates [114, 115]. We co-cultured defined numbers of eGFP-expressing human ESC-ECs and DsRed-expressing human ESC-pericytes at 1:20 ratio in the ultra-low attachment plates. Within 24 hours, the cells rearranged to form multicellular aggregates (vascular spheroids). We used standard ultra-low attachment 96-well round bottom plates (Corning) and Aggrewell™ plates (StemCell Technologies). In the standard ultralow attachment plates, the cells seeded in one well reorganized to form a single standardized spheroid. In the Aggrewell™ plates, the cells reorganized to form ~1200 multicellular spheroids of approximately the same size. In both the systems, the size of the spheroid was dependent on the number of cells seeded at the start of

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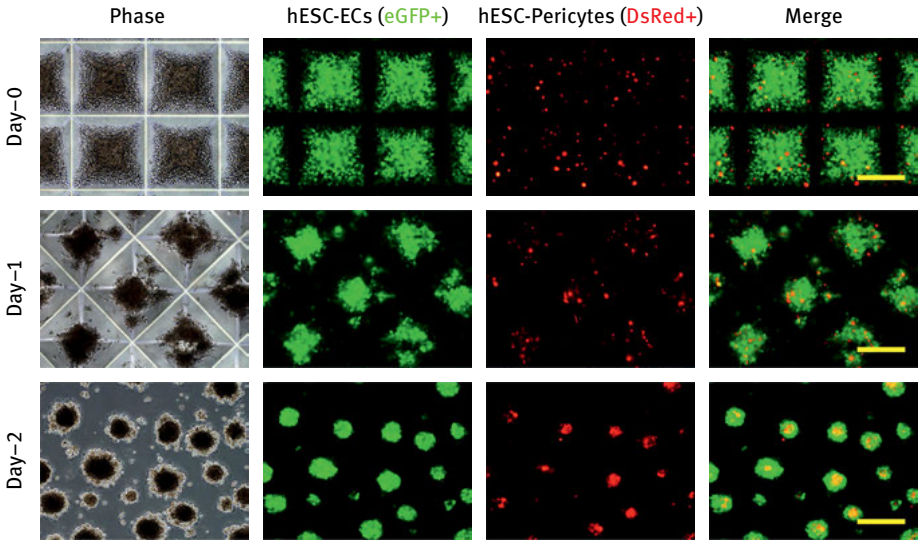

Fig. 1.6.2: 3D vascular spheroidal co-culture model. The photomicrographs show the generation of 3D vascular spheroids using ultra-low attachment Aggrewell™ plates. Forced aggregation of eGFP-expressing human ESC-ECs (green) and DsRed-expressing human ESC-pericytes (red) within ultralow attachment Aggrewell™ plates results in formation of aggregates with random distribution of the cells. Within a day, these aggregates reorganize to form 3D spheroidal aggregates of approximately the same size. Further, the randomly distributed human ESC-ECs and human ESC-pericytes reorganize to form spheroidal aggregates with a central core of human ESC-pericytes surrounded by a surface layer of human ESC-ECs.

the experiment. Within 24–48 hours of seeding, the cells spontaneously rearranged into a core of human ESC-pericytes and a surface layer of human ESC-ECs (Fig. 1.6.2).

These spheroids could be maintained in suspension culture for several days or embedded within a matrix like Matrigel, collagen or fibrin. The later results in sprouting angiogenesis similar to the EB-based angiogenesis model.

The reorganization of ECs and pericytes/vSMCs into 3D vascular spheroids mimics the 3D assembly of a blood vessel inside-out. The media representing the lumen, outer layer of ECs of the spheroids represent the ECs lining the lumen and the inner core of pericytes/vSMCs the vessel wall. Earlier studies have shown that the ECs covering the outer layer of these multicellular spheroids are quiescent, in contrast to monolayer EC cultures and hence more closely mimics quiescent blood vessels [114]. The spontaneous self-assembly of the ECs and pericytes/vSMCs within these multicellular spheroids suggests the distinct morphogenetic interactions between the two cell types. Hence, this 3D model system offers a physiological experimental system to analyze paracrine interactions between ECs and vSMCs/pericytes in a physiological context and also enables the high-throughput screening of proangiogenic/antiangiogenic drug candidates.

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1.6.4 *In vitro* 3D vascularized tissue equivalent (vascular organoids) model

All the above angiogenesis models describe the interaction between ECs and pericytes/vSMCs and to a limited extent interaction with the cell-derived ECM. It is well established that in addition to providing a scaffold for the ECs to form vascular networks, ECM also acts as a growth factor sink by sequestering and releasing various growth factors and other signaling molecules [19, 116–118]. Hence, in order to model a physiologically relevant system, it is important to include ECM in addition to the multicellular microenvironment.

Many studies have demonstrated the ability of primary ECs in the presence of supporting cells (pericytes, SMCs, fibroblasts, MSCs) to form microvascular networks when cultured within 3D microenvironment [119–124]. Recently, self-organization of vascular networks from human PSC-derived vascular cells in a synthetic matrix that integrates with the host circulation has been reported [125]. Harnessing the strengths of human ESC-derived ECs and pericytes, we have recently developed an *in vitro* 3D vascularized tissue equivalent (vascular organoids) model amenable to real-time imaging and high-throughput assessment of angiogenesis [105]. In this *in vitro* 3D vascular tissue equivalent model, eGFP-expressing human ESC-ECs and dsRed-expressing human ESC-pericytes are encapsulated within a polyethylene glycol (PEG)-fibrin based gel in a μ-angiogenesis slide/plate (ibidi GmbH, Germany). Within 24 hours of culture, human ESC-ECs elongate and develop vacuoles within them. In the next 2–3 days, extensive tubulogenesis occurs resulting in extensive sprouting of the human ESC-ECs and formation of thin endothelial cords with branching anastomoses. Further, these cords of human ESC-ECs display focal regions of vacuolization along their length. The vacuolization slowly extended into adjacent regions of the endothelial cords and branches resulting in the formation of lumenized microvessels within 6–9 days. In parallel, the lumenization was associated with increase in the diameter of the microvessels, new vascular sprouts, branching and anastomoses resulting in an increase in the complexity of the microvascular network. Throughout the period of vessel formation, human ESC-pericytes are observed to be associated in close proximity to the endothelial cords, tubules and microvessels (Fig. 1.6.3).

These bi-cellular 3D microvascular networks are stable in culture for a further 2-week period.

The above process of formation of lumenized microvascular network within 3D PEG-fibrin gels is reminiscent of the formation of primitive vascular plexus observed during embryonic vasculogenesis. The 3D co-culture model encompasses all the stages observed in vasculogenesis and angiogenesis, namely *de novo* blood vessel formation from individual ECs, vacuolization and lumenization, sprouting, elongation, branching and anastomoses. The 3D co-culture model also replicates the close interaction between ECs, pericytes and ECM observed *in vivo*. The use of fluorescently labelled human ESC-ECs and human ESC-pericytes enables real-time imaging and visualization of the angiogenic process longitudinally over time. Further, the use

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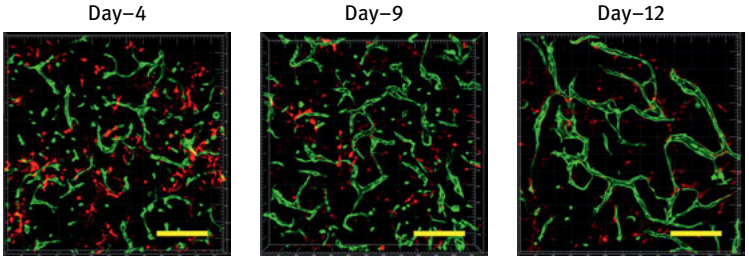

Fig. 1.6.3: *In vitro* 3D vascularized tissue equivalent (vascular organoids) model. The confocal z-stack projections display the kinetics of vascular morphogenesis of human ESC-ECs (green) and human ESC-pericytes (red) cultured within a 3D fibrin-based gel. Within 4 days, the human ESC-ECs undergo morphological changes to form thin cords of ECs that further reorganize to form anastomosing network of lumenized microvascular channels (day 9). The microvascular networks mature further to form thick anastomosing network of endothelial vessels that are supported by a peripheral layer of human ESC-pericytes (day 12).

of μ-angiogenesis slide/plate has the advantage of extremely minimal use of cells, media and ECM components and is adaptable to high-throughput screening. Furthermore, human PSCs provide an inexhaustible, reproducible and human source of vascular cells. Hence, we believe that this human PSC based-3D *in vitro* vascularized tissue equivalent model would be an extremely valuable tool in the high-throughput screening and validation of novel proangiogenesis/antiangiogenesis drug candidates. Further, we believe that this model would be a physiologically more relevant angiogenesis tool in terms of multicellular organization in spatiotemporal dimensions, human origin and for its ease and versatility.

1.7 Conclusions and future outlook

Various studies have highlighted the advantages of 3D cell culture and limitations of the reductionist monolayer culture systems especially in the context of the multicellular organization in 3D space that allows the crucial cell-cell and cell-ECM interactions. It is also increasingly recognized that drug responses are different in 3D culture systems compared to the monolayers which is favorably more close to *in vivo* observations. Further, in contrast to the conventional angiogenesis assays and cell systems frequently used in drug development, human PSC-derived vascular cells and the different 3D angiogenesis models bear the potential to be used in physiologically relevant phenotype-based angiogenesis assays. An important advantage of such phenotype-based angiogenesis assays is that it is possible to evaluate the effect of the investigative drug compound on all the phases of the complex angiogenic process in the *in vitro* settings, without sacrificing the crucial cell-cell and cell-ECM interactions as

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well as the 3D vascular architecture observed *in vivo*. Hence, we propose that human PSC-derived self-organizing organoid models of angiogenesis could be used as appropriate test platforms for future drug discovery and pharmacological safe efforts. Certainly, these miniaturized organoid systems could be used as a significant validation tool that bridges the gap between primary high-throughput screening and expensive animal and human trials. Combining with other human PSC-derived organoid models (i.e. liver, kidney, gut, brain) safety and efficacy of the lead proangiogenic/antiangiogenic compounds could be tested directly on *in vitro* human organoids. Furthermore, such organoid systems could be linked with each other in order to build a "human in a dish" or "human in a chip" system with unprecedented reliability to model human disease and drug response.

It is widely recognized that human PSC-based models have a huge potential to advance drug development and safety pharmacology. However, in order to realize this potential, several important concerns need to be resolved. Standardization in the methods for human ESC and human iPSC generation and maintenance, differentiation to vascular cells and quality control in terms of elimination of animal-derived products, efficiency of differentiation and large-scale translational potential are some of important issues related to the cell source. It is also necessary to realize the need for commercial availability of well-characterized human PSC-derived vascular cells.

Multicellular and 3D microenvironment is obviously relevant and advantageous; however, there are various downstream technical challenges that need to be addressed. The increase in the complexity comes with a challenge, namely different cell types require different growth conditions and the need for complicated micro-engineered pumps and expensive control devices. Similarly, seeding of various cell types in 3D increases the variability between cell culture wells and thus might need higher numbers of experimental replicates to obtain reproducible results. To add to the problem, certain parameters like the ratio between cell types would change with culture duration. Hence, it is necessary to establish quality parameters at different stages of the experiment. With the 3D organoid morphology and function, one of the challenges would be the systematic assessment of function. Probably more advanced readouts would have to be developed to address these challenges. Though confocal microscopy allows the real-time imaging of vessel formation within the vascular organoids, the throughput is certainly limited considering the amount of time needed for each sample. Hence, newer techniques currently used in *in vivo* and whole animal imaging like light sheet fluorescence microscopy, high resolution optical coherence tomography, and/or multi spectral optical tomography could be utilized in this context. Hence, extensive characterization, validation and probably new quality control parameters should be established before their use in drug discovery.

From a pharmaceutical industry point-of-view, one of the important challenges is regulatory acceptance. The regulatory authorities would accept these novel models only after significant amount of published scientific evidence and joint cross-pharma validations across different laboratories and countries. One of the classical successes

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in acceptance of 3D models for toxicity and safety testing by the regulatory authorities is the example of the 3D human skin model. These models are excellent alternatives to human skin, and have been adopted by European Union (under the auspices of the European Centre for the Validation of Alternative Methods, or ECVAM), [126, 127] the US (through the Interagency Coordinating Committee on the Validation of Alternative Methods, or ICCVAM), Japan (through the Japanese Centre for the Validation of Alternative Methods (JaCVAM), and internationally through the OECD [128–131] for screening, testing, safety assessment and validation of chemicals, and biologics [132]. This could serve as a prototype model for development, testing and validation of human PSC-based 3D angiogenesis models. In conclusion, while human PSC-based angiogenesis models are promising candidates for drug discovery and pharmacological safety – the full potential of these model systems remains to be exploited in the future.

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Irfan Khan, Nadia Naeem, Anwar Ali and Asmat Salim

2 Role of small molecules in the cardiac differentiation of mesenchymal stem cells

Abstract: Globally, coronary heart disease is a major life-threatening degenerative disease. The molecular and cellular events in the ischemic myocardium culminate in necrosis of the tissue architecture in the infarct area which eventually leads to heart failure. As current therapeutic approaches are limited in preventing ventricular remodeling following myocardial infarction (MI), a novel therapeutic approach of transplantation of exogenously differentiated cardiomyocytes could be a better option. Mesenchymal stem cells (MSCs) have been used for the treatment of MI to repair the injured myocardium and improve cardiac function as these cells possess the ability to differentiate into multiple lineages *in vitro* and *in vivo*. However, donor cells have limited capacity to differentiate into functional cardiomyocytes in the ischemic microenvironment of the heart. Among other strategies, small molecules have been identified that promote differentiation of MSCs into functionally active cardiac-like cells. One of the best studied examples is that of the demethylating agent, 5-azacytidine and its analogues. Other synthetic and naturally occurring compounds have also been tested and were found to be good candidates for future cell-based therapeutics against cardiovascular diseases. In this chapter, potential role of small molecules on the cardiac differentiation of MSCs will be highlighted.

2.1 Introduction

Myocardial infarction is one of the leading causes of death worldwide. According to the latest report of the American Heart Association, around 8 million deaths were caused in the United States by cardiovascular diseases in 2013 [1]. The healthcare system in the United States invested the gigantic sum of $115 billion in 2012 in treatment costs [1]. Although microinfarcts are repairable by an intrinsic repair mechanism in the heart with the involvement of stem cells as well as re-entry of cardiomyocytes into the cell cycle, large infarcts lead to irreversible loss of functional myocardium which encompasses a massive loss of cardiomyocytes as well as smooth muscle and endothelial cells. The affected portion of the heart is replaced with the non-contractile fibrous tissue as part of the internal repair mechanism [2]. Contemporary therapeutic modalities for MI mostly provide symptomatic relief, without addressing the root cause of the disease process. Neurohormonal inhibition with angiotensin-converting enzyme inhibitors and β-blockers which improve clinical outcomes, as well as the interventional and surgical procedures are limited in obviating the vicious cycle of left ventricular remodeling due to their inability to repair or replace the damaged myocardium [3–5]. Heart transplant remains the gold standard treatment option; nevertheless it is

DOI 10.1515/9783110493764-002

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limited by the availability of donors. Stem cell transplantation therapy in regenerative medicine has emerged as one of the most promising treatment modalities proposed to improve outcomes in patients with heart failure [6]. The potential of stem cells to cross lineage restriction and adopt the desired morphofunctional phenotype has opened new horizons in the field of regenerative medicine. Among the various stem cell types, MSCs have been extensively characterized for their biology and reparability potential and subjected to many pre-clinical as well as clinical investigations. Some of the important characteristics of MSCs are outlined in Tab. 2.1.1 and Tab. 2.1.2

Tab. 2.1.1: Comparison of properties of various stem cells.

Properties	Embryonic stem cells	Mesenchymal stem cells	Hematopoietic stem cells
Potency	Pluripotent	Multipotent	Multipotent
Colony forming unit (CFU)	Very High	High	Limited
Plastic adherence	No	Yes	No
Self-renewal	Very high	High	Restricted
Tumorgenicity	High	No	No
Ethical issues	Yes	No	No
Ease of isolation	Relatively difficult	Easy	Easy
In vitro proliferation	Very high	High	Limited
Immunogenicity	Immunogenic	Non-immunogenic	Non-immunogenic
Clinical application	Applicable	Applicable	Directly transplantable

Table 2.1.2: Surface markers of mesenchymal stem cells from different species.

Species	Positive markers	References
Human	CD146	[7, 8]
	CD106	[9]
	SSEA4	[10]
	CD271	[11]
	HLA-ABC	[12]
	CD29, CD44	[12, 13]
	CD13, CD73, CD90, CD105	[13, 14]
	CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD166, MHC I, α-SMA, Vimentin	[13]
Rat	CD29	[15, 16, 17, 18]
	CD90	[15, 16, 19, 20, 21]
	CD29, CD73, CD90	[15, 16]
	CD54	[19]
	CD44, CD117	[17, 18, 20, 21]

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Tab. 2.1.2 (continued)

Species	Positive markers	References
Mice	Nestin	[22]
	CD51, CD90, CD105	[23]
	CD105	[23, 24, 25, 26, 27]
	PDGFRα	[28]
	Sca1	[25, 26, 27, 28, 29]
	CD29, CD44, CD105, Sca-1	[24, 25, 26, 27]
	CD73, CD80, Sca-1	[29]
	CD29, CD44, CD73, CD105, CD106, Sca-1	[25, 26, 27]
Rabbit	CD44, α-SMA, Desmin, Vimentin	[13]
	CD105, CD106, Collagen type I and II	[30]
	CD29, CD73, CD81, CD90, CD166	[31]

MSCs are multipotent and can be transdifferentiated into mature cell types irrespective of the tissue of their origin [32–33]. Given their ease of availability and *in vitro* expansion, several studies have shown that MSCs are a promising therapeutic option for the treatment of cardiovascular pathologies [34]. Bone marrow derived MSCs have shown the ability to transdifferentiate into cardiomyocytes, both *in vitro* and *in vivo* [35–36]. MSCs constitute a heterogeneous population of cells and this heterogeneity makes it difficult to achieve uniform outcome in terms of their differentiation capacity and reparability. Moreover, an inadequate supply of cells during cell therapy to the target organ is a major concern. The predifferentiation of MSCs into cardiomyocytes *in vitro* for subsequent use as donor cells for transplantation therapy *in vivo* can be an alternative strategy. Various strategies have been adopted to enhance the rate and efficiency of cardiac differentiation of MSCs *in vitro* [18, 37]. In this regard, the use of small molecules has shown promise and is of particular interest in the field of regenerative medicine due to the advantages associated with their use including a high level of purity, low batch-to-batch variation in their quality of preparation, chemical stability, cost effectiveness, ease of cell membrane permeability that allows their entrance into the cell and effect on the desired signaling pathway within the cell, and the ease of modifying their concentration for achievement of the desired level of effect. An additional advantage linked with their widespread application is that they are chemically well defined. Moreover, some small molecules have already been optimized pharmacologically and are in clinical practice for routine therapeutic applications [38]. Many of the small molecules are also being tested and assessed for their potential to induce cardiomyogenic differentiation of stem cells. These are now becoming essential moieties to develop and optimize well-defined, specific, efficient, and cost-effective protocols with higher induction rates for cardiomyogenic differentiation of stem cells.

Besides these small molecules, drugs that are capable of conferring cardioprotection when used concomitantly with stem cells also hold potential in cardiac therapy. Most interestingly, despite achieving the same end results in terms of their ability to

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support cardiogenic differentiation from stem cells, these compounds diverge in the mechanism of achieving the common end result. Of all the small molecules tested so far, 5-azacytidine (5-aza), a DNA methyltransferase inhibitor (DNMTi), appears to be the most powerful inducing agent to promote cardiomyogenic differentiation of stem cells. Since the pioneering work of Makino *et al.* [39], wherein the authors reported more than 30% bone marrow stromal cells undergoing induced cardiomyogenic differentiation after 5-aza treatment, several studies have reported the successful use of this compound and optimized the protocol for an improved rate of cardiomyogenic differentiation of stem cells *in vitro* [17, 37, 40, 41]. The derivative differentiated cells expressed cardiac-specific markers including MEF2A and MEF2D, exhibited spontaneous beating during *in vitro* culture with measurable action potential. Nevertheless, given its low stability in aqueous solution and the toxic nature, 5-aza is now less favored for cardiomyogenic induction of stem cells. Instead, 5-aza has been derivatized to explore newer chemical moieties with superior stability and less toxicity for enhanced rates of cardiomyogenic differentiation [37]. Other small molecules analyzed to improve cardiac function include the cardioprotective agents such as statins, anti-hypertensives and other cardiovascular drugs, peptide hormones, acids, etc. Some of these compounds are specific for cardiac function while others can non-specifically induce cardiomyogenic differentiation. Currently, the search for an ideal compound that could induce efficient cardiomyogenic differentiation of stem cells without being toxic in biological system or incur cardioprotection after MI, remains an important area of research.

2.2 Epigenetic modifiers and cardiomyogenic differentiation of MSCs

In the cardiovascular system, epigenetic regulation mechanisms play a significant role in the lineage commitment of stem and/or progenitor cells. Epigenetic modification can regulate lineage specification by imposing a specific and hereditary pattern of gene expression in their cellular descendants without altering the DNA sequence. Two of the prime epigenetic modifications include DNA methylation and histone deacetylation that play significant role in mammalian growth and development [42], stem cell differentiation [43] and tumor transformation [44].

DNA methylation is a reversible process. It is one of most crucial epigenetic mechanisms mediated by the enzymes DNA methyltransferases (DNMTs), which transfer or covalently link a methyl group ($-CH_3$) to the cytosine residue within 5′-CpG-3′ islands at the promoter region [45]. Subsequent to the successful methylation step, another group of proteins having a methylcytosine binding domain (MBD) is recruited, which joins the methylated CpG sites, causing a barrier to the entry of transcription factors to the DNA promoter. The chromatin becomes more condensed, resulting in inaccessibility of the transcription binding sites thus leading to reduced transcription and gene silencing [42]. DNA methylation is involved in various cellular

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processes and genetic events such as activation of X chromosome, gene expression, chromatin modification, genomic imprinting and endogenous silencing of genes [46]. DNA demethylation status has also been associated with greater cellular plasticity of lineage-committed cells. Various demethylating agents can induce differentiation of bone marrow stromal cells into cardiomyocytes, hepatocytes or neuronal cells [47].

Histone acetylation is another extensively studied epigenetic modification. It regulates gene expression by altering the structure of DNA, thus influencing the binding of DNA to various transcription factors [48]. This reaction involves the addition of an acetyl group to the lysine residue at the N-terminus of the histone proteins catalyzed by the enzyme, histone acetyltransferase (HAT). HATs often work as large multiprotein complexes regulating specific chromatin targeting [49]. The reaction is regulated by HAT as well as histone deacetylase (HDAC) enzymes with opposing effects [50]. HDACs remove an acetyl group from the lysine residue, whereas HATs transfer the acetyl group to lysine. Regulation of gene expression is maintained with the processes of acetylation and deacetylation occurring at various sites on the histone [50, 51]. HDAC inhibitors represent a class of pharmacological agents that modulate gene expression by enhancing histone acetylation. HDAC inhibitors are a class of anticancer drugs which therapeutically act by reducing proliferation and inducing cell-cycle arrest, apoptosis, differentiation, angiogenesis, migration and cell resistance to chemotherapy [52]. Later studies have also explored the significance of HDAC inhibitors in other diseases, including immune disorders, renal, neurological and cardiovascular diseases [53]. In the cardio-cerebrovascular system, HDAC inhibitors show anti-inflammatory and anti-fibrotic effects [54].

Relationships between epigenetic modification and differentiation status of stem cells have initiated new exploration in the field of biomedical research. There is mounting evidence in literature regarding the maintenance of the lineage commitment of stem cells by DNA methylation and histone modification with functionally distinct chromatin structures that influence cell fate [55]. In differentiated mammalian cells, CpG DNA methylation is the principal epigenetic tag [56]. In undifferentiated stem cells, cytosines other than those in CpG can be methylated as well and are crucial for gene regulation in ESCs [57, 58]. The mammalian epigenome undergoes global remodeling during early development. At the blastocyst stage, a genome undergoes both active and passive cytosine demethylation immediately following fertilization followed by up to 60% CpG methylation prior to gastrulation, which reprograms chromatin structure [42, 59, 60]. This regulation is critical for mammalian development, as ESCs with reduced or elevated levels of 5-methylcytosine are viable but unable to differentiate [61]. Histone deacetylation correlates with CpG methylation and the inactive state of chromatin [58]. During *in vitro* differentiation of murine ESCs, histone deacetylases and acetylases transmit differentiation signals to initiate erasure of pre-existing chromatin structure and establishment of new histone modification patterns [62]. Few studies have been conducted to explore differentiation of stem cells into various cell types after treatment with epigenetic modifiers [63–65].

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2.2.1 5-azacytidine

5-aza is one of the well-studied chemical moieties for cardiomyogenic induction of stem cells (Fig. 2.2.1). First synthesized as a nucleoside antimetabolite and effectively used as a chemotherapeutic agent, 5-aza is incorporated into DNA inhibiting DNA methylation, thus leading to loss of methylation in the specific gene regions and their activation [66]. 5-aza and 5-aza 2`-deoxycytidine (decitabine) are both inhibitors of DNA methyltransferases in mammalian cells [66]. 5-aza has the potential of differentiating different cell types to adopt morphofunctionally competent cardiomyocytes, i.e. MSCs [67, 68], human ESCs [69], mouse ESCs [70] and mouse embryonal carcinoma cell lines into cardiac lineage [71]. Most of the studies identified the newly formed cardiomyocytes by morphological observations, expression of cardiomyocyte specific markers and spontaneous beating characteristics *in vitro* and confirmed identification by electrophysiological studies and their responsiveness to adrenergic and cholinergic agonists [72].

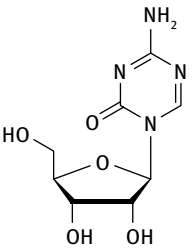

Fig. 2.2.1: 5-azacytidine.

In pursuit of optimization of differentiation protocols to enhance the rate of cardiomyogenic differentiation, different concentrations of 5-aza were used for variable time periods. In studies with murine bone marrow MSCs, differentiation of stem cells into cardiomyocytes was potentiated by 5-aza treatment, which was evident by the significantly increased expression of cardiac-specific genes and proteins in the treated cells *in vitro* [17, 37, 73]. 5-aza also induced differentiation of rat adipose-derived MSCs into cardiomyocytes and improved cardiac function after transplantation in an *in vivo* animal model [74]. It also showed excellent capability of transdifferentiating rabbit bone marrow MSCs into mature cardiomyocytes by expressing cardiac-specific protein cTn-I [75]. These reports clearly show the versatility of the 5-aza-based differentiation protocol to achieve a clinically pertinent and useful source of cardiomyocytes for cell transplantation therapy.

Despite successful cardiomyogenic induction of stem cells with 5-aza treatment, the exact underlying mechanism of induction remains unclear. Experiments conducted to elucidate the mechanism showed that cell proliferation and differentiation after 5-aza induction were regulated through different signaling pathways relevant

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to cytoskeletal, metabolic and calcium-binding proteins [41]. Gene expression profiling experiments showed that the process of cardiomyogenic induction in response to 5-aza treatment was not abrupt or spontaneous, rather it involved sequential events that spanned days and involved temporo-spatial expression of 1814 genes [76]. These genes were differentially expressed in the 5-aza-treated cells and were grouped into five hierarchal clusters of genes related to proliferation, metabolism, development, differentiation and topogenesis. One study demonstrated that the expression of an inotropic peptide, apelin was increased in a time-dependent manner. Apelin is the ligand for the G-protein coupled receptor and plays a role in the regeneration of myocardium through paracrine and autocrine mechanisms [77]. Similarly, a recent study has signified the expression of Nesprin-1 protein in 5-aza treated rodent MSCs derived from bone marrow [78]. Nesprins constitute a family of proteins which are significantly expressed in cardiac, skeletal and smooth muscle cells and form a scaffold that links the nuclear envelope with the nucleus, cytoplasmic organelles and cell membrane *via* actin cytoskeleton. 5-aza treatment of MSCs derived from various human tissue sources including bone marrow [40, 79–81], adipose tissue [82] and umbilical cord and cord blood [83–85] demonstrated successful cardiomyogenic induction. The derivative cardiomyocytes were confirmed by the expression of cardiac-specific genes; alpha cardiac actin, MHC, cTn-T with adjoining cells forming myotubular structures in the culture conditions. Zhang *et al.* extrapolated the data to show enhanced differentiation of first trimester fetal MSCs towards cardiac-like cells after 21 days treatment with 5-aza [86]. In studies conducted with human umbilical cord MSCs, cardiac differentiation by 5-aza was attributed to the involvement of the DLL4-notch signaling pathway [87] and activation of sustained extracellular signal related kinases (ERK) [88].

2.2.1a 5-aza in combination with growth factors and other small molecules

Despite reports of successful cardiomyogenic induction of MSCs from animal and human sources with 5-aza, the efficiency and rate of differentiation has always remained a concern. Moreover, insufficiency of 5-aza alone to prime cardiomyogenic differentiation of MSCs or cell culture passage dependence of 5-aza treatment effect warrants re-assessment and optimization of the induction protocols [89–91]. In pursuit of enhanced efficiency and rate of cardiomyogenic induction, a combinatorial approach based on treatment with 5-aza and growth factors and signaling molecules has been developed. Noticeable amongst these are transforming growth factor-β (TGF-β) [92], insulin-like growth factor-1 (IGF-1) [93], cardiotrophin-1 [94, 95] and bone morphogenic protein-2 (BMP-2) [96]. The selection of growth factors and signaling molecules for use in the combinatorial approach was mainly based on their significant involvement in cellular processes such as cell proliferation, survival and differentiation. Small molecules, i.e. trichostatin A [97] and angiotensin-II [98], stimulated cardiomyocytes differentiation of rat bone marrow MSCs. 5-aza combined

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with dimethylsuloxide (DMSO) and retinoic acid was able to promote differentiation of human fetal liver derived MSCs to cardiac cells [99]. In addition to treatment with recombinant growth factors, the cells were also genetically modified for transgenic overexpression of the growth factor of interest prior to 5-aza treatment. 5-aza treatment of MSCs for cardiomyogenic induction and the efficiency of the derivative cells' culture characteristics on different scaffolds, either synthetic or of natural origin, have been studied. Scaffolds used to culture 5-aza induced MSCs include collagen I nanofibrils [100], natural nanofibers, Hb/ gelatin/ fibrinogen having high oxygen binding capability [101], poly(e-caprolactone)-gelatin (PCL gelatin) nanofibrous scaffolds encapsulated with VEGF [102], and polyurethane, 3-hydroxybutyrate-co-4-hydroxybutyrate and polypropylene carbonate gels [103].

2.2.2 Zebularine

Zebularine was originally identified as an anti-cancer drug [104, 105] (Fig. 2.2.2). It is a cytidine deaminase inhibitor [106, 107]. During DNA replication, it incorporates itself into DNA by substituting cytosine residues. It decreases DNA methylation by making covalent complexes with DNA methyltransferase, inhibiting its activity [108]. *In vitro* experiments confirmed that zebularine causes potent inhibition of DNA methylation by forming tight complexes with bacterial methyltransferases through covalent binding [108–111]. Inhibition of methylation by zebularine is also reported in mammalian cell lines [83, 112, 113].

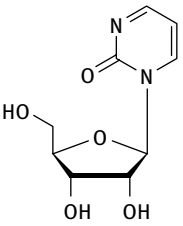

Fig. 2.2.2: Zebularine.

Zebularine is less cytotoxic, has lesser myelosuppressive effect and is more stable both in basic and neutral media [114, 115]. Given its low level cytotoxicity, zebularine can be continuously given at a lower dose to maintain demethylation for extended time periods [116]. Our group reported the cardiomyogenic differentiation potential of zebularine on rat bone marrow derived MSCs *in vitro* at very low concentrations. The treated cells were able to express cardiac markers both at gene and protein levels [17, 37]. A direct comparison of 5-aza and zebularine was carried out by individual treatment with incremental doses of 3, 10, 25 and 50μM concentrations and assessment of cell cytotoxicity and expression of cardiac-specific proteins. In comparison with the

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untreated controls, MSCs treated with either of the two compounds showed significantly higher expression of cardiac-specific proteins including GATA4, Nkx2.5 and cTn-T with concomitant morphological changes and appearance of tubular structures between adjacent cells. Although both the compounds showed cytotoxicity at higher concentrations of 25μM and 50μM, lower concentrations (3μM and 10μM) of zebularine were less toxic. These data support zebularine as a safer alternative to 5-aza as a cardiomyogenic inducer with clinical relevance in future (Figs. 2.2.3 and 2.2.4 reproduced from [37]).

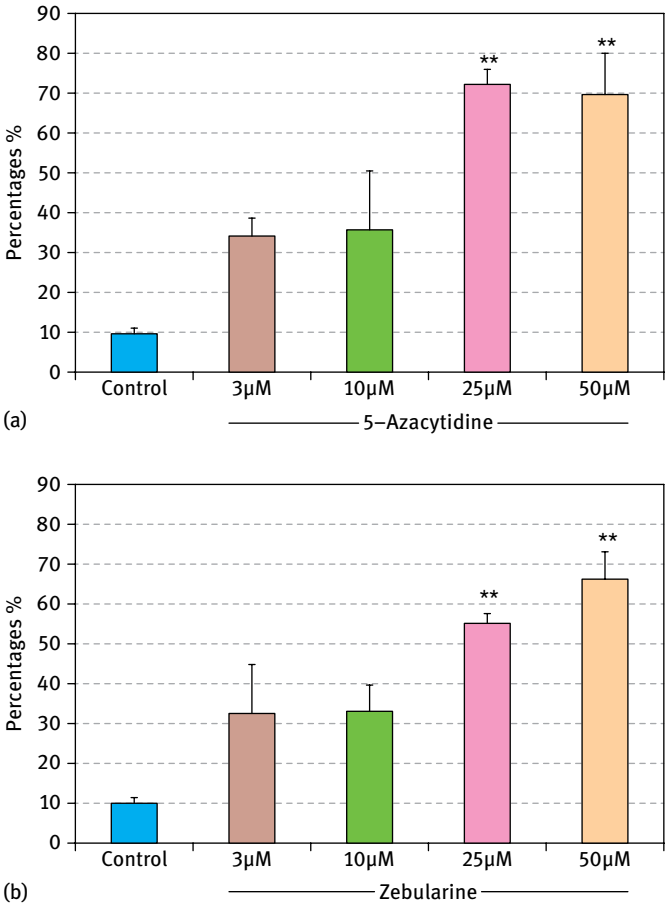

Fig. 2.2.3: Cytotoxicity measurement of MSCs using various concentrations of (a) 5-azacytidine and (b) zebularine: four concentrations (3 μM, 10 μM, 25 μM and 50 μM) of each compound were used for optimization. Data are presented as mean ± standard error of the mean (SEM) and calculated using Microsoft Excel. Statistical significance (*$p<0.05$) was determined by analysis of variance (ANOVA) with Tukey correction using SPSS software. The optimized concentrations of 3 μM and 10 μM were then selected and used in experiments (reproduced from [37] with permission from the publisher).

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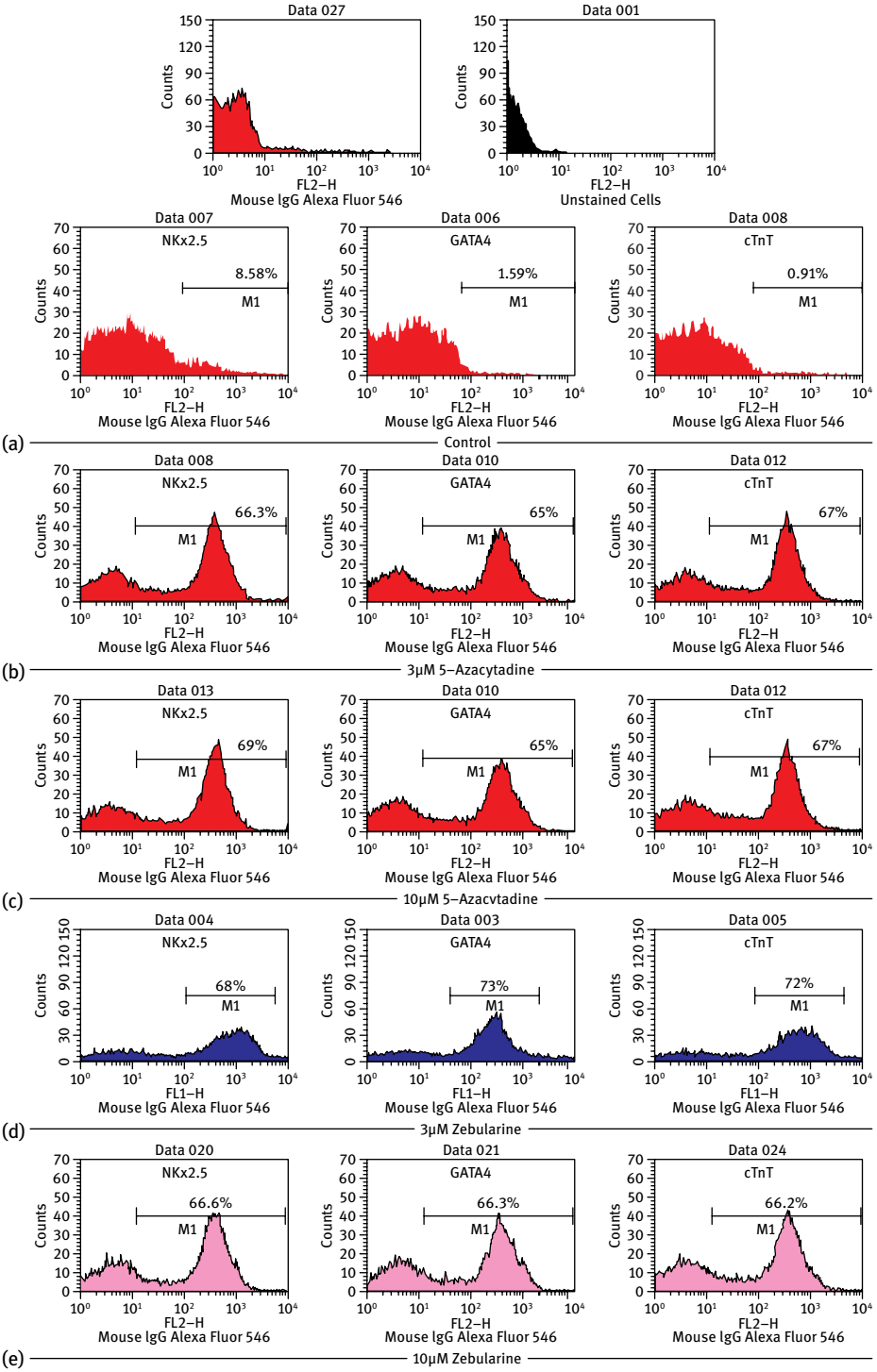

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2.2.3 RG108

RG108 [2-(1, 3-dioxo-1,3-dihydro-2H-isoindol-2-yl)-3-(1H-indol-3-yl) propanoic acid], is a small-molecule DNA methyltransferase (DNMT) inhibitor that blocks the active site of human DNMTs (Fig. 2.2.5). An attractive candidate for new drug development, RG108 has low toxicity, and it significantly demethylates epigenetically silenced genes [117]. It has been successfully used to reprogram mouse SkMs to pluripotency with high efficiency [118].

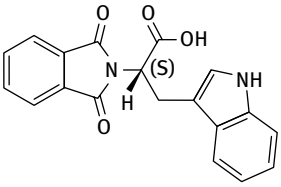

Fig. 2.2.5: RG108.

2.3 Cardioprotective compounds

2.3.1 Statins

Statins are known to exhibit a number of favorable cardiovascular influences. Statins constitute a class of lipid-lowering compounds that inhibit the enzyme, 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA reductase) that contributes significantly to cholesterol synthesis *via* conversion of HMG-CoA to mevalonate, an early and rate-limiting step in the process of cholesterol synthesis [119]. Nevertheless, the mechanism associated with cardioprotection of statins may or may not involve lipid metabolism. The lipid and non-lipid consequences converge in a resolute pattern for the protection of myocardium against injury by lowering its ischemic burden. Statins have been shown to prevent lesion initiation and are involved in repair mechanism, enhancement of myocardial perfusion and prevention of coronary occlusion [120]. Various derivatives of statins are being used in order to explore their role in improving the therapeutic potential of stem cells.

◄ Fig 2.2.4: Cardiac-specific proteins in treated mesenchymal stem cells (MSCs). Flow cytometry analysis showing expression of cardiac-specific proteins, Nkx2.5, GATA4, and cardiac troponin T in MSCs treated with (b) 3 μM and (c) 10 μM 5-azacytidine and (d) 3 μM and (e) 10 μM zebularine, while (a) unstained MSCs, untreated MSCs stained only with secondary antibody and untreated MSCs stained with primary antibodies to Nkx2.5, GATA4, and cardiac troponin T were used as controls. FSC is selected as the threshold parameter and the threshold is set to a value of 52, which eliminates small debris (reproduced from [37] with permission from the publisher).

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2.3.1.1 Simvastatin

Simvastatin is commonly used as a blood cholesterol and triglyceride-lowering agent (Fig. 2.3.1). It is a lactone that is hydrolyzed post-administration to a β–hydroxyacid to perform its pharmacological effects by potent inhibition of HMG-CoA reductase. However, effects of simvastatin beyond classical lipid-lowering activity ranging from cardiomyocyte protection to anti-inflammatory activity and differentiation of stem cells have been reported [121]. To exploit the pleiotropic beneficial effects of simvastatin, intra-myocardial transplantation of MSCs was carried out in an experimental animal model of acute MI in Chinese mini-swines. The experimental animal group was concomitantly administered simvastatin starting 3 days prior to cell transplantation until 4 days post-transplantation and showed improved donor cell survival (up to 4-fold improved survival) and cardiovascular differentiation in comparison with the control group treated with MSCs alone without simvastatin administration [122]. The authors used magnetic resonance imaging (MRI) to observe changes in the infarcted myocardium and reported decreased infarct size, improved cardiac function and decreased perfusion defects.

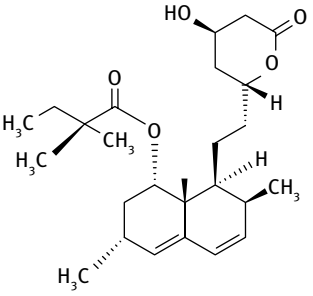

Fig. 2.3.1: Simvastatin.

2.3.1.2 Atorvastatin

Atorvastatin has also been reported to play an important role in the protection of myocardium against acute infarction by stabilizing the environment suitable for the survival and transdifferentiation of MSCs after implantation [123] (Fig. 2.3.2). The authors reported improved cardiac perfusion and contractility in the Chinese swine model of MI after implantation of atorvastatin-treated MSCs. Favorable results from stem cell therapy in combination with atorvastatin may restrain the progression of apoptosis by reducing oxidative stress and inflammation in the infarcted myocardium. Qu *et al.* have shown that atorvastatin can be useful in modulating the microenvironment to improve the therapeutic effects of bone-marrow-derived MSCs (BM-MSCs) in animal models of acute MI [124]. They observed reduction in inflammatory cell infiltration, cardiac fibrosis and derangement of myocardial morphology and also improvement in the survival rate of implanted BM-MSCs. The authors postulated that an approach combining stem cell therapy with pharmacological intervention using

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statins demands future preclinical and clinical studies. Similar therapeutic effects of atorvastatin have also been reported in another study in which atorvastatin was used in combination with adipose-derived MSCs [125]. In an attempt to elucidate the mechanism of pleiotropic beneficial effects of statins, human adipose tissue derived MSCs were treated with clinically relevant concentrations of atorvastatin and pravastatin [126]. The cells were studied *in vitro* for various characteristics including proliferation, differentiation and gene expression profiling. The most interesting observation was their reduced differentiation potential to adopt macrophage phenotype after statin treatment, which provided insight into how statin treatment benefits patients by reducing the macrophage density in the arterial plaque leading to reduced inflammation and improved plaque stability.

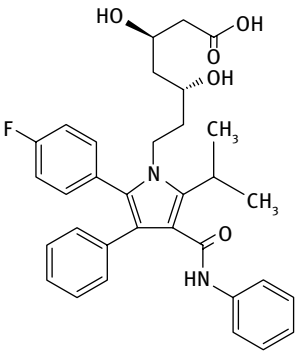

Fig. 2.3.2: Atorvastatin.

2.3.1.3 Rosuvastatin

Similar to other statins, combined treatment of stem cells and rosuvastatin has been shown to enhance the outcome of stem cell therapy in both experimental animal models and human patients. Although the underlying mechanism of the beneficial effects of combined therapy still remains contentious, animals treated with adipose-tissue-derived MSCs showed reduced fibrosis and improved global cardiac function when cell-based intervention was accompanied by daily administration of rosuvastain, 20 mg/kg for 28 days [127] (Fig. 2.3.3). The findings were supported by bioluminescence imaging and histological evidence showing 1.5-fold improved survival of the donor MSCs postengraftment. Molecular studies showed involvement of activated PI3/Akt and reduced levels of pro-apoptotic proteins in the donor cells. Cantoni *et al.* studied the effects of rosuvastatin in patients with chronic heart failure. It was shown to produce myocardial neovascularization and intervene in myocardial remodeling [128]. Rosuvastatin-induced increases in capillary formation were proposed to be dependent on the activation of paracrine signals and other growth factors like VEGF and its receptor, KDR. Another study on the same compound illustrated a mechanism that involves activation of the JAK-STAT pathway [129].

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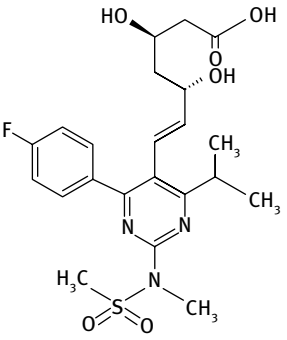

Fig. 2.3.3: Rosuvastatin.

2.3.2 Resveratrol

Resveratrol is a natural non-flavonoid polyphenol compound, having antioxidant properties and is known to produce protecting effects on the body against cancer and in patients with cardiovascular disorders (Fig. 2.3.4). It is a sirtuin1 (SIRT1) activator and can also function as an anti-inflammatory agent. A recent study has shown that MSCs response in culture to resveratrol treatment occurs in a passage-dependent manner and is also influenced by the level of SIRT1 expression by the cells [130]. Whereas resveratrol treatment of early passage MSCs resulted in their enhanced rate of self-renewal and multipotency, the late passage cells responded by undergoing accelerated senescence. Similarly, the early passage MSCs with SIRT1 expression responded with decreased ERK and GSK-3B phosphorylation with concomitantly suppressed B-catenin activity. On the contrary, late passage MSCs responded with enhanced ERK and GSK-3B phosphorylation and enhanced beta-catenin activation. In addition to the known biological functions of resveratrol, its shielding effects were further explored by using an *ex vivo* aortic ring co-culture system and an *in vivo* 3D-scaffold model in mouse [131]. The authors demonstrated that MSCs induced aortic ring sprouting, a feature of neovascularization from pre-existing vessels. In another study, resveratrol in combination with adipose-derived MSCs successfully treated doxorubicin-induced cardiomyopathy in rats [132]. The beneficial effects of the combined treatment with MSCs and intraperitoneal administration of 12mg/kg (t.i.d) resveratrol were attributed to multiple mechanisms including cell differentiation and autocrine and paracrine activity of the transplanted cells.

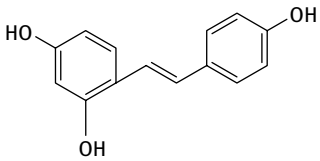

Fig. 2.3.4: Resveratrol.

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2.3.3 Trimetazidine

Trimetazidine (1-[2,3,4-trimethoxybenzyl] piperazine with the trade name Vastarel is clinically used to alleviate ischemia-induced metabolic damage, which it achieves via reducing ischemic tissue oxygen requirements [133] (Fig. 2.3.5). The possible mechanism of its action has been attributed to the 3-ketoacyl CoA thiolase enzyme inhibition, which has a major role in the β-oxidation cycle of fatty acids. Trimetazidine is administered orally as an antianginal agent and is known to increase cells' tolerance against ischemia by retaining cellular homeostasis. Trimetazidine has been used to pharmacologically precondition MSCs to enhance their survival under oxidative stress. The preconditioned MSCs treated with trimetadizine showed significantly upregulated expression of HIF-1α, survivin, phosphorylated Akt and Bcl–2 and helped in functional recovery of the infarcted heart postengraftment [134]. Xu *et al.* have demonstrated the protective effect of trimetazidine on the viability of bone marrow MSCs in an *ex vivo* model of hypoxia and *in vivo* model of myocardial ischemia [135]. Administration of 2.08 mg/kg per day of trimetadizine, starting 3 days prior to and continuing until 28 days after cell transplantation significantly enhanced donor cell viability. The combined treatment decreased the cardiac infarct size and improved cardiac function as compared to MSCs alone. Improved survival of umbilical-cord-derived MSCs pretreated with trimetadizine has also been observed under hypoxic and serum-deprived culture conditions. Molecular studies confirmed the activation of Akt signaling as the underlying mechanism of cytoprotection by trimetadizine treatment [136].

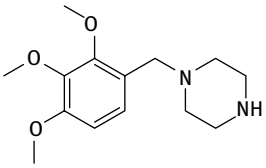

Fig. 2.3.5: Trimetazidine.

2.3.4 Pioglitazone

Pioglitazone is a well known antidiabetic agent that lowers blood glucose levels in type 2 diabetes mellitus (Fig. 2.3.6). It induces a cytoprotective effect against reperfusion injury when administered prior to ischemia [137–140]. Several contributing factors like enhanced COX-2 and phospholipase-A2 expression, decreased expression of intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinase-2 (MMP-2) reduction in infiltrating macrophages and suppression of cardiomyocyte apoptosis have been associated with the protective mechanism of pioglitazone [137–139, 141]. Cardiac function after MI was improved by pioglitazone in rat models [142]. Differentiated MSCs after pioglitazone treatment were shown to significantly improve left ventricular cardiac function after transplantation [143].

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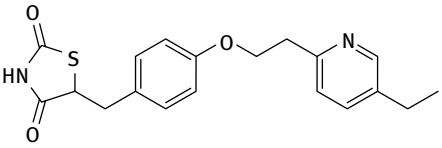

Fig. 2.3.6: Pioglitazone.

2.4 Fatty acids and ipids

2.4.1 Phorbol myristate acetate

Lipid-containing compounds have also been shown to induce differentiation of MSCs into cardiomyogenic lineage. Thus, stem cell efficacy can be improved for the treatment of cardiovascular diseases, especially in the setting of hyperlipidemia. One such compound is phorbol myristate acetate (PMA), which is a protein kinase C (PKC) activator (Fig. 2.4.1). It differentiates adipose-derived MSCs into cardiomyogenic lineage [144]. The cardiomyogenic differentiation of MSCs was confirmed by the protein expression of cardiac troponin T and myosin light and heavy chains in the PMA-treated cells. Preferential expression of β-adrenergic and muscarinic receptors and α_1-adrenergic receptor subtypes was also observed while the levels of mRNA for Ca^{2+}-related factors (sarcoplasmic reticulum Ca^{2+}-ATPase, and L-type Ca^{2+} channel) were found to be similar in PMA-treated ASCs as in the case of cardiomyocytes. Furthermore, overall myocardial function was improved as exhibited by reduced infarct size, decreased interstitial fibrosis and apoptotic index and restoration of cardiac function.

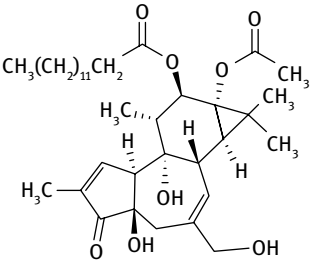

Fig. 2.4.1: Phorbol myristate acetate.

2.4.2 Sphingosine-1-phosphate

Sphingosine-1-phosphate (S1P) is a native circulating bioactive lipid metabolite (Fig. 2.4.2). It was used for the cardiomyogenic differentiation of human umbilical cord derived MSCs [145]. Treatment with SIP induced cardiomyocyte-like morphology and protein expressions of α-actinin and myosin heavy chains in MSCs, thus showing the potential of this lipid molecule as an inducer of cardiac differentiation. In another study, S1P was shown to have potential for cardiac differentiation of human

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adipose-tissue-derived MSCs as well as in conditioned cardiomyocyte culture medium [146]. The differentiation of these MSCs into cardiomyocytes was confirmed by positive expressions of cardiac specific proteins, α-actin, connexin-43 and myosin heavy chain-6. Calcium transients were also observed showing *in vivo* electrophysiological properties.

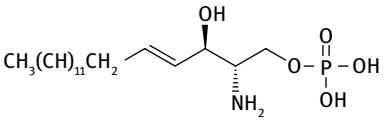

Fig. 2.4.2: Sphingosine-1-phosphate.

2.5 Acids

Some acids have also been used either alone or in combination with other compounds to observe their cardiac differentiation potential.

2.5.1 Salvianolic acid B

Salvianolic acid B extracted from *Salvia miltiorrhiza*, a perennial plant of Chinese origin, has been reported to have a cytoprotective effect on MSCs (Fig. 2.5.1). In cell-transplanted myocardium, it induced differentiation of endothelial cells as evidenced by increased angiogenesis and preserved cardiac function. However, it did not induce differentiation of MSCs into cardiomyogenic lineage. Improvement of cardiac function was attributed to the improved survival of the transplanted cells and their enhanced paracrine activity [147].

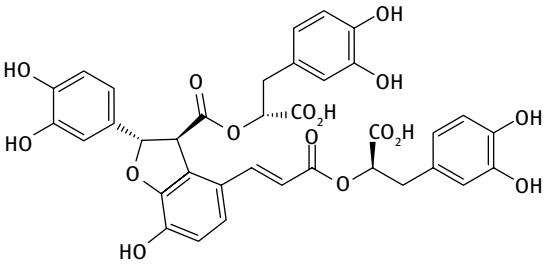

Fig. 2.5.1: Salvianolic acid B.

Salvianolic acid B used in combination with 5-aza in the presence of cardiomyocyte lysate was shown to induce MSCs for cardiomyogenic differentiation by inhibition of the Wnt/ beta-catenin signaling pathway [148]. Gene expression profiling and protein analysis showed that cTn-T, alpha-cardiac actin, MEF-2c, connexin-43 and GSK-3β

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expressions were increased showing that these MSCs acquired the phenotypical characteristics of cardiomyocytes while beta-catenin expression was decreased.

2.5.2 Retinoic acid

Retinoic acid, a metabolite of retinol (vitamin A) (Fig. 2.5.2), differentiated human fetal-liver-derived MSCs to cardiac cells when used in combination with 5-aza and DMSO [149] (also discussed in Section 1.2). Immunocytochemistry and quantitative RT-PCR showed that more than 40% of cells treated with the tri-combination of 50uM 5-aza, 1% DMSO and 10 (-1) retinoic acid showed expression of Nkx2.5, desmin, cTn-I and Oct4 at gene and protein expression levels.

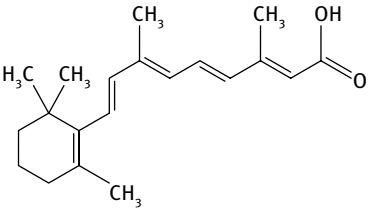

Fig. 2.5.2: Retinoic acid.

2.6 Peptides and peptide hormones

Some peptides and peptide hormones have also been explored for their cardiomyogenic potential.

2.6.1 Triiodo-L-thyronine

In a study, it was shown that triiodo-L-thyronine (T3) was able to differentiate bone-marrow-derived MSCs into cardiomyocytes [150]. The authors observed significant increases in the number of cells positive for cTnI, α-actinin, GATA4 and Cx-43 at both gene and protein levels after T3 treatment while downregulating the pluripotency marker Oct4.

2.6.2 Oxytocin

Oxytocin has been revealed to have several new roles in the cardiovascular system including vasodilatation, lowering of blood pressure, parasympathetic neuromodulation, negative cardiac inotropy and chronotropy and anti-oxidative, anti-inflammatory and metabolic effects. It has been observed to express in early fetal heart development.

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Oxytocin was shown to stimulate cardiomyogenic differentiation of various stem cell types. Treatment of MSCs with oxytocin stimulates paracrine factors beneficial for cardioprotection [151, 152].

In a study, human umbilical-cord-blood-derived MSCs pretreated with oxytocin expressed cardiac markers, connexin-43, cTn-I and α-sarcomeric actin. [153] In the same report, *in vivo* studies demonstrated that in the treated group, transplanted cells were detected in the infarcted areas with a marked lowering in cardiac fibrosis and macrophage infiltration, expressing cardiac proteins and significantly improving cardiac function after cellular transplantation.

2.6.3 Neuropeptide Y

Neuropeptide Y (NPY) induced differentiation of bone-marrow-derived MSCs into cardiomyocytes following their transplantation into infarcted myocardium. NPY directly induced neonatal and adult cardiomyocyte cell-cycle re-entry and enhanced the number of differentiated cardiomyocytes from MSCs in the infarcted myocardium, which corresponded to improved cardiac function, reduced fibrosis, attenuated left ventricular remodeling and increased angiomyogenesis [154].

2.7 Miscellaneous compounds

In addition to the above mentioned compounds, there are some isolated studies that show cardiomyogenic potential of some small chemical compounds. S-nitroso-N-acetyl-D,L-penicillamine (SNAP)-treated adipose tissue-derived stem cells in rat infarcted myocardium showed significant improvement in the ejection fraction in rat hearts with increased expression of cTn-T and von Willebrand factor. SNAP alters the redox environment of adipose tissue stem cells, associated possibly with a predifferentiation state, which results in the improvement of cardiac function after transplantation [155].

Nitric oxide, a free radical signaling molecule and its intermediates also induced increased expression of cardiac-specific genes in bone marrow MSCs [156]. Similarly, difluoromethylornithine can also induce early differentiation of MSCs to cells of myocardial lineage [157].

Sulfonylhydrazones promote myocardial regeneration by activating the earliest genes involved in cardiac differentiation in adult and embryonic stem cells, including peripheral blood mononuclear cells, bone marrow MSCs and induced pluripotent stem cells [158, 159].

Tetrahydroisoquinoline analog of nitrobenzylmercaptopurine riboside (NBMPR) is a nucleoside transport inhibitor. It has shown cardiomyogenic potential and is a promising candidate for the development of novel cardioprotective treatment as there is a lesser chance of losing the nitro benzyl part of NBMPR *in vivo* [160].

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2.8 Conclusions

MSCs can be utilized in combination with small molecules to regenerate damaged heart tissue. Small molecule-based approaches can be employed in stem cell biology because these molecules have characteristics that make them uniquely suited to regulate the complex signaling pathways that control self-renewal, pluripotency, cell fate, behavior and differentiation or reprograming toward cardiomyogenic lineage. Continued research with technological development and application of small molecule-based chemical approaches in stem cells would lead to the discovery of ideal novel candidates that can differentiate MSCs into cardiomyocytes *in vitro*. These predifferentiated cells can be transplanted *in vivo* to regenerate damaged myocardium, and are expected to improve cardiac performance.

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Dursun Gündüz and Muhammad Aslam

3 MicroRNAs as modulators of endothelial differentiation of stem cells: role in vascular regenerative medicine

Abstract: MicroRNAs (miRNAs) are a class of small (~22 nucleotides), widely distributed and highly conserved non-coding RNA molecules, and they play an important role in posttranscriptional regulation of gene expression by directly targeting mRNA. Embryonic and induced pluripotent stem cells (ESCs and iPSC, respectively) hold great promise for vascular regenerative therapies. However, several limitations currently prohibit their therapeutic use. The importance of miRNAs in controlling the gene expression profile of a particular cell type is emerging and a multitude of miRNAs have been identified that play key roles in determining the fate and differentiation of the pluripotent cells and thus govern the vascular development and regeneration. A combination of pluripotency transcription factors and particular miRNAs not only enhances the pluripotency of stem cells but also has been reported to enhance their endothelial differentiation. This chapter will summarize the findings that focus different miRNA clusters in the induction, maintenance and directed endothelial differentiation of pluripotent stem cells.

3.1 Introduction

Endothelial cells (ECs) form the innermost lining of the blood vessels and play an active role in the maintenance of vascular integrity and homeostasis [1] through the synthesis and release of numerous vasoactive molecules [2], and are crucial for vasculogenesis [1] and angiogenesis [3]. In vertebrates, the cardiovascular system is the first functional organ formed during embryonic development. During embryogenesis, the ECs form a rudimentary vascular meshwork that undergoes a series of developmental changes thus culminating in stabilized vessels by the recruitment of mural cells [4–6]. During the establishment of functional vascular networks through the process of vasculogenesis, a plethora of signalling pathways coordinate to warrant the development and maintenance of these vascular networks. [6] Loss of ECs function leads to the development of numerous chronic macro- and microvascular anomalies. During the last decade, it has become clearer that pluripotent stem cells can be directed to differentiate towards EC lineage and may potentially be used for the repair of injured vasculature [7]. By delivering stem/progenitor cells to the injured tissues or vessels, new blood vessels can potentially be formed [8]. The understanding of molecular mechanisms regulating EC differentiation will greatly benefit regenerative strategies

DOI 10.1515/9783110493764-003

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for the treatment of various vascular diseases. To this end, numerous protocols have been optimized and reported to differentiate pluripotent cells to adopt morphofunctional phenotypes of ECs [9]. However, most of these protocols suffer from limited efficiency and low rates of differentiation. Various strategies have been adopted to address these limitations. Micro RNAs (miRNAs or miRs) are relatively new players in the field. Given their involvement in regulation of at least 30% of mammalian genes, miRNAs are being extensively studied to promote directed differentiation of stem cells to adopt EC phenotypes [10]. This chapter presents an overview of various miRNAs involved in the processes of angiogenesis and vasculogenesis, with special emphasis on their potential to induce EC differentiation of stem cells. The chapter also discusses their future potential for application in regenerative vascular medicine in the clinical perspective.

3.2 MicroRNAs

MiRNAs belong to a class of small (~22 nucleotide; nt) noncoding RNAs that regulate gene expression at a transcriptional level [11]. They bind to target mRNA at 3′-untranslated region (UTR), coding region or 5′-UTR thus inducing its degradation. MiRNAs are first transcribed as primary miRNAs (pri-miRNAs) by RNA polymerase II either from specific miRNA genes or intronic sequences of the protein coding genes, which are then processed into ~70 nt precursors (pre-miRNAs) and finally to mature miRNAs by sequential action of two RNase III proteins, *Drosha* and *Dicer*, respectively [12, 13]. One of the duplex strands, the guide strand, is preferentially selected for entry into the RNA-induced silencing complex (RISC), whereas the other strand, known as the passenger strand or miRNA* strand, is typically degraded. However, in some cases, both strands are biologically active as miRNAs. [13] The mature miRNAs are incorporated into RISC consisting of argonautes (AGO) and other RNA binding proteins. The RISC identifies its target mRNA sequences (usually on 3′-UTR) via specific 5′-end "seed sequence" (between nt 2 and 7 on 5′-end of miRNA) and translationally repress the target proteins [14]. Beyond targeting mRNAs, some RISCs may act directly on the genome *via* formation of RNA-induced transcriptional silencing (RITS) complexes consisting of AGO1 with an associated miRNA and chromodomain proteins [15]. Upon target recognition, RITS recruits histone methyltransferases, which modify the histones associated with the target DNA locus [16]. Currently, over 2500 human miRNAs are annotated in the miRBASE database (www.mirbase.org) [17]. Each mRNA can be targeted by multiple miRNAs and each miRNA can target several genes thus simultaneously regulating several signalling pathways. Though smaller in size, they perform bigger functions in the cells ranging from survival to apoptosis to all the metabolic activities and lineage commitment [18]. Fig. 3.2.1 provides an overview of the process of generation of miRNAs and a general schema of their functioning.

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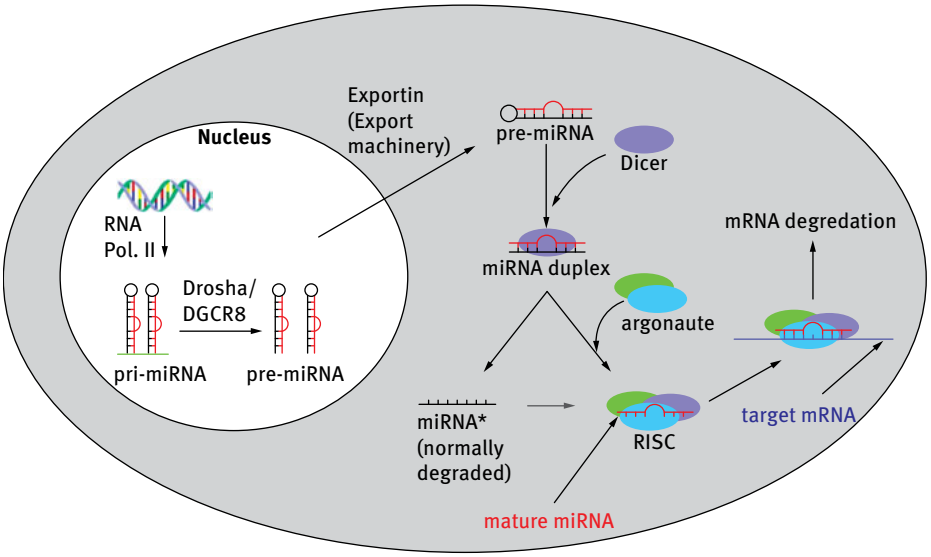

Fig. 3.2.1: A graphical presentation of miRNA production and mechanism of action.

Generally, miRNAs are transcribed as primary transcripts (pri-miRNAs) by RNA polymerase II (RNA Pol. II). Pri-miRNAs usually contain one or more hairpins with miRNAs lying in the double-stranded stem. Pri-miRNAs are processed by the nuclear microprocessor complex comprising the RNase III (Drosha) and DGCR8, leading to generation of precursor miRNA (pre-miRNA). Pre-miRNAs are exported from the nucleus to cytoplasm by nuclear export machinery (Exportin). In the cytoplasm the *Dicer* (RNase III enzyme) cleaves off the loop of the hairpin generating a double-stranded RNA. The mature miRNA strand is subsequently incorporated into the RNA-induced silencing complex (RISC), where it directly binds to a member of the argonaute (AGO) protein family. The other strand is called miRNA* and is normally degraded. However, in some cases, miRNAs* may also be functional.

3.3 Stem cells in vascular regeneration

Vascular diseases encompass diseases of arteries, veins and lymphatics. Noticeable amongst these diseases affecting the circulatory system as the major causes of morbidity and mortality in the Western world are peripheral artery disease, renal artery disease, carotid artery disease and peripheral venous disease [8]. Progression of the macrovascular diseases ultimately leads to the development of microvascular complications and organ failure. The traditional surgical and non-surgical treatment strategies for vascular disease include opening-up or bypassing the heavily blocked vessel using autologous or synthetic grafts. Nevertheless, these procedures are inefficient to

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prevent or reduce target organ damage. Therefore, there is a considerable unmet need for novel therapies addressing the repair of damaged vasculature.

Cell transplantation therapy is an emerging interdisciplinary field that aims to restore normal function of organs through repair, replacement or regeneration of cells, tissues or organs that are lost or damaged due to disease, injury or aging [19]. In this promising technology, a new vascular network can be grown in the ischemic tissues by delivering angiogenic and vasculogenic factors. Alternatively, stem/progenitor cells can be transplanted and act as local reservoirs of vascular repair factors released as part of their paracrine activity, thus modulating the tissue microenvironment to be conducive to vascular regeneration [8] and vasculogenesis [20]. Despite immense progress in the field, the ideal source of the cells for vascular regenerative therapies remains a hot area of research. In this regard, stem cells are considered as the most suitable source of cells and offer an important platform that can be used to progress the field from lab bench to bedside. Stem cells are undifferentiated cells having the capability of unlimited self-renewal and the potential to differentiate into a variety of morphofunctionally competent cell lineages in response to appropriate cues from their microenvironment. Currently, the most commonly used stem cells for vascular regeneration include embryonic stem cells (ESCs), mesenchymal stem cells (MSCs) in addition to circulating endothelial progenitor cells (EPCs) [21]. Other cell sources including induced pluripotent stem cells (iPSCs) and human amniotic fluid stem cells (hAFSCs) are also gaining popularity on account of their pluripotent nature [8, 21]. In line with vascular regeneration, vascular repair is a complex process and requires mobilization, chemotaxis, adhesion, proliferation and differentiation of progenitor cells capable of adopting an endothelial phenotype [22]. The process of differentiation in its own right is multifaceted and involves coordinated involvement of multiple transcription factors, signalling networks, and epigenetic modifications before the cells undergo a tremendous morphofunctional change to adopt derivative endothelial phenotype [23–25]. There is mounting evidence in the published data that circumscribes a highly specialized involvement of miRNAs in every step of the process of vascular regeneration and repair, ranging from self-renewal to differentiation of the stem/progenitor cells, thus suggesting promising prospects of miRNAs in regenerative medicine. In the following paragraphs, we will discuss the recent advances in our understanding of miRNAs in the induction and maintenance of pluripotent stem cell status and their EC differentiation.

3.4 Endothelial enriched miRNAs and their role in angiogenesis

ECs play an important role in vascular development, angiogenesis and in maintaining vascular integrity. ECs receive multiple signals and information from their microenvironment which modulate their EC fate, eventually leading to initiation of the complex process of angiogenesis. Although a repertoire of factors is required to initiate and propagate

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the process of angiogenesis, recent studies have established miRNAs as key regulators of these processes at the transcription level. Likewise, there are no EC-specific miRNAs defined as yet, however several miRNAs have been identified that are enriched in ECs and regulate their angiogenic capacity. The EC-enriched miRNAs relevant to the process of angiogenesis can be classified into two major groups: the pro-angiogenic miRNAs and the angiomiRs, i.e. miR-126, miR-130, miR-21 and the let7 family and the anti-angiogenic or the anti-angiomiRs, i.e. miR-17/92 cluster and the miR-221/222 family [26]. Both of these miRNAs classes are highly expressed in ECs to regulate different genes expression and their activity pertinent to EC survival, apoptosis, senescence and differentiation. The first evidence that miRNAs play an important role in vascular development and angiogenesis was shown by Yang *et al.* using *Dicer* (an enzyme required for the processing of mature miRNAs) knockout mice [27]. Homozygous embryos died between E12.5 and E14.5 due to deregulation of angiogenic factors and signalling pathways leading to impaired vascular development. Similarly, conditional ablation of endothelial-specific *Dicer* resulted in impaired angiogenic response in tumor models and in response to ischemia and impaired wound healing [28]. *In vitro* knockdown of either *Dicer* or *Drosha* negatively regulated miRNAs let7 and miR-17/92 cluster expression and angiogenesis [29, 30]. The anti-angiogenic effect of *Dicer* deletion could be partially corrected by transfection of miR-17/92 cluster [29]. Moreover, knockdown of argonaute 2 (AGO2) in ECs resulted in suppression of their angiogenic capacity *in vitro* [31]. These studies report that suppression of miRNAs processing machinery is sufficient to significantly impair angiogenesis thus demonstrating a crucial role of miRNAs in regulating angiogenesis. A description of individual miRNAs involved in angiogenesis is given below.

3.4.1 miR-126

The first well-characterized miRNA in ECs is miR-126, which plays an important role in the vascular development process and angiogenesis during embryogenesis. EC-specific deletion of miR-126 is embryonically lethal and results in vascular defects and hemorrhages [32]. Loss of function studies involving miR-126 in Zebrafish embryos also causes vascular leakage [33]. On the same note, EC-specific overexpression of miR-126 selectively enhances re-endothelialization of injured vessels and inhibited vascular stenosis *via* inhibition of proliferative vascular smooth muscle cells [34]. These data demonstrate proof-of-concept that vascular stenosis can be alleviated by miR-126 manipulation while still maintaining ECs functioning. In a recently published study, ectopic expression of miR-126 in the placenta enhanced microvascular density and pub survival in a rat model of pre-eclampsia [35]. The ectopic overexpression of miR-126 enhanced ECs proliferation, differentiation and emigrational activity to support proangiogenic activity in the placenta. At a molecular level, the proangiogenic effects of miR-126 overexpression were supported by suppression of antiangiogenic factor p85β (PI3KR2) as the possible mechanism. These data was supported by *in vitro* studies wherein miR-126

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ablation led to the loss of EC function. Moreover, miR-126 targets the negative regulator of VEGF signalling in ECs and Spred1 and promotes VEGF-dependent angiogenesis [33]. The association of Spred-1 expression with miR-126 has also been observed in ECs isolated from diabetes II patients. The functional impairment of ECs in diabetes II patients in terms of proliferation, migration and enhanced apoptosis has been associated with the downregulation of miR-126 via altered expression of Spred-1 [36]. Gain-of-functions studies showed that restoration of miR-126 in the patient-derived ECs *in vitro* helped the cells regain these functions. In addition to proangiogenic activity, mature vessels also respond to the different levels of miR-126 expression. It enhances the integrity of mature blood vessels by targeting p85β (PI3KR2) and inhibiting downstream Ang-1/Tie-2 signalling [37]. These molecular data strongly advocate the significance of miR-126 as a crucial determinant of angiogenic activity of ECs.

3.4.2 miR-17/92 cluster

The miR-17/92 cluster, also known as oncomiR-1, is one of the best-studied miRNA clusters as dysregulation of this cluster leads to a variety of pathological conditions [38]. Nevertheless, their role in developmental angiogenesis is controversial and remains elusive. A polycistronic gene encodes for the seven mature miRNA members of the cluster including miR-17, miR-17*, miR-18a, miR-19a, miR-19b, miR20a, and miR-92a as all of them are processed from the common primary transcript [39]. Earlier studies reported proangiogenic activity of miR-17/92 cluster in tumor vasculature by targeting angiogenesis suppressor-thrombospondin-1 (TSP1) and connective tissue growth factor (CTGF) [40]. Subsequently, the same group of researchers demonstrated that miR-17/92 cluster targeted the TGFβ signalling pathway for activation of antiangiogenic signalling in the tumor vasculature [41]. Research studies focused on determining the effect of individual members of the cluster demonstrated their antiangiogenic activity in the cultured ECs [42]. Epigenetic studies revealed that miR-17/92 cluster was controlled by histone deacetylase 9 (HDAC9). Knockdown of HDAC9 resulted in increased expression of miR-17/92 members with concomitant angiogenic defects while silencing of miR-17-20a rescued these angiogenic defects [43]. Knockdown of individual miRNAs of this cluster promoted angiogenesis, e.g. miR-19b suppressed cell cycle progression which was alleviated by its knockdown [44]. Likewise miR-17* targeted Flk-1 and its downregulation promoted Flk-1 signalling [45]. These data substantiated the antiangiogenic function of the miR-17/92 cluster.

3.4.3 miR-15a/16

miRNA-15a/16 gene cluster is located on the chromosome 13q14 in the humans and 14qC3 in the mouse. The gene resides in the intron of a long non-coding RNA (lncRNA)

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Dleu2 [46, 47]. This miRNA has been extensively studied in tumor-related angiogenesis and is described as tumor suppresser miRNA, which is downregulated in a variety of tumors [48]. The miRNA cluster is negatively regulated by hypoxia and targets VEGF/VEGFR2 signalling [48–50]. Overexpression of this cluster *in vivo* suppressed tumor angiogenesis and growth [51]. In transgenic mice having EC selective overexpression of miR-15a/16, significantly reduced angiogenesis and blood perfusion was observed in the hind-limb of the experimental animals at 1–3 weeks after hind-limb ischemia [52]. The reduced angiogenic potential was attributed to the direct inhibition of the endogenous endothelial FGF-2 and VEGF activities as miR-15 directly targeted the two proangiogenic growth factors to confer its antiangiogenic activity. Conversely, knockdown of miR-15a/16 improved the angiogenic potential of the circulating EPCs [53]. These data suggest that the miR-15a/16 cluster may be an important target to improve organ-specific therapeutic angiogenesis.

3.4.4 miR-130a

MiR-130a is highly expressed in ECs and is known as proangiogenic miRNA. The main targets of miR-130a to incur its proangiogenic activity include antiangiogenic transcription factors, homeobox A5 (HOXA5), growth arrest specific homeobox (GAX) [54] and Runt-related transcription factor 3 (RUNX3) [55]. In cardiomyocytes it activates PI3K/Akt signalling by targeting phosphatase and tensin homolog (PTEN) to promote cell survival [56]. Its expression level in diabetic EPCs is reduced, which results in their impaired angiogenic capacity. Conversely, overexpression of miR-130a improves the angiogenic potential of EPCs [57].

3.4.5 miR-21

miR-21 has been implicated for both pro- and antiangiogenesis effects [58–62]. In tumor vasculature, miR-21 is widely known as proangiogenic miRNA *via* targeting PTEN, PI3K/Akt and HIF1α-dependent VEGF signalling [63, 64]. Transgenic overexpression of miR-21 in ECs reduces proliferation, migration and tube formation *in vitro* by targeting RhoB while its knockdown using a locked nucleic acid (LNA)-anti-miR results in opposite effects [58]. Furthermore, the therapeutic potential of miR-21 as an angiogenesis inhibitor was demonstrated *in vivo* in a mouse model of choroidal neovascularization. Likewise, overexpression of miR-21 in ECs or EPCs resulted in their early senescence and reduced angiogenesis [60, 61]. In contrast, transplantation of human-derived EPCs over expressing miR-21 in a mouse critical limb ischemia model improved angiogenesis and neovascularization *in vivo* accompanied by increased HIF-1α activity [62]. Moreover, short-term exposure to hypoxia significantly induced miR-21 expression in ECs and ESCs [65] and promoted their survival and angiogenic capacity *via* VEGF signalling

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[66]. Proangiogenic and pro-survival activity of miR-21 have also been reported in cells other than ECs in response to pharmacological preconditioning with diazoxide, which induced higher levels of miR-21 in the preconditioned cells [67]. Whereas the pro-survival role of miR-21 was mediated by the extracellular signal-regulated kinase-1/2 (ERK1/2) and signal transducers and activator of transcription-3 (STAT3) downstream of interleukin-11 (IL-11), *in vivo* transplantation of the preconditioned cells significantly enhanced the capillary density in the infarcted myocardium.

3.5 Post-ischemic collateral growth and miRNAs

Although several miRNAs involved in angiogenesis have been identified, little data is available on miRNAs playing a role in arteriogenesis. Following a major artery occlusion, two types of vascular repair responses are activated in the injured ischemic tissue: shear-stress sensitive arteriogenesis or development of collateral arteries from pre-existing arterioles and parenchymal hypoxia-driven angiogenesis or sprouting of capillaries [68]. Shear stress is one of the strongest inducers of arteriogenesis [68, 69]. Besides other factors [68], miR-21 is upregulated in ECs in response to high shear-stress [70]. Given its crucial role as a proangiogenic miRNA, it is generally anticipated that miR-21 would promote arteriogenesis in response to shear-stress-induced collateral development. However, opposite effects were observed by Hutcheson *et al.* as they reported an upregulated myocardial expression of miR-21 in the metabolic syndrome JCR rats leading to enhanced smooth muscle cell (SMC) proliferation but with reduced collateral growth that could be rescued by antagomiR-21 administration [71]. These data implied that miR-21 was a negative regulator of arteriogenesis. Additionally, circulating levels of miR-21 are elevated in hypertensive patients with increased carotid intima media thickness [72]. This suggests that more extensive analysis of miR-21 is required to establish its role in the process of arteriogenesis. Endothelial-specific deletion of miR-17/92 cluster enhanced collateral growth in the cardiac and hind-limb ischemia model thus suggesting the miR-17/92 cluster as a negative regulator of arteriogenesis [73]. Furthermore, it was found that miR-19a/b target frizzled 4 (FZD4) and LRP6, which regulate vascular development *via* Wnt signalling [74, 75]. Another miRNA analysed in the process of arteriogenesis is miR-155. Recent data from Grundmann's group revealed a tissue-specific role of miR-155 in arteriogenesis. In the hind-limb ischemia model, miR-155 is reduced and the knockout mice (miR-155$^{-/-}$) presented a reduced vascularization suggesting a proarteriogenic miRNA in hind-limb ischemia model [76]. This is due to reduced recruitment of inflammatory cells to the ischemic tissue and altered release of pro-arteriogenic cytokines from the miR-155-deficient macrophages. In ECs miR-155 targets AGTR1, which reduces leukocyte-endothelial interaction while in macrophages it targets SOCS-1 which alters their cytokine profile [76]. In contrast, hearts from miR-155$^{-/-}$ mice are protected against ischemia/reperfusion injury [77]. These differential effects seem to be due

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to an altered immune response in a myocardial infarction (MI) model and there is little published data on the role of miR-155 in immune response in MI. MiR-100 is yet another negative regulator of neovascularization [78, 79]. It acts *via* the suppression of mTOR in ECs and SMCs and can be an important target for therapeutic intervention [78]. A recent study has identified the miR-14q32 cluster (miR-329, miR-487b, miR-494 and miR-495) as negative regulators of arteriogenesis in mouse hind-limb ischemia model [80]. Loss-of- function of any or more members of this miRNA cluster results in enhanced vascularization. The only miRNA cluster identified as a positive regulator of arteriogenesis so far is miR-132/212 cluster. The miR-132/212 cluster is upregulated during early the period of hind-limb ischemia and miR-132/212 knockout mice show reduced collateral growth after ligation [81]. The miR-132/212 cluster directly targets the antiangiogenesis factors Spred-1, Spry-1 and Rasa-1, which antagonize VEGF/Ras/Raf signalling, and thus improve neovascularization. However, data on its role in cardiac vascularization in the experimental animal model of MI is still lacking.

3.6 miRNAs regulating endothelial differentiation of EPCs and angiogenesis

EPCs are circulating cells that express different cell surface markers similar to those expressed by ECs, adhere to endothelium at sites of injury and participate in vasculogenesis with an important role of miRNAs at different stages of EPCs differentiation [82].

3.6.1 Role in proliferation

The miR-221/222 family and miR-21 are reported to act as antiproliferative miRNAs in EPCs. The expression of the members of the miR-221/222 family is upregulated in patients with coronary artery disease (CAD) [83], which is weakly negatively correlated with the proliferation of EPCs. Overexpression of miR-221/222 family in healthy EPCs reduced their rate of proliferation [84, 85]. Moreover, the expression of miR-21 is upregulated in EPCs from atherosclerotic patients accompanied by their reduced proliferation rate and emigrational activity while knockdown of miR-21 rescues this phenotype [86]. Unlike miR-221/222 family and miR-21, miR-126 and miR-130a are proproliferative miRNAs in EPCs, which are downregulated in EPCs from diabetic patients [36, 87] and overexpression of miR-126 significantly increased their proliferation rate and emigrational activity while knockdown of miR-126 resulted in reduced proliferation of normal EPCs [36]. Upregulated expression of miR-143 in cells with concomitant transgenic overexpression of Akt and the angiocompetent molecule angiopoietin-1 significantly enhances the proliferation rate in the genetically modified stem cells. Molecular studies revealed phosphorylation of FOXO1 and Erk5 drove the transcriptional activation of cyclin D1 and Cdk4 in the genetically modified stem cells [88].

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3.6.2 Role in senescence

The role of miRNAs in the regulation of EPC senescence is emerging and at least four miRNAs have been identified to either directly or indirectly influence the process of senescence in the EPC. For example, expression of miR-34 was upregulated in EPCs from patients with coronary artery disease [89]. Similarly, overexpression of miR-34a in rat EPCs resulted in reduced expression of SIRT1 accompanied by reduced angiogenesis and enhanced cellular senescence of EPCs [90]. Moreover, the expression of miR-10A* and miR-21 was upregulated in EPCs in aged mice and knockdown of these miRNAs enhanced the proliferation rate of the aged EPCs [60]. On the same note, miR-22 is upregulated in aged donor-derived EPCs as compared to the young donor-derived EPCs. Gain of function *via* overexpression of miR-22 in young donor-derived EPCs induced senescence in these cells with simultaneous downregulation of Akt3 [91].

3.6.3 Role in differentiation

Differentiation of EPCs into mature ECs plays an important role in vascular regeneration and homoestasis. Akin to their functional participation in the other biological processes in the EPCs, miRNAs also play an important and well-defined role in the differentiation of EPCs to mature and morphofunctionally competent ECs particularly during ischemic diseases. Hypoxia is an important factor suppressing EC differentiation of progenitor cells by the enhanced expression of miR-107 which targets HIF-1β [92, 93]. Knockdown of miR-107 promotes differentiation of EPCs into mature ECs. On the same note, non-hypoxic stabilization of HIF-1α has been shown to support endothelial commitment of stem cells [88]. miR-16 acts as a negative regulator of EC differentiation and its knockdown in EPCs enhances the expression of EC markers [94]. Shear stress is another important inducer of EC differentiation of EPCs which modulates the expression of several genes *via* miR-34a [95]. Specific knockdown of miR-34a resulted in the loss of the capacity of EPCs to differentiate into mature ECs but increased the SMC markers suggesting its role in trans-differentiation of EPCs [96]. Like ECs, miR-126 is also highly enriched in EPCs also and is involved in their EC differentiation at several stages. Moreover, the expression level of miR-126 is positively associated with prognosis of MI patients [97]. In EPCs, miR-126 controls their EC differentiation and maturation *via* targeting phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2) and overactivation of PIK3R2 results in enhanced mesenchymal transition of EPCs suggesting PIK3R2 acts as negative regulator of EC differentiation and offers an important target for therapeutic intervention to enhance EC population and reduce fibrosis [98]. However, ectopic expression of miR-126 enhances the angiogenic capacity [99] and vascular repair function of EPCs in a deep vein thrombosis model [100]. A graphical presentation of various miRs involved during the different stages of EPCs differentiation to ECs is shown in Fig. 3.6.1 (modified from Gündüz and Aslam 2016) [101].

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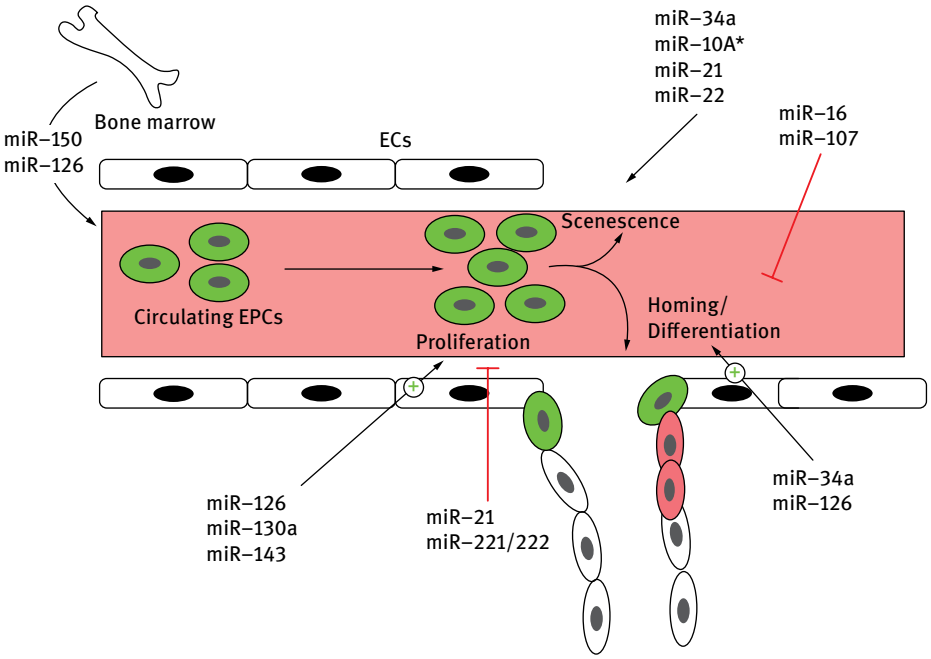

Fig. 3.6.1: A graphical presentation of different miRNAs involved in different stages of EPCs differentiation to ECs. MicroRNAs miR-126 and miR-150 facilitate mobilization of EPCs from bone marrow and thus enhance population of circulating EPCs. The miR-126, miR-130a and miR-143 induce proliferation while miR-126 and miR-34a mediate their homing and differentiation to ECs.

3.7 ESC-specific miRNAs regulating their commitment to ECs

ESCs are the pluripotent cells derived from the inner cell mass of a mammalian blastocyst. Given their capacity of long-term undifferentiated self-renewal and the ability to differentiate into almost any cell type of the three germ layers, ESCs hold the promise of an ideal stem cell for clinical applications [102]. Several studies have carried out miRNA expression profiling of ESCs to identify ESC-specific miRNA clusters, which are preferentially expressed in ESCs and their expression changes, either up or downregulated, during the process of differentiation to adopt lineage commitment [103, 104]. The first evidence that miRNAs are required for self-renewal and differentiation of ESCs demonstrated that ESCs lacking *Dicer* lost the ability to proliferate or form embryoid bodies [105]. Later, it was found that miR-290/295 cluster accounts for ~50% of the miRNA contents in the mouse ESCs and its expression was downregulated during cell differentiation [104]. Interestingly, overexpression of three miRNAs, i.e. miR-291a-3p, miR-294 and miR-295, from this cluster are sufficient to induce pluripotency in somatic cells [106] The human homolog of the mouse miR-290/295 cluster is miR-371-373 cluster, which is specifically expressed in

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human ESCs and is upregulated in some tumors [107, 108]. Another ESC-specific miRNA cluster is miR-302/367, which is highly expressed both in the human and mouse ESCs [109]. This miRNA cluster has been used for rapid and efficient reprogramming of fibroblasts into iPSCs without manipulation of cells with exogenous transcription vectors [110]. The exogenous transcription factor-free iPSCs generated by miRNA manipulation alone showed similarity in their differentiation potential and teratogenicity. Molecular studies showed that iPSCs thus generated had highly induced Oct4 and HDAC2 suppression. Interestingly, all these miRNA clusters possess identical seed sequences (*AAGUGCU*) suggesting that they might be regulating the same pools of mRNAs [111]. During the process of differentiation, a set of miRNAs is upregulated which targets and suppresses the pluripotency genes Oct4, Sox2 and Nanog, thus supporting the ESCs to undergo differentiation. These include the let-7 family of miRNAs, miR-21, miR-145, miR-134, miR-296 and miR-470 [112–114]. Additionally, there is a long list of miRNAs that show differential expression during the process of EC differentiation, however, their role in EC differentiation and vascular development derived from ESCs needs to be better defined and established further. Two novel miRNAs, miR-6086 and miR-6087 have been reported that target the endothelial-specific genes, vascular endothelial (VE-) cadherin and endoglin, respectively, during ESC differentiation to EC phenotype. [115] Based on the miRNA expression profiling of human ESCs at defined stages during endothelial differentiation, level of the three miRNAs, miR-99b, miR-181a and miR-181b was significantly upregulated. Interestingly, the upregulation in their expression occurred in a time- and extent-of-maturation-level-dependent manner. As the level of maturation increased in the ECs, the level of expression of the three miRNAs also increased. Moreover, overexpression of these miRNAs enhanced endothelial differentiation of human ESCs and injection of the derivative ECs overexpressing these miRNAs into a mouse model of hind-limb ischemia significantly improved neovascularization in the ischemic limb [116]. In ECs, miR-99b targets Nox4, [117] while miR-181b targets NF- κB and PHLPP2, a phosphatase regulating Akt phosphorylation at Ser473 [118, 119], thus indirectly activating the PI3K/Akt signalling pathway. However, contrary to the lymphatic ECs, miR-181a negatively regulates their differentiation by targeting homeobox transcription factor Prox1, and knockdown of miR-181a in embryonic lymphatic cells enhances their endothelial differentiation [120]. Although overexpression of these miRNAs promotes EC differentiation, their knockdown has no significant impact on EC differentiation of human ESCs suggesting they are not integral to EC differentiation of ESCs [116]. Kane *et al.* has also developed a feeder-free and serum-free protocol for direct derivation of ECs from human ESCs with a concomitant increase in the induction of angiogenesis-related miRNAs including miR-126 and miR-210 during the process differentiation [121]. The role of miR-210 in myocardial angiogenesis has also been reported in rat model of MI. Although there was little evidence that the transplanted bone-marrow-derived stem cells adopted an endothelial phenotype, regional perfusion and the blood vessel density were

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significantly enhanced in the infarcted hearts transplanted with cells manipulated for transgenic overexpression of miR-210. On the same note, miR-17/92a cluster is upregulated during EC differentiation of ESCs, although forced knockdown of individual members of this cluster in ESCs during the process of differentiation had no impact on their EC differentiation [122]. A recent report demonstrated an upregulation of miR-150 and miR-200c during EC differentiation and antagomirs to these miRNAs reduced the level of vascularization in chick embryos [123]. This data is opposed by another report showing downregulation of the miR-200 family in mouse ESCs during the process of EC differentiation whereas constitutive overexpression of miR-200 family members repressed EC differentiation [124]. In the quiescent ECs, miR-150 is highly expressed and seems to regulate blood vessel stability by targeting angiogenic genes CXCR4, DLL4 and FZD4 whereas its deficiency promotes pathological neovascularization [125]. The opposing functions of miR-150 have been attributed to the differences in the species under study and the experimental protocols. A careful review of miRNAs regulating different transcription factors required for EC differentiation of ESCs will provide a deeper insight into the miRNAs regulating the trans-differentiation. For example, Shi *et al.* demonstrated that GATA2 together with Etv2 regulated ECs and hematopoietic cell differentiation of mouse ESCs [126]. Etv2 controls vasculogenesis during Zebrafish development and its transcription is regulated by the let-7 family of miRNAs [127]. Ectopic expression of let-7 miRNAs leads to downregulation of Etv2 and vascular defects in Zebrafish [127]. Likewise, GATA2 expression in murine cardiac ECs is regulated by miR-24 and its ectopic expression promoted ECs apoptosis thus suggesting let-7 family and miR-24 as negative regulators of endothelial differentiation of ESCs [128]. A graphical presentation of different miRs involved in ESCs differentiation to adopt EC phenotype is shown in Fig. 3.7.1 (modified from Gündüz and Aslam 2016) [101].

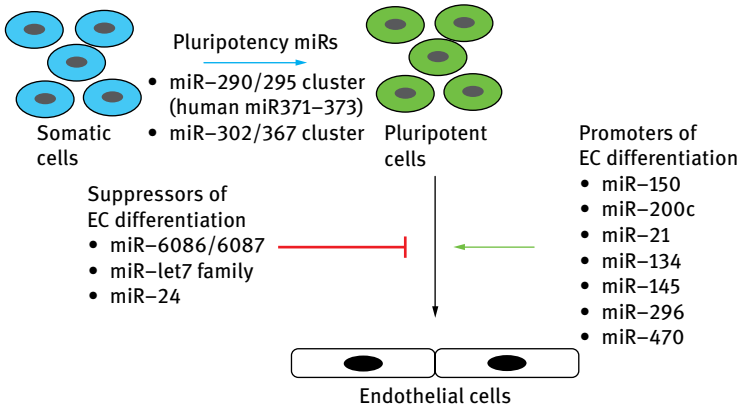

Fig. 3.7.1: A graphical presentation of the different miRNAs that participate during the endothelial differentiation of ESCs at different stages.

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3.8 IPSCs and miRNAs

The clinical application of hESCs is limited by the moral and ethical issues pertaining to their availability and use in addition to teratogenicity and immune rejection of their derivative tissue. In this regard, human-induced pluripotent stem cells (iPSCs) have recently emerged as a promising alternative [129]. In this method, somatic cells can be reprogrammed by forced expression of four pluripotent transcription factors (either Oct4-Sox2-Klf4-c-Myc or Oct4-Sox2-Nanog-Lin28 for animal and human cells, respectively) [130, 131]. The major limitations in this protocol are the poor reprogramming efficiency, which is usually less than 1% in most cases, in addition to the use of viral vectors and random insertion of exogenous transcription factors in the genome of the reprogrammed cells. Using *Dgcr8* (miRNA processing protein) knockout-mouse-derived ESCs, the miR-290 cluster was identified as an important regulator of mouse ESCs pluripotency [132]. Interestingly, forced expression of subsets of this miRNA cluster improved the reprogramming efficiency of mouse embryonic fibroblasts (MEFs) in the presence of four Yamanaka factors, i.e. Oct3/4, Sox2, Klf4 and cMyc [106, 133]. Likewise, overexpression of its human orthologue, miR-371 and miR-302/367 clusters, enhanced reprogramming of human fibroblasts by 10–15-folds [134]. Morrisey *et al.* demonstrated that expression of the miR-302/367 cluster alone successfully induced both mouse and human somatic cells in the presence of HDAC inhibitor without the need of extrinsic Yamanaka factors [110]. Transfection of the miR-302/367 cluster mimetic in the presence of Oct4 and Sox2 overexpression could increase the pluripotency efficiency by 50-fold while knockdown of the miR-302/367 cluster completely blocked iPSC generation thus suggesting an important role of miR-302/367 cluster in the induction of pluripotency [135, 136]. The increased efficiency by miR-290 (miR-371 human homologue) is mediated by mesenchymal-to-epithelial (MET) transition, affecting the cell cycle and inhibiting the TGF-β signalling [133], while miR-302 cluster targets epigenetic regulators responsible for DNA methylation [137]. Recent reports have implied more miRNAs that contribute to somatic cell reprogramming. These include c-Myc regulated miRNAs, miR-21 and miR-29a, which are highly expressed in MEFs. Knockdown of these miRNAs enhanced the reprogramming efficiency of MEFs [138]. Similarly, miR-34-deficient MEFs depicted higher reprogramming efficiency, thus suggesting these miRNAs as negative regulators of pluripotency [139].

The role of miRNAs in endothelial differentiation of iPSCs is still emerging. As discussed earlier, depletion of miR-21 enhanced the reprogramming efficiency of MEFs while re-expression of miR-21 in iPSCs enhanced their endothelial differentiation capacity, thus suggesting miR-21 as an important candidate to reprogram somatic cells into ECs [138, 140]. The controlled temporal expression of miR-21 with other factors may offer an important tool to directly differentiate somatic cells into ECs without iPSC development. The identification of miR-199a and miR-199b as differentially regulated miRNAs during EC differentiation is an important step forward in the field, but their role in EC differentiation needs to be verified [141, 142]. Similarly, the results from an extensive miRNA profiling of human iPSCs and differentiated ECs have revealed several

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of the differentially regulated miRNAs including miR-20a, miR-20b, miR-27b, miR-100, miR-125a-5p, miR-137, miR-149, miR-181a, miR-210, miR-222 and miR-296-5p, however, with an as yet undefined mechanism in endothelial differentiation of iPSCs [143].

3.9 Future applications and outlook

Recent studies provide new strategies to improve reprogramming protocols for vascular regeneration. Almost all of these protocols require an overexpression of two or more pluripotent transcription factors even in the presence of treatment with small molecules. However, miRNA based protocols for pluripotency induction are unique and provide opportunities to completely substitute all the classical transcription factors in the reprogramming of somatic cells and for their EC differentiation. Thus, miRNA-based protocols may prove to be a promising method to generate safe, high quality, patient-specific iPSCs that could be ideal for clinical use. Numerous miRNA clusters and individual miRNAs have been identified that would be potential candidates for induction of pluripotency as well as EC differentiation of the reprogrammed iPSCs. In this regard, the miR-290 and miR-302/367 clusters have been identified for the induction and maintenance of pluripotency while miR-21 supports their EC differentiation. A potential clinical avenue is combining miRNA modulation with stem cell-therapy strategies. For example, transplantation of EPCs or pluripotent cells at the site of injury with modulated expression of miRNAs may promote faster healing and organ recovery *via* paracrine mechanisms.

As miRNA-targeted therapies are being established to enter clinical practice, a detailed understanding of their role in EC differentiation of stem/ progenitor cells and their deregulation in cardiovascular disease is required. A detailed understanding of the role played by specific miRs in regulating endogenous vascular repair responses during injury/disease may lead to discovery of novel therapeutic strategies to activate regenerative potential as well as vascular reparability of progenitor cells. Although, a large number of miRNAs have been identified that are differentially expressed in pluripotent as well as mature ECs, little data are available confirming their role in EC differentiation. Therefore, there is still a need to identify specific miRNAs actually involved in the process of EC differentiation. A big challenge in miR-therapeutics would be the delivery strategies of specific miRNAs to the site of injury. In this regard a combination of cell-therapy with miRNA expression can be exploited [144]. Progenitor cells programmed to over express a specific set of miRNAs may be injected at the site of injury to locally release these endogenous miRNAs synthesized by these progenitor cells.

Funding and acknowledgements

The study was supported by the University of Giessen Anschubsfinanzierung grant to M. Aslam and UKGM grant to D. Gündüz. The authors also wish to thank Prof. Dr. Kh. H. Haider for extensive language editing of the manuscript.

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4 Cells for the repair of damaged skin and cartilage

4.1 Introduction

The human body is composed of trillions of cells, grouped into nearly 200 lineages/cell types and every lineage performs highly specialized functions [1]. All the specialized lineages are developed from a small cluster of cells that originate from a single cell, the zygote. Every cell in this cluster is programmed to develop into any one of the different lineages. The ability of a cell to turn into many different specialized types is called pluripotency, and into all cell types is called totipotency. The mother cell from which a particular specialization originates is called a stem cell. There are four categories of stem cells, namely, i) embryonic, ii) placental/cord blood, iii) adult, and iv) induced pluripotent. Embryonic stem/germ cells are able to develop into most, if not all, specialized cell types. Placenta and cord blood are convenient sources of pluripotent stem cells. Stem cells from the amniotic fluid of the placenta and Wharton's jelly of umbilical cord tissue have gained widespread attention because of an increased ability to differentiate into various specialized cell lineages.

Adult stem cells are present in small numbers in almost every organ of the human body and exhibit varying degrees of plasticity. This allows self-repair of various organs following injury as well as their renewal with regular periodicity. The entire human epidermis is completely replaced every 27 days and the skeleton every seven years. Adipose tissue presents an easily accessible source of adult stem cells that can differentiate into multiple lineages. Wharton's jelly of the umbilical cord and cord blood contains mesenchymal stem cells (MSCs) that are able to differentiate into connective tissues, cartilage, muscle, tendon and fat cells [2, 3]. A fully developed terminally differentiated somatic cell, under defined experimental conditions, can be induced to transform into a pluripotent surrogate embryonic stem cell that is capable to undergo directed differentiation into many different specialized cell types in the human body. The ability to grow *in vitro* just about any somatic cell type, coupled with the discovery of procedures to transform somatic cells into ''induced pluripotent stem cells" (iPSCs) has enabled treatment of many diseases, including some that were previously untreatable, such as heart failure [4], osteoarthritis, ocular defects, diabetes, spinal cord injuries, amyotrophic lateral sclerosis, cartilage defects and adverse skin conditions [5]. Nevertheless, there are some problems associated with *in vitro* generation of iPSCs such as inefficiency of induction (<1%), variability in the reprogrammed clones ranging from genetic, epigenetic or phenotypic, use of viral vectors to enhance the efficiency of induction, random insertion of the transgenes leading to impulsive changes in the genome, etc. The problems are solvable in theory and are currently

DOI 10.1515/9783110493764-004

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being solved in practice. Recent successes in the bulk induction of somatic cells into pluripotent cells and *in vitro* propagation and differentiation into even those tissues that are not adept at repairing themselves have made stem cell regenerative medicine the most exciting of medical sciences as well as a novel therapy. This chapter provides an update on different approaches for the cellular repair of damage and diseases of skin and cartilage. It also includes a review of stem cell sources and clinical perspectives on stem-cell-based therapeutic strategies.

4.2 Stem cells in the repair of damaged skin

4.2.1 Skin structure and function

The skin is the largest organ of the human body and it acts as an effective barrier against environmental insults and protects internal organs. It covers about 1 to 2 m^2 of surface area on the human body and accounts for approximately 12% to 16% of the body weight of an adult [6]. Human skin consists of three anatomic layers, the epidermis, dermis and hypodermis or subcutis. The epidermis is attached to the dermis by the basement membrane. The dermal-epidermal junction provides adhesion, mechanical support, proliferation/differentiation and also mediates the relocation of nutrients and cells between the dermal and epidermal tissues [7].

4.2.1.1 Epidermis

The epidermis is multilayered and its sublayers are arranged from the outermost to innermost as the stratum basal, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. It contains no blood vessels and eliminates waste products by diffusion [8]. Keratinocytes, melanocytes, merkel cells and Langerhans cells are different cell types found in the epidermis. Keratinocytes make up 90% of the epidermis and produce the proteins, including keratin that guard the skin and underlying tissues against microbes, heat and chemicals. The epidermis constantly renews with the synthesis of a new layer of keratinocytes at the stratum basal layer, and with the loss of an older layer of cells (corneocytes) from the stratum corneum [9, 10].

4.2.1.2 Dermis

The dermis accounts for ~90% of the weight of the skin and is divided into the papillary layer and the reticular layer. There are more fibers in the dermis than the epidermis [11, 12]. Numerous structures, including nerve endings, sebaceous glands, lymph and blood vessels, sweat glands and hair follicles are present within or originate from the dermis. Dermal tissue is composed of fibroblast cells, temporary lymphocytes, mono-nuclear phagocytic cells, dermal mast cells and dendritic cells [7]. Fibroblasts secrete extracellular matrix components such as collagen, elastin and

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glycosaminoglycans (dermatan sulfate and hyaluronic acid). Fibroblasts are essential for epithelial-mesenchymal communications and are vitally important in the repair and regeneration of skin [11, 12].

4.2.2 Skin diseases and injuries

4.2.2.1 Skin diseases

There are a number of skin diseases such as vitiligo, diabetic foot ulceration, psoriasis, skin cancers, cutaneous scleroderma and epidermolysis bullosa, caused either by genetic aberrations or bacterial/fungal infection, which have varying degrees of health/medical burden.

4.2.2.2 Skin injuries

Skin has the highest risk of injury in contrast with the other body organs because of its direct exposure to environmental insults [13]. Damage to various layers of skin due to diseases or trauma, especially burns, results in wounds of varying degrees. Skin burns, caused by thermal, chemical, electrical and radiation sources are the second leading cause of accidental death [14]. According to a fact sheet of the World Health Organization (2014), over 265,000 people die worldwide due to burns, and a majority of these burn incidents occur in low- and middle-income countries [15]. Burns may be categorized as superficial (first-degree) burns involving the epidermis only, superficial partial thickness burns (II-a^0) involving the superficial papillary dermis, deep partial thickness burns (II-b^0) affecting the reticular dermis, full thickness burns (third-degree) affecting the entire dermis and subcutaneous fat and fourth degree burns, which destroy all layers of skin and extend into tendon, muscle and/or bone.

4.2.3 Conventional therapy of skin wounds

Wound healing is an intricate process of regeneration and the repair of damaged skin. Conventionally, topical and oral medications, i.e. antibiotics (erythromycin, tetracycline, bactroban), antiviral (valtrex, acyclovir), antifungal (Lamisil, lotrimin, ketoconaole), immunosuppressants (azathioprine and methotrexate) and biologics (enbrel, humira, remicade) have been used to prevent secondary infections [16]. Herbal-derived compounds (aloe vera and marigold) and animal-derived compounds (propolis and honey) have also been used because of their anti-inflammatory, antimicrobial and cell stimulating characteristics. Traditional dressings such as cotton wool, gauzes and bandages are often used in combination with alginate and hydrocolloid dressings to protect wounds from absorbing exudates and intruding pathogens [13]. Silver nitrate solution, silver sulfadiazine (SSD) cream, Urgotul SSD gauze dressing and PolyMem Silver foam are the most common antimicrobial agents in wound care [17].

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Debridement has also been used to improve wound treatment by removing dead, contaminated tissue and foreign materials that may reduce the efficiency of the host immune system.

4.2.4 Cellular therapy of skin wounds

Conventional therapies do not heal chronic wounds, and delayed healing can prime wounds to chronicity, as evidenced by venous ulcers and ischemic wounds. Cellular therapy offers a more effective treatment of skin wounds.

4.2.4.1 Repair of skin wounds by resident skin cells

The epidermis and hair follicles in the skin harbor stem cells that not only help in the maintenance of skin regeneration and homeostasis but also contribute to wound repair. Three distinct stem cell populations in the bulge area, sebaceous gland and interfollicular spaces have a vital role in the homeostasis of the exterior layers of skin. The dermis contains stem cells in the dermal papilla of hair follicles and at dermal perivascular sites. Human dermal sheath stem cells have been shown to accelerate wound healing in diabetic mice [18]. The activation of progenitor cells and MSCs resident in the skin augment wound healing. Skin keratinocytes and fibroblasts have been used in the repair of diseased as well as damaged skin. They are a major component of several commercial bio-engineered skin substitutes (EpiDex, Epicel, ReCell, Laserskin and Myskin) and have also been used with natural or synthetic scaffolds [19]. Such bioengineered substitutes containing skin-derived cells have contributed significantly to effective wound repair, especially full thickness burn wounds, thus reducing the mortality of large, deep body surface burns [19].

4.2.4.2 Repair of skin wounds by mesenchymal stem cells (MSCs)

MSCs are derived from bone marrow, adipose tissues, placenta/cord blood/amniotic fluid and Wharton's jelly. MSCs derived from cord blood have the capability to differentiate into keratinocytes *in vitro* [20], express collagen VII protein and seem to have potential for healing patients with recessive dystrophic epidermolysis bullosa [5]. MSCs derived from amniotic fluid differentiate into keratinocytes and express markers such as Keratin 5, Keratin 10, Keratin 14 and involucrin. MSCs can be injected systemically [21], intra-dermally [22], administered in scaffolds like hydrogels containing collagen [23, 24], fibrin [25] or chitosan [26] for the treatment of cutaneous wounds. MSCs assist in the repair of wounds by immune modulation, cellular differentiation and secretion of growth factors that stimulate re-epithelialization, neovascularization and mobilization of endogenous skin stem cells. The therapeutic benefits of MSCs are mainly attributed to their multilineage differentiation potential and the release of paracrine and autocrine trophic factors [27], which reduce inflammation and exhibit immune modulation [28] and anti-microbial activities [29]. MSCs migrate to the injured or inflamed site, recruit resident progenitor cells, and activate differentiation,

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proliferation and extracellular matrix formation by the secretion of bioactive molecules such as vascular endothelial growth factor (VEGF), epidermal growth factor, stromal cell-derived factor-1 (SDF-1α), insulin-like growth factor (IGF-1), keratinocyte growth factor and matrix metalloproteinase-9 [30]. MSCs can also secrete Prostaglandin E2, which downregulates inflammatory cytokines and attenuates sepsis [31]. Furthermore, MSCs activate the secretion of anti-inflammatory cytokines like interleukin 10, which prevents scar formation [32] and excessive inflammation [33].

Bone marrow-derived MSCs (BM-MSCs)

Bone marrow is the most widely studied source of MSCs. Autologous BM-MSCs infused in a collagen matrix reduced wound size and increased vascularity and dermal thickness in patients with chronic leg ulcers resistant to conventional treatment [34]. Cultured autologous BM-MSCs within a fibrin spray enhanced the healing of acute surgical or chronic lower extremity cutaneous wounds in humans [35]. BM-MSCs laden in a hydrogel accelerated the process of wound closure and increased re-epithelialization and secretion of extracellular matrix when injected in diabetic mice [36]. Transplantation of BM-MSCs efficiently cured burn wounds in mice [37]. Although transplantation of BM-MSCs is more effective than conventional treatments, the invasive procedures of its harvest and the time and costs associated with the proliferation of cells in culture to achieve therapeutic concentrations remain major challenges in the liberal use of these cells.

Adipose-derived MSCs (ADSCs)

In several preclinical studies, ADSCs have been shown to improve healing of cutaneous wounds [38]. In our laboratory, we also studied the therapeutic ability of ADSCs and the effect of ascorbic acid thereon as judged from the repair of acid-inflicted wounds. Our flow cytometry data clearly showed that there was no significant effect of ascorbic acid treatment on the expression of surface markers CD29, CD90, CD34 and CD45 in ADSCs (Fig. 4.2.1). We further observed that transplantation of ascorbic acid-treated ADSCs in acid-inflicted wounds in rats resulted in enhanced wound closure (Fig. 4.2.2). When chronic wounds caused by radionecrosis, drepanocytosis, type 2 diabetes, systemic lupus erythematosus and full thickness burns are treated with autologous ADSCs cultured on human acellular collagen matrix, they have no adverse effects [39]. The use of ADSCs for the repair of chronic wounds and burns is currently being tested in clinical trials [40]. It is expected that these clinical trials will validate the therapeutic feasibility and efficacy of ADSCs in wound treatment.

Placenta/cord blood and amniotic fluid-derived MSCs

Placenta-derived MSCs promote wound healing by augmenting the secretion of angiogenesis-associated factors such as VEGF, upregulating integrin beta1 and beta 3, and decreasing pro-inflammatory cytokines such as the intercellular adhesion molecule (ICAM)-1 [41]. Placenta-derived cells have better proliferation and differentiation abilities than the more widely studied bone marrow and adipose derived stem

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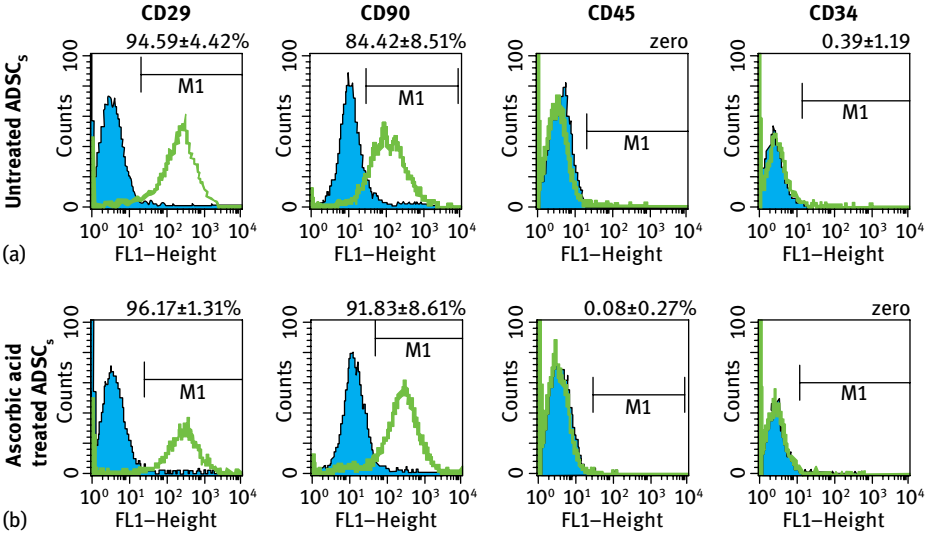

Fig. 4.2.1: Expression of CD29, CD90, CD45 and CD34, in untreated (a) and ascorbic acid treated (b) adipose derived mesenchymal stem cells (ADSCs) as determined by flow cytometry. The results show that treatment of ADSCs with ascorbic acid does not affect the expression of CD29, CD90, CD45 and CD34.

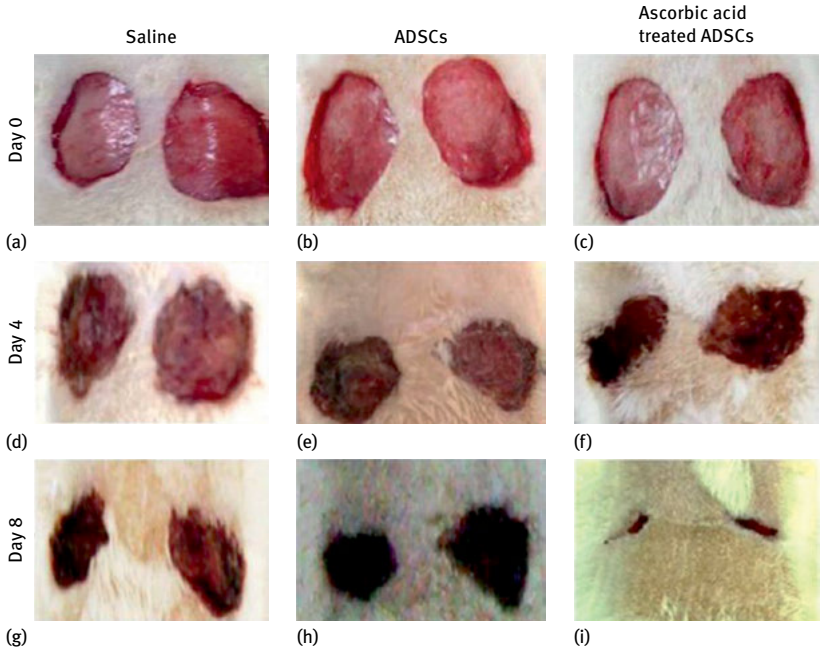

Fig. 4.2.2: Ascorbic acid treatment enhances the wound healing ability of ADSCs. Wound size in rats injected with saline, untreated ADSCs and ascorbic-acid-treated ADSCs, at day 0 (a, b and c, respectively); at day 4 (d, e and f, respectively); and at day 8 (g, h and i, respectively) postinjection. The results show that injection/transplantation of ascorbic acid treated ADSCs resulted in enhanced wound healing.

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cells. Amniotic fluid derived MSCs are known to have remarkable paracrine properties. Conditioned medium of amniotic fluid MSCs has been demonstrated to enhance healing of excisional wounds in mice [42, 43].

Wharton's jelly MSCs (WJ-MSCs)

Wharton's jelly serves as an abundant and readily available allogeneic source of multipotent MSCs for the treatment of burn wounds that require immediate coverage. We have recently shown that Wharton's jelly MSCs and amniotic epithelial stem cells have the ability to differentiate into skin keratinocytes and fibroblast-like cells, respectively, in an *in vitro* setting (Fig. 4.2.3 and Fig. 4.2.4). These differentiated cells offer a potential use as seed cells in the preparation of off-the-shelf bioengineered skin substitutes [44].

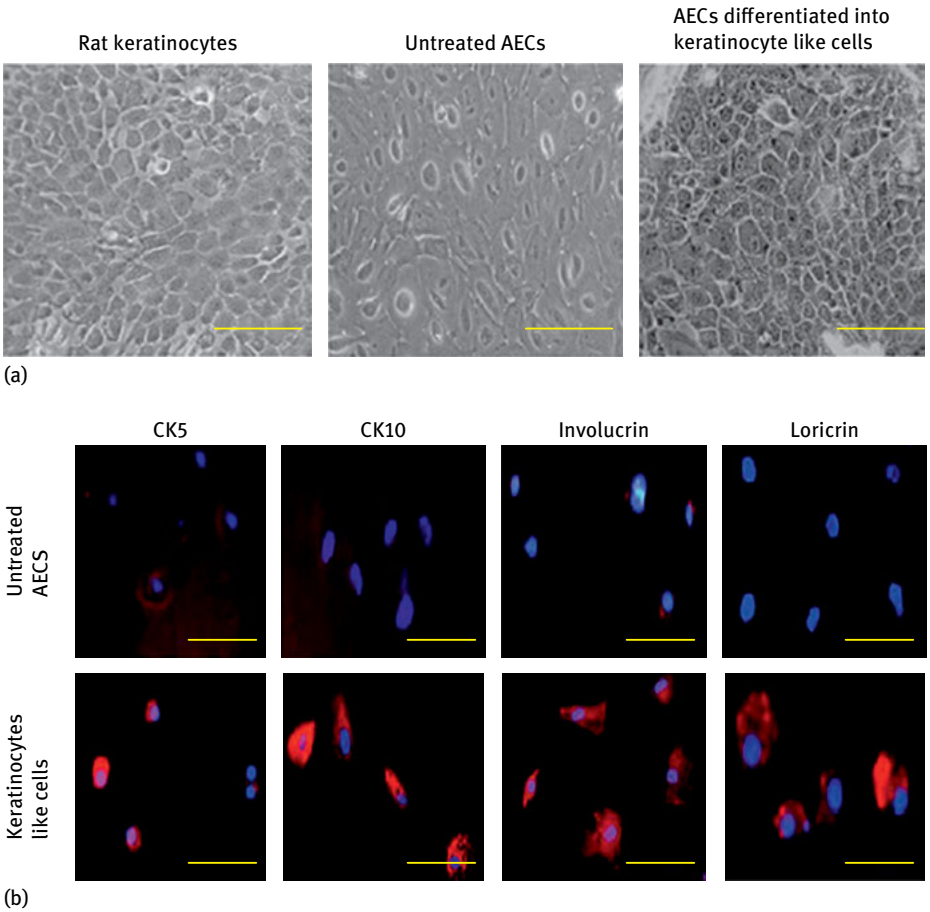

Fig. 4.2.3: Differentiation of amniotic epithelial cells (AECs) into keratinocyte-like cells as shown in phase contrast images of cells (a), and immunostaining for CK5, CK10, involucrin and loricrin, respectively (b). The figure shows that keratinocyte like cells derived by *in vitro* differentiation of AECs show prominent expression of CK5, CK10, involucrin and loricrin [44].

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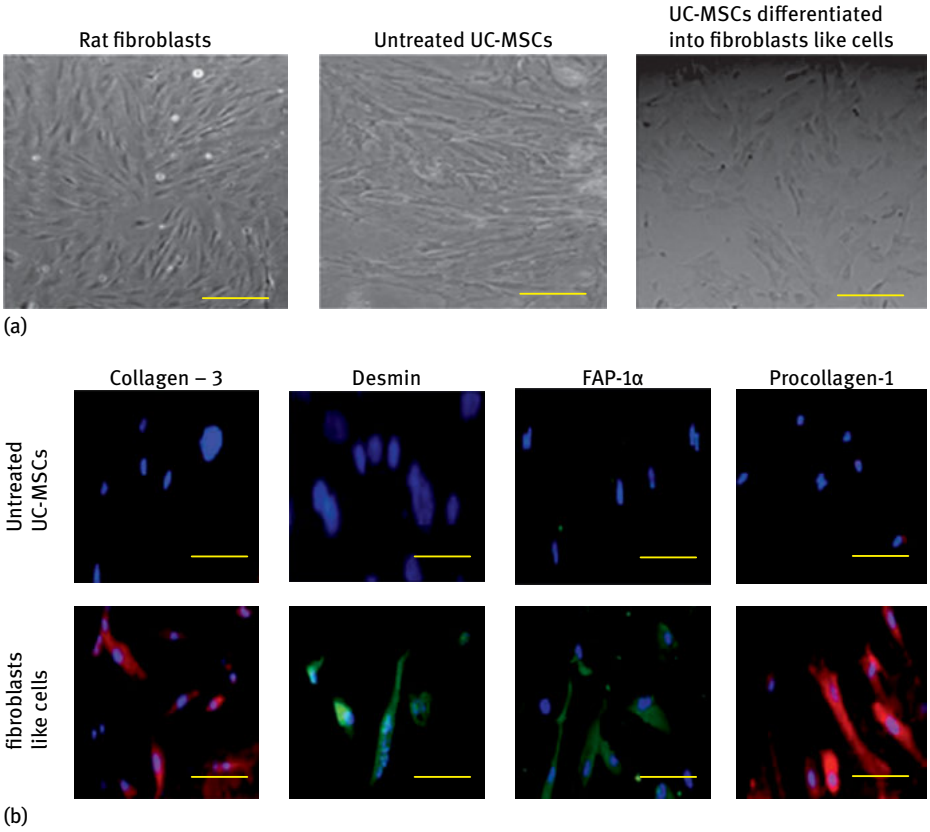

Fig. 4.2.4: Differentiation of UC-MSCs into dermal fibroblasts like cells as shown in phase contrast images (a), and immunostaining for collagen3, desmin, FAP-1α and procollagen-1, respectively (b). The figure shows that UC-MSCs show prominent expression of collagen3, desmin, FAP-1α and procollagen-1 after differentiation into dermal fibroblasts like cells [44].

4.2.4.3 Induced pluripotent stem cells and skin repair

Human iPSCs were first produced in 2007 by viral transduction into human skin fibroblasts. Such iPSCs are akin to ESCs in their morphology, expression of pluripotency genes and the epigenetic memory. Further, these cells have the ability to differentiate into cells of three germ layers [45, 46]. The use of iPSCs will help to overcome moral and ethical issues related to the use of human embryos, as well as immune rejection following transplantation [47]. To avoid any unpredictable transgene insertion by lentiviral or retroviral transduction, several non-integrating methods have been optimized, such as delivery of the transcriptional factors through adenoviral vectors, plasmid vectors, excisable transposons, and the direct provision of recombinant proteins that have the ability to reprogram cells [48]. Some small molecules also improve reprogramming efficiency; therefore, it seems that in the future, iPSCs may

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be established by chemical modifications alone [49]. Recent developments have further allowed the correction of mutations in iPSCs from genetically recessive dystrophic epidermolysis bullosa fibroblasts [50, 51]. iPSCs established from skin fibroblasts can be differentiated into keratinocytes, melanocytes and fibroblasts. iPSC-derived keratinocytes have been used to produce a stratified epidermis *in vitro* (3D skin-like cultures) [50, 52, 53].

4.2.5 Skin bioengineering

Cellular and acellular tissue-engineered skin substitutes have been developed to overcome drawbacks due to poor integration of conventional wound dressings, difficulties in regeneration of skin appendages, inability to replace the lost dermis from severe burns and shortages of donor grafts. Cellular constructs are made from cells obtained from different bases, including autologous, allogeneic and xenogeneic sources. Acellular constructs made from natural or synthetic biomaterials may be used in combination with autografts. Clinically available skin substitutes may be categorized into i) single layered, ii) double layered and iii) complex skin substitutes. The available skin substitutes also suffer from problems of scarring at graft margins and poor integration due to inadequate vasculrization.

4.2.5.1 Single layered substitutes

Epidermal substitutes

Epidermal substitutes are generally used for the treatment of lesions affecting the epidermis. They contain autologous keratinocytes that are generally cultured in the presence of murine fibroblasts. Major difficulties in generating epidermal constructs include fabrication time, high production costs, variable rates of engraftment, and difficulty in handling the thin, fragile constructs. To overcome delays due to the long fabrication time associated with epidermal substitutes, cell populations from a biopsy may be directly sprayed into lesions (ReCell®; Avita Medical, Perth, Australia; Spray®XP; Graco, MN, USA). This strategy allows for a faster epithelialization and epidermal maturation. However, it is not suitable for the treatment of third-degree burn wounds because of a lack of a dermal component [13].

Dermal substitutes

Dermal substitutes are generally used for the treatment of full-thickness lesions, skin ulcers and burns affecting both the epidermis and dermis. Dermal substitutes must be covered by a permanent epidermal substitute, usually an autologous split-thickness skin graft. Dermal substitutes can be produced with natural or synthetic materials. These substitutes prevent wound contraction, provide good mechanical stability and are available in different thicknesses and compositions.

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4.2.5.2 Double-layered substitutes

Double-layered skin substitutes mimic the structure of normal skin without the appendage-like structures, vasculature and rete ridges. The epidermal/dermal composite Apligraf represents the best option for skin repair due to interplay between multiple seed cell types [54]. This substitute shows enhanced wound closure and keratinization capability. A full thickness living skin analogue, Activskin, has been successfully used to treat refractory ulcers in clinical applications [55].

4.2.5.3 Complex skin substitutes

Development of 3D skin equivalents that mimic human skin in structure and function, including hair follicles [56], capillary networks [57], sensory innervations [58], adipose tissue [59] and pigment production [60] have revolutionized cellular therapy of damaged skin. A combination of BM-MSCs with epidermal stem cells greatly accelerates the regeneration of hair follicles and blood vessels when compared to treatment with epidermal stem cells alone [61]. Like BM-MSCs, ADSCs are capable of differentiating into various skin cells [62]. Furthermore, a combination of dermal fibroblasts and ADSCs (1:1) is superior to fibroblasts or ADSCs alone in promoting keratinocyte proliferation and differentiation [63].

4.2.6 Stem cells for cosmetic purposes

It has been shown that epidermal stem cell injection cured vitiligo patches in 80% of treated vitiligo patients [64]. Similarly, autologous injection of MSCs was shown to improve skin tone and reduce expression lines on the face [30]. Autologous fibroblasts have been shown to fade wrinkles, thereby reducing aging effects on skin [65, 66]. Injection of cultured autologous fibroblasts in the dermis is used in the treatment of naso-labial folds around the nose and mouth [67]. It has also been shown that repeated injections of fibroblasts cultivated in human serum improved the flaccidity of periorbital skin [68]. Cell-assisted lipotransfer with freshly isolated ADSCs along with fat grafts is a technique widely used by plastic surgeons [69]. Cultured ADSC-based therapies also seem to have potential for scar reduction [70].

4.3 Stem cells for the repair of damaged cartilage

4.3.1 Cartilage structure and function

Articular cartilage is a connective tissue with unique features to bear load and covers the long bones in synovial joints. It reduces friction between moving bones and provides a cushion to absorb impact. Biological and biochemical physiognomies

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together enable it to perform specialized functions [71]. The three main types of cartilage are hyaline, fibrocartilage and elastic [72], which differ from each other in amounts of collagen fibers, proteoglycans and elastin. The trachea, ribs and bronchial tubes mainly consist of hyaline cartilage. The inter-vertebra of the spine and the pubic symphysis consist of fibrous cartilage. The epiglottis and auricle contain mainly elastin [73].

There are three different zones of cartilage:

4.3.1.1 Superficial zone (tangential zone)

The superficial zone is the thinnest layer, which makes up to 10% of the articular cartilage [74]. The orientation of type II collagen fibers is parallel to the articulating joint. This layer is mainly composed of trodden chondrocytes, compact collagen fibers and thin proteoglycans.

4.3.1.2 Intermediate zone (middle zone)

The intermediate zone is the thickest layer, with random organization of type II collagen and is mainly composed of globular chondrocytes which occur in a vertical form, parallel to collagen fibers [75].

4.3.1.3 Deep zone (basal layer)

In the basal layer, collagen II lies vertical to joints and chondrocytes in a random organization. The tidemark is the zone of classification between the upper articular cartilages and the calcified deeper region. The deep zone performs endochondral ossification in vertical bone formation from the juvenile stage to adolescence [75].

4.3.2 Diseases and injury to cartilage

4.3.2.1 Osteoarthritis

Osteoarthritis (OA), or "wear and tear" is a degenerative process that results from the breakdown of articular cartilage in synovial joints [76]; therefore, inflammation is predominantly found in osteoarthritic joints. OA affects the whole joint systematically and deteriorates the cartilage, subchondral bone, synovium tendons and muscles [77].

The prevalence of OA is significantly higher in women than in men. OA affects 10% of males and 18% of females over the age of 45, especially women with co-morbid metabolic bone conditions such as osteoporosis and after menopause [78].

4.3.2.2 Rheumatoid arthritis

Rheumatoid arthritis (RA), also known as chronic inflammatory polyarthritis, is an autoimmune disease [79]. It causes disability and lowers quality of life [80]. RA is

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an inflammatory disease that affects multiple joints. The etiology of RA is unknown, but it is believed to result from a combination of genetic and environmental factors that include hormone replacement therapy [81], menstrual history [82] and physical activity.

4.3.2.3 Cartilage damage due to injury

Sudden shock, accident, trauma, rotational force in direct trauma, osteochondral lesions, twisting and locking of knees cause chondral injury.

4.3.3 Approaches for repair of cartilage

4.3.3.1 Surgical procedures

Extensive surgical interventions like osteotomy, distraction of joints, autologous tissue transplantation, allogeneic osteochondral and chondral grafting, osteochondral transplantation (mosaicplasty) and microfraction are being performed to treat cartilage defects.

4.3.3.2 Implantation of autologous chondrocytes

The implantation of autologous chondrocytes involves the arthroscopic procurement of a biopsy (8 mm) from the femoral groove of a healthy cartilage area that normally is not subjected to load. Retrieved tissue is enzymatically treated to obtain a population of healthy isolated chondrocytes, which are then *ex vivo* expanded under conditions that preserve cell viability and function. The resulting population of chondrocytes is injected under the periosteum, where they grow and mature over time [83]. However, hypertrophy of regenerative cartilage, disturbed fusion, delamination and inadequate repair of cartilage still remain major concerns in the use of chondrocytes [84, 85]. Oxidative stress, present in osteoarthritic tissue, impedes the use of chondrocytes [86]. Oxidative stress induced by reactive oxygen species (ROS) or free radicals is a major cause of OA pathogenesis [87, 88]. Elevated levels of ROS have also been observed in OA patients [89]. Since chondrocytes are exposed to oxidative stress during OA, we investigated whether Vitamin E confers resistance to oxidative stress induced by hydrogen peroxide (H_2O_2) in chondrocytes. We also examined the effect of preconditioning of chondrocytes with Vitamin E on injuries induced by H_2O_2 [90]. Our results show that there was a decrease in the expression of Acan1, Col2a1 and content of proteoglycan following H_2O_2 treatment as determined by immunocytochemistry and safranin-o staining (Fig. 4.3.1a–d). Administration of Vitamin E ameliorated these effects. The group treated with 100 µM Vitamin E showed proteoglycan contents similar to an untreated control as well as increased immunocytochemical expression of Acan1 and Col2a1 gene as compared with 50 µM Vitamin E (Fig. 4..3.1e–l).

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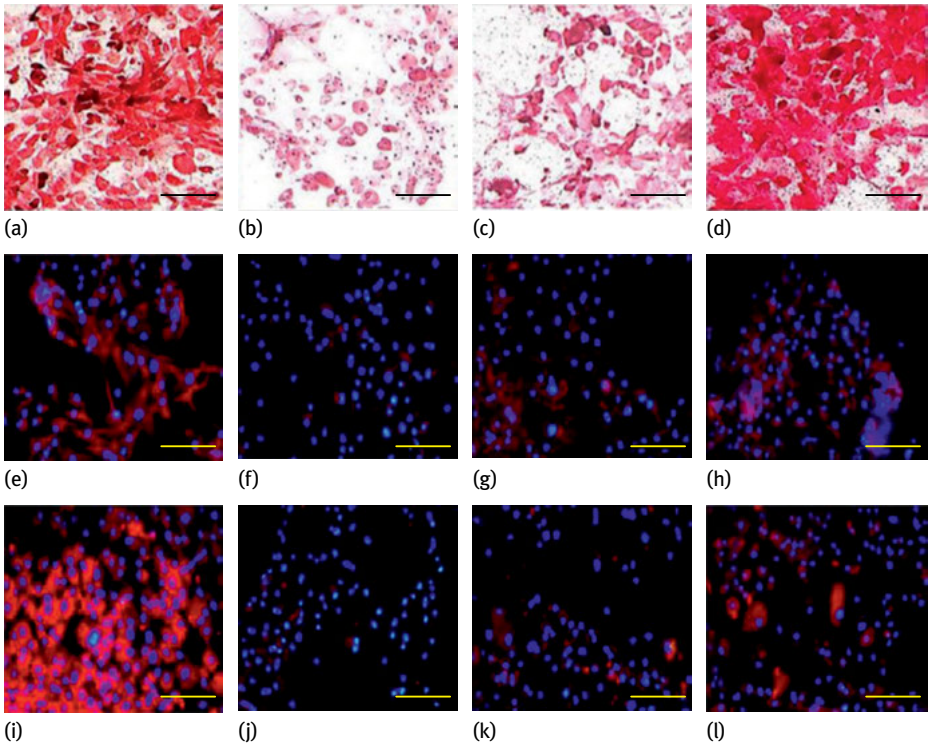

Fig. 4.3.1: Proteoglycan deposition and immunocytochemical expression of Acan1 and Col2a1. Control (C), H_2O_2-treated (H), 50 µM Vitamin E plus H_2O_2-treated (T50), and 100 µM Vitamin E plus H_2O_2-treated (T100) chondrocytes stained with Safranin-O (a–d); immuno-stained with Acan (e–h), and Col2a1 (i–l). Scale bar: 200 µm, 100×. The figure shows preservation of proteoglycan deposition (as observed by Safranin-O staining) and expression of Acan1 and Col2a1 (as observed by immunocytochemistry) in vitamin E pretreated chondrocytes. Whereas, untreated chondrocytes exposed to H_2O_2 exhibited severe depletion of proteoglycan, Acan and Col2a1 contents [90].

4.3.4 Repair of damaged cartilage by implantation of stem cells

4.3.4.1 MSCs for cartilage repair

Mesenchymal cells can differentiate into the three lineages: osteoblasts, adipocytes and chondrocytes [91]. The ability of mesenchymal cells to differentiate into cartilage has been well indicated for the repair of damaged cartilage. MSCs have the potential to generate cartilage, bone interface and ligament tissue [92].

MSCs for the repair of damaged cartilage are derived from bone marrow [93], adipose tissue [94], synovium [95], trabecular bone [96], infrapatellar fat pad [97], placenta [98] and umbilical cord/ Wharton's jelly [99]. BM-MSCs are practical for use in chondrogenesis. However, there are limitations due to the associated pain and other side effects like inflammation at the donor site and also the small number of

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recoverable stem cells. Since full thickness cartilage defects involve cartilage and subchondral bone, the best cell source should have the capability of generating both cell types.

4.3.4.1a ADSCs

ADSCs have been shown to regenerate cartilage in a variety of animal models. Nathan *et al.* (2003) observed the reconstruction of osteochondral defects in rabbit models using allogeneic ADSCs seeded on a fibrin scaffold [100]. Ude *et al.* (2014) reported better chondrogenic induction and gene expressions for BM-MSCs compared to ADSCs. However, no differences were observed in terms of quality of cartilage regeneration in an induced-OA animal model [101]. Frisbie and colleagues (2009) reported that injecting cultured BM-MSCs or ADSCs produces clinical, radiological, histological and biochemical improvements in the treatment of induced OA in a horse model [102]. In our laboratory, we investigated the regenerative potential of ADSCs and chondrogenically induced ADSCs in the repair of cartilage in rats. We observed a significant reduction in the expression of the fibrocartilage-related gene, Col1a1, and the apoptotic associated gene, Casp3, with a corresponding increase in hyaline cartilage specific genes, Col2a1 and Acan, and the proliferation gene, PCNA. A significant increase in the differentiation index was noted in DCs and ADSCs transplanted knee joints ($P = 0.0110$ vs $P = 0.0429$), when compared to those in OA control knee joints (Fig 4.3.2) [103].

4.3.4.1b Synovial fluid-derived MSCs (SF-MSCs)

SF-MSCs are a striking source of cells for the treatment of OA, as compared to MSCs derived from other tissues, because of their high chondrogenic and proliferative activity. Cells are harvested from OA patients during arthrocentesis or routine arthroscopic examination without damaging normal tissues [104]. SF-MSCs showed faster expansion than BM-MSCs when cultured with autologous human serum. Synovial tissue produces a reservoir of MSCs which migrate to the injured site and help in repair in intra-articular tissue diseases [105].

4.3.4.1c Wharton's-jelly-derived stem cells (WJ-MSCs)

WJ-MSCs contain high levels of hyaluronic acid and sulfated glycosaminoglycans (GAGs) and exhibit collagen expression [106]. Differentiation of WJ-MSCs into multiple cell lineages like adipogenic, chondrogenic and osteogenic have been reported [107]. In our laboratory, we have studied the effect of lovastatin on MSCs derived from Wharton's jelly of the umbilical cord during hydrogen-peroxide-induced injury conditions. The *in vitro* differentiated chondrocytes were subjected to oxidative stress by exposure to 200 μM hydrogen peroxide, either in the presence or absence of Lovastatin (2 μM) for 5 hours. We observed that proteoglycan deposition was markedly

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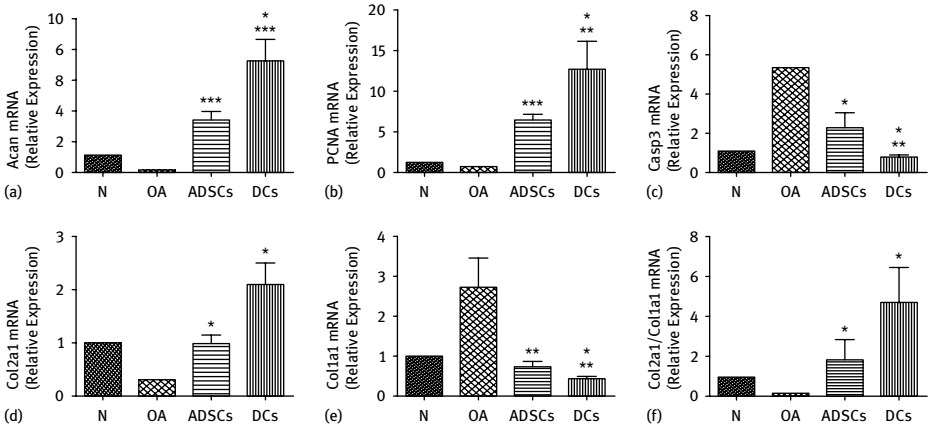

Fig. 4.3.2: Analysis of gene expression in transplanted rat knee: Expression of Acan (A), PCNA (B), Casp3 (C), Col2a1 (D), and Col1a1 (E) in normal control (N group), osteoarthritic (OA group), ADSCs transplanted (ADSCs group) and differentiated chondrocytes transplanted (DCs group) rats. Differentiation index of Col1a1 and Col2a1 in the aforementioned groups is shown as E. Data is presented as mean ± SD (n=3). (*) represents DCs vs. OA and (•) represents DCs vs. ADSCs.*P or •$P < 0.05$, **P or ••$P < 0.01$ and ***P or •••$P < 0.001$. The figure shows that the transplantation of DCs in surgically induced rat models of OA resulted in significant upregulation of Acan, and PCNA, and significant downregulation of Casp3 and Col1a1 when compared with OA rats transplanted with undifferentiated ADSCs (ADSCs group) [103].

reduced in the hydrogen peroxide-treated group and was improved by Lovastatin treatment (Fig. 4.3.3a–c). The expression of COL2A1 and ACAN was also improved in the Lovastatin-treated group compared with the hydrogen-peroxide-treated group without Lovastatin, as assessed by immunocytochemistry (Fig. 4.3.3d–f, g–i). RT-PCR analysis revealed a significant increase in expression of BGN, COL2A1 and SOX9 after Lovastatin treatment (Fig. 4.3.3j–k) [99].

4.3.5 Transplantation of MSCs

Tissue engineering with chondrocytes and MSCs is a promising way of repairing articular cartilage lesions. Several clinical trials successfully achieved the transplantation of MSCs into OA knees, using a scaffold with MSCs alone or mixed with cytokines and/or growth factors.

4.3.5.1 Intra-articular injection of MSCs

Local delivery of MSCs is the simplest strategy for the treatment of OA and has positive effects in the improvement of joint repair and reduction of the degeneration process [108]. Transplantation of human BM-MSCs in a caprine OA model exhibited a therapeutic effect by regenerating meniscal tissue and reducing the progression of damage

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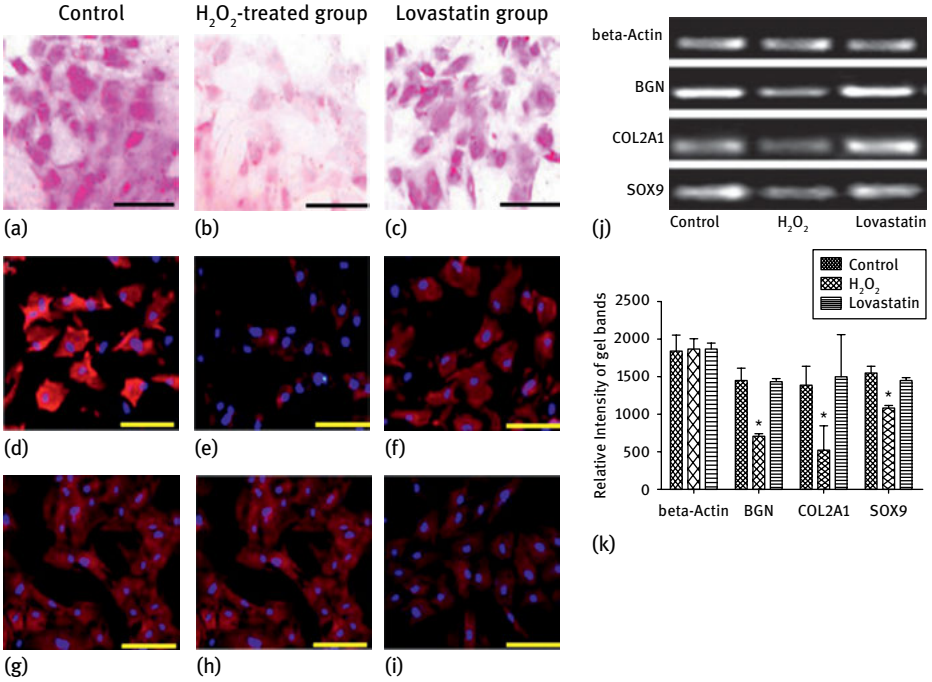

Fig. 4.3.3: Effect of Lovastatin treatment on WJ-MSCs derived differentiated chondrocytes (control), hydrogen-peroxide-treated (H_2O_2-treated) and Lovastatin-treated (Lovastatin) differentiated chondrocytes groups. Safranin-O staining (a–c). 100×, Scale bars: 100 µm. Immunocytochemistry for COL2A1 and ACAN (d–f, and g–i), respectively. J, RT-PCR data for Biglycan (BGN), collagen II (COL2A1) and SRY-box 9 (SOX9) shown as J. Gel quantification by Image J shown as K. *P<0.05. Data are expressed as means ± SD. This figure shows that presence of lovastatin during H_2O_2 treatment of *in-vitro* differentiated WJ-MSCs derived chondrocytes resulted in lesser deterioration of proteoglycan contents, as well as COL2A1 and ACAN expression [99].

in the injured area [109]. Centeno *et al.* (2008) reported that intra-articular injection of autologous MSCs enhance the growth of cartilage and diminish pain in degenerative joint disease [110].

4.3.5.2 Transplantation of MSCs in combination with growth factors, cytokines, and scaffolds

Different approaches have been used to improve the therapeutic potential of stem cells, either by transplantation of MSCs with cytokines/growth factors or with scaffolds. Mrugala *et al.* (2008) demonstrated that transplantation of ovine MSCs had beneficial effects when used in combination with chitosan and TGFβ3 for the cure of OA [111]. Seo *et al.* (2013) reported the therapeutic effects of bilayer gelatin /β-tricalcium phosphate (GT) sponges combined with stem cells, chondrocytes, BMP-2 and platelet-rich plasma (PRP) for the repair of osteochondral defects in horse talus.

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These studies showed that the Ch/MSC/PRP/GT combination stimulated osteochondral regeneration and had a capacity as a useful source for the treatment of osteochondrosis [112]. Another study showed that transplantation of autologous BM-MSCs onto a platelet-rich fibrin glue scaffold is an efficient strategy that promotes the regeneration of articular cartilage defects [113].

4.3.6 Scaffolds for cartilage repair

Articular cartilage has complex architecture. It's a challenge to develop scaffolds that can mimic the biomechanical properties of the resident tissue. Novel scaffolds are now being used to regenerate cartilage. An extensive variety of matrices have been investigated, such as carbohydrate-based scaffolds (agarose, alginate, chitosan/chitin and hyaluronate), protein-based scaffolds (collagen, fibrin and gelatin) and artificial polymers (polyglycolic acid, polylactic acid, poly (lactic co-glycolic acid), polyethylene glycol and polycaprolactone). Nanocomposite scaffolds, which have the structural design, chemical cues and mechanical properties of mature articular cartilage, have also been used. These scaffolds direct the morphology and orientation of cultured chondrocytes in a controlled manner [114].

Among the impressive scaffolds, chitosan-based systems, including physical hydrogels, chemically cross-linked hydrogels or porous scaffolds, show great potential in cartilage tissue regeneration. Chitosan has beneficial characteristics like biocompatibility, biodegradability, bioabsorbability, low immunogenicity and an antibacterial nature. These properties make chitosan a good candidate for potential applications in tissue engineering. Most important, chitosan has the same chemical structure as glycosaminoglycans (GAGs). GAGs play an important part in chondrocyte function and differentiation. Further properties like porosity, cell adhesion and the controlled release of useful growth factors have also been engineered in chitosan-based scaffolds [115].

In another study, human placenta-derived extracellular matrix sponges (PEMS) were used to repair osteochondral tissue by a decellularization procedure. PEMS *in vitro* provided a non-toxic environment for human amniotic-membrane-derived stem cells (HA-MSCs) to proliferate and differentiate into chondrogenic and osteogenic lineages under induction. Histological analysis revealed formation of blood vessels and no severe immune response after 28 days of PEMS subcutaneous implantation in the host. So a human placenta-derived biocompatible scaffold has been effectively developed for osteochondral tissue engineering [116].

In another study, collagen threads were used. Continuous length-aligned collagen threads were woven to emulate the interdigitated arcade structure of cartilage. Unique arcade-like orientation of collagen fibers enabled the cartilage to bear mechanical loads. The weaving pattern provided a macropore network within which micromass cell pellets were seeded to take advantage of mesenchymal condensation-driven chondrogenesis. Robust chondrogenesis was observed, as evidenced by GAGs, type II

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collagen and aggrecan staining. This woven collagen scaffold holds a significant potential for cartilage regeneration with shorter *in vitro* culturing time periods due to functionally sufficient mechanical robustness [117].

Nano-structured cartilage constructs have been made using electro spinning and carbon nanomaterials, which are like cartilage extracellular matrices. Results show enhanced stem cell adhesion, proliferation and chondrogenic differentiation of human BM-MSCs compared to controls without carbon nanomaterials. This suggests the future use of nano-scaffolds for *in vivo* studies and clinical applications for cartilage formation [118].

BM-MSC-derived ECM (BM-MSC-dECM) scaffolds have been fabricated by lyophilization without decellularization. Acellular porcine chondrocyte-derived ECM (AC-dECM) scaffolds were used as a control. Different properties like surface morphology, internal structure, water uptake ratio, biocompatibility of the scaffolds and cell growth on the scaffolds were compared. The BM-MSC-dECM scaffolds supported cell attachment and proliferation more efficiently than AC-dECM scaffolds. Hence, BM-MSC-derived ECM scaffolds can also be used for cartilage tissue-engineering applications in the future [119].

4.4 Whether *in vitro* structures adequately meet *in vivo* functions?

Methods of *in vitro* multiplication and delivery of keratinocytes and chondrocytes through scaffold technologies have resulted in successes in tissue and wound repair [120]. However, *in vitro* development of ready-to-use products for tissue repair or replacement does not always produce tissue architecture for required functions *in vivo*. Aberrations in the architecture of nascent tissue inevitably affect its *in vivo* functions. For example, when articular chondrocytes are cultured under conditions of high density in the presence of TGF-β, it produces cartilaginous tissue with a high degree of similarity in histological hallmarks to hyaline cartilage but it does not completely heal the cartilage defect [121]. This is attributable to the fact that *in vitro* development of the tissue does not permit integration of the cartilage within the damaged host tissue. Introducing cell suspension without extracellular matrix, however, allows formation of undifferentiated cells that attach to the bone to redifferentiate, leading to better integration of the nascent cartilage into the host tissue [122]. These procedures, together with replacement products, have received regulatory approval [123], which has placed cell-based regenerative therapies on a firm footing.

4.5 Future prospects of cell therapies

Stem cells have been used in the treatment of diabetic ulcers, severe full thickness burns and for cosmetic purposes in aged individuals or individuals whose skin has been distorted due to diseases (such as epidermolysis bullosa, vitiligo or epidermal cancer) or accidents. Stem cells also assist in the repair of articular cartilage, meniscus

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tears and in the relief of symptoms of stiffness, pain and disability. The efficacy of stem-cell-based therapy seems superior to all available medicinal and surgical treatment modalities. Despite these advancements, the search continues for optimal therapeutic options for efficient and scar-less healing of wounds with all the properties of intact, healthy skin as well as stiffness and pain free cartilage. The development of cellular screening systems will help in identifying natural or synthetic compounds with therapeutic effects [52, 53]. The availability of human genetic skin and osteoarthritis disease models will help to further improve our understanding of the molecular pathogenesis of these diseases. Simultaneous progress in biology, chemistry and engineering will yield novel clinical uses of various cell sources, smart biomaterials and cytokines and other growth factors to further enhance the existing capabilities of skin and cartilage engineering, and stem cell regenerative medicine.

Acknowledgements: The authors wish to thank. S. Amer Riazuddin, Clare Brooks and S. Gottsch of Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA for critical reading of the manuscript.

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5 The skeletal muscle stem cells: biology and use in regenerative medicine

Abstract: Despite being considered a postmitotic tissue, skeletal muscle shows extensive reparability capacity in the event of injury and repairs itself by intrinsic repair mechanisms. This reparability of skeletal muscle has been attributed to the existence of satellite cells; a pool of muscle resident population of progenitor cells which are now considered as resident stem cells of skeletal muscle on par with resident stem cells in other organs and tissues. Their loss has been shown to significantly impact the capacity of skeletal muscle to repair itself during postnatal life. Satellite cells are mono-nucleated myogenic cells that are located between the sarcolemma and basement membrane, adherent to existing muscle fibers and lying dormant until stimulated by chemical cues emanating from the muscle injury. Once stimulated, satellite cells show extensive proliferation, migration and fusion with the existing fibers to participate in the regeneration process of the muscle. These characteristics strongly support their candidature as one of the choice cells for use in regenerative medicine as well as carriers of transgene delivery *in vivo* for stem-cell-based gene therapy. The present chapter discusses at length the biology of satellite cells, their characterization in experimental studies, their successful reprogramming to pluripotency and applications in regenerative medicine and drug development.

5.1 Introduction

Satellite cells or skeletal muscle stem cells were first reported in 1961 based on their anatomical position during ultrastructure examination of the frog tibialis anticus muscle [1]. Initially considered as dormant cells, experimental data gathered over years since their first reporting has clearly advocated their indispensable role in the skeletal muscle regeneration and repair process [2]. So much so that the loss of satellite cells significantly impacts skeletal muscle repair and regeneration during postnatal life. Genetic labeling studies have reaffirmed their essential participation in the repair process in the event of muscle injury [3]. The data is replete with information about their widespread presence and distribution in various vertebrate species including mouse, rat, ovine, porcine and humans [4, 5, 6, 7, 8]. Human satellite cells are smaller than myonuclei (8 μm *vs*. 11 μm) and are located between the plasma membrane and the basal lamina of muscle fibers [9, 10]. Rich in nuclear heterochromatin, the cytoplasm is scarce in organelles that reflect their transcriptionally less active status and quiescence until they are stimulated to be active [11]. Once stimulated in response

DOI 10.1515/9783110493764-005

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to muscle injury, satellite cells undergo morphological changes including the development of protrusions that facilitate their mobility to home in on the injured muscle site. These morphological changes are accompanied by a reduction in heterochromatin and an increase in cytoplasmic organelles to support their activity and participation in the repair process [10]. Recent studies have shown the presence of non-satellite cell muscle progenitors that also participate in postnatal myogenesis and skeletal muscle repair [12]. This population of progenitors is characterized by the expression of PW1 but lacks the expression of Pax7. Incidentally, PW1 is a novel zinc finger gene implicated in the myogenic and neural lineages, with restricted expression in skeletal muscle and subregions of the nervous system. It is considered as a transcription factor and therefore the PW1 protein is localized in the nucleus [13].

Skeletal muscle is a classic example of an adult tissue that has the capability of generating terminally differentiated cells that are specifically programmed to carry out tissue-specific functions including regeneration of tissue in response to injury [14]. This significant reparability and regeneration of skeletal muscle is ascribed to the existence of satellite cells that contribute to the pool of activated skeletal myoblasts (SkMs) to undertake the formation of new muscle fibers in the event of muscle injury. The genetic studies for ablation of the satellite cell pool in adult mice showed that muscle regeneration was mostly satellite-cell-dependent [15]. Satellite cells become activated and self-renew during the process of muscle repair to ensure that undifferentiated precursors are available for future repair as well [16].

The potential of extensive self-renewal has tipped the satellite cells to be considered as skeletal muscle stem cells. Upon activation, the quiescent satellite cells re-enter the cell cycle and give rise to unipotent skeletal myoblasts which continue to proliferate, increase in number and become committed to myogenic lineage. SkMs undergo fusion with the existing muscle fibers or alternatively fuse with each other to form multinucleated myotubes for myogenic repair [17]. Concomitantly, a population of the newly formed SkMs backslides and replenishes the satellite cell pool. Although the classic model of satellite cell self-renewal and their role in skeletal muscle maintenance has been challenged by bone marrow stem cells and other resident muscle cell populations, satellite cells still remain the workhorse for reparability of skeletal muscle [18, 19]. Nevertheless, various factors compromise satellite cells reparability; the aging process is one of the main factors. Similar to any other cell in the body, the aging of satellite cells leads to their reduced myogenic potential. In an interesting study, it was observed that satellite cells from the aging human muscle were able to proceed through the stages of the myogenic program and form myotubes *in vitro*. However, the rates of their proliferation and differentiation were slower than the satellite cells isolated from the young control group of cells [20].

5.2 An overview of satellite cell markers

Irrespective of their source, satellite cells have a role in myogenic repair and regeneration. A direct comparison of murine and human satellite cells revealed that they

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mostly share the expression of Pax7 as their characteristic marker. However, there are many markers expressed in the murine satellite cells that have not been reported in human satellite cells [21]. Therefore, identification of human satellite cells, especially during quiescence, remains a daunting task due to lack of specific markers and therefore it is difficult at times to discriminate them from the other cells present in the muscle. As Pax7 is the only marker expressed on the quiescent and activated satellite cells, it is usually considered essential for satellite cell formation [22]. However, the expression of Pax7 is higher on the self-renewing satellite cells as compared to the ones that are already activated for differentiation. Pax7 is usually combined with neural cell adhesion molecules (NCAM) to identify human satellite cells. On the same note, within the population of cells with positivity for Pax7 and NCAM, there are also subpopulations with Pax7 positivity but negative for NCAM, whereas there are still others with Pax7 negativity but that are positive for NCAM [21]. The other proposed markers include M-cadherin, CD34 and cMet [23, 24]. The satellite cell activation model is in fact the description of the interplay between induction and repression of certain markers that orchestrate their myogenic commitment. Upregulation of Myf5 in the quiescent satellite cells expressing Pax7 signifies the very early stage of their commitment to a myogenic lineage. Upon activation, they begin to express MyoD and proliferate to form a population of muscle precursor cells (MPC). While a part of the MPC reverts to Pax7 expression and become part of the satellite cell pool, the others continue their onward journey to muscle cell differentiation and express MRF5 and myogenin [21].

The first antibodies used to distinguish satellite cells from other cells were Leu19/CD56, which recognized NCAM in the adult human satellite cells during the early stages of myogenesis [25]. Immunocytochemistry for specific surface markers may discriminate between quiescent and activated satellite cells: the quiescent satellite cells express Pax7 [26] whereas SkMs cultured *in vitro* also express muscle-specific markers such as Pax7, Myf5 and MyoD. Using a rodent model of gastrocnemius and soleus muscle repair *in vivo*, an expression pattern of the two markers Myf5 and MyoD was observed. The results showed that the satellite cells, identified by M-cadherin expression, initially expressed either Myf5 or MyoD and in some cases both Myf5 and MyoD, upon activation [27]. MyoD belongs to the myogenic regulatory factors (MRFs) family of proteins with a crucial role in determining that the myoblasts to follow the myogenic lineage during embryonic development [28]. Both the quiescent satellite cells as well as the adult myofibers lack MyoD but it is continuously expressed during differentiation and after fusion into myotubes. On the same note, myogenin, another member of the MRFs family, is expressed during the early phases of differentiation and during subsequent cell fusion. In fact, the expression of MRF4 and myogenin mark the initiation of terminal differentiation of satellite cells [29]. During the process of differentiation, activated satellite cells fuse together to form multinucleated myotubes that are postmitotic tubular structures and generally express markers of terminal muscle differentiation, i.e. sarcomeric myosin heavy chain (MHC) [19]. MHC is a muscle-specific protein that forms the thick myofilaments in skeletal muscle fibers. It

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consists of two heavy chains and four light chains and is expressed during the early and late stages of myogenic differentiation [30]. Besides these markers, desmin which is present in high abundance as an intermediate filament protein in the cytoskeleton of muscle cells is the most widely used marker for human SkMs in culture [31].

5.3 The origin of satellite cells and their role in muscle repair and regeneration

Similar to other body organs with their own population of resident stem cells, satellite cells constitute the main armory of resident skeletal muscle stem cells to facilitate its postnatal growth, remodeling and regeneration [17]; however their origin has always remained contentious. The lineage tracing studies have shown that satellite cells originate from a Pax3/7 positive cell population of the dermomyotome of the somites [32]. These cells undergo extensive proliferation, marked by extensive muscle protein synthesis that reaches its maximum until the muscle matures subsequent to which the muscle progenitors acquire a quiescence state. An interesting feature of satellite cells is that they comprise a heterogeneous population of cells that clearly suggests that some other tissue sources such as dorsal aorta and other blood vessels besides the bone marrow may also contribute to their pool [33, 34]. Additionally, side population, $PW1^{+}$ cells, pericytes, $CD133^{+}$ circulating cells and β1-integrin-expressing interstitial cells have the potential to fuse with differentiating myofibers and adopt a satellite cell phenotype [35]. Some of these cells even begin expressing Pax7. Moreover, their number/quantity also differ between different muscles and muscle fiber types (slow or fast muscle fibers) at different developmental stages. [36, 37] A significant 2–4-fold increased percentage of satellite cells have been reported in the adult soleus muscle compared to the tibialis anterior muscle [38]. The heterogeneity infiltrates down to a level where even the satellite cells on a single fiber may be delineated into diverging populations of cells either performing the stem cell function or the ones that are readily available for fusion to endure the process of myogenesis [39]. The proliferative behavior of satellite cells within a muscle exhibits a marked difference. Despite heterogeneity in their population, satellite cells do not diverge in their functionality from each other. They all perform the same function of myogenesis irrespective of the muscle of their origin.

Since the pioneering studies by Bischoff and colleagues to elucidate the significant participation of satellite cell in skeletal muscle repair, several studies have reported cellular and bioactive molecular components that participate in the process [40]. Together, these cellular and bioactive molecular components constitute a satellite cell niche that is anatomically well defined and the ultimate determinant of their state of quiescence, activation, proliferation, differentiation or maturation [17]. The most significant difference between the satellite cell niche and an equivalent in other systems of stem cells is that the satellite cells in their niche are quiescent and inactive [41].

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A stem cell niche is typically a collection of cellular components, mediators, extracellular matrix components and other factors, including oxygen tension, pH, etc., that help stem cells to maintain an undifferentiated status. During the process of skeletal muscle regeneration, the activity of satellite cells is strictly controlled and regulated by the interaction between intrinsic factors within satellite cells and the microenvironmental extrinsic factors constituting the muscle stem cell niche. These also include extracellular matrix components [41, 42]. It is important to note that the myofiber in direct contact with the satellite cells is considered as the primary component of the satellite cell niche and produces some signals that help the satellite cells maintain a quiescent state [43]. Abrogation of myofibers from the niche significantly alters satellite cell behavior in terms of their activity and proliferation. Recent studies have confirmed the release of bioactive molecules by myofibers that affect the behavior of satellite cells in the niche. One of the bioactive molecules secreted by the myofibers in the niche is stromal-cell-derived factor-1α (SDF-1α), which interacts with the CXCR4 receptor expressed by the satellite cells to support their emigrational activity [44]. Besides myofibers, different types of cells that contribute to the muscle niche include granulocytes, monocytes, macrophages, fibroblasts and vascular cells [35]. The number as well as the contribution of each cell type to the niche fluctuates in temporal fashion following injury. For example, monocytes/macrophages that home-in on the area of muscle injury ultimately become anti-inflammatory M2 phenotypes to support the healing process and their abrogation may interrupt the healing process [45]. Similarly, a group of transcription factor 4 (Tcf4) expressing fibroblasts are essential for the proper healing of the injured muscle cells thus indicating their participation in the repair [46]. Additionally, transmembrane Notch signaling gets upregulated on the myofibers to support the proliferation of satellite cells whereas the onset of differentiation of the myogenic precursors requires a temporal transitioning to Wnt signaling and increased responsiveness of the progenitors to Wnt [47]. *In vitro* molecular studies have shown significant participation of Notch signaling on the self-renewal capability of $Pax7^+$ cells and the temporospatial Notch-Wnt interplay that regulates the proliferation and differentiation of the progenitor cells.

Like the cellular components, the microenvironment is rich in bioactive molecules secreted by each of the cellular components of the niche. Noticeable among these are insulin-like growth factor-1 (IGF-1), VEGF, fibroblast growth factor (FGF), stromal cell-derived factor-1α (SDF-1α), hepatocyte growth factor-1 (HGF-1), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), interleukin (IL)-4, IL6 and transforming growth factor-β (TGF-β) [48]. These bioactive molecules serve as regulatory factors for satellite cell behavior and functioning in the niche including their quiescence, survival, proliferation and differentiation. Published data shows that satellite cells are generally observed in the vicinity of small vessels where the endothelial cells affect their proliferation by the releasing regulatory molecules such as IGF-1, FGF, HGF-1 and VEGF [49]. *In vitro* studies have shown that satellite cells and their derivative SkMs respond to various growth factors, i.e. TGF-β, IGF-1&2 and FGF in a dose-dependent

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manner [50, 51, 52]. However, the responsiveness and behavior of these cells taken *in vitro* culture conditions is different from their responsiveness in the niche. Gene expression profiling of the cultured activated myoblasts was found to be different than that of activated satellite cells *in vivo* [53]. The reason for these differences has been attributed to a lack of a satellite cell niche in *in vitro* conditions, as simple removal of the cells from their natural habitat severely impacts their "stemness".

5.4 Isolation and culture of muscle stem cells

Lack of definite surface markers and the ability to maintain their undifferentiated quiescent status akin to the one maintained in the skeletal muscle niche make isolation and propagation of skeletal muscle satellite cells a technically demanding and challenging exercise. Several protocols have been optimized to isolate skeletal muscle satellite cells [54, 55, 56]. FACS-based isolation protocols involving the sorting of enzymatically digested skeletal muscle tissue have been extensively reported after immunofluorescence labeling for specific markers to ensure positive and negative selection of the satellite and non-satellite cells [57, 58]. Correspondingly, human skeletal muscle satellite cells have also been isolated and analyzed by a FACS method involving fixed [59] or live cell populations [60]. Bareja *et al.* were able to achieve up to 2% of the total live cell population expressing the positive markers, which is in comparison to the results obtained from the murine satellite cells in the range of 4%–6% [61, 62]. The proliferation of satellite cells was subsequently achieved in Ham's F-10 medium supplemented with 10% horse serum, 1x penicillin-streptomycin and bFGF. Several transgenic mouse strains have been established expressing GFP-Nestin or Pax7-regulatory elements for isolation of satellite cells using FACS [63, 26]. The satellite cells and their derivative myoblasts cultured on collagen-coated flasks are both molecularly and functionally different from the freshly isolated muscle satellite cells [64, 65]. There are only a few studies published so far involving cultured human satellite cells [66, 67, 68]. In one of our recently published studies, needle muscle biopsies from *vastus lateralis* muscle of young and old human donors were obtained. Each biopsy provided 100–300 mg of muscle tissue and was immediately placed in an ice-cold isolating solution. The muscle cells were detached from the tissue fragment and centrifuged. The cells were cultured in skeletal muscle cell growth medium. We observed similar proliferative, differentiation and senescent profiles in the cultures maintaining cells from the young and aged donors. The major challenge for future research, however, remains understanding not only the components but also the role of the satellite cell niche to better control and manipulate their quiescence, activation, proliferation and differentiation.

Culturing and multiplication of the myogenic cells has led to the establishment of various muscle cell lines including L6 and L8 from rats [69]. A control line (C2) from the injured thigh muscle of 2-month-old C3H mice has also been established [70]

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that was later expanded as the C2C12 cell line [71]. Similarly, several mouse cell-lines MM14 [72], normal C3H mouse [73], G-7 and G-8 [74] have also been established for experimental use.

5.5 Proliferation and differentiation of myogenic stem cells

Satellite cells are the primary myogenic stem cells responsible for growth, regeneration and repair of skeletal muscle and for repopulation of the satellite cell niche. Nevertheless, the number of muscle stem cells isolated in small biopsy specimens taken from the donors warrant significant expansion *in vitro* to obtain a sufficient number of cells before they can be of any significance for use in any experimental or clinical application. *In vitro* processing of the cells for expansion in culture significantly affects their proliferative and differentiation capacity unless stringent and highly controlled culture conditions are maintained [65]. Even the best and the most optimal culture conditions are no match for the microenvironment of the niche. For example, the culture of the human SkMs in xenoproteins containing culture medium allows the xenoproteins to settle on the surface of the cells and alter their immunogenic characteristics postengraftment.

As discussed earlier, satellite cells are kept in an intricate and highly sophisticated niche *in vivo* which is influenced by the cellular, chemical and physical characteristics of their microenvironment. For example, in their niche, satellite cells are bound to the basal lamina through integrins [75], a physical interaction between the two that allows extracellular mechanical forces to initiate intracellular signal transduction [76]. When cultured *in vitro*, such physical interaction is lost thus significantly impacting the self-renewal and reparability characteristics of the satellite cells and their derivative myoblasts. Rather, it has been observed that *in vitro* culturing causes rapid loss of their proliferation and differentiation potential. A recent study has reported an optimized protocol to culture murine muscle progenitor/precursor cells [77]. The inclusion of matrigel to the culture medium supported the culture of the cells up to 25 passages without the loss of their "stemness" and engraftment capabilities. The cells cultured on a more elastic cell substrate rather than on hard plastic increase their self-renewal and regenerative capacity postengraftment into injured muscle [78]. Cytokines produced by T-cells, macrophages and eosinophils have been reported as prime regulators of cell proliferation and differentiation in different tissues including skeletal muscle [79]. Based on these observations, the addition of T-cells or their conditioned medium to satellite cell culture *in vitro* supported their phenotypically unchanged proliferation for at least 20 passages without diminishing their engraftment efficiency [80]. Later a cocktail of four cytokines IL-1α, IL-13, tumor necrosis factor- (TNF-α) and interferon- (IFN-γ) was identified to significantly sustain satellite cell expansion. Moreover, the satellite cells expanded with the cytokine cocktail were able to participate in the muscle regeneration that was comparable to the freshly isolated satellite cells [80].

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These results highlight the importance of *in vitro* culture systems for expansion and differentiation of skeletal muscle progenitors satisfying the physical and biochemical requirements of these cells to avoid early undesired differentiation and senescence.

5.6 Skeletal muscle cells in regenerative therapy and drug development

The loss of muscle architecture and reduction in muscle mass are the hallmark of many muscle-related pathologies. Although various cell types including non-muscle derived cells, i.e. bone marrow cells, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been studied for cell-based therapies to restore normal myogenic function, satellite cells and SkMs remain the most preferred ones due to their associated advantages. These include ease of autologous availability, *in vitro* expansion without compromising their myogenic capability, non-immunogenicity, ease of genetic manipulation and potential use as a transgene delivery vehicle and, their commitment to undergo myogenic differentiation. The therapeutic advantages of SkMs and satellite cell-based therapy have already been reported for genetically inherited dystrophies, age-related muscular atrophies, malfunctioning of sphincter and myocardial pathologies as deliberated in the following text.

5.6.1 Satellite cells and SkMs for the treatment of muscular dystrophies

There are over 80 genetically determined muscular dystrophies (some affecting both skeletal and cardiac muscles); of these, Duchenne muscular dystrophy (DMD) is the most common type. Other muscular dystrophies show localized manifestations due to the involvement of regional muscles, of which oculopharyngeal muscular dystrophy (OPMD) is a typical example [81]. DMD is a progressive muscle-wasting pathology that results from lack of dystrophin, a 427kd protein expressed at the sarcolemma of the skeletal muscle. Hence, this can be treated either by genetic restoration of dystrophin expression or by cell-based therapy for the DMD patient using healthy allogenic cells with functional dystrophin. After encouraging results from the experimental animal studies [82, 83, 84, 85], the first successful clinical application of cell transplantation therapy for the treatment of DMD involved the injection of SkMs in the extensor digitorum brevis muscle of the patient. The results showed an increase in voluntary contractions and isometric twitch as compared to non-treated patients. Subsequently, 21 patients were given intramuscular injections of SkMs, as a result of which 81% of patients showed either an increase in or at least preservation of strength [86]. In another clinical study, DMD patients received repeated injections of donor SkMs every month for a period of 6 months in the biceps brachii muscle with significant prognosis [87]. Although clinical studies for the treatment of DMD have been conducted over the last

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3 decades [88], there has been no major breakthrough establishing this approach as a routine clinical modality. One of the many reasons for this less than optimal consequence of the clinical studies has been the non-localized nature of the disease process that requires repeated intramuscular injections of the donor cells at multiple sites. Ideally, the donor cells should be injected systemically to home in on the target muscle by crossing the capillary wall to their niche. However, this requires in-depth animal studies to optimize the delivery strategy in order to overcome the obstacles that hinder the approach of the cells from the systematic circulation to target muscle site [89]. Other important factors impacting the outcome of DMD treatment has been the use of allogenic SkMs, which requires immunosuppressive therapy to support donor cell acceptance postengraftment [84] and extensive death of the transplanted cells during the acute phase after cell engraftment Recent success in the reprogramming of terminally differentiated cells to achieve pluripotent status by induction of a cocktail of exogenous transcription factors, i.e. Oct4, Sox2, Klf4 and cMyc, has given new hope for cell-based therapeutic strategies [90]. The approach has been extended to the reprogramming of disease-specific cells from the patients, genetically correcting the cells for dystrophin expression and transplanting the healthy cells back into the patients. In a recently published study, Tedesco *et al.* have reprogrammed fibroblasts and SkMs into iPSCs from patients with limb girdle muscular dystrophy-2D, which is caused by a mutated gene encoding for α-sarcoglycan [91]. The authors obtained fibroblasts and SkMs from the patient and reprogrammed the cells to generate human iPSCs. The iPSCs thus generated were differentiated to mesoangioblast-like cells, expanded *in vitro* and genetically corrected using lentiviral vector carrying the gene encoding for human α-sarcoglycan. Improved muscle function was witnessed following transplantation of the genetically corrected cells in an experimental animal model of α-sarcoglycan-null-immunodeficient mice [91]. A similar approach has also been adopted by other research groups to identify the mechanism of the disease besides the use of the genetically corrected cells in drug development [92, 93]. Contrary to the outcome of cell-based therapy for DMD, the results of treating a localized dystrophic condition such as OPMD with a cell-therapy approach has been more encouraging [94] in comparison to the treatment of DMD or Becker MD [95, 96]. Autologous myoblast transplantation in cricopharyngeal muscles of phase I/IIa clinical trial showed safety and tolerability in patients affected by OPMD [94]. The ongoing clinical trials are expected to explore the possibility of treatment for various muscular dystrophies using a combination of stem cell and gene transfer approaches.

5.6.2 Satellite cells and SkMs for the treatment of sphincter incontinence

The results from the studies encompassing the treatment of weak anal and urethral sphincters have been more reassuring than the outcome of studies for the treatment of dystrophies [97, 98]. An optimal cell selection, *in vitro* manipulation such that the

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cells retain their differentiation capability and targeted delivery would be imperative to ensure the desired prognosis. The ideal cell for muscle cell therapy would have to satisfy several criteria. The chosen cell should have a high myogenic potential, be easy to isolate, be expandable in number without early senescence or hasty differentiation and pose no significant risk of transformation into a neoplasm. The satellite cells, mesenchymal stem cells, embryonic stem cells and induced pluripotent stem cells are the current main candidates.

5.6.3 Satellite cells and SkMs for cardiomyopathies

Cardiomyocytes are sensitive to hypoxia whereas satellite cells/SkMs possess comparatively more resistance to low oxygen conditions besides being committed to myogenic differentiation. Hence, the skeletal-muscle-derived cells possess great potential for use in cell-based therapies for the ischemically damaged heart. A plethora of experimental studies in both small and large experimental animal models of myocardial ischemia and infarction have shown that SkMs transplantation leads to significantly preserved left ventricle function [99, 100, 101, 102, 103, 104, 105]. Although the exact mechanism of significantly preserved/improved cardiac function after SkMs remains oblivious, it has always been considered as multifactorial wherein the scaffolding effect of the neofibers on the left ventricle and release of paracrine factors by the transplanted cells make the foremost contribution. A recent study has shown that 35 growth factors, 40 cytokines and 36 metallopeptidases in addition to many other substances are secreted by the cells in skeletal muscle tissue [106]. These data led to the first-in-man transplantation of SkMs in a 57-year-old patient as an adjunct to coronary artery bypass grafting [107]. The subsequent clinical studies showed that SkMs transplantation successfully attenuated left ventricular remodeling and preserved global cardiac function in the patient [108, 109, 110]. The promising results of SkMs were short-lived when the 4-year multicenter follow-up of the Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial was abandoned due to concern about arrhythmias [111]. MAGIC was the first randomized placebo-controlled study of myoblast transplantation. Recently, concluded long-term follow-up of seven patients from MAGIC trial by single-center cohort also found no improvement in left ventricular function [112]. Similarly, phase IIa randomized Safety and Effects of Implanted (Autologous) Skeletal Myoblasts (MyoCell) using an Injection Catheter (SEISMIC) trials were safe and provided some symptomatic relief to the patients, however, no global improvement in the global left ventricular ejection fraction was reported [113]. The Myoblast Implantation into Myocardium Post Myocardial Infarction (MARVEL-I) trial involved randomized placebo-controlled image-guided catheter-based intramyocardial delivery of SkMs in patients with class II to IV heart failure, which raised concern for the safety of SkMs transplantation and due to financial reasons this trial was

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stopped [114]. Although immunohistological evidence from the human heart transplanted with SkMs showed the survival of the transplanted SkMs and their myogenic differentiation to form islands of skeletal muscle fibers in the infarcted myocardium, there was no direct evidence that the neomyocytes have electromechanical coupling with the host cardiomyocytes [107, 115]. Such lack of functional coupling with the host cardiomyocytes hindered the synchronous beating of the neomyocytes. Moreover, arrhythmogenicity and poor survival of the transplanted cells were some of the major obstacles in the progress of SkMs transplantation as a routine clinical option. Various strategies have been adopted to overcome the problem of poor survival of the transplanted cells. These strategies include genetic modulation of SkMs for overexpression of pro-survival signaling molecules such as Akt and Bcl2, pharmacological preconditioning, priming the cells with growth factors and genetic manipulation for growth factor overexpression [116, 117, 118, 119, 120, 121, 122]. Arrhythmogenicity of SkMs has been ascribed to the failure of SkMs to differentiate into cardiomyocytes and lack of connexin-43 expression by the neomyocytes to form gap junctions with the host cardiomyocytes [123, 124]. Although, most of the arrhythmias due to SkMs transplantation have been pharmacologically treatable, various strategies have been adopted to overcome this problem [125, 126]. In a recent interesting study, SkMs were genetically modified to overexpress connexin-43 before engraftment in a rabbit experimental model [126]. SkMs overexpressing connexin-43 preserved the left ventricular function postengraftment. The electrical communication between the cardiomyocytes and SkMs was confirmed in the co-culture. The various challenges that need to be overcome before SkMs become acceptable as candidates for cardiac repair have been thoroughly discussed by several authors [127, 128, 129].

Despite the superiority of autologous SkMs for transplantation, there are multiple factors that affect the outcome of the procedure, including logistic issues with the use of autologous SkMs. To ensure their ready-to-use off-the shelf availability, the cells have to be used from the allogenic source. Alternatively, SkMs can be reprogrammed to pluripotency using a classical four-factor protocol to obtain a uniform continuous source of cells for transplantation [90]. During a recent study, purified SkMs were isolated from male Oct4-GFP transgenic mice and were transduced with retroviral vectors encoding for Oct3/4, Sox2, cMyc and Klf4 for reprogramming. The SkMs-derived iPSCs showed spontaneous beating and expression of cardiomyocyte markers and were later used in an experimental model of myocardial infarction. The transplanted cells successfully differentiated into cardiomyocytes postengraftment, reduced infarct size and preserved global cardiac function. Nonetheless, more than 25% of the animals showed myocardial tumor formation that raised safety concerns about their clinical use [130]. Future clinical trials with muscle stem cells reprogrammed to iPSCs and differentiation to various cells or tissues will open up many possibilities in drug screening and treatment of all kinds of diseases.

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5.7 Conclusions

The muscle satellite cells and their derivative SkMs have a commitment to myogenic lineage and show extensive proliferation capacity in response to muscle injury. However, both these characteristics are severely compromised during chronic muscle degeneration. The use of allogenic muscle cells has been seriously considered as an alternative strategy to circumvent this issue but suffers from the immune rejection of the donor cells that warrants immunosuppression to ensure donor cell acceptability. A more recent advancement in the field of somatic cell reprogramming to pluripotent status using the classical four-factor induction of exogenous Oct3/4, Sox2, Klf4 and cMyc has given a new direction to cell based therapies. SkMs are malleable to reprogramming as they intrinsically express Oct3/4 and different groups have already reported their successful reprogramming to pluripotency. Further refinement of the classical protocol of reprogramming is being sought by various research groups to improve the safety and efficiency of reprogramming and include the use of viral/non-viral vectors, chemical treatment, small molecules and microRNAs [131, 132]. Combining genetic correction of the diseased cells followed by reprogramming may provide an unlimited source of cells for cell-based therapies for diseases like DMD. Moreover, SkMs-derived iPSCs have also shown promise in experimental animal models of myocardial infarction albeit with a note of caution about their safety.

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Changfa Guo, Kai Zhu and Khawaja Husnain Haider

6 Nanoparticle-based genetic engineering of mesenchymal stem cells

Abstract: Given their ease of availability, *in vitro* expansion without altering their multilineage differentiation potential and paracrine behavior and, combined with the data from *in vitro* characterization studies, preclinical and clinical studies in human patients, mesenchymal stem cells (MSCs) are being viewed as choice cells for cell-based therapies. Although the results have been reassuring in terms of their ability to cross lineage restriction and adopt different functionally competent phenotypes, MSCs are also being conjoined with gene delivery to augment their therapeutic benefits. In this context, a safe, efficient, biocompatible and regulatable gene transfer vehicle is warranted that might effectively transfer genes of interest into MSCs. In comparison with the conventional gene transfer vectors, which suffer from issues *vis-a-vis* their safety, poor transfection efficiency, toxicity and immunogenicity, nanoparticle-based gene delivery vehicles afford safe, efficient and cost-effective alternatives. Numerous nano-vehicles with particle sizes of less than 100 nm have been designed with superior transgene delivery efficiency and biocompatibility. Likewise, with beacon-like and missile-like modifications, nanoparticles are being used for tracking the fate of transplanted cells in real-time. This chapter provides an overview of the application of nano-vehicles for genetic engineering of cells with a focus on MSCs. Key issues and future directions in nanoparticle-based gene transfer have also critically addressed.

6.1 Introduction

Stem cell transplantation therapy has emerged as a novel therapeutic modality that is already being realized for treatment of a large spectrum of health problems, i.e. cerebral and spinal cord injury, cardiovascular pathologies, cancer [1–4]. To this end, stem cells, including adult, embryonic and induced pluripotent stem cells (iPSCs), have been successfully used. Each of the cell types has its associated advantages and disadvantages, which range from ease of availability to ethical and moral issues surrounding their clinical applications [5–7]. Moreover, safety concerns due to teratogenicity remain a major challenge especially with the use of pluripotent stem cells [8]. The novel strategy of somatic cell reprogramming to pluripotent status has rejuvenated the field as it ensures a continuous autologous source of surrogate embryonic stem cells without ethical and moral issues. However, teratogenicity still remains a serious issue with the use of iPSCs [9]. Bone-marrow-derived mesenchymal stem cells (MSCs) are one of the most extensively studied and well characterized cells for transplantation therapy [10]. They are primarily predestined to maintain bone

DOI 10.1515/9783110493764-006

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marrow homeostasis, however, given their biological characteristics, inherent capacity to undergo multi-lineage differentiation, release of copious amounts of bioactive molecules as part of their paracrine activity, natural emigrational activity to home-in on the site of injury, immunomodulatory properties, reparative potential by undergoing differentiation and by the release of paracrine mediators that have direct cytoprotective effects on the host cells besides mobilization of the resident stem cells to the site of injury make them choice cells for cell-based therapy [11–16]. Besides the characteristics listed above, MSCs are also relatively easy to isolate, culture *in vitro* and show excellent undifferentiated expansion capability to achieve the large number of cells required for transplantation therapy [4]. Other than bone marrow as a major tissue source to harvest MSCs, they have also been successfully isolated and studied for their reparability and regenerative potential from other tissues in the body, i.e. peripheral blood, cord blood and adipose tissue [17–19]. Using fibroblasts as controls, a direct comparison of MSCs derived from bone marrow, adipose tissue and umbilical cord blood showed that MSCs from all the sources shared the osteogenic and adipogenic differentiation characteristics and had the same phenotype as determined by the surface marker analysis by flow cytometry [20]. A recently published study shows that despite sharing similar fibroblast-like morphology, human bone marrow MSCs and human adipose tissue MSCs differ in their osteogenic and chondriogenic differentiation characteristics but both cell types showed similar rates of adipogenic differentiation under xeno-serum-free culture conditions [21]. These cells also diverge in their paracrine behavior to release bioactive mediators. The paracrine activity and differentiation characteristics render MSCs as attractive potential candidates for the regenerative and personalized medicine. Although in most cases, the primary cultured autologous MSCs have been studied for *in vivo* behavior and reparability postengraftment in experimental animal models, a recent double-blinded, placebo-controlled, dose-ranging clinical study has preferred to test allogenic human MSCs for safety and efficacy. The purpose of the study was to assess the acceptability of allogenic MSCs across the histocompatibility barriers so as to overcome the logistic issues and ensure off-the shelf availability of cells for cell-based therapies [22]. The results provided evidence of safety and efficacy of the allogenic cells with significantly improved left ventricular ejection fraction. Currently, MSCs are at the intersection of stem cell therapy and gene therapy because of their associated advantages as discussed in the following text [23].

6.2 Genetic modification of MSCs

MSCs have also been studied extensively as carriers for transgene delivery to the target tissues because of their compliant nature to undergo genetic manipulation and ability to overexpress and translate the transgene/s of interest [24–27]. With the current advancements in understanding the critical participation of microRNAs as regulators of almost every biological function of a cell, MSCs are genetically modified

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to overexpress microRNAs of interest as part of a combinatorial approach that conjoins delivery of microRNAs with cell-based therapy [28]. The genetic manipulation of stem cells in general and that of MSCs in particular have been intended to yield multiple benefits which are accomplished by the correct selection of the gene/s of interest. Likewise, genetic modification of MSCs has been performed to enhance the paracrine behavior of the cells in general and for the gene of interest in particular such that the genetically modified cells serve as tiny little "reactors" to synthesize and reservoirs that continuously secrete the protein/s encoded by the transgene [29–31]. In some other instances, such genetic manipulation initiates survival signaling to precondition the genetically modified cells that become more resistant to stresses emanating from their microenvironment [32, 33], promote differentiation [34–37], allow mobilization and homing-in of the resident stem cells to participate in the repair process [38] and many other similar desired end points [39]. The protocols for genetic modulation of MSCs are generally based on either the viral vectors or the non-viral vectors; the preference of the protocol is influenced by multiple factors ranging from safety concerns to the ultimate desired outcome for which the genetically modified cells will be used in the end. For example, the non-viral methods are predominantly non-integrating strategies as the delivered gene of interest remains episomal without being integrated into the host genome. Hence, the effect of the transgene will be transient and will continue only as long as the cell retains the episomal transgene. Once the cell loses the transgene, it would be no longer proficient to synthesize the protein encoded by the delivered gene and accomplish the desired function. Contrary to this, integrating a viral-vector-based delivered transgene becomes part of the host genome and therefore the effects are durable and longer lasting. A detailed account of the delivery vectors and their transfection/transduction efficiency, safety and cost-effectiveness has been extensively reviewed in the literature [40–44].

6.3 Methods to genetically modify MSCs

The primary cultured MSCs are relatively difficult to transfect without impacting their multipotency or compromising their viability and often conclude in extremely poor efficiencies. MSCs also diverge in their docility to the genetic manipulation protocols and hence the choice of the method will determine the efficiency of the gene transfer [45]. Although retroviral vectors display high efficiency transduction in MSCs due to their high proliferation rate, unnecessarily long-term unregulated expression may lead to deleterious effects linked to the transgene expression product [46]. Similarly, adenoviral and adeno-associated vectors have shown limited applications in gene delivery to the MSCs due to low transfection efficiency unless the protocol involves additional modifications or otherwise specific strains of the virus are used to develop the delivery vectors for manipulation of the cells [47, 48]. Although lentiviral vectors show high efficiency of gene transduction in MSCs, safety due to insertional mutagenesis remains a major concern [40]. On the contrary, non-viral vectors are notorious

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for low transfection efficiency and toxicity to the transfected cells [14]. Therefore, an efficient gene vehicle is extremely important for the engineering of MSCs to achieve the required level of transgene expression without inflicting deleterious effects on the cells [49–51]. From the data accumulated over the past many years, even though it is difficult to rate any of these methods as ideal, the results are distinct about superiority of the nanoparticle-based methods for being more efficient in delivering transgenes into MSCs with little effect on their "stemness", biological characteristics and reparability. Our book chapter reviews the current knowledge and progress in the field of the nanostructured vehicles for genetic engineering of MSCs.

6.4 Exploiting nanoparticle technology for genetic priming of stem cells

Besides their advantages, the use of primary cultures of MSCs has its own limitations and hence, may require further optimization. Although the preclinical and clinical studies with native, genetically unmodified MSCs have shown encouraging results in terms of safety of the treatment method, the limited to moderate therapeutic benefits reported thereof after MSCs transplantation require further optimization of cell therapy protocols to achieve the required prognosis [52]. A way forward in this regard would be a combinatorial approach wherein stem cells for therapy could be genetically engineered to enhance their reparability in terms of a higher rate of differentiation potential. Correspondingly, genetic engineering can be used as a powerful tool to prolong the survival of transplanted MSCs by transferring antiapoptotic genes into cells and making them resistant to microenvironmental stresses [53]. Additionally, MSCs are selectively engineered to achieve improved paracrine activity and express therapeutic proteins targeting specific disease states [54, 55]. Taken together, combining MSCs transplantation with transgene delivery is anticipated to offer unprecedented opportunities to broaden the therapeutic benefits of a patient's own cells to realize the modern concept of personalized medicine in a clinical perspective. As discussed earlier, the primary cultures of MSCs are relatively difficult to genetically modulate without impacting their multipotency or viability. Although the progress made in optimization of gene transfer protocols and refinement of the gene delivery vectors have narrowed down the potential problems of limited gene-packaging capacity, risk of pathogenicity, oncogenic transformation and immunogenicity, it still necessitates a search for new vectors to make the procedure safer for clinical applications. Nanotechnology in this regard has numerous applications and provides a leap forward in this field of medicine by controlling the materials for use on a nanoscale thus enabling the use of nanostructures, i.e. nanoparticles, with broad applications [56]. On the same note, nanomedicine amalgamates the innovations of nanotechnology with the field of medicine for much reliable diagnosis, molecular therapy and tissue engineering [57, 58]. Many of these nanomaterials are already being assessed for routine clinical applications whereas a few of these are already available for use in

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clinical practice [59–62]. Besides numerous potential clinical applications of nanomaterials, nanoparticle-based delivery systems have been designed and synthesized as nanostructured vehicles for therapeutic gene transfer into MSCs and will be discussed further.

6.5 Nanoparticle-based systems for gene transfer

As discussed earlier, genetic modification of cells is mostly intended to achieve various biological endpoints from production of transgenic animals to cell fate determination and reprogramming of somatic cells to pluripotency. In addition to the classical methods of gene transfer, the last decade has seen the emergence of nanoparticle-based gene transfer vehicles, i.e. polymeric nanoparticle, lipid-based nanoparticles, ceramic nanoparticles, magnetic nanoparticles, polymeric micelles and dendrimers. Each one of these exhibits improved efficiency of uptake and better gene transfer kinetics in addition to lower toxicity to the cells, such that their "stemness" characteristics including differentiation capability are not compromised [63]. These gene transfer vehicles or the nano-carriers of the genes have already been characterized and assessed for safety and efficiency during *in vitro* studies and *in vivo* experimental animal models. Although nanoparticle-based delivery systems are not without their limitations, i.e. encapsulation efficiency, cell toxicity, stability issues and aggregation, some of these have already entered into clinical assessment with encouraging results.

6.5.1 Polymer-based nanoparticles

Polymers are highly versatile molecules of natural or synthetic origin with their respective advantages and disadvantages. Whereas the natural polymers are more biocompatible (and hence cytocompatible during cell transfection procedures) and offer in their molecular structures active sites that are easy to manipulate for cross-linking and conjugation reactions for ligand binding, synthetic polymers offer nanocarriers that can be surface shielded and modified for targeted gene delivery and give sustained expression without toxicity and immunogenicity. Different types of polymers, i.e. polyethyleneimine (PEI), chitosan, polylactic acid, collagen, etc., have been used to develop nano-carriers for gene delivery into stem/progenitor cells [64–66]. These polymers contain a high density of positively charged groups, most often primary amines, which can interact with the negatively charged phosphate groups of the DNA to form condensed "polyplexes" with residual positive charges [67]. As part of the underlying mechanism of their uptake, the positively charged nanoparticles of polyplex bind to the anionic sites on cell membranes and are internalized by the cells. Once inside the cell, the cationic polymers also contribute to protecting DNA from degradation and facilitate their escape from endosomes and lysosomes [68]. An important characteristic

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of the cationic polymers is the ease of their surface modification that enhances their flexibility to any required modifications so they may be adjusted for optimal efficiency, low cytotoxicity and specific targeting characteristics [69]. For example, branched polyethyleneimine (bPEI) has a promising level of transfection efficiency in different cell types, the downside of bPEI as a gene delivery vehicle is cytotoxicity, which can be alleviated by the addition of another molecule to the bPEI preparation that may serve as a ligand for binding with the cells. In an interesting study, PEI was modified with the inclusion of hyaluronic acid (HA), a linear non-sulfated glycosamine with wide distribution on cells and tissues, to make the preparation more acceptable for cell binding [69]. By taking advantage of the presence of hyaladherins, i.e. CD44, CD54 and CD168, on the surface of human MSCs, the authors showed improved efficiency of gene transfection using bPEI-HA formulation. Following the same strategy, the authors of this chapter have used hyperbranched poly(amidoamine) (hPAMAM) for genetic engineering of rodent bone marrow MSCs. hPAMAM is a structural analogue of dendrimers and has similar architecture and properties in terms of globular topology, low viscosity, high solubility and high density of functional groups but with a simpler and more economical one-step polymerization technique [70]. Using the hypoxia regulated VEGF expressing plasmid hPAMAM-pHRE-VEGF, 38±1.95% cells were successfully transfected with more than 92% cell viability. Upon subsequent exposure to hypoxia, the transfected MSCs continued to express VEGF for up to 14 days of observation, with peak level expression of VEGF on day 2 after transfection. The conditioned medium from the transfected cells supported endothelial cell proliferation *in vitro* culture. Our results also demonstrated significantly higher level transfection efficiency with hPAMAM as compared to PEI alone as control. From among the natural polymers, chitosan, a linear amino polysaccharide of N-acetyl-Dglucosamine and D-glucosamine, has been extensively studied in the development of a non-viral vector for gene transfer due to its biocompatibility, excellent complexation ability with DNA and facilitation of its endocytosis. In a recent study, human MSCs were successfully transfected with chitosan nanoparticles. Although the highest transfection efficiency level achieved was only 18%, it was significantly less as compared with the commercially obtained Lipofectamine. Likewise, viability of the transfected MSCs was more than 95% with a chitosan nanoparticle preparation as compared to only 60% with Lipofectamine [71]. PEGylation of chitosan-DNA complex using alpha-methoxy-omega-succinimidylpoly (ethylene glycol) significantly enhanced its tumor uptake in addition to increasing its transfection efficiency [72]. Others have attempted to improve chitosan nanoparticles by lactosylation, inclusion of PEI and hyaluron to enhance their gene delivery efficiency with fewer cytotoxic effects [73–75]. Chitosan and chitosan-HA preparations have also been used to serve as substrate to culture MSCs and study the effect of cell culture substrate on nanoparticle uptake by the cells during gene transfection. Interestingly, both the substrates enhanced the uptake of nanoparticles by more than 5-fold, i.e. paramagnetic iron oxide and naked plasmid DNA, which otherwise were poorly taken up by MSCs when cultured on polystyrene dishes [76]. Molecular studies revealed

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increased Rho activity in the cells cultured on chitosan and chitosan-HA substrates, which allowed the cells to adopt a spheroid formation, which supported increased endocytosis. Furthermore, use of specific inhibitors revealed clathrin-mediated endocytosis (CME) on chitosan substrate, whereas caveolae-mediated (CvME) endocytosis was more evident on chitosan-HA substrate. These data clearly show the importance of the nature of the substrate on which MSCs are cultured as a critical determinant of transfection efficiency using nanoparticles for gene transfer. Moreover, it is anticipated that the use of a proper substrate for cell culture would also safeguard cell proliferation and differentiation characteristics of the stem/progenitor cells.

6.5.2 Inorganic nanoparticles

Inorganic nanoparticles can be used either alone or in combination with organic materials as vehicles for gene transfer as they have the capability to load transgene/s of interest *via* adsorption or conjugation for internalization by the cells [77]. As their size nears nano-scale, they start to show distinct properties due to their high surface to volume ratio. With their size ranging from 2–100 nm, their interaction with the biomaterials is generally at a molecular level and allows targeted delivery through multiple biological barriers. Several types of inorganic nanoparticles including calcium phosphate, carbon nanotubes, magnetic nanobeads, silica, gold and quantum dots have been developed as gene vehicles [78]. Although transfection efficiency achieved in most cell types using inorganic nanoparticles is only low to moderate, they are easy to prepare and exhibit low cytotoxicity [77, 78]. Additionally, they can easily be modified to improve their performance as gene delivery vehicles in terms of safety, efficiency of transfection and targeted delivery. Recently, Muroski *et al.* have reported the modified gold nanoparticles as good candidates for genetic engineering of MSCs. The authors showed that Bax-inhibiting peptide was transfected into the rat MSCs with modified gold nanoparticles at a significant rate of transfection efficiency. More than 80% of the treated cells showed the desired protein expression within 4 days of transfection. More importantly, the high efficiency of transfection was achieved with little impact on the surface marker expression of MSCs and without any significant loss of cell viability [51]. We have recently reported the synthesis of an organo-inorganic hybrid mesoporous organosilica nanoparticles (HMONs) based on nano-synthetic chemistry [79]. Our novel organo-inorganic hybrid nanoparticles had pore sizes of 20 nm, small particle sizes and hollow cavities. We successfully used these surface-modified nanoparticles for high efficiency transfection of HGF transgene into MSCs. The gene-transfected bone-marrow-derived MSCs were later transplanted into a rodent model of myocardial infarction. The genetically modified cells significantly reduced cardiomyocyte apoptosis in the recipient heart, attenuated infarct size expansion and improved global cardiac function. Our data signify the use of fabricated organo-inorganic HMONs for gene transfer into MSCs and support in-depth studies to develop hybrid nanoparticle-based vectors for enhanced gene

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delivery performance. Moreover, proper surface treatment of the inorganic nanoparticles with a focus to develop hollow and porous nanomaterials would significantly enhance their performance as gene delivery vehicles.

6.6 The mechanism of nanoparticle-based gene transfer

6.6.1 Uptake pathways

Identification of the underlying mechanism of nanoparticle-based gene transfer into cells in general, and for MSCs in particular, is essential to the design of nano-delivery vehicles as it decides the eventual fate of the nanoparticle-gene complex post-endocytosis. Predominantly, endocytosis remains the foremost method of nanoparticle-gene complex uptake by the cells [80]. This may involve CME, CvME, macropinocytosis and phagocytosis [81]. Nevertheless, there is a dearth of literature on any specific pathway that contributes to gene transfer in MSCs when nanoparticles are used as gene transfer vehicles (Fig. 6.6.1).

CME is a receptor-dependent, clathrin-mediated and dynamin-required mechanism [82]. It involves a series of events that are activated upon interaction of the ligands by receptors present on the cell membrane. Clathrins assemble in the polyhedral lattice on the cytosolic surface of the cell membrane, which helps to deform the membrane into a coated pit. The invagination of the pit continues until the vesicle is pinched from the membrane and subsequently, the endocytosed vesicles get internalized into the cytoplasm and are integrated into endosomes [82, 83]. Likewise, CvME is also a type of cholesterol, dynamin-dependent and receptor-mediated pathway [84]. Flask-shaped caveola, mainly made up of caveolins, are formed on the cell membrane, the fission of which occurs in the presence of dynamins to generate a cytosolic caveolar vesicle.

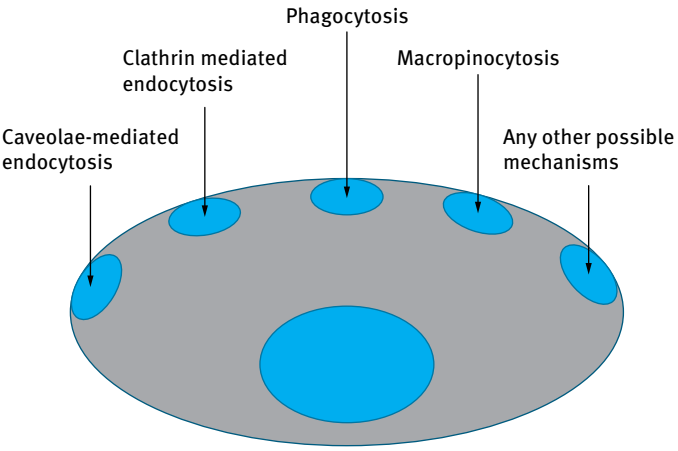

Fig. 6.6.1: Summary of the possible mechanisms of cellular uptake of NPs during transgene delivery.

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Caveosome buds from the vesicle and joins the classical endocytic pathway, and sometimes is directly transported into Golgi and/or endoplasmic reticulum [84]. Macropinocytosis occurs *via* the formation of actin-driven membrane protrusions. The internalized macro-pinosomes have no apparent coat structures and are heterogeneous in size. As similar as macro-pincocytosis, phagocytosis is also an actin-dependent pathway and primarily exists in professional phagocytes, such as macrophages, dendritic cells, monocytes and neutrophils. Although phagocytosis has a limited role in gene transfer by nanoparticles, an understanding of phagocytosis is important to comprehend the whole mechanism of uptake pathways of nanoparticles [85]. There is ample evidence that fusion with the cell membrane contributes minimally to the overall uptake of lipoplexes while the CME plays an important role in the uptake of lipoplexes [86].

6.6.2 Endo-lysosomal escape

The nanoparticle-gene complex eventually fuses with lysosomes after internalization by endocytic pathways [87]. This involves a maturation process involving compartment acidification by proton-pumps located on the cell membrane. Some nanoparticles such as PEI escape from the endolysosomes through the proton sponge effects and the nitrogen atoms of PEI are protonated by endosomal protons [88]. The endolysosomes then break up, and the complexes are released and transported to the appropriate sites to perform their functions. For lipoplexes, the cationic liposome can interact with the anionic cytoplasm-facing monolayer lipid of endosome/lysosome to release the genes through the flip-flop mechanism [89].

6.7 Factors influencing nanoparticle-based gene transfer

In comparison with the viral vectors, nanoparticle-based gene delivery vehicles suffer from low efficiency of transfection and a transient expression of the transgene. The success of the transfection protocol is influenced by multiple factors some of which are relevant to the nanoparticle formulation characteristics, while others are relevant to the cells to be transfected. A brief description of these factors follows.

6.7.1 Characteristics of nanoparticles as determinants of gene transfer efficiency

The characteristics of nanoparticles, i.e. nature and structure, particle size, shape, density and surface charge and the residual nature of the nanoparticle-DNA complex, significantly affect the way they interact with cells [90]. As these characteristics are relevant to their overall design and thus the outcome of design strategy, they seriously impact the transgene loading capacity, uptake efficiency and pathway of complex internalization and stability of the delivered transgene into MSCs [91]. Although the cells do

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not use too many different mechanisms for uptake, phagocytic cells show preferential uptake of anionic nanoparticles whereas the non-phagocytic cells prefer cationic nanoparticles. Similarly, cationic nanoparticles cause more membrane disruption and lysosomal damage than the anionic ones [92]. A direct comparison of three preparations of nanoparticles, similar in all respects except for diverging in their residual surface charge, showed that the rate as well as the level of cellular uptake of each preparation was different [93]. The nanoparticle preparations also differed in the intracellular trafficking after internalization. Interestingly, whereas the positively charges particles were observed with perinuclear localization, both neutral and negatively charged particles co-localized with lysosome. We have recently used a positively charged arginine-terminated generation 4 (Arg-4) poly(amidoamine)-based nanoparticle. We used our nanoparticle preparation for the delivery of propyl-hydroxylase domain-2 (PHD-2)-specific small interfering RNA (siRNA) into MSCs to enhance their survival and paracrine activity (Fig. 6.7.1).

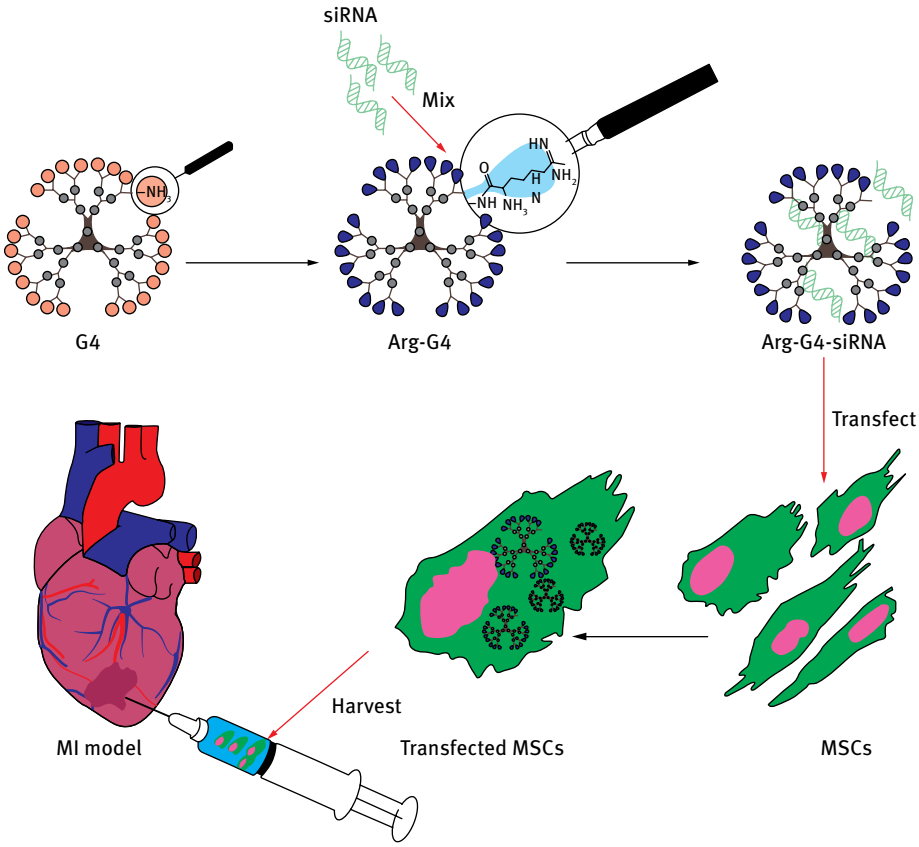

Fig. 6.7.1: Summary of the method for use of Arg-4 poly(amidoamine) based nanoparticle for siRNA transfer into MSCs and subsequent cardiac repair. (Adapted by permission from Dove Medical Press [94], copyright 2014.)

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After cellular uptake, a large number of positive charges from terminated groups could be protonated in acidic endosomes thus causing the endosomes to swell and finally burst to release the cargo. Compared with the unmodified poly(amidoamine), the enhanced surface charge could boost the transfection efficiency significantly [94]. As discussed earlier, we have also developed a novel type of mesoporous organosilica nanoparticles as gene vectors for MSCs [79]. Their large pore size and hollow structure enhanced gene-loading capacity significantly, which contributed higher gene transfer efficiency as compared with commercial liposome and PEI. In addition, the surface charge and size, the heterogeneity of particle size and shape significantly interfere with their functionality. The homogeneity and uniformity of morphological properties in terms of size and shape of the nanoparticles will be helpful to form a stable transgene-nanoparticle complex with high uptake efficiency [95, 96].

6.7.2 Characteristics of cells as determinants of gene transfer efficiency

Not all the cell types would show the same level of allowance to the DNA-nanoparticle complex to penetrate its membrane defenses and enter the cell unchecked. Different types of cells, depending upon a multitude of factors ranging from tissue source to the stage of cell cycle and the culture conditions; everything matters seriously when it comes to gene transfer or genetic modification of the cells. Samples of the human bone marrow MSCs obtained from 30 different patients showed differences in the gene transfection efficiency [97]. As discussed earlier, MSCs obtained from different sources such as bone marrow, adipose tissue and cord blood differ in their basic characteristics encompassing everything from paracrine behavior to differentiation potential, and their response to the same protocol of gene transfection may also diverge. In a study intended for optimization of protocol for transgene delivery to the human adult stem cells and ESC-derived progenitor cells, a comparison of adipose-tissue-derived MSCs, bone marrow MSCs and ESC-derived progenitor cells was carried out in terms of transfection efficiency using a novel nanoparticle formulation. Cells from all three sources differed in their response to the same gene manipulation protocol. Whereas adipose-tissue-derived MSCs showed the lowest transfection efficiency (24±3%), ESC-derived progenitor cells showed the highest transfection of the transgene (56±11%) as compared to human MSCs (27±2%) [98]. The cell viability ranged from 87%–96% in all three cell types. These data show an obvious difference in susceptibility to genetic modification between different types of cells as well as between the adult and embryonic tissue sources. The efficiency of gene transfection also drops significantly with the length of cells in the culture conditions and by the passage number in the culture [99]. Cell cycle is another important parameter that discriminates the cells in their responsiveness to transgene delivery by nano-structured gene vehicles. Increased permeability of the nuclear membrane during mitosis might allow plasmid to enter the nucleus thus improving transfection as well as expression efficiency of the transgene.

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Using a luciferase reporter gene, it was observed that luciferase activity from cells transfected with polycation- or lipid-based transfection systems was 30–500-fold higher when transfection was performed during S or G2 phase as compared with the cells in their G1 phase which showed the lowest expression levels [100]. On the contrary, culture conditions inhibiting cell division of human MSCs also decreased their transfection efficiency [101]. In light of these data, it is imperative to perform transfection when the majority of cells in the culture are in their S or G2 phase to achieve higher transfection efficiency.

In addition to these factors, MSCs derived from different species also differ in transfection behavior even when subjected to the same transfection protocol [102]. Many other factors, including cell density, passage number, transfection duration, transfection medium and gene dosage, make significant impact on the gene transfer. Therefore, while optimizing a transfection protocol for MSCs, it is important to optimize the whole transfection process including the biophysical characteristics of the nanoparticles as well as characteristics of the MSCs to be transfected to achieve the best transfection conditions.

6.8 Recent advances and modifications in nanoparticle-based gene transfer

Despite the positive impact of nanotechnology in terms of genetic modulation of stem cells, there are still many obstacles on the way to clinical application. These obstacles appear as lacunae in the transfection protocol irrespective that these are related with the cell conditions or the design and nature of the nanoparticles. There is a clear lack of technical abilities for real-time monitoring of cellular events that take place during the process. Similarly, the protocols are still less than optimal to achieve site-directed targeted gene insertion into the cells to avoid unwanted effects. Following are some of the important and popular modifications of nanoparticles for gene transfer.

6.8.1 Beacon-like modification

The precise assessment of stem cell-based therapy requires accurate determination of the real time *in vivo* behavior of the transplanted cells. Many types of agents, i.e. superparamagnetic iron-oxide nanoparticles, have been introduced into nanostructured gene vehicles for stem cell tracking *in vivo* [103, 104]. Nevertheless, these nanoparticles should be extensively characterized for their biocompatibility and toxicity before these can be considered for routine clinical use. Another interesting application of these modifications is to observe the intracellular activities of stem cells during the process of differentiation both *in vitro* and *in vivo* to optimize conditions conducive to their activity. Two such molecular beacon probe-modified nanoparticle

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platforms have been reported [105]. Subsequent to their transfer inside the cells, one probe targets the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase as an internal reference while another one detects alkaline phosphatase.

6.8.2 Missile-like modification

The bilayer nuclear membrane is a barrier to the entry of transgenes into the nucleus and its continuity is only interrupted by the nuclear pore complexes (NPCs). NPCs are 9-nm-diameter pores that regulate transport through the nuclear membrane by allowing the free diffusion of small molecules but limiting the free entry of large macromolecules [106]. Therefore, the nuclear uptake of large proteins is mediated by nuclear localization signal (NLS) peptide in an active manner through sequence-specific recognition. Recently, the missile-like modifications have been developed on nanoparticles for targeting genes of interest into the nucleus for desirable results. In a recent development in this regard, peptides containing reiterated motifs of NLS in a cationic lipid were designed for gene transfer into MSCs with significantly increased transgene expression [102]. To this end, the exogenous genes might be constructed on a plasmid with site-specific recombinase (SSR) to realize stable gene expression. The SSR is expressed and recognizes unique recombination sites (RS) within the plasmid and the host chromosome; this enables recombination to take place and consequently integrates the plasmid DNA into the host genome [107].

6.9 Conclusions

Nanoparticles provide an exciting and novel tool for genetic engineering of stem cells for cell-based treatment of different diseases. Although significant progress has been made in designing nanostructured gene vehicles for improved transgene transfer without impacting their "stemness" characteristics, it is important to perform systematically designed, in-depth *in vitro* and *in vivo* studies to assess the cytotoxicity of the nanoparticles and to evaluate their potential influence on the nature of stem cells. When choosing appropriate nanoparticle-based tracking agents, it is important to consider their respective imaging requirements. Recently, superparamagnetic iron oxide nanoparticle-engineered MSCs labeled with lentiviral-GFP vector were transplanted to observe the *in vivo* fate of the cells posttransplantation in adult warmblood horses, which represented great step forward to clinic [108]. An in-depth understanding of nanoparticle-based transgene delivery and transport mechanisms will be helpful for future designing of safer and more efficient vehicles. For MSCs, understanding of their biology, improved protocols of isolation, purification and propagation that ensure their *in vitro* expansion without altering their phenotype and surface characteristics, and a preserved differentiation potential would be a

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prerequisite to guarantee that the therapeutic endpoints are achieved. Same is true for the development of optimized protocols to genetically engineer MSCs without compromising their intrinsic features as a multipotent stem cell with the ability to secrete bioactive mediators. Although we have specifically focused on MSCs, the nanostructured vehicles-based genetic manipulation protocols would be applicable to different cell types with slight modifications. Once protocols with nanoparticles have been optimized for *ex vivo* manipulation of MSCs, the same nanovehicles can also be used for *in vivo* targeting of the transgenes for specific delivery to the organs of interest. To this end, nanovehicles can be surface-modified with specific antibodies, or their fragments, peptides, etc., for their targeted delivery. On the diagnostic side, as the fluorescence nanoparticles mostly depend upon optical imaging systems, the sensitivity of the procedure becomes limited due to poor penetration of the fluorescence from the deeper tissues *in vivo* thus warranting further investigations to enhance the sensitivity of image detection.

6.10 References

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Seyed Mojtaba Hosseini, Seyyed Mohyeddin Ziaee and Parisa Tabeshmehr

7 Neural stem cells in regenerative medicine

Abstract: Neural stem cells (NSCs), reported for the first time in the early 90s, are stem cells of the central nervous system that are capable of self-renewal and tri-lineage differentiation to form neurons, oligodendrocytes and astrocytes. The existence of NSCs spans from the very early stages of embryonic development to adulthood in NSC niches located in specific regions of the nervous system such as the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in hippocampus. NSCs express astrocytic properties in all stages of life; they are distinct from regular astrocytes of the nervous system though. The delicately poised dynamics of the microenvironment of NSCs remains instrumental in determining their stemness characteristics such that they respond to minor changes and stimuli by undergoing significant changes in behavior and differentiation status. NSCs have been extensively characterized *in vitro* and in the experimental animal models for their postengraftment behavior, thus leading to their potential assessment as novel therapeutic modality for treatment of neurodegenerative disorders, i.e. Parkinson's disease and Alzheimer's disease, as well as pathologies like spinal cord injuries and strokes. Although the data generated from these studies has been encouraging, further in-depth mechanistic studies are required for regular use in the clinical settings.

7.1 Introduction

Neural stem cells (NSCs) are self-renewing cells with multilineage differentiation capacity and, in the presence of appropriate cues can undergo tri-lineage differentiation to generate the three main nervous system phenotypes: neurons, oligodendrocytes and astrocytes [1]. In the same context, the primary progenies of NSCs are the progenitors, which are also undifferentiated albeit with more restricted differentiation potential, i.e. the neuron-restricted progenitor cells (NRPs) that can only form functionally competent neurons [2] and the glial-restricted progenitor cells (GRPs) that only differentiate to produce astrocytes and oligodendrocytes via two other subclasses of the precursor cells [3]. Additionally, NSCs can also differentiate into melanocytes and specific mesenchymal cells through different pathways [4, 5]. Taken together, these cells in general, encompass the cellular basis of neurogenesis and are integral to the development of the cellular components of the peripheral nervous system (PNS) and the central nervous system (CNS). At the developmental level during embryogenesis, soon after germ-layer differentiation, the neural plate that later grows into the neural tube is responsible for development of the nervous system. The neuro-epithelial (NEP)

DOI 10.1515/9783110493764-007

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cells lining the central hollow canal of the neural tube are considered as the first generation of NSCs during CNS development, whereas another group of stem cells in the margins of the neural plate, i.e. the neural crest stem cells (NCSCs), are responsible for development of the PNS [6]. It is generally considered that the fate of neuronal cells in the nervous system becomes restricted during postnatal life. Nevertheless, a review of various studies recognizes an evidently more plastic role for NSCs in response to microenvironmental cues to produce diverse neuronal phenotypes [7]. These findings get supported by the widespread distribution and presence of NSCs across the mammalian species during their adult life [8]. These cells generally reside in the subventricular zone (SVZ) of lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. Upon isolation and culture *in vitro*, adult NSCs form neurospheres, the spherical aggregates of NSC populations each of which is formed by a single cell and retain their tri-lineage differentiation potential to develop into neurons, astrocytes and oligodendrocytes. Incidentally, based on neurosphere formation, neurosphere assay has been developed as the gold standard to identify the purity of an NSC-isolated cell culture, although it is not without its limitations [9]. Additionally, adult NSCs undergo neurogenic differentiation *in vivo* in the experimental studies that will be discussed at length in this chapter. The data originating from these experimental studies pertaining to the neurogenic potential of NSCs in the adult nervous system provide an alternative cell-based therapeutic modality for complex neurodegenerative disorders and CNS injuries. Mostly, these studies involve transplantation of *in vitro* expanded NSCs or stimulation of intrinsic NSCs to home-in and participate in the repair process. Although the data from these experimental studies have been encouraging, further systematic in-depth and mechanistic evaluation of NSCs is required for better understanding of the biology and reparability of these cells before they can be used for regular clinical applications.

7.2 Chronology of events involving NSCs

Following two back-to-back publications identifying neurogenic activity in adult rodent brains [10, 11], a later study reported similar observations in the dentate gyrus of the hippocampus and olfactory bulb in rodents [12, 13]. Likewise, proliferating cells were also detected in the SVZ of lateral ventricles in the adult mammalian brain [14]. The pioneering work of Reynolds and Weiss led to the isolation and *in vitro* culture of NSCs from adult murine brains that were responsive to epidermal growth factor (EGF) and showed the potential to adopt neurons and glial phenotypes [15]. Subsequently, these data were reproduced in the presence of basic fibroblast growth factor (bFGF) in place of EGF [16]. The migrating derivative cells of the spontaneously dividing SVZ cells were also reported to differentiate as interneurons in the olfactory bulb [17]. Subsequently, many more studies have reported on NSCs derived from

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various experimental animals to study their biology, *in vitro* characterization, differentiation capabilities and fate *in vivo* postengraftment in experimental animal models.

The first human NSCs isolation and purification was reported from embryonic human brain [18]. The cells were isolated and cultured *in vitro* to achieve stable NSC lines. Unlike murine NSCs, the cells were responsive to EGF and bFGF together to show extensive proliferation. During subcloning experiments, the cells were functionally stable with steady growth and showed neuronal differentiation potential for up to 2 years of observation. Their derivative glial and neural progenies survived post-transplantation into the lesioned striatum in rodents. Subsequently, the same cell lines were genetically perpetuated and transplanted into rodent brains where the cells got integrated non-disruptively within 1 week after transplantation [19]. The transplanted cells also migrated from the transplantation site, downregulated Nestin expression and started to express neural cell markers MAP2, β-tubulin III, neural nuclear antigen (NeuN) and non-specific enolase (NSE). Kukekov *et al.* reported the isolation and culture of progenitor cells from neurospheres obtained from *in vitro* culture of cells using surgical biopsy specimens from the periventricular subependymal zone and hippocampus of adult human brain. The progenitors showed neural and glial differentation potential under optimal culture conditions [20]. Similar results were also obtained using mitotically competent neuronal progenitors from dentate gyrus as well as the ventricular zone of adult human brain [21, 22]. Since these studies, different studies have reported optimized protocols for NSCs isolation, culture, characterization and assessment of *in vivo* behavior as discussed below.

7.3 NSCs in embryonic period

Once the germ layers have been specified, the outer ectoderm becomes distinct as neuro-ectoderm and non-neural ectoderm due to neural plate formation with simultaneous expression of neural markers including the *Sox* gene family [23]. The cells of neuro-ectoderm then proliferate to form the neural tube. The early neural tube has a single layer of NEPs lining a hollow canal that repeatedly proliferate via symmetric cell divisions to form a pseudo-stratified epithelium [24]. NEPs can form both neurons and glia in the embryo and are listed as first generation of NSCs. Nevertheless, temporo-spatial differences are observed in these cells in terms of proliferation and differentiation capacities, with more proliferation and differential potential during early stages as compared to their counterparts during the late stages of embryonic development [25–27]. Neural crest stem cells (NCSCs) constitute another cell population positioned between neural plate and non-neural ectoderm, where neural folds originate to form the neural crest and finally neural tube during the early stages of embryogenesis [28]. Likewise, NCSCs are self-renewing, multipotent cells with a high degree of migratory activity [29]. NCSCs delaminate from the neural crest, begin to migrate, start and undergo epithelial to mesenchymal transition that enables them

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to differentiate and adopt various cell phenotypes: melanocytes in the skin, neurons of the dorsal root ganglia of spinal nerves, the autonomic nervous system, Schwann cells, pericytes and smooth muscle cells of the vascular system including the major vessels of the heart, the adrenal medulla and leptomeninges and a part of the craniofacial mesenchyme [28]. These cells will not be further discussed as this is beyond the scope of this chapter. Lineage tracing and fate mapping studies have also shown the existence of–other radial glia cells (RGCs) in murine embryos on E9–10 during cortical neurogenesis [30–32]. Asymmetric division of the RGCs gives rise to a population of daughter RGCs or their end product are intermediate progenitor cells (IPC) though with restricted differentiation potential [31]. The restricted differentiation potential allows these cells to merely adopt neuronal (NRPs) or the glial (GRPs) lineages [31, 33, 34]. Studies in rodents have shown that NSCs accumulate as secondary proliferative layer above the ventricular zone or the SVZ [32, 35]. As the ventricular zone undergoes changes in structure and function, it gives up generation of IPCs, neurons and glia as it becomes the responsibility of the SVZ. The SVZ lining the lateral ventricles continues to provide a neurogenic niche after birth and throughout the adult life [36].

7.4 NSCs in adults

Cells with characteristics of neural progenitors, including relative immaturity and high proliferation rates, were reported in adult mammalian brains for the first time during mid-60s in a series of studies, mostly dominated by Altman's group [37–39]. Nevertheless, these reports were inconclusive due to insufficient evidence, until 1992 when a study performed by Reynolds *et al.* reproduced these observations and reported the existence of a cell population in the adult rodent brain with the potential to self-renew and the capability to generate the three main cell types in the nervous system: neurons, astrocytes and oligodendrocytes [15]. Since then, many studies have elucidated the phenotypes, biology, specific markers and behavioral patterns of the NSCs.

NSCs exist in two major neurogenic niches in adult mammalian brains: the SVZ of lateral ventricles [40] and the SGZ of the dentate gyrus in the hippocampus [41]. Since these earlier reports, substantial evidence has become available regarding the existence of these NSCs niches, however, the dynamic interaction between NSCs and the components of their microenvironment to maintain their undifferentiated status [42], both cellular and molecular components and the underlying molecular mechanisms involved therein, remain less well-understood and an area of immense interest for researchers. Moreover, it is now being considered that the status of this interaction between NSCs and their microenvironment is altered during pathological conditions [43].

The SVZ is a thin layer of dividing cells adjacent to the ependymal layer lining the ventricles and is separated from ventricular space by this ependymal cell layer. The SVZ is the largest germinal area in the adult mammalian brain [44] and shelters a population of RGC derived NSCs, the B cells [45]. The B cells of the SVZ have an apical

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process with a primary cilium that infiltrates the ependymal cells to contact the intraventricular cerebrospinal fluid CSF and extend end-feet that terminate at blood vessels [46]. Thus within the SVZ, NSCs have two distinct microenvironments: one apical in contact with the CSF and one basal perivascular niche; in addition to the intermediate SVZ niche, where bodies of the NSCs reside [44]. In the SGZ of the dentate gyrus, NSCs reside within the dentate gyrus hilus at the border of the inner granule cell layer [47]. These NSCs are also called type I progenitors or radial astrocytes. The type I progenitors give rise to granule neurons that form the granule cell layer through the IPCs [48].

Earlier studies with adult NSCs clearly showed their tri-lineage differentiation potential to adopt a phenotype of the three cell types in the nervous system as was evident from the simultaneous differentiation of neurospheres or the NSCs in the monolayer cell cultures into neurons, astrocytes and oligodendrocytes *in vitro* [15, 49] Fig. 7.4.1 and Fig. 7.4.2.

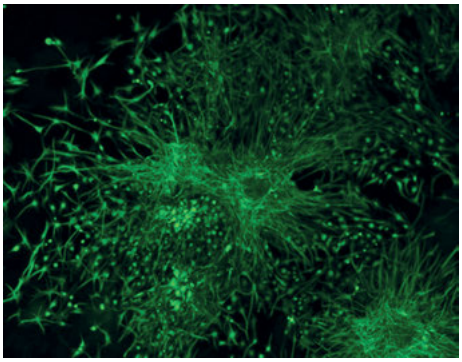

Fig. 7.4.1: Neural stem cells (NSCs) are capable of producing tri-neural lineages, especially the astrocyte. This image shows NSC-derived astrocytes in culture after fluorescence immunocytochemistry for the detection of glial fibrillary acidic protein (GFAP; green) using specific antibodies.

Fig. 7.4.2: Neuronal differentiation is a typical feature of neural stem cells (NSCs). This image shows NSC-derived neurons after fluorescence immunostaining for microtubule associated protein-2 (MAP-2) expression using specific antibodies.

Subsequent studies challenged this view as cell tracing studies demonstrated that NSCs of the adult hippocampus did not give rise to oligodendrocytes *in vivo*; they were only able to generate neurons and astrocytes [50]. In the case of SVZ NSCs, although population fate mapping studies demonstrated differentiation of these cells to both oligodendrocytes and neurons, the *in vivo* clonal studies performed recently demonstrated the generation of neural lineages alone from individual NSCs [51]. In another

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study performed *in vitro* on acutely isolated SVZ precursor cells, neurons or oligodendrocytes could be generated by these cells, but only one of them could be derived from each single cell [52]. There have been some explanations for these contradictory reports that necessitate further investigation and evaluation. One of these models claims that NSCs undergo gradual programming during embryonic development and finally during postnatal life that restrict their capacity to single lineage differentiation [32]. An alternative model believes in inherent tri-lineage potential of NSCs that somehow gets curtailed due to their interaction with the NSC niche over time. It is noteworthy that NSCs obtained from different regions during different stages of the life time have likeness in potency and progeny, these cells diverge from each other in their growth factor dependence, identification markers, proteomics, cell cycle time and differentiation potential [53–55]. Hence, the characteristics of these cells including their cellular identity, their microenvironment and markers of identification will be discussed in more detail.

7.5 Identification of the neural stem cells

After isolation of EGF-responsive NSCs in 1992, intensive efforts were made to identify cells with the capacity to form neurospheres *in vitro* Fig. 7.5.1.

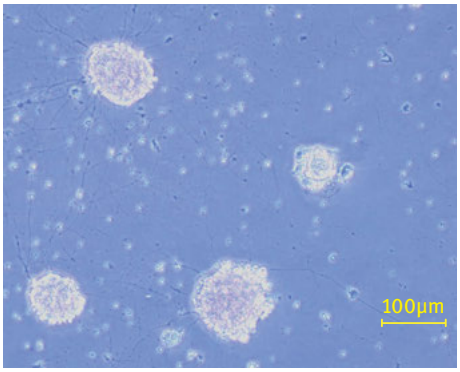

Fig. 7.5.1: Phase contrast images of the neural stem cells (NSCs) cultured under appropriate conditions *in vitro* leads to the formation of 3D neural stem cell aggregates or neurospheres (100–200 µm).

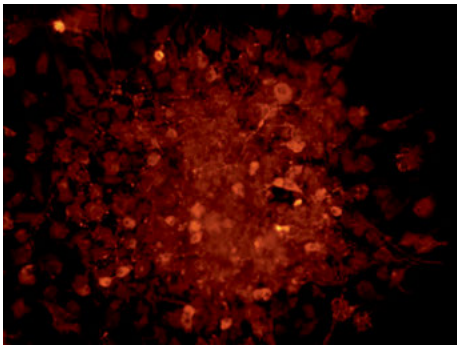

Fig. 7.5.2: In addition to tri-neural lineages production, there are some markers for neural stem cells including nestin. The image shows nestin^{+} (red) neural stem cells after fluorescence immunostaining for the detection of nestin using a specific antibody.

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These efforts resulted in the identification of two populations of NSCs residing in the subependyma of the SVZ of lateral ventricles of adult mice: the first one is a relatively quiescent population of cells, whereas the other consists of constitutively proliferating cells. By excluding the latter population from the culture using high doses of ^{3}H-thy, it was observed that the relatively quiescent population was responsible for neurosphere formation and thus, they were considered as the source of isolated NSCs [56]. It was also inferred that the constitutively proliferating cells were the progeny of the slowly dividing cells and contained precursors of neurons and glia. The idea that in the ependymal cells of the SVZ exists a slowly dividing cell line that generates more rapidly dividing "transit cells", which finally give rise to neurons and glia, was later endorsed by other studies [57]. These quiescent stem cells of the SVZ are called type B cells and the constitutively proliferating intermediate cells (also called transit amplifying precursors) produced by them are known as type C cells, which themselves generate another class of precursor cells termed neuroblasts or type A cells [45]. The neuroblasts finally migrate to the olfactory bulb and generate interneurons [17, 58]. A recent study has reported a different model that reflects an adaptive response of SVZ to maintain endogenous repair of the brain in response to traumatic injury that involves physical expansion through cell proliferation [59]. Two distinct transit amplifying cell populations Mash1^{+} and EGFR^{+} were identified that were responsive to the injury opposite to each other. Although both of them were similar in an uninjured brain, in response to injury, Mash1^{+} showed enhanced proliferation whereas EGFR^{+} cells become mitotically less active.

The fate of NSCs depends on whether they generate NRPs or GRPs but not both [52]. A noteworthy finding about neurogenic NSCs was that these cells display molecular and structural characteristics of astrocytes [45, 47, 60, 61]. In fact, in the embryonic brain, a particular cell type called radial glia from the astro-glial lineage, was first observed in songbirds to proliferate in the ventricular zone during neurogenesis [62]. The neurogenic role for radial glia was later confirmed in the mammalian embryonic brain. It is now generally accepted that these cells act as NSCs in the embryonic brain [63–65]. Lineage tracing analysis demonstrates that B cells, the persisting NSC population in the SVZ, are derivatives of embryonic radial glial cells and maintain astrocytic properties such as expression of specific markers, i.e. glial-fibrillary acidic protein (GFAP), glutamate aspartate transporter (GLAST) and brain lipid binding protein (BLBP) [66–69]. Radial glia cells were first detected when NEP cells extruded processes to pial surface and contact the pial basal lamina. With further growth of the CNS during the histogenesis, the radial processes elongate and thus remain in contact with both the pial basal lamina and the ventricular surface. As NSCs of the embryo, the radial glia undergoes cell division for two purposes: self-renewal and for the production of neurons, probably through their intermediate progeny. In the meantime, brain vasculature starts invading the brain parenchyma through the pial surface, carrying with them the pial basal lamina. The fate of radial glia is to give rise

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to both parenchymal astrocytes (non-stem cell) and neurogenic astrocytes in the SVZ (i.e. the B cells) and SGZ in the adult [70]. Thus, B cells of the SVZ as the descendants of radial glia retain contact with both the ventricular cavity by their apical processes and the basal lamina of the blood vessels by their end-feet on these vessels [46, 70, 71]. At the ventricular surface of the apical process, there is also a single primary cilium that plays a role in signaling cascades associated with NSCs survival and proliferation [46]. Contact with blood vessels through end-feet compensates for an inability of the B cells to reach the pial surface and promotes their maintenance as stem cells [46, 72, 73]. The B cells can exist either in a quiescent or activated state. Of these two states of existence, quiescent B cells apparently do not express Nestin, which is accepted as one of the most trusted NSC markers until now Fig. 7.5.2. Nonetheless, upon activation, B cells appear to express Nestin [74, 75]. The progeny of the activated B cells is an IPC-like transit amplifying precursor or C cell. The rapidly dividing cells undergo three cycles of symmetric cell division before giving rise to neuroblasts or A cells, which divide a couple of times more before reaching their destination [76]. Type A cells migrate through an interconnecting network of rostral migratory streams (RMS) to reach the olfactory bulb where they differentiate into different subtypes of interneurons [77]. However, this is not the only fate of B cells from the SVZ as lineage analysis suggests that they can also produce specific glial derivatives and thus can be considered as truly multipotent stem cells [32].

NSCs are also present in the SGZ of the dentate gyrus of the hippocampus. These NSCs are astrocyte-like cells and are known as radial astrocytes or type I progenitors. These cells are similar to the SVZ B cells in many ways; however, they have more limited developmental potential and their migratory route is also more restricted [36, 78, 79]. There are also non-stem cell astrocytes in the SGZ of dentate gyrus that can be distinguished from radial astrocytes on the basis of GFAP and Nestin expression, in addition to their long radial process [80, 81]. These radial astrocytes or type I progenitors undergo asymmetrical cell division and generate a progeny of more restricted precursors and is termed as type D or type II progenitor. The D cells exist in either immature or mature form; immature D cells are mitotically active whereas the mature D cells differentiate and produce a lineage of migrating neuroblasts, which migrate a short distance towards the granular layer of the dentate gyrus and differentiate into mature granule neurons. This is considered as the only fate of type I progenitors [24].

7.6 Regulation of NSCs

NSCs are always considered as quiescent cells located in specific regions of the CNS. Nonetheless, they have the ability to respond to stimuli such as neural activity, which is reflected in terms of their rapid proliferation and neurogenic differentiation. The loss of quiescence, if allowed unchecked, will result in depletion of the NSCs

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thus causing consequential impairment of neurogenesis in the long run [82–85]. The cellular activity involving quiescence-proliferation-differentiation is tightly regulated to ensure the proper functioning of the system. In response to extracellular stimuli, cascades of signaling pathways, transcription factors, epigenetics and metabolic changes initiate processes that affect gene expression or cell function, either directly or due to cross-talk with other regulators. The most important and well-studied pathways involved therein are Notch, BMP and Wnt signaling besides TGF-β. The activation of Notch and BMP pathways are known to promote quiescence of NSCs [82–84] while the Wnt signaling pathway promotes symmetric division of adult NSCs [86]. Activation of TGF-β also promotes quiescence and survival of NSCs [87]. Moreover, different transcription factors significantly regulate NSCs activity and serve as switches of NSCs activities. The foremost regulator of NSCs activities is SRY (sex determining region Y)-box 2 (Sox2), which requires active Notch signaling for expression and is found in abundance in adult NSCs of both the SVZ and SGZ [88]. So much is the involvement of Sox2 in the functioning of NSCs that Sox2 deletion causes depletion of NSCs [89]. Likewise, in combination with other transcription factors; it regulates the self-renewal mechanism and suppresses their differentiation [90]. More recent studies have shown that Sox2 causes transcriptional repression of many genes in NSCs [91]. Another important factor is achaete-scute homolog 1(Ascl1), which targets cell cycle regulators to activate a subset of adult NSCs that express AscII at a particular time [92]. AscII has been used besides *Brn2* (*Pou3F2*) *and Myt1l* transcription factors for complete reprogramming of fibroblasts to functionally competent NSCs [93]. Other transcription factors with significant influence on NSCs include TLX with a role in cell proliferation and maintenance of NSCs and repressor element-1 silencing transcription/neuron-restrictive silencer factor (REST/NRSF) prevents precocious neuronal differentiation thus helping in the maintenance of the NSC pool in the SGZ [89]. Many more transcription factors have been studied for their influence on regulation of NSCs; however, their tasks were studied individually. More recently, combinatorial transcription factor regulation of NSCs behavior at the whole genome level is being approached [94]. This is based on single-cell RNA-sequence datasets for adult NSCs in both the SVZ and SGZ [95, 96].

Regulation of NSCs by modifications in epigenetics is a field that has attracted attentions recently. The consequences of a balance between methylation and demethylation was shown in a number of studies, for example, the studies performed on DNA methyltransferase DNMT3a [97], TET-GADD45 pathway [98–100] and methyl-CpG binding proteins [101–103]. Additionally, the process of histone modifications also have a role in the epigenetic regulation of NSCs [104, 105]. Other intracellular regulators of epigenetics are non-coding RNAs including mi-RNAs and long non-coding RANs (lnc-RNAs), which regulate adult neurogenesis [106] either by negative effects (such as Six3os and Dlx1as) [107] or by positive effects (such as Pnky) [108]. A succession of studies have reported an association between the behavior of stem

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cells and their energy. NSCs employ glycolytic mechanisms more than neurons and thus are more resistant to hypoxia due to their less frequent use of oxidative mechanisms [109, 110]. Indeed, the activation of quiescent NSCs was accompanied by the downregulation of glycolysis and up-regulation of mitochondrial oxidative mechanisms in both the SVZ and SGZ [95, 96]. In addition to the regulatory effects of these metabolic states, it was demonstrated that physiological, pathological and pharmacological stimuli could also affect neurogenesis in adults [111].

7.7 Neurogenic niche microenvironment

As discussed earlier, neurogenesis is restricted to the SVZ and SGZ in the adult brain. It is imperative that NSCs reside in an optimal microenvironment or the neurogenic niche to maintain their stemness characteristics of self-renewal and multipotency [112]. Understanding the dynamics of NSCs microenvironment/niche at both the molecular and cellular levels is essential to comprehending the regulation and behavior of NSCs. The cellular components of a neurogenic niche include many different lineages of cells including the NSCs, their derivative progenies and many of the differentiated cells. In the SVZ, most NSCs (B cells) are located in the lateral walls of the lateral ventricles, which constitutes a highly vascularized region [113]. These cells are known to have long basolateral processes that terminate on blood vessels (vascular end-feet) [46]. Likewise, in the SGZ of the hippocampus, NSCs are gathered close to the tips of capillaries [49] and therefore, these blood vessels and the contacts between NSCs and ECs are crucial components of the microenvironment. The permeable contacts allow the bioactive soluble molecules in the blood vessels to be more accessible to the NSCs. Thus, these bioactive molecules have a crucial role in the proliferation and positioning of NSCs *in vivo* [72, 73]. More so, the bioactive molecules secreted by the ECs are the significant determinants of the differentiation status and maintenance of NSCs [114, 115]. On the contrary, the vascular endothelial growth factor (VEGF) that induces both angiogenesis and neurogenesis and the pigment epithelium derived factor (PEDF), are released from ECs with significant influence on the self-renewal properties of NSCs [116, 117]. The bFGF-induced Flk1 and VEGF interact to promote NSCs proliferation [118]. The hormone erythropoietin (EPO) and the neurotrophin NT3 are also secreted from the same cells and regulate the induction of neurogenesis [119, 120].

The ependymal cells that line the lateral ventricles as a monolayer and are in direct contact with NSCs in the SVZ represent another component of the niche microenvironment. The ependymal cells affect the self-renewal of NSCs through the production of PEDF and promote neurogenesis by releasing Noggin, a polypeptide that blocks gliogenesis [117, 121]. Furthermore, the apical processes of NSCs that penetrate the ependymal cell layer to contact the CSF [46] provide NSCs with signal

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molecules running through the CSF. The cellular sources of some of the most important niche signals that regulate neurogenesis, i.e. the Notch, Shh and Wnt pathways, have not yet been identified completely. The niche vasculature and the CSF are being implied as the possible source of these signals in addition to niche astrocytes [35]. *In vitro* co-culture involving astrocytes from neurogenic and non-neurogenic niches of the brain with NSCs demonstrated that neurogenic niche astrocytes induced neuronal differentiation of NSCs while the astrocytes from other regions of the brain promoted differentiation of NSCs into cell types in the culture other than neurogenesis. These data suggest that niche astrocytes secrete some of the signaling molecules that determine the differentiation fate of NSCs [122–124]. These data were supported by the *in vitro* studies using A cells of the SVZ and type 2 cells of the SGZ, isolated from their respective niches. Their co-culture with NSCs enhanced the proliferation rate of NSCs that was suggestive of a feedback effect from the niche cells [45, 47]. Another class of niche cells having possible regulatory effects on the NSCs is neurons through their release of neurotransmitters such as GABA which inhibits self-renewal and thus keeps NSCs of the hippocampus in a quiescent state [125].

7.8 NSCs in cell-based therapy

The pathological conditions of the CNS are responsible for a high percentage of morbidity and mortality such that the global economic burden is projected to increase over 6 trillion USD in 2030 [126]. Such a gigantic increase in the prevalence of progressive neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD) and neural damage such as spinal cord injury (SCI) and brain stroke necessitates a search for advanced therapeutic modalities. Cell therapy offers a novel alternative treatment approach to treat these pathologies. Cells from different origins have been used including adult tissue derived stem/progenitor cells and pluripotent stem cells or their derivatives. Given their origin from the nervous tissue, NSCs are considered as the most appropriate cell type for cell-based therapies involving nervous tissue. The possible sources of NSCs for cell therapy include adult nervous system tissue, the developing neural plate, the developing nervous system, ESC-derived NSCs and iPSC-derived NSCs [55].

7.8.1 NSCs for cell-based therapy of Parkinson's disease

The clinical motor dysfunction in PD is primarily the consequence of progressive degeneration of dopaminergic neurons in the substantia nigra of the nigrostriatal pathway. The degeneration of this tract provokes a depletion of dopamine in the striatum where it is required for normal motor function. Despite the availability of effective pharmacological options of intervention for symptomatic relief, there

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is no cure addressing the root cause of PD at present and most attempts to slow neuronal cell loss and degeneration during the disease process have met with little success.

According to earlier studies, neural precursors in the rostral migratory stream (RMS) and SVZ "sense" the dopamine deficiency since increased numbers of new dopaminergic neurons were found in the OB following systemic administration of a dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [127] or 6-hydroxydopamine (6-OHDA) [128]. These data suggest that the OB neurogenesis itself may serve as an inherent mechanism to restore local dopamine neural circuits and therefore, the neural precursors in RMS and the SVZ might provide an ideal sources of adult NSCs for cell-based therapies for PD patients [129].

7.8.2 NSCs for cell-based therapy of AD

AD is one of the most prevalent age-related neurodegenerative disorders characterized by multiple pathologies that afflict several neuronal subtypes across multiple brain regions. Mainly, AD is characterized by the accumulation of two hallmark lesions: plaques and neurofibrillary tangles (NFTs), which are accompanied by gliosis and widespread neuronal and synaptic loss. These molecular and cellular changes lead to progressive loss of memory and cognitive function. Stem cells are being actively studied for their potential to replace dead or diseased cells [130]. In the case of AD, multiple neuronal subtypes are affected within several brain regions. Conceivably for this reason, none of the published studies have as yet explored the potential utility of stem-cell-based therapies for AD. In continuation of early reports that showed significant survival and neurogenic and astrogenic differentiation of NSCs transplanted into the brains of aging rats [131], various research groups have elucidated the beneficial effects of stem-cell-based therapy in experimental animal models of AD. Xuan *et al.* reported that NSCs derived from the hippocampus and their derivative glial cells successfully differentiated into cholinergic neurons post-transplantation in the transected basal forebrain of the rats with an experimental model of AD [132]. While elucidating the underlying mechanism of NSCs transplantation, the bystander effect was proposed as an alternative mechanism that underlies the beneficial effects of treatment with NSCs [133]. For example, transplantation of NSCS can improve function by providing missing or defective enzymes or through modulation of the inflammatory response [134]. Alternatively, NSCs can preserve endogenous neuronal functions by providing trophic support [135]. Likewise, treatment with NSCs produces a marked increase in hippocampal synaptic density that is mediated through increased brain-derived neurotrophic factor (BDNF), which plays a central role in the synaptic remodeling associated with the memory of the animal [136, 137]. Although these data generated significant interest in the research for cell-based therapy with NSCs, isolation and purification of NSCs and in sufficients number required for

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transplantation remains a challenging task. In order to address these availability issues, neurospheres were derived from ESCs for use in the murine experimental model of AD. The transplanted cells differentiated into choline acetyltransferase positive and a few serotonin-positive neurons in and around the area of cell graft [138]. With the aim of enhancing the therapeutic benefits of cell-based-therapy, NSCs have been genetically modified to overexpress choline acetyl-transferase prior to transplantation. A direct comparison of the genetically modified human NSCs with native human NSCs (without genetic manipulation) showed that the former were superior in terms of therapeutic benefits as compared to the native NSCs postengraftment in a rat model of AD that was produced by application of ethylcholine and aziridinium (AF64A; specific denaturant of cholinergic neurons) [139]. These results distinctly showed the advantages of NSC-based cell therapy for the treatment of AD. A recently published systematic review and meta-analysis of 58 (23 of these using NSCs) eligible controlled experimental animal studies in AD models showed that stem cell transplantation helped in significant recovery of learning abilities and memory [140]. The systematic review also underscored the importance of genetic modification of the cells to enhance their therapeutic benefits.

7.8.3 NSCs for cell-based therapy for spinal cord injury

Spinal cord injury (SCI) is a serious medical problem in advanced societies. Worldwide, an estimated 5.3 million people are living with a SCI, which yields an estimated global incidence of 250,000–500,000 new SCI cases per year, and there is no effective therapy at hand as yet [141, 142]. Invariably, SCI is characterized by the extensive loss of neurons and axonal degeneration at the site of the injured spinal cord. Stem cells in general and NSCs in particular have the potential to either regenerate lost neurons or release trophic mediators that are neuroprotective and at the same time would create an environment conducive for the intrinsic repair mechanisms to replace the injured/lost neurons. While various types of stem cells have been used to assess their capability to repair SCI, our focus here is on the use of NSCs for the treatment SCI. Implanted NSCs labeled genetically with green fluorescence protein (GFP) by lentiviral vector transduction showed that the transplanted NSCs were proficient to substitute for the lost neurons in the injured spinal cord regions and successfully formed functional connections with axons to restore the conduction pathway in SCI. Transplanted NSCs produced multiple extracellular matrices to fill-in the nonfunctional spaces left in the injured tissues and provided supportive mass for axon regeneration. Subsequent to cell transplantation in the injured spinal cord areas, NSCs differentiated into neurons with different functions that bridged the broken spinal cord and restored the nerve conduction pathway [143]. These data also showed the possibility of genetically modifying the NSCs to enhance their paracrine activity and release of neurotropic factors. Neurons and glial cells differentiated from NSCs secrete multiple neurotrophic factors that improve the microenvironment of the

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injured tissues and initiate expression of regeneration-related genes, thus triggering the regeneration of new axons [144, 145]. Another possible mechanism by which cell-based therapy with NSCs led to improved prognosis in the SCI is by preventing the broken axons of host neurons from retrograding and necrosis and enables the generation of new myelin sheaths in the existent as well as the newly formed nerve fibers thus maintaining the integrity of nerve fiber functions [146, 147]. In an attempt to enhance donor NSCs survival and differentiation, cell transplantation was combined with intracaudal vein delivery of edaravone, a radical scavenger. The transplanted cells showed improved survival and rate of differentiation into neurons in the presence of edaravone [147]. Similarly, NSCs combined with EPO promoted axonal regeneration that reached the damaged zone [148]. EPO strongly inhibited neuronal apoptosis and curtailed inflammatory activity. With these beneficial effects of EPO administration, NSCs were genetically modified for human *EPO* gene expression that significantly decreased *Caspase-3* mRNA and protein expression while increasing *bcl-2* mRNA and protein expression. The human *EPO* mPRNA-modified NSCs also decreased apoptosis, promoted spinal cord repair, increased the numbers of CM-Dil-positive cells and HRP-positive nerve fibers and contributed to the recovery of hind-limb motor function in SCI [149]. Transplantation of NSCs into the acute and subacute SCI has been shown to generate neurons and glial cells and form a functional relay to connect the lesioned sensory pathway of dorsal column axons [150] and improve function following a contusion injury [151]. However, in case of chronic SCI, a combined treatment using NPCs and neurotrophic support (chondroitinase and neutrophins) showed significant improvement in bladder function [152].

7.8.4 NSCs for cell-based therapy of stroke

Stroke is the second major cause of death in industrial countries and the most significant cause of adult disability around the world, with more than 15 million people suffering from stroke every year [153]. More than 80% of stroke cases are preventable by management of the risk factors, and pharmacological intervention with tissue plasminogen activator (tPA) is effective, however, there is a narrow time-window of only 3–4 hours for a hopeful prognosis. Alternative methods for the treatment of stroke are warranted and researchers are seeking stem cell-based therapy for management of the stroke patients as an alternative treatment modality to secure better neurological outcome. Given their neuronal differentiation potential and biologically useful paracrine activity, both mesenchymal stem cells (MSCs) and NSCs might be useful for preventing complications emanating from brain ischemia, reduce brain lesions via antiapoptotic actions and restore neurological functions [154]. Recently, a novel approach of combination cell therapy has been proposed. The strategy involves simultaneous administration of MSCs either with NSCs [155] or their derivative differentiated cells obtained by adding fetal bovine serum (FBS) to NSCs culture medium followed by removal of astrocytes in animal stroke models showed promise [156].

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An increase in the prevalence of neurodegenerative diseases and neural damage has motivated scientists to search for various approaches such as cell therapy to improve neurogenesis techniques [157]. Data emanating from multiple investigations have confirmed the presence of resident stem cells in breast milk [158], dental pulp and its polyps and many other tissues and organs [159]. Surface marker expression analysis of these studies report that nearly 10%–15% of the cells isolated from fresh breast milk expressed MSC markers and upon *in vitro* culture, a significant increase in the MSC specific marker expressing cells were observed due to their higher proliferative capacity. The presence of $Nestin^+$ sub-population (a neuroectoderm marker) has also been reported in the breast-milk-derived cells, albeit at a very low frequency [160, 161]. The presence of $Nestin^+$ cells indicated that the breast-milk-derived stem cells can be appropriate candidates for differentiation towards neurons or neuroglia. Breast-milk-derived stem cells also express ESC specific markers, i.e. Nanog, Oct4, Sox2, SSEA4, and TRA1–60/81 in addition to the expression of some MSC markers as previously shown [162]. ESCs have a great potential to be differentiated toward neural cell lineages [163, 164] besides spontaneously giving rise to neural progenitor cells [165, 166]. The stability in electrical phenotype of stem-cell-derived neurons has been shown previously revealing that the differentiated cells may be functional [167].

7.9 Conclusion

NSCs are cells of the nervous system with stemness properties that exist throughout the embryonic period to adult life. The existence of NSCs in embryos starts with the appearance of NEP cells lining the neural tube and later in embryogenesis, they are found all along the fetal nervous system in the form of radial glia. During adult life, only specific regions of the brain continue to host NSCs. These regions include the SVZ of the lateral ventricles where relatively quiescent B cells reside; and the SGZ of the dentate gyrus in the hippocampus that hosts type 1 cells or radial astrocytes. The behavior and fate of NSCs is regulated by their niche microenvironments. Many factors such as cascades of signaling pathways, transcription factors, epigenetics and metabolic changes ensure the proper functioning of the system. These advances about properties of NSCs have shed light upon novel cell-based therapies for conditions of the nervous system in both animal models and human patients (Tabs. 7.9.1 and 7.9.2). Transplantation of NSCs, either alone or in combination with other stem cells, has shown promise in neurodegenerative diseases, i.e. PD and AD, and other CNS conditions including SCI and stroke. These positive effects are now attributed to the potential of NSCs to prevent further cell death and secondary injury, and support the intrinsic neurogenesis process rather than their ability to replace lost neurons and axons. Nevertheless, further studies are required to ensure the efficacy and safety of these cells in different CNS conditions, if they are to be used as clinical strategies in the future.

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Tab. 7.9.1: Experimental animal studies using NSCs and their derivative progenitor cells.

	Animal model	Cell type	Route of delivery	Important findings	Teratogenicity	Reference
1	Rats	SVZ-NSCs	Grafting by direct injection into the hippocampus	No difference between young and old recipients. Establishing new neurogenic niches in non-neurogenic regions	N/A	[168]
2	Immune-deficient ATN rat models of TBI	hNSCs	Direct injection into ant. and post. hippocampus and corpus callosum	9%–25% of cells survived for 5 months with tri-lineage differentiation. Cognitive recovery without change in volume of the lesion.	N/A	[169]
3	Mice	iPSCs, iNSCs, ESCs NSCs, MSCs	Direct stereotactic injection into brain tissue	Both iPSCs and ESCs were tumorigenic and immunogenic, NSCs, iNSCs and MSCs showed no tumorigenicity or immune rejection	Yes for iPSC & ESC.	[170]
4	Rats	NSCs MSCs	Direct stereotactic injection into LV	Both NSCs and MSCs protected CNS against ischemia.	N/A	[154]
5	AD rat models	ChAT expressing hNSCs	ICVI	Improved learning & memory; elevation of Ach in CSF, migration and differentiation of hNSCs	N/A	[171]
6	Rat models of stroke (transient MCAO)	hNSCs	MRI targeted injection into peri-infarct vs. ICVI	Cell survival and behavior dysfunctions recovered after IPI, but not any other group.	N/A	[172]
7	Parkinsonian primates	hNSCs	Stereotactic injection into SN and CN	Survival, migration and differentiation of hNSCs along with functional improvement of animals	N/A	[135]
8	Mouse model of ICH stroke	Immortalized hNSCs	Intracerebral injection	NSCs were found in perihematomal regions, differentiated to neurons and astrocytes, improved function with NSCs transplantation.	N/A	[173]

Tab. 7.9.1 (continued)

	Animal model	Cell type	Route of delivery	Important findings	Teratogenicity	Reference
9	Rat model of spinal cord injury	NPC	Intraspinal cord	NPCs + SAP increases NPCs survival, reduced astrogliosis and deposition, and synaptic connectivity increased by NPCs.	N/A	[174]
10	Rat model of SCI	iPSC-derived NSCs	Intraspinal	A late decline in functional recovery	Yes	[175]

Ach= acetyl-choline; AD= Alzheimer's disease; Ant.= anterior; ATN= athymic nude; ChAT= choline acetyl-transferase; CN= caudate nuclei; CSF= cerebrospinal fluid; CSP= chondroitin sulfate proteoglycan; ESCs= embryonic stem cells; hNSCs= human neural stem cells; ICVI: intra-cerebroventricular injection; ICH= intra-cerebral hemorrhage; IPI= intra-parenchymal injection; iNSCs= induced neural stem cells; iPSC= induced pluripotent stem cells; LV= lateral ventricles; MCAO= middle cerebral artery occlusion; MRI= magnetic resonance imaging; MSCs= mesenchymal stem cells; N/A= not assigned; NPC= neural progenitor cell; NSCs= neural stem cells; Post.= posterior; SAP= self-assembling peptide; SCI= spinal cord injury; SN= substantia nigra; SVZ= subventricular zone; TBI= traumatic brain injury.

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Tab. 7.9.2: Clinical studies involving NSCs for different pathological disorders.

Clinicaltrials.gov Identifier	Location and Investigator	Title of the study	Purpose	Start Date	Completion date	Cells involved
NCT01640067	Angelo L Vescovi Terni, Italy	hNSCs transplantation in ALS	Safety and tolerability of expanded fetal hNSCs. Safety and tolerability of a micro surgery fetal hNSCs transplantation model. Change in patient's quality of life. Assessment of human NSCs on the disability of ALS and on their mortality (all causes).	December 2011	December 2015	hNSCs
NCT01172964	Jana Portnow California, USA	Feasibility study of oral 5-FC and NSCs expressing E.coli CD for RHGG treatment.	Genetically-modified NSCs and 5-FC in patients undergoing surgery for RHGG.	August 2010	February 2015	Genetically-modified NSCs
NCT00581113	Mitchell S. Anscher, Virginia, USA	NSCs preserving brain radiation therapy & stereotactic radiosurgery in patients with 1–6 brain metastases	Patients with 1–6 intra-parenchymal brain metastases from various primary histology (except for melanoma), stereotactic radiosurgery (administered upfront or concurrently) or complete surgical resection with NSCs-preserving WBRT results in improved neurocognitive profile over standard WBRT.	March 2007	June 2009	hNSCs
NCT02163876	Stephen Huhn, USA	hNSCs transplantation in CSCI	Safety and efficacy of hNSCs in patients with traumatic CSCI.	October 2014	May 2016	hNSCs
NCT01725880	Stephen Huhn, Zurich, Switzerland	Long-term follow-up of transplanted hNSCs in SCI patients	To determine the long-term safety and preliminary efficacy of intramedullary transplantation of hNSCs for thoracic SCI.	November 2012	May 2016	hNSCs

Tab. 7.9.2 (continued)

Clinicaltrials.gov Identifier	Location and Investigator	Title of the study	Purpose	Start Date	Completion date	Cells involved
NCT01251913	Katherine E Warren, National Cancer Institute	A pilot study of inpatient hospice with procurement of brain tumor tissue on expiration.	Development of tumor and brain stem cell lines for research that will also help identify new targets for treatment.	November 2010	April 2015	hNSCs
NCT01321333	Stephen Huhn, Zurich, Switzerland	Study of hNSCs in patients with thoracic SCI	This study will evaluate the effect of a single transplantation of hNSCs into the thoracic SCI.	March 2011	April 2015	hNSCs
NCT01632527	Stephen Huhn, USA	Study of hNSCs in AMD	Phase I/II study for unilateral subretinal transplantation of hNSCs	June 2012	June 2015	hNSCs
NCT00397423	Dongsheng Fan, China, Beijing	G-CSF treatment for ALS: a RCT for clinical response	Effectiveness of G-CSF in patients with ALS	December 2006	August 2007	Adult NSCs
NCT01343511	Yongtao Lv Shandong, China	Safety and efficacy of stem cell therapy in patients With autism	Neural hypoperfusion and immune deregulation are the two key pathologies associated with Autism. Safety and efficacy of hUC-MSCs and hCB-MNCs transplantation ASD.	March 2009	May 2011	hCB-MNCs and hUC-MSCs

AMD= age-related macular degeneration; ALS= amyotrophic lateral sclerosis; hCB= cord blood; CD= cytosine deaminase; CSCI= cervical spinal cord injury; 5-FC= 5-fluorocytosine; hNSCs= human neural stem cells; MNC= mononuclear cells; MSCs= mesenchymal stem cells; RHGG= recurrent high-grade gliomas; hUC= umbilical cord; WBRT= whole-brain radiotherapy.

***Source**: https://clinicaltrials.gov/ct2/results?Term=Induced+pluripotent+stem+cells&Search=Search*

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Acknowledgment: We would like to express our gratitude to Mr. Pahlavan, our first professor, as well as Dr. Namavar, Prof. Talaei, Dr. Azari, Prof. Imanieh and Prof. Baharvand, who helped us during the writing of this chapter, and finally Prof. Kh Husnain Haider, without whose guidance and help this work would not have been possible.

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Ye Lei and Khawaja Husnain Haider

8 "Paracrining" the heart with stem cells

Abstract: Cell-based therapy is being investigated in preclinical and clinical settings with encouraging results. The beneficial effects of cell-based therapy for the treatment of infarcted hearts are believed to be mediated partially by paracrine mechanism. The paracrine mediators released by donor cells have biological functions to modulate inflammation, inhibit cardiomyocyte apoptosis, promote cytoprotection, proliferation, angiogenesis, reduce fibrosis and enhance myogenic repair of the diseased heart. However, the understanding of the mechanism underlying the observed therapeutic effects exerted by paracrine mediators is still limited. Here, we summarize the paracrine factors and associated mechanisms during cell-based therapy of infarcted hearts based on available data with a special focus on the cell types that, after extensive characterization *in vitro* and during preclinical studies, have progressed to clinical evaluation in the human patients.

8.1 Introduction

Cardiovascular pathologies inflict huge social and economic burdens on healthcare systems worldwide. Coronary heart disease alone was responsible for approximately one in every six deaths in the United States in 2010 [1]. According to the latest figures released by the American Heart Association, the total cost for management of cardiovascular-related pathologies was $656 billion in the United States alone and is expected to double by 2030 [2]. With ever increasing numbers of patients suffering morbidity and mortality caused by coronary artery disease, new therapeutic approaches are urgently required for these patients. Subsequent to a myocardial infarction (MI) episode, regional blood flow to the myocardium is compromised due to coronary artery occlusion that consequently results in irreversible cardiomyocyte apoptosis and necrosis. The heart has always been considered a postmitotic organ with limited inherent capacity to regenerate and replenish any significant loss of cardiac tissue in the event of myocyte injury during postnatal life. Henceforth, restoration of regional coronary blood flow via generation of coronary vasculature and supplementation of the damaged myocardium with functional cardiomyocytes remains a major challenge for addressing the root cause of the problem. In this regard, cell transplantation offers a biological therapeutic approach to complement the pharmacological and surgical options for patients with ischemic cardiomyopathies with the scope of addressing both of the aforementioned issues. The safety profile of cell-based therapy has been generally favorable both from preclinical [3] and clinical perspectives [4–6]. Although the efficacy of cell-based therapy has been well-documented in preclinical studies, the clinical outcome has been inconsistent and overall modest during the clinical trials, irrespective of the cell types used during the trials [7, 8].

DOI 10.1515/9783110493764-008

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The beneficial effects of cell transplantation therapy are considered as multifactorial, extending from differentiation of donor stem cells into functionally competent cardiomyocytes to successful integration with the host myocytes, preserved contractile function and improved regional blood flow in the ischemic heart [9]. The much trumpeted common perception that transplanted cells undergo cardiac differentiation postengraftment has been challenged [10]. This controversy was initiated by the publication of studies that failed to provide evidence of neomyogenesis in the infarcted myocardium despite improvement in cardiac function [11–14]. These studies have not only questioned the cardiac differentiation capacity of stem cells, but there is also skepticism about whether the transplanted cells survive long enough to make any significant impact in terms of preserved cardiac function. Hence, the beneficial effects observed after stem cell transplantation have been partially attributed to the paracrine activity of the transplanted cells that secrete a plethora of bioactive molecules at the site of the cell graft [15]. Donor cells from various sources including embryonic and adult tissues and more recently, induced pluripotent stem cells (iPSCs) have been used in preclinical experimental animal models and some of these have been tried in patients to repair injured hearts, including skeletal myoblasts (SkMs) [16–18], endothelial progenitor cells (EPCs) [19, 20], bone marrow (BM)-derived cells (BMDCs) [20, 21], mesenchymal stem cells (MSCs) [22–25], embryonic stem cells (ESCs) [26–28], iPSCs [29, 30] and cardiac stem cells (CSCs) [31, 32, 33]. This chapter emphasizes the findings from the available data on the paracrine behavior of different stem cells with a special focus on the paracrine mechanism of cell-based therapy for myocardial repair. It is important to note that understanding of the intricacies of the molecular mechanisms underlying the observed therapeutic benefits emanating from stem cell transplantation remain less well understood in general and regarding the paracrine activity of stem cells in particular.

8.2 Overview of cell transplantation for cardiac repair

During intrauterine and the early part of postnatal life, cardiomyocytes are mitotically active and proliferate like any other cell in the body [34–36]. Subsequently, cardiomyocytes withdraw from the cell cycle, thus rendering the mammalian heart mitotically stagnant and incapable of repairing itself in the event of injury [34, 35, 36]. Moreover, myocyte hypertrophy together with growth of the resident non-myocytes solely contributes towards further development of the heart throughout postnatal life in humans [37]. Although various research groups have successfully demonstrated mitotic activity in adult cardiomyocytes in response to ischemic injury [38, 39], little convincing experimental evidence is at hand, either in experimental animals or in humans, to show that such a low level of mitotic activity has any meaningful role in the injured myocardium. The existence and purification of the resident CSCs have also been extensively demonstrated in the mammalian heart, including the human

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heart [32, 40]. Characterization of the resident CSCs has demonstrated the existence of distinct myogenic as well as vasculogenic lineages with the potential to adopt myogenic and endothelial phenotypes [41]. Again, there is little evidence that CSCs could sufficiently contribute to replenish cardiomyocyte loss and prevent scar formation in the event of large infarcts. Given the inadequacy of regenerative ability of the human heart, a more realistic approach has been adopted to regenerate the damaged myocardium through exogenous cell transplantation.

The feasibility of cellular cardiomyoplasty was first demonstrated in early 1990s when mouse fetal cardiomyocytes survived and integrated with the host myocytes postengraftment in the mouse heart [42]. These data paved the way for scores of later studies that reported the feasibility of the approach [43–45] in terms of neomyogenesis, limited scar expansion, and globally preserved heart function. After extensive characterization *in vitro* and experimental animal model studies for *in vivo* behavior and reparability [46, 47], SkMs were the first to enter the clinical phase when Phillip Menasche performed the first-in-man safety assessment studies [18]. The autologous cell transplantation was performed as an adjunct to coronary artery bypass surgery (CABG). Histological studies on the recipient heart nearly 17 months after transplantation revealed islands of myocytes in the infarcted myocardium that were attributed to milieu-dependent myogenic differentiation of the transplanted cells [48]. BM stem cells (BMSC) for cardiac repair were first reported by Tomita *et al.* [49], followed by a pioneering study by Orlic *et al.* [50] which showed that BMSCs differentiated into cardiac-like muscle cells *in vitro* and *in vivo* in ventricular scar tissue and improved global myocardial function. Subsequent to encouraging results in scores of translational studies, both SkMs and BMSCs received the most attention for use from a clinical perspective. Whereas the earlier clinical studies used unfractionated BM stem/progenitor cells, which made it difficult to attribute the beneficial effects of cell therapy to a particular subpopulation of BM cells, recent clinical trials have focused more on the fractionation and purification of cells for engraftment [51]. The resident CPCs are Lin^{-}c-kit^{+} self-renewing, clonogenic and multipotent cells with the capability to form myocytes, smooth muscle cells (SMCs) as well as endothelial cells (ECs). EPCs or angioblasts were first reported for their angiogenic potential by Kocher *et al.* [52], showing that BM derived angioblasts revascularized infarcted myocardium. Gojo *et al.* [53] demonstrated that BM-derived $CD34^{low}$ c-Kit^{+} $CD140a^{+}$ Sca-1^{high} cells were able to differentiate into cardiomyocytes, ECs and SMCs after direct injection into adult hearts. Matsuura *et al.* [54] reported Sca-1^{+} cells in adult mouse hearts had some of the features of stem cells and could differentiate into cardiomyocyte-like cells. Other cell types, including iPSCs [55–59], umbilical cord and cord-blood-derived stem cells [60], very small embryonic/epiblast-like stem cells (VSELs) [61, 62] and adipose-derived stromal cells [63, 64], have been studied to assess their feasibility and efficacy for cardiac repair. Besides direct differentiation, these cells are being scrutinized to support the emerging paracrine paradigm for the release of paracrine factors that promote neovascularization, recruit endogenous cardiac progenitor cells by sending well defined chemical cues, inhibit apoptosis and enhance cardiac cell viability.

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8.3 Cellular cardiomyoplasty and paracrine factors

8.3.1 Skeletal myoblasts

Given that myogenesis in skeletal muscle involves a coordinated involvement of various processes including extracellular remodeling, cell proliferation, cell migration and myogenic differentiation, these processes are delicately balanced and intricately regulated by the myogenic sceretome, part of which is secreted by SkMs. A study on the secreted myokines profile of a mouse-derived C2C12 cell line cultured under serum-free conditions showed the presence of more than 80 non-redundant proteins including matrix metalloproteinase-2 (MMP-2), a secreted protein acidic and rich in cysteine (SPARC) and cystatin-C relevant to TGF-β [65]. Further analysis of the secretome components revealed that its composition is dynamic and changes with the requirement of each component with regard to its need at different stages during the process of myogenesis [66, 67]. In addition to the release of myokines, SkMs also release exosomes as a means of intercellular crosstalk during the process of differentiation [68]. Analysis of the exosomes showed that their composition of contents varied between differentiated and undifferentiated SkMs. These data suggest that SkMs show significant paracrine activity that is dynamic in nature and its composition changes in response to signals from their microenvironment.

When taken from the biological conditions, *in-vitro*-cultured SkMs continue their paracrine activity and secrete a plethora of bioactive molecules in the culture medium [69–71]. Analysis of the cell culture supernatant recognized the presence of growth factors relevant to angiogenesis, i.e. VEGF, placenta growth factor [PIGF], angiogenin, angiopoietin-1 (Ang-1), HGF-1 and platelet-derived growth factor (PDGF), matrix metalloproteinases 2, 9 and 10 (MMP2, MMP9 and MMP10) and their biological inhibitors, that is, tissue inhibitors of metalloproteinase, (TIMPs) [71]. Quantitative analysis of the cell culture supernatant of the C2C12 cell line cultured *in vitro* identified more than 635 secreted proteins including 35 growth factors, 40 cytokines and 36 metallopeptidases during differentiation [67]. Comparative analyses of selected secreted proteins revealed little correlation between the mRNA expression and their encoded protein levels that indicated their pronounced regulation by post-transcriptional mechanisms. During a similar study, proteome analysis of the conditioned medium from cultured human myotubes derived from strength-training individuals showed 236 secreted proteins [72]. Of these 236 proteins identified by proteome analysis, 17 proteins were potentially secreted by the cultured human skeletal muscle cells via the classical secretory pathway. Both studies highlighted the importance of SkMs as a prominent secretory tissue.

Similar to any other cell type, paracrine activity regulating mechanisms in SkMs are responsive to extrinsic stimuli from their microenvironment. Hence, the baseline expression and secretion profile of normal SkMs is only objective and gets altered by multiple factors such as preconditioning, hypoxia, anoxia, chemical stimulus, stress, stretch and genetic modification. The authors have previously reported that

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preconditioning of SkMs enhances their viability and reparability postengraftment. Pretreatment of SkMs with 200 μm diazoxide for 30 minutes protected the cells against oxidative stress upon subsequent treatment with 100 μm H_2O_2 as was shown by decreased lactate dehydrogenase release, reduced apoptosis and preserved mitochondrial membrane potential [73]. Most importantly, preconditioning resulted in enhanced cell engraftment after intramyocardial transplantation in the infarcted heart. At a molecular level, these benefits were contributed to the activation of Akt and enhanced paracrine activity of the preconditioned SKMs. Preconditioned SkMs released a number of cytokines and growth factors that were involved in angiogenesis, cell proliferation, chemotaxis, matrix remodeling, migration and inhibition of apoptosis.

SkMs either alone or synergized with angiogenic growth factor gene therapy have given encouraging results in multiple experimental studies [16, 74–80]. To this end, SkMs transplantation improves both systolic and diastolic function of injured hearts [16, 81–84], attenuate left ventricle (LV) remodeling by reducing the LV diastolic dimensions [82] and increase myocardial wall thickness [85] in experimental animal models. Given that these studies involved transplantation of SkMs in the poorly oxygenated microenvironment of the ischemic heart, different research groups have experimented to predict the effect of anoxia and hypoxia on their functionality postengraftment using *in vitro* experimental models. It is important to note that stem and progenitor cells not only differ in their paracrine behavior under normoxic culture conditions but also diverge in their responsiveness to low oxygen in their microenvironment. Additionally, secretory responses of stem/progenitor cells from the same tissue source show different secretome profiles when exposed to varying levels of oxygen.

SkMs are excellent carriers of transgenes of interest as a combinatorial approach for angiomyogenic repair of the heart [86–89]. For this reason, genetic modification of SkMs with the transgene(s) of interest also amends their paracrine behavior and the genetically modified cells serve as reservoirs of bioactive molecules that are secreted as part of their paracrine activity. For example, tempering the cells with the transgene encoding for hemoxygenase-1 (HO-1) enhanced their angiogenic potential postengraftment. Molecular analysis of the conditioned medium showed altered expression of proangiogenic molecules, especially VEGF and SDF-1α [89]. It was interesting to note that alteration of the expression of proangiogenic growth factors in the genetically tempered cells for HO-1 expression was significantly influenced by the differentiation status of the cells. Whereas the undifferentiated SkMs after HO-1 overexpression secreted significantly higher levels of VEGF, the differentiated SkMs responded to the genetic modification by increased levels of SDF-1α [89]. Mass spectroscopy also revealed that the conditioned medium from genetically modified SkMs was rich in proangiogenic proteins including galactin-1 and haptoglobin. There are many other interesting studies using the approach of genetic modification of SkMs to alter their paracrine activity for the expression of bioactive molecules of interest to promote their

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beneficial effects postengraftment in the heart [87, 90–92]. In some cases, gene deletion rather than overexpression tempers the paracrine activity profile of SkMs besides changing the engraftment characteristics. SkMs derived from satellite cells for depleted *MyoD* function ($MyoD^{-/-}$) showed elevated levels of proangiogenic factors such as PlGF and SDF-1α. Engraftment of $MyoD^{-/-}$ resulted in a significantly higher angiogenic response as compared to wild type SkMs. *In vitro* experiments for *MyoD* gene deletion and gene insertion experiments revealed that the *MyoD* gene negatively regulated the expression of angiogenic factors released as part of the paracrine activity of SkMs. These data infer that beneficial effects of $MyoD^{-/-}$ transplantation postengraftment were more due to the paracrine activity of the transplanted cells [88].

In addition to altered oxygen levels and genetic modification, application of mechanical stretch to the cultured rat SkMs resulted in copious release of HGF-1. Additionally, stretch treatment also accelerated entry of SkMs into cell cycle when medium pH was maintained between 7.1–7.4 [70]. The mechanical strain regimens of negative pressure elevated granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), monocyte chemotactic protein (MCP)-1, IL-6 and IL-8 secretion in the conditioned medium [69]. As an effort to find new methods to enhance the paracrine activity of SkMs, a co-culture system was developed between human adipose tissue derived MSCS and human SkMs by seeding the cell sheets on thermoresponsive culture plates. A commercially available cytokine/chemokine multiplex assay and ELISA were performed on the conditioned medium collected from these cells. The expression of HGF-1, leptin, VEGF and PECAM-1 in the co-cultured cells was significantly higher as compared to each of the cell type cultured alone. Extrapolating these data to an experimental rodent model of left anterior descending (LAD) coronary artery ligation, the survival of co-cultured SkMs was significantly higher after engraftment in addition to higher blood vessel density and improved cardiac parameters depicting global cardiac function [93]. While elucidating the underlying molecular mechanism of the beneficial effects of paracrine activity of SkMs, a recent study has shown that soluble mediators released by SkMs in their conditioned medium protected the cardiomyocytes against oxidative stress. The cytoprotective effect was mediated by EGF interaction with c-met signaling in the cardiomyocytes that was mimicked by neuroglin-1. Microarray analysis of the cardiomyocytes treated with conditioned medium from SkMs showed differential expression of genes associated with antioxidant effects including cystathionine-y-lyase (cst), xanthine oxidase and thioredoxin-interacting protein and tribbles homologue 3 (trib3). In order to study the cytoprotective effects afforded by SkMs on the host cardiomyocytes after transplantation in the infarcted heart, the expression of both cst and trib3 were found to be elevated in the host cardiomyocytes. CST was found more localized around the blood vessels, thus suggesting their smooth muscle localization [94]. Put together, these data vividly support that SkMs secrete copious amounts of soluble bioactive mediators as part of their paracrine activity and significantly contribute towards the therapeutic benefits presented by SkMs postengraftment in the heart.

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8.3.2 BM-derived stem cells

BM constitutes a heterogeneous population of stem and progenitor cells including hematopoietic and non-hematopoietic and the stromal cell subpopulations. All of these subpopulations are heterogeneous and thus their sublineages have different biologies and characteristics and hence, diverge in their differentiation potential and paracrine behavior.

BM-derived MSCs constitute 0.01% of the BM stem/progenitor cells. MSCs are multipotent, lowly immunogenic, compliant to undifferentiated *ex vivo* expansion and easy to genetically modify for genes of interest [95]. BM-derived MSCs have been extensively characterized *in vitro* and robustly employed for therapeutic cellular cardiomyoplasty. In addition to their ease of availability, isolation, purification and *in vitro* expansion, paracrine activity is the hallmark of MSCs and, together with differentiation, is considered as an alternative mechanism of their therapeutic benefit postengraftment [96]. Their secretome includes cytokines, growth factors, exosomes, microvesicles and microRNA as part of their paracrine activity [97–100]. It is noteworthy that composition of the constitutively expressed mediators diverges significantly under the influence of the culture conditions *in vitro* and by the host tissue microenvironmental conditions *in vivo* postengraftment in a temporo-spatial manner. The data pertaining to paracrine secretome ranges from analysis of the conditioned medium from the cells cultured *in vitro* to single cell polymerase chain reaction (PCR) on the micro-dissected MSCs from the ischemic heart 5 days after transplantation by intramyocardial injection [99].

The list of bioactive molecules secreted by MSCs is quite extensive in conjunction with diverse biological functions including angiogenesis, i.e. VEGF, bFGF and HGF-1, IGF-1, PDGF, epiregulin, endothelin [24, 101, 102], anti-apoptosis, i.e. Galactin-3, Smad-5, sRFP, secreted frizzled-related protein-1 and secreted frizzled-related protein-4 [sRFP-1 and sRFP-4) [24], cell mobilization and homing, i.e. HGF-1, LIF, SCF, SDF-1α [103–105], immunomodulation, i.e. suppression of pro-inflammatory molecules, i.e. TNF-α, IL1β, and IL6 [106, 107] and anti-fibrosis, i.e. adrenomedullin, thymosine β, thymorgulin, MMPs [108–110]. Their conjoined contribution encompasses intrinsic repair mechanisms of cardiac repair, promotes angiogenesis, mobilization and homing of resident stem cells, anti-apoptosis, anti-fibrosis, limits LV remodeling thus preserving cardiac function. The secretome profile of MSCs published from various research groups is diverse; however this list may contain many overlapping names of bioactive molecules. There is no single comprehensive list as yet published in the literature. Even the MSCs isolated from different tissue sources differ in their paracrine secretion profile [111]. The possible and the most important reason for such diversity is the sensitivity of the cells to their microenvironment, which is registered by the cells in terms of an altered secretome profile. On the same note, BM MSCs from different species sources also differ from each other in terms of their secretome profile albeit with some shared factors. For example, VEGF, FGF1, FGF2, PlGF, HGF-1, IGF-1,

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PDGF-BB, NGF, CSF1, MCP-1, IL1, IL6, TGFβ, TNF, AGPT2, MMP2, MMP9, AGPT1, BMP4, BMP2, ITGβ1, TIMP1 and TIMP2 have been reported typically in conditioned culture medium from murine MSCs [99, 112]. Similarly, analysis of the conditioned medium collected from human MSCs culture revealed ample secretion of bFGF, HGF-1, IFN, IGF-1, IL, PlGF, SDF-1α, TNFα, VEGF, epiregulin, endothelin, gylpican3, IGFBP7, IL15, LRP-1, LRP-6, sFRP-4, Smad-4, Smad-7, TIMP-1, TIMP-2 and thrombospondin [96]. In both cases, treatment of cells with hypoxia differentially upregulated some of these factors whereas the others remained either unchanged or their secretion was downregulated under hypoxia.

MSCs are amenable to genetic modification and therefore various research groups have genetically modified the cells to enhance their survival and reparability postengraftment besides significantly altering their paracrine secretome profiles [103, 113, 114]. Genetic engineering of MSCs for overexpression of prosurvival Akt, either alone or in conjunction with proangiogenic factors, has been reported by various research groups or analyzed for change in paracrine activity [113, 115, 116]. More recently, hypoxia and Akt-induced stem cell factor (HASF), a secretable factor has been identified as an integral constituent of paracrine secretions of MSCs that is cytoprotective for cardiomyocytes and also promotes their proliferation [117]. Both *in vitro* studies as well as *in vivo* studies have shown that HASF acted via activation of PKCε in the cardiomyocytes treated with conditioned medium from MSCs. When purified HASF protein was injected into the heart immediately after experimental MI, the level of injury and area of fibrosis were significantly reduced [118]. Despite preserved cardiac function at 4 weeks of observation, there was little evidence of the injected MSCs seen incorporated into mature collaterals. The authors inferred that MSCs can contribute to collateral remodeling through a paracrine mechanism [24, 118]. The same group of researchers has also reported that promotion of cytokinesis in neonatal cardiomyocytes treated *in vitro* with recombinant HASF was mediated through the activation of PI3K-Akt-CDK7 signaling [119]. The growth factors contained in the human MSCs conditioned with rat cerebral tissue extract from traumatic brain injury showed a time-dependent increase in VEGF, HGF-1, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) levels in the conditioned medium [120]. Mangi *et al.* genetically engineered rat MSCs to overexpress the prosurvival gene Akt1 (Akt-MSCs) [121]. *In vivo* characterization of the genetically modified Akt-MSCs was carried out in comparison with normal MSCs. The cells were transplanted by intramyocardial injection into the ischemic rat myocardium. The results showed that Akt-MSCs inhibited cardiac remodeling, reduced myocardial inflammation, collagen deposition and cardiac myocyte hypertrophy, regenerated 80%–90% of lost myocardial volume and completely normalized the systolic and diastolic cardiac functions in comparison with the normal MSCs. Rat Akt-MSCs restored 4-fold greater myocardial volume than the control MSCs. The same group further demonstrated that conditioned medium from Akt-MSCs subjected to hypoxic treatment markedly inhibited hypoxia-induced apoptosis and triggered vigorous spontaneous contractions of adult rat cardiomyocytes *in vitro* [122]. The

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genes encoding for the growth factors (VEGF, bFGF, HGF-1, IGF-I and thymosin β4) were reported as possible mediators of the beneficial effects of Akt-MSCs conditioned medium and were significantly upregulated in the Akt-MSCs. When injected into infarcted hearts, the Akt-MSCs conditioned medium significantly alleviated the infarct size expansion and improved the LV contractile function. Analysis of the conditioned medium from Akt-MSCs showed the presence of secreted Frizzled related protein-2 (Sfrp2) as a key component in the conditioned medium that was released in the paracrine secretions [116]. Subsequent molecular studies showed that it could attenuate Wnt3a-induced caspase activities in a concentration-dependent fashion in response to hypoxia reoxygenation [123]. Akt overexpression has also been combined with angiocompetent molecules such as Ang-1 for a two-prong strategy to gain benefits of transgene overexpression [113]. First, transgenic Akt overexpression was part of the preconditioning strategy to support donor cell survival. Second, the secretion of Ang-1 protein was expected to promote maturation of the neovascular structures at the site of cell graft during the process of angiogenesis for improved regional blood flow. In the absence of angiopoietins, the newly formed vascular structures either regress over time or remain leaky due to failure of the maturation process. In the same context, constitutive expression of Pim-1 kinase, which was previously studied for its role in cardiomyocyte survival and proliferation, has been reported to enhance MSCs survival under serum deprivation and hypoxia via cooperation with serum signal in the culture conditions [124]. Molecular studies have shown that Pim-1 kinase actually binds with proapoptotic protein Bad to phosphorylate ser-112, the gatekeeper site for its inactivation, and enhances cell survival in addition to maintenance of prosurvival Bcl2 expression [125]. Pim-1 also gets inducted during preconditioning of MSCs with simultaneous inverse expression of miR-206 in the preconditioned cells. Loss-of-function studies for miR-206 showed prosurvival effects on the preconditioned cells, whereas inhibition of Pim-1 blocked the prosurvival effects [126]. Besides the prosurvival and proproliferative actions, Pim-1 induction in resveratrol-treated adipose-tissue-derived senescent MSCs had anti-senescence effects and promoted their paracrine activity via activation of multiple transcription factors responsible for insulin secretion in the B-cells, i.e. TFAM, PDX1, Glut2, HNF-1 [127].

In addition to altered paracrine response to genetic modification, MSCs have been extensively studied for their sensitivity to the low oxygen concentration in their microenvironment in terms of paracrine activity [128]. Changes in the oxygen concentrations significantly influence the proliferation and differentiation characteristics of MSCs in general [129–132] and their paracrine secretory activity in particular, albeit with an as yet less defined underlying mechanism [133–135]. Stability of HIF-1α and activation of its downstream signaling pathway remain central to the altered responses of MSCs under hypoxia [136]. The hypoxia responsiveness varies with the level of oxygen concentration as well as duration of exposure. In a recent study, exposure to 0.5% oxygen stabilized HIF-1α with a concomitant increase in the paracrine secretions that were rich in BNDF, glial cell-derived neurotropic factor (GNDF), VEGF and its

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receptor Flk-1, erythropoietin (EPO) and its receptor EPOR and SDF-1α and its chemokine receptor CXCR4. The observed expression of proinflammatory cytokines was significantly lower in the hypoxia-treated MSCs as compared to the non-treated cells [137]. Exposure of MSCs to 1% oxygen significantly enhanced HGF-1, bFGF, VEGF and IGF-1 secretions in the culture medium in NFκb dependent fashion [138].

As part of their paracrine activity, MSCs also release exosomes (30–100 nm size microvesicles), which act as carriers of bioactive molecules [139–141]. The release of exosomes is being considered as an essential method of intercellular communication and for horizontal transfer of materials between cells [142]. Upon fusion with cells, exosomes get internalized to transfer their contents as part of the mechanism to enhance intercellular communication. Besides proteins, exosomes are rich in mRNA, microRNAs and other non-coding RNAs, which are taken up by the neighboring or distant cells upon fusion with the exosomes [143]. Given the bioactivity of the vesicular contents, microvesicles released from MSCs are being considered for use in therapeutic applications [97]. In one of the recently published studies, ECs treated with MSC-derived exosomes responded by enhanced tube formation *in vitro*, whereas intramyocardial delivery of the purified exosomes significantly attenuated experimental infarct size expansion via enhanced angiogenesis [144]. MSC-derived exosomes delivery had cardioprotective effects and led to successful attenuation of myocardial remodeling via activation of PI3/Akt signaling that promoted host cardiomyocyte survival after ischemia reperfusion injury [145]. The infarcted animal heart treated with MSC-derived exosomes also showed significantly preserved cardiac function. Given that exosomes released from the stem/progenitor cells have cytoprotective ECs mobilization, cell proliferation and proangiogenic effects, their further characterization, mechanism of release and ability to transfer materials of interest to the target cells need further and in-depth exploration before these can be safely and effectively translated into clinically relevant therapeutic approach [146, 147].

8.3.3 Sca1^{+}/CD31^{-} cells

Stem cell antigen-1 (Sca-1 or Ly6A) is a member of the Ly6 family of glycosyl phostidylinositol (GPI)-anchored cell surface proteins and has long been associated with murine stem/progenitor cells [148]. The presence of Sca-1^{+} cells has been reported in different organs in the body [53, 54, 149–152]. Genetic deletion of Sca-1 resulted in early onset of cardiac function and repair deficiency in addition to impaired cardiac development [153]. A vast majority of the side population of cells housing the interstitial spaces of the skeletal muscle express Sca-1^{+}CD34^{+}CD45 [154]. Gojo *et al.* demonstrated that CD34^{low}c-Kit^{+} CD140a^{+}Sca-1^{high} were able to differentiate into cardiomyocytes, ECs and pericytes or SMCs in the heart after engraftment, whereas the same cell population contributed to formation of the vasculature when they were injected into skeletal muscle and lung and heart [53]. The ability of Sca-1^{+} cells to adopt functionally competent

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phenotypes of cardiomyocytes has been reported from many research groups. Various protocols have been optimized to induce their differentiation into cardiomyocytes and endothelial lineage. In a study involving Sca-1+ cells, the cells were isolated from the adult murine heart expressed genes encoding for cardiac transcription factors and contractile proteins and showed sarcomeric structure and spontaneous beating after treatment with oxytocin. The beating rate of the differentiated neomycytes increased by isoproterenol treatment, which was accompanied by the intracellular Ca^{2+} transients [54]. On the same note, Sca-1$^+$CD34$^-$ cells were treated with VEGF and a combination of Wnt antagonist Dikkoff (DD-1), BMP-2, FGF4, FGF8 and 5-azacytidine to induce their endothelial and cardiomyocyte differentiation, respectively [151]. Co-culture of Sca-1$^+$CD31$^-$ cells have also been co-cultured with neonatal cardiomyocytes for successful induction to cardiomyocyte phenotype. Transplantation of the *in vitro* expanded Sca-1$^+$CD31$^-$ cells successfully integrated into the heart after transplantation and preserved global cardiac function [151]. Besides their ability to engraft after transplantation, Sca-1$^+$CD31$^-$ cardiac progenitor cells are able to migrate into damaged myocardium from a non-ischemic area of the heart and differentiate into both cardiomyocyte and endothelial-like cells following acute ischemic injury through the SDF-1α/CXCR4 system in a murine model of myocardial ischemia [155]. In order to enhance their reparability, Sca-1$^+$CD31$^-$ cells transplantation has been combined with simultaneous injection of combined (IGF-1 + HGF-1) growth factors into a mouse model of MI with an enhanced rate of cell engraftment, better cell viability, increased angiogenesis and minimally stimulated endogenous cardiomyocyte regeneration *in vivo*. Given the encouraging preclinical data, cardiosphere-derived Sac-1$^+$ cells progressed to CADUCEUS phase I clinical studies [156]. In addition to their cadiogenic and vasculogenic ability to participate in the repair process, recent studies have focused on the paracrine activity of Sca-1$^+$ progenitor cells as an alternative contributing mechanism in myocardial repair [157–159].

A direct comparison of different cell types including the BM-derived MSCs, BM mononuclear cells, adipose-tissue-derived MSCs and CDCs with and without sorting of c-kit$^+$ cells have been reported [158]. The cells were compared for the secretion of six selected growth factors, i.e. IGF-1, VEGF, SDF-1α, HGF-1, Ang-1 and bFGF *in vitro* culture conditions. ELISA results showed that the unsorted CDCs secreted the highest level of all six growth factors in comparison with all other cell types including the sorted c-kit$^+$ sub-population of CDCs. Upon engraftment into the infarcted heart, CDCs showed the highest level of survival and engraftment at par with their rate of survival under oxidative stress *in vitro*. Another recently published study has shown differential expression of EGF, TGF-1β, IGF-1, HGF-1, IL6 and MCP-1 which were incidentally the most predominant factors secreted by Sca-1$^+$CD31$^-$ cells in their culture. Of these factors, MCP-1 was found to paly significant role at molecular level to support their survival upon subsequent exposure to hypoxia. Abrogation of MCP-1 drastically reduced their resistance to hypoxic injury [159]. Molecular studies elucidating the mechanism of paracrine secretions by Sca-1$^+$CD31$^-$ showed a critical regulatory role

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of A(2B) receptor expression on the surface of these cells that was responsible for their adenosine responsive release of pro-angiogenic growth factors. An equivalent stromal cell population to murine Sca-1$^+$CD31$^-$ cells has also been identified in the human heart which expressed A(2B) receptors and is being considered as a potential target to upregulate pro-angiogenic factors in the ischemic heart [160].

8.3.4 c-Kit$^+$ cells

The stem cell growth factor receptor c-Kit$^+$ is a cytokine receptor that was first detected on the surface of hematopoietic stem cells (HSCs) [161]. Subsequently, the existence of c-kit$^+$ cells was reported in the heart as c-Kit$^+$/Lin$^-$ cardiac progenitor cells [32]. Although these cells were found in very small in number (one out of 10,000 myocytes), they were often present in small clusters and expressed the transcription factors $Gata_4$, $Nkx_{2.5}$, and Mef_2. The existence of c-Kit$^+$/Lin$^-$ cardiac progenitor cells have been investigated in multiple mammalian species, including mouse [162–164], rat [165, 166], dog [167], pig [168], and human [40,169]. During cell culture *in vitro*, c-Kit$^+$/Lin$^-$ cells were highly clonogenic and showed extensive capacity of self-renewal. In response to appropriate cues, they showed multi-lineage differentiation potential to adopt cardiomyocyte, smooth muscle and EC phenotypes. Experimental animal studies have shown that ckit$^+$/lin$^-$ cells were capable of regenerating cardiomyocytes and new vascular structures in the ischemically damaged heart. Characterization of CSCs in the human heart has shown the existence of a pool of CSCs that has the capacity to regenerate damaged myocardium in the event of MI. However, there is extensive loss of functionally competent CSCs during chronic ischemic cardiomyopathy. It has been found that CSC number increases markedly during acute myocardial ischemia and their growth is correlated with the increase in telomerase-competent dividing CSCs. [40, 169]. Several protocols have been optimized for isolation and expansion of c-Kit$^+$ human CSCs from small myocardial specimens for their characterization. These cells are predominantly myogenic and differentiate to adopt cardiomyocyte phenotypes at a higher rate and with a comparatively lesser rate to become smooth muscle and ECs phenotypes. The human CSCs can also generate a chimeric heart when they are locally injected into infarcted myocardium of immunodeficient mice and immunosuppressed rats wherein they show structural and functional integration with the rodent myocytes. Moreover, the transplanted cells were also found integrated into the coronary blood vessels. Subsequent to encouraging data, c-kit$^+$ cells have progressed to the clinical studies [170]. A new phase II clinical study is in the process of registering patients for participation to assess the combined effect of treatment with BM-derived MSCs and myocardial derived c-kit$^+$ cells in the patients with ischemic heart (https://clinicaltrials.gov/ct2/show/NCT02501811).

Similar to the other stem and progenitor cells, c-kit$^+$ cells secrete many growth factors, i.e. VEGF, SDF-1α, HGF-1 and IGF-1, as part of their paracrine secretome [171].

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The conditioned media of adult human heart derived cardiospheres or the CDCs exerted anti-apoptotic effects on neonatal rat ventricular myocytes, and proangiogenic effects on human umbilical vein ECs *in vitro* culture conditions. *In vivo*, human CDCs secreted VEGF, HGF-1, and IGF-1 when transplanted into the murine model of acute MI. Intramyocardial injection of CDCs in the peri-infarct area increased the expression of Akt with simultaneous reduction in caspase-3 thus preventing the rate of cell apoptosis. Based on the number of human-specific cells relative to the overall increase in the capillary density and myocardial viability, direct differentiation quantitatively accounted for 20%–50% of the observed effects. Maxeiner *et al.* further documented that the supernatant of CPCs contained a diversity of cytokines. It was found that for rat either 8 (juvenile) or 7 (adult) cytokines out of 19, and for human 29 (juvenile) and 18 (adult) out of 507, were significantly released into the cell culture medium [172]. The isolated adult cardiomyocytes in culture responded to treatment with CPCs conditioned medium to show increased contractility in a concentration-dependent manner after incubation for 24 hours. Treatment with the conditioned medium also normalized the angiotensin-II induced contractile dysfunction. A direct comparison of the human CDCs subjected to 60 cycles/min of mechanical stretch giving 120% elongation with non-stretched native cells in culture was performed to show the effect of mechanical stress that the transplanted cells could endure after engraftment in the heart [173]. The results depicted a significant reduction in the total number of surviving cells as well as $Ki67^+$ cells under stretching conditions that decreased significantly after 24 hours of stretching. However, the stretched cells responded by abundant release of inflammatory cytokines IL6 and IL-1β as well as the angiogenic growth factors VEGF and bFGF during 12 hours. Furthermore, there was significant reduction in c-Kit^+ stem cells but with a simultaneous increase in cardiac troponin-I expressing and smooth muscle actin expressing cells 3 days after stretching. It has been proposed that c-Kit^+/Lin^- CPCs constitute a heterogeneous cell population that contains lineage-committed progenitor cells. A distinguishable c-Kit^+CD45^- cell population was isolated from the right atrial biopsies that contained small percentage (1.1%) of NKX2.5 expressing cells while most of the cells (81%) expressed late endothelial gene vWF. The latter population was subcategorized as endothelial genes (FLK-1 and CD31) and the late cardiac genes (TNNT2 and ACTC1) expressing populations [174].

8.3.5 Pluripotent stem cells derived cells

The pluripotent stem cells, i.e. ESCs and iPSCs, and their derivative cells, i.e. MSCs, ECs, SMCs and cardiomyocytes, are being extensively studied for their safety and efficacy in treatment of cardiovascular diseases [57–59]. Their differentiation potential to adopt cardiac and endothelial phenotypes remains unopposed in both *in vitro* as well as experimental animal studies and has been widely recognized in literature. With the emergence of paracrine hypothesis and interest of researchers

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in paracrine activity of stem/progenitor cells as an alternative contributor to heart cell therapy, pluripotent stem cells are in focus for their paracrine secretome and its role in repairing cardiac structure, improving cardiac function and bioenergetics after myocardial injury. The conditioned medium from human iPSC and their derivative MSCs is rich in secreted mediators. A direct comparison of BM-derived MSCs with ESCs showed their responsiveness to hypoxia treatment, which resulted in elevated levels of VEGF and anti-inflammatory cytokine IL-10 expression in the culture conditions [138]. These findings provide strong argument about the inherent capacity of ESCs to respond to innocuous stimulus by secreting paracrine mediators that may also work in an autocrine fashion to help them survive under unfavorable conditions. A direct comparison of BM-derived MSCs with their derivative counterparts from iPSCs on a panel of 507 bioactive molecules, i.e. cytokines, chemokines, adipokines, growth factors, angiogenic factors and some soluble receptors, showed that the conditioned medium from both cell types shared the expression pattern of 214 molecules [57]. Further analysis of the conditioned medium revealed that iPSC-derived MSCs secreted higher levels of 129 factors compared to the BM-derived MSCs, of which macrophage migration inhibitory factor (MIF) and growth differentiation factor-15 (GDF-15) were attributed to antiapoptotic and cardioprotective effects. Treatment of murine heart model of experimental MI with conditioned medium from iPSC-derived MSCs reduced the infarct size expansion, remarkably attenuated left ventricular dysfunction and remodeling after doxycyline-induced cardiomyopathy [57]. Ye *et al.* further determined the paracrine factor profile of human iPSC-derived ECs, SMCs and cardiomyocytes under normoxia [59]. Analysis by protein array showed that human iPSC- derived ECs not only expressed high protein levels of angiogenin, IL-6, IL-8, TIMP-1 and TIMP-2, but also expressed high levels of Ang-2, growth regulated protein and MCP-1, and low levels of Ang-1, PDGF-BB, RANTES and MCP-3. However, iPSC-derived SMCs expressed high levels of angiogenin, Ang-1, MCP-1, TIMP-1 & 2 and low levels of IL-6, growth regulated protein, Ang-2, IL-8, RANTES and uPAR. Similarly, human iPSC derived cardiomyocytes expressed high levels of angiogenin, IL-6, growth regulated protein, MCP-1 & 3, TIMP-1 & 2 and low levels of Ang-1 & 2, uPAR and VEGF. Their biological functions were identified as: inhibition of apoptosis (angiogenin, Ang, IL-6, MMP-1, PDGF-BB, TIMP-1, uPAR and VEGF), induction of cell migration or homing (angiogenin, Ang, IL-8, MCP-1 &3, MMP-9, uPAR, and VEGF), and promotion of cell proliferation (angiogenin, Ang-1, PDGF-BB, and VEGF). The conditioned medium of human iPSC-derived ECs and SMCs protected human iPSC-derived cardiomyocytes from hypoxic injury (exposure to 1% O_2 for 48 hours), suggesting a paracrine mechanism through which human iPSC-derived ECs and human iPSC- derived SMCs could promote cardiomyocytes survival [59]. These are significant data and clearly show vast potential for exploration for clinical application. As is evident from these findings, both pluripotent stem cells and their derivative cells, based on their differentiation potential and paracrine activity warrant further investigation especially their tumorgenicity before they can enter the clinical arena.

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8.4 Conclusion

Each stem/progenitor cell type has inherent capacity to secrete a wide profile of bioactive mediators, i.e. cytokines, chemokines, growth factors, etc., with attributes of biological functions that modulate inflammation, inhibit cell necrosis and apoptosis, promote cell viability, migration, homing, and proliferation, induce neovascularization (angiogenesis and arteriogenesis) and promote neomyogenesis. These may directly or indirectly improve the engraftment rate of transplanted donor cells in addition to involving the resident cardiac stem cells by their mobilization and homing-in to get involved in the endogenous cardiac repair and improve the efficacy of cellular cardiomyoplasty. Therefore, using the conditioned medium for treatment alone or in combination with transplantation of donor cells would be a simple and realistic strategy for cardiac repair and regeneration. With the recent advances in understanding of the paracrine activity of stem cells and release of bioactive molecules that can perform multiple functions, we may be heading towards an era of cell-free regeneration of myocardial tissue *via* supporting the intrinsic repair mechanisms, i.e. protection of cardiomyocytes from apoptosis and mobilizing the resident stem cell pool for participation in the regeneration of the damaged tissue [175]. Each of the stem/progenitor cell used to date has some limitation/s ranging from availability and logistic issues to *in vivo* behavior in terms of engraftment to differentiation and integration after transplantation. Therefore, it requires protocol modifications and adjustments to achieve optimal prognosis but still the outcome of the clinical studies has proven it to be more hype than reality. Researchers are still in quandaries about utilizing the stem cells to their best regenerative potential (Tab. 8.4.1). Given the complexity of the disease process and its multifaceted nature in the infarcted heart, use of paracrine mediator-rich conditioned medium in combination with genetically modified stem cells may provide headway in the otherwise sluggish field of stem cell therapy for myocardial regeneration from a clinical perspective.

Tab. 8.4.1: Summary of the studies focused on paracrine release of bioactive mediators from different types of stem/progenitor cells.

Cell Type	Paracrine factors secreted	Effect	Reference
MSC	BDNF, NGF, bFGF, VEGF and HGF	Neuroprotection and angiogenesis	[120]
	HASF is a stem cell paracrine factor	Antiapoptosis, reduce fibrosis	[118]
	VEGF, bFGF, PlGF, and MCP-1	Collateral remodeling	[112]
	Paracrine factors	Prolong repolarization of neonatal rat cardiomyocytes	[176]
	PDGF	Exert promigratory effect	[102]

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Tab. 8.4.1 (continued)

Cell Type	Paracrine factors secreted	Effect	Reference
	Unknown	Inhibit apoptosis and improve cardiac function	[177]
	VEGF, endothelin, epiregulin, Galectin-3, Smad-5, sRFP-1 and sRFP-4	Promote angiogenesis, antiapoptosis, reduce fibrosis and antiremodeling	[24]
AKT modified MSC	VEGF, bFGF, HGF, IGF-I and TB4	Antiapoptosis, limit infarct, improve heart function	[122]
	Frizzled related protein-2	Antiapoptosis	[123]
HSP-20 modified MSC	VEGF, bFGF, and IGF-1	Improve cell engraftment rate and cardiac function	[101]
Umbilical cord derived MSC	VEGF, angiopoietins, HGF-1, bFGF, TGF-β and PDGF	Promote angiogenesis and mitosis, and antiapoptosis	[178]
Cardiac progenitor cells	VEGF, IGF-1, HGF-1, IGF-1	Promote endogenous regeneration, myocardial protection	[171]
	Rat: 8 (juvenile) or 7 (adult) cytokines Human: 29 (juvenile) and 18 (adult).	Improve cardiomyocyte contractile function	[172]
	IL-6, IL-8, VEGF and bFGF	Mechanical stress increases the release of these factors	[173]
Cardiac explants	IL-6, IL-8, GRO, MCP-1, bFGF, EGF, HGF-1, IGFBP-1 and TGF-β	Promote myocardial differentiation	[179]
Human aortic $CD133^+$ Progenitor Cells	VEGF, IL-8	Activate Wnt signaling pathway	[180]
Hematopoietic progenitor cell	Galectin-3, gelsolin, IL-6, IL-13, MCP-1, MCP-3, MIP-1α, MIP-1β	Promote tissue regeneration	[181]
Bone marrow-derived progenitor cell	HGF-1	Reduce cardiac fibrosis and improve heart function	[182]
Endothelial progenitor cells	TGFβ1, IGF-1	TGFβ1 promote cardiac Hypertrophy	[183]
Skeletal myoblast	VEGF, PIGF, angiogenin, Ang-1, HGF, PDGF-BB, MMP2, MMP9, MMP10 and TIMPs	Promote angiogenesis and antiapoptosis	[71]

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Tab. 8.4.1 (continued)

Cell Type	Paracrine factors secreted	Effect	Reference
	HGF	Stretch triggers intracellular Signaling pathway leading to NOS activity and HGF releasing	[70]
	bFGF and HGF-1	Pre-condition rat SkMs promoted cell enhanced releasing paracrine factors	[73]
	IL-8, GM-CSF, G-CSF, MCP-1 and IL-6	Mechanical strain increases chemokine releasing	[69]
	635 secreted proteins, including 35 growth factors,40 cytokines, and 36 metallopeptidases	These proteins can be potent signaling mediators to other cells	[67]
	236 proteins were detected by Proteome analysis	Strength training induced human muscle cells to secrete a range of proteins	[72]
Sca-1$^+$	FGF11, IGF-1, Kit ligand, Nerve growth factor, PDGF, TGFβ1 and β2, and CD31$^-$ and VEGF-A,-B, and -C	Simultaneous IGF+HGF and Sca-1$^+$ CD31$^-$ transplantation achieved better cardiac function improvement	[184]
Pluripotent stem cells derived *hiPSC-MSCs*	MIF and GDF-15	Attenuated LV dysfunction and dilatation	[57]
hiPSC-ECs	Ang-1,VEGF-A, VEGF-C and PDGF-AA	Increase capillary density and blood perfusion	[185]
	Angiogenin, IL-6, IL-8, TIMP-1, TIMP-2, Ang-2, MCP-1, PDGF-BBm, Growth regulated protein, MCP-3, Ang-1, RANTES and uPAR	Antiapoptosis and cytoprotection, enhancing angiogenesis and blood flow, promoting cell migration and division	[59]
hiPSC-SMCs	Angiogenin, Ang-1, MCP-1, TIMP-1, TIMP-2, IL-6, IL-8, growth regulated protein, RANTES and uPAR	Antiapoptosis and cytoprotection, enhancing angiogenesis and blood flow, promoting cell migration and division	[59]
hiPSC-CMs	Angiogenin, IL-6, MCP-1, MCP-3, TIMP-1 and TIMP-2, Ang-1, Ang-2, uPAR, VEGF and growth regulated protein	Antiapoptosis and cytoprotection, enhancing angiogenesis and blood flow, promoting cell migration and division	[59]
hESC-ECs	Angiogenin, ENA78, IL-6, IL-8, TIMP-1, and TIMP-2, EGF, IGF-1, and VEGFD, PDGF-BB, thrombopoietin, VEGFA	Antiapoptosis and cytoprotection	[58]

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Tab. 8.4.1 (continued)

Cell Type	Paracrine factors secreted	Effect	Reference
hESC-SMCs	TIMP-1, angiogenin, IGF-1, IL-8, MCP-1 and TIMP-2	Antiapoptosis and cytoprotection	[58]
Irradiated ESCs	Unknown	Improve cardiac function and decrease infarct size	[186]

Ang: angiopoietin-1; BDNF: brain-derived neurotrophic factor; ENA78: epithelial neutrophil-activating protein 78; EGF: epithelial growth factor; bFGF: basic fibroblast growth factor; G-CSF: granulocyte-colony stimulating factor; GDF-15: growth differentiation factor-15; GM-CSF: granulocyte macrophage colony-stimulating factor; GRO: growth-regulated oncogene; HASF: hypoxia and Akt-induced stem cell factor; HGF: hepatocyte growth factor; IGF-I : insulin-like growth factor 1; IGFBP-1: insulin-like growth factor binding protein-1; IL: interleukin 6; MCP: monocyte chemoattractant protein 1; MIF: migration inhibitory factor; MIP: macrophage inflammatory protein; MMP: matrix metallopeptidase; NGF: nerve growth factor; PDGF: platelet-derived growth factor; PlGF: placental growth factor; RANTES: regulated on activation, normal T cell expressed and secreted; sRFP: secreted frizzled-related protein; TB4: thymosin β4; TGF-β: transforming growth factor β; TIMP: tissue inhibitor of metalloproteinase; uPAR: urokinase receptor; VEGF: vascular endothelial growth factor.

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