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STEM CELLS: FROM MYTH TO REALITY AND EVOLVING

Edited by Khawaja Husnain Haider

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Preface

Stem cell-based therapy has progressed over years from a myth to reality thus configuring a conceptual change in the contemporary therapeutic approach from symptomatic relief for a patient to modulating his disease course. The field has successfully evolved from an initial hype to a real hope as cell-based therapy has found remedy for various pathologies that were once reckoned as incurable. The credit for this accomplishment is shared by all the institutions, organizations and researchers who have immensely contributed to the cause. In this regard, I am gratified for the professional support rendered by DeGruyter and appreciate their elite team of thorough professionals who accepted my three consecutive book proposals including the previously published: "Stem cells: from drug to drug development" and "Stem cells: from hype to real hope". In continuation with these two books, "Stem cells: from myth to reality and evolving" contains 8 book chapters contributed by cell-based therapy experts from some of the prestigious academic and research institutions around the globe. Chapter-1 has been contributed by a leading research group from the Wilmer Eye Institute, John Hopkins University School of Medicine which discusses pluripotent stem cell-derived corneal cells for the treatment of corneal dystrophies. Chapter-2 has been contributed by a leading Italian group from University of Modena and Reggio Emilia who are researching in the field of cancer stem cells. Chapter-3 has been contributed by a widely published research group from Institute of Atherosclerosis Research, Skolkovo, Russia, regarding the multi-lineage potential of pericytes and their significance in atherosclerosis. Chapter-4 provides an in-depth analysis of the published clinical trial data using bone marrow derived stem cells for cardiac repair in terms of safety and efficacy. Cryopreservation of stem cells is important for their off-the-shelf availability in the clinical perspective, an issue which has been thoroughly discussed by a leading research group from Masaryk University, Czech Republic. The authors have reviewed methods of cryopreservation, post-thaw characteristics of cryopreserved cells in terms of their viability, inner structure, and mechanical properties. Chapter-6 provides an insight into the molecular mechanisms underlying neuronal stem cell differentiation. Chapter-7 discusses adrenomedullins and their receptors for their role in angiogenesis and vasculogenesis, and during fate determination of stem cells. Finally, chapter-8 reviews the translational efforts using bone marrow derived mesenchymal stem cells and their paracrine secretions for the treatment of critical limb ischemia and ischemic heart disease. For all these elegant contributions, I am deeply indebted to the contributing authors and co-authors. In the end, I dedicate this book to my Angel of Paradise Anas whose departure from my life is a source of inspiration for me to do science and Mowahid who is the love of my life.

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List of abbreviations

ABI = Ankle-brachial index
AC = Adenylyl cyclase
ADMs = Adrenomedullins
ADSCs = Adipose tissue-derived stem cells
AFM = Atomic force microscopy
AFP = Antifreeze proteins
AH = Anti-Hem
ALDH = Aldehyde dehydrogenases
AMI = Acute myocardial infarction
ANR = Anterior neural ridge
AP = Anterior-posterior
APs = Apical progenitors
APC = Antigen-presenting complexes
ASCs = Adult stem cells
ASCs = Adipose-derived stem cells
AVE = Anterior visceral endoderm
bFGF = Basic fibroblast growth factor
BLBP = Brain lipid-binding protein
BM = Bone marrow
BMCs = Bone marrow cells
BMMNCs = Bone marrow mononuclear cells
BMPs = Bone morphogenetic proteins
BMSCs = Bone-marrow-derived stem cells
CABG = Coronary artery bypass graft
cADMsP = Cyclic adenosine monophosphate
CAR = Coronary artery reserve
CCR2 = C-C motif chemokine receptor 2
Cdk5 = Cyclin-dependent kinase 5
CECs = Corneal endothelial cells
CGRP = Calcitonin gene related peptide
Cip/Kip = Interacting protein/kinase inhibitory protein
CK1 = Casein kinase-1
CLI = Critical limb ischemia
CLRs = Calcitonin-receptor like receptors
CNS = Central nervous system
CNTF = Ciliary neurotrophic factor
CP = Choroid plexus
CPCs = Cardiac progenitor cells
CSC = Cancer stem cells
CSCs = Cardiac stem cells
CVCs = Calcifying vascular cells
Cx43 = Connexin 43 (Cx43)
DG = Dentate gyrus
DHH = Desert Hedgehog
DMSO = Dimethyl sulfoxide
DSBs = Double-stranded breaks

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DV = Dorsoventral
ECs = Endothelial cells
ECM = Extracellular matrix
EGF = Epidermal growth factor
EMT = Epithelial mesenchymal transition
ENO = Enolase
EPCs = Endothelial progenitor cells
ER = Endoplasmic reticulum
ERK = Extracellular signal-related kinase
ESCs = Embryonic stem cells
FD = Force-distance
FGF = Fibroblast growth factor
FGFR = Fibroblast growth factor receptor
FG2 = Fibroblast growth factor-2
FM = Force-maps
FU = Fused
Fz = Frizzled
GAPDH = Glyceraldehyde 3-phosphate dehydrogenase
GCSF = Granulocyte colony stimulating factor
GE = Ganglionic eminences
GFAP = Glial fibrillary acidic protein
GPCR = GTP-binding protein coupled receptor
GPCRs = G-protein coupled receptors
GREs = Growth hormone response elements
Gro = Groucho
GSK3 = Glycogen synthase kinase-3
HAEC = Human aortic endothelial cells
HAS = Human serum albumin
hCE = Human corneal endothelium
hESCs = Human embryonic stem cells
HGF = Hepatocyte growth factor
HIF-1α = Hypoxia-inducible factor-1α
HIF-1β = Hypoxia-inducible factor-1β
HLA-DR = Human leukocyte antigen–DR isotype
hMSC = Human mesenchymal stromal/stem cells
hPSCs = Human pluripotent stem cells
HRE = Hypoxia response element
HSCs = Hematopoietic stem cells
HSPG = Heparin sulfate proteoglycans
HUVECs = Human umbilical vein endothelial cells
IBP = Ice-binding proteins
IDP = Intrinsically disordered proteins IDP
IFN-γ = Interferon-γ
IGF-1 = Insulin-like growth factor-1
IGF2 = Insulin-like growth factor-2
IHD = Ischemic heart disease
IHH = Indian Hedgehog
IL = Interleukin
IL-6 = Interleukin-6
IL1β = Interleukin-1β

IMD = Intermedin
iPSCs = Induced pluripotent stem cells
IRS-1 = Insulin receptor substrate 1
ISCT = International Society for Cellular Therapy
ISP = Ice-structuring proteins
LC = Lung cancer
LDL = Low-density lipoprotein
LF = Left-right
LGR5 = Leucine-rich-repeat-containing G-protein coupled receptor 5
LIF = Leukemia inhibitory factor
LSCs = Limbal stem cells
LSCD = Limbal stem cells deficiency
LV = Left ventricle
LVEDV = Left ventricular end diastolic volume
LVEF = Left ventricle ejection fraction
LVESV = Left ventricular end systolic volume
MAPC = Multipotent adult progenitor cells
MAPK = Mitogen-activated protein kinase
MCP-1 = Monocyte chemoattractant protein-1
MDR = High levels of multidrug resistance
MDSC = Muscle-derived stem cells
MGE = Medial ganglionic eminence
MHC-II = Major histocompatibility complex class II
MIDCAB = Minimally invasive coronary artery bypass
MLHF = Minnesota Living with Heart Failure
MM = Malignant mesothelioma
mMSCs = Native mouse mesenchymal stromal/stem cells or human
MNC = Mononuclear cell
MOF = Metal-organic frameworks
MSC = Mesenchymal stromal/stem cells
MVs = Microvesicles
NC = Neural crest
NF-kB = Nuclear factor kappa B
NG1 = Neurogenesin-1
NG2 = Neuron-glial 2
NOS3 = Nitric oxide synthase 3
NSCs = Neural crest cells
NSCLC = Non-small cell lung cancer
NYHA = New York Heart Association
OPCs = Oligodendrocyte precursor cells
OPS = Open pulled straws
OSVZ = Outer SVZ progenitors
γH2AX = Phosphorylated histone H2A
PBMC = Peripheral blood mononuclear cell
PCI = Percutaneous coronary intervention
PDGF = Platelet-derived growth factor
PDGF-BB = Platelet-derived growth factor-BB
PDGFR-β = Platelet-derived growth factor receptor beta
PEG = Polyethylene glycol
PGD2 = Prostaglandin D2

PGK = Phosphoglycerate kinase
PGM = Phospohoglycomutase
PHD = Prolyl hydroxylases
PKA = Protein kinase A
PKM2 = Pyruvate kinase m2 isoform
PlGF = Placental growth factor
pMN = Motor neuron progenitors
PORT = Postoperative radiotherapy
PSC = Pluripotent stem cells
Ptch = Patched
PV = Paravalbumin expressed cells
PVP = Polyvinylpyrollidone
RADMsPs = Receptor activity modifying proteins
RGPs = Radial glial progenitors
ROCK = Rho kinase
ROS = Reactive oxygen species
RPCT = Randomized placebo-controlled clinical trials
SDF-1 = Stromal cell-derived factor-1
SGZ = Subgranular zone
SHH = Sonic Hedgehog
SIRS = Systemic inflammatory response syndrome
SkMs = Skeletal myoblasts
SMA-α = Smooth muscle actin alpha
SMCs = Smooth muscle cells
Smo = Smoothened
S1P = Sphingosine-1-phosphate
SST = Somatostatin
SUFP = Suppressor of FU
SVZ = Subventricular zone
TB4 = Thymosin-4
TcPO2 = Transcutaneous oxygen pressure
TGF-α = Transforming growth factor-α
TGF-β = Transforming growth factor-beta
3d = Three-dimensional
3G5 = O-sialoganglioside
THP/F = Thermal hysteresis proteins/factors
TNF = Tumor necrosis factor
TNFα = Tumor necrosis factor α
TNTs = Tunneling nanotubes
TRAIL = Tumor necrosis factor–related apoptosis-inducing ligand
TREs = Thyroid response elements
TSP1 = Thrombospondin-1
UCB = Umbilical cord blood
UCSCs = Umbilical cord stem cells
VEGF = Vascular endothelial growth factor
VESL = Very small embryonic-like stem cells
vHL = von Hippel-Lindau
VSMCs = Vascular smooth muscle cells
WNT = Wingless/integrated

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1 Pluripotent stem-cell-derived corneal cells

Abstract: Corneal dystrophies are the leading cause of corneal transplantations performed in the United States each year. Although keratoplasty has been successful at visual rehabilitation, graft rejection and lack of suitable donor tissue for transplantation are among the obstacles to reduce cornea-related blindness worldwide. As an alternative to donor corneal transplantation for corneal epithelium dysfunction, cultured limbal stem cell (LSC) transplantation has been performed successfully for the treatment of LSC deficiency to replace the degenerated corneal epithelium. Stromal scarring because of trauma, surgery, or infection is responsible for a significant portion of the corneal blindness worldwide. At present, deep anterior lamellar keratoplasty is the only option to treat corneal stromal disease. The therapeutic potential of stromal keratocytes by differentiating adult dental pulp stem cells, human embryonic stem cells (hESCs), and mesenchymal stem cells for corneal stromal dysfunctions have been reported. In contrast to the epithelium, corneal endothelial cells (CECs) have limited proliferative capability *in vivo*, which is the driving force for efforts to generate CECs from pluripotent stem cells. Several groups used hESCs and induced pluripotent stem cells (iPSCs) to generate CECs and characterized the differentiated CECs through next-generation based RNA-sequencing (RNA-Seq) and mass-spectrometry-based proteome sequencing. We previously reported the generation and proteome profiling of CECs using the peripheral blood mononuclear cell (PBMC)-originated, iPSCs and recently extended our analysis through RNA-Seq-based transcriptome profiling of hESC- and iPSC-derived CECs. As an alternative to donor corneas, multiple therapeutic options for corneal endothelial dysfunction, including cultured CECs and hESC- and iPSC-derived CECs, are currently under investigation. Different preclinical studies demonstrated the regeneration of corneal endothelium following the injection of a cultured and pluripotent stem-cell-derived CECs, in combination with a Rho kinase (ROCK) inhibitor. Recently, a clinical trial using human cultured CECs supplemented with a ROCK inhibitor reported an increased CEC density in 11 patients with bullous keratopathy. In this chapter, we will discuss efforts by various research groups to generate pluripotent stem-cell-derived corneal cells and their possible therapeutic applications in corneal dystrophies.

Key Words: Corneal, Dystrophies, Eye, ESCs, iPSCs, Limbal stem cells, Pluripotent.

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1.1 Introduction

The cornea, a transparent, avascular tissue, comprises the outermost layer of the eyeball and consists of three cellular layers (the epithelium, stroma, and endothelium) and two noncellular layers (Bowman's layer and Descemet's membrane) [1]. The corneal epithelium is a stratified, nonkeratinized layer that covers the external surface of the cornea [2, 3]. The corneal epithelium harbors stem cells, known as limbal stem cells (LSCs), are located in a basal layer of the limbus [1]. A loss or dysfunction of LSCs can result in LSC deficiency (LSCD). Pellegrini *et al.* were the first to report autologous transplantation of *ex vivo* cultivated LSCs for the treatment of LSCD [4].

The corneal stroma is a collagenous intermediate connective tissue that constitutes approximately 80%–90% of the total corneal thickness [5, 6]. Stromal scarring caused by ocular trauma, surgery, or infection can result in stromal opacities, which are significant contributors to corneal blindness worldwide [7, 8]. Buznyk and colleagues implanted bioengineered corneal grafts into three patients and reported the restoration of corneal integrity, and improved vision in two out of three patients [9]. The corneal endothelium is a monolayer of hexagonal cells that are critical for maintaining corneal clarity [10]. The most common factors contributing to the loss of corneal endothelial cells (CECs) are hereditary corneal endothelial dystrophies and surgical trauma [11]. Recently, a clinical trial using human cultured CECs supplemented with a Rho kinase (ROCK) inhibitor reported an increase in CEC density in 11 patients with bullous keratopathy [12].

The pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cell (iPSCs), have been used extensively in the generation of multiple corneal cell types, such as corneal epithelial cells [13], corneal stromal cells [14–16], and CECs [17–22]. In this chapter, we will discuss the stem-cell-based therapies for the regeneration of different corneal layers including corneal epithelial, stromal and endothelial layers.

1.1.1 Corneal epithelial stem cells

The corneal epithelium is a stratified, nonkeratinized layer that covers the external surface of the cornea and plays a major role in protection and transparency [2, 3]. The corneal epithelial stem cells, also known as LSCs, are located in a basal layer of the limbus (the transitional zone between the cornea and the conjunctiva) [1]. A loss or dysfunction of LSC can result in LSCD.[2] LSCs reside in the limbal-stem-cell niche, which is protected from UV-radiation by melanocytes [23–25], and keep the cells in an undifferentiated state [26, 27]. While proliferating, LSCs give rise to two daughter cells, an oligopotent LSC and a transient, rapidly dividing cell that subsequently transforms into terminally differentiated cell [13, 28–30].

LSCD may affect the limbus barrier and induce neovascularization by replacing corneal epithelium with conjunctival epithelial cells, which results in reduced corneal transparency and vision impairment [31]. In general, the etiologies of LSCD can be divided into four groups: physical injury to the ocular surface, congenital stem cell aplasia (e.g., aniridia and sclerocornea), stem cell exhaustion (e.g., Stevens-Johnson syndrome and ocular cicatricial pemphigoid), and idiopathic diseases of unknown cause [13, 32–34].

1.1.2 Stem cell-based therapies for corneal epithelium dysfunctions

Several investigations have highlighted the ability of LSCs to treat LSCD by replacing the degenerated corneal epithelium [35–38]. Pellegrini *et al.* were the first to report on the autologous transplantation of cultivated LSCs for the treatment of LSCD [4]. The autologous and allogeneic transplantation of LSCs has been reported [36]. However, these are hampered by both donor shortage and postoperative complications, including immunological rejection and bacterial or fungal keratitis [31, 37–39]. Although the transplantation of autologous LSCs proved successful in unilateral LSCD patients, an alternative source is still necessary for the treatment of severe bilateral LSCD cases. To address this issue, an attempt was made to treat bilateral LSCD patients using epithelial cell sheet derived from adult autologous human buccal epithelial stem cells [40]. In parallel, Homma and colleagues studied the therapeutic efficacy of epithelial progenitors derived from mouse ESCs when transplanted into a damaged cornea [41]. Hayashi *et al.* was the first to demonstrate the differentiation of human iPSCs into corneal-epithelium-like cells [42].

1.2 Corneal stromal stem cells

The corneal stroma is a collagenous intermediate connective tissue that constitutes approximately 80%–90% of the total corneal thickness [5, 6]. The stroma is made up of highly organized collagen proteins, proteoglycans, glycoproteins, and keratocytes [43–45]. The precise alignment and consistent diameter of stromal collagen fibers are crucial to maintaining the transparency and strength of the cornea [45].

Stromal scarring because of trauma, surgery, or infection is responsible for a significant portion of the corneal blindness worldwide [7, 8]. In the process of stromal scarring, quiescent keratocytes migrate to the damaged area and differentiate into fibroblasts, which disrupt the organization of the extracellular matrix [46–49]. Initially, Funderburgh and colleagues identified cells in bovine corneal stroma that exhibit a gene expression profile consistent with the profile of mesenchymal stem cells [50]. Subsequently, Du *et al.* were the first to identify and isolate keratocyte

progenitor cells from human corneal stroma. The keratocyte progenitor cells exhibit several characteristics of stem cells, such as *in vitro* clonal growth, self-renewal capability, and adult-stem-cell marker *ABCG2* [51]. Another study reported an up-regulated expression of keratocyte-specific markers, including a proteoglycan specific to the corneal stroma in human corneal stromal stem cells (hCSSCs) [52].

1.2.1 Stem cell-based therapies for corneal stromal dysfunctions

Deep anterior lamellar keratoplasty is currently the only option to treat corneal stromal disease [53]. However, this strategy has some limitations, including immune rejection and the scarcity of donor corneas [53]. The limited availability of cadaveric human stromal tissue has driven researchers to develop alternative sources, such as bio-synthetic corneas [54], and alternative treatments, such as the generation of normal stromal tissue with hCSSCs and related cell types [14–16, 55–61]. Du *et al.* described their success in restoring corneal transparency by injecting hCSSCs into the stroma of Lumincan-null mice (a corneal opacity model having disrupted stromal collagen organization) [57]. These findings confirm the application of hCSSCs in cell-based therapeutic approaches for the treatment of stromal scarring [62]. Griffith and colleagues reported the generation of functional human corneal equivalents developed from immortalized human corneal cells [63]. Buznyk *et al.* implanted bioengineered corneal grafts into three patients and reported the subsequent restoration of corneal integrity, by enhancing endogenous regeneration of corneal tissues, and improved vision in two out of three patients [9]. Basu and colleagues reported the regeneration of damaged stromal tissue by grafting human limbal biopsy-derived stromal cells into the corneal wounds of mice [58]. Furthermore, the generation of stromal keratocytes by differentiating adult dental pulp stem cells, human ESCs (hESCs), and mesenchymal stem cells has been reported [64–66].

1.3 Corneal endothelium

The corneal endothelium is a monolayer of hexagonal cells that are critical for maintaining corneal clarity, which is largely dependent on the regulation of osmosis by corneal endothelium barriers and pump functions [10]. The CEC density of a healthy human adult is approximately 2500 cells/mm^2 [53]. The most common factors contributing to the loss of CECs are hereditary corneal endothelial dystrophies and surgical trauma [11]. The physiological function of the corneal endothelium becomes substantially compromised when the cell density falls below 500 cells/mm^2; inadequate corneal endothelium function results in corneal edema and loss of vision [53].

1.3.1 Corneal endothelial stem cells

To identify corneal endothelial stem cells, several research teams isolated human corneal endothelial stem cell precursors using the sphere-forming assay [67, 68]. The precursor cells lack the expression of stem cell markers; however, they did exhibit clonogenic and proliferative potential. These precursor cells exhibited the ability to form a hexagonal monolayer of cells having pump function, suggesting the corneal endothelial progenitor-like cells [67, 68]. Further characterizations of the sphere-forming precursor cells revealed high sphere forming potential of peripheral cells in comparison with the cells closer to the center of endothelium [69, 70]. In another study, prospective progenitor-like cells were identified in the transitional zone between the peripheral endothelium (Schwalbe's line) and the anterior portion of the trabecular meshwork [71–74].

In an initial study, Hirata-Tominaga and colleagues detected the expression of leucine-rich-repeat-containing G-protein coupled receptor 5 (*LGR5*), a stem cell marker, in cells isolated from the peripheral border of the corneal endothelium [75]. They observed an increased proliferation capacity of *LGR5* $^{(+)}$ CECs compared to *LGR5* $^{(-)}$ CECs [75]. They also showed that persistent *LGR5* expression not only maintains the endothelial phenotype but also inhibits endothelial to mesenchymal transformation [75]. Neural-crest markers were subsequently used to isolate and characterize the progenitor-like cells from human corneal endothelial tissue [76, 77]. The progenitor-like CECs expressed several neural-crest markers, including *p75*, *SOX9*, and *FOXC2* [76]. Progenitor-like cells from young corneas exhibited higher proliferative potential than those isolated from older corneas [77]. Katikireddy and colleagues investigated the lineage of progenitor-like cells isolated from normal as well as Fuchs corneal endothelial dystrophy corneal endothelium [77]. The cells isolated from normal and Fuchs corneal endothelial dystrophy corneal endothelium revealed characteristic features of neural-crest-derived progenitor cells, including the absence of senescence, a tendency to form spheres, an increased colony-forming ability, and the expression profile of transcription factors regulating multipotency (*SOX2*, *OCT4*, *LGR5*, and *TP63*) and neural crest progenitor markers (*PSIP1*, *PAX3*, *SOX9*, *AP2B1*, and *NES*) [77].

1.3.2 Stem cell-based therapies for corneal endothelium dysfunctions

Although keratoplasty has been successful for visual rehabilitation, graft rejection and the scarcity of suitable donor tissue are substantial obstacles to the further reduction of worldwide corneal blindness [53]. To find an alternative to donor corneal transplantation, multiple therapeutic avenues are currently under investigation to determine their efficacy in treating corneal endothelial dystrophies. The future candidate therapeutic sources include cultured CECs, isolating and

characterizing corneal endothelial progenitor cells, and hESC- and iPSC-derived CECs. In a recent study, the injection of cultured CECs supplemented with a ROCK inhibitor into the anterior chamber resulted in the corneal endothelium regeneration in rabbit and monkey corneal endothelial dysfunction models [78]. Multiple studies reported that the ROCK inhibitor enhances the adhesion potential of cultured CECs [79, 80]. Recently, a clinical trial using cultured human CECs supplemented with a ROCK inhibitor reported an increase in CEC density in 11 patients with bullous keratopathy [12]. As an alternative to cultured cells, CECs have previously been generated from human pluripotent stem cells [17–22]. Fukuta *et al.* differentiated human pluripotent stem cells into CECs under chemically defined conditions [17]. Recently, McCabe and colleagues generated CECs from hESCs through the neural crest cells [19]. Zhang and colleagues reported the derivation of CEC-like cells from hESCs through the periocular mesenchymal precursor phase and reported that the transplantation of these CEC-like cell sheets restored corneal transparency in rabbits [18].

Recently, Ali and colleagues have successfully developed a personalized approach to generate peripheral blood mononuclear cell (PBMC)-originated, iPSC-derived CECs and an unlimited CECs source using hESCs that closely mimic the molecular architecture of human corneal endothelium [22]. They adopted a 20-day procedure modifying the protocol reported by McCabe and colleagues [19] to generate PBMC-originated, iPSC- and hESC-derived CECs. Briefly, the cryopreserved PBMCs were reprogramed into iPSCs using a Sendai virus delivery system, according to the manufacturer's instructions. Both iPSCs and hESCs were seeded on 35-mm Matrigel-coated plates in 1:12 dilution on day 0 using cell dissociation buffer. The iPSCs and hESCs were grown for 4 days in the mTeSR1 medium. On day 4, mTeSR1 media were replaced with dual Smad inhibitor media containing 500 ng/ml human recombinant Noggin and 10 μM SB431542 in a basal media of 80% Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM-F12), 20% knockout serum replacement (KSR), 1% nonessential amino acids, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 8 ng/ml basic fibroblast growth factor (bFGF).

On day 6, dual Smad inhibitor media were replaced by cornea medium containing 0.1 × B27 supplement, 10 ng/ml recombinant human platelet-derived growth factor-BB, and 10 ng/ml recombinant human Dickkopf related protein-2 in a basal media of 80% DMEM-F12, 20% KSR, 1% nonessential amino acids, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 8 ng/ml bFGF. On day 7, the differentiating CECs were transferred to new Matrigel-coated plates (35 mm) and were grown in cornea medium for 13 additional days. The differentiated CECs were harvested on day 20 for mass-spectrometry-based proteome sequencing and next-generation based RNA-Seq.

The morphological examination on day 20 illustrated tightly packed cells with a hexagonal/polygonal appearance for both hESC-derived CECs (Fig. 1.1) and iPSC-derived CECs (Fig. 1.2).

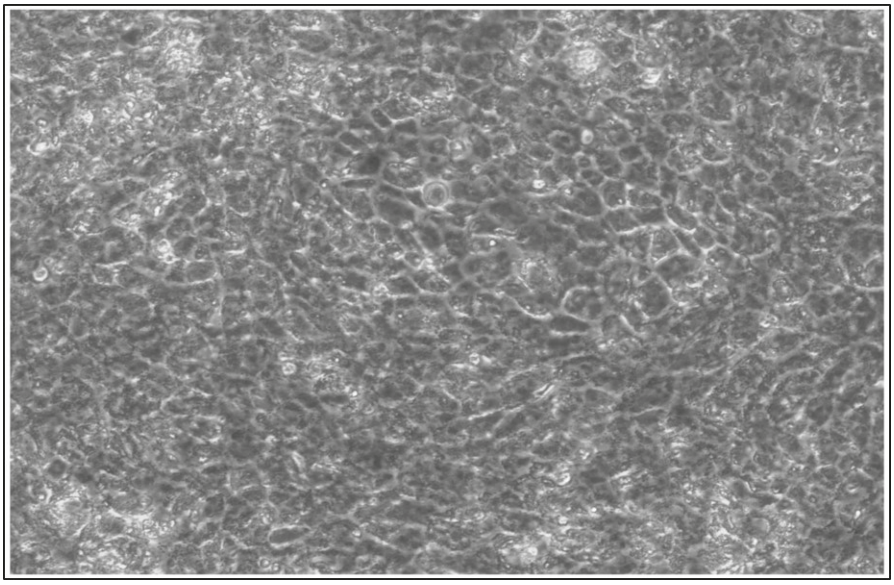

Fig. 1.1: Generation of corneal endothelial cells (CECs) from H9 human embryonic stem cells (hESCs). Phase contrast microscopy of differentiated CECs illustrating CEC-like hexagonal/polygonal morphology at day 20 (D20).

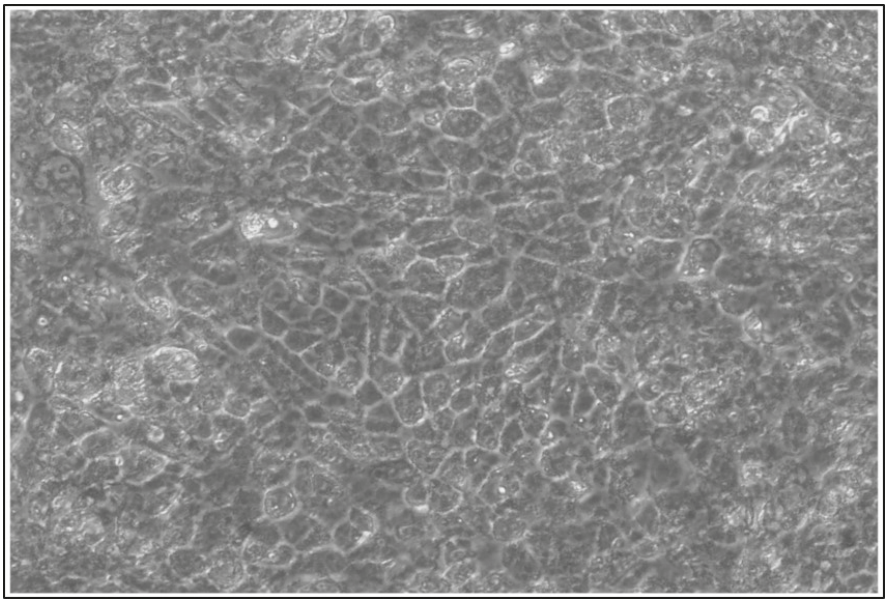

Fig. 1.2: Generation of corneal endothelial cells (CECs) from human peripheral blood mononuclear cell (PBMC)-originated, induced pluripotent stem cells (iPSCs). Phase contrast microscopy of differentiated CECs illustrating CEC-like hexagonal/polygonal morphology at day 20 (D20).

Next, we examined hESC- and iPSC-derived CECs for expression of zona occludens-1 (ZO-1) by immunostaining, which illustrated the expression of ZO-1 at cell boundaries, and importantly, the staining patterns were indistinguishable between hESC- and iPSC-derived CECs (Figs. 1.3 and 1.4). Taken together, the data from phase contrast microscopy and immunostaining confirm the structural integrity of hESC- and iPSC-derived CECs.

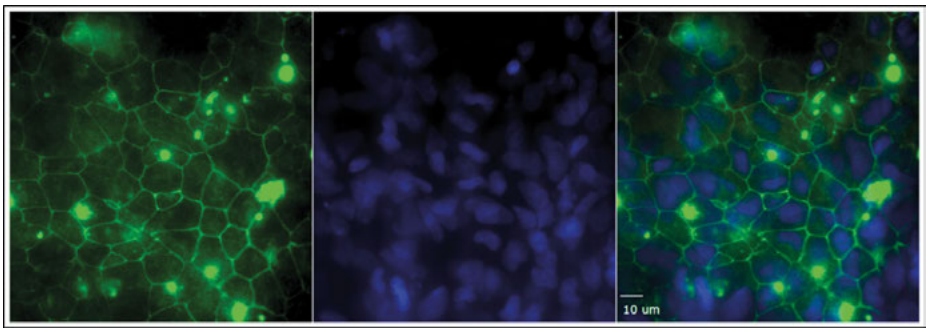

Fig. 1.3: Characterization of H9 human embryonic stem cell (hESC)-derived corneal endothelial cells (CECs) by immunocytochemistry. Immunostaining for zona occludens-1 (ZO-1, a tight junction protein) of hESC-derived CECs. Cell nuclei were counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI). Images are taken at 60× magnification and the scale bar represents 10μm.

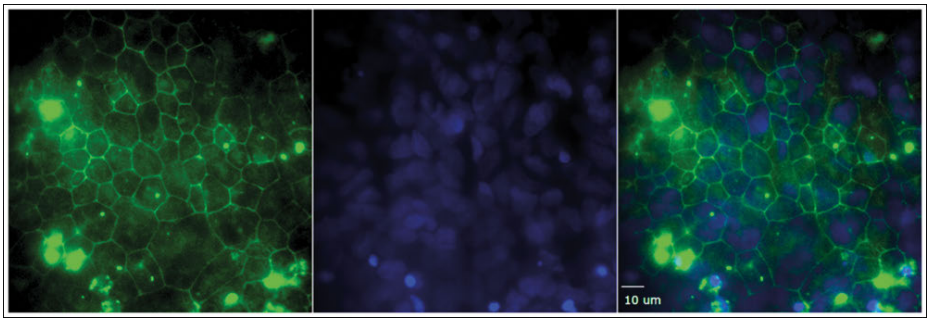

Fig. 1.4: Characterization of human peripheral blood mononuclear cell (PBMC)-originated, induced pluripotent stem cell (iPSC)-derived corneal endothelial cells (CECs) by immunocytochemistry. Immunostaining for ZO-1 of human PBMC-originated, iPSC-derived CECs. Cell nuclei were counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI). Images were taken at 60× magnification and the scale bar represents 10 μm.

Mass-spectrometry-based label-free quantitative proteome profiling was performed to understand the molecular composition of iPSC-derived CECs at day 20. The proteome sequencing generated a total of 463,737 tandem mass spectrometry (MS/MS) counts,

yielding 162,795 total peptides, including 129,750 unique peptides in iPSC-derived CECs. In total, proteome sequencing identified 10,575 proteins in the iPSC-derived CECs. In parallel, a comparative analysis suggested that more than 90% of the human corneal endothelium proteome overlaps with the iPSC-derived CEC proteome. In another study, Ali and colleagues extended their analysis by performing next-generation RNA-Seq profiling of hESC- and PBMC-originated, iPSC-derived CECs [81]. Next-generation RNA-Seq generated a total of 180.18 and 179.01 million reads for hESC- and iPSC-derived CECs, respectively. The raw reads were aligned to the human reference genome (GRCh38.p11), which resulted in the mapping of 176.31 (97.85% of total reads) and 174.82 (97.66% of total reads) million reads for hESC- and iPSC-derived CECs, respectively. The mapped reads represent 251.87× and 249.75× sequence coverage for hESC- and iPSC-derived CECs, respectively, for the 70 Mb *Homo sapiens* transcriptome. The mapped reads (176.31 and 174.82 million) were further assembled into transcripts, and gene expression was determined and normalized using the RPKM (reads per kilobase per million mapped reads; RRKM = calculated as total exon reads/mapped reads in millions × exon length in kb) method. These analyses identified the expression (≥ 0.659 RPKM) of 13,546 and 13,536 genes in hESC- and iPSC-derived CECs, respectively, which suggests that ~68% of the total human protein-coding transcriptome is expressed in CECs. Comparative transcriptome analysis of hESC- and iPSC-derived CECs revealed 13,208 (> 96%) genes common in both transcriptomes. Among the 13,208 genes common in these transcriptomes, 12,580 (> 95%) exhibited a quantitatively similar expression. The analysis strongly suggests that both the hESC- and the iPSC-derived CECs have largely equivalent transcriptomes.

1.4 Future prospects

Corneal dystrophies are the leading cause of corneal transplantation, and so far, only keratoplasty has been successfully applied for visual rehabilitation. However, graft rejection and lack of suitable donor tissue for transplantation are among the obstacles to reduce cornea-related blindness worldwide. The stem-cell-derived corneal cells such as epithelial and endothelial cells can provide an alternative source to donor corneal transplantation with an overall goal to maximize the availability of these cells for treating corneal dysfunctions.

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2 Stem cells and lung cancer: between advanced diagnostics and new therapeutics

Abstract: Lung cancers (LCs) remain a significant and devastating cause of morbidity and mortality worldwide. Despite the very recent success of immunotherapy, the diagnosis and treatment of LC remain one of the greatest challenges in chest surgery, clinical oncology, and molecular medicine. A growing number of investigations on normal/cancer stem cells and cellular therapies are offering exciting new avenues to advance knowledge on LC. Here, we will be focusing on the multiple relationships between LC and stem cells accounting for cancer stem cell (CSC) diagnostics and progenitor-based therapeutics for LC. Cancer cell repopulation after chemotherapy and/or radiotherapy still represents a major factor limiting the efficacy of treatment since CSCs play critical roles during this process by reciprocal connections between CSCs and tumor microenvironment. This calls for new opportunities to integrate advanced CSC diagnostics and targeted approaches also based on immunotherapy. In addition, recent discoveries on malignant pleural and other LC highlight that mesenchymal stromal/stem cells may be a novel platform for drug delivery within still unexplored gene therapy strategies. This chapter will dissect these two apparently distant technologies within a unified stem-cell-based vision aimed at providing better diagnostics and therapeutics for LC at the forefront of modern clinical oncology.

Key Words: Cancer, Carcinoma, Lung, MSCs, Stem cells.

2.1 Introduction

2.1.1 Lung cancer epidemiology

Lung cancer (LC), which has a low survival rate, is a leading cause of cancer-associated mortality worldwide. Reports of LC in the scientific literature date back to the early 1400s, when up to 50% of miners working along the border of Germany and the Czech Republic died of a pulmonary disease called *bergkrankheit* (mountain disease) [1–3]. In 1879, Harting and Hesse performed 20 autopsies on miners and described pulmonary sarcoma in 75% of these patients diagnosed with *bergkrankheit*. It was hypothesized that dust inhalation was a causative factor of this illness, which was later identified as squamous cell carcinoma of the lung [4]. Investigators in the 1920s and 1930s proposed radiation and radon gas as potential etiologic agents. With the incidence

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of LC increasing in the 1930s, Ochsnerand De Bakey reviewed the increasing number of LCs among their patients and concluded that cigarette smoke inhalation was a probable responsible factor [5]. Sir Richard Doll and Austin Hill's landmark article in 1950 described mounting evidence that LC was associated with cigarette smoking [6]. The 1962 report by the Royal College of Physicians and the 1964 warning by the surgeon general of the United States firmly established the correlation between cigarette smoking and LC [6, 7]. It is now known that most deaths from LC (up to 85%), which is now the leading cause of cancer mortality in the United States, are directly attributable to smoking [8, 9].

Incidence and mortality attributed to LC have risen steadily since the 1930s, predominantly due to the popularity of cigarette smoking [1]. In the past 100 years, LC has therefore been transformed from a rare disease into a global problem [1].

In 2012, the world age-adjusted incidence rate of LC was 34.2/100,000 for men and 13.6/100,000 for women. The rate translated to 1.82 million new LC cases (1.24 million men and 0.58 million women), an increase from 2002 estimates (1.35 million for both genders) [10]. Among the geographic regions, males in Central and Eastern Europe had the highest incidence rate (53.5/100,000), followed by males in Eastern Asia (50.4/100,000). The highest incidence rates among females were in North America (33.8/100,000) and Northern Europe (23.7/100,000).

With regard to mortality, in 2012, the world age-adjusted mortality rate of LC was 30.0/100,000 for men and 11.1/100,000 for women. There were 1.59 million deaths attributable to LC, and the number has increased from 1.18 million deaths in 2002 [10]. Among the geographic regions, males in Central and Eastern Europe had the highest mortality rate (47.6/100,000), followed by males in Eastern Asia (44.8/100,000). The highest mortality rates among females were in North America (23.5/100,000) and Northern Europe (19.0/100,000). Smoking and air pollution are the major causes of LC; however, numerous studies have demonstrated that genetic factors also contribute to the development of LC. Despite very recent success of immunotherapy, the diagnosis and treatment of LC remain one of the greatest challenges in chest surgery, clinical oncology and molecular medicine.

In fact, the low survival rate in LC patients is related to the stage of LC at diagnosis. In the United States during 2005–2011, LC patients diagnosed when localized and regional had moderate 5-year survival rates (55% and 27%, respectively); however, this decreased to 4% for those with distant cancer [11]. Due to the nonspecific nature of LC symptoms, the majority of LCs are typically diagnosed after it has advanced (57% of LCs in the United States are detected with metastases) [11].

2.1.2 LC *standard* treatments

Surgery is the cornerstone of management for patients with early-stage (I–II) non-small cell lung cancer (NSCLC) and selected patients with stage IIIA disease (T3N1)

according to TNM (T= Primary Tumor; N= involvement of lymph node; M= metastasis) 8th edition [12, 13]. The Lung Cancer Study Group concluded that lobectomy is a superior operation for T1N0 NSCLC, based on a randomized trial of lobectomy versus more limited resection [14]. Thus, in patients with stage I and II NSCLC who are medically fit for conventional surgical resection, lobectomy or greater resection is recommended rather than sublobar resections (wedge or segmentectomy) [12]. In patients who, for medical reasons (severely compromised pulmonary function, advanced age, or other extensive comorbidity), cannot tolerate a full lobectomy, a more limited operation (sublobar) is recommended [10]. For patients with more advanced tumor in whom complete cancer resection cannot be achieved with lobectomy, sleeve lobectomy is recommended over pneumonectomy because it preserves pulmonary function [12].

Although surgery is the treatment of choice for NSCLC patients with early-stage disease, some never undergo surgery. Common reasons for not undergoing surgery are older age, the presence of serious comorbidities, and patient refusal. For those patients who do not undergo an operation, radiotherapy can be administered with curative intent, albeit with lower survival rates when compared to surgery [15, 16].

Recent data from randomized adjuvant clinical trials [17–19] and a recent meta-analysis [20] have changed the standard of care for patients with completely resected NSCLC. The survival benefit observed with adjuvant chemotherapy was confirmed by a meta-analysis of five randomized trials [17, 18, 20–22] with 4584 patients registered in the Lung Adjuvant Cisplatin Evaluation database [19]. This meta-analysis demonstrated a 5.4% increase in 5-year survival in favor of adjuvant chemotherapy compared with observation (hazard ratio: 0.89; 95% confidence interval: 0.82–0.96) [20]. The survival benefit varied according to stage and was most pronounced for patients with stage II and IIIA disease. The improvement in survival in patients with stage IB disease did not reach statistical significance. Patients with stage IA disease appeared to do worse with adjuvant chemotherapy. Some retrospective data suggest that patients with stage IB disease and tumor ≥4 cm may also benefit from adjuvant chemotherapy [23]. With regard to postoperative radiotherapy (PORT) meta-analysis [24, 25], which included 2128 patients, it has been demonstrated that the use of PORT was associated with a detrimental effect on survival, which was more pronounced for patients with lower nodal status. This analysis has been criticized, however, for its long enrolment period and use of different types of machines, techniques, and doses.

Up to one-third of patients with NSCLC present with disease that remains local to the thorax but is considered too extensive for surgical treatment (stages IIIA and IIIB). Concurrent chemoradiotherapy is considered the standard therapy for unresectable stage III NSCLC [26]. The concurrent administration of chemotherapy plus radiotherapy results in a modest but statistically significant survival benefit compared with sequential administration, as demonstrated by randomized phase III trials

[27, 28]. This approach is, however, associated with significant toxicity and it applies only to patients with good performance status [29].

There is another issue influencing the battle against cancer: cancer heterogeneity. Essentially, not all tumor cells are identical for biology and chemosensitivity. This is one of the reasons contributing to the treatment failure and disease progression. Surgery can successfully remove cancer from the body, while combining radiotherapy with chemotherapy can effectively give better results for treating many types of cancer [30, 31]. However, chemotherapy can also induce tumor heterogeneity, resulting in suboptimal anticancer action, ultimately contributing to treatment failure and disease progression [32, 33]. Chemoresistance is a major problem in the treatment of cancer patients, as cancer cells become resistant to chemical substances used in treatment, which consequently limits the efficiency of chemo agents [34]. It is also often associated with tumors turning into more aggressive form and/or metastatic type [35–38].

2.1.3 LC and immuno-target therapy

During the past few decades, research has provided breakthroughs that have enhanced our understanding of the mechanisms and pathways that regulate the immune system's response to cancer [39]. However, despite these advances, obstacles still exist in cancer immunotherapy [39]. These include the inability to predict treatment efficacy and patient response, the need for additional biomarkers, the development of resistance to cancer immunotherapies, the lack of clinical study designs that are optimized to determine efficacy, and high treatment costs [40–49]. Future advances in cancer immunotherapy are expected to overcome and resolve many of these challenges. A major challenge for cancer immunotherapies is the need to develop agents that are consistently effective in a majority of patients and cancer types [40]. Dramatic results have been observed in some patients treated with cancer immunotherapies, indicating that it is feasible to restore effective antitumor immune surveillance [40]. However, to date, many immunotherapy treatments have demonstrated efficacy in only a select group of cancers, and usually in a minority of patients with those cancers [41, 42, 48].

Reasons for the variability in patient response to cancer immunotherapies have been proposed, including the need to identify additional biomarkers and cancer pathways, again tumor heterogeneity, variability in cancer type and stage, treatment history, and the still largely obscure immunosuppressive biology cancer [40, 43]. Treatments that target single molecular mutations or cancer pathways have only modestly affected survival in some cancers [44]. This approach, which has been described as "reductionist," might be improved by administering drug combinations that target multiple mutations and cancer pathways [44]. In addition, a large number of the

mutations found in human tumors do not occur with meaningful regularity among different patients [50]. Therefore, immunotherapies directed at molecular mutations most likely need to be customized and patient specific in order to be more effective [50–52].

One major limitation of cancer immunotherapy is the availability of known targetable tumor-specific antigens (TSAs), also called "neoantigens," that are solely expressed by tumor cells [39, 40]. Tumor-associated antigens, which are expressed by both tumor and normal tissues, also provide an option for immunotherapy, but targeting them is likely to cause off-target toxicities and has achieved little success [39, 40].

Moreover, it is important to develop cancer immunotherapies that enhance TSA-specific T-cell reactivity [39]. Identifying biomarkers that have predictive or prognostic value for use in selecting patients who will benefit from treatment with cancer immunotherapy is a lengthy and difficult process [44]. To date, few predictive biomarkers for cancer immunotherapy treatments have been robustly validated [44]. Still, a predictive benefit has been observed for certain biomarkers with respect to response rate in patients with oncogene addicted tumors when those patients receive matched targeted immunotherapies [44]. For example, human epidermal growth factor receptor 2 amplification has been found in 20% of patients with gastric cancer; these patients have been found to exhibit a response rate of 40% to 50% when treated with the monoclonal antibody trastuzumab [44]. PD-L1 has been perhaps the most investigated biomarker with regard to potential predictive capabilities; it has been studied in numerous randomized controlled trials [44]. Evidence in different tumor types has suggested that the higher the PD-L1 expression by the tumor, the better the response rate and survival rates with PD-1/PD-L1 ICB treatment [43, 44]. Interestingly, however, it has been found that treatment benefits with PD-1/PD-L1 ICBs are not solely restricted to PD-L1-positive patients [43, 44].

However, TSAs have been extensively investigated and are thought to be a promising category of immunotherapy targets [40, 43]. The features making TSAs potentially optimal biomarkers for cancer immunotherapy include highly selective expression in tumor versus normal tissues, broad expression in a variety of human cancers of different histological origins, and remarkable "immunogenicity" allowing the induction of humoral and/or cellular immune responses in cancer patients [40, 43]. TSAs may also be optimal targets for cancer immunotherapy directed at cancer stem cells (CSCs) [40]. TSAs are expressed by CSCs and play a role in CSC differentiation and biology [40]. Most cells comprising a tumor mass are thought to result from the differentiation and cloning of a small number of CSCs that maintain and constantly "feed" the growth of the tumor [40]. With evidence that CSCs exist in many different tumors, it is imperative to identify and understand tumor antigens expressed by CSCs.

2.2 CSC role in LC

CSCs (Fig. 2.1), also known as tumor-initiating cells, have been intensively studied in the last decades, focusing on the possible source, origin, cellular markers, mechanism study, and development of therapeutic strategy targeting their pathway [53–55].

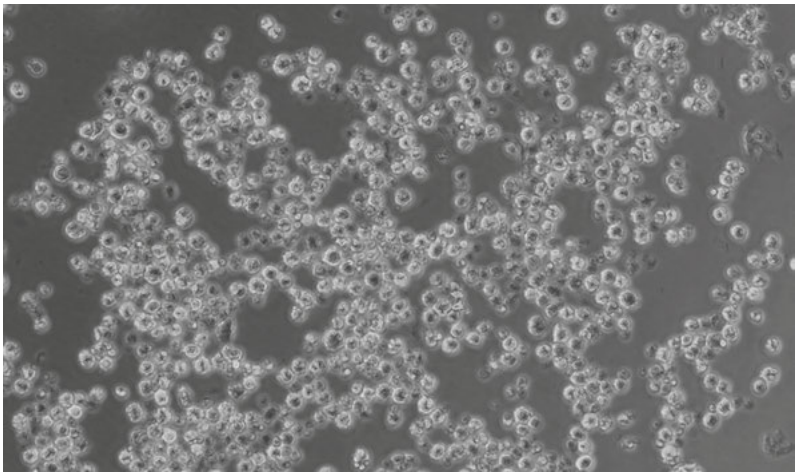

Fig. 2.1: How cancer stem cells look like before sphere formation.

In 1963, Bruce *et al*. observed [56] that only 1%–4% of lymphoma cells (not all cancer cells) can form colonies *in vitro* or initiate carcinoma in mouse spleen (Fig. 2.2). However, the first compelling evidence proving the existence of CSCs is generally acknowledged to have been provided in 1997 when scientists discovered that only the CD34+/CD38– cells from acute myeloid leukemia patients could initiate hematopoietic malignancy in NOD/SCID mice and showed that these cells had the characteristics of stemness: self-renewal, proliferation, and differentiation [55]. Thus, CSCs had the ability to differentiate into the spectrum of all cell types observed in tumors and the ability for the growth of the primary cancer tumor as well as the development of new tumors [57, 58].

CSCs are also able to induce cell cycle arrest (quiescent state) that supports their ability to become resistant to chemotherapy and radiotherapy [59–64]. Common chemotherapeutic agents target the proliferating cells to lead their apoptosis [63]. Although successful cancer therapy abolishes the bulk of proliferating tumor cells, a subset of remaining CSCs can survive and contribute to cancer relapse due to their ability to establish higher invasiveness and chemoresistance [65, 66]. Understanding the features of CSCs is important to establish the foundation for new era in the treatment of cancer. In this review, we address the detailed mechanisms by which CSCs display the resistance to chemotherapy and radiotherapy and their implication for

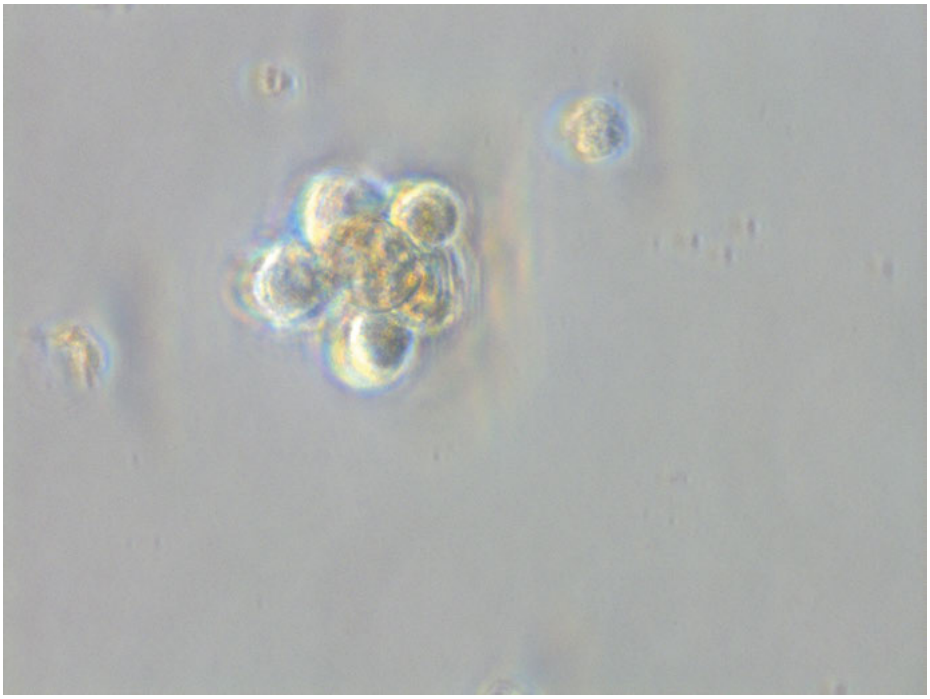

Fig. 2.2: Cancer stem cell spheroid.

clinical trials. Of note, CSCs can be identified by specific markers transferred from normal stem cells, which are commonly used for isolating CSCs from solid and hematological tumors [67]. Several cell surface markers have been verified to identify CSC enriched populations, such as CD133, CD24, CD44, EpCAM (epithelial cell adhesion molecule), THY1, ABCB5 (ATP-binding cassette B5), and CD200 [68–71]. Additionally, certain intracellular proteins also have been used as CSCs markers, such as aldehyde dehydrogenase 1 (ALDH1), which is used to characterize CSCs in many types of cancer such as leukemia, breast, colon, liver, lung, pancreas, and so forth [72, 73]. The usage of cell surface markers as CSC markers might differ from each cancer types depending on their characteristics and phenotypes (Tab. 2.1).

In recent years, there has been an increasing amount of evidence to support a CSC phenotype in human LC [74–76]. Many of these markers have also been found in other tumors and, indeed, in normal stem cells; in fact, they are now widely regarded to be stem cells in a number of malignancies, such as lung, breast, and glioblastomas, as well as in normal hematopoietic cells [77–82]. Although recent studies have contributed to a better understanding of CSC surface molecules, the picture is not yet complete. It is often observed that CSCs do not express the same markers, or that normal cells also express these surface antigens. Therefore, it is not possible yet to

Tab. 2.1: Overview of cancer stem cell markers and their functions [69].

Cell surface marker	Cancer types	Functions
CD44+	Breast, ovarian, prostate, colon, pancreatic, lung	Glycoprotein involved in migration, cell adhesion, and chemoresistance
CD24–	Breast	Down regulates the CXCR4/SDF-1 pathway
CD133	Ovarian, glioblastoma, lung, prostate, colon, renal, melanoma	Glycoprotein involved in cell growth, metastasis and chemoresistance
CXCR4	Pancreatic	Metastasis
ALDH1	Breast, head and neck, lung	CSC self-protection, differentiation, expansion, and chemoresistance

certainly isolate CSCs, but only to identify a CSC-enriched population. Consequently, identification of CSCs must be based on additional functional assays, such as the ability to form spheres in serum-free medium and to initiate tumor growth after serial transplantation in immunocompromised animal models, based on their self-renewal capacity. However, these assays also have limitations due to microenvironment. Therefore, to specifically address CSCs in further experiments, it is necessary to sort cells based on surface markers and subsequently to assess their functional abilities by *in vitro* and *in vivo* assays.

The surface markers used for the identification and isolation of CSCs are also important targets for therapy [83–91]. Immunotherapy that involves antibodies targeting CSC-specific markers is often used as an adjunct to chemotherapy, radiotherapy and surgery [92]. The most important CSC-associated markers, together with strategies for targeting them, are, for example, CD133 and CD44, even if they are not restricted to CSCs [93]. One of the mechanisms by which CSCs manage to avoid or to survive cancer treatments seems to be represented by signals generated within the tumor microenvironment, due to dysregulation of signaling pathway networks [83]. Like normal stem cells, CSCs use signaling pathways that are essential for self-renewal, proliferation, and differentiation in order to preserve stem cell properties, but the final result is carcinogenesis. Many studies have also focused on signaling pathways to deregulate CSCs attempting to find new strategy for cancer therapy; this line of research is promising mainly because many cancers present up- or down-regulation of the same signaling cascades. In this regard, CSCs can be identified by surface markers but also by the signals they send in tumor microenvironment [84]. The major involved pathways in the regulation of self-renewal and differentiation of normal and CSCs are Notch, Hedgehog, Wnt/b-catenin, nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), phosphatidylinositol 3-kinase/ Protein kinase B (Akt), and Phosphatase and tensin homolog (PTEN); sustained by aberrant activation of these pathways, CSCs have the capacity to initiate cancer and promote recurrence after the surgical removal of tumor [85].

It has been shown that CSCs may express ABC transporters such as ABCG2, MDR1, ABCA2, etc., which have important roles in chemoresistance by active efflux of the drug from within the cell [80], and they have been successfully identified in both NSCLC and small-cell LC cell lines [74–82]. Ho *et al.* examined CSC fractions in six NSCLC cell lines and a small number of clinical samples [74]. They found that this fraction was the tumorigenic population in a xenotransplantation model requiring far fewer cells to initiate a tumor than the non-CSC fraction. Subsequent analyses of the cancer stem like cell-derived tumors also showed their differentiation into both CSC and non-CSC cells. This repopulation ability was also confirmed *in vitro*.

2.3 Role of chemotherapy and radiotherapy on CSC and tumor microenvironment

Understanding the mechanisms of chemoresistance in cancer could help to predict disease progression, develop new therapies, and personalize systemic therapy. In the last few decades, CSCs have proven to play a key role in "tumor initiation" and may also act as a key factor for chemoresistance and recurrence of the disease following chemotherapy. Resistance to anticancer therapy in patients has been attributed toward a number of factors controlling the stemness character of the CSCs that leads to therapeutic resistance.

Recent studies suggest that CSCs are enriched after chemotherapy, because a small subpopulation of cells remaining in tumor tissue that can survive and expand through most chemotherapeutic agents kill bulk of the tumors [91, 94, 95]. For instance, preleukemic DNMT3Amut hematopoietic stem cells, which can initiate clonal expansion as the first step in leukemogenesis and regenerate the entire hematopoietic hierarchy, were found to survive and expand in the bone marrow remission after chemotherapy [94]. Similarly, exposure to therapeutic doses of temozolomide, the most commonly used antiglioma chemotherapy, consistently expands the glioma stem cell (GSC) pool over time in both patient-derived and established glioma cell lines, which has been shown to be a result of phenotypic and functional interconversion between differentiated tumor cells and GSCs [96]. Therefore, by understanding the mechanisms and oncogenic drivers by which the CSCs escape radiotherapy and chemotherapy, we can develop more effective treatments that could improve the clinical outcomes of cancer patients. In order to survive during and after therapy, CSCs display many responses, including epithelial mesenchymal transition, self-renewal, tumor environment, quiescence, multidrug resistance, and dormancy. Moreover, tumor microenvironment may play a crucial role in protecting CSCs from the cytotoxic effect of chemotherapeutic drugs [97–102]. In fact, cells within the CSC microenvironment are capable of stimulating signaling pathways [82], such as Notch [103–105] and Wnt [106–108], which may facilitate CSCs to metastasize, evade anoikis, and alter divisional dynamics, achieving repopulation by symmetric division [106, 109–111].

2.4 New generation therapies based on normal stem cells to target CSC

Traditional therapies against cancer, chemotherapy and radiotherapy, have multiple limitations that lead to treatment failure and cancer recurrence. These limitations are related to systemic and local toxicity, while treatment failure and cancer relapse are due to drug resistance and survival also associated with CSC self-renewal. Therefore, in order to develop efficient treatments that can induce a long-lasting clinical response preventing tumor relapse, it is important to develop drugs that can specifically target and eliminate CSCs. Combined therapy using conventional anticancer drugs with CSC-targeting agents may offer a promising strategy for management and eradication of different types of cancers [112–114].

Besides the possibility of developing new therapies targeting CSCs, a normal population of progenitor/stem cells, namely, mesenchymal stromal/stem cells (MSCs), has been recently used as cellular vehicle for therapeutic compounds [115]. MSC can carry anticancer agents allowing a revision of the old chemotherapy-based paradigms [114]. This introduced novel therapeutic opportunities based on genetically engineered MSCs whose properties make them a unique and promising option in cancer therapy [114].

Some of the distinct properties of MSCs, such as nonimmunogenicity, stimulatory effect on the anti-inflammatory molecules, inhibitory effect on inflammatory responses, nontoxicity against normal tissues, and easy processes for clinical use, have been important prerequisites for MSCs clinical translation for cancer [114]. In 2015, Lathrop *et al.* focused the attention on malignant mesothelioma (MM), a still highly deadly lung malignancy with poor treatment options [116]. MM cells further promote a highly inflammatory microenvironment, which contributes to tumor initiation, development, severity, and propagation. This group engineered MSCs in order to overexpress tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) protein (MSC-TRAIL), which would effectively inhibit mesothelioma growth [116]. Using a mouse xenograft model of intraperitoneal human mesothelioma, native mouse (mMSCs) or human (hMSC) MSCs were administered systemically (intravenously or intraperitoneally) at various times following tumor inoculation [116]. Both mMSCs and hMSCs localized at the sites of MM tumor growth *in vivo* and decreased local inflammation. Parallel studies of *in vitro* exposure of nine primary human mesothelioma cell lines to mMSCs or hMSCs demonstrated reduced tumor cell migration [116]. However, there is an aspect that has to be better defined regarding the role of MSC in tumor tropism. This seems to be related to the type of tumor and histotype, as demonstrated by tumor development after subcutaneous coadministration of MSCs with allogeneic melanoma cells [117, 118]. This effect was attributed to the immunosuppressive effect of MSCs, which suppressed the host immune reaction to the allogeneic melanoma cells. [117]. Engineered MSC-TRAIL served as a platform for an efficient and targeted form of therapy [112, 116, 117].

In particular, TRAIL can bind to a receptor preferentially expressed on tumor cells, causing them to undergo apoptosis, and engineered MSCs have proven highly effective against cancer cell lines and animal models of cancer, including LC [112]. Recently, Janes and colleagues have developed clinical-grade engineered MSCs showing that they retain their potency even after freezing [119]. Patients with advanced metastatic LC are now being recruited for a phase I/II trial that will compare a combination of TRAIL-armed MSCs and chemotherapy with the current chemotherapy standard of care. Even if MSC-TRAIL can target CSCs, which are resistant to many conventional chemotherapies, and act synergistically with chemotherapy, the presence of TRAIL-resistant CSC clones shall be taken into account for more effective treatment [113, 120]. Nonetheless, with the discovery of small molecular inhibitors that could target CSCs and tumor signaling pathways, a higher efficacy of MSC-TRAIL-mediated tumor inhibition can be achieved [114]. This might pave the way for a more effective form of combinatory therapies, which shall lead to a better treatment outcome [113]. However, the major problem regarding LC diagnosis is that the patients received a diagnosis in an advanced-stage disease and that a large part of these patients did not survive despite treatment. Similarly, the prognosis remained poor even in locally advanced disease because of the high relapse rate and early formation of micro-metastases. CSCs were thought to be a primary obstacle to cancer therapy; for this reason, great effort has been lavished for the development of anti-CSC strategies [121]. Recently, researchers focused their attention in the delivering or administration of drugs that eliminate CSCs, which might represent a more efficient therapeutic approach for the treatment of patients with recurrent or advanced stage LC [121]. Studies have been published to find out new efficient drugs targeting CSCs, especially due to the fact that CSCs possessed drug resistance granted by their ability to actively expel therapeutic drugs *via* transport proteins, such as ATP-binding cassette. These proteins use ATP-dependent efflux pumps to eliminate drugs into the extracellular space [122]. Methods for the administration of anticancer drugs have been evaluated in order to maximize their effects, minimizing side effects in normal tissues and damage in normal stem cells. Scientists had to take into account some important aspects, such as the vascular endothelial thinness in a cancer cell with respect to normal ones, that facilitate the delivering and, in addition, the lack of an effective lymphatic drainage ensured drugs to be much more easily retained in cancer than in normal tissues. This last feature was called a retention effect used extensively in anticancer drugs modified with liposome, nanomaterials, or high–molecular weight polymers [123].

Currently, there were two methods used for discovering new efficient drugs, one was based on validation of old drugs targeting CSCs and the other one relied on a traditional method build on a high-throughput screening, which is profitable for discovering new drugs among many compounds [124]. Drugs should impact CSCs, inhibiting their self-renewal activity, inducing apoptosis, oxygen reactive species, and ALDH, moreover inactivating the ubiquitin-proteasome pathway. An example was the case of

the drug thioridazine, an antipsychotic, which selectively targets leukemia stem cells via the dopamine receptors, without being cytotoxic to normal blood stem cells [125], and its anticancer potential was also reported in breast and gastric carcinoma [126]. Studies made on disulfiram, a drug used for treating alcoholism, showed anticancer activity *in vitro* and *in vivo*, further potentiating the chemotherapeutic response. Its effectiveness has been demonstrated on paclitaxel resistant triple-negative breast cancer cells, in NSCLC cells, and glioblastoma [127, 128].

Another new branch regarding the identification of new drugs anti-CSCs is represented by oncolytic viruses, which differ from those of conventional therapies and represent a completely different class of therapeutics that can kill cancer cells in a variety of ways. Unlike radiation and chemotherapeutics, many oncolytic viruses, including vaccinia virus, adenovirus, HSV, and retrovirus, can infect both quiescent and dividing cells and replicate efficiently in those cells. Consequently, most oncolytic viruses tested against CSCs have been found to have more or less similar efficacy in killing CSCs and non-CSCs. Recent studies have shown that oncolytic viruses can efficiently kill CSCs in many types of cancer [129]. While several preclinical studies have shown that oncolytic virus as a monotherapy may be effective against some malignancies, it would be logical to combine oncolytic virus with traditional therapies to achieve greater therapeutic benefits [129]. Given the fact that oncolytic viruses and traditional therapies exert their antitumor effect through different mechanisms, one would expect to achieve additive, if not synergistic, antitumor effect from combination therapies. Indeed, several studies have shown that combination of oncolytic virus with chemotherapy or radiation therapy results in synergistic antitumor effect in animal models [130–133]. Additionally, transgenes ranging from toxic genes for direct killing of cancer cells to immune-stimulatory genes for activation of antitumor immunity could be inserted into oncolytic viruses to further increase the overall efficacy of oncolytic viruses. One major concern in the use of oncolytic virus for killing CSCs is that CSCs have many properties in common with normal stem cells. Therefore, oncolytic viruses may kill CSCs and normal stem cells to similar levels. However, several studies have shown that despite similarities between normal stem cells and CSCs, oncolytic viruses specifically kill CSCs while leaving normal stem cells unharmed [128–136].

In conclusion, CSCs in LC are important diagnostic and therapeutic targets to consider (Fig. 2.3).

They also represent a relevant cell type to study focusing on drug resistance and cancer heterogeneity accounting also for the immunological pressure due to the latest checkpoint inhibitor therapy. Other stem cell types may have to be accounted for by therapeutic purposes. This is the case of normal MSCs that are now the delivery vehicles of therapeutic genes as a new approach in the treatment of various types of cancers. In addition, the distinct properties of MSCs, such as tumor-tropism, non-immunogenicity, stimulatory effect on the anti-inflammatory molecules, inhibitory

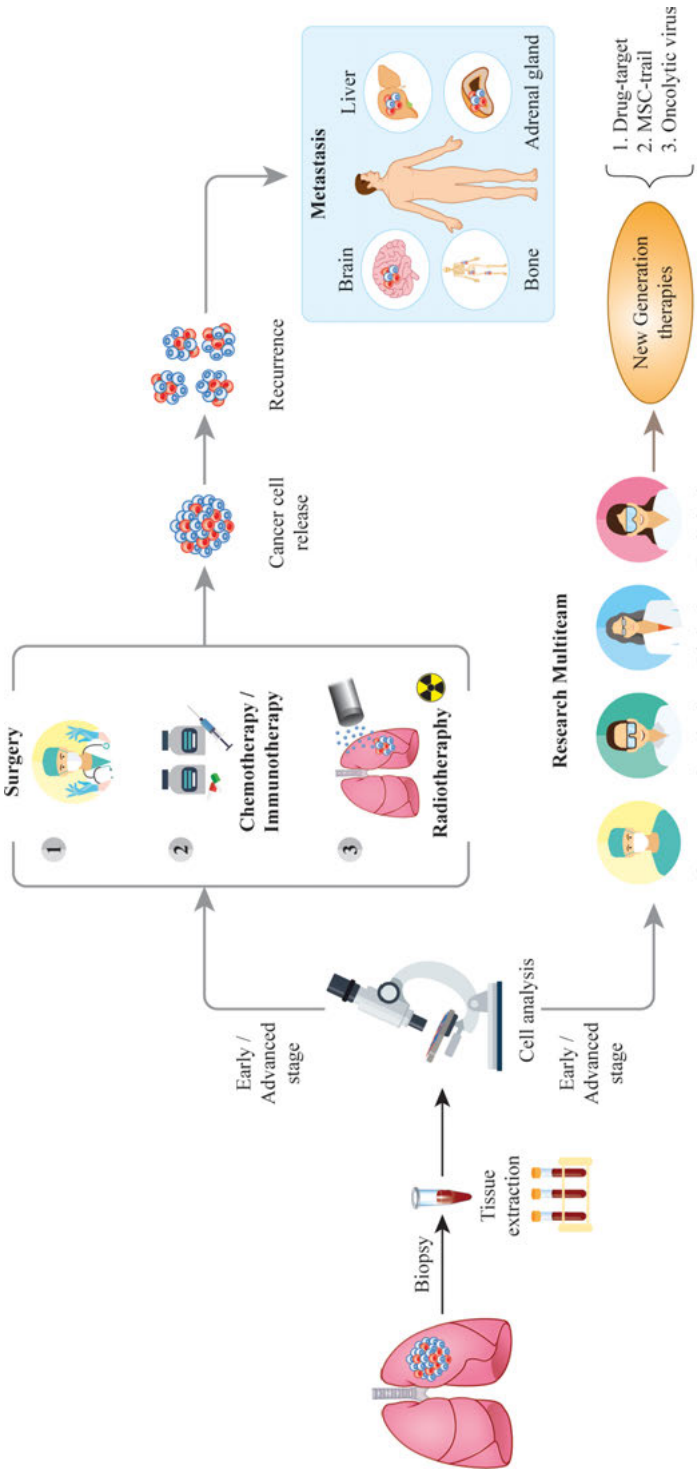

Fig. 2.3: From the operative room (OR) to the bench: a new era of clinical approaches and therapies in early and advanced lung cancer.

effect on inflammatory responses, nontoxicity against the normal tissues, and easy processes for clinical use, have set the basis of their attention.

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3 The significance of pericytes in health and disease: the role of pericytes with special focus on atherosclerosis

Abstract: Pericytes are mural pluripotent cells surrounding the endothelium of both capillaries and large blood vessels. They are highly heterogeneous cells because they exhibit significant diversity in their phenotype, tissue distribution, origin, cell-surface expression proteins, and functions. According to current understanding, they are essential for the regulation of morphogenesis and function of the vasculature. Pericytes were demonstrated to be implicated in tissue development and homeostasis, as well as in the development of vascular disorders, including atherosclerosis. Both microvascular and macrovascular pericytes form the cellular network of the arterial wall, profoundly contributing to lipid accumulation, local inflammation, growth and neovascularization of the atherosclerotic plaque, and thrombosis. The evidence accumulated to date suggests that pericytes originate from the multipotent stem cells committed to the mesenchymal differentiation into oligopotent lineages, including osteoclasts, chondrocytes, and adipocytes, and also may serve as local mesenchymal progenitors in tissues. Moreover, in anticipation of unambiguous perceptions of pericyte origin, further views on pericyte derivation has recently emerged exploring the relationship between mesenchymal stem cells, pericytes, and other cell types. Pericyte multilineage potential is fundamental for the vascular pathology, including the formation of the atherosclerotic lesion. Pericytes can represent useful cellular models, which would potentially help to facilitate the advances in the research of the atherosclerotic process and other pathologies, in order to develop novel diagnostic and therapeutic approaches.

Key Words: Pericytes, Pericyte-like cells, Mesenchymal stem cells, Differentiation, Vasculature, Atherosclerosis.

3.1 Introduction

Pericytes are mural pluripotent cells surrounding vascular endothelium. In the literature, pericytes are often referred to as Rouget cells, named after their discoverer [1–3]. In 1873, French scientist Charles Rouget first described them as capillary contractile cells that encircle the endothelium of small blood vessels [4]. Fifty years later, another researcher, Karl Zimmerman, distinguished three subtypes of these cells, that is, precapillary, true-capillary, and postcapillary, and renamed them pericytes because of their perivascular location (*peri*—around, *cyte*—cell) [5].

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Isolation from a tissue culture and electron microscopy enabled the determination of the morphological structure of pericytes. Overall, pericytes can be recognized by a cell body with a prominent round nucleus with a relatively small content of cytoplasm. They possess several long extensions that encircle the endothelium of the vessel wall [6]. Being present in tissues of nearly all vertebrates, pericytes are mural cells located on the abluminal side of the endothelial cells [7]. Both endothelial cells and pericytes are situated within the basement membrane of vessels, where they form direct intercellular connections facilitated by paracrine signaling pathways. Pericytes make contacts with their long cytoplasmic processes with neighboring cells or even other pericytes by forming peg-and-socket arrangements and gap junctions that permit interchange of ions and small molecules [2, 8, 9]. The endothelial cell/pericyte interactions govern maintenance of the endothelial structure and function. The population of pericytes is a heterogeneous population of cells incorporating pericytes and pericyte-like cells [1, 2, 7]. Pericytes were found in the *vasa vasorum* of microvessels, providing sustenance of the endothelium of large blood vessels [10]. Moreover, several authors reported the presence of stellate pericyte-like cells in the intima of the large arteries and veins [11, 12]. These data clearly indicated that pericytes form a continuous subendothelial network covering the entire human vasculature. Due to their high heterogeneity and the lack of a single specific marker, pericytes have proven difficult to identify and quantify precisely. Because of these challenges, the role of pericytes in health and disease remains to be studied in detail. In particular, the participation of pericytes in the development of atherosclerotic process attracted special attention. In this chapter, we will discuss the biological role of pericytes and their involvement in various aspects of atherosclerosis pathogenesis. Also, we will summarize the recent advancements of the understanding of the pericyte derivation and identification exploring the relationship between pericytes, mesenchymal stem cells (MSCs), and other cell types. This would help to reinforce the role of pericytes as cellular models for atherosclerosis research, as well as to establish potential cell targets for the specific cell-based therapeutic intervention in the regenerative medicine.

3.2 Biological role of pericytes

Pericytes or pericyte-like cells are the most enigmatic cell type populating the subendothelial layer of the vascular intima due to a broad diversity in phenotype, location, gene/protein expression pattern, tissue distribution, and functions. In different organs, pericytes are morphologically distinct depending on the vessel type. For example, central nervous system (CNS) pericyte is an elongated solitary cell wrapping up an extensive vessel area with its multiple cytoplasmic processes [9], whereas kidney glomerular mesangial cell is round and compact and makes only focal microvascular beds and a small number of attachments to the basal membrane [7]. Moreover, in terminal arterioles and venules, cells with transitional phenotypes

featuring both pericytes and vascular smooth muscle cells (VSMCs) were described [7]. Pericyte coverage of blood vessels varies by organ. Thus, the endothelial cell-to-pericyte ratio is the highest (1:1) in the CNS and retina. Significantly lower proportions were reported for lung and skin tissues (1:10) and skeletal muscles (1:100) [13]. It was suggested that the variation in pericyte tissue distribution may be associated with the tissue's function [1, 14]. For instance, blood vessels of larger diameter are more abundant in pericytes maintaining a higher blood pressure in the organ [13, 14]. Interestingly, the studies revealed that pericytes regulate the vascular tone via pericyte-specific regulatory and signaling pathways, which are functionally associated with active regulation of blood flow throughout the body [1, 2, 15]. Thus, in capillaries, pericytes can participate in regulating the blood flow by their contractile ability, carried out by the expression of varying amounts of essential contractile proteins, such as actin, myosin, smooth muscle actin alpha (SMA-α), tropomyosin, and desmin [16]. At the same time, consistent with the concept of pericyte heterogeneity, there are data supporting the hypothesis that pericyte constriction may not be a universal property of all pericytes. Thus, SMA-α-negative noncontractile brain and retinal capillary pericytes were described [16]. Noteworthy, pericyte contractility determines the regulation of capillary blood flow in pathological conditions, such as ischemia [17, 18]. The contractility of pericytes is less important in the aorta and other large blood vessels, as the vascular tone is mainly regulated by other mechanisms [19]. Alterations in the count and size of endothelial cell/pericyte interfaces, pericyte distribution, and contractility leading to distorted vessel sprouting, remodeling, maturation, and stabilization accompany vascular disorders, including atherosclerosis [19–21].

Current evidence suggests that pericytes are pluripotent cells with mesenchymal plasticity and that their multiple tissue-specific properties determine their functional plasticity to a high degree [14]. However, pericyte contribution to MSC-derived mesenchymal cells can be variable and possibly dependent on the extent of the vascularity of a tissue [22]. Lineage tracing studies indicated that pericytes containing heterogeneous populations differ in embryonic origin [1, 14]. Nevertheless, provided that pericyte heterogeneity depends on the vascular bed, their exact origin is still uncertain.

In addition, pericyte diversity in morphology, distribution, origin, and marker expression mirrors the diversity in their functions. To date, providing the evidence for better understanding of the beneficial and negative roles of pericytes in health and disease, several vasculature-related physiological functions of pericytes have been described: (i) angiogenesis and vessel stabilization [23], (ii) capillary blood flow regulation [24], (iii) vascular morphogenesis and maturation [25], (iv) vascular remodeling and architecture [26], (v) vascular permeability [27], (vi) maintenance of the functional integrity of the blood-brain barrier [28], (vii) regulation of blood coagulation [29], (viii) lymphocyte activation [30], (ix) phagocytic activity [31], and (x) immunomodulatory activity [32]. However, because of pericyte's great heterogeneity and

the lack of appropriate methods to isolate and exemplify these cells, many aspects of the multipotent pericyte biology remain obscure.

3.3 Identification of pericytes

Identification and count of pericytes across organs and tissues are challenging, because of their great heterogeneity and the absence of a single specific expression protein marker for all pericyte subtypes. The expression patterns of the pericyte antigens are tissue specific and can be associated with pericyte functional heterogeneity [2]. Protein marker expression can be up- or down-regulated by a pathological condition. For example, lipid accumulation in atherosclerosis may influence pericyte antigen expression [33]. Other factors, such as the developmental stage of a blood vessel, culture conditions (*in vitro* or *in vivo*), and pericyte functional state, can also affect the expression of marker proteins [2, 6, 33]. Several proteins are coexpressed in pericytes and other cell types. The most common example is SMA-α, which is typically expressed in VSMCs; pericytes of large human arteries are frequently found to be positive for SMA-α [34]. Moreover, using the immunocytochemical analysis, a population of SMA-α-positive intimal cells also expressing CD68 marker protein was identified in the subendothelial aortic intima [35]. CD68 is atypical for VSMCs and usually regarded as a macrophage marker. Interestingly, the ratio of double positive SMA-α+CD68+ cells was increased in atherosclerotic lesions, as well as in primary cell cultures, exposed to the atherogenic modified low-density lipoprotein (LDL) [35]. It addition, it was shown that the special subpopulation of pericyte-like cells existing in the peripreluminal proteoglycan-rich sublayer of the subendothelial aortic intima apart from SMA-α and CD68 can also express both specific 3G5 (O-sialoganglioside) and 2A7 (melanoma chondroitin sulphate proteoglycan) antigens [33]. In association with lipid accumulation, the expression of these markers was altered [36]. The expression of 3G5 antigen was found in microvascular pericytes and considered to be characteristic for dormant pericytes [33, 37]. The expression of 2A7 antigen was found to be typical for macrovascular and activated pericytes, including proliferating cells during the active angiogenesis [33]. Importantly, due to diversity in the distribution of stellate cells and the insufficient amount of knowledge about the expression range of other pericyte-associated markers in the arterial wall, it is reasonable to avoid identifying SMA-α-positive/3G5 antigen-positive stellate-shaped cells as true pericytes [38].

Another example of a common expression marker between pericytes and other cells, including endothelial cells, fibroblasts, astrocytes, and some tumor cells, is platelet-derived growth factor receptor-beta (PDGFR-β) [39, 40]. Many reports have described the pericyte expression of other discriminating proteins, including aminopeptidase A and N (CD13), neuron-glial 2 (NG2), desmin, CD146, endoglin, nonmuscle myosin, nestin, and vimentin, which are also related to other cell lineages [8, 9,

34, 39, 40]. Several markers, albeit not pericyte specific, including SMA-α, PDGFR-β, CD13, NG2, and desmin, were validated and approved for common pericyte identification [1]. Therefore, immunocytochemical identification of pericytes is lacking a definitive pan-marker and hence should be based on the use of combinations of markers. For example, perivascular cells (pericytes) in multiple human organs, such as skeletal muscle, pancreas, adipose tissue, and placenta, were identified on the expression of CD146, NG2, and PDGF-Rb markers and the absence of markers expressed by other cells [41]. Moreover, in identifying pericytes, a combination of criteria, including morphology, tissue localization, functional characteristics, and gene expression, should be considered in order to produce unambiguous results [10]. Additionally, the assessment of cell-to-cell communications such as Connexin 43 (Cx43)-mediated contacts via gap junctions between pericytes and other vascular cells was suggested to discriminate pericytes in cultures [42].

3.4 Origin of pericytes

The developmental origin of pericytes is rather complex, and the absence of any unique single marker to identify all pericytes makes it difficult to study the ontogeny of pericytes and their differentiation capacity. The best understood and the most commonly accepted concept of the pericyte origin considers pericytes as pluripotent stem cells that exhibit multilineage developmental features of MSCs. Based on the match in the location (perivascular niche), multilineage developmental potential, and immunophenotype, animal and human studies presented the evidence demonstrating the direct relationship between MSC and perivascular pericytes [41–44]. MSC is capable of differentiation into tissues originating from the three germ layers. Also, numerous *in vivo* and *in vitro* experiments demonstrated pericyte ability for differentiation into several oligopotent lineages, giving rise to osteoblasts, chondrocytes, adipocytes, smooth muscle cells (SMCs), fibroblasts, and Leydig cells [14, 45]. Pericytes can give rise to multiple mesodermal tissues *in situ* in response to PDGFR-β [46]. PDGFR-β signaling is important for the proliferation and recruitment of pericytes to blood vessels [6]. The multilineage potential of pericytes may contribute to bone and muscle regeneration, including cardiac muscle, as well as fat accumulation [47–51]. Moreover, MSCs are involved in the maintenance of high-turnover tissues like liver, skin, skeletal muscles, adipose tissue, and dental pulp. Consequently, the study demonstrated that pericytes may serve as a source for local mesenchymal progenitor cells in these tissues in adults [52]. MSC can be induced into neural-like phenotype differentiation [44], generating brain cell neurogenesis, which probably confirms the neuroectodermal origin of CNS pericytes [53]. Similarly, during developmental stages, neuroectodermal cells can possibly also differentiate into the VSMCs of embryonic cerebral vessels [54]. In addition, it was found *in vitro* and *in vivo* that, promoted by transforming growth factor-beta (TGF-β), some pericytes can be derived from myeloid

progenitors in the developing skin and brain tissues [6]. The above studies clearly indicate that the developmental origin of pericytes is heterogeneous and tissue specific, but whether pericytes have different functions in different tissues remains to be elucidated.

Moreover, it was established that MSCs obtained from multiple tissues share important functional properties for tissue maintenance and repair and protein-expression profile not only with pericytes but also with fibroblasts [55]. MSCs can regenerate tissues in either direct differentiation or indirect ways via stimulating angiogenesis, limiting inflammation, and recruiting tissue-specific progenitors [56]. Also, several authors reported *in vitro* and *in vivo* experiments describing vascular pericytes as the important source of stromal fibroblasts contributing to some pathological conditions, such as acute skeletal muscle injury, tumor invasion and metastasis, and fibrotic responses [57–59]. In addition, an experimental model demonstrating the MSC/pericyte positive role during tissue repair was described [44]. Multilineage pericyte plasticity denoting their capacity for endogenous tissue regeneration and repair makes them attractive cells for clinical applications in regenerative medicine.

In order to achieve consistent MSC identification across different studies, the International Society for Cellular Therapy (ISCT) created a position statement formulating three minimum regular MSC criteria. First, MSCs must be adherent to plastic when maintained in standard culture conditions. Second, MSCs must express the following cell surface antigens: CD105, CD73, and CD90, and not express CD45, CD34, CD14, CD11b, CD79α, CD19, or human leukocyte antigen–DR isotype (HLA-DR). Third, MSCs must be capable of differentiation into osteoblasts, adipocytes, and chondroblasts in vitro [60]. These criteria summarized the best data currently available; however, they do not encompass MSC behavior *in vivo*.

The relationship between pericytes and MSCs was studied in more detail. It was demonstrated that not all pericytes are MSCs and that not all MSCs can behave as pericytes [61]. The ISCT stated that MSCs represent a heterogeneous population of adventitial cells with wide differentiation capacity [60]. In support of this statement, many authors presented studies describing the pericyte-like cellular population in the tunica adventitia of vessels in different tissues [10, 61–63]. These cells share their antigenic profile with conventionally defined pericytes expressing NG2 and PDGFRβ, but they did not express CD146. Also, they can express CD34, a typical marker of hematopoietic progenitor cells [64, 65]. Apart from a common marker expression, adventitial cells demonstrated a multipotent differentiation capability similar to MSCs [66]. To what extent this adventitial population is related to microvascular pericytes remains uncertain. In that regard, a group of researchers divided pericytes in two distinct *in vitro* populations: type 1 pericytes, generating adipocytes and fibroblasts but not neural cells, and type 2 pericytes possessing neurogenic and myogenic potential [67]. Moreover, as demonstrated by the characterization of MSCs *in situ*, those expressing CD146 resemble pericytes in their position (peri-endothelial), angiocrine activity, and phenotype [43, 68]. Thus, on the basis of localization and

multipotency, two distinct cell populations (perivascular MSC progenitors), first, CD146+, CD34–, and CD45– pericytes surrounding capillaries and micro-vessels and, second, CD146–, CD34+ and CD45– adventitial cells of large arteries and veins, were grouped together as perivascular stem cells [41, 62].

Another point of view that all pericytes are not MSCs was reported [69]. This opinion was based on the fact that perivascular cells encircling both large and small vessels have highly discriminating other functions, such as immunomodulatory, secretion of trophic factors, and tissue repair, in response to local injury, which are rather separate from the actions associated with the mesenchymal differentiation. Moreover, the resent experiments using Tbx18-CreERT2 mouse model showed that endogenous adult pericytes of some organs, such as heart, brain, skeletal muscle, and fat tissue do not behave as MSCs under certain conditions, and their plasticity can be limited [70]. In these experiments, pericyte plasticity demonstrated *in vitro* or following transplantation *in vivo* might be a consequence of the artificial cell manipulations *ex vivo* conditions. Also, another study presented the evidence that not all pericytes are MSCs [71]. Investigating the role of pericytes in wound healing, this study revealed that despite pericyte inherent MSC potential, some pericytes may not have beneficial properties for the wound healing process; i.e., pericytes were not differentiating into promoting contraction myofibroblasts but instead were producing collagen and promoting fibrosis. Most biological aspects of perivascular MSCs are still obscure, from the pericyte emergence in the embryo to molecular control of their behavior in adult tissues.

Furthermore, there is still uncertainty regarding the true identity of the host pericyte progenitors with wide differentiation capacity continuously present in developed adult tissues. To clarify this uncertainty, it was reviewed whether multilineage MSCs, multipotent adult progenitor cells, muscle-derived stem cells, or adipose tissue-derived stem cells share a common progenitor in multiple developed organs [72]. Unfortunately, it was left undiscovered, as all these multipotent cells were identified only on reflection of primary cultures in donor tissues, but not on purified pericytes. Moreover, several independent studies described novel subsets of endothelial cells in embryonic tissues that contribute to blood vessel architecture and develop into non-vascular cell lineages promoting postnatal growth and regeneration of tissues [73–75]. In addition, it was found that some adult tissue-resident progenitors contribute to pericytes in pathological conditions, such as the growth of tumors [76, 77]. Further research is necessary that would help to solve this intriguing question of whether pericytes of the heterogeneous origins at embryonic stages remain in adults.

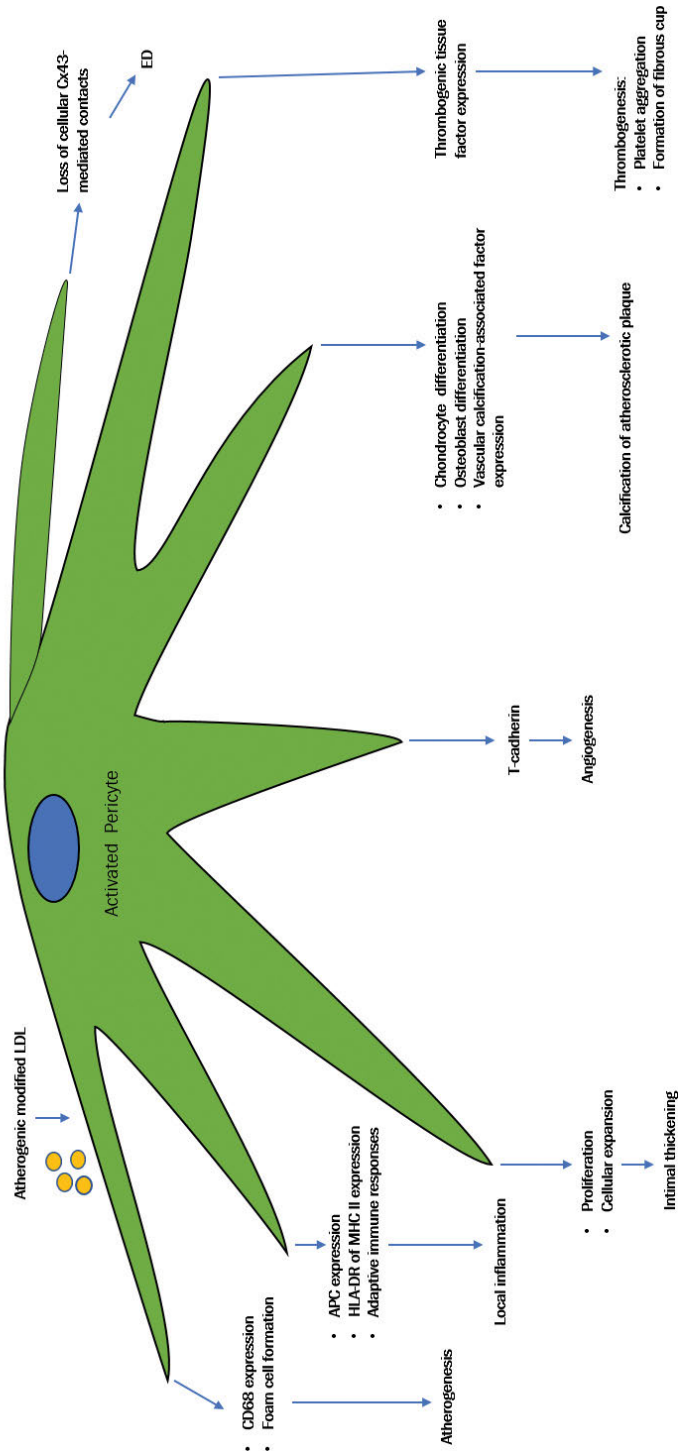
Taking into account that throughout fetal and adult life the vasculature revealed a prominent dynamic capacity for growth and repair, the assumption that several distinct stem/progenitor populations may reside within the blood vessel wall was possible. At present, the understanding of mural stem/progenitor populations, as well as their potential biologic function, is unclear. It was suggested that any of multipotent and lineage-specific progenitor populations, including pericytes, are associated with

a stem cell niche [78, 79]. Moreover, the systematic review summarized the scientific evidence available that various stem cell types can serve as a potential source for functional pericyte differentiation [80]. However, all reviewed studies were limited by the lack of standardized guidelines; therefore, further studies are needed to validate this stem cell niche for pericyte hypothesis considering the diverse array of stem cells used and the inconsistent experimental protocols.

To date, relying on the comprehensive and detailed comparison of the *in situ*, *in vitro*, and *in vivo* characteristics of MSCs and pericytes, the considerable amount of evidence supports the origin of pericytes in favor of the MSC family in human tissues. Further studies are required to support or challenge this concept.

3.5 The pathogenic impact of pericytes on the development of atherosclerotic lesion

Atherosclerotic lesion development is associated with the pronounced qualitative and quantitative changes in the cellular composition of the arterial intima and lipid accumulation leading to the formation of foam cells. Most of these changes occur in the innermost layer (proteoglycan-rich layer) of the intima, involving the endothelium and subendothelial space. Distorted morphology of the endothelium, often seen in atherosclerosis affected sites, compromises its functions, leading to inadequate vasoconstriction, leukocyte infiltration, coagulation, increased permeability that facilitates the entry of LDL in the subendothelial space, and accelerated proliferation or apoptosis [81]. Since pericytes reside in the subendothelial space and are important for the maintenance of endothelial function [34], it can be suggested that they are implicated in atherogenesis-induced endothelial alterations inducing the formation of early atherosclerotic lesions, such as fatty streaks. In this view, macrovascular pericytes were found to be susceptible to the typical for early stages of atherosclerosis atherogenic stimuli, such as the presence of atherogenic modified LDL and proinflammatory signaling, and can actively accumulate lipids in the subendothelial layer of the arterial intima [82]. That leads to their activation, proliferation, and differentiation to other cell types, subsequently contributing to plaque growth and vascular calcification [82]. Moreover, early microscopic studies made evident that lipid accumulation can cause pathological alterations in pericyte phenotype, such as an increase in cell size, acquisition of irregular shape, and loss of cellular Cx43-mediated contacts that, in turn, can cause disruption of the continuous subendothelial network with the reduction in the proportion of cell-to-cell contacts and the number of gap junctions [83, 84]. The expression of cell surface Cx43 protein, the essential component of the intercellular contacts, can determine the extent of the intercellular interactions via the gap junctions. It was observed that in atherosclerotic lesions, the number of Cx43 plaques per cell was considerably decreased in lipid-laden cells, compared with lipid-free cells, and with a smaller amount toward the lumen that



Note: LDL- low-density lipoprotein, APC-antigen-presenting complexes, HLA-DR-Human Leukocyte Antigen-DR isotype of MHC II-major histocompatibility complex class II, Cx43-Connexin 43, ED-endothelial dysfunction

Fig. 3.1: Diagram summarizing the functions of pericytes in the atherosclerosis pathogenesis.

was not the case in the grossly normal intima [19]. These findings suggested that functional changes in the gap junctions are one of the causes of atherosclerosis-related disintegration of the cellular network formed by the proteoglycan-rich layer intimal pericytes and associated with the formation of foam cells, the process recognized as a key event of atherogenesis. Nevertheless, the exact role of pericytes in the endothelial dysfunction associated with the accumulation of lipids is still vague. Endothelial injury is likely to occur from the luminal side because of the disturbed blood flow, especially at branching localities, where blood flow is nonlinear and undertaking shear stress leading to an increased inflammatory signaling and the recruitment of inflammatory cells [85].

The intracellular lipid retention triggers changes in the functional state of pericytes, i.e., from dormant to active (proliferative). Upon activation, pericytes may turn into aberrant differentiation to chondrogenic, osteogenic, macrophage, and myofibroblast lineages contributing to the pathogenesis of atherosclerosis and vascular calcification [82]. Moreover, it was noticed that during the formation of the early atherosclerotic plaques, the functional changes of pericytes occur in association with variations in the antigenic expression [33]. Accordingly, the expression of 2A7 antigen was assigned to the activated pericytes, since the presence of 2A7+ cells was observed in atherosclerotic plaques using the anti-2A7 antibody that adheres to activated pericytes, whereas none of the 2A7+ cells were detected in the normal intima [33]. Moreover, the special subpopulation of SMCs (likely pericyte-like cells) expressing CD68 macrophage-associated antigen (a scavenger receptor), simultaneously acquiring the phagocytic phenotype, was identified in the atherosclerotic plaques [11, 35, 86]. The scavenger receptors of pericyte-like cells promote uptake and subsequent accumulation of LDL particles that indicates their participation in active phagocytosis leading to the development of foam cells and the thickening of the arterial wall. Further lipid accumulation via phagocytosis impairs the situation in the atherosclerotic lesion, leading to a segregation of cells, the breakdown of cell-to-cell contacts, and the cellular network disruption. Like fibroblasts, pericytes can rapidly proliferate, accelerating thickening of the arterial wall and contributing to the extracellular matrix synthesis [82]. Continuously growing atherosclerotic plaque is the main cause of the arterial stenosis that precedes vascular ischemia and its severe consequences. The potential plaque rupture can lead to life-threatening conditions, such as atherothrombosis, stroke, acute coronary syndrome, and, in many cases, fatality [87].

Proven by the comparative studies of grossly normal aortic intima and atherosclerosis-affected intima, intracellular accumulation of lipids also triggers the cellular expansion of the intimal cells associated with both enhanced proliferation of resident intimal cells (pericytes) and the increased total cell count in the atherosclerotic plaque [88, 89]. Confirming this, the robust data indicating the direct correlation between the number of pericytes and the content of cholesteryl esters, as well as total lipids in the vascular wall, were obtained [33]. For the reason that intimal

resident cells account for a bulk of the intimal cell population (84–93%), the increase in their number is accountable for the increase in cellularity of the atherosclerotic lesions [82]. Morphological analysis demonstrated cellular expansion in the atherosclerotic intima as following: a 6-fold increase in stellate cell (likely pericytes) count and a 2-fold increase in the number of the elongated cells (likely SMCs) and in total cell count [38]. According to current understanding, the increased cellularity in the proteoglycan-rich layer of atherosclerotic intima is also determined by the recruitment of circulating hematologous immune (inflammatory) cells. The study showed an increase in the number of inflammatory cells in the atherosclerotic intima, despite that their proliferative index (the proportion between proliferating cells and the total cell number) was not increased [89]. In fact, the increased number of inflammatory cells was a result of their migration into subendothelial intima from the blood circulation [89]. Unlike pericytes, the proliferative activity of inflammatory cells is not stimulated by the atherogenesis [90]. In addition, in the zone of atherosclerotic plaque formation, migrating inflammatory cells release proinflammatory cytokines and chemokines, stimulating proliferation of the resident intimal cells [91]. Notably, the topmost number of cells was detected in lipid-rich lesions, i.e., early-stage lesions (fatty streaks and lipofibrous plaques) [33]. In early atherosclerotic lesions, increased cell proliferation is considered as an important event in atherogenesis contributing to the intimal thickening. However, the proliferative index of resident intimal cells can be variable depending on plaque developmental stage and its location. It was shown that the amount of resident proliferating cells in lipid-laden atherosclerotic lesions was 10-fold to 20-fold greater than in uninvolved intima [38]. As well, the number of resident proliferating cells in the later stage lesions (fibrous plaques) was lower than in lipid-rich lesions, but substantially higher than in uninvolved intima [90]. It is worth mentioning that the correlation coefficients between the number of pericyte-like cells, collagen content, and intimal thickness were more significant than those in other intimal cells [33]. These findings indicate that the increased cellularity of the atherosclerotic arterial intima is a result of both a proliferative "splash" of resident cells and the infiltration of inflammatory cells. Relying on these data, pericytes were suggested to be the key cells driving intimal thickening in the atherosclerotic process and the subsequent growth of atherosclerotic lesion [33].

Furthermore, lipid accumulation caused by circulating LDL can orchestrate proinflammatory environment in the arterial intima, stimulating both adaptive and innate immunity [91]; thus, the growth of atherosclerotic plaque is accompanied by the accumulation and activation of local immune-inflammatory cells [89, 92]. Consequently, it was suggested that upon lipid accumulation, pericytes, along with macrophages and dendritic cells, can express antigen-presenting complexes that is indicative of their participation in the antigen presentation and the inflammatory progression in the vascular wall [82]. In this view, the immunofluorescent analysis demonstrated a population of stellate subendothelial cells (pericyte-like cells) expressing HLA-DR molecule of the major histocompatibility complex

class II that clearly correlated with a number of immune-inflammatory cells in the atherosclerotic lesion [92, 93]. Immunocytochemical typing and morphological analysis identified these cells as pericytes in observance of the cellular network with pericyte-characteristic intercellular contacts, typical cell morphology, prevalent number, and locality in the close proximity of endothelial cells [5, 37]. Noteworthy, as the microscopic study revealed, some HLA-DR-positive cells contained apolipoprotein B that is possibly indicative of the early phase of lipid accumulation in the intimal cells; therefore, it is possible that pericyte-like cells play a double role in the atherogenesis, participating in both lipid accumulation and local inflammation [93].

Interestingly, by participating in the processes of immune-mediated inflammation, pericytes may have important protective functions in the blood vessel wall. The study showed that pericytes were able to interact with and alter the behavior of infiltrating T-cells, revealing their positive role in adaptive immune responses [94]. Moreover, cytokine production by stellate pericyte-like cells was detected in atherosclerotic plaques [38]. However, it remains to be explored whether macrovascular pericytes have immune-modulating properties *in vivo*.

Apart from lipid accumulation, intimal thickening, and inflammation, processes that directly contribute to atherosclerotic lesion progression, pericytes play additional roles in atherosclerosis, including plaque neovascularization, calcification, and regulation of thrombogenesis. It was revealed that the expression of the unique cell adhesion molecule T-cadherin is up-regulated in atherosclerotic pericytes that potentially can mediate LDL-induced recruitment of microvascular pericytes to the angiogenesis [95]. It is likely to be involved in LDL-induced phenotypic changes and activation of pericytes via both processes of disturbed signaling, such as Erk1/2 tyrosine kinase pathway and nuclear factor kappa B translocation [96]. Also, in atherosclerotic plaques, pericytes can be recruited to the formation of neo-vessels via c-Met-PI3K/Akt pathway triggered by the activation of hepatocyte growth factor signaling [97]. Additionally, in complicated atherosclerotic plaques, pericytes can be engaged in the angiogenesis as a part of the angiogenic response [11]. Moreover, T-cadherin can mediate the LDL-induced chondrogenic differentiation of pericytes and vascular wall remodeling via Wnt/β-catenin pathway during the development of atherosclerotic plaque [98]. The molecule of cadherin is an important component of the Wnt/β-catenin signaling [99], and some of Wnt receptors, including LDL-receptor-like proteins 5 and 6 were identified in pericytes [98]. Induction of pericyte chondrogenic differentiation pathway is followed by the inhibition of their adipogenic differentiation and associated with the increased Sox-9 expression and accumulation of glycosaminoglycans in the extracellular matrix [98]. The enhanced signaling of the TGF-β expressed in abundance by macrophages, foam cells, and VSMCs in the atherosclerotic plaque can further promote Wnt/β-catenin-dependent chondrogenic differentiation of pericytes [100–102]. In addition, in valve myofibroblasts and mural pericytes, TGF-β_3 was demonstrated to be responsible for vascular calcification via bone morphogenetic protein 2/4-mediated mineralization [103].

The fact that pericytes can differentiate into osteoblasts and chondrocytes is suggestive of their potential involvement in the maladaptive ectopic calcification of atheromatous vessels, where they serve as a source of osteoprogenitor cells in lesions [104]. The maladaptive ectopic calcification is followed by the matrix remodeling and the intense recruitment of calcifying vascular cells in atherosclerosis-affected vessels. Moreover, intimal pericytes were found to be capable of the expression of vascular calcification-associated factor, further promoting vascular calcification in the proinflammatory milieu of the plaque [104]. Ectopic calcification of blood vessels is a typical complication of advanced atherosclerotic lesions. Moreover, at advanced stages of atherosclerotic plaque development, pericytes are capable of the expression of thrombogenic tissue factor in the endothelium-uncovered arterial wall, leading to platelet aggregation and the formation of so-called fibrous cap wrapping up the plaque [105]. According to current knowledge, the fibrous cap has protective function isolating the plaque from the blood circulation. Its damage, however, can lead to a rapid formation of thrombus, often with severe consequences.

In addition, pericytes and pericyte-like cells can be used as cellular models for atherosclerosis research and drug development. For example, pericytes are capable of active lipid accumulation, so they were used as the *in vitro* model for the evaluation of blood serum atherogenicity and testing potential active substances with direct antiatherosclerotic action (reducing blood atherogenicity and lipid accumulation in the vascular wall) [106]. Moreover, further administration of the substances effective *in vitro* (recorded a decrease in lipid accumulation by cultured cells) to the study subjects (*ex vivo* model) showing a decrease in blood serum atherogenicity was reported [106]. These models were successfully used for the development of several plant-derived nonpharmaceutical products, which can aid in the control of atherosclerosis risk factors, as well as applied for a long-term antiatherosclerotic treatment [82].

Taken together, macrovascular pericytes can actively accumulate lipids, which leads to their activation, proliferation, and differentiation to other cell types, contributing to plaque growth, vascularization, calcification, and extracellular matrix synthesis. Therefore, they play a key role in the development of atherosclerotic lesions of all stages at the cellular level, which makes them an attractive potential therapeutic target. Regarding microvascular pericytes of *vasa vasorum* of large vessels, they can also contribute to atherosclerotic plaque progression by orchestrating angiogenesis in the growing plaque [11].

3.6 Conclusion and future perspectives

Accumulated data indicated that continuous networks of pericytes and pericyte-like cells are present in the healthy intima, where they play important roles in

tissue homeostasis and development. Moreover, pericytes as a part of cellular rearrangements in the vascular wall were demonstrated to be involved in all the stages of the atherosclerotic lesion development, including atherogenesis, local inflammation, vascular remodeling, intraplaque neovascularization, ectopic calcification, and thrombosis. Numerous lines of experimental evidence support the concept of the mesenchymal origin of pericytes. Therefore, it would be important to further explore the relationship between MSC and pericytes, in anticipation of unequivocal perceptions of pericyte origin that would help in a more precise understanding of pericytes' distinct roles in human health and disease, including atherosclerosis. In particular, comparative studies analyzing the functional characteristics of the special population of aortic stellate cells, pericytes, and MSCs, using pericytes or MSCs as cellular models, and the combination of lineage tracing and surface marker expression approaches are required, so that it would be useful to support the distinguishing role of stellate pericyte-like cells in atherogenesis. At present, research into the relationship between these cells has produced controversial results. As mentioned above, immunocytochemical typing and morphological analysis showed stellate cells to be similar to pericytes. Moreover, based on the similarities between stellate pericyte-like cells and MSC in the mesenchymal differentiation capacity, perivascular location, and common marker expression (SMA-α), the presence of subendothelial niche for MSC-like cells was suggested [107]. On the other hand, it was found that stellate cells are able to express HLA-DR markers [92, 93], which, according to ISCT, MSCs must not express. This made it possible to make a speculative suggestion that stellate cells are not MSCs. Therefore, further studies are needed to clarify the origin of stellate subendothelial cells. Additional *in vivo* studies would help to establish a detailed understanding of pericyte behavior in disease models, including studies that would enable translating animal research to humans. Furthermore, reliable pericyte markers or their combinations should be established. Such markers may serve as potential molecular targets in aid of diagnosis and therapeutic strategies in atherosclerosis and other vascular pathologies.

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4 Bone stem cell therapy in the clinical perspective: a focus on nonrandomized and randomized trials

Abstract: Following encouraging data from translational studies in experimental animal models, contemporary stem cell research is more fixated on their assessment in human patients. Successful safety studies during early-phase clinical trials using unfractionated whole bone marrow (BM) or its derivative sublineages have paved the way for phase 3 clinical trials. Most of these trials are focused on the management of ischemic heart disease in both its acute and chronic stages either as a stand-alone procedure or as an adjunct to routine reperfusion strategies including percutaneous coronary intervention or coronary artery bypass grafting. Largely, the use of BM cells (BMCs) has been reported as safe regardless of the cell type, cell source (i.e., autologous or allogenic), their manipulation prior to transplantation, delivery strategy, etc. Moreover, most of the clinical trials have reported no major adverse cardiac events directly or indirectly related to BMC therapy during both short-term and long-term follow-ups. However, their efficacy has been reported as modest and considerably less than the purported hype and hence warrants further investigation in larger study population. This chapter critically reviews the published data pertaining to the clinical studies with BM-derived cells to ascertain their safety and efficacy profile in both acute and chronic myocardial infarction patients.

Key Words: Bone marrow, Clinical trials, Heart failure, Hematopoietic, Infarction, Ischemia, MSC, Mononuclear, Randomized, Stem cells.

4.1 Introduction

From among the cardiovascular diseases that account for every one out of three deaths, ischemic heart disease (IHD) is a principal cause of morbidity and mortality and epitomizes a major clinical challenge worldwide [1]. Acute myocardial infarction (AMI) most often occurs when there is a rupture of an atherosclerotic plaque into a coronary artery, which causes thrombosis and occlusion of the artery, diminishing blood supply in the affected region of the heart and causing necrosis and death of cardiomyocytes [2]. Postischemia and postinfarction episode management, as well as rate of patient survival, has significantly improved during the recent years due to the advancements in the revascularization strategies, including percutaneous coronary intervention (PCI) and coronary artery bypass graft (CABG) [3]. While PCI entails flow-limiting aspect of coronary artery stenosis using stents with or without drug elution,

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CABG accomplishes surgical by-passing of the occluded vessel/s. A hybrid approach of revascularization involves minimally invasive coronary artery bypass surgery in conjunction with PCI [4, 5]. As an alternative to invasive revascularization approaches, pharmacological intervention provides symptomatic relief to enhance the quality of patient life [6]. Furthermore, standard pharmacological therapies are preferentially aimed to reduce the rate of mortality, prevent the expansion of ischemic myocardial damage, and alleviate the chance of further attacks [7].

The ischemic myocardium may undergo a detrimental cascade of events, including the formation of a noncontractile scar tissue, which replaces the damaged myocardium along with an overload of blood flow and pressure capacity, and overstretching of viable cardiomyocytes in an attempt to sustain cardiac output, thus eventually leading to chronic ischemic changes and heart failure [8]. Nevertheless, contemporary therapies are lacking the potential to replenish dead cardiomyocytes. Hence, the concept of cardiac repair/regeneration after myocardial infarction (MI) exploiting the exclusive abilities of stem cells to differentiate into new contractile cardiomyocytes and regenerate deceased cells to restore the lost function is innovative and revolutionary [9]. This chapter critically reviews the published data focusing on the clinical studies pertaining to bone-marrow-derived cells (BMCs) in terms of their safety and efficacy in both acute and chronic myocardial infarction patients.

4.2 Types of stem cells that reached clinical use

Stem cells possess unlimited potential of self-renewal, the capacity to cross lineage restriction and differentiate into an array of specialized cells during the post-natal growth and development [10]. They are instrumental in the intrinsic repair process in various tissues and organs, including muscles, heart, liver, and kidneys [11]. Given the reparability and regenerative potential of stem cells, research pertaining to their preclinical and clinical use has remained an area of intense investigation during the last two decades, while the development of the protocols to reprogram somatic cells to pluripotency has given new impetus to their application in regenerative medicine. Stem/progenitor cells have been categorized in various ways: anatomically, based on functionality, and on the basis of their surface marker expression. According to the tissue of origin, stem cells can be classified into two main categories: embryonic stem cells (ESCs) and adult stem cells (ASCs), which are isolated from the inner cell mass of an embryo and from the adult tissues, respectively. Both ESCs and ASCs have been extensively characterized *in vitro* as well as *in vivo* during cell-based therapy in preclinical animal models, while ASCs have progressed to clinical trials in human patients for myocardial repair and regeneration.

ESCs are undifferentiated cells derived from an embryo during the preimplantation blastocyst stage. They are pluripotent and can develop into any cell type of the

three primary germ layers, i.e., endoderm, mesoderm, and ectoderm. Given optimal culture conditions, ESCs are capable of undifferentiated self-renewal for a prolonged period in culture [12]. There is experimental evidence that ESCs continue to proliferate undifferentiated for up to 300–400 population doublings [13]. Molecular studies have shown that pluripotency determining genes, i.e., Oct4 and Nanog, have a key mechanistic role in suppressing differentiation associated genes in ESCs, thus maintaining their pluripotent status [14]. *In vitro* studies have shown their vasculogenic and cardiomyogenic differentiation potential in response to appropriate culture conditions [15, 16]. A similar study has subsequently reported successful differentiation of ESCs under serum-free and feeder cell-free well-defined culture conditions, which involved sequential modulation glycogen synthase kinase-3, basic fibroblast growth factor (bFGF), bone morphogenetic protein-4, and vascular endothelial growth factor (VEGF) signaling [17]. Transplantation studies in both small and large experimental animal models of myocardial ischemia and infarction have generated promising data in terms of myocardial structural repair by repopulating the infarct and peri-infarct areas with morpho-functionally competent myocytes and preserved global cardiac function [18–20]. However, the clinical use of ESCs in human patients is challenging because of the predicted tendency of immunological rejection of their derivative tissue, as they are allogenic in origin [21]; the anticipated risk of teratoma [22]; as well as moral and ethical concerns shrouding their availability [23]. However, ESCs are slowly progressing to clinical applications. Some of these clinical trials have been tabulated in a recent publication [24] and are available at https://clinicaltrials.gov/ct2/show/.

The development of reprogramming protocols for induction of pluripotency in terminally differentiated somatic cells has reinvigorated interest in the use of pluripotent stem cells. The innovative protocol to develop induced pluripotent stem cells (iPSCs) from mouse skin fibroblasts provided a breakthrough discovery for the generation of cells that possessed peculiar morphological features of ESCs and could act as surrogate ESCs [25]. Subsequently, various research groups were able to successfully develop iPSCs from different somatic cell types, including skeletal myoblasts (SkMs), BMCs, dental pulp, peripheral cells, T-cells, various other cell types, etc. [26–30]. iPSCs provide a renewable source of pluripotent stem cells, but without any moral, religious, or ethical issues [31]. Currently, their potential is being extensively explored for use as a platform for drug discovery and development [32, 33] in addition to extensive characterization in cell therapy for myocardial repair and regeneration in experimental animal models [34–38]. However, despite all the refinement and improvement in the reprogramming protocols, their use is not without the fear of tumorigenesis [39].

On the other hand, ASCs are adult-tissue-resident cells that have traversed deep down the differentiation pathway in comparison with their embryonic-tissue-derived counterparts and reside in various adult body tissues [40]. They possess a much more limited capacity of self-renewal as well as differentiation under physiological

conditions that is limited to the cell types present in the tissue of their origin [41]. Bone-marrow-derived stem cells (BMSCs), SkMs, resident cardiac stem cells (CSCs), adipose-tissue-derived stem cells, endothelial progenitor cells (EPCs), and umbilical cord stem cells are some of the most extensively characterized ASCs for use in cell-based therapies [42, 43]. One of the major advantages of ASCs in cell-based therapy is their autologous availability, which enhances their immunologic acceptance after engraftment and alleviates the problem of rejection of their derivative tissue graft. Moreover, given their robust nature, ASCs can be easily expanded *in vitro* to obtain a large number of cells required for transplantation. More recent research is directed toward reprogramming of ASCs to pluripotency to generate disease- and patient-specific autologous iPSCs [44, 45]. Preclinical experimental studies have clearly profiled ASCs' safety for treatment of IHD, and hence, some of these cell types have already progressed to clinical assessment in human patients for myocardial repair and regeneration [46, 47]. Although the mechanism of their involvement in myocardial repair remains contentious, it is generally considered to be multifactorial, including transdifferentiation to adopt cardiac phenotype [48–50], fusion with host cardiomyocytes [51–54], and host cardiomyocyte protection by paracrine and mircrine activity [55, 56].

Another main advantage of ASCs is that they can be genetically modified without compromising their "stemness" due to their robust nature [57–60]. They have been extensively studied for their reparability after genetic manipulation for cell-based gene therapy of the heart. The genetically modified cells serve as a continuous source of bioactive molecules encoded by the transgene in regulated and unregulated fashion as long as the transgene expression is sustained in the genetically modified cells [61]. Genetic manipulation of ASCs has also been performed with transgenes encoding for prosurvival proteins to enhance their survival after transplantation. These genetic modification protocols may include viral as well as nonviral vector-based protocols [62–65]. Alternative to genetic modifications, ASCs have been preconditioned by physical as well as pharmacological manipulations to enhance their paracrine activity, survival, and differentiation potential [66, 67]. More recently, ASCs have been used to dispense microRNAs (miRNAs) to the heart [68–70]. Based on these data, delivery of miRNA to the heart has shown salutary effects on infarct size and global cardiac function [71]. ASCs have also progressed to clinical trials for assessment of their safety and efficacy, including SkMs, BMSCs, and more recently, CSCs [72, 73]. Although SkMs were the first cell type to be tested in patients for heart cell therapy as an adjunct to CABG [74], which was followed by similar attempts by many other research centers [75–78], SkMs have lost their way in the journey from bench to bedside due to the risk of arrhythmias owing to their inability to integrate with the host cardiomyocytes [79].

The bone marrow (BM)-derived stem/progenitor cells have taken lead over any other cell type due to their safety, feasibility, superior characteristics in terms of

Tab. 4.1: Types and main origins of stem cells.

Stem cell type	Main origin
Embryonic stem cells (ESCs)	Inner cell mass of blastocyst embryo
Bone marrow progenitor cells (PMPCs)	Bone marrow
Hematopoietic stem cells (HSCs)	
Mesenchymal stem cells (MSCs)	
Skeletal myoblasts (SMs)	Adult skeletal muscle
Endothelial progenitor cells (EPCs)	Bone marrow/peripheral blood
Cardiomyocyte progenitor cells (CMPs)	Adult/fetal heart
Umbilical cord stem cells (UCSs)	Fetal umbilical cord

myogenic and vasculogenic differentiation potential, better immunologic properties, and superior paracrine activity due to which they secrete plethora of useful cytokines, growth factors, and miRNAs with multifactorial cardioprotective mechanisms. The resident CPCs are new entrants in the field of heart cell therapy [80, 81]. Given their origin from the heart tissue and electrical conditioning from the neighbor cardiomyocytes in their niche, they are being considered as an exceptional choice, even ahead of their counterparts from skeletal muscle as well as BM [82]. They can be isolated easily and expanded from human myocardial samples obtained using a minimally invasive biopsy procedure and can be used for transplantation with minimal risk of immune rejection or teratoma formation. Moreover, their differentiation *in vitro* is highly efficient, and hence, they are being extensively characterized in preclinical studies nowadays [83, 84]. Table 4.1 lists the various types of cells and their respective tissue of origin. The following text will focus only on the application of BM-derived cells in the clinical perspective.

4.2.1 BM stem cells

Human BM is composed of various lineages of cells in a growth factor and cytokine-rich extracellular matrix. Besides differentiated cells (e.g., monocytes, lymphocytes, fibroblasts, osteoblasts, osteoclasts, chondroblasts, and adipocytes), the cellular component of the BM has a small but diverse population of undifferentiated stem cells that includes hematopoietic stem cells (HSCs) and nonhematopoietic mesenchymal precursors that give rise to a heterogeneous population of mesenchymal stem cells (MSCs). Density-gradient centrifugation of the isolated human BM allows purification of BM mononuclear cell (BMMNC) fraction that mainly contains both HSCs (including EPCs; 2–4%) and 0.01% MSCs [85, 86]. A relatively rare adherent population of multipotent adult progenitor cells (MAPCs) has also been reported using

special culture conditions in the presence of specific growth factors, i.e., epidermal growth factor and Platelet-derived growth factor (PDGF). MAPCs have shown the capacity to transdifferentiate into classical endodermal, mesodermal, and ectodermal cell types *in vitro* [87]. Additionally, Ratajczak and colleagues have reported the presence of very small embryonic-like (VSEL) stem cells in the young BM, which share pluripotent stem-cell-like characteristics [88, 89]. The cultured VSEL stem cells were able to differentiate into all three embryonic germ cell lineages and expressed Nestin, PDX-1, NKX2.5, DAZL, CD45, and other relevant markers [90]. We have reported the existence of similar small juvenile cell population in old rat BMCs that express the cardiac lineage markers GATA-4 and MEF-2c and possess enormous potential for the repair of ischemic heart [91].

4.2.2 BM cell lineages and their characteristics

Characteristically, HSCs form all the hematopoietic cell lineages for continuous renewal and replenishment of red blood cells, platelets, monocytes, and granulocytes. HSCs are normally identified by positivity for cell surface marker expression, including CD31, CD34, CD45, CD133, and KDR, and the absence of CD38 [92]. They have been extensively studied clinically for BM transplant for a variety of hematologic disorders. EPCs constitute an integral derivative of HSCs with an inherent as well as indispensable role in endothelial cell function and angiogenesis. Although there is no one single identification surface marker for EPCs, they are identified by the coexpression of CD34, Flk-1, Tie, and VEGR2 surface markers and secretion of various bioactive molecules as part of paracrine activity to help in their functionality [86, 93].

On the other hand, human MSCs (hMSCs) form multiple mesoderm-type cell lineages including adipocytes, chondroblasts, and osteoblasts. They are essentially identified by surface antigens including CD90 and CD105, besides CD17, CD29, CD44, CD106, CD120a, and CD124. In addition, they are negative for HSC hematopoietic lineage markers CD31, CD34, CD45, CD133, CD14, CD19, and KDR [94, 95]. They can evade immune recognition due to HLA-DR negativity and exert immunomodulatory effects postengraftment [96–98]. Besides BM as the main source of MSCs, they are also located in the adipose tissue, heart, blood, brain, liver, and skeletal muscle. They constitute and maintain a supportive microenvironment in the BM hematopoietic niche, remain in close contact with HSCs, and provide them an essential support and play a vital role in their function [99]. Table 4.2 summarizes the surface markers and special characteristics of HSCs, EPCs, and MSCs.

Although both MSCs and HSCs possess multilineage differentiation potential to become skeletal muscle, bone, hepatocytes, and neurons [100–102], it remains contentious whether HSCs can truly transdifferentiate to adopt cardiac phenotype

Tab. 4.2: Surface markers and characteristics of the main bone marrow stem cells.

Bone marrow stem cell type	Surface markers	Special characteristics
Hematopoietic stem cells (HSCs)	CD31, CD34, CD45, CD133, KDR	Differentiate to endothelial cells and to all hematopoietic lineage cells (red blood cells, platelets, monocytes, and granulocytes)
Endothelial progenitor cells	CD34 Flk-1, Tie VEGR2	Possible to isolate from sources other than bone marrow (i.e., peripheral blood) Secrete various bioactive molecules Intrinsic endothelial differentiation and repair Essential role in angiogenesis
Mesenchymal stem cells	CD90, CD105, CD17, CD29, CD44, CD106, CD120a, CD124	Differentiate to adipocytes, chondroblasts, and osteoblasts Support hematopoietic niche necessary for hematopoiesis by HSCs Easily isolated and expanded Immunomodulatory properties

[103–105]. Contrary to the contentious cardiomyogenic potential of HSCs, a large number of *in vitro* and *in vivo* experimental studies have shown that both naïve as well as genetically modified MSCs transdifferentiated into cardiomyocytes post-engraftment into the experimentally injured heart with a resultant improvement in left ventricle (LV) function and attenuation of LV-remodeling studies [106–111]. The following section discusses the proposed mechanisms by which transplanted BM-derived stem/progenitor cells contribute to the attenuation of cardiac remodeling and preservation of global cardiac function after cell therapy.

4.2.3 Proposed mechanisms of cardiac repair and regeneration with BMSCs

How BMSCs participate in the repair process after homing-in or transplantation in the damaged heart remains a debatable issue. It is considered to involve more than one mechanism encompassing from myogenic and vasculogenic differentiation to fusion with host myocytes, paracrine signaling, and supporting the intrinsic repair process by the activation of CSC niches. The holistic mechanism is summarized in Fig. 4.1.

4.2.3.1 Cardiogenic and vasculogenic differentiation

Orlic and colleagues were the first to postulate cardiogenic differentiation of BMSCs as the key mechanism involved in salvaging global heart function subsequent to

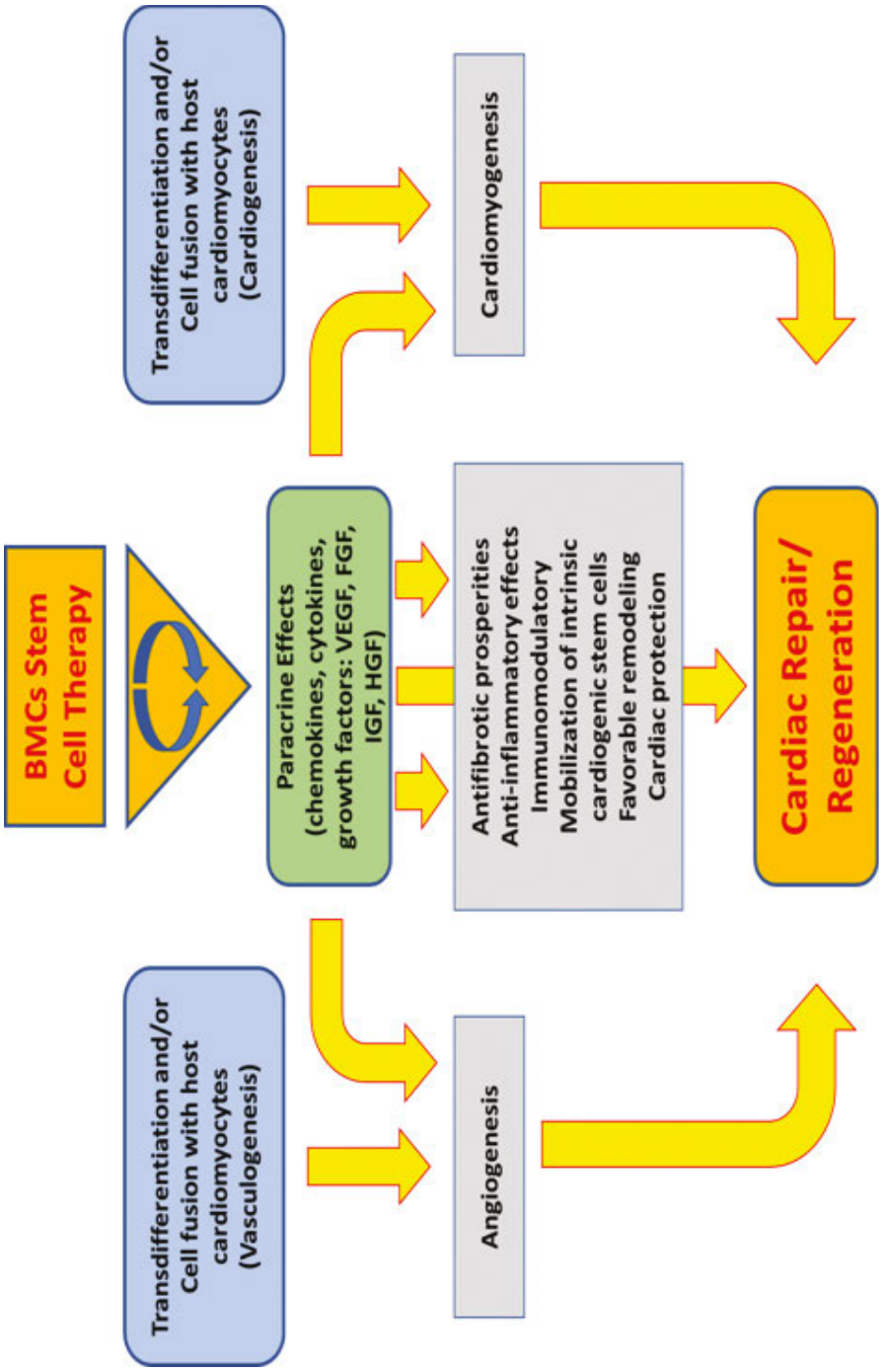

Fig. 4.1: The holistic approach shows that differentiation processes, cell fusion, and paracrine effects activate a cascade of events that interact actively to create new blood vessels and cardiomyocytes, with the final objective of functional cardiac repair. (Reproduced from Haider *et al.* [41])

heart cell therapy using BMCs [112]. The authors reported that the intramyocardially delivered Lin-ckit+ cells reconstituted 68% of the experimentally infarcted murine heart. These data were subsequently emphasized by many other research groups [113–115]. However, the rate of cardiomyogenic differentiation of BMCs has been very low and only limited. Additionally, there are only a few studies that have reported the long-term fate of donor cells and their derivative graft [116–118]. Hence, various strategies have been adopted to promote their rate of differentiation, including genetic, physical, or miRNA manipulation [119–121].

4.2.3.2 Fusion with the host myocytes

Fusion between the donor BMCs and recipient cardiomyocytes posttransplantation has been proposed as an alternative mechanism to cardiomyogenic differentiation as the underlying mechanism contributing to preserve or improve the infarcted heart function. In order to understand the *in vivo* fate of the donor BMCs, an *in vitro* model was developed between fluorescently labeled neonatal cardiomyocytes and human sternal BMCs. Laser scanning cytometry showed the existence of both differentiated and fused cell populations in the culture [122]. A recent study has used time-lapsed microscopy to study the fate of cocultured rat cardiomyocytes with human BMCs and observed that 25–40% of BMCs (from four different donors) acquired spontaneous Ca++ transients indicative of fusion with cardiomyocytes within few hours of coculture [123]. Immunostaining for connexion-43, Ki67, and α-sarcomeric actinin showed that the fused cells remained coupled with the surrounding cardiomyocytes and accomplished a nonproliferative and noncontractile phenotype. We have reported similar observations from experiments wherein donor SkMs formed heterokaryons in the recipient heart postengraftment [124, 125]. Thus, chimerism between the recipient cardiomyocytes and donor SkMs or their differentiation independent of chimerism or both remain the possible mechanisms contributing toward improvement of global cardiac function [126, 127]. However, the frequency of cell fusion (chimerism) and its mechanistic contribution to improved heart function remain undefined.

4.2.3.3 The paracrine hypotheses/immunomodulatory and anti-inflammatory effects/mobilization of endogenous stem cells

In the midst of the debate regarding cardiomyogenic differentiation of donor BMCs or their low rate of fusion with host cardiomyocytes, the paracrine hypothesis has drawn the attention of researchers with substantial supportive evidence to explain improved heart function after BMC transplantation. The BMCs secrete a broad array of soluble factors, i.e., cytokines, chemokines, interleukins, growth factors, etc., that are integral to the intrinsic cardiac repair mechanism [128–130]. Once released, these bioactive molecules contribute to the intrinsic repair process by multiprong

mechanisms that encompass from cytoprotection to mobilization and homing-in of resident stem cells to the site of injury to participate in the repair process [131]. The paracrine activity of cells differs between the types of the stem cells and their response to various physical, chemical, genetic manipulation, and stimuli from their microenvironment, including hypoxia, heat shock, electrical stimulus, etc. Although there is no all-inclusive comprehensive list of paracrine factors released from BMCs, some of the important identified growth factors include VEGF, bFGF, hepatocyte growth factor, insulin-like growth factor-I, and adrenomedullin [130]. Also included in the paracrine secretions are prosurvival molecules; antifibrotic, anti-inflammatory, and immunomodulatory agents; and microRNAs [130, 132]. Moreover, the paracrine factors influence the adjacent cells and initiate a signaling cascade that improves host cardiomyocyte survival, induce angiogenesis and neovascularization, and stimulate resident CSCs to proliferate, mobilize from their niche, and home into the ischemic regions [133]. Besides acting in a paracrine fashion, these released factors also act in an autocrine manner on the stem cells themselves, thus promoting their survival, proliferation, and differentiation besides supporting their paracrine activity [134].

4.3 BMCs in clinical trials

Following extensive characterization *in vitro*, and during the preclinical small and large animal studies, BMCs are the second cell type to enter clinical settings for safety and efficacy assessment in human patients after pioneering clinical use of SkMs by Menasché and colleagues [74, 135]. Many human trials have been conducted thereafter to assess BMCs for the treatment of acute and chronic IHDs. The versatility of the design of these clinical trials demonstrates the seriousness of the researchers involved to devise an optimal protocol for patient use. This versatility of the protocols includes cell preparation (use of whole BM for purified sublineages, naïve, or expanded *in vitro*), source of the cells (i.e., autologous to allogenic), variety of delivery strategies (i.e., transepicardial, transendocardial, intracoronary [I/C], intravenous infusion, etc.), variation in cell dosing (i.e., number of cells), time of injection (acute phase or chronic phase postinfarction episode, etc.), and variety of the pathological conditions (ischemia, infarction, etc.) to ensure the consistency in terms of safety and effectiveness of cell therapy. This section discusses the salient features of the clinical trials for their design and functional outcome in the patients included therein. Table 4.3 and Table 4.4 list the randomized clinical trials and their main results in acute and chronic forms of IHD.

Tab. 4.3: Clinical trials in different phases using bone marrow stem cells in the treatment of acute MI.

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
Phase 1	Hamano *et al.* (2001) [136]	Clinical trial	A total of 5 patients Aged between 59 and 73 years	Autologous BMMSCs	Intramyocardium injection	CABG	–	An improvement in coronary perfusion in three of the five patients after 1 month. No calcification or teratogenic tumors in the BMC injected area. BMCs were safe to use.
Phase 1	Strauer *et al.* (2002) [137]	Clinical trial	A total of 20 patients $n = 10$ for cell therapy (49 ± 10 years) $n = 10$ for standard therapy (50 ± 6 years)	Autologous BMMSCs	Intracoronary	PTCA	–	Cell therapy group has significant reduction of the infarct region, with a significant increase of infarction wall movement velocity, stroke volume, LVESV, and contractility and myocardial perfusion of the infarct region. Safety profile established.
Phase 1	Hare *et al.* (2009) [138] Prochymal	Randomized, double-blind, placebo-controlled, multicenter study	A total of 53 patients $n = 39$ for the hMSC group (59.0 ± 12.3 years) $n = 21$ for the placebo group (55.1 ± 10.2 years)	Allogeneic bone-marrow-derived hMSCs	Intravenous	–	CD105+, CD166+, and CD45–.	Adverse event rates were similar between the hMSC (5.3%) and placebo (7%) groups, and renal, hepatic, and hematologic laboratory indexes were not different. Reduced ventricular tachycardia episodes ($p = 0.025$) in hMSC. Global symptom score ($p = 0.027$) was significantly better in hMSCs. Increased left ventricular ejection fraction and reverse remodeling in hMSC, not placebo. Intravenous allogeneic hMSCs are safe in patients after acute MI.

Tab. 4.3 (continued)

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
Phase 1	Xinyang *et al.* (2015) [139] CHINA-AMI	Prospective, randomized and double-blind controlled trial	A total of 36 patients *n* = 11 for N-BMCs (61.2 ± 12.8 years) *n* = 11 for HP-BMCs (59.7 ± 11.7 years) *n* = 14 for control (60.62 ± 10.85 years)	Autologous HP-BMCs and N-BMCs	Intracoronary Infusion	Post PCI with stent implantation or thrombolysis	CD34+, CD133+, CD309+, CD117+, CD29+, CD166+, mSca-1+	– No differences in the occurrence of major adverse cardiovascular events among three groups. – Significant improvement in LVEDV and LVESV in HP-BMC group – No differences in LVEF or WMSI among three groups. – WMSI was improved in HP-BMCs and NBMC group but not in control.
Phase 1/ phase 2	Wollert *et al.* (2004, 2006) [140, 141] BOOST trial and follow-up	Randomized, blinded clinical trial	A total of 60 patients *n* = 30 for BMCs (53.4 ± 14.8 years) *n* = 30 for control (59·2 ± 13·5 years)	Autologous BMCs	Intracoronary infusion	Post-PCI	CD34+	After 6 months, mean global LVEF increased significantly in the BMC group ($p = 0.0026$) vs. the control group, but the increase was not significant after 18 months. LV systolic function improved in myocardial segments adjacent to the infarcted area in the BMC group. After 6 and 18 months, BMC transfer is safe and did not increase the risk of adverse events, restenosis, or arrythmias.

Tab. 4.3 (continued)

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
Phase 1/ phase 2	Hirsch *et al.* (2011) [142] HEBE	Multicenter, randomized clinical trial, no placebo group, control group received standard therapy	A total of 200 patients *n* = 69 for mononuclear BM cells (56 ± 9 years) *n* = 66 for mononuclear peripheral blood cells (57 ± 9 years) *n* = 65 for standard therapy (55 ± 10 years)	Mononuclear stem cells from BM or peripheral blood	Intracoronary Infusion	PCI and stent	CD34+ and CD14+	No significant difference in improvement of dysfunctional LV segments, LVEF, LV mass, or infarct size among the three groups. Intracoronary infusion of mononuclear cells from BM or peripheral blood following AMI does not improve regional or global systolic myocardial function.
Phase 2	Lunde *et al.* (2006) [143] ASTAMI	RCT	A total of 100 patients *n* = 50 BMC (58.1 ± 8.5 years) *n* = 50 control (56.7 ± 9.6 years)	Autologous BMCs	Intracoronary infusion	Post PCI	CD34+	The two groups did not differ significantly in changes in LVED volume, LVEF, or infarct size and had similar rates of adverse events. No effects of intracoronary injection of autologous mononuclear BMC on global left ventricular function.

Tab. 4.3 (continued)

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
Phase 2	Janssens *et al.* (2006) [144]	Randomized, double-blind, placebo-controlled trial	A total of 67 patients *n* = 34 for placebo (57·9 ± 10 years) *n* = 33 for BMC (55·8 ± 11 years)	Autologous BMC	Intracoronary infusion	PCI	CD34, CD133, CD90, CD105, CD117/ c-kit, CD73	LVEF, myocardial perfusion, and metabolism increased similarly in both groups. Significant reduction in myocardial infarct size and a better recovery of regional systolic function in BMC, denoting a possible favorable effect on infarct remodeling.
Phase 2	Mansour *et al.* (2011) [145] COMPARE-AMI	Randomized, double-blinded, placebo-controlled trial	*n* = 20 hBMCs *n* = 20 placebo group (52.2 ± 8.9 years)	Autologous hematopoietic BMCs	Intracoronary injection	Successful PCI with stent, persistent LV dysfunction	Selected CD133+	At 1 year, there were no complications (death, myocardial infarction, stroke, or sustained ventricular arrhythmia). LVEF significantly improved at 4 months and remained significantly higher at 1 year. Selected CD133+ stem cells are safe and feasible in patients with LV dysfunction following acute MI.
Phase 2	Qiu *et al.* (2016) [146] COMPARE-AMI IVUS substudy	Prospective, double-blinded, placebo-controlled trial	*n* = 17 hBMCs with average age of 51.0 (43.0–60.5) years	Autologous hematopoietic BMCs	Intracoronary injection	Successful PCI with stent	Selected CD133+	No incidence of death, MI, stroke, or major arrythmias in both groups.

Tab. 4.3 (continued)

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
			n = 20 placebo group, with average age of 50.5 (48.3–63.3) years					No detectable effects on neointimal hyperplasia or atherosclerosis progression between both groups in the infarcted-related or confraternal coronary arteries.
Phase 2	Roncalli *et al.* (2011) [147] BONAMI	Multicenter, randomized controlled trial	A total of 101 patients *n* = 49 for control group (55 ± 11 years) *n* = 52 for BMC group (56 ± 12 years)	Autologous BMCs	Intracoronary infusion	PCI with bare metal stent implantation	CD34+, CD45+, CD133+, KDR+, CXCR4+	Significant improvement in myocardial viability in the BMC vs. the control group ($p = 0.03$) in a multivariate analysis. LVEF did not differ significantly between the BMC and control groups ($p = 0.62$), at 3 months.
Phase 2	Traverse *et al.* (2012) [148] The TIME	Randomized, double-blinded, placebo-controlled trial	A total of 120 patients randomized to receive BMC and placebo at day 3 or day 7 (56.9 ± 10.9 years)	Autologous BMCs	Intracoronary infusion	Following PCI	CD34+, CD34+/ CD133+	LVEF increased similarly in both groups. No detectable treatment effect on regional LV function in either infarct or border zones. No significant differences between groups in the change in global LV function over time. Timing had no detectable effect on recovery of regional LV function. Rare major adverse with no difference between groups.

Tab. 4.3 (continued)

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
Phase 2	Choudry *et al.* (2016) [149] REGENERATE-AMI	Randomized, double-blind, multicenter and placebo-controlled trial	A total of 100 patients $n = 45$ placebo (56.7 ± 10.7 years) $n = 55$ BMC (56.4 ± 10.4 years)	Autologous BMC	Intracoronary infusion	Infusion within 24 h of successful PCI	CD34+	LVEF increased compared with baseline in both groups, between-group difference for BMC was small ($p = 0.10$). Significantly greater myocardial salvage index in the BMC group. Major adverse events were rare in both groups. Early infusion leads to a small, nonsignificant improvement in LVEF; it may play a role in infarct remodeling and myocardial salvage.
Phase 2/ phase 3	Huikuri *et al.* (2008) [150] FINCELL	Randomized, placebo-controlled, double blind	A total of 80 patients $n = 40$ for intervention group (60 ± 10 years) $n = 40$ for control group (59 ± 10 years)	Autologous BMCs	Intracoronary injection	Thrombolysis followed by PCI 2–6 days after STEMI	CD34+	The BMC group had a greater absolute increase in global LVEF than the placebo group did. No differences in adverse clinical events, arrhythmia risk, or restenosis of the stented coronary lesions.
Phase 2/ phase 3	Lee *et al.* (2014) [151]	Randomized, pilot, multicenter trial	A total of 80 patients (58 completed)	Autologous MSC	Intracoronary infusion	Successful revascularization by PCI or thrombolysis	CD79+ and CD105+, CD14–, CD34–, CD45–	Absolute improvement in LVEF was modest but greater in the MSC group than in the control group ($p = 0.037$).

Tab. 4.3 (continued)

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
			n = 26 MSC (65 ± 10.3 years) *n* = 28 placebo (54.2 ± 7.7 years)					Safe, no toxicity or adverse cardiovascular event.
Phase 3	Schächinger (2006) [152] REPAIR-AMI	Randomized, placebo-controlled trial	A total of 204 patients *n* = 103 placebo (57 ± 11 years) *n* = 101 BMC (55 ± 11 years)	Autologous BMC	Intracoronary infusion	PCI with stent	CD34+/, CD45+, CD34+/, CD133+/, CD45+	Absolute improvement in the global LVEF and contractile function was significantly greater in the BMC group than in the placebo group. Significant reduction in clinical end-point of death, recurrence of myocardial infarction, and any revascularization procedure in BMC group ($p = 0.01$).

MI = myocardial infarction; BMMSC = bone marrow stem cells; CABG = coronary artery bypass graft; BMC = bone-marrow-derived cell; PTCA = percutaneous transluminal catheter angioplasty; LVESV = left ventricular end systolic volume; hMSC = human mesenchymal stem cell; BOOST = Bone Marrow Transfer to Enhance ST-Elevation Infarct Regeneration; PCI = percutaneous coronary intervention; LV = left ventricle; LVEF = left ventricle ejection fraction; BM = bone marrow; AMI = acute myocardial infarction; ASTAMI = Autologous Stem-Cell Transplantation in Acute Myocardial Infarction; hBMC = human bone marrow-derived mesenchymal cells; BONAMI = Bone Marrow in AMI; REGENERATE-AMI = •••; STEMI = ST-elevation myocardial infarction; REPAIR-AMI = Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction.

Tab. 4.4: Clinical trials in different phases using bone marrow stem cells in the treatment of chronic ischemic heart disease and HF.

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
Phase 1	Pokushalov *et al.* (2010) [153] DOBLON-DIB	Randomized, single blinded, controlled	A total of 109 patients $n = 55$ for BMC group (61 ± 9 years) $n = 54$ for control group (62 ± 5 years)	Autologous BMMCs	Intramyocardial injections	None	CD34	Significant improvement in CCS class and NYHA in the BMMC group. LVEF increased significantly in the BMMC group ($p = 0.04$), decreased in the control group ($p = 0.61$). The improvement in stress score was more noticeable. BMMC therapy is safe and improved survival, clinical symptoms, and has beneficial effect on LV function.
Phase 1/ phase 2	Hare *et al.* (2012) [154] POSEIDON	RCT	A total of 30 patients randomized to receive three different MSC concentrations Autologous (62 ± 10.5 years) Allogenic (63.7 ± 9.3 years)	Allogeneic and Autogenic MSCs	Transendocardial injection	None	CD105, CD166, CD45	Similar low rates of adverse events including immunologic reactions, safe, and well tolerable. Better functional capacity, quality of life, and ventricular remodeling in cell therapy group. LVEF increased by 1.96 but not statistically significant ($p = 0.11$).

Tab. 4.4 (continued)

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
Phase 1/ phase 2	Hare *et al.* (2017) [155] POSEIDON-DCM	Randomized, open-label, pilot study	A total of 37 patients $n = 18$ auto-hMSC (57.4 ± 11.0) $n = 19$ allo-hMSC (54.4 ± 11.5)	Allogeneic and autogenic hMSCs	Transendocardial injections at 10 left ventricular sites	None	CD19+ (B-cell) CD3+ (T-cell)	No 30-day SAEs. After 1 year, allo had fewer SAEs (28.2%) as compared to auto (36.5%). EF increased in both groups but more significantly in the allo ($p = 0.004$) vs the auto ($p = 0.116$) group. 6MWT increased for the allo but not for the auto group, with a reduction in TNF.
Phase 1/ phase 2	Assmus *et al.* (2013) [156] CELLWAVE	A randomized, double-blind, placebo-controlled trial	A total of 103 patients $n = 42$ for LV shock wave with low dose (65 ± 12 years) $n = 40$ for high dose (58 ± 11 years) $n = 21$ for placebo	Autologous BMCs	Intracoronary infusion	Shock wave to left ventricular anterior wall	CD34+/ CD45+ CD34+/ CD133+/ CD45+	Significant but modest improvement in LVEF in the shock wave + BMC group ($p = 0.02$). Regional wall thickening improved significantly in the shock wave + BMC group ($p = 0.01$). Overall major adverse cardiac events was significantly less in the shock wave + BMC group ($p = 0.02$).

Tab. 4.4 (continued)

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
Phase 1/ phase 2	Karantalis *et al.* (2014) [157] PROMETHEUS	Prospective randomized study	A total of 6 patients $n = 2$ low-dose MSC $n = 4$ high-dose MSC (54.9 ± 4.2 years)	Autologous MSCs	Intramyocardial injection	CABG	CD45–/ CD105+, CD45+/ CD105+	The MSC group had increased LVEF ($p = 0.0002$) and decreased scar mass ($p < 0.0001$) compared to baseline. MSC-injected segments had concordant reduction in scar size and improvement in perfusion and contractile function.
Phase 1/ phase 2	Heldman *et al.* (2014) [158] TAC-HFT	Randomized, double-blind, placebo-controlled trial	$n = 19$ for MSC (57.1 ± 10.6 years) and $n = 11$ placebo (60 ± 12 years) $n = 19$ for BMC (61.1 ± 8.4 years) and $n = 10$ placebo (61.3 ± 9 years)	Autologous MSC and BMC	Transendocardial injection	None	–	Similar safety profile for MSC and BMC. MLHF score improved with MSCs ($p = 0.02$) and BMCs ($p = 0.005$) but not placebo. 6MWD increased with MSCs only ($p = 0.03$). Infarct size was reduced by MSCs ($p = 0.004$) but not by BMCs or placebo. Regional myocardial function improved with MSCs ($p = 0.03$) but not BMCs or placebo. LV volume and EF did not change.

Tab. 4.4 (continued)

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
Phase 1/ phase 2	Mathiasen *et al.* (2015) [159] MSC-HF trial	Randomized, double-blind, placebo-controlled trial	A total of 60 patients (55 completed) $n = 37$ for MSCs (66.1 ± 7.7 years) $n = 18$ for placebo (64.2 ± 10.6 years)	Autologous MSCs	Intramyocardial injection	VEGF-165 treatment	CD45, CD34, CD13, CD73, CD90, CD105	LVESV was reduced in the MSC group and increased in the placebo group ($p = 0.001$). Significant improvements in LVEF, stroke volume, and myocardial mass. No differences in NYHA class and 6MWT. No side effects were identified.
Phase 2	Losordo *et al.* (2011) [160] ACT34-CMI	Randomized, double-blinded, placebo-controlled prospective trial	A total of 168 patients $n = 56$ for each group CD34+ 1×10^5 cells/kg (61.3 ± 9.1 years) CD34+ 5×10^5 cells/kg (59.8 ± 9.2 years) Placebo (61.8 ± 8.5 years)	Autologous CD34+ HBMCs	Intramyocardial injections	G-CSF	CD34+	Weekly angina frequency was significantly lower in the low-dose group vs. the placebo group; it was also lower in the high-dose group but not significantly. Exercise tolerance improved significantly in low-dose patients than in placebo patients; it was also better in the high-dose group but not significantly. No deaths among cell-treated patients at 12 months, while mortality was 5.4% in the placebo group.

Tab. 4.4 (continued)

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
Phase 2	Henry *et al.* (2016) [161] ACT34-CMI 2-year outcome	Randomized, double-blinded, placebo-controlled prospective trial	A total of 167 patients $n = 55$ for CD34+ 1×10^5 cells/kg (61.3 ± 9.1) $n = 56$ for CD34+ 5×10^5 cells/kg (59.8 ± 9.2) $n = 56$ for placebo (61.8 ± 8.5 years)	Autologous CD34+ HBMCs	Intramyocardial injections	G-CSF	CD34+	At 24 months, patients treated with both low- and high-dose CD34+ cells had a significant reduction in angina frequency ($p = 0.03$). At 24 months, there were a total of seven deaths (12.5%) in the control group versus one (1.8%) in the low-dose and two (3.6%) in the high-dose ($p = 0.08$) groups.
Phase 2	Perin *et al.* (2012) [162] FOCUS-CCTRN	Randomized, double-blinded, placebo-controlled trial	A total of 92 patients $n = 61$ in BMC group $n = 31$ in placebo group Average age: 63 years	Autologous BMCs	Transendocardial injection	None	CD34+ and CD133+	No significant improvement in LVESV, maximal oxygen consumption, or reversibility on SPECT in the cell therapy group as compared to the placebo group.

Tab. 4.4 (continued)

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
Phase 2/ phase 3	Bartunek *et al*. (2013) [163] C-CURE	Prospective, open-label, randomized, multicentre trial	A total of 47 randomized, $n = 21$ cell therapy group (77.5 ± 10.4 years) $n = 15$ in control group (59.5 ± 8 years)	Autologous BMC	Endomyocardial (endoventricular) injections	None	CD34+	No adverse events or toxicity. LVEF increased significantly by 7% ($p < 0.0001$) and LVESV significantly reduced ($p < 0.001$) in the cell therapy group. Significant improvement in 6MWD and superior scores regarding NYHA functional class, quality of life, physical performance, hospitalization, and event-free survival.
Phase 2/ phase 3	Nasseri *et al*. (2014) [164] CARDIO133	Randomized, double-blind, placebo-controlled trial	A total of 60 patients $n = 30$ CD133+ BMC (62.7 ± 10.6 years) $n = 30$ placebo (61.9 ± 7.3 years)	Autologous CD133+ BMC	Intramyocardium injection	Adjunct to CABG	CD133+	Similar adverse events in both groups. No difference in 6MWT, Minnesota score, or CCS class between groups. NYHA class improved more in the placebo group ($p = 0.004$). LVEF at 6 months was 2% higher in the placebo vs. cell therapy group ($p = 0.3$). Regional wall motion and myocardial perfusion at rest recovered in more LV segments in the cell therapy vs. the placebo group (9 vs. 2%; $p = 0.001$). Scar mass decreased in CD133+ patients ($p = 0.05$) but unchanged in placebo patients.

Tab. 4.4 (continued)

Phase	Study details (authors/ year)	Study type	Sample size and age	Type and source of stem cells	Rout of delivery	Pre/ concomitant treatment	Surface markers	Results
Phase 3	Bartunek *et al*. (2017) [165, 166] CHART-1	Prospective, randomized, double blind, sham controlled	A total of 271 patients $n = 120$ cardiopoietic cells (61.6 ± 8.6 years) $n = 151$ sham procedure (62.1 ± 8.7 years)	Autologous MSC Cardiopoietic cells	Intramyocardial injections	None	–	Primary outcome was neutral (LVESV, LVEF, all-cause mortality, worsening heart failure, 6MWT) at 39 weeks. ($p = 0.27$ for cell therapy). Safety was demonstrated across the cohort.

HF = heart failure; BMMC = bone marrow mononuclear cells; CCS = Canadian Cardiovascular Society; NYHA = New York Heart Association; LVEF = left ventricle ejection fraction; POSEIDON = Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis; RCT = randomized controlled trial; MSC = mesenchymal stem cell; POSEIDON-DCM = Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis-Dilated Cardiomyopathy; hMSC = human mesenchymal stem cell; SAE = serious adverse event; EF = ejection fraction; 6MWT = 6-minute walk test; TNF – tumor necrosis factor; BMC = bone-marrow-derived cell; PROMETHEUS = Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery; CABG = coronary artery bypass graft; TAC-HFT = Transendocardial Autologous Cells in Ischemic Heart Failure Trial; MLHF = Minnesota Living with Heart Failure; 6MWD = 6-minute walk distance; LV = left ventricle; MSC-HF = bone marrow-derived mesenchymal stromal cell treatment in patients with severe Heart Failure; VEGF = vascular endothelial growth factor; LVESV = left ventricular end systolic volume; ACT34-CMI = Adult Autologous CD34+ Stem Cells for Reduction of Angina Episodes in Patients With Refractory Chronic Myocardial Ischemia; HBMC = human bone marrow-derived mesenchymal cells; G-CSF = granulocyte colony stimulating factor; FOCUS-CCTRN = First Mononuclear Cells injected in the United States conducted by the Cardiovascular Cell Therapy Research Network; SPECT = single photon emission computed tomography; C-CURE = Cardiopoietic Stem Cell Therapy in Heart Failure; CARDIO133 = Bypass Surgery and CD133 Marrow Cell Injection for Treatment of Ischemic Heart Failure; CHART-1 = Congestive Heart Failure Cardiopoietic Regenerative Therapy.

4.3.1 Clinical trials in patients with AMI

Phase 1 clinical trials have been primarily envisioned to determine the safety of BMCs either unfractionated or its fractionated sublineages, unexpanded or expanded, *in vitro* to experiment different methods of delivery and explore more possibilities for future studies. Hamano and colleagues were the first to perform a clinical study using BMCs for myocardial repair in five patients with IHD. The patients received 5×10^8–1×10^9 cell autologous BMMNCs by multiple intramyocardial (I/M) injections under direct vision as an adjunct to routine CABG [136]. One month later, radionuclide cardiac imaging showed three out of the five patients having cell therapy with improved regional coronary perfusion, while 1-year follow-up showed that BMMNC-based cell therapy was safe for human use. Postoperative assessment using echocardiography, electrocardiogram, radiography, and blood tests did not reveal any detrimental effects of cell transplantation and none of the cell transplanted hearts had any calcification or teratoma in any of the five patients. Although the results were encouraging, the lack of a control group of patients was a major drawback in the study.

A year later, Strauer and colleagues reported the results of a single pilot patient study that involved I/C delivery of BMMNCs in the infarct-related artery 6 days after percutaneous transluminal catheter angioplasty (PTCA). Results showed a significant reduction in transmural infarct area and improved LV ejection fraction (LVEF) and stroke volume [167]. Later, they included 10 more patients with AMI and treated them with BMMNCs by the I/C cell delivery approach as an adjunct to PTCA. A control arm of 10 patients was included in the study wherein the patients received standard treatment but without the BMC therapy. Besides indicating the safety of BMMNCs, a significant reduction in the infarct size, enhanced regional myocardial perfusion, and significantly preserved indices of LV-pump function were observed in the experimental group of patients [137]. These data from the pioneering clinical studies, which demonstrated the safety of BMMSCs for human use, paved the way for subsequent clinical research in the field of stem cell therapy for cardiovascular pathologies. Although devoid of any histological evidence, these studies reported regeneration of the infarcted heart and angiogenesis as the contributory underlying mechanisms for the improved regional myocardial perfusion and preserved cardiac function observed in the patients receiving BMMNCs treatment.

More structured trials followed the earlier safety assessment studies. The Bone Marrow Transfer to Enhance ST-Elevation Infarct Regeneration (BOOST) trial [140] was one of the important earlier studies that proved the safety and effectiveness of BMMCs for the treatment of patients with AMI. The study included 60 patients who were randomized into the experimental group (n = 30 patients), who received I/C BMMNC delivery 4–8 days after PTCA besides standard therapy, and to the control group (n = 30 patients), who received standard treatment without cell therapy. The 6-month follow-up revealed improved LV contractility in the infarct border zone and 6% increase in global LVEF (p = 0.0026) as compared to the control group of patients.

Nevertheless, the observed statistical benefit was lost at 18 months and 5 years follow-up [141]. Only patients with larger transmural infarcts showed sustenance of benefits in terms of LVEF during the longer follow-up as compared to those with smaller infarcts. The BOOST trial was unique in providing one of the longest periods of safety as it demonstrated the safety of I/C BMMNC transfer, which did not increase the risk of major adverse cardiac events (MACEs), restenosis, or arrhythmias even after 6 months, 18 months, and 5 years despite loss of significant statistical benefits of cell therapy over a longer period of time. Encouraged by the safety data, the same group of researchers has conducted BOOST-2 randomized placebo-controlled trials (Clinical Trial Identifier SRCTN17457407), which included a bigger group of ST-elevation myocardial infarction (STEMI) patients (n = 153) having a larger infarct size [168]. Besides a placebo-treated group of patients, the experimental groups of patients received either low or high doses of BMCs, which were either irradiated or nonirradiated at 8 ± 2 days after PTCA. The trials were envisioned to determine the effect of cell dose as well as the effect of mitotically inactive T-lymphocyte depletion to enhance the efficacy of cell preparation. The primary end-point of the study was to determine the change in LVEF from the baseline values at 6 months after cell therapy. The results of the study clearly showed the safety of I/C BMCs transfusion in terms of the absence of MACE. However, there was little benefit for the patients with STEMI with moderately reduced LVEF (showing an improvement in LVEF of only 1 percentage point in the experimental group as compared to the placebo-treated control). Moreover, irradiation of the cell prior to use failed to enhance their therapeutic efficiency. The study data do not support the use of nucleated cells for therapy of STEMI patients with moderately declined LVEF.

Despite ample safety data from various clinical studies, the choice of the cell source remains an arguable issue. On the basis of the results of a pilot HEBE study including 26 patients with large AMI who had received primary PCI and stent placement [169], Hirch and colleagues designed a phase 1/phase 2 randomized and controlled HEBE study (International Standard Randomized Controlled Trial Identifier #ISRCTN95796863). The study was intended to compare the reparability of autologous BMMNCs isolated from peripheral circulation (PBMNCs) [142]. The multicenter study included 200 patients with large AMI who were treated with primary PCI and stent placement. The patients were randomized to the control group (n = 65; receiving standard therapy without placebo treatment), the BMMNC-treated group (n = 69), or the PBMNC-treated group (n = 66). The primary objective of the study was to assess whether cell therapy of AMI patients is superior to standard therapy for restoration of normal myocardial function. Magnetic resonance imaging (MRI) at 4 months after treatment showed no significant improvement in dysfunctional LV segments, LVEF, LV mass, and infarct size among the three groups. Nevertheless, improvement in segmental wall thickening was observed in the cell therapy group of patients as compared to their respective baseline reading. A Doppler flow measurement substudy of HEBE in all three groups of patients (BMMNC group, n = 23; PBMNC group, n = 18; and control group, n = 19) reported insignificant recovery of microcirculation in all

three groups of treatment at 4 months after their respective treatment [170]. Long-term follow-up MRI at 2 years was performed, while 5-year follow-up was performed to assess MACE, i.e., reinfarction, rehospitalization for heart failure, or death [171]. The results showed that a total of nine patients died during the trials. On the functional note, BMMNC-treated patients showed a trend for decreased LV-end systolic volume (LVESV) as compared to the control patients, while the PBMNC-treated group had statistically insignificant difference as compared to the control group. Similarly, the combined end-point of death and hospitalization for heart failure was insignificantly less frequent in the BMMNC-treated patients. Unlike most clinical studies reported in the literature, the HEBE study is one of the very few clinical studies wherein functional assessment of the cells was performed as part of the quality control. The major study limitations included the lack of placebo and sham treatment groups for comparison. Moreover, MRI was not performed at a fixed time in all patients after AMI, which hampered a precise comparison of the results between groups.

Subsequent to the successful I/C use of autologous BMMSCs for the treatment of AMI by Chen *et al.* (2004) [172], Hare *et al.* experimented with allogenic BMMSCs for the treatment of patients with MI [138]. The study was designed to establish the safety of Prochymal (Provacel), an hMSC preparation during a randomized, double-blinded, placebo-controlled multicenter trial including 53 patients (Clinical Trial Identifier NCT00114452). The experimental group of patients was divided into three cohorts, each one of them receiving single I/C infusion of either 0.5, 1.6, or 5×10^6 cells suspended in 1.9% human serum albumin (HSA) and 3.8% dimethylsulfoxide (DMSO), while the placebo-controlled patient group received the same preparation of HSA and DMSO without cells. Follow-up assessments at 1, 3, and 6 months showed the safety of allogenic hMSC preparation for heart cell therapy. The study also demonstrated that the global symptom score in all patients ($p = 0.027$) and LVEF in a subset of patients with anterior MI ($p = 0.004$) were both significantly better after hMSC treatment. Moreover, MRI also revealed reversed myocardial remodeling. Although LVEF improved significantly in MSC-treated patients as compared to their baseline readings, it remained insignificantly changed as compared to their placebo-treated counterparts during the 6-month follow-up.

Given that cellular preconditioning approach has gained popularity for its effectiveness to enhance the donor cell survival postengraftment as well as to improve their differentiation potential and paracrine activity [66, 67], a recently published CHINA-AMI phase 1 trial (Clinical Trial Identifier NCT01234181) has reported the use of preconditioned cells for the treatment of acute MI patients [139]. The study enrolled 36 AMI patients who were randomized to the control group ($n = 14$, without cell therapy, placebo treated), normoxia-treated BMMNC group ($n = 11$), and 24-hour hypoxia-treated BMMNC group ($n = 11$) during a double-blinded trial. The primary end-points of the study were the safety and feasibility of the preconditioned BMMNCs after I/C administration. Follow-up on day 30 and 1 year revealed no MACE in any of the three groups, while a significant improvement in LVEDV and

LVESV was observed in the hypoxia-treated BMMNC group both at 6 months and 1 year after cell therapy. However, there was no difference in LVEF among the three groups. Single photon emission computed tomography (SPECT) showed a significant reduction in perfusion defect in the cell therapy group as compared to their respective baseline level; however, intergroup perfusion defect remained insignificantly altered. These data provided the first direct evidence of the safety of cellular preconditioning approach for use in the human patients.

4.3.2 Trials in patients with chronic myocardial ischemia

Trials in patients with chronic myocardial ischemia have been primarily designed to determine the therapeutic benefits of BMSC-based therapy in the ischemic myocardium. Most of these clinical trials are recent and have focused on I/M administration of BMSCs rather than I/C infusions, as was the case in patients with AMI. Pokushalov and colleagues reported the results of a single-blinded phase 1 trial that included 109 patients [153]. The patients were randomized to the control group (n = 55) or the experimental group (n = 54) wherein the patients received I/M injection of autologous BMMNCs. The primary aim of the study was to evaluate the therapeutic benefits of BMMNC transplantation in patients with severe ischemic heart failure. The NOGA system (Biosense-Webster) was used to ensure infarct border zone delivery of $41 \pm 16 \times 10^6$ cells at multiple injection sites. The results showed no periprocedural complications or safety issues in both groups of patients. There was significant improvement in the Canadian Cardiovascular Society (CCS) class as well as New York Heart Association (NYHA) class scores in BMMNC-treated patients at 6 and 12 months of follow-up. Additionally, LVEF significantly increased in the BMMNC-treated patients (27.8 ± 3.4% to 32.3 ± 4.1%; p = 0.04), while it tended to decrease in the control group of patients (26.8 ± 3.8% to 25.2 ± 4.1%; p = 0.61). Repeated NOGA mapping showed improved electrical activity and myocardial contractility in the cell transplanted as well as their adjoining myocardial segments. Rate of death after 12-month follow-up was significantly lower in the BMMNC group (log-rank test, p = 0.0007) as 6 (10.9%) BMMNC-treated patients died as compared to 21 deaths (38.9%) in the control group. Lack of functional assessment of the isolated BMMNCs, missing placebo treatment group for comparison, and smaller cohorts in the control and experimental groups are some of the limitations of the study. Moreover, there is no explanation for the observed functional improvement in the cell therapy patients.

Based on the translational studies in a porcine model of ischemic cardiomyopathy that showed reversal of remodeling and restoration of myocardial function [173, 174], various studies were designed to assess the therapeutic efficacy of MSCs in human patients suffering from ischemic cardiomyopathy. Williams and colleagues enrolled eight patients with ischemic cardiomyopathy to validate their hypothesis that transendocardial delivery of BMMSCs (n = 4 patients) or BMMNCs (n = 4 patients)

could cause functional recovery of the ischemic myocardium as well as reversal of remodeling [175]. All patients well tolerated the cell therapy procedure without any untoward events. Cardiac MRI (CMRI) at 3 and 6 months after the procedure revealed decreased infarct size and improved regional LV function while the chamber dimensions insignificantly changed until 1 year. Although the results were encouraging, the number of patients included in the study and the fact that there was no control group necessitated the designing of further studies using larger cohorts of the patients. Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis (POSEIDON) phase 1/phase 2 trials (Clinical Trial Identifier NCT01087996) were designed to compare autologous versus allogenic BMMSCs in patients with IHD [154]. The study included 30 patients with LV dysfunction due to ischemic dilated cardiomyopathy who received increasing doses of 20, 100, or 200 million cells (5 patients per dose level of each cell type) at multiple (~10) endocardial sites. The primary objective of the trials was to compare the effectiveness of transendocardial injection of allogenic and autologous BMMSCs in patients with chronic ischemic cardiomyopathy. One-year follow-up showed no adverse effects related to cell therapy. Only four patients in the autologous and one patient in the allogenic MSC group developed arrhythmias. Additionally, LVEF increased by 1.96%, but it was statistically insignificant (p = 0.11). Interestingly, the low-dose cell treatment group (20 million) showed the highest benefits in terms of reduction in LV volumes and an increase in LVEF. In conclusion, treatment with BMMSCs was associated with an acceptable safety profile, better functional capacity, improved quality of life, and reversed ventricular remodeling. More recently, the same research group has published the data of updated POSEIDON trials [155]. The rationale and study design of the phase 1/phase 2 POSEIDON-DCM trials were modified to include patients with nonischemic cardiomyopathy [176]. A total of 37 patients (average age, 55.8 ± 11.2 years) were included in the study, who were randomized into allogenic MSC or autologous MSC treatment groups. Transendocardial stem cell injections were carried out using NOGA catheter on 10 sites in the LV and the patients were followed up for the assessment of safety and efficacy end-points at baseline and 30 days, 3 months, 6 months, and 1 year after cell therapy. The overall safety of the procedure was evident from the low rate of MACE after 1 year, which was 28.2% in the allogenic MSC-treated group as compared to 36.5% in the autologous MSC-treated group. Similarly, 6 minute-walk time (6MWT) and LVEF were significantly improved in allogenic MSC-treated patients as compared to their counterparts (p = 0.004). Although the safety and efficacy data are encouraging, the study has its limitations, including the absence of a placebo-treated control group of patients for comparison and the lack of functional characterization of the cells before transplantation. The data also point to the significance of using young donor cells as compared to autologous cells obtained from the patients [177].

Given that there is a good correlation between granulocyte colony stimulating factor (G-CSF) concentration and progenitor cell mobilization from the BM [178], a recent advancement in the clinical studies has been the use of a combinatorial

approach in which cytokine treatment is combined with BMSC transplantation [179]. A single-center, randomized, placebo-controlled REGENERATE-IHD was designed that included 90 patients with IHD. The patients were randomized to receive either G-CSF (10 mg/kg/day) or serum for five consecutive days, subsequent to which BMMNC or placebo (serum) treatment was given either by peripheral injection or I/M or I/C injections. CMRI at 1 year showed significantly improved LVEF (4.99%; 95% confidence interval [95% CI], 0.33–9.6%; $p = 0.038$) in the I/M treated BMMNC group while no other groups showed a significant change in LVEF.

CELLWAVE (Clinical Trial Identifier NCT00326989), a randomized double-blinded, placebo-controlled trial, was designed to validate the hypothesis that concomitant cardiac shock-wave pretreatment followed by I/C infusion of BMSCs will improve the recovery of LVEF in patients with chronic ischemic heart failure [156]. The underlying anticipated mechanism was that shock-wave pretreatment would enhance the homing factors in the heart (which otherwise get depleted in the failing heart) that would be supported by I/C infusion of BMSCs. A total of 103 patients were randomized in a single-blinded manner into low-dose shock wave ($n = 42$), high-dose shock wave ($n = 40$), and placebo ($n = 21$) pretreatment groups. Subsequently, I/C infusion of cells or placebo treatment was given to the patients who were assessed for primary and secondary study end-points at 4 months after their respective treatment. A significant but modest improvement in LVEF (3.2%; 95% CI, 2.0–4.4%, $p = 0.02$) and regional wall thickness and a significantly low rate of MACE ($p = 0.02$) were observed in the shock wave + BMC group. Unlike CHINA-AMI, wherein a cellular preconditioning approach was used to precondition the donor cells prior to transplantation, CELLWAVE trials are unique in target tissue preconditioning with shockwave treatment prior to cell therapy.

4.3.3 BMSC trials in patients with heart failure

Karantalis and colleague have reported a small prospective randomized study (PROMETHEUS; Clinical Trial Identifier NCT00587990) in six patients ($n = 2$ for low-dose MSCs, $n = 4$ for high-dose MSCs), two of whom had a history of congestive heart failure and all of them were in New York Heart Association (NYHA) class 1 and 2. Autologous cell therapy was carried out as an adjunct to CABG. CMRI was carried at baseline and 3, 6, and 18 months to assess structural and functional cardiac parameters, i.e., scar size, regional perfusion, wall thickness, and contractility. The study showed increased LVEF ($p = 0.0002$) and decreased scar mass ($p < 0.0001$) as compared to their baseline values. Moreover, MSC-injected segments had concordant reduction in scar size and improvement in regional perfusion and contractile function [157].

Unlike PROMETHEUS trials, the MSC-HF trials (Clinical Trial Identifier NCT00644410) used culture-expanded autologous MSCs in 55 patients who were randomized in double-blind manner (2:1) to cell-treated ($n = 37$) and placebo-treated ($n = 18$) groups [159]. The primary end-point of the study was to measure change in

LVESV besides safety of I/M delivery of autologous BMMSCs. LVESV was favorably reduced in the cell therapy group ($p = 0.001$), whereas it increased in the placebo group ($p = 0.07$) in comparison with their respective baseline values [180]. Additionally, cell therapy significantly improved LVEF by 6.2% ($p < 0.0001$), stroke volume by 18.4 mL ($p < 0.0001$), and myocardial mass by 5.7 g ($p = 0.001$) as compared to the baseline, albeit without improvement in NYHA class or 6MWT.

The phase 1/phase 2 randomized, double-blinded Transendocardial Autologous Cells in Ischemic Heart Failure Trial (TAC-HFT; ClinicalTrials.gov NCT00768066) was designed to compare autologous BMMSCs with BMMNCs using placebo-treated patients as control [181]. BMMSCs were found as superior in terms of improved 6MWT ($p = 0.03$), reduced infarct size ($p = 0.004$), and improved regional myocardial function ($p = 0.03$), which were not observed in either the BMMNC or placebo group [158]. Nevertheless, BMMSCs and BMMNCs had a similar safety profile and Minnesota Living with Heart Failure score improved significantly in both of them ($p = 0.02$ for BMMSCs and $p = 0.005$ for BMMNCs).

4.4 Results from phase 2 randomized clinical studies

4.4.1 Trials in patients with AMI

Reassuring observations and the published data emanating from phase 1 and phase 1/phase 2 trials warranted cell therapy trials on a larger scale. Various phase 2 trials were designed to test BMSCs in patients with AMI using autologous BMCs by I/C delivery after successful PCI. A communal feature of most phase 2 trials is validation of BMSCs' safety feature, which was reported during the previous trials, while some phase 2 trials showed a modest positive outcome in terms of efficacy.

Subsequent to a feasibility and safety study involving I/C injection of $CD133^+$ BM-derived cells in AMI patients [182], the COMPARE-AMI trial was one of the earliest studies to test BM-derived $CD133^+$ in a small group of patients ($n = 20$) using placebo-treated patients ($n = 20$) as a control [145]. The double-blinded, randomized study was intended to ascertain the safety, feasibility, and functional effects of I/C administration of CD133+ BM cell lineage following AMI. Four-month follow-up showed a significant improvement in LVEF as compared to the baseline that persisted for 1 year without any protocol-related complications or safety issues ($p < 0.001$) [183]. A substudy of the COMPARE-AMI trial analyzed the effects of autologous $CD133^+$ BMCs on in-stent restenosis and atherosclerotic progression using serial intravascular ultrasound [146]. A total of 17 patients treated with CD133+ BMSCs and 20 placebo-treated patients were included in the study for analysis of various stented and non-stented segments of infarct-related coronary artery. It was observed that CD133+ cells failed to show any detectable effect on neointimal hyperplasia or atherosclerotic progression between both groups in the infarcted-related or contralateral coronary arteries.

The multicenter, randomized, double-blinded, placebo-controlled REGENERATE-AMI (Clinical Trial Identifier NCT00765453) trial was designed to test early delivery of stem cell therapy within 24 hours of successful reperfusion therapy in 100 patients (BMC group, n = 55 vs. placebo group, n = 45) [149]. One year later, although LVEF values in both the groups were improved as compared to their respective baseline values, the intergroup difference was insignificant (2.2%; 95% CI, –20.5 to 5.0; p = 0.10). Nevertheless, the authors observed a significantly greater myocardial salvage index in the BMSC group as compared with the placebo group of patients (p = 0.048) that was attributed to cell therapy in infarct remodeling and myocardial salvage. In addition, the Bone Marrow in AMI (BONAMI) trial (Clinical Trial Identifier NCT00200707) was designed to assess the treatment of AMI patients by I/C injection of BMMNCs [147]. The primary end-point of the study was improved myocardial viability assessment by thallium scintigraphy at 3 and 12 months after cell therapy. After successful PCI, a total of 100 patients were randomized to receive either autologous BMMNCs or optimal medical treatment without cell therapy. SPECT showed that BMMNC infusion significantly improved myocardial viability (p = 0.03) in a multivariate but not univariate analysis. However, LVEF was insignificantly different between the BMMNC and control groups (p = 0.62) at 3 months.

Lunde and colleagues conducted a randomized controlled trial (RCT), Autologous Stem-Cell Transplantation in Acute Myocardial Infarction (ASTAMI; Clinical Trial Identifier NCT00199823), that included 100 patients (n = 50 each for the control and BMC groups) [143]. BMSCs were delivered by I/C injection at 4–8 days after MI in the patients reperfused with PCI. The patients included in the study were having anterior wall infarct only to facilitate LV function by imaging. When assessed by echocardiography, SPECT, and CMRI, the two groups of patients did not differ significantly in terms of LVEDV, LVEF, or infarct size at 2–3 weeks as well as 6 months after treatment. Moreover, patients from both groups had similar rates of MACEs. It was concluded that intracoronary injection of autologous BMMNCs had little therapeutic benefit in terms of global LV function. A substudy of ASTAMI (Clinical Trial Identifier NCT00199823) was designed to measure global and regional LV strain and twist by CMRI at 2–3 weeks and 6 months after BMMNC treatment (n = 15) and sham treatment (n = 13) [184]. The results showed only a subtle difference in the global as well as regional LV strain, which was rather less in the control patients as compared to the BMMNC-treated patients. Similarly, LV twist and LV mass changed insignificantly between the two groups. These data clearly vindicated the findings of the earlier concluded ASTAMI trials. Similar to ASTAMI findings, Janssens and colleagues have reported similar an increase in LVEF, myocardial perfusion, and metabolism in both control and BMMNC-treated patients during a randomized, double-blind, placebo-controlled trial (Clinical Trial Identifier NCT00264316) [144]. However, there was a significant reduction in myocardial infarct size and a better recovery of regional systolic function after treatment with BMMNCs, thus pointing to reversal of infarct remodeling. A total of 67 patients were randomized to the placebo-treated (n = 33) and BMMNC-treated

(n = 34) patient groups, who received I/C intervention. No MACEs were reported and all but one patient completed the stipulated study time duration.

Time of donor cell infusion after infarction is considered a crucial factor in terms of outcome of the procedure; whereas acute phase cell transplantation will expose the donor cell to the harsh microenvironment of the infarcted myocardium, delayed cell therapy may compromise the full benefits of the procedure. Traverse and colleagues designed a randomized, double-blind, placebo-controlled TIME trial (Clinical Trial Identifier NCT00684021) during which 150×10^6 BMMNCs were infused early (3 or 7 days) after PCI in 120 patients with STEMI [148]. The cells were infused fresh, within 12 hours of BM aspiration. The coprimary end-point included CMRI measurement of LVEF and LV function in the infarct and border zones while assessment of MACE was part of the secondary end-points. Six-month follow-up showed that LVEF improved in both groups without any significant difference, while time of intervention on day 3 or 7 showed no significant impact on the outcome of cell therapy. Similarly, there was no detectable difference in the effects on recovery of regional LV function in both the BMMNC- and placebo-treated groups. The same group later designed a randomized, double-blind, controlled trial, LATE-TIME (Clinical Trial Identifier NCT00684060), to determine the beneficial effects of late administration of BMMNCs in STEMI patients [185]. The study included 87 patients who were randomized between the cell therapy and placebo treatment groups. Again, the primary end-points were LVEF and regional LV function in the infarct and border zones using CMRI, while the secondary end-points included LV volumes and infarct size. There were insignificant changes in both primary and secondary endpoints between the control and cell therapy groups at 6 months after treatment in the respective groups. Similarly, no significant differences were observed between the two groups for the secondary end-points. SWISS-AMI (Clinical Trial Identifier NCT00355186) combined the early and late cell therapy approaches in 200 patients who were randomized in a 1:1:1 pattern into an open-labeled control group, an early TIME BMMNC group (2–7 days after AMI), and a LATE TIME BMMNC group (3–4 weeks after AMI) [186]. Results at the 4-month follow-up substantiated the findings of the previous time studies.

The FINCELL trial reported the efficacy and safety of I/C delivery of BMMNCs in a group of 40 STEMI patients after thrombolytic therapy followed by PCI with an equal number of patients receiving placebo treatment [150]. Autologous BMMNCs were freshly isolated and were used within 3 hours. The patients in BMMNC group showed a greater absolute increase in the global LVEF than the placebo group did, measured either by angiography (increase of 7.1 ± 12.3% vs. 1.2 ± 11.5%; p = 0.05) or by two-dimensional echocardiography (increase of 4.0 ± 11.2% vs. –1.4 ± 10.2%; p = 0.03). Lee and colleagues used autologous BMMSCs for I/C delivery in the infarct-related artery 1 month after their successful reperfusion (Clinical Trial Identifier NCT01392105). A total of 80 patients were randomized, 40 each in the cell therapy and control groups [151]. SPECT at 6 months in 58 patients after their respective treatment showed absolute improvement in the LVEF, which was modest but greater in the BMMSC group

than in the control group (5.9 ± 8.5% vs. 1.6 ± 7.0%; p = 0.037). In terms of safety, cell therapy was well tolerated by all the patients as no MACEs were observed in any patients.

4.4.2 Trials in patients with chronic myocardial ischemia

Refractory angina is a widespread clinical problem owing to improved survival rate among coronary artery disease patients and in the aging population [187]. Stem cell therapy is emerging as an alternative treatment modality for patients with refractory angina. Preclinical studies have shown the safety and feasibility of CD34+ HSCs to enhance regional neovascularization in the ischemic tissue [188–190]. With encouragement from preclinical data, the ACT34-CMI trial (Clinical Trial Identifier NCT00300053) was designed as a randomized, double-blinded, placebo-controlled, prospective study that included 168 patients with chronic refractory angina [160]. The patients were grouped to receive either 1×10^5 or 5×10^5 cells/kg dose of GCSF-mobilized autologous $CD34^+$ HSCs or placebo treatment by electromechanical endocardial mapping. The results of the study revealed significantly reduced weekly angina frequency in the low-dose group than in placebo-treated patients at both 6 months (6.8 ± 1.1 vs. 10.9 ± 1.2; p = 0.020) and 12 months (6.3 ± 1.2 vs. 11.0 ± 1.2; p = 0.035). The high-dose group patients also had lower weekly angina frequency at both 6 and 12 months, albeit insignificantly. Similar observations were made regarding exercise tolerance test. No deaths were reported in the cell-treated patients until the 12-month follow-up, while mortality was 5.4% in the placebo group of patients. Henry and colleagues have reported 2-year follow-up data for ACT34-CMI patients [161]. Patients treated with both low- and high-dose $CD34^+$ cells had significant reduction in angina frequency (p = 0.03), while both the low-dose (one death; 1.8%) and high-dose (two deaths; 3.6%) treatment groups had significantly lower rate of deaths as compared to a total of seven deaths (12.5%) in the placebo-treated control group of patients. In conclusion, autologous $CD34^+$ treatment showed stable therapeutic benefits in refractory angina patients for up to 2 years with a concomitant tendency in rate of mortality. The same group of researchers has earlier reported a phase 1/phase 2 study, however, by using I/M delivery approach [191].

In continuation of their previous nonrandomized open-label study in patients with chronic ischemic heart failure who were treated by transendocardial delivery of BMMNCs [192], Perin and colleagues designed the FOCUS-CCTRN phase 2 trial (Clinical Trial Identifier NCT00824005). The study determined the effectiveness of BMMNCs in 92 patients with chronic IHD. The patients were randomized to either the BMMNC group (n = 61) or the placebo group (n = 31) [162]. Unfortunately, the study concluded that transendocardial injection of BMMNCs did not improve LVESV, maximal oxygen consumption, or myocardial defect reversibility on SPECT at 6 months after treatment in comparison with placebo.

Experimental animal studies have shown the induction of directed cardiopoiesis by pretreatment of stem/progenitor cells with growth factors or cocktail of growth factors to enhance their angiomyogenic restoration of the myocardial function [193, 194]. The Cardiopoietic Stem Cell Therapy in Heart Failure (C-CURE) trial (Clinical Trial Identifier NCT00810238) was the first prospective, multicenter, randomized trial in patients with chronic myocardial ischemia that used preconditioned cardiopoietic MSCs. The cells were preconditioned by culturing in platelet extract supplemented culture medium and primed by treatment with cocktail of cardiogenic growth factors before use for patient therapy [163]. The preconditioned cells were later delivered by endomyocardial injections. The results of the study showed the feasibility and safety of the procedure as it showed significant improvement in LVEF in the cell therapy group (from 27.5 ± 1.0% to 34.5 ± 1.1%) versus standard of care alone (from 27.8 ± 2.0% to 28.0 ± 1.8%; $p < 0.0001$). Furthermore, the autologous cardiopoietic MSC therapy was associated with a significant reduction in LVESV ($p < 0.001$) and a significant improvement in the 6MWT ($p < 0.01$). It was also associated with better clinical scores encompassing cardiac functionality, i.e., change in NYHA class, improved quality of life, enhanced physical performance, low hospitalization rate, and event-free survival.

The CARDIO133 randomized, double-blinded, placebo-controlled trial (Clinical Trial Identifier NCT00462774) was conducted on 60 patients with chronic myocardial ischemia and impaired LV function [164]. The patients undergoing CABG were randomized to either placebo treatment or $CD133^+$ BMC injection in the nontransmural, hypokinetic infarct border zone. There was no difference in the functionality scores of 6MWT, Minnesota, or CCS class between groups, while NYHA class improved significantly more in the placebo group ($p = 0.004$). Moreover, LVEF at 6 months was 2% higher in placebo-treated patients as compared to the cell therapy group ($p = 0.3$). Nevertheless, regional wall motion and myocardial perfusion at rest were recovered in more LV segments in the cell therapy group as compared to the placebo-treated patients (9 vs. 2%; $p = 0.001$).

4.5 Results from phase 3 randomized clinical studies

4.5.1 Trials in patients with AMI

The REPAIR-AMI was started as a phase 3 clinical trial (Clinical Trial Identifier NCT00279175) to investigate whether I/C infusion of autologous BM-derived progenitor cells might improve the global LV-contractile function [152]. The trial included a total of 204 STEMI patients who had been successfully reperfused. The patients were randomized in a double-blinded fashion to receive either placebo treatment ($n = 101$) or BM progenitor cell treatment ($n = 103$). A 4-month follow-up showed improved safety parameters, i.e., reduction in rate of death, low recurrence of MI, and alleviation of

need for revascularization procedure, after cell therapy as compared to placebo treatment. Besides, the absolute improvement in the global LVEF was significantly greater in the cell therapy group (5.5 ± 7.3% increase in cell therapy patients vs. 3.0 ± 6.5% in placebo group; $p = 0.01$). More interestingly, the patients with worse baseline LVEF (at or below the median value of 48.9%) benefitted more from cell therapy, showing an absolute improvement of 5.0% in LVEF. Both 1-year and 2-year follow-ups showed the persistence of beneficial effects in the cell therapy group [195, 196]. A Doppler substudy of REPAIR-AMI was designed to include a total of 58 patients ($n = 30$ in the cell therapy group and $n = 28$ in the placebo group) after their respective treatment [197]. The aim of the substudy was to determine the coronary artery reserve (CAR) in the infarct-related vessel in comparison with a reference coronary artery. Initially, CAR declined in the infarct-related artery in both the treatment groups. Nevertheless, it increased significantly (>90%) in the cell therapy group as compared to the placebo group during the 4-month follow-up. Similarly, adenosine-induced vascular resistance also decreased significantly in the cell therapy group, thus signifying microvascular therapeutic functional benefits. A similar substudy was designed in 54 patients ($n = 27$ each in the cell therapy and placebo treatment groups) [198]. Serial CMRI at baseline and at 4 and 12 months showed significant improvement in the indices of LV function and LV remodeling.

4.5.2 Trials in patients with chronic myocardial ischemia

The encouraging experience of using preconditioned/primed cardiopoietic BMCs has been further tested in one of the largest multicenter (US), double-blinded, randomized trials, the Congestive Heart Failure Cardiopoietic Regenerative Therapy (CHART-1) trial (Clinical Trial Identifier NCT01768702) [165]. A total of 271 patients were randomized to the cardiopoietic MSC ($n = 120$) and placebo ($n = 151$) treatment groups. Cell delivery was carried out endomyocardially with an enhanced-performance retention catheter. The 39-week follow-up showed one death in the cell therapy group and nine deaths in the sham group [166]. Similarly, primary outcome was neutral, with no significant difference in LVESV, LVEF, all-cause mortality, worsening heart failure, or 6MWT ($p = 0.27$ for cell therapy). Exploratory analysis favored cell therapy using cardiopoietic MSCs.

Based on the published data from phase 1 and phase 2 clinical trials, phase 3 Transplantation of Bone Marrow Stem Cells for Improvement of Post-Infarct Myocardial Regeneration (PERFECT) trials (Clinical Trial Identifier NCT00950274) were designed to determine the therapeutic efficacy of BM-derived $CD133^+$ cell-based therapy as an adjunct to the surgical revascularization in patients with chronic myocardial ischemia [199]. The patients included in the study had chronic ischemic cardiomyopathy subsequent to MI. The prospective placebo-controlled, double-blinded randomized trial was conducted in six centers across Germany. Although

the trials have been terminated due to slow enrollment of the patients, the published data from the study show that LVEF improved from the baseline after cell therapy. However, there was an insignificant change in LVEF between patients treated with cell therapy and those treated with placebo. Long-term follow-up perfusion score at 36 months after treatment was improved in the cell therapy group but lacked significant functional improvement in the small study cohort of patients [200].

REVEW trials (Clinical Trial Identifier NCT01508910) were initiated as multicentered, placebo-controlled, randomized trials to study whether I/M administration of mobilized CD34+ would improve total exercise time and reduce the frequency of angina episodes in patients with refractory angina [201]. The trials, which were anticipated to include 444 patients, have been terminated after enrolment of only 112 patients. The early results published from the study, however, were consistent and showed that angina frequency was significantly improved in the cell therapy group [202].

Mathur and colleagues have reported the study design of the ongoing BAMI trial, the largest BMMNC phase 3 trial in patients with AMI (Clinical Trial Identifier NCT01569178). The study is still enrolling patients and is expected to enroll 3000 patients in 11 European countries and 17 centers [203]. The eligible patients will have LVEF <45% and will be treated with I/C autologous BMMNCs 2–8 days after reperfusion. The primary endpoint of the study is to determine the time from randomization to all-cause-death and ascertain whether treatment with BMMNCs will reduce all-cause mortality in patients with AMI.

4.6 Future perspective

BMC-based therapy for myocardial repair and regeneration has progressed to randomized clinical trials that have been mostly restricted to either phase 1 or phase 2, with sporadic attempts to phase 3 trials. Diverse in many aspects, from inclusion and exclusion criteria to cell type selection and study end-point assessment, these clinical trials have benefitted from this diversity in terms of finding the best conditions for optimal prognosis. All that these trials have accomplished to show is that no MACE was directly or indirectly related to the use of BMC therapy after variable periods of follow-up. However, it has remained a challenge for researchers to obtain a concrete inference in terms of efficacy. Despite reporting statistically significant improvement in cardiac function indices, i.e., LVEF, infarct size, remodeling, and different functionality system scores, the effectiveness appears to be too modest. The unassertive efficacy observations have been attributed to the clinical settings and characteristics of the studied population, the types of BMC lineage used, methods of cell isolation and preparation, time and routes of administration, different methods of assessment and follow-up etc., which make comparison of trials problematic and erratic. Moreover, the use of cell therapy as an adjunct procedure to the routine revascularization

methods, including PCI, thrombolysis, or CABG, in majority of the trials adds to the difficulty in delineating the effectiveness of cell therapy as a stand-alone intervention. These less-than-expected outcomes have been supported by a series of recent systematic reviews and meta-analyses. A 2015 Cochrane systematic review of 41 RCTs with a total of 2732 participants concluded that BMC treatment for AMI [204] had no effect on LVEF, morbidity, or quality of life as measured by MRI. Although the meta-analyses of LVEF showed some evidence of a 2–5% difference in mean LVEF between treatment groups, this change is too meager to be considered as clinically relevant. On the contrary, a 2016 systematic review of 38 RCTs involving 1907 patients of chronic IHD and congestive heart failure reported low-quality evidence that BMC therapy improves LVEF over short- and long-term follow-up with concomitantly improved NYHA functional class [205]. However, the authors also cautioned interpreting these data, as "the event rates were generally low, leading to a lack of precision." In conclusion, these observations warrant the need for more adequately powered trials with better rationale, superior quality of cells ("super cells") combined with preconditioning of both donor cells and the recipient's heart, and to uniform cell delivery and functional study endpoint assessment methods such that the efficacy of BMC therapy is proven.

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5 Influence of selected cryoprotectants on regeneration of cryopreserved cells properties

Abstract: The cryopreservation of cells is a fundamental task for a wide range of applications, from basic storage of cells to fertilization. Cells must be protected against damage during freezing by application of specific cryoprotectants and freezing/melting protocols. This chapter presents a brief overview of the predominant cryopreservation methods and most common cryoprotectants. Furthermore, it explores the specifics of cryopreservation of stem cells with various types and levels of multipotency. Special attention is dedicated to the effect of cryopreservation on human pluripotent stem cells, including the effect of different cryoprotectants used to prepare fibroblast feeder layer for the cultivation of pluripotent cells. Finally, the chapter concludes with a review of selected methods that have been successfully used to probe the postthaw state of cryopreserved cells through their viability, inner structure, and mechanical properties.

Key Words: Cryopreservation, Cryoprotectants, Freeze, Multipotent, Pluripotent, Saccharose, Stem cells, Therapy, Trehalose.

5.1 Introduction

5.1.1 Methods of cells cryopreservation

Long-term preservation of cells by their freezing – cryopreservation – is widely utilized; however, it affects cells in a number of negative ways (e.g., structural damage and change in gene expression) [1, 2], and further optimization of cryopreservation is needed. Special care belongs to gametes, stem cells, and embryo cryopreservation, where negative effects have to be minimized. Cell therapies and assisted reproduction are having an increasing role in clinical practice and are further being studied in basic research. The occurrence of live birth defects in neonates, possibly related to *in vitro* fertilization, has been observed, and there are several factors influencing the success of cryopreservation of cells [3, 4]. Optimization of cell cryopreservation methods is essential.

Cryopreservation has a number of tunable parameters, such as freezing medium composition, cryoprotectants and their concentrations, and cooling and warming speed. The overall scheme of the cell freezing procedure and basic equipment is shown in Fig. 5.1.

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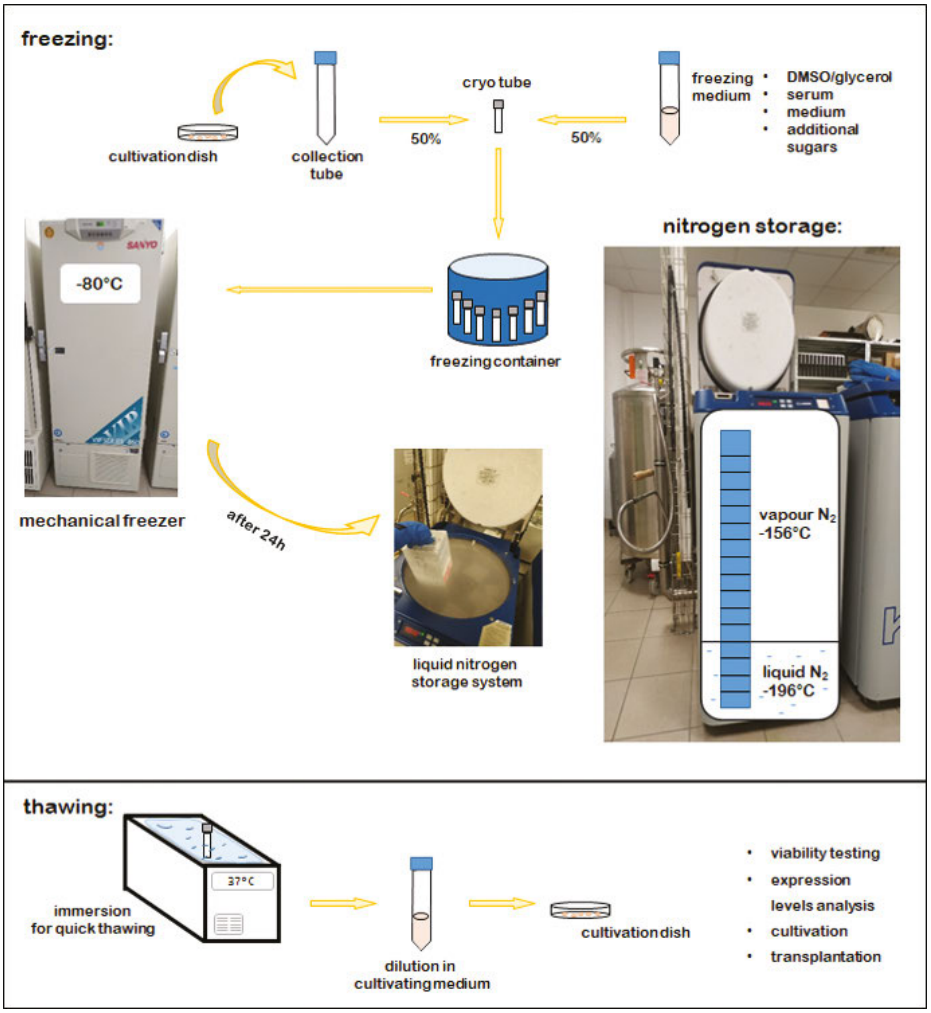

Fig. 5.1: Scheme of slow rate freezing and thawing procedure.

Different cell types have a wide variety of optimal cryopreservation settings, since they differ in internal structure, membrane permeability, and overall cell size. The amount and speed of reactions in the cell drop down, while temperature decreases below 37°C. This decrease varies widely, especially if the reactions are not interconnected. Eventual metabolic imbalance may lead to cell damage [5]. Furthermore, a decrease in temperature may result in protein denaturation [6] and structural changes, possibly cell membrane phase transitions [7]. However, at temperatures close to 0°C, damage decreases, and cells may survive for hours without significant changes.

Temperature drops below 0°C may initiate ice crystallization; nevertheless, as there are solvents present in cultivation media, the freezing point of media is

frequently reduced. By approximately –20°C, ice nucleation typically starts in most aqueous solutions. Nucleation commonly begins on the surface of various impurities in a solution [8]. In tissues and cellular suspensions, crystallization starts in the extracellular space, as there are a number of potential crystallization nuclei. At the same time, there are lower concentrations of macromolecules and eventual solvents than inside the cells [9]. Extracellular solution freezing effectively leads to a rapid increase in the concentration of the remaining liquid solvents. This creates an osmotic imbalance, resulting in an outward "solvent" drag of water from the cytoplasm. Supercooled protoplasm content has a higher chemical potential than outside crystalline ice does. This difference further dehydrates the cell and further increases the concentration of ions and other solvents. Gradual osmotic changes represent chemical stress, eventually changing the solvation layer around biomacromolecules, resulting in their denaturation [10].

Slow cooling progression typically results in extracellular water crystallization, while constricted areas of concentrated solvents and dehydrated cells remain between the crystals. The cytoplasmic membrane dehydrates and, typically at temperatures between –3 and –10°C, undergoes a phase transition from liquid crystalline to gel phase [11]. Hyperosmotic stress leads to membrane ion leakage to the intracellular space (later on causing hypotonic stress during thawing).

Further remnants of liquid eventually create a eutectic mixture of ice and salt hydrates or finally vitrify into an amorphous glass-like structure. Although intracellular ice formation may be avoided during slow cooling, chemical and mechanical stress heavily affects cell survival.

Chemical stress can be decreased by a rapid cooling rate. However, a too quick process results in intracellular water retention and cell exposure indeed to very low temperatures still having a high water content, directly linked to intracellular ice formation. This damages the intracellular fiber networks and other structures and often leads to cytoplasmic membrane rupture [12].

Glassy state formation – vitrification – is an important phenomenon in frozen solutions. It relates to a rapid temperature decrease. The glass transition temperature of pure water is –137°C [13]. It represents loss of rotational and translational degrees of freedom and only the vibration of bonds within a fixed molecular structure is preserved. Cryoprotectants and/or intracellular macromolecules move this value closer to 0°C. Vitrification seems to be a solution of the cryopreservation problem: water molecules are fixed in place, with neither chemical nor mechanical stress occurring. However, to perform the vitrification process, the cooling rate needs to exceed 10^7 K/s [14]. This is difficult to obtain, namely, for nonmicroscopic samples, because of heat conduction. There are various cryoprotectants available, changing the viscosity of the solution, glass transition temperatures, etc. Cryoprotectant concentrations necessary for vitrification are usually higher compared to those used for slow freezing. At cryogenic temperatures (–196°C, temperature of liquid nitrogen), biological processes are stopped, and the cells can be stored long-term without noticeable

degradation. The original approach included the procedure performed in open pull straws for cell manipulation mostly used in gamete cryopreservation [1, 2]; novel methods are, however, developed with a surface-based approach with potential use for *in vitro* cultivated cells [3].

Thawing of a cryopreserved sample to physiological temperatures often includes ice recrystallization (IRI) or secondary crystallization. This causes mechanical injury to cells that survived the initial freezing stage. Larger ice crystals have a tendency to increase its size even when smaller ones start to melt. Similarly, devitrification during rewarming is linked to secondary crystallization as the energetic barrier separating the crystalline and amorphous states is lower than the barrier to a liquid aqueous state. The optimal thawing rate depends on the initial rate of cooling. Quickly cooled cells benefit from rapid thawing, since the time for ice recrystallization is limited. In case of slow cooling, a number of protocols (fast thawing presented as superior/equal/inferior to slow thawing) have been published, with results heavily dependent on the specific cell lines and cryoprotectants used, etc. [15, 16]. Thawing of the cryopreserved cells exposes them to oxidative stress due to mitochondrial respiratory chain disruptions as well as uneven reactivation of coupled chemical reactions [12].

5.2 Cryoprotectants and their effects on living cells

5.2.1 Small permeating cryoprotectants

Cell-permeating substances with cryoprotective properties decrease the freezing point of cryopreservation media. Although these substances seem to merely postpone the onset of crystallization, dehydration, and chemical stress to later cooling phases, such a delay is in fact beneficial. The chemical stress resulting from extracellular and/or intracellular freezing occurs at low temperatures. Thus, the rates of all chemical reactions are slowed down, and the harmful effect of these reactions is decreased [12].

In this group of reagents, dimethyl sulfoxide (DMSO) is very common. DMSO is a polar, aprotic, and highly hygroscopic solvent. It forms hydrogen bonds with water molecules; however, its hydrogen atoms are not sufficiently acidic, hence its aprotic character. The second important property of DMSO resulting from its membrane permeability is its tendency to partially substitute the intracellular water that leaves the intracellular space during freezing. This substitution decreases the negative consequences of cellular dehydration and simultaneously limits the mechanical stress resulting from changes in the volume and shape of cells [17].

DMSO molecules combine well and homogeneously with water counterparts. On a molecular level, DMSO forms various structures with water molecules depending on the relative concentration ratio [18]. At low concentrations, the $(DMSO)_1(H_2O)_2$ clusters are preferably formed (with one water molecule possibly participating in two such clusters). These clusters can radically distort the native hydrogen bond

network, and the lower interactions can explain the observed deep depression of the freezing point in case of eutectic mixtures [19]. Furthermore, at mixtures near the eutectic point, a large increase in viscosity occurs upon cooling and the mixture eventually vitrifies. This process makes the DMSO-water combination useful for cryopreservation purposes, as the final vitrified, amorphous solid phase causes much less mechanical stress to the present cells compared to crystalline ice [20]. However, the desired concentration of DMSO is rather high and it might cause a chemical stress of its own. Consequently, a suitable composition must be optimized for particular types of cells.

For slow-freezing cryopreservation, lower levels of DMSO around 10% w/w are typically used. Furthermore, as the aqueous part in the mixture undergoes slow freezing, the DMSO content in the remaining liquid fraction increases. The ability of DMSO-water mixtures to vitrify can thus contribute to cell survival even in the case of slow freezing of cryoprotective solutions with a low DMSO content. DMSO interacts with cell membranes exposed in the extracellular space depending on its concentration [21]. At low concentrations, membrane thinning, undulation, and increased fluidity are observed. Higher concentrations enhance membrane permeability for water as well as ions, even inducing transient pores. Finally, disintegration/dissolution of the membrane begins. In this context, a higher membrane fluidity/elasticity is likely beneficial when conforming to mechanical stresses exerted on cells during freezing. Increased permeability helps ameliorate osmotic stresses, otherwise presenting another significant source of damage.

In addition to its cryoprotective role, DMSO can facilitate membrane fusion and drug penetration through the skin. A mechanism of the interaction of DMSO with lipid membranes was suggested in [22]. Leaving aside the exact effects of DMSO on cells on the molecular level, there is no doubt that especially at high concentrations and long incubation times, DMSO negatively affects cells [23, 24]. For instance, DMSO has been shown to impede cell proliferation [25].

DMSO permeates cell membranes and consequently affects osmotic processes in the course of freezing. Without DMSO, the concentration of ions in the yet liquid fraction of extracellular volume increases after the onset of extracellular freezing. As a result, intracellular water gradually starts to flow out of the cells in order to compensate for the emerging osmotic imbalance. This efflux of water, in turn, increases intracellular osmolality beyond physiological concentrations and subjects the cytoplasmic membrane to mechanical strain. DMSO decreases the freezing temperature of the surrounding solution, which means that any osmotic imbalance takes effect at low temperatures, where the rates of harmful chemical reactions are slower [12].

5.2.2 Saccharides

The principles underlying the functions of saccharides as glucose, saccharose, and trehalose are the very similar, but the natural ability of these molecules

to penetrate the cell varies [7]. Saccharides contribute to the stabilization of biological molecules. There is significant evidence that trehalose partially substitutes the surrounding solvation shell of proteins. During the phase transition, this shell remains amorphous and thus protects the proteins from dehydration and denaturation [26]. Trehalose is also able to intercalate the outer phospholipid layer of the cell membrane and stabilize this layer by hydrogen bonding to the polar parts of the phospholipids [27]. In this way, saccharides provide cryoprotective effects even if present only extracellularly.

From the mentioned saccharides, most attention has been focused on trehalose as it yields results equal to or superior to those of other cryoprotectants. The reason for the cryoprotective behavior of trehalose can be its rather large stability [17]. It is a nonreducing saccharide (unlike glucose) that does not induce the Maillard reaction in the presence of proteins. Saccharose is also a nonreducing sugar, but its glycosidic bond is vulnerable to hydrolysis, and its monosaccharide products can react with proteins. Organisms using glucose, saccharose, etc., for cryoprotection probably employ supporting protective mechanisms that counter this instability [12].

5.2.2.1 Trehalose

Among the saccharides, trehalose is perhaps the most potent cryoprotectant, being nearly as effective and universal as DMSO [28]. It is a disaccharide consisting of two glucose subunits linked by an alpha-1,1-glycosidic bond. The glycosidic bond joins and blocks the anomeric carbons of its glucose subunits and prevents the subunits from unfolding into linear, open-chain forms with free aldehyde groups. The low energy of this bond makes trehalose quite stable (at increased temperatures or extreme values of pH) and resistant to hydrolysis. As a result, trehalose practically does not interact with proteins (i.e., nonenzymatic Maillard-type browning), unlike glucose and sucrose [17, 29, 30], although sucrose belongs to nonreducing disaccharides and it performed similarly to trehalose in other tests of stability.

Trehalose can form a glassy state in bulk and in solution. Compared to similar behavior of other sugars, however, the glass transition temperature of trehalose is high, which possibly relates to its stabilizing properties [31].

In aqueous solutions, trehalose forms hydrogen bonds with water molecules, but also with other trehalose molecules; molecular dynamics simulations indicate the formation of trehalose clusters [32, 33]. Trehalose helps to prevent denaturation of proteins in aqueous solutions (distortion of their secondary and tertiary structure) initiated by a range of environmental changes (freezing, heating, desiccation, etc.) [34–36]. Generally, it exhibits a strong stabilizing effect on the structure of biomolecules that is superior among saccharides. Trehalose molecules form a cage around proteins, where water molecules are locked with substantially slowed dynamics. This arrangement does not allow crystallization of the trapped water

molecules and, therefore, dehydration and denaturation of the protein in the center of the cluster. Other considered models of stabilization include direct vitrification of proteins within the glassy trehalose matrix that directly shields the protein from environmental stress, preferential exclusion theory, and water replacement theory [31, 37]. In addition to stabilizing proteins, trehalose also contributes to the stability of lipid membranes [38]. Several other studies concerned the positive effects of trehalose on liposome integrity during lyophilization and freeze-drying, which are similar to cryopreservation [39].

Trehalose cannot readily penetrate the membranes of mammalian cells [40]. Therefore, it acts only extracellularly, which also contributes to osmotic stress [41].

There are ways to load trehalose inside the cell – after longer incubation times, trehalose does in fact enter the intracellular space, but the long loading time (for up to 24 h) is a slight practical disadvantage. Other alternatives include incubation with the copolymer PP-50, which unfolds at low pH, bursting the encapsulating endosome and releasing all endocytosed trehalose into the cytoplasm [42] or engineering membrane pores. The genes responsible for trehalose synthesis have been transfected into human cells, conferring resistance to desiccation [43]. An acetated analog of trehalose (with retained cryoprotective ability) has also been engineered, with much higher permeation rates [12].

5.2.3 Polymeric cryoprotectants

The cellular membrane is stabilized and/or mechanically protected in the presence of various nonpermeating cryoprotective polymers [44], including polyethyleneglycol (PEG)-1500 [45] and polyvinylpyrrolidone (PVP) [46]. Such polymers can affect the transport of water from the cell during cooling and may impede the recrystallization of ice when thawing COOH-polylysine, thus inhibiting IRI. A rather extreme but functional approach is encapsulation of the cells in an alginate hydrogel layer [47].

5.2.4 Ice-structuring proteins and their analogs

An alternative different mechanism of cryoprotection is interference of the relevant cryoprotecting proteins with the growth of emerging ice crystals. This is a key property in several (not necessarily disjunctive but rather largely overlapping) terms referred to as antifreeze proteins (AFPs), ice-structuring proteins (ISPs) [48], ice-binding proteins, thermal hysteresis proteins/factors, IRI inhibitors, and more [49].

The common structural denominator is the existence of a planar “ice-binding” domain with regularly distributed amino acid residues, especially threonine and alanine [50].

If temperature falls below zero, proteins start to adsorb on the surface of ice crystals via the ice-binding domain and block further growth of the crystals. In this way, phase transition does not initiate, and the temperature at which the solution remains in the supercooled state is depressed until the level of supercooling is too large and the solution flash-freezes. During rewarming, the melting point will not be affected accordingly. The mixture melts close to or even above the original freezing point because the proteins adsorbed to the ice crystal nuclei impede the dissolution of these ice crystals by a process inverse to the one occurring during cooling [51].

The resulting difference between the freezing and melting points is "thermal hysteresis," and the ability to induce hysteresis is typical for ISPs. The specific arrangement of amino acids in the ice-binding domain defines the crystal plane where the protein can bind, as well as the strength of such binding.

The actual benefit of ISPs is not their ability to introduce thermal hysteresis of bodily/intracellular fluids but rather their ability to limit and suitably shape the growth of emerging ice crystals. A second major potential benefit of ISPs is the suppression of IRI during thawing; this is at least as crucial as the cooling phase of cryopreservation, and IRI may present (depending on the adopted protocol) a serious source of cryodamage. If the crystals are covered with adsorbed proteins, IRI becomes strongly suppressed.

Many natural cryoprotectants and additional of freezing protection mechanisms existing within freeze-tolerant organisms likely remain undisclosed. A case in point is the recent explanation of the role of intrinsically disordered proteins (IDPs) in the tolerance of tardigrades toward desiccation [52].

Proteins with loose structures are able to form amorphous solids (vitrify) during desiccation and thus protect other simultaneously present macromolecules from denaturation. Until now, the knowledge base has established that the most important cryoprotectant in tardigrades by far is trehalose. IDPs have not yet been clearly considered in cryoprotection; however, because many aspects (dehydration, osmotic imbalance) of desiccation stress and low-temperature stress are similar, such a role is expected.

The ApAFP752 AFP originates from a desert beetle *Anatolica polita*, living in the Chinese Xinjiang province [53]. Its molecular weight is approximately 20 kDa, and the active part consists of a set of β-sheet domains with regularly spaced threonine residues, aligned in a helical superstructure ("β-helix"). The structure of the homolog of the ApAFP752 protein (68.97% sequence homology), named as TmAFP AFP [4], is shown in Fig. 5.2 (by use of PDB file "1EZG").

The ApAFP752 undergoes thermal hysteresis and affects ice-structuring, consistently with its expected IDP. The magnitude of hysteresis ranges between 0.5 and 5.5°C, depending on the pH and the presence of various ions and concentration of the protein itself [54]. ApAFP752 significantly slows down the dynamics of water molecules, which are in its close proximity. This is consistent with AFP having an

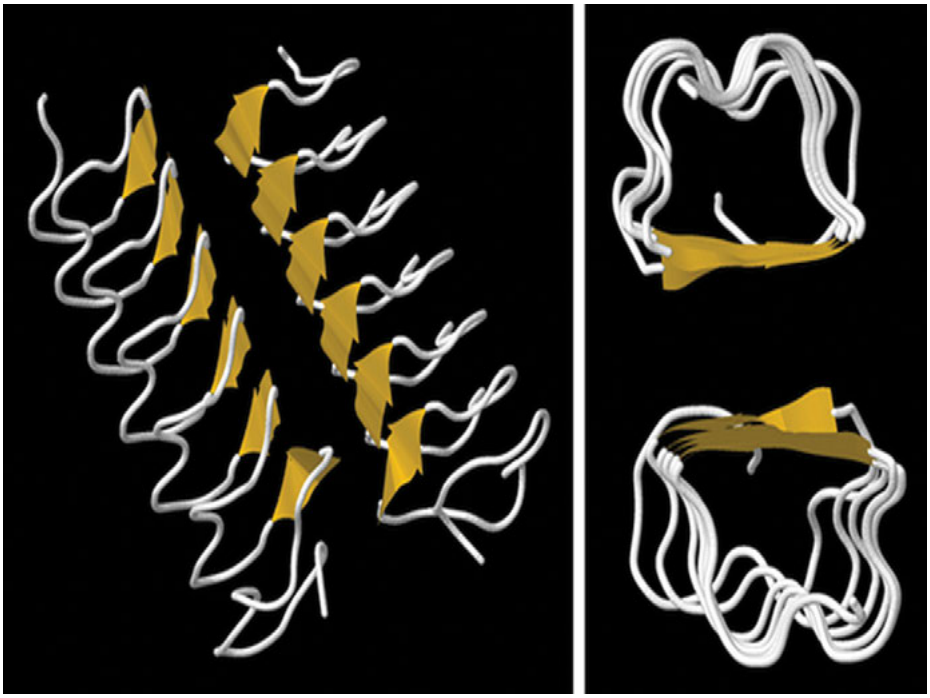

Fig. 5.2: Structure of the antifreeze protein TmAFP, a structural homolog of ApAFP752 (68.97% sequence homology). Image constructed by use of PDB file "1EZG."

ice-binding surface. Despite its inability to permeate through the cell membrane, ApAFP752 increased chromatin condensation and postthaw cell viability, but at a lower level than all the cryoprotectants under study.

Trehalose-treated thawed cells were similar to AFP-treated thawed cells. They both act as a natural osmolyte (osmoprotectant) and stabilize phospholipid membranes and the three-dimensional structure of proteins [26].

Despite not penetrating into the cell nuclei, trehalose and AFP increase chromatin condensation in the thawed cells. One can hypothesize that chromatin condensation is influenced by changes in nucleus hydration and ionic balance. Extranuclear cryoprotectants perhaps act by changing the intracellular osmotic pressure, followed by dehydration of the nucleus. Osmotic pressure (concentration of solutes) during freezing further depends on the ice formation dynamics. Consequently, extracellular cryoprotectants are not limited to favorably changing the shape of ice crystals or protect the cell membrane, but they can also play an indirect protective role at the nuclear level [12].

5.3 Cryopreservation of various cell types

5.3.1 Hematopoietic stem cells from bone marrow and umbilical cord

Bone marrow stem cell units of collected hematopoietic stem cells (HSCs) are commonly preserved from patients for autologous stem cell transplantation prior to administering high doses of chemotherapy or/and radiotherapy. Allogenic stem cell transplantation also represents an important therapeutic approach in malignant and nonmalignant hematologic diseases [5, 6]. Cryopreservation of somatic stem cells is an integral part of allogenic and autologous transplantations, and effective cryopreservation techniques are critical in these approaches. HSCs are multipotent stem cells capable of differentiation into all types of blood cells and are commonly isolated from bone marrow and umbilical cord and are most commonly defined by the presence of CD34 [7, 8]. For HSCs, at least 2.5×10^6–5×10^6 $CD34^+$ cells/kg body weight are necessary for collection and centrifugation for generating a cell-rich pellet [9]. For autologous transplantation, donor plasma is used for resuspension together with heparinized plasmolytic solution (multiple electrolytes injection, type 1) and 10% DMSO, yielding in a suspension of concentration of at least 500×10^6 cells/ml [10]. Plasma proteins having cryoprotectant abilities improve the survival rate of HSCs [11]. Similar to other cell types, concentrated HSCs are frozen down at a controlled rate of 1–2°C/min up to a temperature point of about –40°C [12]. Further freezing down to a target of –120°C is performed at a faster pace, about 3–5°C/min. Long-term recommended optimal storage conditions are in the vapors of nitrogen with a temperature around –156°C [13–16]. HSCs can be stored this way for more than 6 months while preserving optimal viability and desired stem cell properties [17]. Further studies have shown that the cells can be stored frozen for a longer period of time and retain their properties [18–21].

5.3.2 Umbilical cord blood

Umbilical cord blood (UCB) is a source of HSCs as well as mesenchymal stem cells (MSCs) [22, 23]. It has been used as a source for replenishment of bone marrow or peripheral blood progenitor cells since the 1980s [24]. Cord blood from neonates contains substantial numbers of HSCs [25], which can be harvested at delivery, cryopreserved, and later transplanted to patients [26–28]. Cord blood banks store these units worldwide and perform cell counts and virologic screening and inventories of HLA types, in order to be available to transplant centers. Cord blood transplants have a slightly delayed engraftment but a lower risk of graft-versus-host disease [29]. A single cord blood unit is usually insufficient by absolute

amount of cells to be transplanted; thus, individual storage remains a controversial approach [30].

Most freezing protocols for UCB cells currently use 10% ethylene glycol (EG) and 2.0% DMSO (v/v) [31], 10% DMSO (v/v) and 2.0% dextran-40 [32], and 2.5% DMSO (v/v) + 30 mmol/l trehalose [32]. Due to DMSO toxicity after transplantation, novel methods of cryopreservation are being developed without the use of DMSO. Wang *et al*. developed a freezing medium containing 5% EG, 35% 1,2-propylene glycol, and 5% sucrose supplemented with 1% polyvinyl alcohol prior to vitrification process in open straws [33]. This protocol resulted in 95% viability of the cells, with a possible nontoxic use in further transplantations. Nicoud *et al*. [34] developed DMSO-based cryopreservative CryoStor that reduces the DMSO concentration to ≤5% with better reproducibility, cell viability, and lower toxicity.

5.3.3 Mesenchymal stem cells

Bone marrow isolated MSCs are a source of self-renewing, multipotent stem cells capable of differentiating into adipocytes, osteoblasts, and chondrocytes [35–39]. An invasive puncture aspiration of MSC from flat hip bones as a primary source resulted in a search for other ways of obtaining MSCs, such as adipose tissue [40] and UCB [22].

The efficacy of MSC isolation is, however, strongly dependent on the harvesting method [41–43]. Moreover, further cryopreservation may result in a loss of up to 50% of harvested cells [44]. DMSO is still used widely in cryopreservation protocols due to its availability and low cost. However, decline in survival [45] and DNA methylation-induced differentiation [46] have been reported and thus render DMSO less suitable for clinical use. Other freezing supplements, such as PVP suitable for cornea cryopreservation [47] or a mix of PVP and horse serum, did not exceed the survival rate of DMSO and thus was not considered a suitable candidate [48].

Amniotic fluid (AFL) consists of several undifferentiated fetal cell types, including MSCs [49, 50]; they may be considered an alternative cell source for cell/tissue engineering in regenerative medicine [51, 52]. For the cryopreservation of AFL-MSC, supplementation of the freezing suspension with the originating AFL resulted in 98% viability after thawing [53]. There was no increase in chromosomal aberrations compared to nonfrozen cells [53, 54]. The stemness properties after thawing procedures remained high in terms of CD44, CD73, and CD90 expression, as well as the immunoprivileged marker HLA-ABS, while a very low expression was observed for CD31, CD34, CD45, and CD117, as well as HLA-DR [53].

5.3.4 Pluripotent stem cells

Human embryonic stem cells [55] and induced pluripotent stem cells [56] (PSCs; human pluripotent stem cells [hPSCs]) have the highest potential of all of the

above-mentioned stem cells for use in regenerative medicine. They serve as a tool for translational research and disease modeling [57, 58] but also have been proven as a powerful tool in the treatment of degenerative diseases such as age-related macular degeneration [59–61], with other diseases closely behind (Parkinson´s disease [62–65], Huntington´s disease [66, 67], or type 1 diabetes [69, 70]).

However powerful, the cryopreservation of PSCs has been proven challenging as PSCs are highly sensitive to freezing and thawing procedures, with low survival rate (bellow 20%), low reattachment, and spontaneous differentiation of hPSCs [1, 55, 71]. PSCs require physical intercellular contact and paracrine signaling to maintain viability and undifferentiated state in *in vitro* culture as well as during freezing due to their collaborative nature, with a high presence of gap junctions and connective proteins [55, 72, 73]. While it is known that prolonged cultivation decreases DNA repair protein expression [73] and increases mutation frequency [74] and influences overall genome instability [75], little is still known if the freezing procedure escalates these changes.

PSCs are usually cultured and frozen in cell clumps [55] as a lack of cell-cell interaction decreases the survival and triggers anoikis/apoptosis [77], but this method poses a problem with proper saturation of cryoprotectants throughout the whole clump. The addition of a Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor (Y27632) into the culture medium for the first 24 h increases the survival rate of pluripotent stem cells during the thawing procedure [78–81]. Nevertheless, its addition can lead to morphological or metabolic changes [82]. Further analyses are thus necessary involving not only PSC viability after the freeze/thaw cycle but also the effects of cryopreservation on the PSC biology.

The most common freezing procedure used for PSCs involves 10% DMSO as a cryoprotectant and a freezing rate of 1°C/min to decrease the chance for ice crystal formation [83]. Fetal bovine serum (FBS) of up to 30% is usually added into the freezing suspension. However, the FBS concentration with the best effectivity on cell survival is at 50% with subsequent 5% DMSO or 5% EG in the freezing medium [84]. Since FBS triggers differentiation [71], the serum used must be tested for suitability for use in stem cell cultures without spontaneous differentiation that makes the PSCs freezing procedures less cost effective. However, other methods such as vitrification have also been developed [1, 85–87].

The vitrification process for stem cells consist of exposure to two different cryoprotective solutions with 10% DMSO and 10% EG for 1 min and 20% DMSO and 20% EG for 25 s prior to flash freezing in liquid nitrogen [3]. The best results have been obtained using sucrose in both of the cryopreservation solutions. The process is done either in straws similar to the gamete freezing [1] or, more recently, with the novel surface approach [3, 88, 89], which avoids the use of serum and increases the survival rate (above 90%) as well as expression of pluripotent markers. The hPSC colonies are cultivated on unique double-sided culture dishes (TWIST), in which one side serves for culture only and the other side serves as surface for liquid nitrogen for the process of freezing. When the colonies achieve the required size, the culture undergoes cryoprotective chemical treatment as described above, the culture dish is flipped,

and nitrogen is poured on the nitrogen side. Subsequently, the dish is immediately stored at the desired temperature, usually in the vapor phase of nitrogen. Alternative cryopreservatives are being developed to avoid the use of DMSO and serum [90, 91].

5.3.5 Germ stem cells

Cryopreservation of testicular tissue or spermatogonial stem cells is a promising strategy for male patients undergoing gonadotoxic chemotherapies. Tissue or cells can be retransplanted after the treatment, restoring the fertility of the patient. Solely, a whole-tissue alternative is available for prepubertal patients due to lack of spermatogenesis. Such therapy is most beneficial [92–94] and effective in postpubescent candidates [95, 96]. In postpubertal patients, whether testicular tissue or germ stem cell suspension is more suitable for the procedure remains to be clarified. Cryopreservation of the whole tissue [91, 92, 96–98] or the dissociated germ cells [98–101] has been a subject of extensive debate for their respective advantages and disadvantages. Open pulled straw vitrification has been tested for the nonhuman [102, 103] and human [102, 104] testicular tissue, with promising results with preservation of the tissue architecture and observable gametogenesis after retransplantation. Trehalose addition into the freezing medium has been shown to have a beneficial effect on after-thaw survival of the spermatogonia [105].

5.3.6 Oocyte cryopreservation and vitrification

The cryopreservation of unfertilized oocytes has been studied since 1986. However, oocyte storage remains suboptimal due to the low maturation and low pregnancy rates of preserved oocytes. Thus, fresh oocytes are far more routinely used in assisted reproduction. Mice models have shown that ultrarapid freezing of metaphase II oocytes results in a significant decrease in total blastocyst cell number, due to a significant decrease in inner cell, possibly also disruption of polarization, demonstrated in human embryos and a high rate of aneuploidy (–20%). Culturing conditions and the presence of DMSO are also suspected to have a detrimental effect, but neither propanediol (at about 20°C) nor cryopreservation significantly increased the abnormalities of the spindle. Propanendiol was combined with sucrose in order to decrease variation in water permeability kinetics between individual mature humans. A gradual increase in sucrose concentration used for the cryopreservation of oocytes could be also traced in the literature (0.2 to 0.3 mol/l). Finally, a major advancement in oocyte cryopreservation was a shift from the slow-freeze method of cryopreservation to vitrification [106]. These reports claimed similar fertilization success and pregnancy rates as with the use of fresh oocytes; nevertheless, available long-term data revealed lower pregnancy rates compared to cycles involving freshly inseminated oocytes [107].

5.3.7 Sperm cryopreservation

Assisted reproduction, insemination of animals and humans, routinely use cryopreserved spermatozoa [108]. Contrary to oocytes, sperms are small cells with a large surface area that makes them less susceptible to potential water-inflicted damage [109]. Initial studies have shown that glycerol is preferable to DMSO as a cryoprotectant to protect sperm structures [110]. Motility, plasma membrane functionality, acrosome integrity, and overall viability of spermatozoa postthaw significantly decrease in comparison with the prefreeze controls [111]. Therefore, novel cryoprotective supplements such as soybean, lecithin, and low-density lipoprotein have been evaluated in human and animal sperm freezing [112]. These cryoadditives impart mild nitric-oxide-induced oxidative stress [113]. Slow freezing, rapid freezing, and ultrarapid freezing (i.e., kinetic vitrification) are conventional cryopreservation methods. Slow freezing works over a period of 2–4 h, in order to reduce osmotic injury. The rapid freezing technique represents use of cryoprotectant mixture, storage in straw or cryovial, and exposition to a liquid nitrogen vapor phase for at least 10 min and liquid nitrogen. Kinetic vitrification was performed even without cryoprotectants, as the sperm suspension is directly loaded into liquid nitrogen and thus cooled in a few seconds. Nevertheless, this approach is considered obsolete by some authors who prefer to add sucrose or use of 10% DMSO. Some studies have shown a reduction in sperm quality after vitrification as compared to the slow freezing strategy [114]. On the contrary, spermatozoa are separated from seminal plasma before cryopreservation and warmed spermatozoa do not need an additional centrifugation step for plasma removal [115].

Another important technique for sperm cryopreservation is freeze-drying (lyophilization). Lyophilized sperms can be kept at 4°C and transported at room temperature. This method has been also used for humans [116]. The spermatozoa recovered following this method are immotile, but the observed DNA damage was decreased as compared to the other methods that use liquid nitrogen [117]. However, low cost and safety remain the hallmarks of this method for bio-banking but requires intracytoplasmic sperm injection (ICSI) for successful fertilization.

5.4 Methods for the study of cryoprotectant effect

While vitally important, the quality control of cells after cryopreservation often includes solely viability assay. For HSCs and PSCs, live imaging, cytoskeleton analysis, genome integrity, microRNA profiling, transcriptome, and proteome analysis and DNA methylation profile can also be employed. Overall novel methods for quality control are not standardized. The effect of cryoprotectants on the mechanical and structural properties of the cells can be studied by several methods. A combination of atomic force microscopy (AFM) indentation with high-resolution fluorescence

microscopy was shown as an optimal method for simultaneous study of mechanical and structural properties of the living cells [56, 57]

5.4.1 Viability determination

The viability of cells should be always tested after freeze/thaw cycles. For this purpose, microscopy, combined with flow cytometry, can be used to quantify cell survival and apoptosis, especially in a setup that allows discrimination between live, early apoptotic, late apoptotic/necrotic, and dead cells such as the Muse® Cell Analyser (Merck Millipore) and Muse® Annexin V & Dead Cell Assay Kit (MCH100105, Millipore). The viability of the adherent cell culture of fibroblasts as well as various types of stem cells can also be tested by standard Trypan Blue test. Time points of the test should correspond to the experimental or application setup.

The effect of treatment with different cryoprotectants on the cellular structures (such as membrane, nuclear envelope, cytoskleteton, etc.) can be visualized under a confocal fluorescent microscope after fixation. An example of such analysis is shown in Fig. 5.3, where staining of cytoskeleton with phalloidine was carried out to observe cytoskeletal changes after thawing. The process can be automated with robotized confocal microscope equipped with capture and image analysis software, i.e., (Altrincham, Great Britain) [118].

5.4.2 Live imaging

The dynamics of the tissue regeneration process using thawed cells in terms of individual cell fate determination can be studied by live cell imaging. For simultaneous AFM measurements and cytoskeletal observations, or simply bioimaging of cytoskeleton development of the freshly thawed cells, live dyes are available for actin, tubulin, or even lysosomes (e.g. from Cytoskeleton Inc., Denver, CO, USA). The adherent cell culture can be incubated with live dyes, such as sir-actin for 1 h prior to the freezing process, enabling observation of the dynamics of the cytoskeleton remodeling as early as 30 min after leaving the thawed cells to attach (90-min sequence of live cytoskeleton imaging of fibroblast upper panel of the Fig. 5.3). The labeling is stable for at least 8 weeks of observation after the initial freezing.

5.4.3 Atomic force microscopy

Cell mechanical properties, usually described as the stiffness of the cell, is mainly affected by cytoskeleton, i.e., by a network of actin fibers and intermediate filaments. Disruption and remodeling processes of such fibers may lead to cell

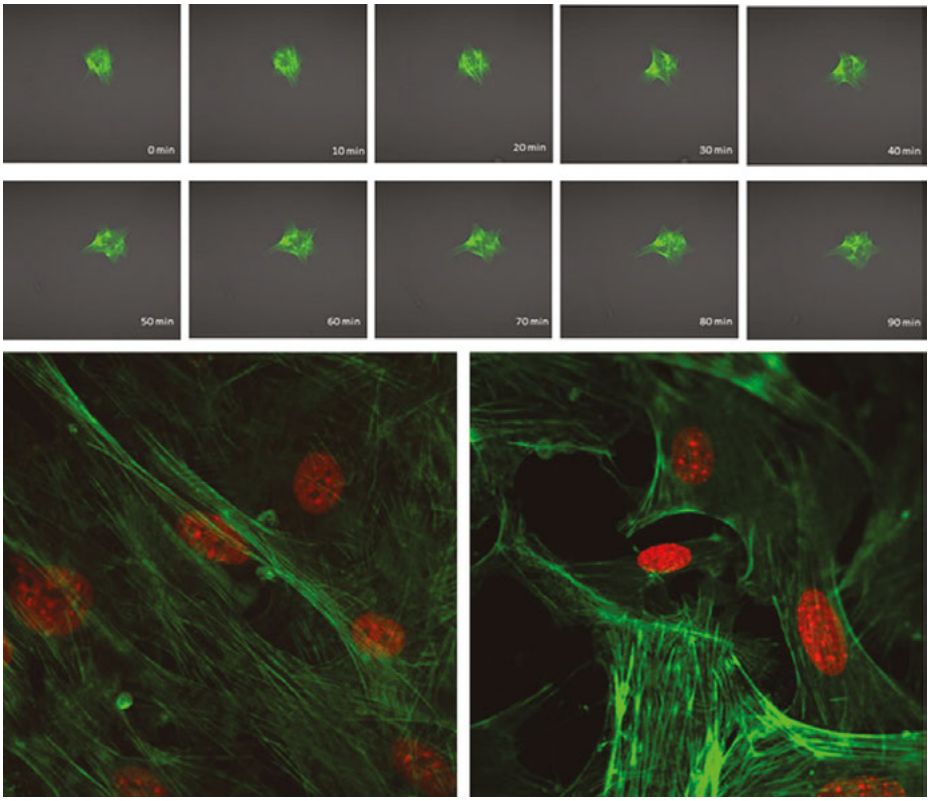

Fig. 5.3: Confocal fluorescence imaging of cell cytoskeleton. Time-lapsed sequence of images on the top shows changes in the cell cytoskeleton during 90 minutes. Two images below illustrate differences in the actin structure of fibroblast cells, actin fibers (green) were stained with phalloidine conjugate, and cell nuclei can be observed as red objects (stained with DAPI, 4′,6-diamidino-2-phenylindole.

stiffness changes [119]. A limited number of methods suitable for noninvasive investigation of live cell stiffness are available. AFM as a nanoindentation technique was widely used to determine the mechanical properties of cells and tissues with submicron-level resolution [58, 120]. Moreover, AFM allows mapping the topography of a live cell; in the spectroscopic mode (nanoindentation), the complete elastic response of the cell is recorded, thus leading to the construction of a stiffness map. The ability to work under near-physiological conditions favors AFM-based stiffness mapping when compared with other methods.

Changes in the cells' mechanical properties as a result of their exposure to cryogenic temperatures and to different cryoprotectants were studied by many authors [121, 122]. We have recently shown that AFM spectroscopy combined with fluorescence microscopy [123, 124] is a useful tool used to study the effect of selected

cryoprotectants (low and high molecular mass molecules) on the cytoskeletal remodeling and mechanical properties of cryopreserved mouse embryonic fibroblasts. Time-lapsed differences in investigated properties were found as a result of the freezing process. Fig. 5.4 depicts a schema of the overall process of our study. Briefly, thawing of the cryopreserved cells is initiated by placing the cryovial into warm water until a small ice crystal is observed in the cryovial, subsequent to which the suspension is transferred to a falcon tube wherein a 10–15 times higher amount of cold (5°C) culturing media is added dropwise. The Falcon tube containing the diluted cell suspension is centrifuged at low speed (2000 rpm, 5°C) for 5 min and the supernatant is immediately transferred to the preheated (37°C) medium. The cells are then seeded in sterile glass-bottom Petri dishes (suitable for fluorescence microscopy and imaging with oil-immersion objective). The cells are left to adhere to the bottom of the dish for 15–20 min in a standard CO_2 incubator. Immediately after adhesion of the cells (30 min postthawing), the media in the dish are exchanged, and fluorescence stack images are recorded. This is periodically followed by the force-mapping process, for the period of 4.5 h.

The cell culture medium in the Petri dish is periodically exchanged with fresh medium from an incubator after each force-mapping procedure. Either the pyramidal or spherical indenter can be used to map the mechanical properties of the cell.

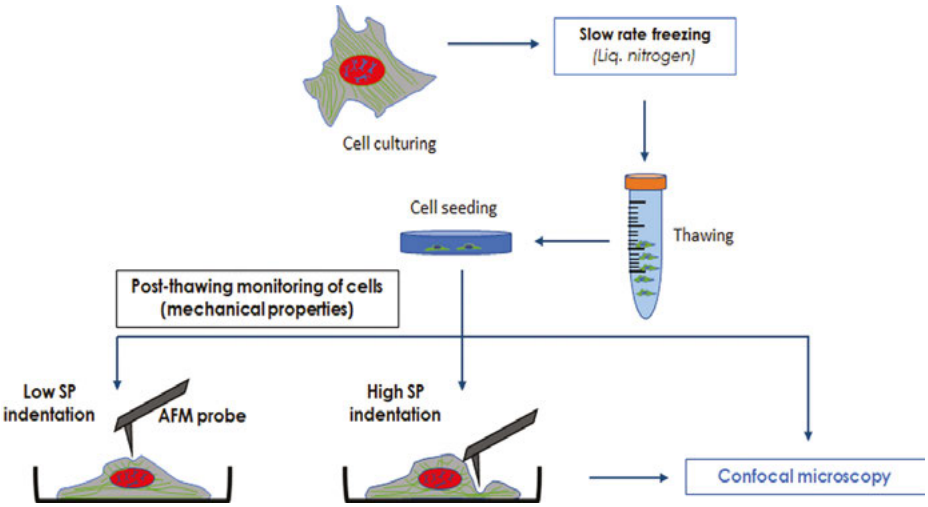

Fig. 5.4: Schematic representation of the testing of freeze-thaw cycle on the mechanical properties of live cells. The cultured cells were frozen in liquid nitrogen, when slow freezing protocol was used. The cell suspension was then thawed by subjecting to a stream of hot water followed by immediate 1–15 times dilution using cell culture medium. The cells were later seeded in Petri dishes and allowed to adhere to the culture vessel for short time period. The postthaw mapping of the mechanical properties of the cells was subsequently recorded by AFM based nano-indentation process, when low set-point (Low SP) and high set-point (High SP) values were used. The actin fiber structure of the fixed cells was subsequently visualized by confocal microscopy.

Operational parameters are used according to tip choice – setpoint value shall be either 0.75 nN (pyramidal tip) or 1.0 nN (spherical indenter). The extension time per curve is 0.5 seconds, the length of the Z-axis is 15.0 μm, and the time of curve recording is 0.5 s; the sampling rate of FD (force-distance) curves recording was 5 kHz. Grid of 64 × 64 points on 100 × 100 μm^2 are is sampled during the nanoindentation process, as a step-by-step recording of FD curves in the space covering either single or multiple fibroblasts. The area is kept constant during the whole period of the nanoindentation process. The above-mentioned process gives a network of FD curves (i.e., tip-sample distance vs. force between the tip and surface), called force maps [58]. The value of Young's modulus (stiffness) can be calculated by fitting the FDC with Equation 5.1 derived from the Hertz-Sneddon model [59]:

$$P = \frac{E}{(1-\nu^2)}\left(\frac{R^2+a^2}{2}Log\left(\frac{R+a}{R-a}\right) - aR\right), \delta' = \frac{a}{2}Log\left(\frac{R+a}{R-a}\right), \tag{5.1}$$

where P is the loading force, E is Young's modulus, ν is the Poisson's ratio (0.5; incompressible materials), δ is the indentation depth, a is the radius of contact, and R is the spherical probe radius.

5.4.4 Observation of genomic and nuclear envelope integrity

One of the important characteristics of a successful freeze/thaw process, especially in the case of stem cells, is preservation of the genomic integrity of the cells. The use of various cryoprotective agents might be associated with the induction of the DNA damage [118], which, in combination with postthaw selection for apoptosis-resistant clones, might lead to mutagenesis. One of the most dangerous and highly toxic DNA lesions, the double-stranded breaks (DSBs), can be visualized as DNA damage response foci of the phosphorylated histone H2A (further referred to as γH2AX). Colocalization of γH2AX and early response end-joining protein 53BP1 in DNA repair foci point at the mutagenic end-joining DNA repair, which might lead to mutagenesis in stem cells [125].

An even more precise analysis of the preservation of the structurally and functionally distinct chromatin domains during the freeze/thaw process can be observed by confocal microscopy and by quantifying the intensity profiles of 53BP1, gH2AX, and TO-PRO3 costaining [118]. While intensive γH2AX peaks colocalizing with 53BP1 indicate DSBs, the constant γH2AX free of 53BP1 indicates apoptotic and/or necrotic DNA damage. The damage to the nuclear envelope can be quantified by fluorescence microscopy after laminin A/C antibody staining combined with chromatin (TO-PRO3) leakage out of the nucleus, which shows diverse effect of cryoprotectants and cryopreservation on the higher-order chromatin structure and shrinking of the nuclear envelope [118]. An important observation was that the survival of the cryopreserved cells correlated well with the

postthaw chromatin condensation. Falk *et al*. showed that the highest viability was achieved with a mixture of DMSO and trehalose [118].

Falk *et al*. also showed a novel type of chromatin damage resembling replication stress-associated damage, which could not be prevented by any of the tested cryoprotectants [118]. This type of damage is serious and most likely lethal, but a fraction of the S-phase cells may survive the replication fork collapse. Even in such cases, however, complete genome reparation is unlikely [63]. Hence, the surviving cells pose a danger by propagating the DNA defect through the cell population, a scenario that is especially worrying in the context of assisted reproduction [12].

5.5 Conclusions

Cryopreservation of cells has growing potential for preservation of the clinically relevant cell types for regenerative medicine in general and for cell therapy in particular. Protection of structural and functional characteristics of the cells during cryopreservation is possible by number of specific cryoprotectants and freeze/thaw protocols. Cell survival and quality maintenance are assessed by viability evaluation and should be accompanied by more detailed quality control mechanisms. Quantification of DNA damage and cytoskeletal regeneration are progressive methods of postthaw monitoring, thus allowing optimization of protocols and selection of novel prospective cryoprotectants.

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6 Molecular mechanisms of neural stem cells differentiation

Abstract: Stem cells persist in specialized niches in discrete regions of the central nervous system, which supports their self-renewal and differentiation throughout the life span. The subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the subventricular zone (SVZ) of the lateral ventricles contain neural stem cells (NSCs) and act as NSC niches in the adult brain. In the SGZ niche, NSCs, as multilineage cells, differentiate into interneurons that finally generate the neurons and astrocytes. Moreover, NSCs of the SVZ niche are capable of generating neurons, oligodendrocytes, and astrocytes. Thus, active neurogenesis continues throughout their life in the mammals, including humans. Emerging findings have shown that intrinsic and extrinsic regulators play a crucial role in NSC differentiation and fate determination. Gene expression, epigenetic remodeling of genes, and various gene boxes such as HOX and SOX genes regulate neurogenesis and differentiation of NSCs. Additionally, several signaling pathways, including JAK/STAT, Notch, MAP Kinases, and Wnt, assist in the development of this trend. Furthermore, different signaling molecules and proteins such as transforming growth factor-β, bone morphogenetic proteins, or Smad control the progression of this process. Metabolic changes and physiological, pathological, and pharmacological stimuli can also affect neurogenesis in the adult brain. This chapter focuses on the special conditions and molecular mechanisms that direct neural differentiation and specify the ultimate fate of NSCs.

Key Words: Molecular, Neurodevelopment, Neurogenesis, Neural, Progenitors, Signal, Stem cells, Transduction.

6.1 Introduction

6.1.1 Subtypes of neural progenitor subtypes in brain development

Neural stem cells (NSCs) are a group of cells that are capable of producing oligodendrocytes, astrocytes, and different neural subtypes. Culturing this type of cells could be performed in two ways, adherent or nonadherent. By nonadherent culture, they form a well-rounded three-dimensional structure that is around 150–250 μm and called neurosphere (Fig. 6.1) [1, 2].

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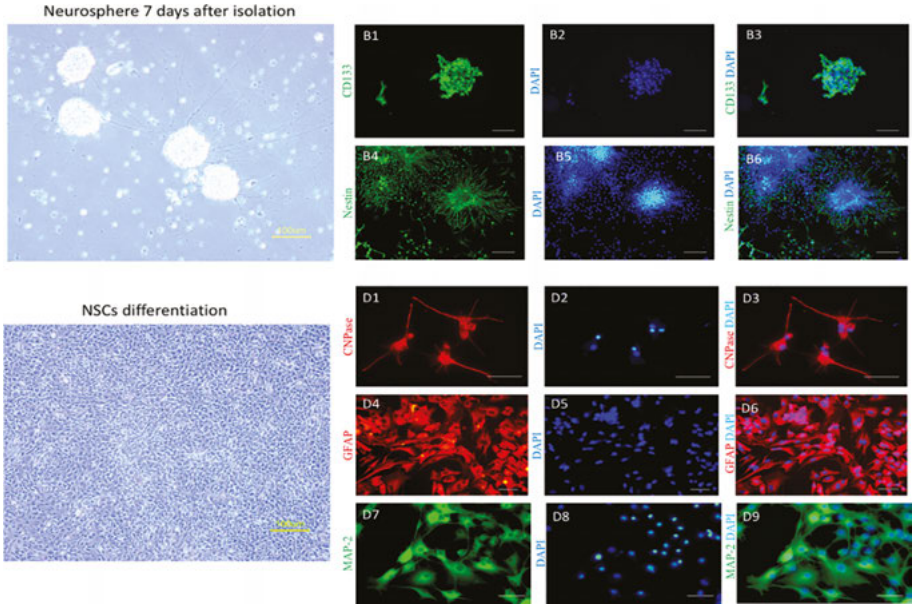

Fig. 6.1: Neural stem cell isolation and trilineage differentiation [1]. (A) The process of neural stem cell isolation, expansion, and differentiation: the neurospheres differentiated from NSCs isolated from 14-day-old rat embryos. (B) The trilineage differentiation of purified NSCs into neurons, oligodendrocytes, and astrocytes.

NSCs can be harvested from different central nervous system (CNS) regions such as the spinal cord and subventricular zone (SVZ). Besides forming a unique structure *in vitro*, they also express some specific markers, including *Sox1*, *CD133*, *Nestin*, and *Sox2*. Brain development, as a complex fundamental process, is initiated to generate various parts of the neural system. The neural progenitors provide a distinct cell source to govern neural plasticity and proliferation [3]. As proliferating cells, the neural progenitors control the size of the developing cortex. During this pathway, the variable alterations in cell types, cell maintenance, their division, and specifications due to the transient intrinsic and extrinsic factors, establish the complex dynamic process of cortical development. The neural progenitors affect the neurodevelopmental processes by producing various types of progenitors, including apical progenitors (APs) and basal progenitors. The APs contain cells lying adjacent to the ventricular (apical) surface, and as the first population of neuroepithelial progenitors, they proliferate to generate the early neuroepithelium and subsequently appearing radial glial cells. These cells demonstrate apical-basal polarity and side-to-side contacts with neighboring cells via adherent junctions [4]. Neuroepithelial progenitors divide both symmetrically and asymmetrically to produce the early developing neural progenitors, some early neurons, or intermediate (basal)

progenitors [5]. Radial glial progenitors, the alternative cell population originating from APs, tend to proliferate asymmetrically to form a pair of daughter cells with distinct progenitor or early neuronal cell fate [6]. Another population of APs, the intermediate progenitors, prefer to divide in a symmetric fashion [7]. This type of progenitors may be described as the SVZ or the nonsurface progenitor cells. The outer SVZ progenitors (OSVZs) have a modified radial morphology and may cleavage randomly. They may indicate proliferative and self-renewing cell divisions like the APs, or otherwise they tend to divide asymmetrically and provide an apical daughter cell or intermediate progenitor [8]. The ultimate cerebral cortical size directly results from this symmetric cell division in the APs, which leads to generating an increasing number of postmitotic daughter neurons [9]. A variety of molecular markers on neural progenitors have been identified to illustrate different stages of cortical neurodevelopment. For instance, the presence of Pax6 confirms the APs' generation, Tbr2 is detected to report the population of basal intermediate progenitors, and Tbr1 acts as the early born neuronal marker. Specifically, Pax6, brain lipid-binding protein, glial fibrillary acidic protein, and phospho-vimentin are the expression markers of OSVZ progenitors [8].

6.2 Role of Cdk5 in NSC differentiation

The differentiation of NSC starts after the completion of their final cell division. The cell division cycle of NSC, as well as other proliferating eukaryotic cells, is driven by cyclin-dependent kinases (Cdks), which are a group of Ser/Thr protein kinases activated at the particular phase of cell cycle by cyclin proteins. Downregulation of cell cycle Cdks and the resulting exit from the cell cycle are required for the commitment of NSC for neuronal differentiation. The accumulation of Cip/Kip Cdk inhibitors is thought to prevent their entry into the next cell cycle, although the precise mechanism remains to be investigated. Interestingly, Cdk5, a unique member of the Cdk family, is oppositely activated in postmitotic neurons by noncyclin protein p35 or p39 [10, 11]. Importantly, Cdk5-p35 plays a variety of roles from the development to pathological loss throughout the whole lifespan of neurons [12].

Using a Cdk5 or p35 knockout (KO) mouse model, the regulatory role of Cdk5-p35 in the migration of newborn neurons from the place of birth to that of residence in brains has been clearly demonstrated in the published data. For example, cerebral cortex of mouse brain is composed of six layers of neurons in the order of inside-out depending on their birthdate. Genetic ablation of Cdk5 or its activator p35 results in the reverse layer structure because late-born neurons cannot get through the previously migrated neurons [13, 14]. Cdk5-p35 regulates this radial migration of cortical neurons by acting on several different proteins at multiple time points of the migration [15]. It has already been established that Cdk5-p35 regulates axon and dendrite

outgrowth through the organization of cytoskeleton and trafficking of endosomal vesicles [15, 16].

On the contrary, the role of Cdk5 in the earlier phase of neuronal differentiation, such as cell cycle exit and differentiation commitment, remains less clear and warrants further investigation. An interesting report is that Cdk5 prevents the cell cycle reentry of the already differentiated neurons by disrupting the E2F1-DP1 complex in the nucleus [17]. This is the kinase-activity-independent function of Cdk5 that may be an event before the initiation of p35 biosynthesis. In the differentiating NSCs, after removal of fibroblast growth factor (FGF), gradual expression of p35 begins from day 1 to day 7, which leads to the activation of Cdk5 with a concomitant decrease in Cdk2 activity [18]. The NSCs prepared from Cdk5 KO mouse embryos generate a fewer number of differentiated neurons labeled with a neuron marker TuJ1 as compared to wild-type NSCs. The expression mechanism of p35 has also been investigated in other systems. For example, p35 expression is induced in PC12 cells by sustained activation of ERK1/2 with NGF. It is also induced in the cultured primary neurons treated with BDNF. ERK1/2 phosphorylate Egr1 transcription factor to induce p35 mRNA expression.

Cdk5-p35 has a consensus phosphorylation sequence (S/T)Px(K/R) similar to those of cell cycle Cdks [19], thus implicating that both will phosphorylate the same proteins if they are placed in the same conditions. Given that the activation of cell cycle Cdks in the nucleus of postmitotic neurons causes cell death, Cdk5-p35 should be excluded from the nucleus not to access the cell cycle machinery. In fact, p35 is a cytoplasmic protein associated with membrane organelles, such as recycling endosomes, through its N-terminal myristoylation [20], and therefore anchoring the activated Cdk5 on membranes. This is one of reasons that Cdk5-p35 mainly functions in the migration and neurite outgrowth, which are regulated by extracellular signaling. Nevertheless, there are a number of reports showing the phosphorylation of cell-cycle-relevant transcription factors by Cdk5-p35, i.e., Rb and p53, and Sox6 related to neuronal differentiation [21–23]. Neuronal commitment of the relevant progenitor cells is principally under the transcriptional regulation. Cdk5-deficient NCSs generate fewer neurons [24], suggesting that Cdk5 critically participates in the fate determination of NSCs, although Cdk5 is not an absolute requirement for neuronal commitment. This is an interesting aspect of Cdk5 that warrants further investigations in the future (Fig. 6.2).

6.3 Sonic Hedgehog signaling during NSC development

Hedgehog signal transduction controls the patterning of neural progenitor cells as well as their neuronal and glial progeny during development. The precise temporal role of this signaling pathway in telencephalic development has been proposed as one of the initiating factors of gastrulation [25] and a key effector of postnatal neurogenesis [26]. Mammals have three Hedgehog homologues, Desert, Indian, and

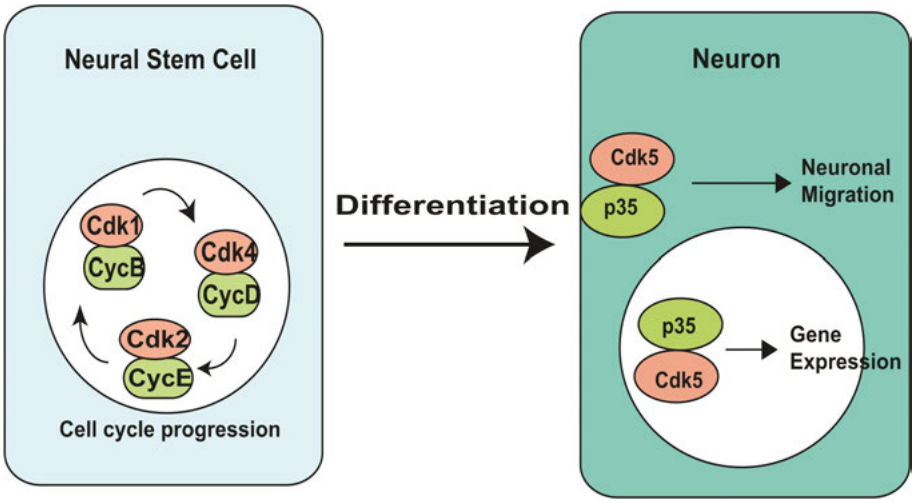

Fig. 6.2: Role of cyclin-dependent kinases in neural stem cells and differentiation to neurons. Cell cycle Cdks promote cell cycle progression in the nucleus of neural stem cells. Upon differentiation to neurons, expression levels of cell cycle Cdks are decreased and neuronal Cdk5 is increased. CDk5-p35 complex may promote neuronal differentiation by regulating gene expression in the nucleus and regulates migration of newborn neurons in the cytoplasm.

Sonic (SHH), of which the SHH is the best studied and well defined in terms of its structure and function [27]. The SHH signaling pathway is critical for appropriate development of limbs, skeleton, lungs, and gut during the human embryonic development [28]. Prenatal SHH expression occurs in the notochord, the floor plate of neural tube, and brain. This expression causes dorsoventral (DV) patterning, specification of oligodendrocytes, proliferation of neural precursors, synaptogenesis, synaptic plasticity, and control of axon growth [28]. Moreover, in an adult mammalian nervous system, this secreted protein can increase cell proliferation in the SVZ and SGZ [26, 29] and may be required for normal proliferation in the SVZ [26].

From molecular point of view, SHH signaling pathway has several aspects. Smoothened (Smo) and Patched (Ptch) are two essential membrane proteins that mediate SHH signal transduction. SHH binds directly to its plasma membrane receptor, Ptch. In the absence of SHH, Ptch inhibits Smo activity, a membrane G-protein-coupled receptor (GPCR)-like protein. Smo inhibition prevents target genes' expression, so the pathway is switched off in this situation. Smo activity is followed by recruitment and activation of a downstream protein complex, including the kinesin-like protein Costal2, a kinase called Fused (FU), and a suppressor of FU (SUFU). This protein complex function leads to the activation of one or more of three transcription factors, Gli1, Gli2, and Gli3. Consequently, target genes will be induced (through Gli1 and Gli2 activities) or repressed (through Gli3 function). Their expression implies the critical role of HH signal transduction [30, 31]. The mammalian genome contains three HH genes and

two ptc genes. Additionally, they express three Gli transcription factors (Gli1-3), and all HH signal transduction particles have been evolutionary preserved.

In 2015, Huangfu and Anderson proposed that normally structured primary cilia are essential organelles for total activity of HH pathway in neural tube [32]. Accordingly, the SHH signaling plays a vital role in oligodendrocyte differentiation. Recently, Mariyath *et al*. concluded that Nkx2.2 homeodomain transcription factor, which is positively regulated by SHH during normal growth, is a critical effector molecule for oligodendrocytes and maturation [33]. In addition, in 2017, Yang *et al*. have studied the regulatory effect of SHH on chicken optic tectum development. Their results confirmed that SHH overexpression may control neural precursor cell fate during chicken optic tectum development. This event may impair neuronal migration and may affect the fate determination of transfected neurons [34]. In the mouse neural tube, the SHH secreted from the notochord seems to be essential for oligodendrocyte precursor cell generation. Furthermore, Hashimoto *et al*. suggested that strong SHH input to the motor neuron progenitor notochord domain is not required for the generation of oligodendrocyte precursor cells, while it is necessary for their proliferation [35]. Recent studies have demonstrated that the HH pathway can regulate cell division. In particular, in 2015, Haldipur *et al*. and, in 2017, Miyashita *et al*. observed randomized orientation of granule cell precursor division in clustered cerebellar slices treated by SHH inhibitor [36, 37]. On the same note, in 2017, Wang *et al*. have indicated that conditional activation of Smo, the HH effector, may affect the orientation of division in apical radial glia in the ventricular zone [38], while Kawano *et al*. (2017) have discussed the role of SHH in regulation of DV patterning of the neural tube. They have demonstrated that SHH acts through Cdh7, a factor that is expressed in the intermediate neural tube region, to limit intracellular movement of Gli3 protein and production of Gli3R [39]. SHH participation in midline brain patterning was suggested by Belloni *et al*. and Roessler *et al*. in 1996 [40, 41]. Moreover, SHH signaling pathway can conduct early steps of forebrain development. In the diencephalon, SHH expression extends dorsally along the zona limitans intrathalamica, dividing the rostral prethalamic region from the more caudal thalamic and pretectal regions. Within the hypothalamus, only the anterior and dorsal regions express SHH at this stage to regulate its DV patterning [42]. SHH signaling can also influence the fate determination of medial ganglionic eminence (MGE)-derived GABAergic cortical interneurons. In 2015, Tyson *et al*. investigated that SHH differentially specifies the fate of the two main MGE-derived interneuron subgroups, including somatostatin and paravalbumin expressing cells from embryonic stem cells [43]. During postnatal development, SHH acts as a regulator of oligodendrocyte production for extensive myelination. More recently, a postnatal cell population with transient SHH signaling has been reported that contributes to the oligodendrogenesis during corpus callosum myelination and gives rise to cells that continue to proliferate in adulthood and contribute to corpus callosum remyelination (Fig. 6.3) [44].

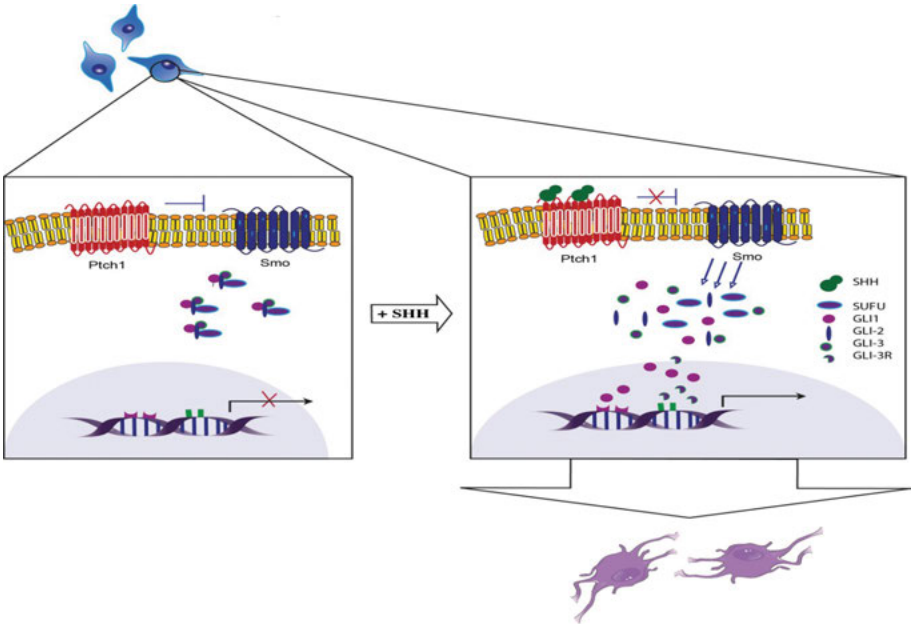

Fig. 6.3: Smoothened (Smo) and Patched (Ptch) are two essential membrane proteins mediating SHH signal transduction. SHH binds directly to its plasma membrane receptor, Ptch. In the absence of SHH, Ptch inhibits Smo activity, a membrane GPCR-like protein. Smo inhibition prevents target genes' expression, so the pathway is off in this situation. Smo activity is followed by recruitment and activation of a downstream protein complex, including the kinesin-like protein Costal2, a kinase called Fused (FU), and a suppressor of FU (SUFU). This protein complex function leads to activation of one or more of three transcription factors, Gli1, Gli2, and Gli3. Consequently, target genes will be induced (through Gli1 and Gli2 activities) or repressed (through Gli3 function). Their expressions result in indicating the critical roles of HH signal transduction.

6.4 Wnt signaling during NSC development

The Wnt signaling is an evolutionary preserved pathway for regulating key actions in embryonic development in general and in the development of almost every aspect of CNS development, including cell fate determination, cell migration, cell polarity, and neural patterning [45]. In vertebrates, β-catenin as a transcription factor is the major effector of canonical Wnt signaling pathway. In the absence of Wnt signal, cytoplasmic β-catenin molecules combine with Adenomatous Polyposis Coli (APC) and Axin scaffold proteins. During the maintenance stage of the affected cell, two kinases of the mentioned complex, casein kinase-1 (CK1) and glycogen synthase kinase-3 (GSK3) phosphorylate β-catenin through several serine and threonine residues. Phosphorylated β-catenin is then ubiquitinated and destroyed by proteasome. On the other hand, by binding Wnt ligand to Frizzled (Fz) family receptor and coreceptor of the LRP-5/6/arrow family, the cytosolic domain of LRP is phosphorylated. This process may be the consequence of free CK1 and GSK3

activities and leads to binding of Axin to cytosolic domain of LRP coreceptor. Inhibition of the APC/Axin/CK1/GSK3β destruction complex causes β-catenin stabilization and translocation to the nucleus, where it interacts with TCF/LEF family of transcription factors. β-Catenin binding to TCF/LEF proteins provides a transcription activation domain that initiates target gene expression activation. In the absence of Wnt signal, TCF binds to promoters or enhancers of target genes and TCF binding to Groucho [46] or histone deacetylase as transcription suppressors to inhibit target gene activation [47].

CNS development processes can be divided into early and late stages. During the early stage of CNS development, the anterior-posterior (AP) and DV patterning is regulated through Wnt signal transduction. This highly dynamic signaling pathway affects all stages of human development by various ligands expressed in a temporal and regionally specific manner in early embryonic neurodevelopment. The neural specification of embryonic ectodermal cells and the subsequent formation of the neural plate were identified as the first principle neurodevelopmental events [48]. In 2006, Heeg-Truesdell *et al.* and, in 2011, Min *et al.* suggested that Wnt signaling can interfere with neural induction [49, 50]. Additionally, researches revealed that the absence of this signal transduction causes FGF upregulation, thereby leading to the generation of neural cell fates [51]. McGrew *et al.* (1997) concluded that the generation of complete AP neural pattern may require the co-operative actions of the Wnt and FGF pathways [52]. Moreover, it has been demonstrated that vertebrate head formation and patterning are subsequent events to the repression of Wnt signal transduction [53] and Wnt signals act directly and in a graded manner on anterior neural cells to induce their progressive differentiation into caudal forebrain, midbrain, and hindbrain cells [54]. Besides, the role of Wnt signaling in forebrain AP patterning in the course of the diencephalic development was discussed by Wilson and Houart. They proposed that the telencephalic development may be heavily dependent on Wnt antagonism [55]. Importantly, during the telencephalon development, Wnt signaling may exert a considerable influence on DV cell fates (i.e., pallidal and subpallidal) [56]. Different studies suggested significant role of Wnt signal transduction in hind-brain specification and segmentation, spinal cord patterning, late stages of neural development including corticogenesis, neural crest (NC) emergence, eye morphogenesis, axon growth and guidance, synaptic formation and function, and adult neurogenesis (Fig. 6.4) [57].

6.5 Bone morphogenetic protein signaling during NSC development

Bone morphogenetic proteins (BMPs) induce bone formation and organogenesis and engage in regenerative and developmental processes. These protein factors contain almost one third of the transforming growth factor-β (TGF-β) superfamily members, and over 30 TGF-β-like homologues of BMPs have now been identified in a broad range of species [58].

The molecular mechanism of BMP signaling pathway commences with ligand secretion and its binding to a heterodimeric complex of two transmembrane receptors,

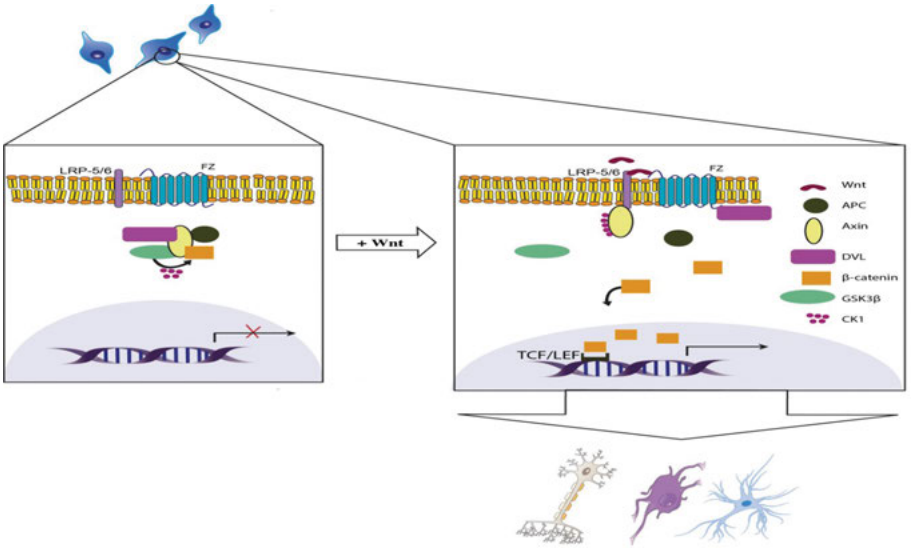

Fig. 6.4: β-catenin as a transcription factor is the major effector of canonical Wnt signaling pathway in vertebrates. In the absence of Wnt signal, cytoplasmic β-catenin molecules join to APC and Axin scaffold proteins. In the maintenance stage of the affected cell, two kinases of the mentioned complex, casein kinase-1 (CK1) and glycogen synthase kinase-3 (GSK3), phosphorylate β-catenin through several serine and threonine residues. Phosphorylated β-catenin is then ubiquitinated and destroyed by proteasome. On the other hand, by binding Wnt ligand to Frizzled (Fz) family receptor and coreceptor of the LRP-5/6/arrow family, the cytosolic domain of LRP is phosphorylated. This process may be the consequence of free CK1 and GSK3 activities and leads to binding Axin to cytosolic domain of LRP coreceptor. Inhibition of the APC/Axin/CK1/GSK3β destruction complex causes β-catenin stabilization and translocation to the nucleus, where it interacts with TCF/LEF family transcription factors. β-catenin binding to TCF/LEF proteins provides a transcription activation domain so target gene expression is activated. In the absence of Wnt signal, TCF binds to promoters or enhancers of target genes and TCF binding to Groucho or histone deacetylase as transcription suppressors inhibits gene activation.

termed type I and type II, with serine-threonine kinase activity. Upon ligand binding, the type II receptor transphosphorylates the type I receptor at the juxtaposition region, thus activating type I kinase. The latter then phosphorylates the members of the Smad family of transcription factors that are subsequently translocated to the nucleus, where they activate the expression of target genes in association with other transcription factors or coactivators [58].

Bennett *et al.* proposed that BMP-2 and BMP-4 contribute to the regulation of orofacial development [59]. Subsequent studies by *Furuta et al.* investigated the crucial role of BMPs in vertebrates' brain development by comparing the expression of five BMP genes, BMP-2 to BMP-7, in the dorsal midline, a region that could act as a signaling center. The data showed that BMPs function during regional morphogenesis of the dorsal telencephalon by regulating specific gene expression, cell proliferation, and local cell death [60]. NC induction can be separated into the gastrula and

the neurula stages. The first step, the gastrula induction, requires Wnt activation and BMP inhibition, whereas the subsequent maintenance step requires activation of both pathways. The dynamic expression of BMP-4 and its antagonists in NC cells have been identified as a conducting factor in BMPs' presence [61]. Moreover, the DV polarity and the epidermal promotion over neural cell fate are prominent consequences of the BMP signaling pathway during the early embryonic development. These outcomes are highly influenced by a gradient of BMP activity, the lateral/ventral expression of BMP ligands, and the dorsal/medial expression of BMP antagonists [62]. BMP signaling is required for the formation of the most dorsal telencephalic derivative, the choroid plexus (CP) thus providing impetus for local patterning of the dorsal midline [63]. BMP signaling through BMPRIA in the epiblast negatively regulates the expansion of anterior visceral endoderm [41], and that in the anterior mesendoderm negatively regulates the expansion of prechordal plate (Fig. 6.5) [64].

6.6 FGF signaling during NSC development

FGF and their specific cell surface receptors (FGFRs) form a major but complicated signaling pathway that appears to participate in a variety of processes during embryonic development and tissue homeostasis processes.

The signaling cascade of 22 FGF ligands is triggered to work by activation of the numerous cell surface FGFRs. These single-pass transmembrane proteins act as tyrosine kinase receptors. Hence, the signal transduction progresses through ligand binding to the extracellular domain of the receptor to induce receptor dimer formation. This step is followed by phosphorylation of multiple tyrosine residues on the receptors to activate their intrinsic tyrosine kinase domain. Consequently, a cascade of phosphorylation events will be triggered by transient assembly of various intracellular adaptors and effectors. This activation of multiple signal transduction leads to the modified gene expression. FGF activities are regulated in different aspects. In addition to the growth factor expression, their activities may be affected by variable FGF:FGFR binding affinities and interaction with heparin or heparin sulfate proteoglycans to form stable receptor dimers [65].

FGF transduction takes part in mesoderm formation during embryonic development [66]. Furthermore, its considerable impact on the neuroectoderm, neural plate, neural specification, and CNS AP patterning has been distinguished by several investigations. Lamb and Harland (1995) concluded that FGF signal affects posterior neural induction and the AP patterning might be influenced by noggin and FGF activities and ectoderm changes [67]. Additionally, it can be a prominent cause of controlling the cerebral cortex size [68]. The idea of FGF role in neural patterning has been supported by different findings [46, 69–71]. For instance, *it has been* indicated that the FGF pathway may regulate cortical neurogenesis and control cortical surface expansion [72]. In another investigation, it has been revealed that intermediate neural progenitor production may be affected by increased FGF signaling. This event is widely influenced by gyri formation in the rostrolateral neocortex (Fig. 6.6) [73].

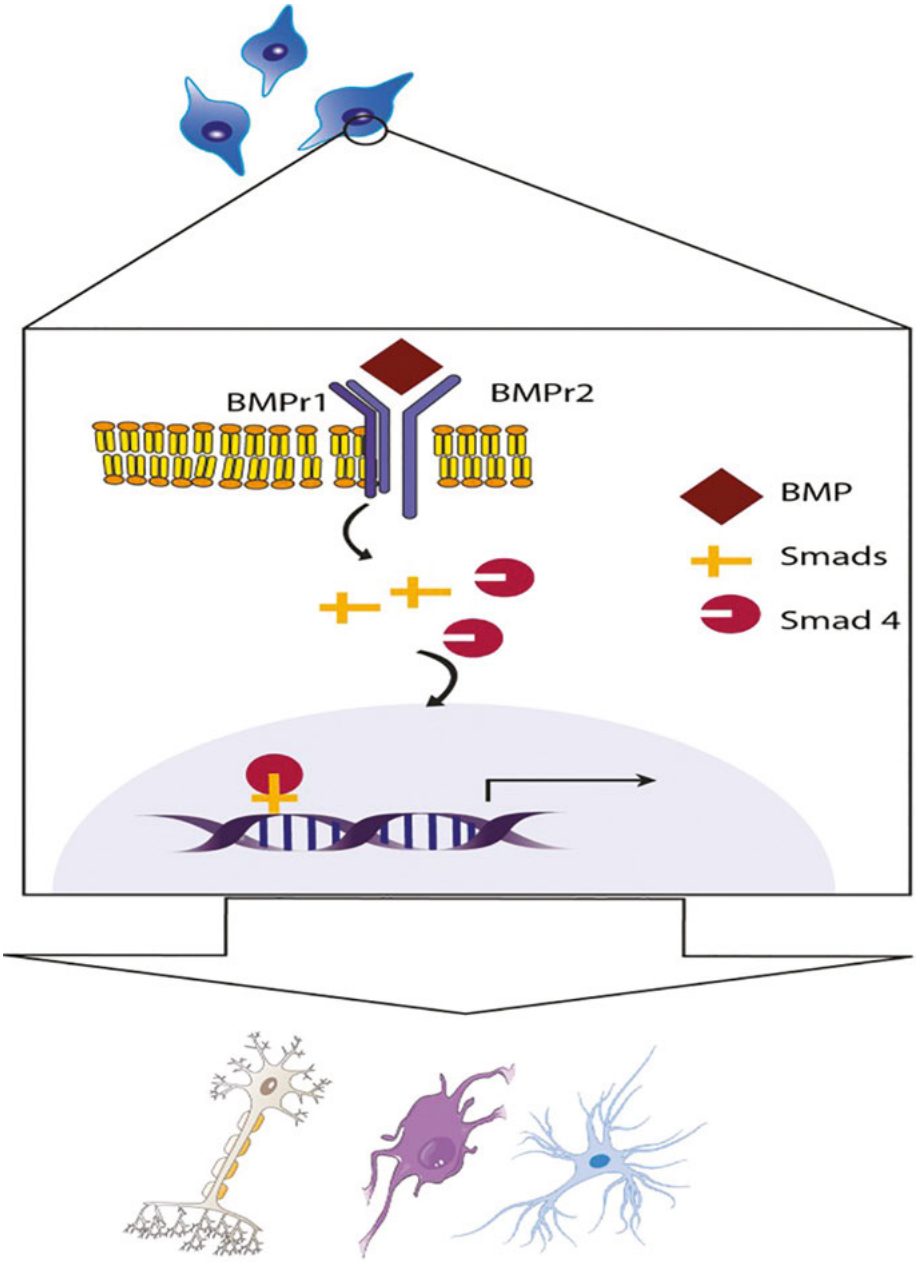

Fig. 6.5: Molecular mechanism of BMP signaling pathway commence with ligand secretion and its binding to a heterodimeric complex of two transmembrane receptors, termed type I and type II, with serine-threonine kinase activity. Upon ligand binding, the type II receptor transphosphorylates the type I receptor at the juxtaposition region activating the type I kinase. The latter then phosphorylates members of the Smad family of transcription factors that are subsequently translocated to the nucleus, where they activate the expression of target genes in concert with other transcription factors or coactivators.

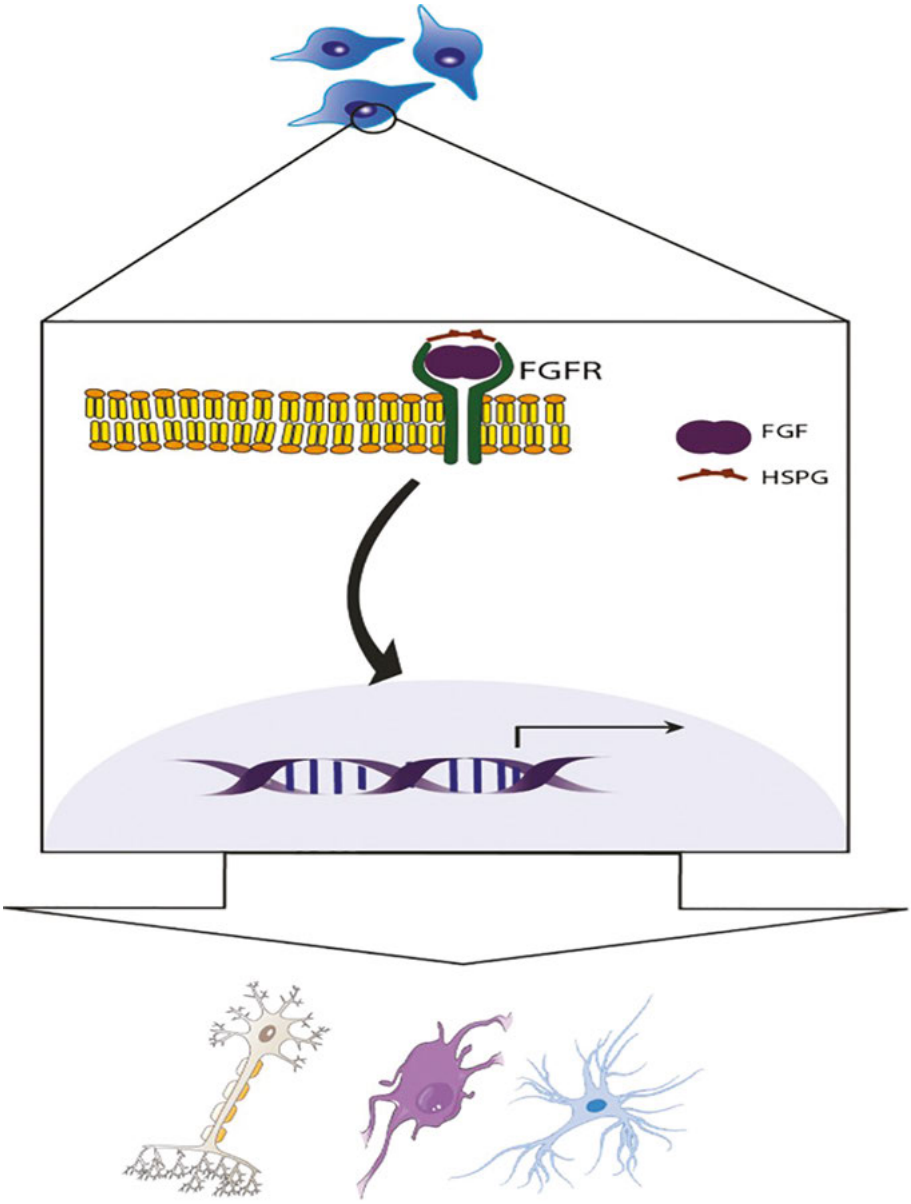

Fig. 6.6: The signaling cascade of FGF ligands is activated by activation of the cell surface receptors (FGFRs). In these tyrosine kinase receptors, the signal transduction progress through ligand binding to the extracellular domain of the receptor to induce receptor dimers formation. This step is followed by phosphorylation of multiple tyrosine residues on the receptors to activate their intrinsic tyrosine kinase domain. Consequently, a cascade of phosphorylation events will be triggered by transient assembly of various intracellular adaptors and effectors. This activation of multiple signal transduction leads to the modified gene expression. FGF activities are regulated in different aspects. In addition to the growth factor expression, their activities may be affected by variable FGF:FGFR binding affinity and interaction with heparin or heparin sulphate proteoglycans (HSPGs) to form stable receptor dimers.

6.7 Cross-talk between signaling pathways in NSC development

According to a wide range of investigations, the embryonic neural development progress toward a regulated complex is a complex chain of molecular events. The precise activation and deactivation of various signaling aspects, including TGF-β/BMP, Wnt/Wg, Hedgehog, Notch, mitogen-activated protein kinase, Cdk5, and other signal transduction, enhance the overwhelming nature of cell fate determination, organogenesis, body patterning, neural development, and stem cell maintenance. In particular, appropriate cell growth and homeostasis are controlled by the cross-talk between these signaling pathways.

The developmental events in the embryogenesis of CNS are followed by patterning along three main axes, including AP, DV, and left-right [54]. Two reverse signaling pathways, SHH and BMP/GDF, control cell fate determination along DV patterning within the neural tube closure. The notochord induces SHH signal transduction ventrally, while the ectoderm neural and nonneural boundaries are responsible for BMP/GDF induction in the dorsal axes. Similarly, BMP/GDF signaling will continue through the roof plate. Recently, there have been few indications of Wnt and retinoic acid signaling role in DV patterning. The neural tube is formed through AP induction of different signaling pathways. This formation can be detected in four major primary vesicles, namely, the forebrain, the midbrain, the hindbrain, and the spinal cord. The specific CNS structure results from multiple development processes, including forebrain conversion into the telencephalon/diencephalon and hindbrain changing into the myelencephalon/metencephalon. During neurodevelopmental pathways, the cerebral cortex originates from the dorsal part of the telencephalon. However, the ganglionic eminences (GEs) are generated by the ventral telencephalon development. The telencephalon, the location of organizing centers, acts as the brain development nucleus. Besides these organizing centers, the anterior neural ridge, cortical hem, and anti-hem release various morphogens such as Wnt/BMPs and FGFs (especially FGF15) to coordinate the developmental process. The ventral part of the telencephalon is responsible for GEs' differentiation and contributes to the increase in interneuron population. The SHH is involved in this progression. The tangential cell migration leads interneurons to reach their final location and promote cortical development. The neural tissue caudalization happens due to the combined involvement of FGF, Wnt, and retinoic acid signaling pathways. Another effective factor along the AP axis patterning can be the Hox gene expression. These preserved gene boxes play a crucial role in hindbrain and spinal cord development. By the formation of AP and DV patterning during gastrulation, LR patterning may be induced as the result of activin presence [74].

Apart from embryonic and perinatal neurogenesis, young neurons of the adult brain have the potential for differentiation [75]. The NSCs proliferate in the neurogenic niche, and their proliferation is regulated by various signals, including increasing FGF-2, insulin-like growth factor-2, leukemia inhibitory factor, ciliary neurotrophic factor, and amphyregulin. Sphingosine-1-phosphate and prostaglandin D2 are two factors that

are implicated in NSCs' quiescent maintenance. Furthermore, the self-renewal and neurogenesis features of adult NSCs are managed through various elements such as juxtacrine and paracrine signals and Notch ligands including Jagged1, Jagged2, and also Delta-like-4 from vascular endothelial cells of the niche. The astrocytes in close proximity to the NSCs may modulate their plasticity potential. Neural proliferation and hippocampal neuro-differentiation are promoted via ATP release and induction of Wnt-3, neurogenesin-1, thrombospondin-1, interleukin (IL)-1β, and IL-6 by astrocytes. The ependymal and meningeal cells may affect NSCs' activities by releasing various morphogens into cerebrospinal fluid (CSF). These different signaling effectors, including FGF2, IGF2, Wnt, and SHH, can be detected by stem cells' primary cilia. Ultimately, the mechanical signal processing leads to modulating NSCs' proliferation and differentiation [76]. On the other hand, increased BMP2/4 expression in adult NSCs' niche causes their impaired proliferation [77].

6.8 Adult neurogenesis

Historically, adult neurogenesis in rodents was confirmed two decades ago, thanks to new biological assessment tools such as immunolabeling and confocal imaging [78, 79]. Subsequently, it has been reported that adult neurogenesis in humans also occurs in the dentate gyrus of the hippocampus, which is a responsible brain area for memory in humans and SVZ of lateral ventricles [50]. Thus, it has been assumed that adult neurogenesis in DG is a key player in neural plasticity and human high-order cognition [80, 81]. Notably, gradual neural production over a whole lifetime in human DG results in the formation of new hippocampal neural circuits and plasticity [81]. Adult NSCs are situated in a limited area between the granular cell layer and the hilus, namely, the subgranular zone (SGZ), and they produce neurons and maintain the stem cells pool according to their regulators such as extrinsic and/or intrinsic morphogens and cytokines; synaptic and non-synaptic transmission; and an individual's behaviors, emotions, and experiences [82]. Since adult neurogenesis is regulated by a wide spectrum of factors, it might be interesting to investigate to what an extent the newborn neurons are involved and/or integrated in new and old neural circuits, which is in progress in many laboratories by employing different techniques of *in vivo* imaging and cell-type-specific calcium indicators.

6.9 Conclusion

The fertilization event triggers a massive array of dynamic process involved in embryogenesis with the allocation of primary stages to the pre-natal neurodevelopment and neurogenesis. These intricate processes highlight their vital importance in organisms'

survival. Stem cells actually supply the raw material for the CNS development. The neurodevelopmental procedures and stem cell differentiation into different subtypes of neurons and other cell types need a variety of intrinsic and extrinsic regulators that control various signaling pathways, their adaptors and effectors and the cross-talk among them are the influential CNS developmental aspects. In addition to the prenatal neurogenesis, the hippocampal NSCs in adulthood provide the opportunity for more extensive neurogenesis.

6.10 References

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7 Adrenomedullins and their emerging role in regenerative medicine

Abstract: The use of stem cells as a strategy for tissue repair and regeneration is an area of fast-growing research with a therapeutic potential for the treatment of heart diseases. The therapeutic potential of stem cells is mainly based on their capability for self-renewal and to produce progenitor cells by producing survival/regeneration factors or by acting as building blocks, that may participate in tissue repair mechanisms. The processes of tissue regeneration within an organism are precisely orchestrated by specific molecular cues also known as niche. One class of factors that is emerging as regulators of stem cell proliferation and progenitor cell fate determinants is the members of calcitonin-gene-related peptide family known as adrenomedullins (ADMs). ADMs regulate numerous cellular functions in a variety of cell types, and recent studies demonstrate their role as regulatory peptides in the behavior of stem and progenitor cells. This chapter presents an overview of the role of ADMs and their receptors in the processes of angiogenesis and vasculogenesis, with special emphasis on their potential to regulate the fate of stem and progenitor cells.

Key Words: Adrenomedullins, Gene, HSCs, Mesenchymal, Myocardial, Receptors, Therapy.

7.1 Introduction

Adrenomedullin (ADM), first isolated from pheochromocytoma (neuroendocrine tumor of adrenal gland medullae) [1], is a small peptide hormone. It belongs to the calcitonin gene related peptide (CGRP) superfamily consisting of calcitonin, amylin, and CGRP; ADM; and ADM2/intermedin (IMD). The members of this superfamily are structurally and functionally related. All members of the peptide family have been known to exert diverse biological actions in various tissues, including the cardiovascular system; however, in this chapter, we will limit discussion to the functions of ADM and ADM2/IMD. Based on phylogenetic studies, ADM and ADM2/IMD fall into two distinct but closely related groups with 33% structural homology.

Human ADM is a 52-amino-acid peptide generated from a 185-amino-acid precursor peptide known as prepro-ADM containing a 21-amino-acid N-terminal signal peptide. Upon proteolytic cleavage, mature 52-amino-acid ADM is released. The mouse and rat ADM consists of 50 amino acids. The prepro-ADM gene is mapped to chromosome 11 and 7 in humans and mice, respectively. ADM2 also, known as IMD (owing to its high expression in the intermediate lobe of pituitary), is a 53-amino-acid peptide and shares 28% sequence homology to ADM. Like ADM, ADM2/IMD is also

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secreted as prepro-peptide consisting of 148 amino acids that, after series of proteolytic cleavages, yields the mature 53-amino-acid ADM2/IMD$_{1–53}$. Some shorter variants of ADM2 consisting of 47 (ADM2/IMD$_{1–47}$) and 40 (ADM2/IMD$_{8–47}$) amino acids have also been reported. In humans, the ADM2 gene is mapped to chromosome 22, while in mice, to chromosome 15.

A global knockout of ADM gene is embryonically lethal due to vascular developmental defects, hemorrhages, and edema formation [2]. Heterozygous ADM$^{+/-}$ survive, but adult mice develop accelerated arteriosclerosis, cardiac hypertrophy and fibrosis, and renal failure. Endothelial specific ADM knockouts are also viable and present no overtly pathophysiological phenotype [3]; however, the adults show infiltration and accumulation of inflammatory cell around the vasculature, spontaneous vasculitis, and accelerated renal failure [4]. ADM2/IMD knockouts are viable; however, the mice show leaky vessels and an enhanced reaction to VEGF [5, 6]. Interestingly, the vessels could be normalized by reinjecting the exogenous ADM2/IMD [5].

7.2 Gene expression and protein secretion

ADMs are secreted peptides that are detectable in blood [7] and tissue fluids [8, 9]. The basal plasma levels of ADM in healthy individuals range from 10 to 25 pg/mL [7, 10, 11]. The plasma levels of ADM2/IMD are relatively lower at 6.3 pg/mL than that of ADM [11]. The plasma levels of both peptides are elevated in hypertensive patients, particularly with concomitant renal insufficiency [7, 11]. The expression of ADM and ADM2/IMD mRNA and precursor peptides has been reported in several tissues, including brain, lungs, heart, kidney, and placenta [12–14]. Endothelial cells (ECs) are one of the main sources of plasma ADM [15], and coronary EC-secreted ADM2/IMD modulates cardiac function in a paracrine fashion [16]. ADM gene expression and production are mainly regulated by inflammatory- and oxidative-stress-related signals. ADM expression and release are increased in inflammation-related pathologies such as sepsis, pancreatitis, systemic inflammatory response syndrome, hypertension, cardiac failure, and hypoxia. Growth hormone response elements, thyroid response elements, hypoxia response elements, and nuclear factor-κB sites have been found in the promoter of human ADM gene. Little is known about the regulation of ADM2/IMD expression. ADM2/IMD is upregulated in response to hypoxia in mouse lungs and pulmonary microvascular ECs [17]. Likewise, it was upregulated in human aortic and umbilical vein ECs under oxidative stress [18].

7.3 ADM receptors and their distribution

Both peptides share the same class B G-protein coupled receptors known as calcitonin-receptor like receptors (CLRs; CRLRs according to old nomenclature).

The receptors alone show little affinity toward these peptides; however, they become ligand selective receptors in association (forming heterodimers) with accessory proteins named receptor activity modifying proteins (RAMPs). To date, three RAMP isoforms (RAMP1–3) have been identified. Structurally, RAMPs comprise a single membrane-spanning domain with an extracellular N-terminal domain of approximately 100–120 amino acids and a short intracellular C-terminal domain of approximately 10 amino acids [19–21]. The CLR may bind to one of the RAMPs, forming three different CGRP family receptors. CLR in combination with RAMP1 gives rise to CGRP receptors, whereas CLR in combination with RAMP2 and RAMP3 forms ADM_1 and ADM_2 receptors, respectively. ADM has high affinity toward ADM_1 and ADM_2 receptors but low affinity toward CGRP receptors. ADM2/IMD exhibits greater affinity toward ADM_2 but low affinity to both ADM_1 and CGRP receptors.

Although CLR and RAMPs are ubiquitously expressed throughout the body, there are differences in their tissue distribution and abundance. CLRs and RAMP2 are highly expressed in the heart, vasculature, lungs, brain, spinal cord, thymus, skeletal muscle, stomach, liver, spleen, female reproductive system, and testis. RAMP1 is also expressed in all these tissues except, of the kidney and heart. RAMP3 is highly expressed in the brain, cerebral arteries, and female reproductive system and moderately expressed in the heart and kidney. Accumulating data reveal differential expression of all RAMPs in stem/progenitor cells of various origins. The tissue-specific expression pattern of RAMPs is differentially regulated under various disease conditions. Since RAMPs determine the ligand and signaling specificity of the agonists, their expression pattern during the course of patho-(physio)-logical conditions regulates the functional specificity of the ADMs under these conditions.

A whole-body, as well as EC-specific CLR gene "*calcrl*," knockout is embryonically lethal [22] due to vascular defects and edema presenting a similar phenotype as ADM. RAMP1 knockout mouse is viable; however, adult $RAMP1^{-/-}$ mouse shows lymphatic defects and increased lymphatic vascular permeability [23]. Like ADM and *calcrl*, knockout mice lacking RAMP2 ($RAMP2^{-/-}$) die *in utero* [24] mainly due to vascular defects and EC deformity [22, 25]. Likewise, most of the EC-specific $RAMP2^{-/-}$ mice die *in utero* and a few surviving develop vasculitis, liver cirrhosis, cardiac fibrosis, and hydronephrosis [3]. Conditionally, drug-inducible $RAMP2^{-/-}$ mice survive; however, on induction of knockout after the drug administration, mice develop edema and vascular leakage occurs [3]. EC-specific overexpression of RAMP2 in whole-body RAMP2 knockouts rescues embryonic lethality; however, mice develop cardiac hypertrophy [26]. Likewise, heterozygous $RAMP2^{+/-}$ mice survive; however, the adult animals show increased permeability and macrophage infiltration [27]. In contrast to RAMP2, RAMP3 knockouts are viable, develop normal vasculature, present normal angiogenesis, and do not present discrete developmental phenotype. However, adult $RAMP3^{-/-}$ mice show some lymphatic abnormalities [28].

7.4 Receptor antagonists

N-terminally truncated analogs of CGRP and ADM have traditionally been used as specific antagonists of these primary receptors—$CGRP_{8–37}$ and $CGRP_{19–37}$ act as antagonists for all CGRP, while $ADM_{22–52}$ and $IMD_{17–47}$ for ADM_1 and ADM_2 receptors have been used. Additionally, some nonpeptide antagonists like BIBN4096BS for CGRP receptor have also been reported.

7.5 Signaling pathways activated by ADM and ADM2/IMD

Although RAMP isoforms differ in their tissue distribution, no pharmacological differences between these receptors have been identified so far, and the functional significance of this receptor redundancy remains unclear. ADM and ADM2/IMD activated signaling pathways vary among species, tissues, organs, and various cell types within an organ/tissue. In many cell types, ADM and ADM2/IMD receptors are coupled to G_s proteins and adenylyl cyclase (AC). Binding of ligands to these receptors leads to the activation of coupled AC and leads to increased production of cyclic adenosine monophosphate (cAMP). This AC/cAMP is considered to be the major signal pathway activated by the ADM-CLR/RAMP system, although some other messengers (like nitric oxide [NO] and Ca^{2+}) have also been reported to be activated/generated in response to ligand binding. Nevertheless, the downstream signaling events/elements are relatively less investigated. Elevation of cAMP could activate one or both of its downstream, i.e., protein kinase A (PKA), and exchange protein directly activated by cAMP (Epac) [29]. Activation of PKA leads to interaction with several other signaling pathways depending on the PKA isoform activated and the availability of other signaling partners. For example, activation of type I PKA correlates with enhanced cell growth and transformation, while activation of type II PKA results in cAMP-mediated growth inhibition and differentiation [30]. PKA inhibits APC/C(Cdh1) ubiquitin ligase and an indirect activation of G9a, thus modulating DNA methylation, and induces cell differentiation [31, 32]. PKA can interact with phosphoinositide-3 kinase (PI3K)/protein kinase B (PKB or Akt) signaling. Both activation and inhibition of PI3K/Akt by cAMP/PKA signaling have been reported in a context-dependent manner. ADM dilated rat thoracic aorta via PI3K/Akt-dependent NO production, induced endothelial progenitor cell (EPC) and leukemic stem cell proliferation, induced oligodendrocyte differentiation, and protected cardiomyocytes against ischemic injury via activation of PI3K/Akt signaling. Likewise, ADM2/IMD regulates uterus relaxation via PI3K/Akt-dependent NO production, *in vitro* and *in vivo* angiogenesis, and coronary blood flow; reduces cardiomyocyte endoplasmic reticulum (ER) stress; and normalizes tumor vessel via activation of the PI3K/Akt pathway. However, it antagonized Ang II-induced Akt phosphorylation and increased blood pressure in a rat model of hypertension [33]. Similarly, ADM2/IMD is highly expressed

during various phases of the estrous cycle, where it regulates uterus contractility via PI3K/Akt-dependent mechanisms [34]. Both ADM- and ADM2/IMD-mediated vasorelaxation is at least in part dependent on endothelium-dependent NO production [35–39]. ADM-mediated activation of PI3K/Akt causes increased phosphorylation of NO synthase 3/endothelial NO synthase (eNOS), leading to its activation and enhanced NO production. A second mechanism of ADM and ADM2/IMD-mediated NO production is via an increased cytosolic Ca^{2+} concentration either via release from ER or increased influx from extracellular room [35, 40, 41]. This leads to Ca^{2+}/calmodulin-dependent activation of eNOS [42], which may regulate ADM-mediated endothelium-dependent vasodilation [36, 39, 43, 44]. However, ADMs also induce vasodilation in an endothelium-independent manner. This effect is triggered via an activation of cAMP/PKA-mediated inhibition of vascular smooth muscle contractile machinery [45]. However, ADM- and ADM2/IMD-mediated endothelial barrier stabilization seems to be independent of NO production [46, 47].

In ECs, both ADM and ADM2/IMD mediate cell proliferation, migration, and angiogenesis via an activation of the PI3K/Akt pathway [48, 49]. The mechanism of PKA-triggered activation of PI3K/Akt is yet not explored in ECs; however, recent reports suggest an indirect activation via PKA-mediated activation of PP1, which causes inhibitory serine phosphorylation of insulin receptor substrate 1, which in turn causes an activation of PI3K in Fischer rat thyroid cell line (FRTL) thyroid and breast cancer cells [50, 51]. Whether this mechanism also exists in ECs or other cells of the cardiovascular system is not known and needs to be investigated.

A mixed effect of ADMs on smooth muscle cell (SMC) proliferation and migration has been reported. Although ADM induces basal SMC proliferation and migration via an activation of the PI3K/Akt and extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) pathways [52, 53], it antagonizes Ang II, serum, and PDGF-induced SMC proliferation and migration via inhibition of PI3K/Akt signaling in a cADMP/PKA-dependent manner [54–57]. Ectopic overexpression of ADM inhibits rat SMC hyperplasia [56] and reduces carotid artery neointima formation in a rat model of wire injury [58]. Likewise, ADM2/IMD also inhibits SMC proliferation and pathologic phenotypic remodeling, as well as ameliorates neointima formation *in vivo* in various models of vascular injury [59, 60].

The small GTPase Ras-dependent activations of MAPK/ERK and p38 MAPK pathways are involved in the regulation of cell proliferation, differentiation, senescence, and apoptosis [61, 62]. Both ADM1 and ADM2/IMD seem to activate the p38 MAPK pathway in different cell types [63–65] and thus may participate in cell differentiation. However, the data about ERK1/2 MAPK activation is conflicting dependent on the cell type studied. In SMCs where both ADM and ADM2/IMD inhibit SMC proliferation and ameliorate pathologic vascular neointima formation, both peptides inhibit both basal as well as agonist-induced ERK activation [33, 59, 60, 66, 67]. However, both peptides activate ERK1/2 and PI3K/Akt signaling in ECs in a coordinate manner and thus regulate cell proliferation and angiogenesis [48, 68] and osteogenesis [69].

The mechanisms responsible for ADM and/or ADM2/IMD-mediated inhibition or activation of MAPK signaling have not yet been explored. ADM and ADM2/IMD-induced inhibition of ERK/MAPK is probably mediated via cAMP/PKA signaling [66, 70, 71]. ADM-mediated activation of both p38 and ERK1/2 MAPK signaling can be speculated to be via cAMP/Epac-mediated Rap1 activation, which integrates cAMP signaling with the Ras/Raf signaling pathway. Both ADM and ADM2/IMD activate the cAMP/Epac signaling [47, 72] but is almost completely neglected.

7.6 ADMs and tumor angiogenesis

In addition to the cardiovascular system, ADMs are highly expressed in tumors, particularly tumor vasculature, and are involved in the regulation of tumor angiogenesis. ADM is considered as a hypoxia-regulated gene, and overexpression of ADM in the endometrial cancer cell line "Ishikawa" protected cells against hypoxia-related apoptosis [73] and hyperactivation of tumor-related Ras/Raf and PKC signaling pathways in breast cancer cell line [74]. Accordingly, ADM receptor antagonism reduced tumor vessel density [75] and inhibited the growth of pancreatic cancer cells [76], glioblastoma, and colorectal cancer cell lines [77]. Little data related to ADM2/IMD expression in tumors are available and still emerging. ADM2/IMD is highly expressed in hepatocellular carcinoma cell lines [78] and pancreatic adenocarcinomas, and ADM2/IMD expression level was correlated with poor survival rate [79]. Small interference (si)RNA-mediated suppression of ADM2/IMD resulted in reduced growth of hepatic cancer cell line [78] and inhibition of ADM2/IMD signaling-ameliorated tumor angiogenesis [80]. In contrast, data on the anticancerous effects of ADM, particularly on colorectal cancers, are also emerging. ADM treatment reduced intestinal inflammation and maintained epithelial barrier in a mouse model of inflammatory bowel disease [81] and reduced inflammatory burden and caused regeneration of intestinal epithelium in patients with inflammatory bowel disease [82]. Moreover, ADM treatment improved disease activity index and mucosal healing and ameliorated ulcers in patients with ulcerative colitis [83]. A recent study using a mouse model of colitis-associated colon cancer demonstrated that treatment with ADM or a positive modulator of ADM reduced tumor burden and improved healing [4]. Data pertaining to the mechanisms of how ADM is upregulated and the mechanistic aspects of ADM-mediated tumor progression or suppression are still lacking. However, it is known that both ADM and ADM2/IMD are hypoxia/hypoxia inducible factor 1α (HIF-1α) regulated genes [17, 84], and tumors (particularly solid tumors) create a hypoxic microenvironment leading to HIF-1α stabilization and, hence, ADM upregulation. Recently, it has been shown that a micro-RNA (miR-126) targets ADM and thus suppresses the expression of ADM mRNA. In cancerous stromal cells of the uterine cervix, miR-126 is downregulated, leading to upregulation of ADM [85]. ADM causes an upregulation of the PI3K/Akt signaling pathway in most of cancer cells and tumor endothelium. Recently, it has been demonstrated that in

prostate and urothelial cancer cells, ADM upregulates the membrane expression of transient receptor potential V2 channel in a PI3K/Akt-dependent manner, which is responsible for tumor progression and invasion [86].

7.7 Role of ADMs in stem cell growth and differentiation

Stem cells are undifferentiated cells, possessing the capacity for self-renewal and differentiating into different lineages under appropriate conditions. Accumulating data suggest in addition to main source i.e. bone marrow, stem cells exist in several tissues such as peripheral blood, vasculature, skeletal muscle, adipose tissue, pancreas, prostate, ovary, and testis [87]. These tissue-specific stem cells are important components of tissue homeostasis and replace the damaged cells due to normal wear and tear or tissue injury. Stem cells are therefore indispensable for the integrity of the complex organisms [87]. The maintenance/self-renewal and differentiation of these tissue-resident stem cells to the specific cell type are dependent mainly upon the specific local tissue microenvironment or niche. Cues in these microenvironments are complex and are a blend of mechanical, physical, chemical, spatial, and temporal cues ranging across many magnitudes. Adult stem cell-mediated tissue regeneration is accompanied by environmental changes in the niche and orchestrated by several autocrine/paracrine growth factors, including epidermal growth factor, wingless ligand, Notches, bone morphogenic proteins, HIF-1α , sonic Hedgehog, and stromal cell-derived factor-1α. In this context, ADM secreted by vascular endothelium is considered an important component of the hematopoietic stem cell (HSC) niche [88]. In the following section, we will discuss the role of ADMs and their receptors in the regulation of apoptosis, proliferation, growth, and differentiation of a variety of stem cells of different origins.

7.7.1 Mesenchymal stem cells

Mesenchymal stem (or stromal/progenitor) cells (MSCs) or nonhematopoietic cells expressing the stem-cell markers (e.g., CD90, CD105, and CD73) and are able to differentiate into adipocytes, chondrocytes, and osteocytes [89–91]. However, controversy about their name and stemness has existed since the early 2000s [92, 93]; from a therapeutic perspective, MSCs are emerging as a biological treatment for different tissue injury syndromes such as stroke, myocardial infarction, sepsis, and several other diseases [94]. Marrow- and fat-derived MSCs have been the subject of clinical trials for more than a decade. Recently, MSC-based therapy (Alofisel) has been approved for the treatment of fistular Crohn's disease in Europe [95, 96].

Accumulating data suggest that ADM plays a positive role in the growth and differentiation of MSCs of different origins. ADM expression is increased during

adipogenesis of human MSCs [97], and enhanced production and release of ADM are observed during macrophage differentiation of THP-1 cells [98]. This secreted ADM is considered to regulate the differentiation process. This assumption is further supported by a couple of recent studies. Exogenous ADM enhanced the oligodendrocyte differentiation of oligodendrocyte precursor cells [99] and odontogenic differentiation of dental pulp stem cells [100]. Several groups have tried to exploit the prosurvival and prodifferentiation effects of ADM for therapeutic usage. Intravenous (IV) administration of bone-marrow-derived MSCs in a rat model of cerebral occlusion improved neurological score. Coadministration of ADM enhanced the survival of engrafted MSCs and potentiated their protective effects significantly [101]. Transplantation of non-viral-vector-based ADM-engineered marrow MSCs improved their survival and their ability to improved cardiac function in a rat model of myocardial infarction [102]. Si and coauthors further investigated this approach using viral-vector-mediated ADM-engineered marrow MSCs [103]. ADM overexpression enhanced the survival of MSCs against hypoxic injury and serum derivation via an activation of PI3K/Akt pathway and reduced caspase 3 activity and, thus, apoptosis. These ADM-engineered MSCs improved cardiac function and reduced cardiac fibrosis in a rat model of myocardial infarction [104]. Similarly, viral-vector-based ADM-engineered adipose-derived stem cells (ASCs) improved erectile dysfunction in a rat model of diabetic erectile dysfunction [105]. Accordingly, knockdown of ADM transcript in ASCs resulted in loss of their ability to improve erectile dysfunction [105].

Compared to ADM, the role of ADM2/IMD in mesenchymal growth/differentiation is not well studied. Recently, a Chinese group (article in Chinese) in an *in vitro* study has demonstrated the antiapoptotic and prosurvival effects of ADM2/IMD in bone-marrow-derived MSCs [106]. Whether these MSCs preconditioned with ADM2/IMD have better survival and clinical outcome needs to be further investigated.

7.7.2 Endothelial progenitor cells

EPCs are the multipotent heterogeneous population of cells found in circulation [107] and the bone marrow [108], identified both by flow cytometry and cell culture techniques, capable of differentiating into ECs and thus participate in vascular repair and regeneration. They express CD133, CD34, and VEGFR2 surface markers [109]. At least two phenotypes of EPCs have emerged from studies: one that incorporates into the vessel wall, directly contributing to vessel repair/regeneration, and one that homes-in to the neo-vessel but locates behind the endothelial wall, supporting the viability of the newly formed vessels via the release of autocrine/paracrine factors. ECs actively synthesize and secrete ADM [15, 110, 111] and ADM2/IMD [17], and ADM receptor signaling is of particular importance in EC physiology [112]. ADMs promote EC proliferation [6, 113], migration [49, 114], and angiogenesis [48, 114–116]. Moreover, ADMs reduce vascular tone [117–119], protect ECs against apoptosis [18, 120, 121], and are involved in the maintenance of endothelial barrier integrity [5, 17, 46, 47, 122].

In an elegant study, Nagaya and colleagues demonstrated that IV administration of human EPCs could incorporate into the pulmonary vasculature of nude rats and ameliorated monocrotaline-induced pulmonary hypertension [123]. This protective effect of EPCs was not only doubled, but also their survival was significantly enhanced when EPCs were engineered to overexpress ADM [123]. In the same direction, IV administration of bone-marrow-derived mononuclear cells (MNCs) enhanced angiogenesis and improved blood flow in a rat model of hindlimb ischemia [124]. The beneficial effects of MNC therapy were enormously enhanced by coadministration of ADM. Moreover, ADM increased the number of MNC-derived EPCs *in vitro* and vWF^+ cells *in vivo*, suggesting that ADM may accelerate MNC differentiation of endothelial lineage [124, 125]. This strategy was extended to other types of multipotent cells for the treatment of injured vessels. Administration of ADM-engineered ASC suspension or ASC sheets at the site of the injured vessel enhanced their survival and accelerated reendothelization of the vessel wall in a rat model of vascular injury [126]. The *in vitro* data from several studies demonstrate that ADM activated PI3K/Akt signaling in peripheral blood MNCs, accelerated their differentiation to EPCs, and enhanced their angiogenic potential [127, 128]. This was accompanied by a reduction in apoptosis and increased survival and proliferation of EPCs [127, 128]. Embryonic stem cells (ESCs) expressing VEGFR2 are another rich source of EPCs, and depending upon the concentration of exogenously added VEGF, they can be differentiated to venous or arterial ECs. At low VEGF concentrations, these $VEGFR2^+$ cells differentiate to venous ECs, while at higher concentrations, they commit to arterial ECs [129]. Interestingly, their arterial but not venous differentiation could be enhanced by cotreatment with ADM [130]. This was accompanied by an activation of Dll4/Notch1 signaling; however, ectopic expression of Notch1 alone was not sufficient for arterial differentiation and additionally required the presence of ADM, suggesting that Notch1 may only be partly involved [130]. Moreover, the authors demonstrated that hypoxia-mediated arterial differentiation is indeed ADM signaling dependent [131].

Embryonic loss of ADM signaling is lethal due to vascular defects, in particular the defects in lymphatic vasculature [22]. During rodent embryogenesis, the expression of both ADM and TGF-β is spatially and temporally regulated in such a manner that their expression patterns overlap at the same stage of development in several tissues, including lymphatics [132]. It is therefore believed that during the development of lymphatic vasculature, endothelial ADM and TGF-β signaling plays an important role [22]. An active TGF-β signaling suppresses the differentiation of mouse ESCs [133] and MSCs [134] to lymphatic ECs. A moderate inhibition of TGF-β signaling in the presence of VEGF-C promotes the lymphatic EC differentiation of both ESCs and MSCs and thus enhances lymphangiogenesis and lymphatic development [133, 134]. Interestingly, the addition of ADM enhanced the VEGF effect on differentiation of embryoid bodies to lymphatic ECs and closely related liver sinusoidal ECs [25].

Few data are available about the role of ADM2/IMD in endothelial differentiation of multipotent cells. Recently, data from ADM2/IMD knockout mice reveals the critical role of vessel enlargement and stabilization. ADM2/IMD knockout mice are viable

but show reduced vessel diameter and leaky vessels [6]. These vascular defects were due to a reduced number of ECs covering the vessel, suppressed EC proliferation, and reduced pericyte coverage of the vessels. Replenishing the ADM2/IMD with exogenous infusion normalized these vascular defects [6], suggesting that ADM2/IMD may be playing a positive role in endothelial differentiation of multipotent cells. However, more studies directly addressing this question are required to support the hypothesis.

7.7.3 Hematopoietic stem and progenitor cells

HSCs are the primary source of mature hematopoietic and immune cells. They possess the unique capacity to undergo self-renewal throughout an individual's life. The idea that ADM may be involved in the differentiation of hematopoiesis came from the early reports by Kubo and colleagues [98], who showed that ADM is secreted by monocytic THP-1 and HL-60 cell lines during their differentiation to macrophages and scavenger cells. Moreover, retinoic-acid- and lipopolysaccharide (LPS)-induced macrophage differentiation was also accompanied by enhanced accumulation of ADM in the medium [98, 135]. Likewise, ADM secretion by monocytes is increased in the acute phase of Kawasaki disease in patients [136], suggesting that ADM may be regulating the macrophage differentiation of monocytes. Later, Del Pup *et al.* demonstrated that ADM enhanced the clonal growth and proliferation of cord-blood-derived MNCs [137] and pharmacological inhibition of ADM receptors abrogated its effects [137, 138]. In contrast, a recent study demonstrates that ADM induced the proliferation of promyelocytic HL-60 cells but inhibited their differentiation to monocytes. However, pharmacological inhibition of ADM signaling promoted differentiation toward monocytic and granulocytic lineage [139]. In vasculature, ECs are the major source of ADM. Using human brain ECs in *ex vivo* culture, Chute and colleagues demonstrated that EC-derived factors amplified human bone-marrow- and cord-blood-derived cells. Gene expression data and functional analysis demonstrated ADM as one of the important factors mediating this effect [88].

7.7.4 Osteogenesis and odontogenesis

Osteogenesis and bone repair are a complex process requiring balanced activities of osteogenic osteoblasts and osteoporotic osteoclasts. The precise series of ordered events required for osteogenesis are modulated by a repertoire of systemic and local factors [140]. Accumulating data suggest that ADM and its receptors are expressed by osteoblasts [141] and ADM signaling functions locally to promote bone growth by regulating osteoblastic activity via its antiapoptotic and mitogenic actions [69, 142–144]. *In vivo* data from animal studies demonstrate that systemic administration of ADM or its truncated peptide $ADM_{22–52}$ can promote

osteogenesis [145–148]. These data propose that ADM, or its peptide fragments, could be used for the treatment of osteoporosis [147, 149]. The expression of ADM was detected in secretory odonblasts in dental pulp, promoted cell growth, and enhanced dental pulp mineralization in rats [150]. Moreover, implantation of chitosan microsphere containing ADM into the rat socket accelerated alveolar bone remodeling and reduced residual ridge resorption [151]. Surprisingly, data from ADM knockout mice are in sharp contrast to the above-described reports. Conditional inducible knockout of ADM in adult mice results in increased bone mass, and the animals present denser bones compared to wild type (WT) littermates [152]. Furthermore, using a postmenopausal mouse model of osteoporosis, the authors demonstrated that the administration of a nonpeptide ADM receptor inhibitor protected against ovariectomy-induced bone resorption. Interestingly, the animals with reduced ADM levels exhibited a reduced lifespan [152]. These contradictory data may be due to differences in local and systemic effects of ADM signaling; however, further studies are required to clarify this discrepancy.

7.8 Role of ADMs in cardiac repair and angiogenesis

As discussed earlier, ADM is a potent vasoactive peptide and have proangiogenic and antiapoptotic properties that have been exploited by various research groups for myocardial injury. At molecular levels, the antiapoptotic properties of ADMs after adenoviral transgene delivery to the heart have been assigned to the Akt-glycogen synthase kinase 3b (GSK3b) signaling pathway, wherein GSK3b and caspase-3 activities become significantly lowered [153]. Overexpression of ADM also increased the phosphorylation of Akt and Bcl2 [154]. The involvement of PI3K-Akt signaling has also been reported by other research groups [155]. Treatment with ADMs also activates signaling pathways that lead to the generation of NO and peroxynitrite that alleviates myocardial ischemia-induced antiarrhythmicity in anesthetized rats [156]. More recent studies have combined ADM and antimicrobial peptide PR39 transgene delivery using adeno-associated viral vector to the infarcted heart, which promoted cardiomyocyte survival and reduction in ischemic injury [157]. Molecular studies revealed that co-overexpression of ADMs and PR39 led to increased phosphorylation of Akt, Bcl2, HIF-1, and VEGF, while Bax expression was significantly reduced in the infarcted myocardium.

Encouraged by these data, the delivery of ADM has also been combined with stem/progenitor cell transplantation to enhance their survival and angiogenic potential postengraftment for the treatment of ischemic injury to the heart as well as hind-limb [124, 158]. Using an experimental rat model of acute coronary artery ligation, Fujii and colleagues reported that direct intramyocardial injection of bone-marrow-derived MNCs, combined with simultaneous subcutaneous infusion of ADMs, significantly reduced the infarct size besides a concomitant increase in

capillary density in the ischemic myocardium. Histological studies at 72 hours after cell delivery revealed significantly reduced Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in animal hearts. The authors attributed the beneficial effects of concomitant treatment using MNC and ADM administration to the proangiogenic and antiapoptotic properties of ADMs. On the same note, ADMs have also been shown to enhance the proliferation and migrational activity of ECs both *in vitro* as well as *in vivo* in murine gel plugs [113]. More recent studies have developed chitosan-based microspheres for ADM delivery to osteoblasts and vascular ECs with the aim to avoid the use of viral vectors [159]. MSCs are excellent carriers of transgene due to their robust nature as genetic manipulation did not interfere with their stemness. Hence, bone-marrow-derived MSCs have been genetically modified to enhance their survival, differentiation capacity, proliferation, and paracrine activity for myocardial repair [160–164]. ADM is also secreted by transplanted MSCs as part of their paracrine secretions, which mechanistically contributes in alleviating cardiac fibrosis in the ischemic myocardium via modulation of cardiac fibroblast function [165]. Various studies have therefore concluded that genetically modified bone-marrow-derived MSCs accentuate ADM secretion to enhance the donor cell survival and potentiate their reparability by releasing ADM [102–104].

7.9 Future perspectives

In this review, we have learned that ADMs and ADM receptors are widely distributed and play a major role in regulating stem cell behavior. Several studies have demonstrated that ADM and ADM2/IMD act as growth factors stimulating the proliferation and migration of stem cells of various origins and also as cell fate determinant for a number of stem and progenitor cells. Conditioning with ADM or ADM2/IMD can be used to propagate and enhance the survival of various stem/progenitor cells. This may be of particular importance in clinical settings where stem/progenitor cells are propagated for injection of cells or cell-releasate. Injection or transplantation of ADM-engineered stem/progenitor cells has shown success in various disease models. In this strategy, a limiting factor may be the expression of ADM receptors. ADM-receptor-engineered stem/progenitor cells have not yet been tried or investigated. Overexpression of ADM receptors, alone or in combination with ADM or ADM2/IMD, may enhance the beneficial effects. This maneuver may be of particular importance in myocardial ischemia and in tooth regeneration as several studies have shown beneficial effects in mouse models of diseases *in vivo*. However, a conditional adult knockout mouse model shows reduced bone resorption in animals with reduced ADM production, and the same beneficial effects on bone resorption in a postmenopausal mouse model could be obtained using a pharmacological inhibitor of ADM receptors [152]. Further studies are required to investigate the mechanisms and whether these beneficial effects of ADM inhibition can be extended to other disease models of bone resorption.

7.10 References

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8 Mesenchymal stem cells and their role in hypoxia-induced injury

Abstract: Mesenchymal stem cells (MSCs) reside in the stromal region of various organs that can be triggered to home-in to injured tissues and differentiate into a specific cell type to replace the damaged ones. Many tissues undergo oxygen deprivation during trauma due to the disruption of the existing vascular network that promotes reparative functions involving MSCs. It is believed that apart from acting as progenitors, MSCs orchestrate a series of functions *in vivo* to mediate tissue repair through their secreted bioactive factors, which suppress inflammation, and upregulate mitogenic, angiogenic, neuroprotective, and antioxidative effects. Coculture of MSCs with hypoxic cells promotes mitochondrial transfer from MSCs to the hypoxic cells to support their rescue. MSCs have also been preconditioned under hypoxic conditions to increase their therapeutic efficacy. It is anticipated that MSCs' therapeutic effect is an artifact of *in vitro* culture. MSCs also extracellularly release a plethora of bioactive molecules, including growth factors, cytokines, microRNAs, and mRNAs. Moreover, exosomes have been derived from MSCs under hypoxic conditions. In this chapter, we discuss in detail the mechanisms by which MSCs exposed to hypoxic conditions increase the survival rate and rescue of ischemic myocytes at the site of the cell graft. The chapter also reviews current translational efforts using MSCs and their derivatives for treating ischemic heart disease and critical limb ischemia.

Key Words: Cytokines, Growth factors, Hypoxia, MSCs, Secretome, Paracrine.

8.1 Introduction

8.1.1 Hypoxia-induced cell injury

Oxygen is required for cell metabolism, in which fats, carbohydrates, and amino acids are broken down in a series of biochemical reactions to generate adenosine triphosphate (ATP), the energy required to drive the various processes in cells. The required oxygen is derived from the air we breathe, where passive diffusion across the membrane of the alveoli in the lung into the blood stream occurs. In the blood, most of the diffused oxygen will bind to hemoglobin and is then carried to the cells in different regions of the body via a complex vascular system.

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Hypoxia is a state whereby there is insufficient supply of oxygen for the demand of the physiological activity of the cells. This can occur when there is a sudden demand for energy, such as when performing strenuous physical activity, or when the ambient O_2 level decreases, such as at high altitudes. Cells in such condition will trigger a series of adaptation mechanisms such as anaerobic metabolism (lactic fermentation). Such a condition may not be pathological but, when prolonged, will lead to irreversible damage to the cells and, eventually, cell death. Ischemia refers to the condition where there is insufficient blood flow to a tissue, for instance, when there is embolism or trauma to the vascular system, which is a common cause of tissue or organ damage.

A number of parameters are affected during hypoxia, i.e., expression of cell surface markers, cell viability, and cellular secretions. Many of these responses to hypoxia are orchestrated by the heterodimeric transcription factor hypoxia-inducible factor (HIF). Under physiological oxygen levels (normoxia), HIF-1α is continuously synthesized and is hydroxylated by prolyl hydroxylases (PHDs) at proline residues using oxygen as a substrate [1]. Hydroxylated HIF-1α binds to von Hippel-Lindau, leading to polyubiquitination, which is quickly degraded by the ubiquitin-proteasome system [2].

In the event of hypoxia, the PHDs are inactive and HIF-1 accumulates. The HIF-1α is then stabilized and forms a heterodimer with HIF-1β, which then translocates into the nucleus and binds to the hypoxia response element in the regulatory region of an array of HIF target genes. This allows the cell to enact a transcriptional program that involves various cellular functions, i.e., metabolism, cell proliferation, survival, migration, angiogenesis, apoptosis, and autophagy, which is appropriate to thrive in the hypoxic environment [3–5]. The PI3K/Akt signaling pathway is important for controlling HIF-1α protein levels by increasing HIF-1α synthesis during hypoxia [6].

A classic example of hypoxia-induced injury is ischemic heart disease (IHD), which is caused by occlusion of the coronary artery. This very quickly leads to apoptosis (programmed cell death) and cell necrosis (acute cell death) in the core region of the infarct in the region surrounding it [7, 8]. The programmed cell death can be reversible and hence can be a potential target of cell rescue strategies.

8.2 Mesenchymal stem cells and their therapeutic effects

Mesenchymal stem cells (MSCs) and their secreted products have been widely explored for preclinical study and various clinical applications. They possess properties that potentially can treat inflammatory, autoimmune, and malignant diseases and regenerate damaged tissues via cellular and acellular approaches. The properties exerted by MSCs may vary as a response in accordance with the microenvironment. Similarly, in response to the hypoxic conditions, MSCs secrete a plethora of factors that are associated with angiogenesis, growth promotion, and antiapoptosis [9]. Hence, MSCs have been proposed as a promising therapeutic tool especially for inflammatory and ischemic disorders either as a cell-based treatment or cell-free treatment,

i.e., MSC-sourced secretome. In the following sections, the strategies using MSCs and their derivative secretome for hypoxic cell rescue will be elaborated, with a special interest in their application in hypoxic cardiomyocytes in IHD.

8.2.1 Cell-mediated repair by MSCs

MSCs are known for their capacity to differentiate into multiple lineages, including osteocytes, adipocytes, chondrocytes, cardiomyocytes, etc., in the presence of suitable microenvironment stimuli [10]. The mechanisms of MSC-mediated tissue repair are complex. It involves diverse mechanisms including the differentiation of MSCs to replace damaged cells according to their cell types. MSCs actively respond or home-in to the injury site, similar to when immune cells sense a pathogen. MSCs residing near the injury site migrate to the injured area and differentiate into mature cells and regenerate new tissues to replace the injured ones. MSCs also mediate tissue repair through cell fusion with the damaged cells [11]. Furthermore, MSCs are able to modulate the immune regulatory system as they secrete immunosuppressive and cytoprotective factors in the body [12]. They have the ability to evade allo-recognition by host immune cells due to the absence of MHC-II antigens on their surface [13].

8.2.2 MSC paracrine effect

There is an increasing body of evidence demonstrating that MSCs have the ability to promote host cell regeneration, tissue repair, and remodeling due to their paracrine signaling. The release of paracrine factors is now recognized as the primary mechanism by which MSCs promote a regenerative environment that is conducive for tissue regeneration [13–15]. MSCs release a set of growth factors, such as epidermal growth factor, fibroblast growth factor (FGF), platelet-derived growth factor, transforming growth factor (TGF-β), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF)-1, in a nuclear factor-κB-dependent manner. MSCs are shown to produce numerous growth factors, cytokines, chemokines, extracellular matrix proteases, and hormones [15]. VEGF, HGF, IGF-1, TGF-β, leukemia inhibitory factor, stromal cell-derived factor-1 (SDF-1), etc., are few examples that are involved in angiogenesis, immunomodulation, antiscarring, and antiapoptotic properties [16].

8.2.3 Tunneling nanotube formation by MSC

Another mechanism of rescuing damaged tissue is the transfer of organelles such as mitochondria and/or molecules through tunneling nanotubes (TNTs). In fact, cells naturally transport the intracellular material, especially mitochondria, through

different processes such as cell-to-cell contact, nanotubular structures, and cell-cell fusion to stabilize and improve the bioenergetic properties of damaged cells. Mitochondrial transfer was initially reported by Spees and colleagues, where healthy mitochondria from human stem cells rescued mitochondrial respiration in mitochondria-depleted recipient cells [17]. Besides organelles, MSCs may transfer proteins/peptides, RNA, hormones, and/or chemicals through these tunnels. MSCs also have been shown to improve survival and restore cell damage through TNTs to cardiomyocytes cultured under glucose-oxygen deprivation in an *in vitro* model of ischemia-reperfusion [18].

8.3 Hypoxic cardiomyocyte rescue strategies by MSCs

Cell-to-cell communications between grafted MSCs and injured cardiomyocytes are necessary for rescuing the damaged cardiomyocytes and regenerating the myocardium. Communication between MSCs and injured cardiomyocytes may be broadly realized by three mechanisms: cardiomyocyte differentiation, paracrine signaling, and transport of intracellular component especially mitochondria via partial and complete cell fusion [19–23]. Fig. 8.1 summarizes the cell-mediated rescue mechanisms of MSCs on hypoxic cardiomyocytes.

8.3.1 Cardiomyocyte differentiation

Mutagenic factors, 5'-azacytidine, bioactive compounds such as basic FGF (bFGF), and hydrocortisone are shown to facilitate the cardiomyogenic differentiation of MSCs [10]. Evidence of cardiomyocyte differentiation from MSCs includes the expression of myotube phenotype, cardiac-specific genes and proteins such as troponin-T and -C, expression of connexin 43 for gap junction, and spontaneous cell contractibility [10, 24]. However, *in vitro* investigations have used cardiomyocyte secreted factors (conditioned medium) or cardiomyocyte lysate or coculture of MSCs and cardiomyocytes and demonstrated successful cardiomyogenic differentiation of MSCs [25–27]. Subsequently, transplantation of xenogeneic, allogenic, or autologous MSCs for cell therapy in small as well as large animals has shown cardiomyogenic differentiation of the transplanted cells and their ability to regenerate the damaged myocardium [20, 28, 29]. However, evidence regarding the persistence of the derivative myocytes is lacking.

8.3.2 MSC paracrine effect on cardiomyocytes

Recent evidence suggests that paracrine signaling from MSCs and the surrounding cells in a cardiac microenvironment facilitates cross-talk and communication between

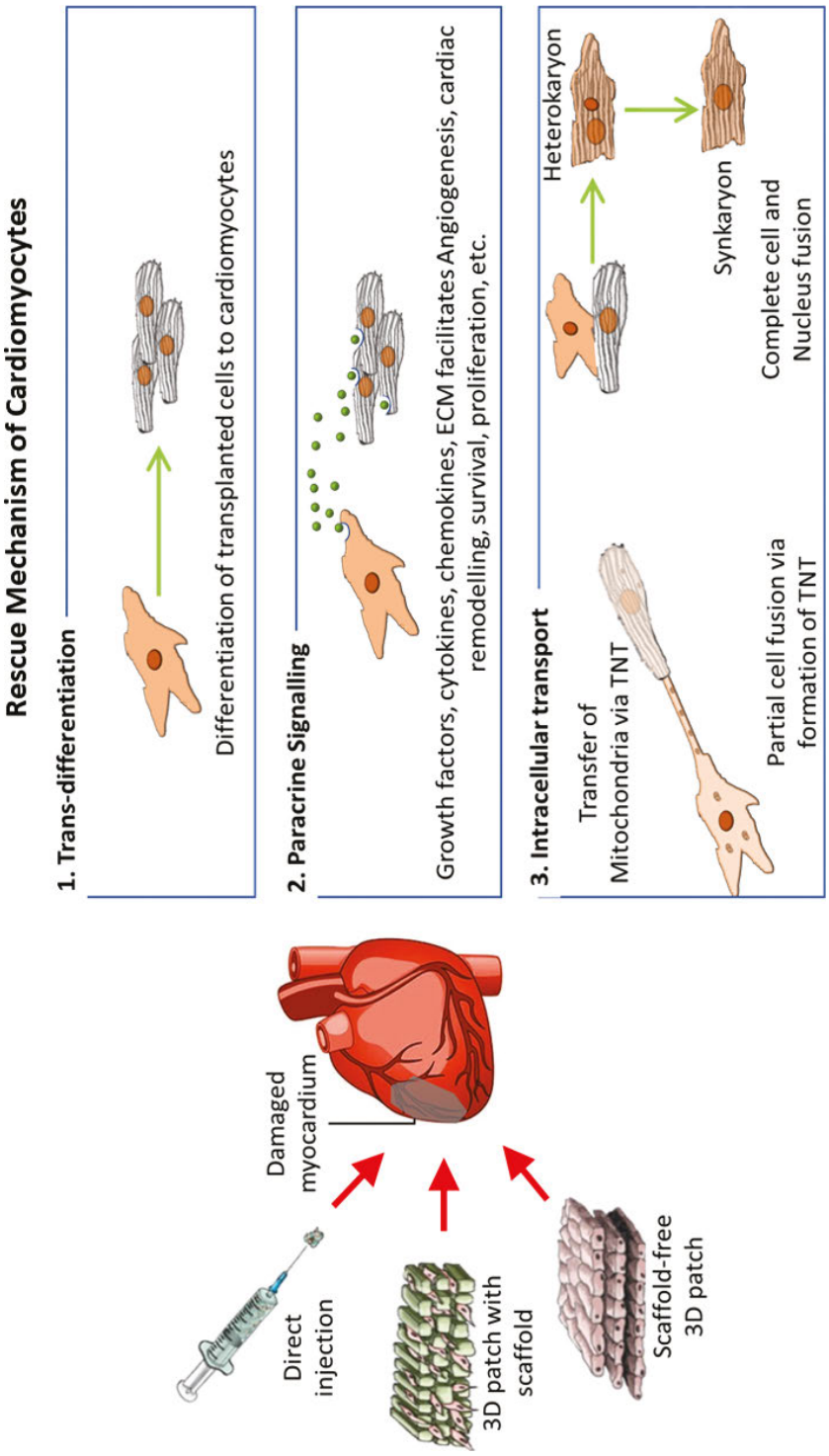

Fig. 8.1: MSC-mediated rescue mechanisms of hypoxic cardiomyocytes.

the cells [30]. Angiogenesis and arteriogenesis are considered as the principal mechanisms to regenerate damaged myocardium post-IHD. MSCs are shown to secret a high level of proangiogenic and proarteriogenic factors like angiopoietins, VEGF, bFGF, and HGF-1, which are associated with neovascularization. Markel and colleagues have shown the significant role of VEGF as MSC-mediated effects in injured rat myocardium [31]. Several studies in rodent models of permanent occlusion showed the impact of MSC implantation on increasing capillary density [32]. Lately, Zhou *et al.* have shown cardiac function improvement following increased vascularity in the porcine model of chronic ischemia subsequent to autologous MSC transplantation [15].

8.3.3 Mitochondrial transfer from MSCs to hypoxic cardiomyocytes

Mitochondrial transfer from MSCs to the damaged cardiomyocytes has been studied widely and shown to rescue cardiomyocytes and improve cardiac functionality [33]. A few studies have stated mitochondrial development, maturation, and expansion in size and shape as well as an increase in ATP production during early differentiation of stem cells into cardiomyocytes [10]. Recent evidence suggests that rescue of cardiomyocytes could be efficient using cardiomyogenic differentiation of MSCs with mature mitochondria and favorable bioenergetics as compared to undifferentiated MSCs [10].

8.3.3.1 Mitochondrial transfer via TNTs

TNTs are formed by connecting two cells via an extension of the plasma membrane and cytoplasm that allows transfer of cellular component and organelles, especially the mitochondria between donor and recipients cells. Usually, TNTs are 5–100 μm long and 50–1500 nm in diameter, and it is continuous between the membranes of the connected cells irrespective to their distance. TNT formation is myocyte reaction to stress. Therefore, mitochondrial exchange through these tunnels can be one of the mechanisms of bioenergetics and survival improvement in stem cell therapy [34]. *In vitro* and *in vivo* studies demonstrated the formation of TNTs between cardiomyogenic differentiated and undifferentiated MSCs and cardiomyocytes for transportation of mitochondria [10]. Studies on ischemic/reperfusion models [35], inflammation, or oxidative stress have also confirmed mitochondrial transfer via TNTs. Consequently, the transfer of mitochondria has restored aerobic respiration and ATP production in the cardiomyocytes. It has been reported that mitochondrial transfer is unidirectional: from MSCs to the cardiomyocytes, regardless of the direction of tunnel and source of its membrane. Although the mechanism is not fully understood, evidence from several studies suggested that the signals originated from recipient cells to initiate the mitochondrial transfer [36].

8.3.3.2 Mitochondrial transfer via permanent cell fusion

Permanent cell-to-cell fusion happens by joining plasma membranes of neighbor cells and forming binucleated heterokaryons. In some cases, fusion has been observed between the nucleus and forming mononucleated hyperploid synkaryons. Permanent cell fusion between MSCs and ischemic cardiomyocytes allows the transfer of mitochondria and phenotypic traits that ultimately improve the biogenesis, proliferation, and survival of the injured myocytes [37]. Permanent cell fusion in the cardiac tissue has been distinguished after implantation of MSCs in different settings of myocardial infarction [38] and cardiomyopathy [39].

8.3.4 MSC secretome-mediated cell repair

Secretomes are proteins derived from secretions of MSCs during culture. The secretome contains a broad range of bioactive soluble factors with antiapoptotic, antifibrotic, angiogenetic, chemoattractive, and immunomodulation properties. It also contains proinflammatory factors such as interferon-γ and tumor necrosis factor α [40]. The balance between pro- and anti-inflammation is crucial for tissue repair [41]. Hence, the various therapeutic benefits of MSC secretome have been intensely investigated for the past two decades. It was found that variations in the MSC culture condition could affect the composition of the secretome; thus, the secretome of MSCs can be modified to a certain extent by modulating the culture conditions.

One of the modulated conditions of the MSC culture is oxygen concentration as oxygen concentration may be modified according to the physiological niche in the human body. Although MSCs are cultured at 21% oxygen concentration in static cultures, many groups have studied the effect of hypoxia on MSCs as most of the tissues in human body have an oxygen concentration less than 10% and the cells tend to have an oxygen concentration of 1.3–2.5% only.

8.3.4.1 Preconditioning of MSCs in hypoxic culture

MSCs cultured under hypoxic conditions proliferate faster, produce a higher number of cells, had more population doublings before senescence, and remain longer in an undifferentiated and multipotent state [42–46]. Thus, many studies compared the conditioned medium of MSCs cultured in hypoxic condition with that of normoxic condition, in terms of their content and effect on alleviating various diseases. The level of growth factors, cytokines, and chemokines such as HGF and VEGF, bFGF, placental growth factor, VEGF-A, TGF-b2, TGF-b3, IGF-1, HGF, interleukin(IL)-1β, IL-6, and IL-8 increases significantly in the secretome of MSCs cultured in hypoxic condition [47–51]. Hypoxic MSC secretome also contains a higher level of angiogenesis promoting factor such as monocyte chemoattractant protein-1 and angiogenin [52].

MSCs treated with secretome collected from MSCs cultured in hypoxic condition were found to enhance adhesion, spreadability, and migration of MSCs *in vitro*.

8.3.4.2 Secretomes from hypoxic MSC for hypoxic cell rescue

Hypoxic MSC secretome was found to enhance vessel formation and wound closure, which are important in wound healing [47, 53]. Studies also showed that hypoxic MSC secretome significantly increased the proliferation of skin cells and human umbilical vein endothelial cells (HUVECs) and migration of skin cells, HUVECs, and monocytes *in vitro*, relative to normoxic MSC secretome. The *in vivo* wound healing was promoted by the proliferation, neovascularization, and mobilization of inflammatory macrophages and the decrease in collagen I and III [47].

MSC secretome attenuates traumatic brain injury in rats, and the secretome of MSCs cultured in hypoxic condition performed significantly better than that of secretome of MSCs in normoxic condition in terms of enhanced rate of generation of neurons by increased secretion of VEGF and HGF [49]. The conditioned medium from MSCs incubated under hypoxia was more effective as compared to the conditioned medium from MSCs incubated under normoxic conditions in rescuing the hypoxic human aortic endothelial cells (HAECs) by inhibiting apoptosis, increasing cell survival, and enhancing tube formation [52]. It was postulated that the effects of conditioned medium from hypoxic MSCs on HAECs were mainly facilitated through the activation of the PI3K-Akt by synergistic action of MSCs secretory proteins.

Gnecchi *et al.* (2005) had initially found that intramyocardial injection of MSCs overexpressed prosurvival gene Akt and greatly enhanced the regeneration of damaged tissue [54]. They further studied whether the effect was due to the engraftment or paracrine effect of the implanted cells and they concluded that it was predominantly due to the paracrine effect as tissue regeneration took less than 72 hours [54]. Furthermore, the hypoxia cultured cells produced a significantly higher amount of VEGF, FGF-2, HGF, IGF-I, and thymosin 4 (TB4) as compared to normoxia cultured cells. It was found that conditioned medium from hypoxic Akt-modified MSCs protected cardiomyocytes exposed to hypoxia *in vitro* by inhibition of apoptosis and induced spontaneous contraction of the cells. Injection of the conditioned medium into infarcted heart was found to reduce infarct size and improve the ventricular function [55].

Overall, secretome collected from MSCs cultured in the hypoxic condition was found to contain more growth factors, cytokines, and chemokines, which were proven to be responsible for the beneficial effects of the conditioned medium. The conditioned medium derived from hypoxic MSCs was found to promote proliferation, survival, and migration at cellular level and also rescue tissues from injuries such as in myocardial infarction, liver injury, and radiation injury. The effectiveness of conditioned medium from MSCs cultured in hypoxia further supports the applicability of conditioned medium for treatment due to advantages such as minimal invasiveness, efficiency, and cost-effectiveness.

8.3.5 MSC exosome-mediated cell rescue

The constituents of the cell secretome include free nucleic acids, soluble proteins, lipids, and extracellular vesicles, i.e., microvesicles and exosomes besides the plethora of bioactive molecules. Exosomes are extracellular vesicles (approximately 40–100 nm) secreted by cells as a means of interaction with the surrounding cells. Exosomes are formed through several cellular processes, starting from the formation of intraluminal vesicles (endocytic pathway) in multivesicular bodies to the transport and fusion of the multivesicular bodies with the plasma membrane and release through exocytosis [56].

Exosomes contain abundant bioactive molecules, including proteins, lipids, RNAs, and miRNAs [57–59]. The therapeutic effect of exosomes is manifested by the bioactive molecules it carries, especially the miRNAs. Several mechanisms have been proposed for the internalization of exosomes by cells, including direct fusion with plasma membrane, phagocytosis, micropinocytosis, as well as receptor- and Raft-mediated endocytosis [60]. After internalization, exosomes will release its content to modulate cell biological activities. In addition, the surface markers on exosomes can also immunomodulate and affect cellular uptake [61, 62].

MSC-derived exosomes have been found to ameliorate ischemia-reperfusion injury of cardiac tissue. Prolonged cardiac ischemia is detrimental as the reduced ATP production by mitochondria through oxidative phosphorylation and the increased ATP production via the ineffective anaerobic glycolysis will disrupt the normal cellular biological activities and eventually lead to cell apoptosis. Reperfusion of ischemic cardiac tissue will exacerbate the injury due to the production of reactive oxygen species [63]. At the same time, the proapoptotic proteins will accumulate in the reperfused cells. MSC-derived exosomes have been reported to protect reperfused cells by increasing ATP production, reducing oxidative stress, and decreasing cell apoptosis [64]. MSC-derived exosomes contain all five enzymes in the ATP-generating stage of glycolysis, i.e., glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phospohoglycomutase, enolase, and pyruvate kinase m2 isoform, and PFKFB3, which regulate the glycolysis [65]. Transfer of these enzymes from exosomes helps to replenish the depleted enzymes within the reperfused cells and increase ATP production, thus alleviating the ATP deficiency within these cells. It has been reported that exosomes are rich in peroxiredoxins and glutathione S-transferases that reduce oxidative stress [64]. In the same study, the authors found that exosomes activate the PI3K/Akt pathway to improve cell survival and suppress cell death. One of the mechanisms of PI3K/Akt pathway activation by exosomes is through the conversion of extracellular AMP to adenosine by the CD73, an ecto-5'-nucleotidase, present on the exosomal membrane surface [65]. Adenosine is an activator that triggers ERK and Akt phosphorylation.

During the regeneration of cardiac tissue subsequent to hypoxia-reperfusion injury, MSC-derived exosomes reduce cardiac fibrosis and inflammation and promote

angiogenesis, thus improving the cardiac function [66–68]. Shao and colleagues (2017) reported that MSCs and MSC-derived exosomes are equally effective in repairing the ischemic heart [68]. Preconditioning of MSCs may have a big impact on the therapeutic potential of their derivative exosomes in treating hypoxia-reperfusion injured cardiac tissue. For example, ischemic preconditioning of bone-marrow-derived MSCs increase the miR-22 load in the exosomes, and injection of these exosomes significantly reduces fibrosis of the infarcted heart [69]. Apart from preconditioning, gene modification is also used for overexpression of miRNA in the cells to enhance the therapeutic potential of their derivative exosomes. Luo and colleagues (2017) transfected adipose tissue-derived MSCs with miR-126 and found that miR-126 enriched exosomes reduce apoptosis, inflammation, and fibrosis as well as improve angiogenesis in *in vitro* and *in vivo* models of myocardium ischemia [70]. In another study, researchers transfected bone-marrow-derived MSCs with GATA-4 to enhance the expression of antiapoptotic miRNA, including miR-19a, and found that the transplantation of exosomes produced by MSCs overexpressing GATA-4 promotes cardiomyocyte survival in an *in vitro* hypoxic model [71].

Damania and colleagues (2018) used a rodent model to study the effect of MSC-derived exosomes in treating liver with hypoxia-reperfusion injury and found that the healing is faster when the animals were treated with exosomes [72]. Results from an *in vitro* study suggested that this might be due to the antiapoptotic and antioxidative properties of the exosomes. For other tissues, MSC-derived exosomes have been demonstrated to promote the healing of ischemia-reperfusion injured kidney and nerve [73, 74]. Shen and colleagues (2016) reported that MSC-derived exosomes express C-C motif chemokine receptor 2 (CCR2) on the membrane surface and this receptor reduces the free CCL2 [73]. By doing so, it reduces the recruitment and activation of macrophage, thus protecting the kidneys with hypoxia-reperfusion injury via suppression of inflammation. Knockdown of CCR2 expression on the exosome membrane diminished the protective effect of exosomes toward the kidney with ischemia-reperfusion injury. Jiang and colleagues (2018) compared MSC-derived exosomes and exosomes overexpressing miR-30d-5p for the treatment of acute ischemic brain injury [74]. The results showed that exosomes overexpressing miR-30d-5p are more effective in suppressing nervous tissue inflammation by reversing the oxygen- and glucose-deprivation-induced and autophagy-mediated M1 macrophage/microglia conversion.

MSC-derived exosomes have also shown promise in protecting and promoting the regeneration of hypoxic cells. The mechanisms of action involved therein include reducing cell apoptosis, inflammation, oxidative stress, and fibrosis while increasing ATP production, cell survival, and angiogenesis. Nonetheless, the bioactive molecules and pathways involved have yet to be elucidated in detail. The finding of the bioactive molecules and pathways involved is very important as it will provide guidance to determine the right modification to the cells or the culture environment to produce exosomes with better therapeutic potential against the hypoxia-reperfusion injury.

8.4 Delivery of MSCs & their secreted products using biomaterials

Majors hurdles in cell transplantation include poor engraftment efficiency and cell retention at the site of injury [3]. Even though differentiation of MSCs is known as a major factor in the therapeutic mechanism of action in early paradigm, current findings have shown that the secreted products include bioactive molecules and extracellular vesicles as the major player [2]. The advantage of cell transplantation is the continuous release of secreted factors under appropriate conditions *in vivo*. Both bioactive molecules and extracellular vesicles have a local and distant effect for the different applications [4]. The efficient delivery of MSCs and their derivatives to the site of damage and its sustained effect are crucial for maximizing their therapeutic potential. Therefore, the use of biomaterial technology as a carrier for the MSCs and secreted products to deliver efficiently at the targeted site has been studied widely to ensure its delivery effectiveness and survival to defect sites. Hence, a range of biomaterials has been explored as temporary containment system and functional three-dimensional (3D) microenvironment for tissue repair used in preclinical and clinical applications [75].

The common 3D design of the biomaterials that have been used include hydrogels, microspheres, sponges, nanofibers, or thin film, which basically depends on the tissue-specific applications. It has been reported that the hydrogels and stem cells could be beneficial in enhancing cell transplantation *via in situ* polymerization for ischemia stroke model [76]. Another study suggests that MSCs in 3D culture, i.e., cultured with a microsphere or microcarrier, stimulate higher levels of anti-inflammatory and antiapoptotic proteins such as STC-1, CXCR4, and anticancer proteins such as TRAIL, IL-24, and CD82 as compared to a monolayer culture, which is of interest for cell therapy [77].

Different designs of 3D biomaterials have been applied from previous studies by using a combination of natural materials together with MSCs. This combination provides better tissue repair and wound healing by enhancing better cell delivery, migration, survival, homing effect, immunomodulation, and angiogenesis [78]. Current clinical studies have revealed the use of hyaluronic acid solution and collagen membrane as MSC carriers to the damaged site. The study performed by Gupta *et al.* demonstrated a better therapeutic effect on osteoarthritis by the injection of hyaluronic acid together with bone-marrow-derived MSCs [79]. In another study that aimed for the treatment of osteoarthritis, a similar approach was used but with a different cell type (synovial MSCs) and biomaterial (collagen membrane) to secure therapeutic benefits postimplantation [80]. The progressive pain reduction and cartilage regeneration from the preclinical model, together with its safety profile from human observation, shows the effectiveness of the treatment approach. Additionally, prolonged benefit from clinical study on osteoarthritis has been explored by Park *et al.* (2017), who demonstrated better safety evaluation of injections of MSCs derived from human umbilical cord blood mixed with hyaluronic acid hydrogel after

7 years of treatment [81]. The study reported no case of tumorigenicity and osteogenesis during the follow-up.

MSC-derived exosomes contain miRNA, various genetic products, proteins, and other essential factors that can elicit various therapeutic benefits. In addition, exosome provides a novel, safe biological regiment for tissue repair and regeneration, which involves immune rejection, tumorigenicity, embolism, and infection transmission [82]. The first results of MSC exosomes were reported in 2010 during the treatment of experimental myocardial ischemia-reperfusion injury [83]. The finding of MSC exosome eventually opened a new era for the development of acellular therapeutic interventions for various diseases such as cancer, ischemia, rheumatoid arthritis, osteoarthritis, inflammatory, bone fractures, and other maladies [84]. In addition, the use of exosomes could potentially produce an off-the-shelf product for immediate treatment in acute conditions such as traumatic injury and ischemic disease. However, several critical issues should be considered prior to clinical use, including the long-term safety evaluation of exosome postadministration, disease specificity, biodistribution, and persistency of its biological effect [85].

Currently, two clinical trials using MSC exosomes have been registered under the US National Library of Medicine Clinical Trials for different targeted applications: ischemic stroke (trial phase I/II) and type I diabetes mellitus (trial phase II/III) [86]. Moreover, autologous dendritic cell-derived exosomes loaded with melanoma associated antigen peptides are being used in a phase II non-small cell lung cancer trial (clinicaltrials.gov/NCT01159288) [87]. Most of the MSC exosomes are still undergoing proof-of-concept in *in vivo* models for various applications such as retinal ischemia [88], articular cartilage defect [89], and bone defect [90]. Retinal ischemia is a critical vision loss or impairment caused by several factors, including diabetic retinopathy, glaucoma, and retinal artery occlusion. Therefore, a study by Mathew *et al.* (2019) demonstrated the administration of MSC-derived exosomes in a biomaterial to evaluate its neuroprotective and regenerative properties for the treatment of retinal impairment. A positive outcome has been reported from the study, which significantly demonstrated an increment in functional recovery and decreased neuroinflammation and apoptosis in the rat model [88]. The MSC-derived exosomes also provide a better outcome in the regeneration of articular cartilage even though they show an innate healing capability that is a great challenge in their clinical application. However, a previous study has unraveled that the combination of MSC exosomes with photo-induced imine cross-linking hydrogel glue as acellular tissue patch successfully demonstrated excellent performance ability and good biocompatibility and promoted cartilage integration [91]. To date, many cases of bone defect due to traumatic injury and other factors have a prolonged healing process. The intervention of MSC exosomes, combined with biomaterials, increases bone regeneration capability and its osteo-integration. Recently, the exosome-integrated titanium nanotube has been developed as a functional material for bone regeneration [90]. The study showed an

improvement in the bio-functionality of the nanotube material and demonstrated better osteogenesis.

In addition, an improvement of MSC exosome is widely explored to ensure the effectiveness of its delivery to the targeted injury site. Recently, a finding has been reported regarding a simple, rapid, and efficient approach to modify surface capability by conjugation of functional ligands using bio-orthogonal copper-free azide-alkyne cycloaddition for cerebral ischemia treatment [92]. The result showed better accumulation of exosomes at the targeted site as compared to nonmodified exosomes. In another study, exosomes were modified with polyethylene glycol and AA (ligand), which improved their circulation time in the blood flow and finally allowed them to target pulmonary metastases [93]. In addition, the concoction enhanced the specific drug delivery to target cancer cells and prolonged the survival rate of lung cancer patients. Current conventional vehicles for drug delivery, including liposomes, micelles, and dendrimers, are inefficient in the controlled drug release properties. Thus, an innovated technology using metal-organic frameworks is being used in nanomedicine with intrinsic biodegradability and high loading capacity [94]. In conclusion, the introduction of biomaterials in MSC exosome delivery provides better exosome stability, controlled site-specific delivery site, and increased accumulation capability that finally expedites the tissue repair and regeneration.

8.5 Clinical trials using MSCs or their derivatives for treating IHD and critical limb ischemia

IHD and critical limb ischemia (CLI) are consequences of atherosclerosis. Atherosclerosis occurs when plaque builds up in the blood vessel, thus restricting blood flow and reducing oxygen transfer to the heart and limb muscle. These, in turn, create a hypoxic condition for cells locating subsequent to the area of the plaque. Most MSC clinical applications initially were on the basis of MSC multipotency. Therefore, MSCs were hypothesized to mediate cardiac regeneration through the replacement of necrotic myocardium in myocardial infarction with MSC-differentiated cardiomyocytes. This hypothesis leads to the delivery of MSC to the site of infarct through several delivery approaches, including intracoronary [95, 96], intramyocardial [97, 98], and intravenous [99]. Even though MSC's ability to differentiate to cardiomyocytes has been proven *in vitro* [10, 100, 101, 102], its delivery *in vivo* shows limited engraftment especially in animal models [20, 103, 104]. A completed randomized placebo-controlled clinical trial, intramyocardial injection of autologous MSC in the PROMETHEUS trial, showed increased left ventricular ejection fraction (LVEF) and decreased scarring [105]. The Transendocardial Autologous Cells (hMSC or hBMC) in Ischemic Heart Failure Trial (TAC-HFT) trial, with 65 patients enrolled, showed improved exercise tolerance and reduction in infarct size after a year of MSC injection [106]. Presently, there are about 25 ongoing phase II, II, and III clinical trials registered with the clinicaltrials.gov

with a range of delivery methods (either intracoronary, intramyocardial, or intravenous) with sample sizes of up to 600 patients [107].

Likewise, for CLI, clinical trials with MSC injection show no difference in amputation rate but improved ulcer healing, ankle-brachial index (ABI), and transcutaneous oxygen pressure (TcPO2) [108–110]. Remarkably, a significant increase in collateral vessel score was observed in MSC-injected CLI patients as compared to a bone marrow mononuclear cell-injected group, thus demonstrating the role of MSCs in promoting angiogenesis [108]. Alternatively, targeted delivery of therapeutic growth factors, e.g., VEGF and bFGF, which are produced by MSCs, is proven to reverse ischemia and improve blood perfusion [111]. Unfortunately, these factors have poor stability and short biological activity *in vivo*. Therefore, the ongoing clinical trials are more focused on intramuscular delivery of MSCs, such as the Clinical and Histologic Analysis of Mesenchymal Stromal Cells in Amputations (CHAMP) trial [112], Allogeneic Mesenchymal Stromal Cells for Angiogenesis and Neovascularization in No-option Ischemic Limbs (SAIL) trial [113], and MarrowStim Pad Kit for the Treatment of CLI in Subjects With Severe Peripheral Arterial Disease (MOBILE) trial [114], to test the efficacy of *in vitro* expanded MSC in treating CLI.

Generally, MSC delivery does improve cardiac and limb function *in vivo*, but not exclusively, through replacement of the injured cell. Paracrine effects and mechanisms are more likely to play a major role in improving the IHD and CLI clinical trials outcome [107,115]. These paracrine effects were studied with MSC conditioned media that are rich with growth factors, which include IL-1 [116] and IL-6 [117], SDF-1 [118, 119], and a few others that have potential therapeutic value. Ther therapeutic value of these factors' involvement in immunomodulation is via suppression of inflammatory cells, angiogenesis, and positive remodeling besides tissue preservation [120].

The development of scaffold-based cell delivery such as cardiac patch and cell-sheet was first devised to achieve better targeted cell delivery and engraftment while providing additional structural support to the infarcted area [121, 122]. Engineered cardiac patch sutured on the infarcted myocardium allowed a 10-fold increase in engraftment rate when compared to direct cell injection to the myocardium [122]. MSCs were delivered in an animal model in the form of cardiac-patch [123, 124] and cell-sheet [125, 126] because of the beneficial 3D structure that allows better survival of MSCs while promoting paracrine mechanism and recruiting endogenous host stem cells for repair. Transplantation of autologous skeletal myoblast sheets into chronic IHD patients showed improved LVEF and New York Heart Association functional class [127].

Similarly, for CLI therapy, the problem of cell survival and engraftment *in vivo* was addressed by scaffold development. The choice of scaffold material ranged from natural autologous, allogenic, or xenogeneic decellularized blood vessels to woven, electrospun, or 3D printed synthetic scaffold [128, 129]. These scaffolds showed a promising approach for the delivery of MSCs to improve cell survival and increase cell engraftment, thus allowing longer pro-regenerative factors' exposure to the ischemic region.

8.6 References

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