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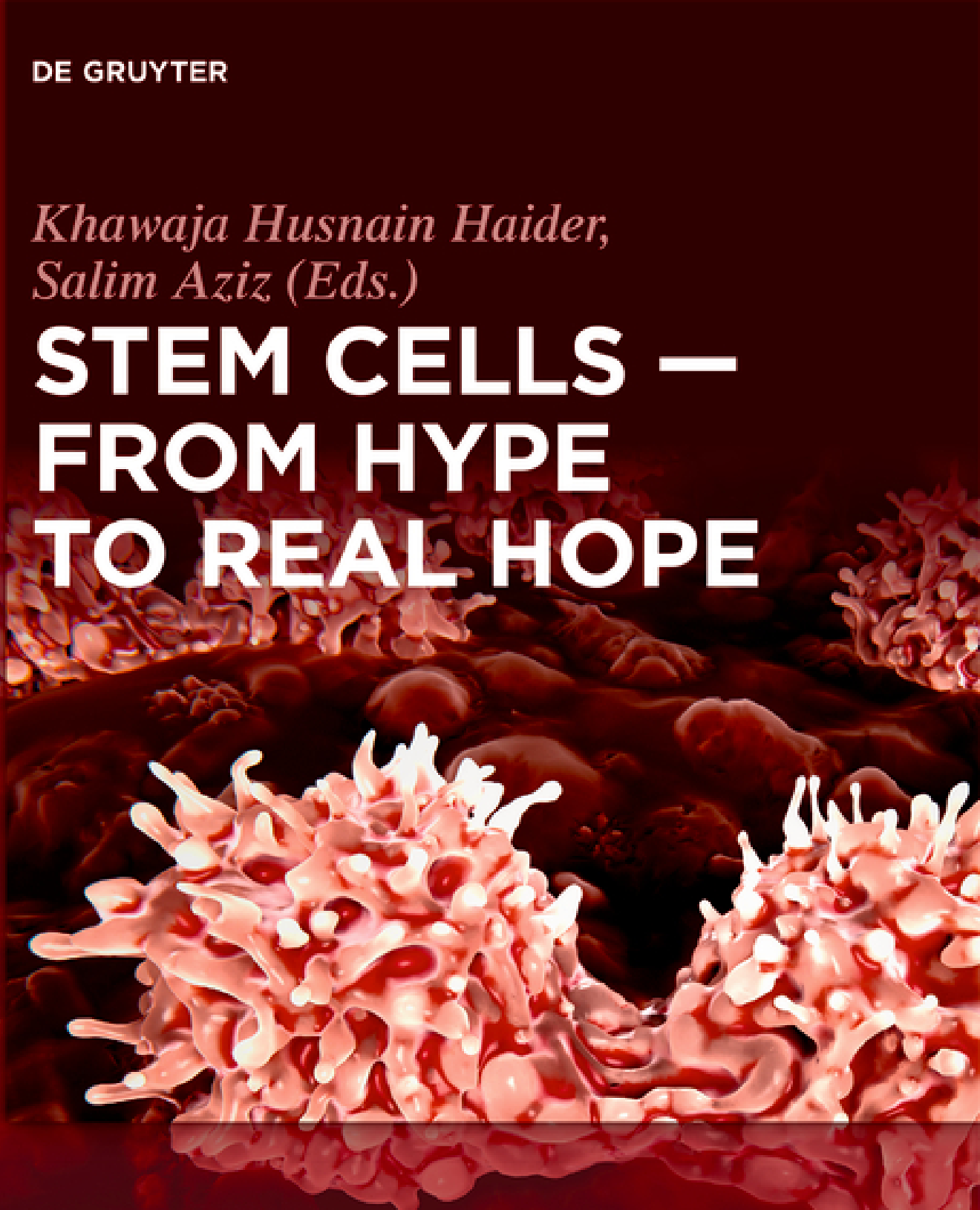
Khawaja Husnain Haider, Salim Aziz (Eds.)

STEM CELLS – FROM HYPE TO REAL HOPE



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Many of my loved ones deserve this dedication, i.e., my parents who groomed me to what I am, my brothers each one of whom I cherish as my role model, my sisters who love me more than myself, my son Mowahid and wife who were there with me in the most trying moments in my life. However, I dedicate this book to my little “Angle of Paradise” whose departure from my life continuously inspires me to contribute to science.

Preface

The fields of stem cells and regenerative medicine are in the process of rapid transformation thus bringing much more hope than hype for the patients. Although it would be a daunting task to cover all aspects of the fields, in this edition the editors have sought to get experts from around the world to cover a number of topics encompassing from the use of stem cells as *in vitro* platform to conduct early potential therapy testing to their various applications in regenerative medicine.

The book is a compilation of topics of interest for the audience who aspire to discover more about the multifaceted applications of this promising approach to treat/cure diseases. Chapter-1 describes the advent of induced pluripotent stem cells (iPSCs) derived cardiomyocyte platforms to perform early phase diagnostic and therapy assessment in patient specific manner. While chapter compares different platforms and charters pathway for improving iPSC derived cardiomyocyte engineered hearty tissues for patient specific theranostics, chapter-2 provides an overview of large experimental animal models for translational assessment of cell-based therapies with a focus on the hurdles that need to be overcome for their clinical use. Chapter-3 provides an in-depth overview of the newly emerging concept of cell-free therapy based on the paracrine activity of stem cells. The authors have also provided mechanistic insight into how the cytokine and growth factor rich conditioned medium from stem cells can be beneficially support the intrinsic repair process. Chapter-4 describes the multifarious applications of skeletal muscle stem cells. Headed by a leading expert in myoblast therapy, the group of authors describes the clinical significance of myoblast implantation with focus on muscle regeneration in muscular dystrophy patients, ischemic cardiomyopathy and for the treatment of diabetes and cancer. Chapters-5 and -6 describe the novel emerging use of stem cells in ophthalmology, an area wherein safety and feasibility of stem cells has already assessed in the clinical settings. Chapter-7 addresses the conventional therapies used to treat prevalent liver diseases along with their limitations and further discusses the potential of various cell types such as hepatocytes, hepatic progenitor cells, mesenchymal stem cells, and embryonic stem cells for liver regeneration. It also reviews the studies related to liver bioengineering and scaffolds assisted liver regeneration. Chapter-8 highlights the importance of patient specific iPSC-derived cardiomyocytes as *in vitro* platforms to study channelopathies and cardiac arrhythmias and their potential application in the clinical perspective. The book is concluded with an interesting hypothesis regarding the role of stem cells in evolutionary process.

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Contents

Preface —— vii
Contributing authors —— xv
List of abbreviations —— xix

Valeria A. Tsvelaya, Anna Gam, Jenna Aziz and Igor R. Efimov
1 Induced pluripotent stem-cell-derived cardiomyocytes (iPSC-CMs): novel diagnostic platform —— 1
1.1 Rationale for the emerging need for iPSC-CMs —— **1**
1.2 Cardiogenesis and iPSC differentiation —— **2**
1.3 Reprogramming limitations and improvements —— **4**
1.4 Disease modeling platform —— **7**
1.5 Drug testing platform —— **9**
1.6 Transplantation of *in vitro* derived CMs into injured areas —— **11**
1.7 Conclusion and future directions —— **13**
1.8 References —— **13**

Keron Navarengom, Elisa A. Ferrante, Todd E. Rasmussen and Manfred Boehm
2 Preclinical large-animal models of cardiovascular regeneration —— 20
2.1 Introduction —— **20**
2.2 Regenerative strategies for HF —— **21**
2.3 Cardiac regeneration using PSCs —— **22**
2.4 Translational models of HF —— **25**
2.5 Large animal models —— **26**
2.5.1 Dilated cardiomyopathy models —— **26**
2.5.2 Surgical induction of myocardial ischemia or infarction —— **28**
2.6 Assessment of animal models —— **29**
2.7 Conclusions —— **29**
2.8 References —— **29**

Khawaja Husnain Haider and Muhammad Aslam
3 Cell-free therapy with stem cell secretions: protection, repair, and regeneration of the injured myocardium —— 34
3.1 Introduction —— **34**
3.2 The secretome of each stem cell type is unique —— **35**
3.2.1 Paracrine factors and angiogenesis —— **39**
3.2.2 Paracrine factors and cytoprotection —— **40**
3.2.3 Paracrine factors and immunomodulation —— **42**
3.2.4 Paracrine factors and cell migration and homing —— **44**

3.2.5 Paracrine factors and antifibrosis — **46**
3.3 Tempering the secretome composition with outside intervention — **48**
3.3.1 Genetic modification of stem cells — **48**
3.3.2 Chemical and pharmacological manipulation of cells — **51**
3.3.3 Physical manipulation of cells — **53**
3.3.4 Heat shock and shock wave treatment to precondition stem cells — **55**
3.3.5 Modification of the ECM — **56**
3.4 Conclusion and future perspective — **57**
3.5 References — **58**

Peter K. Law, Danlin M. Law, Lei Ye, Khawaja Husnain Haider, Shi Jun Song, Ping Lu, Jian-Hua Ma and Jun Ren

4 Myoblasts provide safe and effective treatments for hereditary muscular dystrophies, cardiomyopathies, type 2 diabetes, solid tumors, and aging — 71
4.1 Introduction — **72**
4.2 Gene therapy in the United States — **72**
4.3 What constitute genetic diseases? — **73**
4.4 Two kinds of gene therapies — **73**
4.4.1 Single gene transduction — **73**
4.4.2 Single gene transduction deficiencies — **75**
4.4.3 How does single gene transduction fare? — **76**
4.5 Human genome therapy — **76**
4.5.1 Myoblasts are not stem cells — **77**
4.5.2 World's first human gene therapy — **78**
4.5.3 HMGT/MTT corrects gene defects in DMD clinical trials — **78**
4.5.4 HMGT/MTT versus Exondys 51 — **80**
4.5.5 HMGT/MTT in heart disease clinical trials — **81**
4.5.6 Autonomous cell injection catheter — **83**
4.5.7 HMGT/MTT corrects gene defects in type 2 diabetes — **84**
4.5.7.1 Human study — **84**
4.5.7.2 Animal studies — **85**
4.5.8 Myoblast implantation inhibits metastasis and tumor growth and induces cancer apoptosis — **87**
4.5.9 Anti-aging aesthetica — **88**
4.5.10 Skin cover — **89**
4.5.11 Body sculpture — **91**
4.5.12 Anti-aging angiomyogenesis — **92**
4.6 Conclusion — **92**
4.7 References — **93**

S. Amer Riazuddin, Shahid Y. Khan, Muhammad Ali and John D. Gottsch
5 Stem cells in ophthalmology — 98
5.1 Introduction — **98**
5.2 Cornea — **99**
5.2.1 Corneal epithelium — **99**
5.2.2 Corneal stroma — **100**
5.2.3 Corneal stromal stem cells — **100**
5.2.4 Corneal endothelium — **101**
5.2.5 Corneal endothelial stem cells — **101**
5.2.6 Conjunctiva — **102**
5.2.7 Conjunctival stem cells — **102**
5.3 Iris — **103**
5.3.1 IPE stem cells — **103**
5.4 Ciliary body — **104**
5.4.1 Ciliary epithelial stem cells — **104**
5.5 Trabecular meshwork — **104**
5.5.1 TM stem cells — **104**
5.6 Lens — **105**
5.6.1 Lens stem cells — **105**
5.7 Retina — **106**
5.7.1 Retinal stem cells — **106**
5.7.2 Retinal pigment epithelial stem cells — **106**
5.7.3 Retinal Müller stem cells — **106**
5.8 References — **107**

S. Amer Riazuddin, Shahid Y. Khan, Muhammad Ali and John D. Gottsch
6 Stem cells for ocular therapies — 116
6.1 Introduction — **116**
6.2 Cornea — **117**
6.2.1 Stem cell-based therapies for corneal epithelium — **117**
6.2.2 Therapeutic applications of stem cells to corneal stroma — **118**
6.2.3 Stem cells and corneal endothelium treatment — **118**
6.3 TM and stem cell therapy — **119**
6.4 Ocular lens and stem cell treatment — **120**
6.5 Stem cell-based retinal therapy — **120**
6.6 References — **123**

Sheikh Riazuddin, Maria Tayyab Baig and Azra Mehmood
7 Cell therapy for liver regeneration — 130
7.1 Introduction — **130**
7.1.1 Worldwide prevalence of liver diseases — **130**
7.2 Structure and function of the liver — **131**

7.3 Liver diseases — **131**
7.3.1 Progression of liver fibrosis to liver cirrhosis — **131**
7.3.2 Liver failure — **132**
7.3.3 Liver failure causes malfunctioning of other organs — **132**
7.4 Conventional therapies for liver diseases — **132**
7.5 Cellular therapies for liver diseases — **132**
7.6 Stem cell therapy for liver diseases — **133**
7.6.1 Liver stem/progenitor cells — **133**
7.6.2 MSCs in the therapy of liver diseases — **134**
7.6.2.1 Bone marrow-derived MSCs — **134**
7.6.2.2 Adipose (tissue)-derived MSCs — **137**
7.6.2.3 Umbilical-cord-tissue-derived MSCs — **138**
7.6.2.4 Umbilical cord blood stem cells — **138**
7.6.3 A comparison of the various sources of MSCs — **138**
7.6.4 Clinical promises of MSCs — **139**
7.6.5 Induced pluripotent stem cells — **139**
7.7 Liver bioengineering/scaffold-assisted liver regeneration — **140**
7.8 Future prospects of cellular therapies for the repair of damaged livers — **141**
7.9 References — **141**

Esra Cagavi, Tuba Akgul Caglar, Khawaja Husnain Haider and Gokce Ilayda Soztekin

8 Patient-specific induced pluripotent stem cells for cardiac disease modeling — 146
8.1 Introduction — **146**
8.2 Methods for electrophysiological analysis — **148**
8.2.1 Electrocardiogram — **149**
8.2.2 Patch clamp analysis — **150**
8.2.3 Multielectrode array (MEA) — **151**
8.2.4 Impedance and field potential (FP) recorders — **151**
8.2.5 Voltage-sensitive and Ca^{2+}-sensitive dyes — **151**
8.3 Channelopathies — **152**
8.3.1 Long QT Syndrome Type-1 (LQTS-1) — **152**
8.3.2 LQTS-2 — **158**
8.3.3 LQTS-3 (Gain-of-Function of Na^{+} Channel) — **161**
8.3.4 Brugada syndrome — **162**
8.3.5 Timothy syndrome (LQTS-8) — **163**
8.3.6 LQTS-14 — **164**
8.3.7 LQTS-15 — **164**
8.4 Catecholaminergic polymorphic ventricular tachycardia — **165**

8.5 Conclusion — **167**
8.6 References — **168**

Newton Howard, Abderrahmane Hamlat, Julie Dubourg, Mahmoud Messerer and Eduardo Pasqualini

9 Role of stem cells on evolution: a hypothesis — 173
9.1 Introduction — **173**
9.2 Stem cells and evolution — **174**
9.3 Evolution stages — **176**
9.4 Evolution and update of genetic database — **176**
9.4.1 Evolution of genetic database — **176**
9.4.2 Role of GSCs in genetic database update — **178**
9.4.3 Memory box and update of genetic database — **179**
9.5 Evolution and human emergence — **179**
9.5.1 Brain evolution — **180**
9.5.2 Differences between human and mammal brains — **181**
9.6 Conclusions — **183**
9.7 References — **183**

Index — 188

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List of abbreviations

AAA= Antiaging aesthetica
ABCG2= ATP-binding cassette transporter G2
Ad-phVEGF$_{165}$= Adenoviral transduced plasmid of vascular endothelial factor 165 (human)
ADA= Adenosine deaminase
ADSCs= Adipose-tissue-derived mesenchymal stem cells
AF= Ankle plantar flexors
AIDS= Autoimmune deficiency syndrome
ALLN= N-[N-(N-acetyl-L-leucyl)-L-leucyl]-L-norleucine
ALT= Aminotransferase
AMD= Age-related macular degeneration
AMSCs= Adipocyte-tissue-derived MSCs
Ang-1= Angiopoietin-1
APD= Action potential duration
ARVC= Arrhythmogenic right ventricular cardiomyopathy
AST= Aspartate aminotransferase
ATCC= American tissue culture collection
bFGF= Basic fibroblast growth factor
BMCs= Bone marrow cells
BMD= Becker muscular dystrophy
BMMNCs= Bone marrow mononuclear cells
BM-MSCs= Bone-marrow-derived mesenchymal stem cells
BMP4= Bone morphogenic protein 4
BrS= Brugada syndrome
CABG= Coronary artery bypass grafting
CACNA1C= L-type cardiac voltage-gated Ca^{2+} channel
CaM= Calmodulin
CASQ2= Calsequestrin 2
Ca_v= Voltage-gated Ca^{2+} channel
CBER= Center for Biologics Evaluation and Research
CCS= Canadian Cardiovascular Society
CD-phVEGF$_{165}$= CD liposome transduced plasmid of vascular endothelial growth factor 165 (human)
CDCs= Cardiosphere-derived cells
CE= Corneal endothelium
CEC= Corneal endothelial cell
CFs= Cardiac fibroblasts
CFR= Code of federal regulation
cGMP= Current good manufacturing practices
CIPA= Comprehensive *in vitro* proarrhythmia assay
CJSCs= Conjunctival stem cells
CK= Creatine kinase
CM= Conditioned medium
CMs= Cardiomyocytes
CMT= Cardiac microtissue
CPE= Ciliary pigment epithelial
CPTV= Catecholaminergic polymorphic ventricular tachycardia
CRISPR= Clustered regularly interspaced short palindromic repeats

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CsA= Cyclosporine A
CSCs= Cardiac stem cells
CTRF= Cell Therapy Research Foundation
CX-43= Connexin-43
Cy= Cyclosporine
DAD= Delayed afterdepolarization
DALYs= Disability-adjusted life years
DCM= Dilated cardiomyopathy
DKK1= Dickkopf 1
DMD= Duchenne muscular dystrophy
DMEM= Dulbecco's Modified Eagle's Medium
DMF= Drug master file
DNA= Deoxyribonucleic acid
EAD= Early afterdepolarization
ECs= Endothelial cells
ECG= Electrocardiogram
ECM= Extracellular matrix
ECR= Extracellular recording
EDB= Extensor digitorum brevis
EHT= Engineered heart tissue
18FDG PET= 18F-fluoro-deoxy-D-glucose
ELISA= Enzyme-linked immunosorbent assay
EMA= European Medicine Agency
EPA= European Patent Agency
EPCs= Endothelial progenitor cells
EpCAM= Epithelial cell adhesion molecule
ESCs= Embryonic stem cells
ESC-CMs= Embryonic stem-cell-derived cardiomyocytes
ESLD= End-stage liver disease
FDA= Food and Drug Administration
FG= Fluoro-gold
FGF= Fibroblast growth factor
FHF= First heart field
FIM= First-in-man
FPD= Field potential duration
FRZP2= Frizzled-related protein 2
G-CSF= Granulocyte colony stimulating factor
GF= Growth factor
GFP= Green fluorescence protein
GLUT4= Glucose transporter-4
GPI= Glucose-6-phosphate isomerase
GSCs= Germ stem cells
HCM= Hypertrophic cardiomyopathy
hCSSCs= Human corneal stromal stem cells
HCT= Heart cell therapy
hERG= Human ether-a-go-go-related gene
hESCs= Human embryonic stem cells
HF= Heart failure
HGF= Hepatocyte growth factor

HIF-1α Hypoxia inducible factor-1α
hiPSC-CMs= Human iPSC-derived CMs
HLCs= Hepatocyte-like cells
HMGT= Human Myoblast Genome Therapy
HNSCC= Head and neck squamous cell carcinoma
HRP= Horseradish peroxidase
HSCs= Hematopoietic stem cells
HSPs= Heat shock proteins
HUVECs= Human umbilical vein endothelial cells
IDO= Indoleamine 2,3-dioxygenase
IGF-1= Insulin-like growth factor-1
I_{Kr}= Rapidly activating delayed rectifier potassium current
I_{Ks}= Slowly activating delayed rectifier potassium current
IL= Interleukin
IND= Investigational New Drug
IOP= Intraocular pressure
IPE= Iris pigment epithelial
iPSCs= Induced pluripotent stem cells
ISO= International Organization for Standardization
KE= Knee extensors
KF= Knee flexors
K_s= Slow rectifier K^+ channel
K_V= Voltage-gated K^+ channel
LAD= Left anterior descending
LBSCs= Limbal biopsy-derived stromal cells
LBT= Lower-body treatment
LCA= Leber congenital amaurosis
LCM= Laser capture microdissection
LECs= Lens epithelial cells
LPL= Lipoprotein lipase
LQTS= Long QT syndrome
LSCs= Limbal stem cells
LSCD= Limbal stem cell deficiency
LSPCs= Liver stem/progenitor cells
LV= Left ventricle
LVEF= Left ventricular ejection fraction
MAGIC= Myoblast autologous grafting in ischemic cardiomyopathy
MAPCs= Multipotent adult progenitor cells
MB= Memory box
MEA= Multielectrode array
mESCs= Mouse embryonic stem cells
MHC= Myosin heavy chain
MHC-I= Major histocompatibility class-I
MI= Myocardial infarction
MIBI-Tc99m= Technetium (99mTc) Sestamibi
mirs= microRNAs
MSCs= Mesenchymal stem cells
MPSCs= Multipotent stem cells
MTF= Muscular thin films

MTT= Myoblast transfer therapy
NIDDM= Non-insulin-dependent diabetes mellitus
Na_v= Voltage-gated Na^+ channel
NMDA= N-methyl-d-aspartate
PBS= Phosphate-buffered saline
PCs= Preconditioned cells
PCT= Patent Cooperation Treaty
PDGF= Platelet-derived growth factor
PDMS= Polydimethylsiloxane
PEI-phVEGF$_{165}$= Polyethylenimine-25 nanoparticle transduced plasmid of vascular endothelial growth factor 165 (human)
PEG= Polyethylene glycol
PET= Positron emission tomography
PlGF= Placental growth factor
PIP_2= Phosphatidylinositol 4,5-biphosphate
PKU= Phenylketonuria
PLGA= Polylactic glycolic acid
PLLA= Poly-L-lactic acid
POAG= Primary open angle glaucoma
POMP= Periocular mesenchymal precursor
PSCs= Pluripotent stem cells
ROCK= Rho kinase
RP= Retinitis pigmentosa
RPE= Retinal pigment epithelial
RT-PCR= Reverse transcription polymerase chain reaction
SAN= Sinoatrial node
SCs= Somatic cells
SCID= Severe combined immunodeficiency disorder
SDF-1α= Stromal-cell-derived factor-1α
SFDA= State Food & Drug Administration
sfrp= Secreted frizzled related protein
SkMs= Skeletal myoblasts
SHF= Second heart field
SMs= Skeletal myoblasts
SMCs= Smooth muscle cells
SMT= Single muscle trial
SOPs=Standard operation procedures
SPECT= Single-photon emission computed tomography
SR= Sarcoplasmic reticulum
TALENs= Transcription activator-like effector nucleases
TdP= Torsades de pointes
TGFβ= Transforming growth factor beta
TGF-1β= Transforming growth factor-1β
3D-MIMs= Three-dimensional multifunctional integumentary membranes
TM= Trabecular meshwork
TS= Timothy syndrome
UBT= Upper body treatment
UCB-MSCs Umbilical cord blood-MSCs
USP= United States Pharmacopeia

USPTO= United States Patent Office
UTM= University of Tennessee Memphis
VEGF= Vascular endothelial growth factor
$VEGF_{165}$= Vascular endothelial growth factor 165 (human)
WBT= Whole body treatment
WJMSCs= Wharton jelly mesenchymal stem cells
WT= Wild type
ZFNs= Zinc finger nucleases

Valeria A. Tsvelaya, Anna Gam, Jenna Aziz and Igor R. Efimov

1 Induced pluripotent stem-cell-derived cardiomyocytes (iPSC-CMs): novel diagnostic platform

Abstract: There is growing concern regarding the clinical relevance of therapy testing in immortalized cell lines and animal models. The advent of induced pluripotent stem cells allows conduction of early diagnostics and potential therapy testing in cell derived from a specific patient retaining his/her genetic background. This is especially important in studies of various heritable cardiomyopathies, which are difficult to recapitulate in animal models. A number of methods have been developed building upon the pioneering work of Yamanaka laboratory, aiming at reprogramming various somatic cells to cardiomyocytes and creating two- and three-dimensional models for investigation of cardiac physiology in a particular genetic background. Conventional models, such as short-lived primary cell preparations, monolayer cell cultures, coronary-perfused hearts and preparations, and, more recently, heart slices have unique advantages and limitations, especially in studies of long-term chronic conditions. The emerging induced pluripotent stem-cell-derived cardiomyocyte (iPSC-CM) platform holds significant potential to overcome these limitations while allowing patient-specific diagnostics and therapy development. This chapter compares conventional models with iPSC-CM, reviews cardiogenesis as the basis for reprogramming protocol development, focuses on the iPSC-CM disease modeling platforms for drug testing and patient specific theranostics, and concludes with clinical studies on transplantation of *in vitro* derived CMs.

Key Words: Cardiomyocytes, Cardiogenesis, Differentiation, Drug discovery, Engineered, Heart, iPSCs, Model.

1.1 Rationale for the emerging need for iPSC-CMs

Cardiomyocytes (CMs) are the major cell type that determines cardiac function. CMs have low proliferation and turnover rates, which make them hard to generate, grow to maturity, and study *in vitro*. In addition to those constraints, there is limited availability of both healthy and diseased human CMs, which makes it hard to construct *in vitro* models of the diseases, for drug testing, and design methods for *in vivo* applications [1, 2]. Other models that can be derived from human donor hearts, such as wedge preparations and cardiac slices, have limited availability and low throughput and often lack the desired genetic background. Animal models have significant species-dependent differences; for example, mouse heart rate is 10 times faster, which results in 5–10 times shorter QT-interval compared with human hearts. Murine CMs

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express species-specific ion channels and structural proteins. Additionally, increased heart rate in mice correlates with decreased contraction force, which is opposite to humans [1, 3]. Similar variability across various species renders animal models not entirely suitable for drug testing since the results do not always correlate with the drug response in humans. Additionally, animal cells and tissues cannot be safely used for implantation into humans due to immunogenic response. These constraints stimulated the research in human stem cells, which in turn led to the development of induced pluripotent stem cells (iPSCs). Pluripotent stem cells (PSCs) can be either embryonic stem cells (ESCs) or iPSCs. The first human iPSC-CMs (hiPSC-CMs) were reported in 2009 by Zhang *et al.* [4, 5]. Here, mainly iPSCs are discussed since the goal of this chapter is to describe the-state-of-art research with a focus on personalized medicine. Table 1.1.1 summarizes the current models used to study cardiac functions and highlights the advantages and limitations of each model.

1.2 Cardiogenesis and iPSC differentiation

In order to differentiate stem cells into CMs, it is necessary to know how this process naturally occurs *in vivo*. To this day, the biggest challenge of the field is the inability to obtain *mature CMs* of a *single phenotype*. Therefore, here, the different stages of cardiogenesis are reviewed in parallel with what has been recapitulated *in vitro* to highlight the missing steps that could improve current reprogramming practices and address the aforementioned challenges.

The heart consists of several cell types, including CMs, fibroblasts, neurons, and vascular cells. CMs separate further into four lineages: atrial, ventricular, Purkinje, and nodal cells, each having unique electrophysiology, mechanics, and structure. Since during iPSC differentiation, it is unclear which CMs lineage is obtained, it is necessary to tune the differentiation protocol for a specific CMs lineage that recapitulates its embryonic developmental process.

Developmentally, cells destined for cardiogenesis arrive from a mesodermal germ layer, which are modified by transcriptional signaling molecules such as wingless/integrated (Wnt), fibroblast growth factor (FGF), transforming growth factor beta (TGFβ), bone morphogenic protein 4 (BMP4), activin, and nodal [6]. The gradients of these molecules either promote or inhibit different pathways based on spatiotemporal and functional cues. Wnt/β-catenin signaling contributes to the formation of cardiac progenitor cells, and its inhibition by adjacent cells through frizzled-related protein 2 (FRZP2) and Dickkopf 1 (DKK1) allows for further differentiation and specification into progenitor cells [6–9]. The heart is formed by contribution from two types of progenitor cells: first heart field (FHF) and second heart field (SHF). The cardiac field is specified by downstream transcription events that lead to the expression of cardiac-specific transcription factors [6, 9]. The FHF forms the primitive heart tube, which later transforms to the left ventricle (LV) and parts of both atria. The SHF forms the

Tab. 1.1.1: Comparison of models to study human cardiac function.

Model	Advantages	Limitations
Single cell	– Study of single ion channels	– No cell-cell interactions – Low viability
Cell monolayer (cell line)	– Cell-cell interactions – High viability – High reproducibility	– No 3D interactions – Change of phenotype over time
Primary cells	– Person specific/precision medicine	– Low viability – Limited availability
iPSC monolayer (can be incorporated in EHT, CMT, and biowires)	– Relatively easy to obtain – Potentially large quantities – Patient specific – Retain genetic background – Enables studying of heritable diseases – Enables early diagnostics	– Challenging to purify – Difficult to classify – Hard to obtain 100% of desired phenotype – Full maturity has not been reported
Slices	– 3D morphology – Accurate model for acute studies – Genetic modification (e.g., siRNA transfection)	– Limited availability – Cannot perform chronic studies
Wedges	– 3D physiology studies	– Low throughput – Limited availability

CMT= cardiac microtissue; iPSC= induced pluripotent stem cell; EHT= engineered heart tissue; 3D= three dimensional.

right ventricle (RV) and outflow tract and contributes to both atria as well [6, 10]. FGF, BMP, Hedgehog, noncanonical Wnt, and Notch are also involved in various stages of FHF and SHF differentiation [6, 11]. For example, the main signaling that has a proliferative effect is produced by FGF, BMP, and Notch from endocardium [12–14]. Retinoic acid also has an effect on atrial differentiation vs. ventricular differentiation or the conduction system via Notch activation [6, 15, 16]. Insulin growth factor (IGF) and canonical Wnt play a role in terminal differentiation of the CMs [6, 17]. Both pathways stabilize β-catenin via glycogen synthase kinase 3 beta (GSK3β) [18–20]. The key transcription factors that play a role in cardiac development are GATA4, NKX2.5, TBX1, TBX5, TBX20, Mef2c, Bry, Mesp1 and 2 [6, 21, 22], and Baf60c [6, 21, 23]. Baf60c triggers Nkx2.5, which in turn acts with Gata4 to initiate cardiac reprogramming while Tbx5 continues further differentiation [6]. CMs reach terminal differentiation after birth and become acytokinetic, which results in polyploidy and multinucleation [6, 24–28].

To recapitulate cardiogenesis *in vitro*, biphasic canonical Wnt signaling is required to achieve a high yield of CMs: it is first activated, to recapitulate gastrulation, and then inhibited [6, 29]. It has been shown that addition of FGF2, vascular endothelial growth factor-A (VEGFA), and DKK1 with inhibition of Nodal or TGFβ receptor 2 signaling enhanced CMs differentiation by 50% [6, 30–34]. Sequential and dose-controlled addition of growth factor BMP and activin A has also been shown to result in a 30% increase in differentiation efficiency yield [6, 32]. TGFβ improves the efficiency of differentiation in the multifunctional growth factor involved in cardiogenesis. It was shown that newly derived CMs had an excitation-contraction coupling that involved L-type calcium channel activity and also responded to β-adrenergic stimulation [35]. Additionally, FGF2, which plays a role in driving mesodermal cells to the cardiogenic lineage during embryogenesis, might be in full control of the differentiation from iPSC cells to CMs [36]. The sequential treatment of iPSCs with GSK3 inhibitors, followed by inducible expression of β-catenin shRNA or chemical inhibitors of Wnt signaling, also showed a high differentiation yield [37].

Besides reprogramming from iPSC, there has been an upcoming trend to directly convert lineage from one somatic cell type to another bypassing the PSC state. CMs can be transdifferentiated from fibroblasts by overexpressing core cardiac transcription factors such as Gata4, Mef2c, Tbx5, and Hand2 [38, 39]. Figure 1.2.1 shows a summary of the cardiac development and schematic of signaling pathways contributing toward the development of each cell lineage and compares them to current reprogramming protocols.

1.3 Reprogramming limitations and improvements

It should be kept in mind that in addition to the differentiation protocol, other factors, such as organ of cells' origin, passage number, and sensitivity to growth factors, can affect reprogramming efficacy; therefore, the reprogramming results in terms of yield and efficiency should be viewed with caution [6, 40]. Besides the promises of

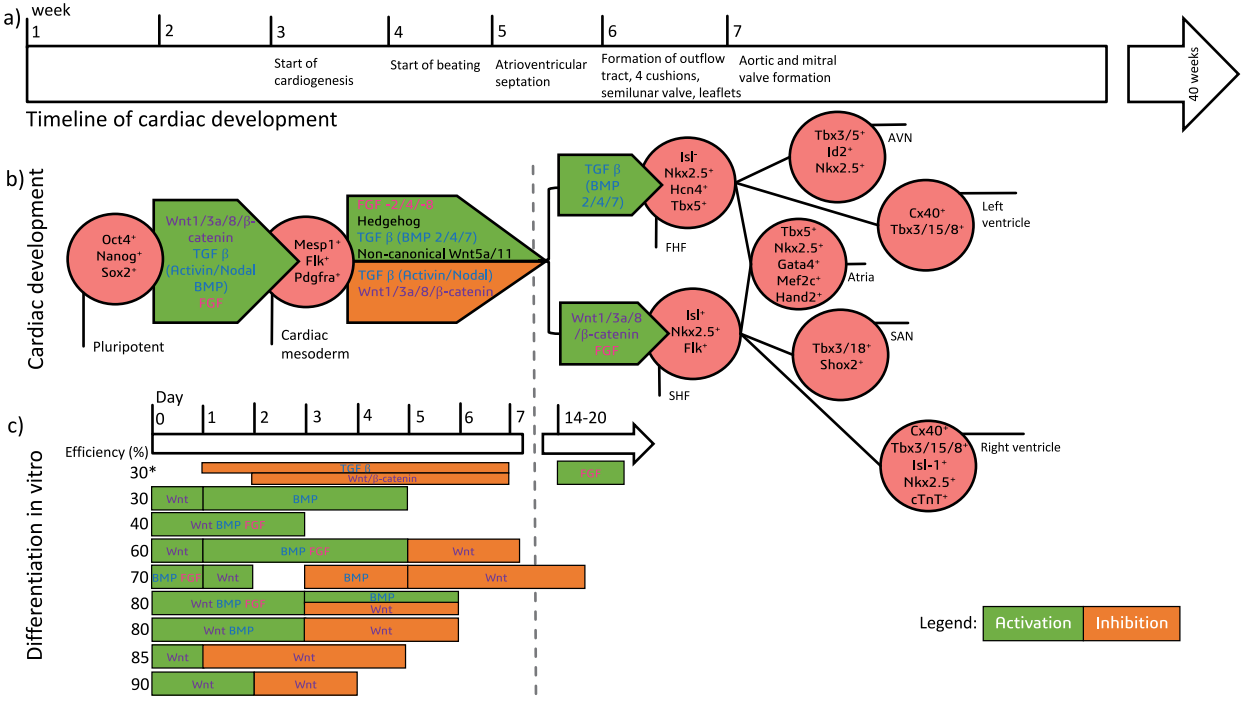

Fig. 1.2.1: Summary of current reprogramming protocols and their relation to developmental pathways. (a) Major cardiac development events within the human embryonic development timeline. (b) Signaling pathways during cardiac cell lineage development. Each intermediate stage has its unique molecular signature. Cardiac development is roughly aligned with the timeline from panel a. (c) Reprogramming protocols (iPSC to iPSC-CM) are plotted in increasing efficiency of differentiation with respect to the signaling pathways they activate and/or inhibit. The differentiation *in vitro* timeline is purposefully plotted below panel b. As it can be seen, the pathways that take several weeks *in vivo*, reprogramming protocols try to recapitulate in about 7 days. The protocols finish their reprogramming before separating to FHF or SHF (dashed line). This might be one of the reasons why iPSC-CMs remain immature and have a mixed phenotype. AVN, atrioventricular node; SAN, sinoatrial node; *Direct reprogramming protocol (fibroblasts to iCM). [41–43], [1], [6]

reprogramming technique, the two main downsides of reprogramming are that immature CMs and yield of single phenotype have not been achieved. This raises the question about their functionality and physiological responses compared to adult CMs. Mixed atrioventricular and nodal CMs are an issue for cell-lineage-specific models as well as for transplantation, since such CMs increase the risk of arrhythmias [6, 44]. At the same time, it has been reported that immature CMs have a higher engraftment potential after implantation compared to integration of more mature CMs [6, 45]. Phenotype studies have demonstrated that current differentiation protocols yield CMs at the first trimester (up to week 12) or midgestation of human fetal heart development (20 weeks) [1, 2]. iPSC-CMs have a high expression level of I_f peacemaking ion channel and low expression rate of I_{K1} that is responsible for resting potential maintenance. iPSC-CMs rely mainly on the human ether-a-go-go-related gene (hERG)/I_{Kr} channel for repolarization.

Variables that play a role in maturation include genetic, chemical, physical, and environmental factors. One way to improve and induce maturation is to overexpress the components of the CMs machinery, such as miR-1 and Kir2.1 [1, 2]. The same approach is used to directly reprogram fibroblasts to CMs by inducing/overexpressing CMs-specific key transcription factors. Maturation can be improved with medium additives. For example, triiodothyronine (T3) helps cardiac development during the perinatal period (20th–28th week) and regulates the switching of several myocardial proteins, including myosin heavy chain (MHC) and titin, which enhance twitch force and upstroke [1, 46].

The heart is an electrically active organ; therefore, electrophysiology needs to be guided to recapitulate more closely its normal physiology. Electrical stimulation has been shown to induce the elongation of the cells as well as to increase sarcomere length and mitochondrial volume, to enhance the development of intercalated disks and gap junctions, and to improve contractility [1, 47]. Additionally, electrical stimulation induces IKr and IK1 as well as enhances the expression of α-actinin, actin, T-tubules, and expression of cardiac-specific genes: HCN1, MLC2V, SCN5A, SERCA, Kv4.3, and GATA4.1 [48, 49]. For this quantification, several platforms have been developed, which include multielectrode arrays with external electrical stimulation and, the more recent, Pulse, an all-in-one platform that has video microscopy, image-analysis algorithms, automatic capturing up to 50 frames per second, and stimulation of hiPSC-CM tissue patterns [50]. Another platform that can be used for stimulation and measurement simultaneously is three-dimensional (3D) multifunctional integumentary membrane (MIM), which represents a 3D elastic membrane with ultrathin electronic/optoelectronic and sensor systems [51].

The application of mechanical stress that CMs normally experience, such as mechanical load, cyclic stress, and substrate stiffness, can play a big role in a maturation process. This was first shown during coculture of human ESC (hESC) with a visceral-endoderm-like cell line called END-2 [52]. Based on that, various platforms have been developed to provide mechanical cues that help to maintain the cocktail of signaling factors that also have tunable stiffness and flexibility. Patterning of the

substrate can improve maturation even further; for example, polyethylene glycol (PEG)-patterned substrates, along with signaling molecules, produced beating cardiospheres by geometrically confining iPSCs [53]. Fibronectin-coated microgrooved polydimethylsiloxane (PDMS) scaffolds improve cellular alignment, sarcomere organization, and Ca^{2+} handling [1, 54]. Several substrates such as gelatin, collagen, fibrin, polyacrylamide, Matrigel™, and laminin scaffolds, with altered mechanical properties, have shown improvement in cell elongation, ion-channel expression, and gap-junction formation [1, 55]. Substrates, besides providing support and mechanical stiffness, also influence cells with extracellular matrix signaling cues (biochemical and biophysical cues); it was found empirically that 4% PEG:96% carboxylated polycaprolactone (PCL) improves contractility and mitochondrial function [1, 56].

The key to full maturation is the combination of medium supplements with electrical pacing and mechanical cues. Additionally, long-term culturing of iPSC-CMs has shown an improvement in structural and functional maturation [6, 57]. Table 1.3.1 summarizes the structural, metabolic, and functional differences of adult-CMs from iPSC-CMs.

1.4 Disease modeling platform

Finding the right protocol that recapitulates cardiogenesis and successfully yields in mature single lineage CMs, in the future, will help to modulate various diseases by altering signaling factors and mechanical environments (e.g., substrate stiffness that mimics myocardial infarction (MI)). For example, some researchers were able to induce arrhythmogenic phenotypes by altering the substrate stiffness [1, 58, 59]. Models help to understand disease mechanisms and the efficacy of novel therapeutics. At the same time, reprogramming and disease modeling have to be done with care; otherwise, the results are not meaningful and do not recapitulate the real response *in vivo* [1, 60]. *In vitro* drug testing with patients' own cells is currently being experimentally used to improve drug administration, thus developing a new field of personalized medicine [1, 61]. Patient-specific iPSCs were used to study phenotypes and drug effects on various diseases, such as long-QT syndrome (LQTS), LQTS3/Brugada overlap, catecholaminergic polymorphic ventricular tachycardia (CPVT), Duchenne muscular dystrophy (DMD), dilated cardiomyopathy, hypertrophic cardiomyopathy (HCM), Leopard syndrome, Barth syndrome, and arrhythmogenic right ventricular cardiomyopathy (ARVC) [1].

Electrophysiological changes, such as prolonged action potential duration (APD) due to inherited cardiomyopathy or shortened APD due to β-adrenergic stimulation, have been observed using single cells. In 2012, the first iPSC-CM monolayer was developed by sequential application of cytokines and matrigel. Cardiac sheets generated functional syncytia, electrically paced after 2 days of seeding; gap junctions were formed (Cx43-ventricular); and rotors could be induced, which confirms it as a relevant model to study arrhythmias [4, 32, 62, 63]. Two-dimensional muscular thin film (MTF) has been validated as a potential platform to model and study the

Tab. 1.3.1: Summary of the structural, metabolic, and functional differences between adult-CMs and iPSC-CM.

	Adult-CMs	iPSC-CM
Structure	– Highly organized	– Disorganized
	– Large rod shaped	– Round or polygonal
	– Polynuclear	– Mononuclear
	– Longitudinal alignment	– Chaotic alignment
	– Sarcomere length: 2.2 µm	– Sarcomere length: 1.6 µm
	– Prominent T-tubules	– No T-tubules
Metabolism	– Mitochondria occupies 20–40% of cell volume	– Mitochondria is perinuclear
	– Energy substrate: oxidative phosphorylation and fatty acids	– Energy substrate: mostly glucose and lactate
Electrophysiology	– No spontaneous beating	– Spontaneous beating can occur
	– Capacitance: 150 pF	– Capacitance: 20–50 pF
	– Max diastolic potential: –85 mV	– Max diastolic potential: –63 mV
	– Upstroke velocity: 150–350 V/s	– Upstroke velocity: 1–50 V/s
	– Conduction velocity: 60 cm/s	– Conduction velocity: 10–20 cm/s
	– dV/dt: 120–200	– dV/dt: 17–45

CMs= cardiomyocytes; iPSC= induced pluripotent stem cell.

diseases: cells from a patient with Barth syndrome (mutation in X-linked gene) were transdifferentiated into iPSC-CMs and compared with healthy human PSC (hiPSC)-CMs. Cells with mutations showed poorer sarcomere alignment and lower twitch force, which are both markers of Barth syndrome [1, 64]. Further improvement with field stimulation and drug loading channels can make this model very promising for disease study and drug testing [1, 65]. Maturation over time (1 month) improved conduction velocity (gap junctions), which was measured by an increase in calcium wave velocity. Besides those promising outcomes, the model lacks 3D; thus, cellular attachments to the substrate and two-dimensional (2D) force load are not exactly physiologically correct [1, 66]. Mechanotransduction in 2D models is different since sarcomeres in adult CMs align perpendicularly to the cell axis, which enables higher probability of actin-myosin cross-bridge formation compared to the MTF iPSC-CMs that do not have structured sarcomere alignment; thus, their contractile force, as well as other parameters, is lower [1].

Additionally, due to poor cell-cell interactions, structural heart diseases such as ARVC and HCM cannot be studied using this model. Due to the shortcomings of MTF, 3D structures have been developed, such as cardiac microtissue (CMT), "micro-heart muscle" (μHM), cardiac biowires, and one of the most established models – engineered heart tissue (EHT) [1, 47, 67–69]. Both EHT and CMT are made by casting hydrogel with cells into molds that have elastic anchors. These help to organize and align the cells by contracting cells against them, which resembles physiologically relevant auxotonic tension. In EHT, CMs are embedded in fibrin gel and placed between silicon posts. In CMT, CMs are seeded into fibrin/collagen and linked to PDMS cantilevers. Contraction is analyzed by cantilever/postdeflection [48]. μHM merges both EHT and self-organizing cardiospheres by miniaturizing the platform and seeding cells into PDMS stencils [53, 69]. In biowires, cells are seeded onto collagen-covered surgical sutures into a PDMS mold. A summary of the advantages and disadvantages is described in Tab. 1.4.1 3D platforms lack the formation of T-tubules and expression of sarcomere proteins such as α-sarcomere protein and myosin-binding protein C and demonstrate low contraction forces [1, 70].

1.5 Drug testing platform

One of the main side effects of drugs is cardiotoxicity, since they affect cardiac structure integrity and CMs viability [1, 76–78]. Cardiac toxicity is caused by the interaction of the drugs with one or more ion channels that regulate contractility, excitability, and cardiac performance in general [4, 79, 80]. Platforms that could accurately predict these interactions are in high demand. This also inspired the development of a comprehensive *in vitro* proarrhythmia assay (CIPA) to be started [1, 81]. Direct comparison of animal cells to human cells (e.g., dog and rabbit hearts to hPSC-CMs) has shown that cells of human origin predict cardiotoxicity more accurately (e.g., IKr blocker moxifloxacin that has proarrhythmic effects) [1, 82]. While testing for doxorubicin toxicity, hPSC

Tab. 1.4.1: Summary of advantages and disadvantages.

	Advantages	Limitations
EHT [1, 67, 69]	– 3D cell-cell interactions – Enables chronic studies – Better longitudinal alignment of sarcomeres – Coculture of CMs with fibroblasts – Validated platform for physiological and pharmacological studies/ stimulations: responds to increased extracellular Ca^{2+} or β-adrenergic agonists by increasing contractile forces [1, 71] – Twitch forces are higher compared to nonstimulated CMs: hiPSC-CM EHT 0.08–4.4 mN/mm^2 compared to hiPSC-CM 2–260 nN/mm [2, 72–74]	– Needs more cells compared to or platforms (>1 million cells/tissue) – More expensive compared to CMT – Gap junction formation along the periphery rather than on intercalated disks – Twitch forces low: (single cell) 0.08–4.4 mN/mm^2 compared to adult-CM (muscle strip) 40–80 mN/mm^2 – Negative force frequency relationship observed
CMT [1, 68, 75]	– 3D cell-cell interactions – Needs fewer cells compared to EHT – Less expensive compared to EHT – Coculture of CMs with endothelial cells	– Only acute studies
Biowires [1, 47]	– Electrical stimulation of biowires induced I_{Kr} and I_{K1} as well as enhanced expression of α-actinin, actin, and T-tubules [1, 48]	– Cannot record contraction forces
μHM [69]	– 3D cell-cell interactions – Needs fewer cells compared to EHT (<10,000 cells/stencil) – Coculture of CMs with fibroblasts – Uniaxial alignment and robust sarcomere alignment – Twitch force can be measured – High-throughput system – Validated by exhibiting Frank-Starling response and ionotropic response to β-adrenergic stimulation with isoproterenol	– Negative force frequency relationship observed – Small size makes it challenging to measure tissue conduction velocity (especially reentries) and contraction forces

CMs= cardiomyocytes; CMT= cardiac microtissue; h-iPSC= human iPSC derived; iPSC= induced pluripotent stem cell; EHT= engineered heart tissue; μHM= micro-heart muscle; 3D= three dimensional.

helped to guide the decision of administering the drug via Her2-targeted liposomal pathway, which led to phase I testing [1, 83]. Currently, to test for cardiotoxicity, only one ion channel, I_{Kr} (hERG gene, Kv11.1 protein), is considered. This model is applied by genetically modifying to overexpress the hERG channel in Chinese hamster ovary cells. hERG current is recorded through a high-throughput patch clamp system. The channel is responsible for repolarization, and the model is based on the assumption that I_{Kr} inhibition causes prolonged APD and longer QT, which can result in polymorphic ventricular tachyarrhythmia or torsades de pointes (TdP). This method raises many questions in terms of its relevance since cells lack all of the cardiac structure (sarcomeres, T-tubules, other ion channels, and a large amount of mitochondria) that also can have an effect on the outcome. Since there are no APD measurements, the model is based on a causation link between the drug effect on the channel and its relationship to APD. There is evidence that inhibition of hERG channel does not always correlate to APD prolongation and TdP. For example, antidepressant Prozac (fluoxetine) is a potent hERG blocker but does not affect APD duration; thus, if it were tested on this platform, it would be classified incorrectly and not reach the market [4, 84, 85]. Ideally, isolated human CMs would be the most accurate platform since they have relevant phenotype, electrophysiology, and calcium handling. The downside of such a model is low viability and survival (challenging to keep cells alive in a culture) and limited supply and throughput, which are very unattractive in the early stages of drug development and screening. Therefore, human induced pluripotent stem cells (hiPSC) are considered a better alternative since they are easier to obtain and maintain. Additionally, they offer the benefit of being patient specific, giving an attractive benefit of precision medicine. This approach was validated by showing that iPSC-CM conduction decreased when stimulation decreased and when sodium channel blockers such as lidocaine and tetrodoxin were applied; rapid pacing induced arrhythmia and was terminated by applying antiarrhythmic drugs. On a downside, conduction velocity propagation was 10 times slower than in a whole heart, which can be attributed to an immature phenotype of PSC [4, 63]. Additionally, iPSC-CMs rely almost exclusively on hERG/I_{Kr} for repolarization, which might not offer more advantages compared to established assay with overexpressed hERG in Chinese hamster ovary cells.

1.6 Transplantation of *in vitro* derived CMs into injured areas

Multiple animal studies have shown that cell therapies improve myocardial wall stress, contractility, and metabolism (Tab. 1.6.1) [1]. Interestingly, as a result of the transplantation, macaques were more prone to arrhythmia development compared to pigs [1]. The first clinical trial implanting hESCs into a human patient has been performed. In this clinical trial, non-patient-specific hESCs were transdifferentiated using BMP-2 and FGF inhibitor, then purified and embedded into fibrin scaffold. After 3 months, there were some recovery in contractility, no signs of arrhythmias, and no other

Tab. 1.6.1: Transplantation of *in vitro* derived CMs into the injured area of different animal models.

Animal model	Graft integration into host tissue	Side effects
Macaque [88]	– Extensive remuscularization of infarcted area – Formation of electromechanical junctions between host tissue and graft – Electromechanical coupling that is demonstrated by regular Ca^{2+} transients	– Nonfatal ventricular arrhythmias
Guinea-pig [89]	– Synchronous contraction of graft and host cells – Improved mechanical function – Arrhythmia suppressive effect	– Full integration of the grafts not achieved
Rat [90, 91]	– Successful engraftment, cell proliferation, increased sarcomere organization – Formation of gap junctions with host tissue – Effective for acute model of myocardial infarction – Attenuated cardiac remodeling process	– No effect on cardiac remodeling of chronic myocardial infarction – Teratoma formation after nondifferentiated hESC implantation
Mouse [92]	– Improved cardiac function – Organization and maturation of the graft over time (12 weeks)	– Improved cardiac function not sustained after a 12-week period

Cell delivery method for all studies was direct intramyocardial injection of hESC-CM.
CMs= cardiomyocytes; hESC-CM= human embryonic stem cell derived cardiomyocytes

complications [1, 86]. The challenges of such procedures in the clinic are large-scale production of cardiac progenitors, immunosuppression, surgical procedure, and patient selection for such therapy. An additional concern is low cell survival: 20–35% in EHT. If 10^9 cells are lost during infarction, 5×10^9 cells are needed [1, 6, 32]. There are still debates whether cells should be fully differentiated prior to transplantation. Another future direction is a coculture with fibroblasts and endothelial cells that could potentially lead to better revascularization and integration with the host tissue [6, 87].

1.7 Conclusion and future directions

hiPSCs are a very promising tool in the future of biology and medicine. Various animal models have shown that the intramyocardial delivery of the cells improves cardiac function and regeneration, However, cell retention, maturation, and delivery require further improvement. The main challenge in the iPSC field is the maturation of the cells. Full differentiation of iPSC-CMs can potentially be achieved by combining not only chemical components (plasmids, small molecules) but also electrical (field stimulation) and mechanical (stretching of the tissue) components. This can be done by combining the expertise of different research groups to develop a more accurate and enhanced differentiation protocol that better recapitulates the processes *in vivo*. For example, extension of the time span of the differentiation might help to achieve a high yield of specific phenotype since it has been shown that cells keep on maturing after several months. Additionally, coculture of multiple cell types might create a more enhanced and physiologically relevant tissue construct for implantation. Perhaps, computer modeling that can take into consideration all the data on different platforms and the specificity factors of each experiment could be the future of diagnostic platforms. However, only several models are biophysical and take into account the signaling pathways or anatomical features of the structure of cardiac tissue [93–95].

hiPSCs are already being used in clinics for personalized drug screening, and the first human clinical trials of cell graft implantation have shown to be successful. This leads to a promise that one day, patients might be able to not only use their own cells to receive medication that is specific to them but also get tissue implants that cause no immune response and regenerate their body naturally.

1.8 References

[1] Denning C, Borgdorff V, Crutchley J, et al. Cardiomyocytes from human pluripotent stem cells: from laboratory curiosity to industrial biomedical platform. Biochim Biophys Acta Mol Cell Res 2016;1863(7):1728–48. Available from: http://dx.doi.org/10.1016/j.bbamcr.2015.10.014

[2] Rajamohan D, Matsa E, Kalra S, et al. Current status of drug screening and disease modelling in human pluripotent stem cells. Bioessays [Internet] 2013;35(3):281–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22886688

[3] Nerbonne JM. Studying cardiac arrhythmias in the mouse—a reasonable model for probing mechanisms? Trends Cardiovasc Med 2004;14(3):83–93. Available from: https://www.sciencedirect.com/science/article/pii/S1050173803002147?via%3Dihub
[4] William G. Cardiac electrophysiology: from cell to bedside. In: Zipes DP, Jalife J, Stevenson WG, eds. 7th Ed. Philadelphia, PA: Elsevier, 2018: 1345–89.
[5] Zhang J, Wilson GF, Soerens AG, et al. Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res 2009;104(4):e34–41.
[6] Spater D, Hansson EM, Zangi L, Chien KR. How to make a cardiomyocyte. Development [Internet] 2014;141(23):4418–31. Available from: http://dev.Bio logists.org/cgi/doi/10.1242/dev.091538
[7] Barrow JR, Howell WD, Rule M, et al. Wnt3 signaling in the epiblast is required for proper orientation of the anteroposterior axis. Dev Biol 2007;312(1):312–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18028899
[8] Foley AC, Mercola M. Heart induction by Wnt antagonists depends on the homeodomain transcription factor Hex. Genes Dev 2005;19(3):387–96. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15687261
[9] Noseda M, Peterkin T, Simoes FC, Patient R, Schneider MD. Cardiopoietic factors: extracellular signals for cardiac lineage commitment. Circ Res 2011;108(1):129–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21212394
[10] Brade T, Pane LS, Moretti A, Chien KR, Laugwitz K-L. Embryonic heart progenitors and cardiogenesis. Cold Spring Harb Perspect Med 2013;3(10):a013847. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24086063
[11] Cohen ED, Tian Y, Morrisey EE. Wnt signaling: an essential regulator of cardiovascular differentiation, morphogenesis and progenitor self-renewal. Development 2008;135(5):789–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18263841
[12] Collesi C, Zentilin L, Sinagra G, Giacca M. Notch1 signaling stimulates proliferation of immature cardiomyocytes. J Cell Biol 2008;183(1):117–28. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18824567
[13] Hotta Y, Sasaki S, Konishi M, et al. Fgf16 is required for cardiomyocyte proliferation in the mouse embryonic heart. Dev Dyn 2008;237(10):2947–54. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18816849
[14] Qi X, Yang G, Yang L, et al. Essential role of Smad4 in maintaining cardiomyocyte proliferation during murine embryonic heart development. Dev Biol 2007;311(1):136–46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17869237
[15] Jiang J, Han P, Zhang Q, Zhao J, Ma Y. Cardiac differentiation of human pluripotent stem cells. J Cell Mol Med 2012;16(8):1663–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22248065
[16] Rentschler S, Yen AH, Lu J, et al. Myocardial notch signaling reprograms cardiomyocytes to a conduction-like phenotype. Circulation 2012;126(9):1058–66. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22837163
[17] Buikema JW, Mady AS, Mittal NV, et al. Wnt/-catenin signaling directs the regional expansion of first and second heart field-derived ventricular cardiomyocytes. Development 2013;140(20):4165–76. Available from: http://www. ncbi.nlm.nih.gov/pubmed/24026118
[18] Heallen T, Morikawa Y, Leach J, et al. Hippo signaling impedes adult heart regeneration. Development 2013;140(23):4683–90. Available from: http://www. ncbi.nlm.nih.gov/pubmed/24255096
[19] von Gise A, Lin Z, Schlegelmilch K, et al. YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. Proc Natl Acad Sci 2012;109(7):2394–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22308401

[20] Xin M, Kim Y, Sutherland LB, et al. Regulation of insulin-like growth factor signaling by yap governs cardiomyocyte proliferation and embryonic heart size. Sci Signal 2011;4(196):ra70. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22028467
[21] Bruneau BG. The developmental genetics of congenital heart disease. Nature 2008;451(7181):943–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18288184
[22] Srivastava D. Genetic regulation of cardiogenesis and congenital heart disease. Annu Rev Pathol Mech Dis [Internet] 2006;1(1):199–213. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18039113
[23] Takeuchi JK, Bruneau BG. Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. Nature 2009;459(7247):708–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19396158
[24] Brodsky VYa, Chernyaev AL, Vasilyeva IA. Variability of the cardiomyocyte ploidy in normal human hearts. Virchows Arch B Cell Pathol Incl Mol Pathol 1991;61(4):289–94. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1685821
[25] Li F, Wang X, Capasso JM, Gerdes AM. Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. J Mol Cell Cardiol 1996;28(8):1737–46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8877783
[26] Olivetti G, Cigola E, Maestri R, et al. Aging, cardiac hypertrophy and ischemic cardiomyopathy do not affect the proportion of mononucleated and multinucleated myocytes in the human heart. J Mol Cell Cardiol 1996;28(7):1463–77. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8841934
[27] Schmid G, Pfitzer P. Mitoses and binucleated cells in perinatal human hearts. Virchows Arch B Cell Pathol Incl Mol Pathol 1985;48(1):59–67. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2858939
[28] Bergmann O, Zdunek S, Alkass K, Druid H, Bernard S, Frisén J. Identification of cardiomyocyte nuclei and assessment of ploidy for the analysis of cell turnover. Exp Cell Res 2011;317(2):188–94. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20828558
[29] Lian X, Zhang J, Azarin SM, et al. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. Nat Protoc 2012;8(1):162–75. Available from: http://www.nature.com/doifinder/10.1038/nprot.2012.150
[30] Cai W, Albini S, Wei K, et al. Coordinate Nodal and BMP inhibition directs Baf60c-dependent cardiomyocyte commitment. Genes Dev 2013;27(21):2332–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24186978
[31] Kattman SJ, Witty AD, Gagliardi M, et al. Stage-specific optimization of activin/nodal and bmp signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell 2011;8(2):228–40. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21295278
[32] Laflamme MA, Chen KY, Naumova AV, et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 2007;25(9):1015–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17721512
[33] Willems E, Cabral-Teixeira J, Schade D, et al. Small molecule-mediated TGF-β type II receptor degradation promotes cardiomyogenesis in embryonic stem cells. Cell Stem Cell 2012;11(2):242–52. Available from: http://www.ncbi.nlm. nih.gov/pubmed/22862949
[34] Yang L, Soonpaa MH, Adler ED, et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature 2008;453(7194):524–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18432194
[35] Goumans M-J, de Boer TP, Smits AM, et al. TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. Stem Cell Res 2008;1(2):138–49. Available from: https://www.sciencedirect.com/science/article/pii/S1873506108000068?via%3Dihub

[36] Rosenblatt-Velin N, Lepore MG, Cartoni C, Beermann F, Pedrazzini T. FGF-2 controls the differentiation of resident cardiac precursors into functional cardiomyocytes. J Clin Invest 2005;115(7):1724–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15951838
[37] Lian X, Hsiao C, Wilson G, et al. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. Proc Natl Acad Sci U S A 2012;109(27):E1848–57. Available from: http://www. ncbi.nlm.nih.gov/pubmed/22645348
[38] Ieda M, Fu J-D, Delgado-Olguin P, et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell 2010;142(3):375–86. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20691899
[39] Song K, Nam Y-J, Luo X, et al. Heart repair by reprogramming non-myocytes with cardiac transcription factors. Nature 2012;485(7400):599–604. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22660318
[40] Kim K, Doi A, Wen B, et al. Epigenetic memory in induced pluripotent stem cells. Nature 2010;467(7313):285–90. Available from: http://www.nature.com/articles/nature09342
[41] Sahara M, Santoro F, Chien KR. Programming and reprogramming a human heart cell. EMBO J 2015;34(6):710–738.
[42] Xin M, Olson EN, Bassel-Duby R. Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. Nat Rev Mol Cell Biol 2013;14(8):529–41.
[43] McNally EM, Svensson EC. Setting the pace: Tbx3 and Tbx18 in cardiac conduction system development. Circ Res 2009;104(3):285–7.
[44] Chen JX, Krane M, Deutsch M-A, et al. Inefficient reprogramming of fibroblasts into cardiomyocytes using Gata4, Mef2c, and Tbx5. Circ Res 2012;111(1):50–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22581928
[45] Reinecke H, Zhang M, Bartosek T, Murry CE. Survival, integration, and differentiation of cardiomyocyte grafts: a study in normal and injured rat hearts. Circulation [Internet] 1999 [cited 2018 Feb 14];100(2):193–202. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10402450
[46] Yang X, Rodriguez M, Pabon L, et al. Tri-iodo-l-thyronine promotes the maturation of human cardiomyocytes-derived from induced pluripotent stem cells. J Mol Cell Cardiol 2014;72:296–304. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24735830
[47] Nunes SS, Miklas JW, Liu J, et al. Biowire: a platform for maturation of human pluripotent stem cell–derived cardiomyocytes. Nat Methods 2013;10(8):781–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23793239
[48] Stoehr A, Neuber C, Baldauf C, et al. Automated analysis of contractile force and Ca^{2+} transients in engineered heart tissue. Am J Physiol Circ Physiol 2014;306(9):H1353–63. Available from: http://www.physiology.org/doi/10.1152/ajpheart.00705.2013
[49] Bian W, Badie N, Himel HD, Bursac N. Robust T-tubulation and maturation of cardiomyocytes using tissue-engineered epicardial mimetics. Biomaterials 2014;35(12):3819–28. Available from: https://www.sciencedirect.com/science/article/pii/S0142961214000611?via%3Dihub
[50] Maddah M, Heidmann JD, Mandegar MA, et al. A non-invasive platform for functional characterization of stem-cell-derived cardiomyocytes with applications in cardiotoxicity testing. Stem Cell Rep 2015;4(4):621–31. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25801505
[51] Xu L, Gutbrod SR, Bonifas AP, et al. 3D multifunctional integumentary membranes for spatiotemporal cardiac measurements and stimulation across the entire epicardium. Nat Commun 2014;5:3329. Available from: http://www.nature.com/doifinder/10.1038/ncomms4329
[52] Mummery CL, Ward D, Passier R. Differentiation of human embryonic stem cells to cardiomyocytes by coculture with endoderm in serum-free medium [Internet]. In: Current

Protocols in Stem Cell Biology. Hoboken, NJ: John Wiley & Sons, Inc., 2007: Unit 1F.2. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18785170
[53] Ma Z, Wang J, Loskill P, et al. Self-organizing human cardiac microchambers mediated by geometric confinement. Nat Commun 2015;6(1):7413. Available from: http://www.nature.com/articles/ncomms8413
[54] Rao C, Prodromakis T, Kolker L, et al. The effect of microgrooved culture substrates on calcium cycling of cardiac myocytes derived from human induced pluripotent stem cells. Biomaterials 2013;34(10):2399–411. Available from: https://www.sciencedirect.com/science/article/pii/S0142961212013142?via%3Dihub
[55] Ruan JL, Tulloch NL, Razumova MV, et al. Mechanical stress conditioning and electrical stimulation promote contractility and force maturation of induced pluripotent stem cell-derived human cardiac tissue. Circulation 2016;134(20):1557–67.
[56] Chun YW, Balikov DA, Feaster TK, et al. Combinatorial polymer matrices enhance in vitro maturation of human induced pluripotent stem cell-derived cardiomyocytes. Biomaterials 2015;67:52–64. Available from: https://www. sciencedirect.com/science/article/pii/S0142961215005803?via%3Dihub
[57] Lundy SD, Zhu W-Z, Regnier M, Laflamme MA. Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. Stem Cells Dev 2013;22(14):1991–2002. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23461462
[58] Engler AJ, Carag-Krieger C, Johnson CP, et al. Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. J Cell Sci 2008;121(Pt 22):3794–802. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18957515
[59] Wen J-Y, Wei C-Y, Shah K, Wong J, Wang C, Chen H-SV. Maturation-based model of arrhythmogenic right ventricular dysplasia using patient-specific induced pluripotent stem cells. Circ J 2015;79(7):1402–8. Available from: https://www. jstage.jst.go.jp/article/circj/79/7/79_CJ-15-0363/_article
[60] Sheridan SD, Theriault KM, Reis SA, et al. Epigenetic characterization of the fmr1 gene and aberrant neurodevelopment in human induced pluripotent stem cell models of fragile x syndrome. PLoS One 2011;6(10):e26203. Available from: http://dx.plos.org/10.1371/journal.pone.0026203
[61] Terrenoire C, Wang K, Tung KWC, et al. Induced pluripotent stem cells used to reveal drug actions in a long QT syndrome family with complex genetics. J Gen Physiol 2013;141(1):61–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23277474
[62] Zhang J, Klos M, Wilson GF, et al. Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method. Circ Res 2011;111(9):1125–36. Available from: http://www.ncbi.nlm.nih. gov/pubmed/22912385
[63] Kadota S, Pabon L, Reinecke H, Murry CE. In vivo maturation of human induced pluripotent stem cell-derived cardiomyocytes in neonatal and adult rat hearts. Stem Cell Reports 2017;8(2):278–89. Available from: http://dx.doi.org/10.1016/j.stemcr.2016.10.009
[64] Wang G, McCain ML, Yang L, et al. Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and heart-on-chip technologies. Nat Med 2014;20(6):616–23. Available from: http://www.nature. com/articles/nm.3545
[65] Grosberg A, Nesmith AP, Goss JA, Brigham MD, McCain ML, Parker KK. Muscle on a chip: in vitro contractility assays for smooth and striated muscle. J Pharmacol Toxicol Methods 2012;65(3):126–35. Available from: https://www. sciencedirect.com/science/article/pii/S105687191200041X?via%3Dihub
[66] Kresh JY, Chopra A. Intercellular and extracellular mechanotransduction in cardiac myocytes. Pflügers Arch Eur J Physiol 2011;462(1):75–87. Available from: http://link.springer.com/10.1007/s00424-011-0954-1

[67] Schaaf S, Shibamiya A, Mewe M, et al. Human engineered heart tissue as a versatile tool in basic research and preclinical toxicology. PLoS One 2011;6(10):e26397. Available from: http://dx.plos.org/10.1371/journal.pone.0026397
[68] Boudou T, Legant WR, Mu A, et al. A microfabricated platform to measure and manipulate the mechanics of engineered cardiac microtissues. Tissue Eng Part A 2012;18(9–10):910–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22092279
[69] Huebsch N, Loskill P, Deveshwar N, et al. Miniaturized iPS-cell-derived cardiac muscles for physiologically relevant drug response analyses. Sci Rep [Internet] 2016;6(1):24726. Available from: http://www.nature.com/articles/srep24726
[70] Cao H, Kang BJ, Lee C-A, Shung KK, Hsiai TK. Electrical and mechanical strategies to enable cardiac repair and regeneration. IEEE Rev Biomed Eng 2015;8:114–24. Available from: http://ieeexplore.ieee.org/document/7105365/
[71] Eder A, Vollert I, Hansen A, Eschenhagen T. Human engineered heart tissue as a model system for drug testing. Adv Drug Deliv Rev 2016;96:214–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26026976
[72] Ribeiro MC, Tertoolen LG, Guadix JA, et al. Functional maturation of human pluripotent stem cell derived cardiomyocytes in vitro – correlation between contraction force and electrophysiology. Biomaterials 2015;51:138–50. Available from: https://www.sciencedirect.com/science/article/pii/S0142961215000848?via%3Dihub#fig1
[73] Liu J, Sun N, Bruce MA, Wu JC, Butte MJ. Atomic force mechanobiology of pluripotent stem cell-derived cardiomyocytes. PLoS One 2012:7(5):e37559. Available from: http://dx.plos.org/10.1371/journal.pone.0037559
[74] Rodriguez ML, Graham BT, Pabon LM, Han SJ, Murry CE, Sniadecki NJ. Measuring the contractile forces of human induced pluripotent stem cell-derived cardiomyocytes with arrays of microposts. J Biomech Eng 2014;136(5):51005. Available from: http://biomechanical.asmedigitalcollection.asme.org/article.Aspx?doi=10.1115/1.4027145
[75] Giacomelli E, Bellin M, Sala L, et al. Three-dimensional cardiac microtissues composed of cardiomyocytes and endothelial cells co-differentiated from human pluripotent stem cells. Development 2017;144(6):1008–17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28279973
[76] Qureshi ZP, Seoane-Vazquez E, Rodriguez-Monguio R, Stevenson KB, Szeinbach SL. Market withdrawal of new molecular entities approved in the United States from 1980 to 2009. Pharmacoepidemiol Drug Saf 2011;20(7): 772–7. Available from: http://doi.wiley.com/10.1002/pds.2155
[77] Braam SR, Passier R, Mummery CL. Cardiomyocytes from human pluripotent stem cells in regenerative medicine and drug discovery. Trends Pharmacol Sci 2009;30(10):536–45. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19762090
[78] Pereira GC, Silva AM, Diogo CV, Carvalho FS, Monteiro P, J. Oliveira P. Drug-induced cardiac mitochondrial toxicity and protection: from doxorubicin to carvedilol. Curr Pharm Des 2011;17(20):2113–29. Available from: http://www. eurekaselect.com/openurl/content.php?genre=article&issn=13816128&volume=17&issue=20&spage=2113
[79] Stummann TC, Beilmann M, Duker G, et al. Report and recommendations of the workshop of the European Centre for the Validation of Alternative Methods for Drug-Induced Cardiotoxicity. Cardiovasc Toxicol 2009;9(3):107–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19572114
[80] Pacher P, Kecskemeti V. Cardiovascular side effects of new antidepressants and antipsychotics: new drugs, old concerns? Curr Pharm Des 2004;10(20):2463–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15320756
[81] Sager PT, Gintant G, Turner JR, Pettit S, Stockbridge N. Rechanneling the cardiac proarrhythmia safety paradigm: a meeting report from the Cardiac Safety Research

Consortium. Am Heart J 2014;167(3):292–300. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24576511
[82] Nalos L, Varkevisser R, Jonsson M, et al. Comparison of the IKr blockers moxifloxacin, dofetilide and E-4031 in five screening models of pro-arrhythmia reveals lack of specificity of isolated cardiomyocytes. Br J Pharmacol 2012;165(2):467–78. Available from: http://doi.wiley.com/10.1111/j.1476-5381. 2011.01558.x
[83] Reynolds JG, Geretti E, Hendriks BS, et al. HER2-targeted liposomal doxorubicin displays enhanced anti-tumorigenic effects without associated cardiotoxicity. Toxicol Appl Pharmacol 2012;262(1):1–10. Available from: https://www. sciencedirect.com/science/article/pii/S0041008X12001366?via%3Dihub
[84] Fermini B, Fossa AA. The impact of drug-induced QT interval prolongation on drug discovery and development. Nat Rev Drug Discov 2003;2(6):439–47. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12776219
[85] Zhang S, Zhou Z, Gong Q, Makielski JC, January CT. Mechanism of block and identification of the verapamil binding domain to HERG potassium channels. Circ Res 1999;84(9):989–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10325236
[86] Menasché P, Vanneaux V, Hagège A, et al. Human embryonic stem cell-derived cardiac progenitors for severe heart failure treatment: first clinical case report: Figure 1. Eur Heart J 2015;36(30):2011–7. Available from: https://academic. oup.com/eurheartj/article-lookup/doi/10.1093/eurheartj/ehv189
[87] Stevens KR, Kreutziger KL, Dupras SK, et al. Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue. Proc Natl Acad Sci U S A 2009;106(39):16568–73. Available from: http://www. ncbi.nlm.nih.gov/pubmed/19805339
[88] Chong JJH, Yang X, Don CW, et al. Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. Nature 2014;510(7504):273–7. Available from: http://www.nature.com/articles/nature13233
[89] Shiba Y, Fernandes S, Zhu W-Z, et al. Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts. Nature 2012;489(7415):322–5. Available from: http://www.nature.com/articles/nature11317
[90] Fernandes S, Naumova AV, Zhu WZ, Laflamme MA, Gold J, Murry CE. Human embryonic stem cell-derived cardiomyocytes engraft but do not alter cardiac remodeling after chronic infarction in rats. J Mol Cell Cardiol 2010;49(6):941–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20854826
[91] Caspi O, Huber I, Kehat I, et al. Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. J Am Coll Cardiol 2007;50(19):1884–93. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17980256
[92] van Laake LW, Passier R, Monshouwer-Kloots J, et al. Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction. Stem Cell Res 2007;1(1):9–24. Available from: https://www.sciencedirect.com/science/article/pii/S1873506107000025?via%3Dihub
[93] Fomovsky GM, Thomopoulos S, Holmes, J. W. Contribution of extracellular matrix to the mechanical properties of the heart. Journal of molecular and cellular Cardiol 2010;48(3);490–6. Available from: http://www.jmmc-online.com/article/S0022-2828(09)00325-3/abstract
[94] Kudryashova N, Tsvelaya V, Agladze K, Panfilov A. Virtual cardiac monolayers for electrical wave propagation. Sci Rep 2017;7:7887. Available from: https://www. nature.com/articles/s41598-017-07653-3
[95] Graner F, Glazie JA. Simulation of biological cell sorting using a two-dimensional extended Potts model. Physical Rev Lett 1992;69(13):2013–6. Available from: https://journals.aps.org/prl/abstract/10.1103/PhysRevLett. 69.2013

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2 Preclinical large-animal models of cardiovascular regeneration

Abstract: The Centers for Disease Control estimates that about 5.7 million adults in the United States have heart failure (HF) and more than 550,000 new cases are diagnosed every year. The definitive treatment of end-stage HF remains orthotopic heart transplantation (OHT). The mortality rate while waiting for OHT is high (35% in 4 months) due to shortage of donor hearts. Prolonged wait periods also impose significant constraints on the health care system and patient quality of life. Despite recent advances in medical and device therapies, there are no effective long-term treatment strategies for heart attacks. To develop novel therapeutic approaches to improve blood flow to the heart, the scientific community has increasingly relied on preclinical large-animal models to test the feasibility of innovative treatments for cardiovascular regeneration. In this chapter, we provide an overview of the large-animal models available and the therapies being investigated and review the results obtained that pave the way for breakthrough treatment strategies, with a discussion on the hurdles that must be overcome for implementation in clinical trials.

Key Words: Experimental, Heart, Infarction, Models, Regeneration, Stem cells.

2.1 Introduction

Heart failure (HF) is the final common pathway for all pathological processes affecting the myocardium, ultimately leading to the replacement of cardiomyocytes (CMs) with scar tissue and often followed by compensatory changes in surrounding tissues, also known as adverse cardiac remodeling. Clinical stages can progress from minimal symptoms of limitation in exercise ability to eventually complete dysfunctional state, leading to cardiogenic shock and death. Although population statistics vary depending on the definitions applied for diagnosis, the European Society of Cardiology estimates an HF prevalence of 26 million (1–2%) in developed countries, with a significant (10%) rise in population aged above 70 years [1]. In addition to the considerably high mortality rate (up to 40% in the first year after diagnosis), this disease also strains health care systems across the globe [2]. Despite significant advances being made in the management of advanced HF, such as development of left ventricular assist devices, (LVAD) the underlying pathophysiology remains elusive and continues to be an area of active cardiovascular research. Conceptually, if we could replace a significant number of

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damaged/lost cells with cellular therapeutic interventions and were able to electromechanically couple them to the existing myocardium, HF could be potentially cured.

2.2 Regenerative strategies for HF

Due to the improvements in percutaneous revascularization technology in the last decade, the mortality rate after myocardial infarction (MI) has improved considerably, with a paradoxical increase in the number of patients with postinfarction HF [3, 4]. Prior to the beginning of the last decade, the heart was considered as an organ with limited regenerative potential. However, recent studies using genomic ^{14}C (radiocarbon birth dating) have provided indisputable evidence for the first time that there is a continuous, but rather slow-paced, replacement of CMs in the human heart in normal physiology [5–7]. The TACTICS (Transnational Alliance for Regenerative Therapies in Cardiovascular Syndromes) Alliance, the first international alliance on cardiac regenerative medicine, consequently defined the heart as a self-renewing organ [8]. Nevertheless, the adult human heart does not have the capability of replacing lost cells after a severe acute insult, such as MI or other processes that accelerate cell death and induce adverse remodeling. For this reason, the scientific community is currently focused on therapeutic strategies that might accelerate the self-renewal process of native CMs and/or replace CMs with cells that are capable of replicating and supporting the heart so the natural replacement process can eventually resume.

The TACTICS Alliance consensus statement divides the available therapeutic interventions into two complementary groups: interventions based on exogenous regenerative responses and those based on endogenous regenerative responses. Methods described under the exogenous response include implantation of differentiated CMs, endothelial progenitor cells (EPCs), and engineered tissues or patches. On the other hand, therapeutic strategies that target stimulation of endogenous responses may involve the following:

i. Implantation of embryonic-stem-cell-derived CMs (ESC-CMs), cardiosphere-derived cells (CDCs), bone-marrow-derived (BM) mononuclear cells (MNCs), BM mesenchymal stem cells (MSCs), and induced pluripotent stem-cell-derived CMs (iPSC-CMs).
ii. Injections of biological and synthetic factors (microvesicles, exosomes, etc.).
iii. Genetic and epigenetic modification of genes and/or mRNA involved in the regulation of endogenous regenerative processes.
iv. Tissue engineered grafts.

Additionally, stem cell therapies for treating cardiac dysfunction could be classified broadly into therapies using first-generation cell types and second- (or next-) generation cell types. First-generation cells include skeletal myoblasts (SMs), EPCs, BMMNCs, hematopoietic stem cells, and MSCs. These cells impart their regenerative

effects by stimulation of endogenous repair mechanisms in heart. Although preclinical studies showed promise, particularly with BMMNCs, clinical trials focused on treating acute MI failed to demonstrate a significant clinical benefit [9, 10].

Second-generation cell therapies focus on exogenous methods by using pluripotent stem cells (PSCs) and cardiopoetic MSCs for cardiac tissue differentiation. Cardiac stem/progenitor cells (including CDCs), which are derived from cardiac biopsies, are also included under second-generation cell types. In contrast to other cell therapies, these cells modulate endogenous stimulation or other paracrine mechanisms for regeneration. The first phase 1 clinical trial with 17 patients CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction (CADUCEUS) using CDCs has resulted in structural but not functional improvement, which was also observed in prior studies with MSCs [11]. Other promising cell therapies are iPSC-CMs and iPSC-derived cardiac tissue grafts containing CMs, endothelial cells (ECs), and mesenchymal cells.

2.3 Cardiac regeneration using PSCs

Bergmann *et al.* identified by postmortem analysis of 29 hearts using stereology that close to 3 billion CMs are present in a healthy heart throughout adult life [7]. In addition, multiple studies have corroborated that an increase in the size of the human heart after the perinatal period is a result of hypertrophy of individual CMs and a stark increase in the number of endothelial and mesenchymal cells by sixfold and eightfold, respectively. While the number of CMs remains stable in otherwise healthy hearts, there is a declining trend in the number of ECs and mesenchymal cells in adults after their 30s [7]. The constant turnover of CMs at the rate of 1% per year in young adults, which declines to 0.5% per year in elderly individuals, is normally adequate to maintain homeostasis. However, an MI that damages up to 25% of LV mass, which may result in a loss of close to a billion cells, can easily tip this balance, leading to severe symptoms of HF [12]. Replacement of the lost cell mass is the underlying principle of interventions focused on exogenous cardiac regeneration. The discovery that adult cells could be reprogrammed to pluripotent state was a major breakthrough in regenerative medicine [13]. This was soon followed by the generation of functional iPSC-CMs [14].

Kehat *et al.* first reported the generation of CMs from embryoid bodies, albeit with low efficiency, in 2001 [15]. However, the process of generation of these cells has significantly improved over the years by the development of protocols using iPSCs that produce CMs with higher efficiency and purity (up to 95%) in serum-free media with chemically defined factors [16, 17].

The development of these reproducible, cost–effective, and scalable protocols set a new milestone toward meeting the objectives of cardiac regeneration, allowing the replacement of close to a billion ventricular-type CMs in heart tissue. The earliest reported regeneration experiments using PSC-derived CMs (ESC-CMs and iPSC-CMs)

were performed on noninjured rodent hearts and primarily focused on cell engraftment [18]. A series of experiments followed these studies by inducing MI in rodents [19, 20]. Almost all these studies were conducted in the acute and subacute post-MI period, with analysis within 4 weeks of cell injections and eventually translated to large-animal models. However, preclinical studies in both small and large animals, including nonhuman primates, were inconclusive, with varying results for functional recovery [21–24]. In fact, although most studies showed graft survival and some structural improvement, it remained unclear whether the cells electrically and mechanically coupled with the host myocardium.

The first evidence of electrical/mechanical coupling of injected CMs resulted from experiments conducted by Shiba *et al.* [25]. Using human-embryonic stem cells (h-ESCs) with a genetically encoded fluorescent calcium sensor (GcAMP3), they demonstrated that there was a modest degree of coupling of injected CMs in the subacute phase of myocardial damage in guinea pigs. Chong *et al.* from the same group later replicated this study using pigtail macaques (*Macaca nemestrina*) – *M. nemestrina*, with evidence of more consistent engraftment, electrical integration, and a modest improvement in functional performance [23]. In additional experiments, Dr. Shiba *et al.* additionally confirmed a moderate to significant improvement in cardiac function and electrical integration of allogenic transplanted iPSC-CMs in cynomolgus monkeys using further enhanced methods [24]. However, while electromechanical synchrony was observed during spontaneous beating in these experiments, there was evidence of dyssynchrony and delayed propagation in grafts with higher pacing rates. In fact, one of the main concerns from this type of cell therapy is the increased arrhythmogenicity, which is commonly observed and most likely arises from dyssynchrony of the engrafted cells. Additionally, there is conflicting evidence for increased arrhythmogenicity after cell injections, with more prevalence noted with delayed injections (28 days vs. 7–14 days). This observation is, perhaps, also related to the animal model used and the number of cells injected with each experiment since incidence was observed to be higher in models using rhesus monkeys with a large number of injected cells (10^9). Moreover, existing studies were limited by a lack of protocols for the generation of ventricular specific CMs, so injected cells were a mix of nodal, atrial, and ventricular CMs in these experiments. Ongoing development in this field continues to show promise and is aimed at the development of protocols for generating the specific subtypes of CMs, such as sinoatrial-node-like pacemaker cells [26].

As research in this field and protocols for specific cell subtypes continue to be developed, an additional hurdle to keep in mind in cell therapy experiments is the level of maturation of PSC-derived CMs that can be achieved in tissue culture, which have shown more fetal-like than adult-like phenotypes [27–29]. In fact, low engraftment, lack of functional and structural improvement, as well as increased arrhythmicity may be attributable, at least in part, to the immaturity of PSC-CMs prior to implantation. As differentiation protocols have continued to be developed and while a small degree of maturation can be observed postimplantation, efforts are being

made to promote the maturation of PSC-CMs both *in vitro* and *in vivo*. Some of the suggested strategies have included coculture with other cell types (i.e., fibroblasts, ECs) [30–32], activation of cardiac maturation pathways with hormones [33, 34], electrical/mechanical stimulation [35, 36], and culture/codifferentiation in a microtissue environment constructed with different cardiac cell types [37, 38].

An additional barrier to implementation has been the lack of information on methods for optimal cell delivery, cell retention, and survival. Currently, direct injection into the myocardium is the most commonly used mechanism for implanting cells to the host tissue. Physical stimulation, such as heat-shock treatment and hypoxic preconditioning, has also been used to improve cell survival once injected. Using these methods, most studies estimate an average long-term survival and engraftment of approximately 5–10% of injected cells [39]. Laflamme *et al.* have developed a "pro survival cocktail" that has been subsequently shown by multiple groups to be effective in increasing the survival of implanted grafts [19, 23, 25]. One of the key components in this cocktail is Matrigel, a gelatin protein mix that can prevent cell death due to anoikis, a programmed cell death of attachment-dependent cells that is induced by detachment and that occurs if these cells are removed from their normal extracellular matrix (ECM) environment. However, Matrigel is generally derived from mouse sarcoma cell cultures and thus is an unlikely candidate for clinical application in human cell therapies. Other matrix alternatives such as hyaluronan hydrogels (such as Hystem-C) are promising replacements for Matrigel, and a variety of such hydrogels are currently in various stages of approval by regulatory agencies in the United States and European Union (https://www.accessdata.fda.gov/cdrh_docs/pdf13/K134037.pdf). Other types of three-dimensional (3D) scaffolds that mimic ECM are also being evaluated, with promising results as far as retention of injected cells is concerned. Some of these strategies have included the use of decellularized scaffolds (bovine hearts) [40, 41], natural biomaterials such as collagen [42], or synthetic materials such as polyethylene glycol [43].

In healthy cardiac tissues, the ECM serves as an anisotropic structural scaffold that guides cellular alignment and organization to facilitate force transduction, intracellular communication, electrical conduction, recruitment of progenitor cells, and metabolic exchange. However, the ECM also contributes to adverse cardiac remodeling associated with HF [44]. Most recently, Chiou *et al.* suggested that the mechanical properties imparted by the ECM are critical in coordinating the heartbeat in embryonic hearts, rather than spreading of electrical activity through gap junctions, as currently believed [45]. This group further proposed that a minimum level of ECM stiffness by maturation is necessary for these cells to drive cardiac tissue regeneration. To address this, commercially available products such as the CorMatrix ECM delivery system could potentially be modified for coinjection of CMs with tissue scaffold materials [46]. There exist a variety of natural and synthetic materials that could be used as cellular scaffolds, but as they are made up of complex materials, it is possible that they would induce foreign body reactions and stimulation of fibroblasts in

human systems [47]. Additionally, most synthetic materials such as poly-L-lactic acid or polylactic glycolic acid do not possess the tensile strength of native cardiac ECM [48]. As such, these materials can be successfully and reproducibly made to deliver cells to the affected tissue but may not provide long-term support. For these reasons, Ng *et al.* reported the importance of native cardiac ECM in directing differentiation of PSCs as follows: decellularized hearts retain not only the composition but also their mechanical properties, which can enhance cell differentiation [49].

As an added hurdle to implementation, immunogenicity from injected IPS cells is expected but unavoidable in allogeneic cell transplantation experiments. Current immunosuppressive regimens, although effective in preventing rejection of these cells, come with the price of an increased risk for infections. Consequently, scaffold-free approaches with microtissues and cell sheets are also being investigated in parallel.

Further efforts are being made at 3D bioprinting of patches that may not only support cellular differentiation and vascularization of the patches but also better mimic the mechanical properties and architecture of the damaged organ, allowing for enhanced grafting [50]. In fact, Zhang *et al.* are exploring multiple 3D bioprinting technologies such as stereolithography, bioplotting, electrospinning, and inkjet bioprinting that may offer better control over the graft composition, mechanical properties, biocompatibility, and biodegradation of these structures over time that may enhance cell differentiation and improve damaged tissue regeneration [51–53]. At the moment, delivery of cells as microtissues appears to have an advantage in terms of handling and delivery at the target area using minimally invasive techniques [54], but further studies are needed to confirm.

As a final consideration, while teratoma formation from injected cells has always been a concern for the scientific community as its potential increases with enhanced cell retention and engraftment, to date, none of the research groups have identified cardiac or extracardiac abnormal tumors due iPSC-CMs, even after long-term follow-up in animal models [39]. Teratoma formation is expected when non-CM-type cells are present in the injected cell solution. Therefore, achieving very-high-purity cell therapy injections will be a critical prerequisite for human experiments.

2.4 Translational models of HF

Advanced HF is a condition with very high mortality involving multiple organ systems that has limited nonsurgical treatment options [55]. Clinical research in HF patients is challenging due to the severity of their illness and acuteness of their initial presentation. The introduction of any new therapeutic interventions should be evaluated with the utmost scrutiny as even minor adverse events can significantly increase mortality and morbidity outcomes in this patient population. Often, the slow progression of the disease prevents patients from seeking medical care in the early stages, where it could potentially be reversed with optimal medical treatment. HF in humans

is the result of a myriad of complex pathologies and clinical conditions, with the most common etiologies being ischemic heart disease and primary hypertension. In most cases, there are multiple risk factors involved, which in turn alter disease progression and outcomes. For these reasons, development of any novel HF therapy has involved extensive translational studies with animal models as a substitute for testing in human systems. While it is easy to replicate an acute coronary event to induce HF in an otherwise healthy animal, this consists only of a single time point in the myriad of events that contribute to the progression of coronary artery disease that develops over a human lifetime. Genetic, physiologic, and structural dissimilarities may also impede the development of successful animal models for HF. Despite these limitations, an adequately designed animal model can not only help identify novel pathways of disease progression but also aid in developing targeted therapeutic interventions. Ideally, such an animal model would replicate changes in autonomous system responses (increased sympathetic tone), volume and pressure changes inside the heart, as well as changes at the cellular level (cytokines, differential gene expression, etc.).

2.5 Large animal models

Although mouse models provide significant insights into cardiovascular biology, there exist significant differences in heart rate, oxygen utilization, adrenergic receptor ratios, and functional/regulatory proteins between mouse and larger primates [56–58]. Murine models are relatively inexpensive, and their genetic manipulation can be easily achieved, but it can be difficult to use them to draw parallels to human studies. However, murine studies should still be preferred as preliminary experiments before more complex and expensive larger animal models can be implemented.

2.5.1 Dilated cardiomyopathy models

Dilated cardiomyopathy (DCM) is the most common terminal phenotype of human HF. A plethora of clinical conditions, the most common being coronary disease, MI, and primary cardiomyopathies from genetic mutations in CMs, lead to DCM, which is a pathological condition characterized by a reduction in systolic function, ventricular dilatation, higher filling pressure in ventricles, and often reduced ventricular wall thickness. With increased disease severity, there might also be an associated dilatation of mitral and tricuspid valve annuli, leading to mitral and tricuspid regurgitation. The adverse remodeling and elevated filling pressures observed in DCM are driven by an activation of neurohormonal systems, which helps maintain cardiac output at the cost of structural damage that often continues even after resolution of the initial insult

sometimes involving even remote myocardium [59, 60]. The most notable phenotypic feature of a failing LV is the change in shape from the usual prolate ellipse to a spherical geometry.

An ideal DCM animal model should replicate the structural and molecular changes involved in the human pathology of the condition. Key features of the model should include ventricular wall dilatation, relative wall thinning with eccentric hypertrophy, diminished contractility and lusitropy, and reduced contractile and lusitropic reserve under stress [61]. LV remodeling in animals often happens without antecedent clinical features from elevated filling pressures with compromised blood flow. Experimental DCM phenotypes also respond differently to interventions. For example, tachycardia-induced cardiomyopathy is reversed, at least partially, when controlling the original arrhythmia or cessation of pacing as soon as the condition is identified [62]. However, myocyte hypertrophy and fibrosis are not typically seen in pacing-induced DCM despite phenotypic and hemodynamic changes similar to DCM induced by other type of injuries [63].

Regional injury by epicardial coronary ligation is routinely used in large-animal models to study post-MI remodeling and progression of DCM. Porcine and ovine models are generally used because they have a predictable area of distribution of coronaries compared to the canine myocardium, which has extensive collaterals making the defined area of injury practically difficult to obtain [64].

Alternatively, microembolization induced by polystyrene beads has also been used successfully in sheep and dogs to induce DCM. In contrast to epicardial ligation, microembolization causes a severe perfusion contraction mismatch and profound inflammatory reactions in the involved coronary distribution [65]. The changes from a single embolization injury are mostly reversible, and repeated microembolization is often necessary over a prolonged period (10 weeks) to induce a DCM model that closely resembles ischemic cardiomyopathy in humans.

Chronic tachycardia-induced cardiomyopathy is a well-documented clinical occurrence in humans [66]. Rapid pacing in atria or ventricle for 3–4 weeks has been used successfully in large animals to induce biventricular dysfunction. By adjusting the pacing rate or duration, the degree of mechanical dysfunction can be controlled in a highly predictive manner. Pacing-induced cardiomyopathy causes severe mechanical dysfunction involving both chambers with spherical dilatation. The structural, hemodynamic, and mechanical changes in this model closely resemble human chronic HF. Unfortunately, most of the changes induced by this model are reversible on cessation of pacing and there is a significant recovery of ejection fraction with 24–48 hours and it nearly normalizes within 2 weeks, precluding the use of this model in the evaluation of regenerative therapies [62].

Lastly, administration of doxorubicin by either the intracoronary or the intravenous route has been used to induce toxic-DCM models in sheep and cows [67]. However, this model is limited by its variable reproducibility and severe systemic

side effects, leading to increased animal death, which are major hurdles to implementation.

2.5.2 Surgical induction of myocardial ischemia or infarction

Coronary artery ligation has been used habitually for inducing ischemia and infarction in various large-animal models. Although technically easy, acute coronary ligation is often associated with fatal ventricular arrhythmias and significant mortality intraoperatively in large animals. There are substantial intraspecies variations in the incidence of fatal arrhythmia in acute coronary ligation, with up to 50% incidence in dogs and sheep [68, 69] but only in 20% of pigs. In pig models, the use of prophylactic beta-blocker therapy has been shown to reduce the incidence and duration of these arrhythmias substantially [70]. On reviewing a variety of animal models, it can be observed that the prevalence of fatal arrhythmias is inversely correlated to the resting heart rate of a given species. Rapid resting heart rate maintains ventricular capture from native conduction pathways over the reentrant circuits or increased automaticity induced by acute infarction. Prophylactic use of a lidocaine bolus (1 mg/kg), followed by its maintenance administration, has also been routinely used in the prevention of arrhythmias in rhesus MI models [23, 24]. Permanent coronary ligation of either the left anterior descending artery or its branches can induce a predictable area of infarction involving a defined LV mass, but in addition to the high perioperative mortality from arrhythmia and surgical complications, permanent coronary occlusion also leaves a poorly perfused scar tissue possibly impeding further regenerative experiments.

At this time, ischemia-reperfusion (I/R) models may be the most comparable to human MI as they show promise with rapid revascularization of culprit lesions in infarct-related coronaries, either percutaneously or by open heart surgery. Ischemic preconditioning was first identified by Murry *et al.* in experiments on canine myocardium that resulted in reperfusion injury after revascularization [71–73]. The microvascular injury following reperfusion is believed to increase the area of myocardial damage and infarct size. In animal models, this can be reproduced either with a catheter-based intervention or by open thoracotomy using controlled ligation of the selected coronary vessel/s [23, 24]. However, animal studies have failed to show consistent and reproducible areas of damage affected by the I/R model [74]. Nevertheless, a close resemblance to post-revascularization myocardium in humans and the return of macroscopic circulation with the residual damage that leads to LV remodeling make I/R an attractive model of cell-based regenerative experiments.

HF models in large animals using volume overload to mimic volume overload hypertrophy leading to LV dysfunction observed in humans typically involves the creation of an arteriovenous fistula and disrupting mitral or aortic valves [75]. The utility of this model is, however, is limited to evaluation of specific type of HF associated with disruption of mitral valve apparatus in humans.

2.6 Assessment of animal models

Phenotypic evaluation in animal models typically involves morphological assessment using echocardiography or magnetic resonance imaging at selected intervals after induction of myocardial damage. *In vivo* functional assessment using invasive hemodynamic monitoring with dobutamine stress is necessary if the study seeks to evaluate regenerative therapeutics that aims at improving cardiac reserve and function [61]. Isolated myocyte, muscle, or perfused heart preparations can also be employed to prove myocyte contraction status along with appropriate molecular, biochemical, and histopathologic studies to document any effects from therapeutic intervention. After an MI, the remaining CMs compensate for the reduced function provided that the loss of ventricular mass is less than 50% [76]. From clinical studies in humans, a loss of more than 40% of LV mass may precipitate cardiogenic shock from decompensation. Since the ejection fraction is considered constant across mammalian species, this should be considered in study design [77]. Careful planning of experiments is needed to limit the area of myocardial damage to avoid precipitation of cardiogenic shock and arrhythmias. At the same time, the myocardial injury should be significant enough to cause a marked reduction in ejection fraction so that improvement can be demonstrated with the specific intervention or therapy.

2.7 Conclusions

As HF remains one of the leading causes of death in the United States and globally, with no effective long-term treatment strategies, parallel efforts are being made to develop novel therapeutic approaches to improve blood flow to the heart. The scientific community has increasingly relied on preclinical large-animal models to test the feasibility of innovative treatments for cardiovascular regeneration and is continually developing new technologies to address the issue, making significant strides toward improved solutions. In this chapter, we have provided an overview of the large-animal models available and the therapies being investigated, as well as reviewed the results obtained that pave the way for breakthrough treatment strategies with a discussion on the hurdles that must be overcome for implementation in clinical trials.

2.8 References

[1] Ponikowski P, Voors AA, Anker SD, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure. Rev Esp Cardiol (Engl Ed) 2016:69:1167.
[2] Braunschweig F, Cowie MR, Auricchio A. What are the costs of heart failure? Europace 2011;13(Suppl 2):ii13–7.
[3] Spoon DB, Psaltis PJ, Singh M, et al. Trends in cause of death after percutaneous coronary intervention. Circulation 2014;129:1286–94.

[4] Jhund PS, McMurray JJ. Heart failure after acute myocardial infarction: a lost battle in the war on heart failure? Circulation 2008;118:2019–21.
[5] Mollova M, Bersell K, Walsh S, et al. Cardiomyocyte proliferation contributes to heart growth in young humans. Proc Natl Acad Sci U S A 2013;110:1446–51.
[6] Lazar E, Sadek HA, Bergmann O. Cardiomyocyte renewal in the human heart: insights from the fall-out. Eur Heart J 2017;38:2333–42.
[7] Bergmann O, Zdunek S, Felker A, et al. Dynamics of cell generation and turnover in the human heart. Cell 2015;161:1566–75.
[8] Fernandez-Aviles F, Sanz-Ruiz R, Climent AM, et al. Global position paper on cardiovascular regenerative medicine. Eur Heart J 2017;38:2532–46.
[9] Traverse JH, Henry TD, Pepine CJ, et al. Effect of the use and timing of bone marrow mononuclear cell delivery on left ventricular function after acute myocardial infarction: the time randomized trial. JAMA J Am Med Assoc 2012;308:2380–9.
[10] Sürder D, Manka R, Lo Cicero V, et al. Intracoronary injection of bone marrow–derived mononuclear cells early or late after acute myocardial infarction clinical perspective. Effects on global left ventricular function 2013;127:1968–79.
[11] Majka M, Sulkowski M, Badyra B, Musialek P. Concise review: mesenchymal stem cells in cardiovascular regeneration: emerging research directions and clinical applications. Stem Cells Transl Med 2017;6:1859–67.
[12] Gepstein L. Derivation and potential applications of human embryonic stem cells. Circ Res 2002;91:866–76.
[13] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–76.
[14] Zhang J, Wilson GF, Soerens AG, et al. Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res 2009;104:e30–41.
[15] Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 2001;108:407–14.
[16] Burridge PW, Matsa E, Shukla P, et al. Chemically defined generation of human cardiomyocytes. Nat Methods 2014;11:855–60.
[17] Chen VC, Ye J, Shukla P, et al. Development of a scalable suspension culture for cardiac differentiation from human pluripotent stem cells. Stem Cell Res 2015;15:365–75.
[18] Laflamme MA, Gold J, Xu C, et al. Formation of human myocardium in the rat heart from human embryonic stem cells. Am J Pathol 2005;167:663–71.
[19] Laflamme MA, Chen KY, Naumova AV, et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 2007; 25:1015–24.
[20] Caspi O, Huber I, Kehat I, et al. Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. J Am Coll Cardiol 2007;50:1884–93.
[21] Nelson TJ, Martinez-Fernandez A, Yamada S, Perez-Terzic C, Ikeda Y, Terzic A. Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. Circulation 2009;120:408–16.
[22] Shiba Y, Filice D, Fernandes S, et al. Electrical integration of human embryonic stem cell-derived cardiomyocytes in a guinea pig chronic infarct model. J Cardiovasc Pharmacol Ther 2014;19:368–81.
[23] Chong JJ, Yang X, Don CW, et al. Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. Nature 2014;510:273–7.
[24] Shiba Y, Gomibuchi T, Seto T, et al. Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. Nature 2016;538:388–91.

[25] Shiba Y, Fernandes S, Zhu WZ, et al. Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts. Nature 2012;489:322–5.
[26] Protze SI, Liu J, Nussinovitch U, et al. Sinoatrial node cardiomyocytes derived from human pluripotent cells function as a biological pacemaker. Nat Biotechnol 2017;35:56–68.
[27] Fijnvandraat AC, van Ginneken ACG, de Boer PAJ, et al. Cardiomyocytes derived from embryonic stem cells resemble cardiomyocytes of the embryonic heart tube. Cardiovasc Res 2003;58:399–409.
[28] Feric NT, Radisic M. Maturing human pluripotent stem cell-derived cardiomyocytes in human engineered cardiac tissues. Adv Drug Deliv Rev 2016;96:110–34.
[29] Lundy SD, Zhu W-Z, Regnier M, Laflamme MA. Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. Stem Cells Dev 2013;22:1991–2002.
[30] Souders CA, Bowers SLK, Baudino TA. Cardiac fibroblast: the Renaissance cell. Circ Res 2009;105:1164–76.
[31] Pedrotty DM, Klinger RY, Kirkton RD, Bursac N. Cardiac fibroblast paracrine factors alter impulse conduction and ion channel expression of neonatal rat cardiomyocytes. Cardiovasc Res 2009;83:688–97.
[32] Pasquier J, Gupta R, Rioult D, et al. Coculturing with endothelial cells promotes in vitro maturation and electrical coupling of human embryonic stem cell–derived cardiomyocytes. J Heart Lung Transplant 2017;36:684–93.
[33] Krüger M, Sachse C, Zimmermann WH, Eschenhagen T, Klede S, Linke WA. Thyroid hormone regulates developmental titin isoform transitions via the phosphatidylinositol-3-kinase/AKT pathway. Circ Res 2008;102:439–47.
[34] Lin Y, Linask KL, Mallon B, et al. Heparin promotes cardiac differentiation of human pluripotent stem cells in chemically defined albumin-free medium, enabling consistent manufacture of cardiomyocytes. Stem Cells Transl Med 2017;6:527–38.
[35] Ruan J-L, Tulloch NL, Saiget M, et al. Mechanical stress promotes maturation of human myocardium from pluripotent stem cell-derived progenitors. Stem Cells (Dayton, Ohio) 2015;33:2148–57.
[36] Chan Y-C, Ting S, Lee Y-K, et al. Electrical stimulation promotes maturation of cardiomyocytes derived from human embryonic stem cells. J Cardiovasc Translation Res 2013;6:989–99.
[37] Vuorenpää H, Penttinen K, Heinonen T, et al. Maturation of human pluripotent stem cell derived cardiomyocytes is improved in cardiovascular construct. Cytotechnol 2017;69:785–800.
[38] Sekine H, Shimizu T, Hobo K, et al. Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts. Circulation 2008;118:S145–52.
[39] Riegler J, Tiburcy M, Ebert A, et al. Human engineered heart muscles engraft and survive long term in a rodent myocardial infarction model. Circ Res 2015;117:720–30.
[40] Fong AH, Romero-López M, Heylman CM, et al. Three-dimensional adult cardiac extracellular matrix promotes maturation of human induced pluripotent stem cell-derived cardiomyocytes. Tissue Engineering Part A 2016;22:1016–25.
[41] Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: Structure and function. Acta Biomaterialia 2009;5:1–13.
[42] Perea-Gil I, Prat-Vidal C, Gálvez-Montón C, et al. A cell-enriched engineered myocardial graft limits infarct size and improves cardiac function. pre-clinical study in the porcine myocardial infarction model. J Am Coll Cardiol 2016;1:360–72.
[43] Ferreira LS, Gerecht S, Fuller J, Shieh HF, Vunjak-Novakovic G, Langer R. Bioactive hydrogel scaffolds for controllable vascular differentiation of human embryonic stem cells. Biomaterials 2007;28:2706–17.
[44] Libby P, Lee RT. Matrix matters. Circulation 2000;102:1874–6.

[45] Chiou KK, Rocks JW, Chen CY, et al. Mechanical signaling coordinates the embryonic heartbeat. Proc Natl Acad Sci U S A 2016;113:8939–44.
[46] Slaughter MS, Soucy KG, Matheny RG, et al. Development of an extracellular matrix delivery system for effective intramyocardial injection in ischemic tissue. ASAIO J 2014;60:730–6.
[47] Kelm JM, Fussenegger M. Scaffold-free cell delivery for use in regenerative medicine. Adv Drug Delivery Rev 2010;62:753–64.
[48] Taylor DA, Parikh RB, Sampaio LC. Bioengineering hearts: simple yet complex. Curr Stem Cell Rep 2017;3:35–44.
[49] Ng SL, Narayanan K, Gao S, Wan AC. Lineage restricted progenitors for the repopulation of decellularized heart. Biomaterials 2011;32:7571–80.
[50] Cui H, Zhu W, Holmes B, Zhang LG. Biologically inspired smart release system based on 3D bioprinted perfused scaffold for vascularized tissue regeneration. Adv Sci 2016;3:1600058.
[51] Zhu W, Webster TJ, Zhang LG. How can 3D-printing be a powerful tool in nanomedicine? Nanomed 2018;13:251–3.
[52] Miao S, Castro N, Nowicki M, et al. 4D printing of polymeric materials for tissue and organ regeneration. Materials Today 2017;20:577–91.
[53] Shida M, Haitao C, Margaret N, et al. Photolithographic-stereolithographic-tandem fabrication of 4D smart scaffolds for improved stem cell cardiomyogenic differentiation. Biofabrication 2018;10:035007.
[54] Gunter J, Wolint P, Bopp A, et al. Microtissues in cardiovascular medicine: regenerative potential based on a 3D microenvironment. Stem Cells Int 2016;2016:9098523.
[55] Yancy CW, Jessup M, Bozkurt B, et al. 2013 ACCF/AHA guideline for the management of heart failure: executive summary: a report of the American College of Cardiology Foundation/ American Heart Association Task Force on practice guidelines. Circulation 2013;128:1810–52.
[56] Stucker S, Kresin N, Carrier L, Friedrich FW. Nebivolol desensitizes myofilaments of a hypertrophic cardiomyopathy mouse model. Front Physiol 2017;8:558.
[57] Ginis I, Luo Y, Miura T, et al. Differences between human and mouse embryonic stem cells. Dev Biol 2004;269:360–80.
[58] Haghighi K, Kolokathis F, Pater L, et al. Human phospholamban null results in lethal dilated cardiomyopathy revealing a critical difference between mouse and human. J Clin Invest 2003;111:869–76.
[59] Jessup M, Brozena S. Heart failure. N Engl J Med 2003;348:2007–18.
[60] Mann DL, Bristow MR. Mechanisms and models in heart failure: the biomechanical model and beyond. Circulation 2005;111:2837–49.
[61] Houser SR, Margulies KB, Murphy AM, et al. Animal models of heart failure: a scientific statement from the American Heart Association. Circ Res 2012;111:131–50.
[62] Shinbane JS, Wood MA, Jensen DN, Ellenbogen KA, Fitzpatrick AP, Scheinman MM. Tachycardia-induced cardiomyopathy: a review of animal models and clinical studies. J Am Coll Cardiol 1997;29:709–15.
[63] Falk V, Garbade J, Walther T. Experimental Models of Heart Failure. In: Dhein S, Mohr FW, Delmar M, eds. Practical Methods in Cardiovascular Research. Berlin, Heidelberg: Springer Berlin Heidelberg, 2005, 83–110.
[64] Maxwell MP, Hearse DJ, Yellon DM. Species variation in the coronary collateral circulation during regional myocardial ischaemia: a critical determinant of the rate of evolution and extent of myocardial infarction. Cardiovasc Res 1987;21:737–46.
[65] Heusch G, Kleinbongard P, Bose D, et al. Coronary microembolization: from bedside to bench and back to bedside. Circulation 2009;120:1822–36.
[66] Dandamudi G, Rampurwala AY, Mahenthiran J, Miller JM, Das MK. Persistent left ventricular dilatation in tachycardia-induced cardiomyopathy patients after appropriate treatment and normalization of ejection fraction. Heart Rhythm 2008;5:1111–4.

[67] Bartoli CR, Brittian KR, Giridharan GA, Koenig SC, Hamid T, Prabhu SD. Bovine model of doxorubicin-induced cardiomyopathy. J Biomed Biotechnol 2011;2011:758736.
[68] Hood WB, Jr., McCarthy B, Lown B. Myocardial infarction following coronary ligation in dogs. Hemodynamic effects of isoproterenol and acetylstrophanthidin. Circ Res 1967;21:191–9.
[69] Dardenne A, Fernandez C, Wagner A, et al. Benefits of standardizing the treatment of arrhythmias in the sheep (*Ovis aries*) model of chronic heart failure after myocardial infarction. J Am Assoc Lab Anim Sci 2013;52:290–4.
[70] Muller CA, Opie LH, Pineda CA, Peisach M. Bucindolol, a beta blocker, decreased ventricular fibrillation and maintained mechanical function in a pig model of acute myocardial ischemia. Cardiovasc Drugs Ther 1992;6:233–7.
[71] Reffelmann T, Kloner RA. The no-reflow phenomenon: a basic mechanism of myocardial ischemia and reperfusion. Basic Res Cardiol 2006;101:359–72.
[72] Hausenloy DJ, Barrabes JA, Botker HE, et al. Ischaemic conditioning and targeting reperfusion injury: a 30-year voyage of discovery. Basic Res Cardiol 2016;111:70.
[73] Hausenloy DJ, Yellon DM. Ischaemic conditioning and reperfusion injury. Nat Rev Cardiol 2016;13:193–209.
[74] Sjaastad I, Grund F, Ilebekk A. Effects on infarct size and on arrhythmias by controlling reflow after myocardial ischaemia in pigs. Acta Physiol Scand 2000;169:195–201.
[75] Kleaveland JP, Kussmaul WG, Vinciguerra T, Diters R, Carabello BA. Volume overload hypertrophy in a closed-chest model of mitral regurgitation. Am J Physiol 1988;254:H1034–41.
[76] Anversa P, Beghi C, Kikkawa Y, Olivetti G. Myocardial infarction in rats. Infarct size, myocyte hypertrophy, and capillary growth. Circ Res 1986;58:26–37.
[77] Holt JP, Rhode EA, Peoples SA, Kines H. Left ventricular function in mammals of greatly different size. Circ Res 1962;10:798–806.

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3 Cell-free therapy with stem cell secretions: protection, repair, and regeneration of the injured myocardium

Abstract: Experimental animal studies conducted during the last two decades have supported and reflected on the myocardial regenerative potential of bone-marrow-derived stem/progenitor cells. Although the encouraging results emanating from clinical trials on bone marrow cells, which are now in phase III, provide little evidence to support their cardiogenic differentiation post transplantation in the patient heart, the beneficial effects of stem-cell-based therapy are being predominantly attributed to the paracrine activity of the donor bone marrow cells. Bone marrow cells secrete a plethora of trophic factors, including chemokines, cytokine, growth factors, microRNAs, microvesicles, etc., as part of their secretome, which is unique for each of its constituent cell lineages but variable in composition due to altered responsiveness of the secreting cells to their ever-changing microenvironment. Given the important role of paracrine secretions in various stem cell functions, there is a growing inclination to use stem cell conditioned medium either alone or in combination with cell-based therapy for myocardial regeneration. This chapter discusses in depth the usefulness of cell-free therapy for myocardial regeneration and elaborates on how various processes ranging from host cardiomyocyte survival to mobilization and homing-in of the host progenitor cells to heart are stimulated by treatment with a cell-free conditioned medium to ensure preservation as well as recovery of the recipient heart function.

Key Words: Bioactive, Cytokines, Differentiation, GF, Heart, Infarction, Microenvironment, Paracrine, Stem cells

3.1 Introduction

The existence of resident cardiac stem cells (CSCs) in the heart [1, 2] and the ability of cardiomyocytes to reenter into the cell cycle in response to myocardial injury have challenged the long-standing dogma that the heart is a postmitotic organ [3]. Nevertheless, both of these activities, in tandem, can only inefficiently regenerate the injured heart and replenish the dead myocytes [4]. Among the emerging modern-day therapeutic strategies, cell therapy has progressed to clinical applications as a therapeutic intervention to support the insufficient intrinsic repair mechanism in the injured heart [5–8]. Despite that cell therapy approach has shown promise, its ability to adopt cardiac phenotype and functionally integrate with the host myocytes remains contentious [9–12]. Moreover, there are also reports that the transplanted cells are lost

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due to either apoptosis or leakage from the site of the cell graft in a few weeks' time after transplantation [13]. On the same note, although increased angiogenic response is observed in the heart after native or genetically modified stem cell transplantation, most studies have failed to evidence their actual integration into the neovascular structures [14–16]. Despite these limitations, improved cardiac function after stem cell transplantation has been observed. Recent studies suggest that the transplanted stem cells interact with the host cardiac tissue *via* the release of bioactive molecules as part of their paracrine activity that modulate the microenvironment at the site of the cell graft and contribute toward protection, repair, and regeneration processes in the injured heart [17]. Similarly, in the event of myocardial injury, the cardiomyocytes themselves release trophic factors *via* a feedback loop between cardiomyocytes, resident CSCs, and bone marrow cells (BMCs) to ensure that the latter two cell types get activated, home in to the site of injury, and participate in the repair process [18, 19].

The paracrine activity of cells, besides endocrine and juxtacrine mechanisms, constitutes an integral part of cell-to-cell communication and is being exploited in regenerative medicine as an alternative mechanism to explain the beneficial effects of stem cell therapy [20]. Moreover, cells also manage their microenvironment by the release of biologically active molecules and cross-talk with their counterparts in the nearby vicinity by sending chemical signals [21]. However, the effectiveness of the secreted bioactive molecules is restricted to a very short distance. This cross-talk between cells *via* chemical signals tightly regulates most biological processes, including cell protection, survival, apoptosis, growth, proliferation, and differentiation by initiating signaling pathways that are specifically needed for each of these processes. For example, coculture between nonmyocytes and cardiomyocytes significantly reduces doxorubicin-induced cardiomyocyte apoptosis [22]. While elucidating the underlying mechanism of the cytoprotective effects of nonmyocytes on cardiomyocytes, it was observed that nonmyocytes secreted endothelin-1, which phosphorylated extracellular signal-related kinases (ERK)/cAMP response element-binding protein (CREB) antiapoptotic signaling and increased the prosurvival B-cell lymphoma 2 (Bcl2) protein in the cardiomyocytes to support their survival. Similarly, cardiac ECs secrete neuregulin-1β (NRG) to promote cardiomyocyte survival *via* activation of NRG/erbB4 signaling [23].

3.2 The secretome of each stem cell type is unique

The different cell types constituting the heart exhibit specific proteomic constitution [24]. Similarly, each stem cell type secretes a wide array of trophic factors that together constitute the secretome unique for that stem cell population under a given set of conditions. The cell-type-specific composition of secretome is further supported by recent data that showed that trophic factors secreted by the undifferentiated bone-marrow (BM)-derived mesenchymal stem cells (MSCs) were different from their chondriogenic and osteogenic derivative cell lineages [25]. Whereas undifferentiated MSCs secrete

many proangiogenic factors, the osteogenic and chondriogenic derivatives stop secreting these factors, and instead, their secretome is rich in antiangiogenic growth factor (GF). Similarly, adipocytes show a secretome profile, which is diverse from their preadipocyte precursors. Adipocytes and preadipocytes also diverge in the secretome composition in response to hypoxia mimetics [26]. Adipose-tissue-derived stem cells (ADCs) also secrete adipokines as part of their secretome, which act in an autocrine fashion to promote adipogenic differentiation of the cells [27]. These data provide evidence of dynamic changes in paracrine secretions of the stem cells depending upon their differentiation status. Interestingly, these cells also have different secretome profiles in response to different levels of oxygen in their microenvironment [28]. Even the same cell types isolated from two different animal strains have different paracrine activity profiles. For example, paracrine secretions from MSCs isolated from C57/BL6 and Balb/c mouse strains differ in vascular endothelial growth factor (VEGF) expression after exposure to ischemia [29]. Henceforth, post engraftment in an experimental model of hindlimb, the angiogenic response was significantly higher with C57/BL6-derived MSCs as compared to Balb/c-derived MSCs. These differences are attributed to the genetic makeup of the donor animal from which the cells have been obtained; i.e., polymorphism in the *cis*-acting VEGF gene in Balb/c mice on chromosome 17 significantly reduces VEGF gene transcription as well as expression under ischemic conditions. Similarly, paracrine secretions also differ between MSCs derived from different tissues of the same animal. Adipose-tissue-derived MSCs (AMSCs) have completely different angiogenic GF expression profiles in comparison with BMMSCs under the same set of culture conditions [30]. A comparative analysis between human MSCs isolated from different tissue sources, i.e., adipose, BM, and dermal tissues, differ in their cytokine and GF expression profile [31]. AMSCs secrete copious amounts of insulin-like growth factor-1 (IGF-1), VEGF, and interleukin (IL)-8 and therefore show superior functionality as compared to their counterparts in terms of endothelial tubulogenic response. Such differences have been attributed to a specific niche-dependent programming of the cells in their natural habitat.

Advancement in proteome profiling techniques has enabled the analysis of conditioned media (CM) from stem cell culture and shows that the secretome mostly includes GFs, cytokines, and chemokines [32]. Additionally, release of microRNAs as an integral part of their paracrine activity has also been reported [33]. Although there is no comprehensive secretome profile of stem cells available so far, some of the commonly reported trophic factors secreted by different stem cells are listed in Tables 3.2.1 and 3.2.2 [34–50]. The variability observed in the secretome profile of different stem cells is obvious and may be related to a multitude of factors ranging from extreme sensitivity of the cells to the ever-changing microenvironmental cues and their differentiation status (as discussed in the last paragraph) to the suboptimal detection protocols that still suffer from technical and methodological inadequacies. However, studies published to date have reported the composition of the secretome restricted to its relevance with the working hypothesis of the study, and the detected trophic

Tab. 3.2.1: Proteome profiling from different stem cell types without stimulation.

Stem cells	Trophic factors (in CM)	Reference
Amniotic fluid MSC	IL-8, IL-6, TGF-β, TNFRI, VEGF, EGF	Yoon et al., 2010 [34]
Murine BM-MSC	VEGF-α, IGF-1, EGF, KGF, Ang-1, SDF-1, PDGF, MIP-1α and -β, TGF-β1, sTNF-1, and EPO	Chen et al., 2008 [35]
Rat BM-MSC	VEGF, CINC-3, osteopontin, GH, EG-VEGF/PK1, BNDF, FGF-BP, Fas ligand/TNFSF6, TIMP-1 and -2, NGF, MMP-13, TAL-1A, CXCR4, MDC, TRAIL, TROY	Cantinieux et al., 2013 [36]
Pig MSC	VEGF, endothelin, epiregulin, galectin-3, Smad-5, sFRP-1 and -5	Nguyen et al., 2010 [37]
Human cord blood MSC	ENAP-78, GM-CSF, GRO, IL-1β, -6, and -8, MCP-1, OSM, VEGF, FGF-4, FGF-7, FGF-9, GCP-2, IGF-BP-1, -2, -3, and -4, PlGF, TIMP-1 and -2	Liu et al., 2005 [38]
Human BMC	IP-10, LIF, MIF, MIP-3α, osteoprotegerin, PARC, TGF-β2/3, FGF-9, angiogenin, VEGF, HGF, IGF-1, IL-10, CCL2, 23, and 24, CXCL6, 12, and 13	Korf-Klingebiel et al., 2008 [39]
Human CF	VEGF, GRO/KC, MCP-1, leptin, MIP-1α, IL-6, IL-10, -12, and -17, TNF-α	LaFramboise et al., 2007 [40]
Human EPC	VEGF, HGF, PDGF, SDF-1, IL-8, angiogenin	DI Santo et al., 2009 [41]
Human ESC	TSP-1, IGF-BP-1 and -2, AFP, MMP-1, -2, and -9, TIMP-1, tPAI-1, MCP-1, HGF-1, kallikrein 10, TGFb-l, HSP-70, MIF, CAM, IL-1a, 6 & 8, IL-6 and -8, IL-1RA	LaFramboise et al., 2010 [42]

AFP= alpha fetoprotein; Ang-1= angiopoietin-1; BMC= bone marrow cell; BM-MSC= bone marrow derived mesenchymal stem cell; BNDF= brain-derived neurotrophic factor; CAM=cell adhesion molecule; CCL= chemokine ligand (C-C motif); chemokine (C-X-C motif) ligand; CF= cardiac fibroblast; CINC-3= cytokine-induced neutrophil chemoattractant-3; CXCL=C-X-C motif ligand CXCR4=C-X-C chemokine receptor type 4; EGF=epidermal growth factor; EG-VEGF/PK1=endocrine gland-derived vascular endothelial growth factor/prokineticin 1; ENAP= endothelial neutrophil activating protein-79; EPC= endothelial progenitor cells; EPO= erythropoietin; ESC= embryonic stem cell; FGF= fibroblast growth factor; FGF-BP= fibroblast growth factor-binding protein; GCP= granulocyte chemotactic protein; GH= growth hormone; GM-CSF=granulocyte-macrophage colony-stimulating factor; GRO= growth-related oncogene; GRO/KC= growth-regulated α-protein; HGF= hepatocyte growth factor; HSP= heat shock protein; IGF= insulin-like growth factor; IGF-BP= insulin-like growth factor binding protein; IL= interleukin; IP= interferon inducing protein; KGF= keratinocyte growth factor; LIF=leukemia inhibitory factor; MCP-1= monocyte chemotactic protein-1; MDC=macrophage-derived chemokine; MIF= macrophage migration inhibitory factor; MIP= macrophage inflammatory protein; MMP= matrix metalloproteinase-13; NGF= nerve growth factor; OSM= oncostatin M; PARC= pulmonary and activation regulated chemokine; PDGF= platelet-derived growth factor; PlGF= phosphatidylinositol glycan complementation class F; sFRP= secreted frizzled proteins; SDF-1= stromal-cell-derived factor-1; TAL1A=T-cell acute lymphocytic leukemia protein 1A; TGF= transforming growth factor; TIMP= tissue inhibitor of metalloproteinase; TNF= tumor necrosis factor; TNFRI=TNF receptor I; TNFSF6=TNF ligand superfamily member 6; tPAI-1: tissue plasminogen activator inhibitor-1; TRAIL=TNF-related apoptosis-inducing ligand; TROY=TNF receptor superfamily member 19; TSP-1=thrombospondin 1; VEGF= vascular endothelial growth factor.

Tab. 3.2.2: Proteome profiling from different stem cell types after stimulation.

Stem cells	Treatment	Trophic factors (in CM)	Reference
AF-MSCs	Hypoxia (1 or 5% O_2)	VEGF-R, FLT1, IL-4, -6, -15, -17A, -32, and -33, FGF-20, FGFR-2 and -3, FGF-1, MMP-3, collagen-3, fibronectin	Jun et al., 2014 [43]
BM-MSCs	Hypoxia	HSP-90b1, Tpm1 and 2, annexin-1, secretogrannin-3, HIF-1α.	Song et al., 2016 [44]
BM-MSCs	Single gene modification	sFRP-2 and -3, VEGFa, adrenomedullin, HGF-1, PDGF Ang-4, MMP-13, CCL2, angiogenin, MMP-11, TSP-1, CTGF-1	Mirotsou et al., 2007 [45]
Sk. myoblasts	Multi-gene modification	IGF-1, SDF-1, VEGF, HGF-1, sFRP-1, -2, -4, and -5, MMP-3 and -9	Konoplyannikov et al., 2013 [46]
BM-MSCs	Growth factor treatment	IGF-1 and HGF-1	Hahn et al., 2008 [47]
MSCs	Transgenic HSP-20	IGF-1, VEGF, FGF-2	Wang X et al., 2009 [48]
EPCs	Pharmacological	VEGF, IGF, and SDF-1α	Mehmood et al., 2015 [49]
Human CSCs	Mechanical stress	VEGF, bFGF, IL-6, IL-1β, SDF-1α, HGF, IGF-1, and TGF-β1	Kurazumi et al., 2011 [50]

Ang-4= angiopoietin-4; bFGF= Basic fibroblast growth factor; BM-MSCs= bone-marrow-derived mesenchymal stem cell; CCL= chemokine ligand (C-C motif); chemokine (C-X-C motif) ligand; CTGF-1= Connective tissue growth factor-1; EPCs= endothelial progenitor cells; FGF= fibroblast growth factor; FGFR= fibroblast growth factor receptor; FLT1=FMS-like tyrosine kinase 1 (VEGF receptor 1); HGF= hepatocyte growth factor; HIF-1α= hypoxia inducible factor-1α; HSP= heat shock protein; IGF= insulin-like growth factor; IL= interleukin; MMP= matrix metalloproteinase; PDGF= platelet-derived growth factor; sFRP= secreted frizzled protein; SDF-1= stromal-cell-derived factor-1; TGF= transforming growth factor; TSP-1= thrombospondin-1; VEGF= vascular endothelial growth factor; VEGF-R= vascular endothelial growth factor receptor.

factors have been grouped on the basis of their biological activity and functions [36, 51]. Some of the important groups of the trophic factors secreted by stem cells include angiogenic factors, antiapoptotic factors, immunomodulators, antifibrotics, and stimulants of cell migration, homing, proliferation, and differentiation.

3.2.1 Paracrine factors and angiogenesis

Trophic factors with proangiogenic activity, the group of angiocrine factors, constitute one of the most widely studied components of stem cell secretome [52–54]. Each of the different stem cell types secretes a distinct spectrum of angiogenic factors. Some of the more commonly detected proangiogenic factors in the CM include VEGF, angiopoietin-1 (Ang-1), basic fibroblast growth factor (bFGF), IGF-1, stromal-cell-derived factor-1α (SDF-1α), platelet-derived growth factor (PDGF), and hepatocyte growth factor-1 (HGF-1) [41, 53, 55, 56]. The expression of angiotropic factors can be accentuated by physical, pharmacological, and genetic manipulation of the cells. Given the inherent potential of stem cells to serve as a source of angiogenic GFs and cytokines, extensive angiogenic response has been reported during *in vitro* experimental models. These data have formed the basis of stem-cell-based intervention in both small and large experimental animal models with promise as a therapeutic option to achieve biological bypass and restore regional blood flow in the ischemic myocardium [17, 57]. In this regard, endothelial progenitor cells (EPCs) and MSCs have already progressed to clinical trials for therapeutic assessment in patients, albeit with some modest outcome. Both the cell types potentially endure vasculogenic differentiation and get incorporated into the architecture of the newly formed vascular structures [58–61]. Nevertheless, poor survival of the cell graft significantly undermines the effectiveness of cellular angiogenesis. These cells also secrete copious amounts of angiocrines, including GFs, cytokines, and extracellular matrix (ECM) molecules, to promote angiogenic response at the site of the cell graft [62]. Hence, there is a shift in focus to adopt a cell-free approach based on treatment with CM from stem cells [41, 57]. Mechanistically, angiogenesis encompasses a cascade of tightly regulated events that involve the interplay between proangiogenic and antiangiogenic factors and different types of cells, i.e., ECs, smooth muscle cells (SMCs), and fibroblasts. Besides physical participation in the formation of new vascular structures, these cells also secrete various angiogenic trophic factors [63]. For example, fibroblast is one of the major cell types that infiltrate the injured myocardium and secrete various ECM proteins, proteases, angiogenic GFs, and cytokines [64]. Hence, single GF protein or gene therapy based on DNA encoding for a single gene may be less efficient to achieve the desired prognosis. Therefore, a multiple gene delivery approach has been reported to enhance angiomyogenic repair of the infarcted heart, with encouraging results [46]. The cardiomyocytes themselves secrete ischemic stress-induced angiocrine factors, i.e., VEGF, *via* activation of p38

mitogen activated kinase (MAPK), which help them to cross-talk with the repair mechanism in the heart, including neoangiogenesis [65].

Treatment with CM may be a more pragmatic approach due to the concerted participation of its constituent factors to achieve clinically relevant angiogenic responses. CM from a STRO-3$^+$ subpopulation of human BMMSCs is rich in secretable GFs and cytokines with antiapoptotic and proangiogenic potential [66, 67]. Noticeable among the long list of secretable factors are IL-6, VEGF, and monocyte chemoattractant protein-1 (MCP-1) [68] Treatment of human aortic ECs with the CM collected from multipotent stromal cells cultured under hypoxia protected the cells upon subsequent exposure to hypoxic injury and enhanced their angiogenic response *in vitro* [68]. Study of the molecular events showed the activation of phosphatidylinositol-3-kinases (PI3K)/protein kinase B (Akt) signaling pathway in the cells treated with CM. Intramyocardial injection of STRO-3$^+$ cells or treatment with their CM significantly reduced cardiomyocyte apoptosis as well as cardiac fibrosis [69]. More importantly, increased angiogenesis was observed in the infarcted rat heart in response to CM treatment, with clear participation of EPCs and circulating progenitor cells. More recently, the cell-sheet transplantation approach is being preferred to intramyocardial injection of the donor cells [70–72]. Transplantation of a cell sheet of skeletal myoblasts (SkMs) genetically modified to overexpress Bcl2 improved donor cell survival, induced a concomitant improvement in angiogenic response, reduced cardiac fibrosis, and preserved cardiac function [73, 74]. Analysis of the Bcl2 overexpressing SkMs showed that the genetically modified cells had altered paracrine secretions as compared to the wild-type SkMs and expressed a higher level of angiocrine factors, including VEGF and placental growth factor (PlGF). Molecular studies determined Flk1/Flt1 signaling pathway activation as responsible for angiogenesis in response to the release of angiocrine factors. The same group of researchers has reported a cell sheet developed from genetically modified SkMs overexpressing human HGF-1 [75]. The genetically modified SkM cell-sheet was transplanted in an experimental rat model of myocardial infarction (MI). The cell sheet with HGF-1 overexpressing SkMs significantly enhanced angiogenic response in the heart and preserved global cardiac function. As an alternative to genetic modification of stem cells to develop a cell sheet, more recent experimental studies have exploited angiocrine activity of MSCs to stimulate other cell types to improve their therapeutic efficacy. The addition of MSCs to SkM cell-sheets improves their angiogenic mechanics, in addition to improving cell survival in the infarcted rat heart [76]. Parallel *in vitro* experiments showed increased presence of rat HGF-1 and VEGF in the CM from cell sheets containing both MSCs and SkMs. Similar results have also been reported with tissue engineered SMC and EPC bilevel sheets in experimental rodent model of type-I-diabetes induced cardiomyopathy [77].

3.2.2 Paracrine factors and cytoprotection

In addition to angiogenesis, treatment with GF and cytokine-rich CM is cytoprotective for the host cardiac cells. Besides necrosis, which results from loss of cellular

homeostasis in the event of myocardial injury, apoptosis is a predominant mechanism of cardiac cell death which is highly regulated cascade of energy requiring events [78]. It is characterized by cellular and nuclear shrinkage, condensation of chromatin, blebbing of the cell membrane, detachment of cell from its surrounding, and eventual degradation. Various strategies have been adopted to prevent cardiomyocyte apoptosis. In this regard, stem cell therapy is generally perceived to influence the intrinsic repair process *via* the release of paracrine factors that bind with its specific receptors expressed on cardiac cells and induce signal transduction to elicit a diverse array of downstream responses, including antiapoptotic signaling [79]. The antiapoptotic and proliferative effects of the paracrine factors secreted by stem cells have been extensively studied either by treatment of cardiomyocytes with CM from stem cells or in a direct or indirect coculture system between stem cells and cardiomyocytes [80–83]. *In vitro* treatment of rat ventricular cardiomyocytes with CM from hematopoietic stem cells previously cocultured with cardiomyocytes activated Akt to impart cytoprotection. Analysis of the CM showed that it was rich in fibronectin, CCL12, macrophage inhibitory factor, and connexin-40 [84]. Mechanistic studies show that CMs from MSCs activate divergent signaling pathways to support angiogenesis and migration besides improving survival in ECs and cardiomyocytes [54]. Proteomic analysis of the CM showed that it was rich in VEGF, MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, and monokine induced by interferon gamma (IFN-γ) compared to the control media. Similar results were obtained using CM from AMSCs which were genetically modified using nanoparticle-based transgenic overexpression of VEGF [85]. Treatment with the CM significantly enhanced *in vitro* angiogenic response in human umbilical vein endothelial cells. Coculture between $ckit^{+}GATA^{high}$ CSCs and cardiomyocytes significantly improved the survival and contractility of cardiomyocytes [79]. The $ckit^{+}GATA^{high}$ CSCs secreted IGF-1, which acted in a paracrine fashion to activate Akt signaling downstream of IGF-1/IGF-1R ligand/receptor interaction in the cardiomyocytes to promote their survival. Analysis of CM from the cocultured cells showed significantly elevated concentration of IGF-1 protein. These data also point toward the presence of a corresponding interaction between resident CSCs in the heart and their salutary paracrine interaction on cardiomyocytes in the event of myocardial injury as part of the intrinsic mechanisms of cardiac repair and regeneration. Extrapolation of *in vitro* data contributes to understanding the mechanism of *in vivo* antiapoptotic effects and ultimate cardioprotection following stem cell transplantation [81, 86]. The transplanted stem cells release cytokines and GFs to reduce cardiomyocyte apoptosis. As a proof-of-concept, various studies have used cell-free CM for the treatment of injured myocardium [37, 87]. Treatment with concentrated CM revealed significantly reduced apoptosis of cardiomyocytes in the infarct and peri-infarct regions [37].

Apoptosis not only affects cardiomyocytes, it also has an imminent role in loss of cell graft post transplantation. Various strategies have been adopted to enhance donor cell survival in the harsh environment of the infarcted heart. This can be achieved either by preconditioning of the donor cells to boast their resilience [88] or

by using strategies such as immune isolation to enhance their acceptance by the host tissue [89]. Unlike the immuno-isolation approach, which has little effect on the paracrine activity profile of the donor cells, the preconditioning approach significantly impacts their GF and cytokine profile besides activation of survival signaling in the preconditioned cells (PCs) [90]. In an interesting study, 5 million cell beads containing alginate encapsulated MSCs engineered to secrete glucagon-like protein-1 (GLP-1) were transplanted in an experimental pig heart *via* intracoronary injection. Encapsulation was intended to immuno-isolate the cells postengraftment and to enhance cell retention at the site of the cell graft and to serve as a source of GLP-1 as well as the paracrine factors released by the encapsulated MSCs over an extended time period [91, 92]. Histological studies showed significant reduction in deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cardiomyocytes in the infarct and peri-infarct regions, thus attenuating infarct size expansion in GLP-1 secreting MSCs treatment group of pigs.

3.2.3 Paracrine factors and immunomodulation

MSCs derived from various tissue sources such as BM, adipose tissue, umbilical cord Wharton's jelly, etc., also exert immunomodulatory effects besides other therapeutic benefits [93]. These effects have been extensively studied during *in vitro* experimentation and require cell-to-cell contact or it may also occur *via* paracrine release of GFs and cytokines [94]. The array of immunosuppressive factors expressed by MSCs include indoleamine 2,3-dioxygenase (IDO), human leukocyte antigen G (HLA-G), nitric oxide, and ILs. Additionally, MSCs inherently secrete a sufficient amount of transforming growth factor-1β (TGF-1β), a soluble factor, for functional activation of T-cells to induce Treg [94]. The phenotypic and functional characteristics, including the immunomodulatory properties, of MSCs are influenced by various factors ranging from the tissue source of the cells to the method of their isolation and preparation [95, 96]. BMMSCs and the ones isolated from exfoliated deciduous teeth differ from each other in their interaction with co-cultured T-lymphocytes [97]. Similarly, AMSCs possess better immunomodulatory characteristics as compared to the ones isolated from BMMSCs [98]. However, when compared with AMSCs and BMMSCs, human umbilical cord MSCs have more biological advantages as compared to the adult counterparts [99, 100]. For example, umbilical-cord-derived MSCs released significantly lower amount of inflammatory cytokines, including IL-1, IL-8, and IL-10, when challenged with alveolar macrophages treated with lipopolysaccharides.

In another study, the influence of the differentiation status of MSCs on immunomodulatory characteristics was investigated using human Wharton's Jelly MSCs in comparison with the derivative cartilage cells [101]. The cell characterization included determination of the expression of major histocompatibility complex (MHC)-I/II and

costimulatory molecules CD40, CD80, and CD86, besides the expression of immune inhibitor molecules IDO, HLA-G, nitric oxide, and ILs. Human Wharton's Jelly MSCs lack expression of MHC-II and costimulatory molecules, only moderately express MHC-I, and strongly release HLA-G, IDO, and prostaglandin E2, thus supporting the immunosuppressive properties of undifferentiated Wharton's Jelly MSCs. Moreover, unlike their derivative cartilage cells, undifferentiated MSCs also released HGF-1, VEGF, TGF-1β, and IL-10 [101]. Another factor affecting the immunomodulatory behavior of MSCs is the quality of cell preparation. The gene expression profile in MSCs differs with a change in the culture and expansion conditions. For example, the gene expression profile of MSCs plated at low density for expansion is different from that of those cultured at higher cell density for equal length of time duration. These cells also differ in the expression of factors having a critical role in cell migration and tissue regeneration, i.e., podocalyxin-like protein, alpha4 integrin, alpha6 integrin, and leukemia inhibitory factor, in addition to factors that are determinants of immunomodulatory characteristics of MSCs [102]. Therefore, it is important to optimize isolation, purification, and *in vitro* culture expansion protocols to ensure uniform and best-quality cells with the required immunomodulatory properties [103]. Besides other factors, the age of the donor has a significant influence on the immunomodulatory properties of MSCs [95, 104]. An interesting aspect of MSC transplantation for cardiac repair is that post transplantation, allogenic MSCs enjoy an immuno-privileged status. However, postdifferentiation, the loss of the cell graft is more imminent owing to the altered immunogenicity and immunosuppressive properties after differentiation. In a recent study, transplantation of MSCs altered the inflammatory response in the infarcted heart, which was associated with attenuated levels of proinflammatory cytokines tumor necrosis factor-α (TNF-α), IL-1, and IL-6, whereas IL-10 expression was significantly increased in the peri-infarct region [105]. The altered expression of proinflammatory cytokines also reduced type I/III collagen deposition. A comparison of human and porcine BMMSCs ($CD90^+$, $CD44^+$, and $CD29^+$) showed smaller immunophenotypical differences, but their overall functionality with respect to anti-inflammatory and immunosuppressive properties was similar without much difference [106]. A recent study has shown that genetic modulation of BMMSCs for hypoxia inducible factor-1α (HIF-1α) overexpression also alters their immunomodulatory properties [107]. An important aspect of research relevant to the immunomodulatory effects of MSCs is that one has to be careful in extrapolating *in vitro* data to *in vivo* transplantation experimentation. Porcine MSCs tested *in vitro* showed little evidence of immunogenicity [108, 109]. When challenged with IFN-γ, MSCs did not show any proliferative response nor did they secrete IFN-γ or IL-2 in the culture medium. However, upon transplantation in the infarcted heart, allotransplanted MSCs showed moderate immunogenicity, which was evident from donor MSC-specific humoral and cellular responses. These data highlight the importance of simultaneous *in vitro* and *in vivo* experimentation to assess the behavior and functionality of MSCs.

3.2.4 Paracrine factors and cell migration and homing

Subsequent to myocardial injury, the interaction between the infiltrating cells and the nonmyocytes in the heart is an integral part of the adaptive response extended by the cardiomyocytes under stress as part of the intrinsic repair process. Cardiomyocytes constitute nearly 25% of the total number of constituent cells in the heart and are quite capable of secreting a plethora of bioactive molecules during their cross-talk with other cells in both paracrine and autocrine manner [110]. This group of bioactive molecules is referred to as the cardiokines, which, besides others, also include both CC and CXC chemokines that regulate cell trafficking to the myocardium under stress [111]. SDF-1α is one of the chemokines responsible for extravasation of BMCs into peripheral circulation [112]. Released from cardiomyocytes under stress, favorable SDF-1α gradient is established in the heart to serve as a chemical cue for the $CXCR4^+$ cells in the peripheral circulation to home in to the injury site [113]. The ligand/receptor interaction between SDF-1α and CXCR4 is central to the retention of $CXCR4^+$ cells after migration and homing-in to ensure that these cells stay at the site of injury long enough to participate in the repair process. Therefore, SDF-1α is also known as a retention factor [114]. Similar to the cardiomyocytes under stress, normal cardiomyocytes also secrete SDF-1α and express CXCR4 on their plasma membrane [115]. However, the SDF-1α level in the event of myocardial injury is elevated and continues to remain above the baseline levels at least for the first 7 days after MI [116]. According to one estimate, SDF-1α level increases by 67% in the infarcted heart and reduces by 43% in the BM, thus creating a gradient for the progenitor cells to extravasate into the peripheral circulation and home in to the infarcted heart [117]. Given the importance of SDF-1α/CXCR4 axis in stem cell homing and retention, various studies have attempted to enhance SDF-1α expression in the heart or enhance CXCR4 on the progenitors to exploit the SDF-1α/CXCR4 axis for efficient myocardial repair [116, 118–120]. Additionally, SDF-1α has cytoprotective effects and improves cell survival by acting *via* the CXCR4 receptor present on the surface of the cells [118, 121]. In an interesting study, BMMSCs were genetically modified for transgenic accentuation of IGF-1 expression to temper their paracrine activity [122]. The IGF-1-expressing BMMSCs showed elevated SDF-1α levels, both *in vitro* and after transplantation in the infarcted heart. Histological studies revealed extensive mobilization of BMCs into the injured myocardium and implied vital consequences of IGF-1 gene delivery. IGF-1 overexpression in the heart accelerated stem cell migration, reduced cardiomyocyte apoptosis, and increased neo-angiomyogenesis, with resultant preservation of global cardiac function. In another similar study, *in vitro* treatment of cardiomyocytes with CM from VEGF transgene overexpression significantly enhanced SDF-1α release [86]. Similarly, transplantation of BMMSCs genetically modulated to overexpress VEGF markedly increased SDF-1α expression in the infarcted heart and promoted CSC migration to the injury site, with a concomitant increase in the angiomyogenic response. Pretreatment with SDF-1α-specific antibodies or SDF-1α-specific antagonist successfully blunted

the SDF-1α/CXCR4 axis and abrogated the beneficial effects of treatment with VEGF overexpressing MSCs. Similar to BMCs, ASCs also secrete multiple GFs as part of their secretome, which include VEGF, bFGF, and SDF-1α. Transplantation of multipotent ASCs create a favorable chemokine gradient *via* the release of SDF-1α for mobilization of BMCs to home in to the infarcted heart [123–125].

Besides SDF-1α, paracrine secretions also contain many other bioactive molecules with direct or indirect supportive role in stem cell mobilization and migration. For example, the CM from mouse BMMSC culture for 48 hours was found to be rich in VEGF, MCP-1, MIP-1α, MIP-1β, and monokine induced by IFN-γ compared to the control media [54]. An *in vitro* cell migration assay showed that both MCP-1 and MIP-1 were actively involved in cell migration. On the same note, a chemotaxis chamber assay showed that CM from BMMSCs enhanced CSC mobilization besides up-regulating cardiomyocyte-specific genes [126]. However, CM from BMMSCs failed to support EC proliferation despite improving their resistance to apoptosis and adhesion characteristics. This deficiency in CM from BMMSCs may be compensated by mixing with CM from EPCs [56]. Like other cell types, EPCs from BM and peripheral circulation actively participate in neovascularization by differentiating into mature ECs. EPCs respond to different GFs and chemokines and are recruited to contribute in the neovascularization and repair of the injured myocardium *via* recruitment of CSCs with their unique secretome profile [127–129]. A key feature of the secretome profile of stem cells is its dynamic composition, which continues to change with the cells' response to its microenvironment. CM obtained from three-dimensional (3D) cultured (spheroids) human MSCs is more concentrated for bioactive molecules, i.e., VEGF, angiogenin, IL-11, procathapsin, and bone morphogenetic factor-2 as compared to human MSCs cultured as monolayer [130]. Hence, the functional outcome in terms of cell migration, proliferation, and survival may be observed more with CM obtained from spheroids as compared to the CM from the monolayer culture. Similarly, the differentiation status of the cells significantly influences their secretome profile. BMMSCs undergoing osteogenic differentiation also alter their secretome profile at specific time points during the course of differentiation to adapt to their new morphofunctional role. The release of trophic factors supportive of osteogenic differentiation by the differentiating BMMSCs induces osteoblast lineage-specific markers in exogenous BMMSCs besides their recruitment to the site of injury. The health status of the donor can also affect the secretome profile of stem cells. Most clinical studies for myocardial repair and regeneration have used SkMs and BMCs for transplantation, albeit with few evidence of their adoption of cardiac phenotype. Therefore, the preserved global cardiac function in most studies has been attributed to the paracrine activity of the donor cells [131]. Secretome profiling of BMCs isolated from 15 patients with MI was rich in multiple prosurvival and proangiogenic GFs [39] (Tab. 3.2.2). Compared with BMCs, peripheral-blood-derived leucocyte CM was rich in IL-11 and PlGF. *In vitro* studies showed that the angiogenic response observed with CM from patient-derived BMCs during angiogenesis assay was equivalent to the angiogenic response observed

with combined use of recombinant VEGF and bFGF [39]. Similarly, CM derived from peripheral blood leukocytes has a significant effect on the emigrational activity, proliferation, and survival of ECs. The CM from peripheral blood leucocytes and BMCs together exerts synergistic angiogenic response *in vitro*. These data are in harmony with the previously published study and not only establish the importance CM as a therapeutic option but also point to the concomitant use of CM from different cell types to exploit their synergistic therapeutic effects, including enhanced emigrational and homing-in response of the intrinsic stems cells [56].

3.2.5 Paracrine factors and antifibrosis

As discussed earlier, due to the limited recycling ability of the cardiomyocytes and scanty participation of the resident CSCs in the repair process, noncontractile fibrotic tissue remains integral to the intrinsic repair mechanism in the heart to substitute the dead cardiomyocytes. The activation of cardiac fibroblasts (CFs) is central to the process of cardiac fibrosis and involves intimate cross-talk between cardiomyocytes and CFs *via* release of trophic factors [132]. During this cross-talk mediated by as-yet unidentified paracrine factors, both cell types significantly influence each other. Various studies have attempted to determine the essential role of CFs as the possible inducers of cardiomyocyte hypertrophy and determinants of fibrotic response in the heart [133]. While elucidating the mechanism, it was observed that Kruple-like factor-5 (KLF5) in CFs transactivated IGF-1 in the CFs, which was released and acted in a paracrine fashion to cause cardiomyocyte hypertrophy. Manipulation of KLF5 in cardiomyocytes failed to elicit the same response, thus showing the importance of CFs as essential determinants of cardiomyocyte hypertrophy and fibrosis [133]. In addition, paracrine factors released by CFs have also been shown to alter conduction velocity, prolong action potential duration, and increase membrane resting potential [134]. In a coculture system between CFs and cardiomyocytes, cardiomyocytes became hypertrophied, whereas the cocultured CFs were activated to show a higher rate of proliferation and adhesion. Analysis of the CM from the cocultured cells showed increased levels of IL-6 [135, 136]. In a similar study involving coculture between cardiomyocytes and CFs, analysis of CM from the coculture showed elevated expression of at least 10 GFs and cytokines, including VEGF, MCP-1, leptin, MIP-1α, IL-6, IL-10, IL-12, IL-20, and TNF-1α [40]. CFs constitute majority of the nonmyocyte population in the heart. Upon activation, the CFs undergo phenotypic transition to become cardiomyofibroblasts, which can be distinguished from fibroblasts in the expression of SMC-specific markers. Once activated, fibroblasts undergo rapid proliferation and secrete ECM proteins, matrix metalloproteinases (MMPs), and profibrotic GFs and cytokines [137]. These phenotypic and molecular changes then cause disproportionate accumulation of fibrillar collagen type I, stiffening of the fibrotic myocardium with impaired electrical coupling between myocytes, and reduced vascular

structures leading to decreased regional perfusion. Treatment with stem cells or their CM significantly reduces cardiac fibrosis. The antifibrotic activity of either of the two approaches is attributed to the release and presence of paracrine factors, respectively [138–140]. HGF-1 is one of the potent antifibrotic factors that are released by the transplanted stem cells as part of their paracrine activity. Induction of HGF-1 also prevents endothelial-to-mesenchymal transition in the heart, thus preventing perivascular fibrosis [141]. Following the antifibrotic activity of HGF-1, the therapeutic effectiveness of stem-cell-based transgenic overexpression of HGF-1 has been assessed in different experimental studies [142–144]. There is a long list of other paracrine factors more commonly studied for their role in cardiac fibrosis including cytokines (IL-13, IL-21, TGF-1β), endothelin-1, chemokines (MCP-1, MIP-1), angiogenic factors (VEGF, PDGF, peroxisome proliferator-activated receptors (PPARs), acute phase proteins (e.g. SAP), caspases, and components of the renin-angiotensin-aldosterone system (such as angiotensin II; ANG II) [145, 146]. A comparison of the antifibrotic activity of CM derived from BMMSCs and CFs in a cellular model showed that treatment with the former significantly reduced CF proliferation [139]. BMMSC-derived CM significantly upregulated antifibrotic genes, including elastin, myocardin, and DNA damaged inducible transcript-3. Moreover, treatment with BMMSC-derived CM also reduced the synthesis of type I and type III collagen in CFs. Contrary to this, CF-derived CM significantly induced the expression of pro-proliferation genes such as macroglobulin and v-kit Hardy-Zuckerman-4 feline sarcoma viral oncogene homolog in CFs. It has been observed that CFs treated with ANG-II in the presence of BMMSC-derived CM showed significantly reduced proliferation and a low-level expression of collagen I and II, besides significantly reduced adrenomedullin, an important antifibrotic agent [147]. Transplantation of MSCs or treatment with MSC-derived CM significantly reduced myocardial fibrosis, with a simultaneous increase in adrenomedullin expression, in an experimental rat model of isoproterenol-induced heart failure. Although there is an inconclusive underlying mechanism, these data showed the importance of adrenomedullin expression in the heart. Hence, a recent study has effectively used adrenomedullin overexpressing BMMSCs for antifibrotic activity to treat infarcted myocardium [148]. With increasing evidence about the presence of secretable factors in the CM, a cell-free approach as a therapeutic strategy is gaining popularity to curtail fibrosis in the diseased heart. As proof-of-concept, intracoronary treatment with BMMSC-derived CM, rich in antiapoptotic and antifibrotic agents, significantly reduced fibrosis in a porcine model of acute MI [37]. Stem cells have also been modified to alter their paracrine secretion profile to enhance their antifibrotic efficacy. CM from genetically modified MSCs for integrin-linked kinase overexpression significantly reduced collagen I and III, TIMP-1 and -2, and connective tissue GF expression in CFs both *in vitro* as well as post transplantation in the infarcted heart [149]. Moreover, treatment with the genetically modified cells reduced the area of fibrosis. Among many other factors, hyperglycemia has been shown to increase the incidence of cardiac fibrosis. This involves the activation of CFs to proliferate and excessive

synthesis of collagen with synergistic activation of STAT3 and MAPK signaling as the underlying molecular mechanism [150].

3.3 Tempering the secretome composition with outside intervention

Besides vasculogenic and cardiomyogenic differentiation potential of stem cells, there is plausible evidence in the literature supporting that their therapeutic effectiveness is due to paracrine activity. Each stem cell type has a distinct secretome profile; nevertheless, its composition is variable. This may also be tempered with to achieve the desired composition of the paracrine secretions to maximize their therapeutic efficacy post-transplantation.

3.3.1 Genetic modification of stem cells

Genetic modification of stem cells for overexpression of a therapeutic gene of interest is generally intended to modulate stem cell functioning to accomplish the desired prognosis [151]. Moreover, the combinatorial approach based on stem-cell-based gene delivery to the organ of interest facilitates the exploitation of the better one of the two approaches, which were once considered as competitive with each other. The genetically modified cells serve as small "depots," which continuously secrete the product of the transgene/s. Additionally, the genetically modified cells show an altered paracrine profile that is aimed to improve cell survival, create a favorable GF gradient in their vicinity that favors stem cell mobilization and homing from the intrinsic pool, promote angiogenesis and regional blood flow, and improve cardiogenic differentiation (both vasculogenic and myogenic) of the transplanted as well as mobilized stem cells. The length of time for which these cells continue to secrete the transgene product depends upon many factors. Among the delivery strategies, viral vectors are more popular for gene modification of stem [122, 152–154]. Although viral-vector-based DNA delivery results in a more stable transgene expression, the safety issues associated with their use have hampered their progress in the clinical perspective [155, 156]. Non-viral-vector-based delivery strategies are safer for human use but suffer from poor transfection efficiency [46, 157]. Irrespective of the method of gene delivery into the stem cells, there are two important aspects that require special consideration: first, that genetic modification of stem cells alters their paracrine behavior and second, the paracrine release of secretable factors is one of the prime mechanisms of action of the genetically modified stem cells postengraftment in the infarcted heart [15]. For example, tail vein injection of SDF-1α expressing BMMSCs 1 day after MI protected the cardiomyocytes *via* the release of trophic factors besides increasing angiogenic response in the infarcted heart [154]. Similarly, adenovirally transduced BMMSCs overexpressing the prosurvival gene *Akt1* secreted a wide array

of soluble factors, which also included secreted frizzled related protein (sfrp)-2, which was analyzed as the key determinant of the antiapoptotic effects in the infarcted heart [45, 80, 158]. Sfrp-2 is an inhibitor of Wnt signaling, and its level, besides that of BMP-1, significantly increases to support collagen deposition in the infarcted heart. Treatment with recombinant sfrp-2 significantly reduced collagen deposition and preserved global cardiac function [45, 159]. Despite poor cardiomyogenic differentiation in the heart post transplantation, globally preserved cardiac function was reported in the animal hearts treated with Akt overexpressing BMMSCs as compared to the control animals treated with native BMMSCs. In a subsequent study, BMMSCs with simultaneous transgenic overexpression of Ang-1 and Akt were transplanted in a rat model of acute MI, which significantly reduced the host cardiomyocyte apoptosis, attenuated fibrosis, enhanced myocardial angiogenic response, and preserved global cardiac function [58, 160]. Simultaneous overexpression of Ang-1 and Akt also promoted BMMSC cell cycle activity with the downstream involvement of mir-143 [161]. A direct comparison of BMMSCs cultured *in vitro* and post transplantation in the infarcted heart was carried out to study the paracrine effect of the transplanted cells [162]. The single cell samples for paracrine factor profiling were obtained by single cell nucleus laser capture microdissection (LCM) of the heart tissue samples on day 3 after cell transplantation. The results showed a heterogeneous paracrine profiling between subpopulations of the transplanted BMMSCs. For example, a $CD45^{+}$ subpopulation of the cells isolated from the heart by LCM was rich in angiogenic GF expression as compared to the other subpopulations. These data constitute the first report showing differential pattern of paracrine expression in BMMSCs postengraftment in the heart. Similar results have also been reported by another research group after LCM of transplanted BMMSCs combined with single cell nucleus polymerase chain reaction (PCR) profiling for paracrine factors [163]. Gene manipulation of stem cells not only increases the expression and release of the protein but also may affect the global paracrine expression profile of the genetically modified cells. The hypoxia responsiveness of cells significantly alters their paracrine profile *via* activation of HIF-1α signaling. Given the critical participation of HIF-1α in the direct and indirect regulation of various GFs, stem cells have been genetically modified to overexpress HIF-1α transgene, which alters their proangiogenic paracrine secretions but without promoting their endothelial differentiation [164]. It is noteworthy that uncontrolled continuous transgenic overexpression of HIF-1α may unfavorably tailor the secretome profile of the genetically modified cells. Constitutive overexpression of HIF-1α in cardiosphere derived cells significantly increased endothelin-1 and VEGF secretion upon exposure to hypoxia, but such manipulation blunts the beneficial effects of the heart cell therapy [165]. These data imply the need for regulated and controlled expression of transgenes in the donor stem cells, thus providing the opportunity to indirectly regulate their secretome profile. On the same note, hypoxia-regulated VEGF gene delivery has shown the importance of regulated transgene expression in the heart [166, 167].

Given the new insights into IGF-1 signaling in the heart pertaining to its pleiotropic effects in general and protective effects in terms of preventing MI [168], delivery of IGF-1 transgene to the heart is being studied extensively either alone or in combination with other proangiogenic GFs [122, 169–171]. Stem-cell-based transgenic overexpression of IGF-1 in the infarcted heart significantly reduces cardiomyocyte apoptosis, attenuates infarct size expansion, and preserves global cardiac function [122]. More importantly, IGF-1 overexpression increases the downstream expression of SDF-1α in the infarcted heart that creates a favorable gradient for stem cell mobilization from the BM and peripheral circulation. The mobilized cells participate in the angiomyogenic repair of the infarcted heart. In line with these data, VEGF overexpressing BMMSCs or VEGF gene delivery followed by BMMSC transplantation successfully enhances mobilization and homing-in of stem cells into the heart *via* downstream involvement of SDF-1α axis [172, 173]. *In vitro* transduction of H2C9 myoblast cell line supported the *in vivo* observations of SDF-1α induction downstream of VEGF overexpression. Moreover, CM from VEGF overexpressing cells caused significant mobilization of progenitor cells in response to SDF-1α/CXCR4, as well as VEGF-R1 and VEGF-R3 ligand-receptor interaction. RNA interference studies conducted to block SDF-1α/CXCR4 axis showed decreased therapeutic benefits of treatment with VEGF overexpressing BMMSCs [174]. As an alternative to manipulating the secretome profile of stem cells, the therapeutic benefits from paracrine activity of stem cells can be improved by enhanced receptor expression. As a proof-of-concept to this approach, cardiac-specific abrogation of CXCR4 significantly reduced the therapeutic benefits of BMMSC treatment in the infarcted heart. It was observed that the number of $TUNEL^{+}$ cardiomyocytes was significantly increased in CXCR4-null animal hearts as compared to wild-type animals subsequent to BMMSC transplantation [175]. These data show the importance of receptor induction on the cardiomyocytes that may be specific for interaction with the secretome factors of the transplanted stem cells. Stem cells are also genetically modified to enhance their adhesion characteristics post transplantation in the heart. BMMSCs genetically modified to overexpress periostin not only show better survival than their native counterparts do, but also their paracrine secretions were cytoprotective for the juxtaposed host cardiomyocytes [176]. These data were confirmed by *in vitro* experiments when cardiomyocytes were treated with CM from periostin overexpressing BMMSCs. Molecular studies have shown that CM treatment activated PI3K *via* adhesion-related integrins.

As an alternative to transgenic overexpression of GFs and cytokines, the approach of abrogation of intrinsically expressed proteins with vital role as determinants of paracrine activity is gaining popularity. For example, propyl hydroxylase domain protein-2 (PHD-2) is an integral player in the oxygen sensing mechanism in cells and regulates the activity of two important transcription factors, HIF-1α and nuclear factor kappa B (NF-κB). Manipulation of cells for abrogation of PHD-2 alters their secretome profile and the paracrine secretions become rich in IGF-1 [177]. Hence, AMSCs with PHD-2 abrogation showed significantly higher survival post transplantation in the infarcted heart.

Moreover, transplantation of PDH-2 abrogated cells or treatment with their derived CM significantly reduced host cardiomyocyte apoptosis, which was attributed to the release of IGF-1. These data highlight the importance of finding some novel targets that are intrinsic to the cells to alter their expression and activity in combination with transgenic induction of GFs and cytokines for a combinatorial therapeutic option.

3.3.2 Chemical and pharmacological manipulation of cells

Although the combinatorial approach of gene manipulation of stem cells successfully and efficiently alters their secretome profile, the protocols for transgene transfer into cells are either safe but inefficient or efficient but unsafe for clinical application. This situation warrants the development of protocols that are more efficient but safe for use in humans. Moreover, the uncontrolled robust expression of the transgene may lead to undesired effects. In this regard, the strategy of chemical and pharmacological manipulation of stem cells is a simpler and safer option as it involves treatment with chemicals/drugs that have already been assessed for safety in humans. Additionally, the strategy hardly alters the genetic character of the cells during their conditioning before use. A wide variety of chemicals and pharmacological agents have been used to precondition the cells for their improved functioning. Each one of these preconditioning agents primes the cells with a distinct mechanism, which may conclude with overlapping outcomes such as survival, proliferation, metabolism, differentiation, and of course paracrine behavior. GFs and cytokines are among the commonly used preconditioning agents for cellular and subcellular preconditioning due to the wide distribution of their specific receptors on the cells [178–182]. For example, treatment of AMSCs with bFGF stimulates HGF-1-rich secretome, which increases hematopoiesis and vasculogenesis [51]. However, when challenged with lipopolysaccharides, AMSCs secrete copious amounts of hematopoietic, i.e., granulocyte/monocyte, and macrophage stimulating factors and IL-7 and proinflammatory factors, i.e., IL-6, IL-8, IL-11, and TNF-1α. A coculture with umbilical vein ECs increases hematopoiesis *in vitro*. TGF-1β is a multifunctional polypeptide that regulates many cellular functions, including cell proliferation and differentiation. It is synthesized and secreted by many cell types as a part of their secretome and has a wide distribution of its specific receptors. Taking advantage of the expression of TGF-1β-specific receptors by embryonic stem cells (ESCs), the cells were preconditioned by TGF-1β treatment, and the secretome profile of the PCs was compared with that of the non-PCs [183]. Analysis of the CM from the PCs showed that CM contained multiple cytoprotective factors such as IL-10, stem cell factor, tissue inhibitor of MMP-1, and VEGF. The cytoprotective effects of CM from TGF-1β-treated cells were determined both *in vitro* as well as after treatment of the infarcted heart. It was observed that cardiomyocytes survived better after treatment with the CM from TGF-1β PCs as compared to the control non-PCs upon subsequent treatment with oxidant stress and in the infarcted heart via activation of

Akt signaling. Similar observations have also been reported by other research groups [178]. The effect of TGF-1β has been potentiated by costimulation of stem cells with IL-6, a pleiotropic cytokine with multiple biological activities [178]. The strategy of costimulation of MSCs with TGF-1β and IL-6 additively enhances VEGF secretion by the PCs, which was significantly higher as compared to the treatment of the cells with either of the two factors alone. Molecular studies showed the involvement of ERK, c-Jun N-terminal kinases (JNKs), and PI3K signaling mechanisms.

In view of the encouraging data from the preconditioning of stem cells with GFs, a multiple-GF treatment strategy has been adopted to augment the expression and release of angiogenic, prosurvival, pro-proliferation, and proadhesion factors from the PCs [184, 185]. Stimulation of the umbilical-cord-derived mononuclear cells increased their migrational and angiogenic characteristics. Analysis of the mononuclear cells cultured in specific medium containing multiple GFs showed a significantly higher expression of angiogenic and vascular maturation factors. In line with these data, GF-rich tissue protein extract or CM from non-PCs or PCs has been used to manipulate stem cells to modify their functioning [186–188]. The advantage of CM-based treatment strategy is the ease and simplicity of the manipulation protocol besides the strong and synergistic effect of multiple GFs present in the whole protein extract or the CM. While the whole cell or tissue protein extract is rich in nonsecretable GFs, the CM is mostly rich in secretable fraction of the bioactive molecules. A comparison of these two sources of GFs and their combined biological effect will be interesting for the development of noncellular therapeutic approach.

In addition to GFs, treatment with hormones has effectively conditioned the cells to alter their paracrine secretome. Oxytocin has commonly and traditionally been identified to have a role in female reproductive system functioning. However, with the recognition of its specific receptor distribution both in the heart as well as in the vascular bed, there is increasing interest in oxytocin for its role in cardiovascular system [189]. Receptors specific for interaction with oxytocin have also been indentified on the surface of BMMSCs, and therefore, recent studies have focused on determining oxytocin as a preconditioning agent to prime the cells and enhance their functional properties [158]. Oxytocin preconditioned BMMSCs showed significant secretion of antiapoptotic and angiocrine factors, including various heat shock proteins (HSPs), TIMP-1 and TIMP-2, MMP-2, TGF-1β, VEGF, and endothelin-1. Coculture with oxytocin-primed MSCs enhanced the survival of cardiomyocytes under hypoxic culture conditions besides supporting EC migration and angiogenic response. Similarly, estradiol treatment modulates the expression of estrogen receptors alpha and beta on BMMSCs and alters various cellular functions ranging from proliferation to differentiation, in addition to increasing the expression of alkaline phosphate, TGF-1β, collagen I, and BMP-2 [190, 191]. In a recent report, estradiol treatment increases VEGF release from MSCs, thus increasing their angiogenic behavior [192].

Besides the use of bioactive molecules including GFs, cytokines, and hormones, preconditioning mimetics including chemicals and pharmacological agents are being

assessed to prime the cells and modify their biological characteristics [90, 153, 193–195]. Among the pharmacological agents, defroxamine is an important heavy metal ion antagonist that is commonly used for iron chelation. When used for preconditioning of cells, it efficiently induces HIF-1α and modifies the paracrine secretion from AMSCs to become rich in proangiogenic VEGF [196]. Defroxamine treatment significantly increases VEGF in a dose-dependent manner when used in concentrations of 30–500 μM without showing signs of cell toxicity. Diazoxide, a known activator of ATP-sensitive K^+ channels, is a preconditioning mimetic and is well studied for priming stem cells to improve their resistance to ischemic stress [153, 193]. Treatment of BMMSCs with diazoxide significantly enhances their survival upon subsequent exposure to lethal anoxia, with concomitant activation of PI3K, Akt, Gsk3, and NF-κB [90]. The most interesting aspect of the study was the upregulation of prosurvival GFs, i.e., IGF-1, VEGF, HGF-1, and Ang-1 in a NF-κB-dependent manner with a significant role for NF-κB-dependent mir-146 [197]. Not only did transplantation of preconditioned MSCs in the infarcted heart enhance the survival of donor transplanted cells, but also the release of prosurvival and angiogenic factors significantly reduced host cardiomyocyte apoptosis and increased neovascularization in the infarct and peri-infarct areas in the heart. The activity of the enzyme phosphodiestrase-5 (PDE5) is a specific determinant of cyclic guanosine monophosphate activity in the cells, and therefore, inhibition of PDE5 with specific inhibitors such as sildenafil and tadalafil is gaining popularity [194, 198]. Tadalafil treatment provides sustained protection of transplanted stem cells in the infarcted rat heart, as was evident from improved survival of PC as compared to the non-PC stem cells. Preconditioning with PDE inhibitors also altered the paracrine secretion profile of stem cells [198]. The sildenafil-treated AMSCs released increasing amounts of VEGF, bFGF, and IGF-1. More interestingly, the levels of VEGF, bFGF, and Ang-1 remained elevated in the infarcted hearts until 4 weeks after treatment with sildenafil PCs as compared to the non-PCs.

3.3.3 Physical manipulation of cells

Besides genetic and pharmacological methods of priming the cells to modulate their paracrine behavior, different physical methods of manipulation have been devised to maximize their efficacy [88]. More recently, exposure to low-oxygen culture has gained popularity due to the ease and simplicity of the protocols involved therein to modify stem cell characteristics, including their secretome profile and surface marker expression [199–203]. Stem cells are maintained in a strictly regulated oxygen condition in their natural habitat, which is delicately maintained with a critical involvement of HIF-1α signaling mechanism. Isolation and culture under ambient oxygen levels (20% oxygen) significantly influence the signaling pathways relevant to cell survival, proliferation, migration, and differentiation, in addition to changing their paracrine secretome profile. When cultured under low-oxygen conditions that simulate their

natural habitat, it alters their biological behavior *in vitro* and characteristics as well. Hence, high or low oxygen levels in the microenvironment of stem cells remain critical determinants of their biological characteristics during *in vitro* culture. However, the intensity of cell response to hypoxic culture conditions is affected by many factors, including the level of hypoxia, time of exposure, composition of the culture medium, etc. It is prudent to consider that cells from different tissue origins differ in their hypoxia responsiveness. Moreover, the effect of continuous exposure to low oxygen may be different from exposure to short intermittent cycles of low oxygen presence. Nevertheless, a common feature of cellular response to hypoxia treatment is the activation of HIF-1α signaling that regulates its downstream target proteins, including GFs and cytokines. The effect of hypoxia on cells can be simulated by pharmacological treatment of cells that stabilize HIF-1α [204]. Hypoxia treatment causes the release of multiple prosurvival and proangiogenic factors, in addition to induction of SDF-1α/CXCR4 signaling, which allows their retention and participation in the cardiac repair process [205]. Despite plausible evidence in the literature that hypoxia significantly alters the secretome profile of stem cells, differences in the protocols of hypoxia treatment make it challenging to deduce and predict a definite outcome of exposure to low levels of oxygen. Hypoxia (5% oxygen) treatment of human ASCs from female donors significantly reduced proteins involved in ECM remodeling, i.e., fibronectin-1, TGF-1β-induced protein (βig-h3), osteonectin, and collagens type 1α1 and 1α2. IL-6 was upregulated in the hypoxia-treated cells [28]. On the contrary, one-time exposure of human AMSCs to severe hypoxia (0.1% oxygen) enhanced the secretion of VEGF and angiogenin [31]. Transplantation of polyvinyl alcohol sponges soaked in CM from the PCs demonstrated higher angiogenic response, which was abrogated by pretreatment of the sponges with antibodies specific for VEGF and angiogenin. These data underscore the emergence of hypoxia treatment as an effective strategy for preconditioning of stem cells prior to transplantation [88]. Treatment with hypoxia also primes the cells to improve their resistance to any innocuous stimulus post engraftment in the ischemic heart *via* activation of survival signaling. Preconditioning of MSCs by hypoxia (0.5% oxygen) for 24 hours significantly upregulated HIF-1α, Ang-1, VEGF and its receptor Flk-1, erythropoietin, Bcl-2, and Bcl-xL expression in the PCs [206]. The effectiveness of hypoxia-treated stem cells has also been determined in preclinical large experimental animal models. Hypoxia (1.5% oxygen) pretreated MSCs showed significantly enhanced survival and angiogenic potential post engraftment in a porcine heart of chronic ischemia [207]. As an alternative to one-time long-term exposure to hypoxia, preconditioning protocols have been developed based on exposure to short, intermittent, repeated cycles of anoxia-reoxygenation [208]. MSCs preconditioned with the anoxia-reoxygenation approach show significantly higher survival upon subsequent exposure to lethal anoxia as well as post transplantation in a rodent model of acute MI. Mechanistic studies revealed an integral role of mir-210 downstream of HIF-1α activation [209]. Transplantation of preconditioned MSCs significantly increased neovascularization in the infarcted myocardium through the

release of multiple angiocrine factors [210]. However, hypoxia treatment may not always result in paracrine secretions with beneficial effects. Hypoxia (4% oxygen) causes a significantly increased release of TNF-1α in CF culture [211]. Treatment of cardiomyocytes with the CM from hypoxia-treated CFs significantly enhances their susceptibility to reactive-oxygen-species-induced mitochondrial permeability transition and reduced their viability, whereas treatment of CFs with the CM led to their reduced DNA synthesis. These are important findings that highlight the role of CFs as the source of paracrine factors that are instrumental in injury to the cardiomyocytes during myocardial ischemia.

3.3.4 Heat shock and shock wave treatment to precondition stem cells

HSPs are cellular molecular chaperones that regulate protein folding and clearance under stress conditions [212]. Heat shock treatment, gene manipulation of cells for modified expression of HSPs, and treatment of cells with HSPs are gaining popularity as important and effective strategies to manipulate progenitor cells to improve their therapeutic efficacy [213–215]. Treatment of ECs with recombinant HSP-65 alters their adhesive characteristics by induction of CD62E (E selectin), CD106 (vascular cell adhesion molecule-1), and CD54 (intercellular adhesion molecule-1) [216]. Given that different cells differ in their response to heat shock treatment, protocols based on transgenic overexpression of HSPs have been developed [48]. Overexpression of HSP-20 significantly enhances the paracrine secretion of various GFs, including VEGF, FGF, and IGF-1. Transplantation of MSCs with overexpression of HSP-20 protects the host cardiomyocytes *via* release of various paracrine factors. *In vitro* coculture studies of cardiomyocytes or their treatment with CM from HSP-20 overexpressing MSCs confirmed these data.

Like any other cell, mechanical stress triggers various kinds of biological responses in stem cells from altered morphology to differentiation and paracrinicity [50, 217–219]. Defocused low-energy shock-wave treatment of precursors of cardiomyocytes, SMCs, and ECs significantly enhanced their differentiation [220]. Interestingly, the effect of shock waves was more pronounced on the precursors obtained from biopsy samples from normal hearts as compared to the pathologic hearts. In another study, defocused low-energy shock-wave treatment of BMMSCs and AMSCs before every passage significantly enhanced the secretion of VEGF and CXC ligand-5 [221, 222]. Western blot studies also determined the induction of Ki67 in response to shock-wave treatment. *In vitro* functional studies showed that CM from shock-wave-treated cells enhanced tubulogenesis on the Matrigel surface. In a hind-limb model, shock-wave treatment enhanced the tissue levels of VEGF and SDF-1α with a concomitant increase in EPCs in the peripheral circulation [223]. These molecular and cellular events increased regional vascular density as well as blood flow. Similar results have also been reported by the same research group in the treatment of infarcted heart [224].

3.3.5 Modification of the ECM

From their tissue source to the differentiation status and the genetic composition, cells diverge in their paracrine activity. Similarly, from the time of the harvest until the time of transplantation and posttransplantation in the infarcted heart, stem cells experience biophysically and biochemically different microenvironments, and hence, their paracrine behavior also keeps changing accordingly. A friendly and conducive environment is imperative for optimal functioning of stem cells and critically determines their stemness. While elucidating the effect of ECM on secretome profile of stem cells, it was observed that BM stromal cells cultured on fibronectin-peptide-supplemented gellan gum hydrogel not only affected the morphological and differentiation characteristics, but also their secretome profile was significantly altered in comparison with control cells cultured on unaltered gellan gum hydrogel. Moreover, multipotent adult progenitor cells (MAPCs) secrete bioactive molecules with immunomodulating properties. When challenged with inflammatory triggers like INF-γ and lipopolysaccharide or tolerogenic CD74 ligand RTL1000, MAPCs gave differential response to each stimulus, which was obvious from their altered secretome profile, including proteases and protease inhibitors, chemokines and cytokines that influence the emigrational activity of neutrophils, macrophages, and T-cells. Besides chemical cues and composition, the elasticity of the ECM used to culture the stem cells has profound effects on their secretome profile [225]. In an *in vitro* study designed to determine the effect of substrate elasticity on the paracrine activity profile of stem cells, secretion of more than 90 paracrine factors from MSCs was observed at protein level for their release profile [226]. By varying the hydrogel compliance to mimic the elasticity of brain and muscle tissue, it was observed that the secretion of IL-8 was significantly upregulated on hard surface as compared to the soft substrates. Given that ECM has a significant influence on stem cell behavior in terms of their adhesion, proliferation, and differentiation characteristics, instructive ECMs are engineered to modulate the culture characteristics of stem cells *in vitro*. However, the composition of engineered ECMs is developed such that their components such as fibronectin, fibrillar collagen I, tropocollagen, and collagen-I/heparin do not alter the immunomodulatory characteristics of MSCs [227]. With the recent progress in 3D cell culture technique that closely mimics the natural habitat in the tissue, 3D cell culture significantly alters their differentiation potential and response to extrinsic cues (both physical and chemical) in terms of altered secretome profile. Encapsulation of AMSC cells in a water-soluble thermos-responsive hyperbranched polyethylene glycol (PEG)-based polymer significantly improved their paracrine activity, as evidenced by copious secretion of these GFs [228]. A comparison of the behavior of rat ESCs in 2D and 3D cultures using different matrices showed that in the absence of any instructive outside stimulus, the cells cultured on different matrices differed in their paracrine behavior and secreted different levels of GFs [229].

Among the physical factors, tissue ischemia has significant bearing on cell survival, differentiation and myocardial reparability posttransplantation in the infarcted

heart. Hence, exposure to low oxygen is being pursued as an effective strategy to condition the cells prior to transplantation [28]. Glucose concentration in the culture conditions also impacts cell behavior. Whereas short-term exposure to high glucose concentration does not alter their paracrine behavior, long-term culture significantly alters their paracrine profile in terms GFs secretion especially for VEGF, HGF-1 and bFGF [230]. Preconditioning by treatment with GFs and by gene modification also alters the paracrine activity of stem cells [178].

3.4 Conclusion and future perspective

As discussed earlier, stem-cell-mediated preservation and recovery of cardiac function involves paracrine release of GFs as an integral component of the multifactorial mechanism, in addition to the differentiation, fusion, and activation of intrinsic cells *via* cell-cell interaction [231]. The use of cell-free therapy based on MSC secretome for the repair of injured myocardium provides some key advantages over stem-cell-based therapy, such as safety, reproducibility, cost, and possibility of mass production [232].

Despite technological and methodological advancements in proteomic studies, we are still short of developing a distinct and comprehensive secretome profile of a typical stem cell type [233]. The secretome of an individual cell type is unique and is sensitive to changes in the microenvironment of the cell. Similarly, as discussed earlier, the composition of the secretome can be modified by genetic/pharmacological conditioning of the cells. Hence, the therapeutic potential of the secretome will largely be dependent on its composition, which implies the significance of standardization of the secretome for optimal prognosis. The standardization necessitates further studies to define the culture conditions, culture duration, and media supplements. Furthermore, if genetic and/or pharmacological manipulation is used to modify the composition of the secretome, the safety and robustness of the method need to be established.

An important factor that needs further consideration is the route of administration. Although intravenous application would be an easy choice for multiple applications, it is linked with the risk of side effects. On the other hand, intracoronary application will limit its effects to local tissue requiring low dosage but multiple applications will be complicated. Another possibility is to apply patches of biodegradable hydrogels loaded with the MSC secretome for local delivery to the affected tissue. Clinical usage of cell-free secretome would require mass production of the secretome, which must follow biopharmaceutical scale good manufacturing practice (GMP) guidelines. Although there are numerous academic and clinical centers across the United States and Europe with GMP compliance, insufficient GMP guidelines are available for the mass-scale production of cell-free secretomes. Likewise, there is the need to develop standardized protocols for the uniform production of stem cell CM.

3.5 References

[1] Oh H, Bradfute SB, Gallardo TD, et al. Cardiac progenitor cells from adult myocardium: Homing, differentiation, and fusion after infarction. PNAS 2003;100(21):2313–8.
[2] Nadal-Ginard B, Ellison GM, Torella D. The cardiac stem cell compartment is indispensable for myocardial cell homeostasis, repair and regeneration in the adult. Stem Cell Res 2014;13(3),Part B:615–30.
[3] Yuasa S, Fukuda K, Tomita Y, et al. Cardiomyocytes undergo cells division following myocardial infarction is a spatially and temporally restricted event in rats. Mol Cell Biochem 2004;259(1–2):177–81.
[4] Ali SR, Hippenmeyer S, Saadat LV, Lu L, Weissman IL, Ardehalid R. Existing cardiomyocytes generate cardiomyocytes at a low rate after birth in mice. Proc Natl Acad Sci USA 2014;111(24):8850–5.
[5] Povsic TJ, Junge C, Nada A, et al. A phase 3, randomized, double-blinded, active-controlled, un-blinded standard of care study assessing the efficacy and safety of intramyocardial autologous CD34+ cell administration in patients with refractory angina: design of the RENEW study. Am Heart J 2013;165(6):854–61.
[6] Mathiasen AB, Qayyum AA, Jørgensen E, et al. Bone marrow-derived mesenchymal stromal cell treatment in patients with severe ischaemic heart failure: a randomized placebo-controlled trial (MSC-HF trial). Eur Heart J 2015;36(27):1744–53.
[7] Qiu F, Maehara A, El Khoury R, et al. Impact of intracoronary injection of CD133+ bone marrow stem cells on coronary atherosclerotic progression in patients with STEMI: a COMPARE-AMI IVUS substudy. Coron Artery Dis 2016;27(1):5–12.
[8] Hare JM, DiFede DL, Castellanos AM, et al. Randomized comparison of allogeneic vs. autologous mesenchymal stem cells for non-ischemic dilated cardiomyopathy: POSEIDON-DCM Trial. J Am Coll Cardiol 2017;69(5):526–37.
[9] Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 2004;428(6983):668–73.
[10] Detena A, Volza HC, Clamorsa S, et al. Hematopoietic stem cells do not repair the infarcted mouse heart. Cardiovasc Res 2005;65:52–63.
[11] Scherschel JA, Soonpaa MH, Srour EF, Field LJ, Rubart M. Adult bone marrow-derived cells do not acquire functional attributes of cardiomyocytes when transplanted into peri-infarct myocardium. Mol Ther 2008;16:1129–37.
[12] Nygren JM, Jovinge S, Breitbach M, et al. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med 2004;10:494–501.
[13] van den Akker F, Feyen DAM, van den Hoogen. Intramyocardial stem cell injection: go(ne) with the flow. Eur Heart J 2017;38(3):184–6.
[14] Haider HK, Akbar SA, Ashraf M. Angiomyogenesis for myocardial repair. Antiox Redox Signal 2009;11(8):1929–44.
[15] Haider HK, Mustafa A, Feng Y, Ashraf M. Genetic modification of stem cells for improved therapy of the infarcted myocardium. Mol Pharm 2011;8(5):1446–1457.
[16] Huang M, Du W, He ZX, Li Z. Molecular imaging of angiogenesis in cardiovascular diseases. J Mol Biol Mol Imag 2015;2:1012.
[17] Duran JM, Makarewich CA, Sharp TE, et al. Bone-derived stem cells repair the heart after myocardial infarction through transdifferentiation and paracrine signaling mechanisms. Circ Res 2013;113(5):539–52.

[18] Hatzistergos KE, Quevedo H, Oskouei BN, et al. Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. Circ Res 2010;107(7):913–22.
[19] Ramkisoensing AA, de Vries AAF, Atsma DE, Schalij MJ, Pijnappels DA. Interaction between myofibroblasts and stem cells in the fibrotic heart: balancing between deterioration and regeneration. Cardiovasc Res 2014;102(2):224–231.
[20] Hodgkinson CP, Bareja A, Gomez JA, Dzau VJ. Emerging concepts in paracrine mechanisms in regenerative cardiovascular medicine and biology. Circ Res 2016;118:95–107.
[21] Sassoli C, Zecchi-Orlandini S, Formigli L. Trophic actions of bone marrow-derived mesenchymal stromal cells for muscle repair/regeneration. Cells 2012;1(4):832–50.
[22] Tokudome T, Horio T, Fukunaga M, et al. Ventricular nonmyocytes inhibit doxorubicin-induced myocyte apoptosis: involvement of endogenous endothelin-1 as a paracrine factor. Endocrinol 2004;145(5):2458–66.
[23] Kuramochi Y, Cote GM, Guo X, et al. Cardiac endothelial cells regulate reactive oxygen species–induced cardiomyocyte apoptosis through neuregulin-1beta/erbB4 signaling. J Biol Chem 2004;279(49):51141–7.
[24] Stastna M, van Eyk JE. Investigating the secretome: lessons about the cells that comprise the heart. Circ Cardiovasc Genet 2012;5(1):8–18.
[25] Bara JJ, McCarthy HE, Humphrey E, Johnson WEB, Roberts S. Bone marrow-derived mesenchymal stem cells become antiangiogenic when chondrogenically or osteogenically differentiated: implications for bone and cartilage tissue engineering. Tissue Eng (Part A) 2013;20(1–2):147–159.
[26] Rosenow A, Noben JP, Bouwman FG, Mariman EC, Renes J. Hypoxia-mimetic effects in the secretome of human preadipocytes and adipocytes. Biochim Biophys Acta 2013;1834(12):2761–71.
[27] Hemmingsen M, Vedel S, Skafte-Pedersen P, et al. The role of paracrine and autocrine signaling in the early phase of adipogenic differentiation of adipose-derived stem cells. PLoS One 2013;8(5):e63638.
[28] Frazier TP, Gimble JM, Kheterpal I, Rowan BG. Impact of low oxygen on the secretome of human adipose-derived stromal/stem cell primary cultures. Biochimie 2013;95(12):2286–96.
[29] Cunha FF, Martins L, Martin PKM, Stilhano RS, Han SW. A comparison of the reparative and angiogenic properties of mesenchymal stem cells derived from the bone marrow of BALB/c and C57/BL6 mice in a model of limb ischemia. Stem Cell Res Ther 2013;4(4):86.
[30] Ikegame Y, Yamashita K, Hayashi S, et al. Comparison of mesenchymal stem cells from adipose tissue and bone marrow for ischemic stroke therapy. Cytother 2011;13(6):675–85.
[31] Hsiao ST-F, Asgari A, Lokmic Z, et al. Comparative analysis of paracrine factor expression in human adult mesenchymal stem cells derived from bone marrow, adipose, and dermal tissue. Stem Cells Develop 2012;21(12):2189–203.
[32] Vodicka P, Skalnikova H, Kovarova H. The characterization of stem cell proteomes. Curr Opin Mol Ther 2006;8(3):232–9.
[33] Haider HKh, Khan M, Sen CK. MicroRNAs with mega functions in cardiac remodeling and repair: the micro management of the matters of the heart. In “MicroRNA in Regenerative Medicine” Ed. Sen CK. 2015:569–600.
[34] Yoon BS, Moon J-H, Eun Kyoung Jun EK, et al. Secretory profiles and wound healing effects of human amniotic fluid–derived mesenchymal stem cells. Stem Cells Dev 2010;19(6):887–902.
[35] Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One 2008;3:e1886.
[36] Cantinieaux D, Quertainmont R, Blacher S, et al. Conditioned medium from bone marrow-derived mesenchymal stem cells improves recovery after spinal cord injury in rats: an original strategy to avoid cell transplantation. PLoS One 2013;8(8):e69515.

[37] Nguyen BK, Maltais S, Perrault LP, et al. Improved function and myocardial repair of infarcted heart by intracoronary injection of mesenchymal stem cell-derived growth factors. J Cardiovasc Trans Res 2010;3:547–58.
[38] Liu CH, Hwang S-M. Cytokine interactions in mesenchymal stem cells from cord blood. Cytokine 2005;32(6):270–9.
[39] Korf-Klingebiel M, Kempf T, Sauer T, et al. Bone marrow cells are a rich source of growth factors and cytokines: implications for cell therapy trials after myocardial infarction. Eur Heart J 2008;29(23):2851–8.
[40] LaFramboise WA, Scalise D, Stoodley P, Graner SR, Guthrie RD, Magovern JA, Becich MJ. Cardiac fibroblasts influence cardiomyocyte phenotype in vitro. Am J Physiol Cell Physiol 2007;292:C1799–808.
[41] Di Santo S, Yang Z, Wyler von Ballmoos M, et al. Novel cell-free strategy for therapeutic angiogenesis: in vitro generated conditioned medium can replace progenitor cell transplantation. PLoS One 2009;4(5):e5643.
[42] LaFramboise WA, Petrosko P, Krill-Burger JM, et al. Proteins secreted by embryonic stem cells activate cardiomyocytes through ligand binding pathways. J Proteomics 2010;73(5):992–1003.
[43] Jun EK, Zhang O, Yoon B-Y, et al. Hypoxic conditioned medium from human amniotic fluid-derived mesenchymal stem cells accelerates skin wound healing through TGF-β/SMAD2 and PI3K/Akt pathways. Int J Mol Sci 2014;15(1):605–28.
[44] Song S-W, Kim KE, Choi J-W, et al. Proteomic analysis and identification of paracrine factors in mesenchymal stem cell–conditioned media under hypoxia. Cell Physiol Biochem 2016;40:400–10.
[45] Mirotsou M, Zhang Z, Deb A, et al. Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. Proc Natl Acad Sci USA 2007;104(5):1643–1648.
[46] Konoplyannikov M, Haider HKh, Ahmed RPH, Jiang S, Vien KL, Ashraf M. Activation of diverse signaling pathways by delivery of multiple cytokines for myocardial repair. Stem Cell Dev 2013;22(2):204–15.
[47] Hahn J-Y, Cho H-J, Kang H-J, et al. Pre-treatment of mesenchymal stem cells with a combination of growth factors enhances gap junction formation, cytoprotective effect on cardiomyocytes, and therapeutic efficacy for myocardial infarction. J Am Coll Cardiol 2008;51(9):933–43.
[48] Wang X, Zhao T, Huang W, et al. Hsp20-engineered mesenchymal stem cells are resistant to oxidative stress via enhanced activation of Akt and increased secretion of growth factors. Stem Cells 2009;27:3021–31.
[49] Mehmood A, Ali M, Khan SN, Riazuddin S. Diazoxide preconditioning of endothelial progenitor cells improves their ability to repair the infarcted myocardium. Cell Biol Int 2015;39(11):1251–63.
[50] Kurazumi H, Kubo M, Ohshima M, et al. The effects of mechanical stress on the growth, differentiation, and paracrine factor production of cardiac stem cells. PLoS One 2011;6(12):e28890.
[51] Kilroy GE, Foster SJ, Wu X, et al. Cytokine profile of human adipose-derived stem cells: Expression of angiogenic, hematopoietic, and pro-inflammatory factors. J Cell Physiol 2007;212:702–9.
[52] Kinnaird T, Stabile E, Burnett MS, et al. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. Circulation 2004;109:1543–9.
[53] Ratajczak J, Kucia M, Mierzejewska K, et al. Paracrine proangiopoietic effects of human umbilical cord blood-derived purified CD133+ cells – implications for stem cell therapies in regenerative medicine. Stem Cells Dev 2013;22(3):422–30.

[54] Boomsma RA, Geenen DL. Mesenchymal stem cells secrete multiple cytokines that promote angiogenesis and have contrasting effects on chemotaxis and apoptosis. PLoS One 2012;7(4):e35685.
[55] Kinnaird T, Stabile E, Burnett MS, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 2004;94:678–85.
[56] Burlacu A, Grigorescu G, Rosca AM, Preda MB, Simionescu M. Factors secreted by mesenchymal stem cells and endothelial progenitor cells have complementary effects on angiogenesis in vitro. Stem Cells Dev 2013;22(4):643–53.
[57] Timmers L, Lim SK, Arslan F, et al. Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. Stem Cell Res 2007;1(2):129–37.
[58] Jiang S, Haider HKh, Idris NM, Salim A, Ashraf M. Supportive interaction between cell survival signaling and angiocompetent factors enhances donor cell survival and promotes angiomyogenesis for cardiac repair. Circ Res 2006;99(7):776–84.
[59] Feygin J, Mansoor A, Eckman P, Swingen C, Zhang J. Functional and bioenergetic modulations in the infarct border zone following autologous mesenchymal stem cell transplantation. Am J Physiol Heart Circ Physiol 2007;293(3):H1772–80.
[60] Duong Van Huyen JP, Smadja DM, Bruneval P, et al. Bone marrow-derived mononuclear cell therapy induces distal angiogenesis after local injection in critical leg ischemia. Mod Pathol 2008;21(7):837–46.
[61] Rufaihah AJ, Haider HK, Heng BC, et al. Therapeutic angiogenesis by transplantation of human embryonic stem cell-derived CD133+ endothelial progenitor cells for cardiac repair. Regen Med 2010;5(2):231–44.
[62] Haider KH, Aziz S, Al-Reshidi MA. Endothelial progenitor cells for cellular angiogenesis and repair: lessons learned from experimental animal models. Regen Med 2017;12(8):969–82.
[63] Ucuzian AA, Gassman AA, East AT, Greisler HP, Molecular mediators of angiogenesis. J Burn Care Res 2010;31(1):158.
[64] Newman AC, Nakatsu MN, Chou W, Gershon PD, Hughesa CCW. The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. Mol Biol Cell 2011;22(20):3791–800.
[65] Rose BA, Yokota T, Vishnu Chintalgattu V, et al. Cardiac myocyte p38α kinase regulates angiogenesis via myocyte-endothelial cell cross-talk during stress-induced remodeling in the heart. J Biol Chem 2017;292:12787–800.
[66] Martens TP, See F, Schuster MD, et al. Mesenchymal lineage precursor cells induce vascular network formation in ischemic myocardium. Nat Clin Pract Cardiovasc Med 2006;3(Suppl 1): S18–22.
[67] Gronthos S, Fitter S, Diamond P, Simmons PJ, Itescu S, Zannettino AC. A novel monoclonal antibody (STRO-3) identifies an isoform of tissue nonspecific alkaline phosphatase expressed by multipotent bone marrow stromal stem cells. Stem Cells Dev 2007;16(6):53–63.
[68] Hung SC, Pochampally RR, Chen SC, Hsu SC, Prockop DJ. Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. Stem Cells 2007;25(9):2363–70.
[69] See F, Seki T, Psaltis PJ, et al. Therapeutic effects of human STRO-3-selected mesenchymal precursor cells and their soluble factors in experimental myocardial ischemia. J Cell Mol Med 2011;15(10):2117–29.
[70] Shudo Y, Miyagawa S, Fukushima S, et al. Novel regenerative therapy using cell-sheet covered with omentum flap delivers a huge number of cells in a porcine myocardial infarction model. J Thorac Cardiovasc Surg 2011;142(5):1188–96.

[71] Akiyama H, Ito A, Kawabe Y, Kamihira M. Genetically engineered angiogenic cell sheets using magnetic force-based gene delivery and tissue fabrication techniques. Biomaterials 2010;31(6):1251–9.
[72] Hamdi H, Planat-Benard V, Bel A, et al. Epicardial adipose stem cell sheets results in greater post-infarction survival than intramyocardial injections. Cardiovasc Res 2011;91(3):483–91.
[73] Kitabayashi K, Siltanen A, Pätilä T, et al. Bcl-2 expression enhances myoblast sheet transplantation therapy for acute myocardial infarction. Cell Transplant 2010;19(5):573–88.
[74] Siltanen A, Kitabayashi K, Pätilä T, et al. Bcl-2 improves myoblast sheet therapy in rat chronic heart failure. Tissue Eng Part A 2011;17(1–2):115–25.
[75] Siltanen A, Kitabayashi K, Lakkisto P, et al. hHGF overexpression in myoblast sheets enhances their angiogenic potential in rat chronic heart failure. PLoS One 2011;6(4):e19161.
[76] Shudo Y, Miyagawa S, Ohkura H, et al. Addition of mesenchymal stem cells enhances the therapeutic effects of skeletal myoblast cell-sheet transplantation in a rat ischemic cardiomyopathy model. Tissue Eng Part A 2014;20(3–4):728–39.
[77] Kawamura M, Paulsen MJ, Goldstone AB, et al. Tissue-engineered smooth muscle cell and endothelial progenitor cell bi-level cell sheets prevent progression of cardiac dysfunction, microvascular dysfunction, and interstitial fibrosis in a rodent model of type 1 diabetes-induced cardiomyopathy. Cardiovasc Diabetol 2017;16(1):142.
[78] Krijnen PA, Nijmeijer R, Meijer CJ, Visser CA, Hack CE, Niessen HW. Apoptosis in myocardial ischaemia and infarction. J Clin Pathol 2002;55(11):801–11.
[79] Kawaguchi N, Smith AJ, Waring CD, et al. c-kit+ GATA-4 high rat cardiac stem cells foster adult cardiomyocyte survival through IGF-1 paracrine signalling. PLoS One 2010;5(12):e14297.
[80] Gnecchi M, He H, Liang OD, et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 2005;11:367–8.
[81] Gnecchi M, He H, Noiseux N, et al. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 2006;20:661–9.
[82] Yang Z, Von Ballmoos MW, Faessler D, et al. Paracrine factors secreted by endothelial progenitor cells prevent oxidative stress-induced apoptosis of mature endothelial cells. Atherosclerosis 2010;211(1):103–9.
[83] Sassoli C, Pinia A, Mazzantib B, et al. Mesenchymal stromal cells affect cardiomyocyte growth through juxtacrine Notch-1/Jagged-1 signaling and paracrine mechanisms: clues for cardiac regeneration. J Mol Cell Cardiol 2011;51(3):399–408.
[84] Rosenberg M, Lutz M, Kühl C, et al. Coculture with hematopoietic stem cells protects cardiomyocytes against apoptosis via paracrine activation of AKT. J Transl Med 2012;10:115.
[85] Deveza L, Choi J, Imanbayev G, Yang F. Paracrine release from nonviral engineered adipose-derived stem cells promotes endothelial cell survival and migration in vitro. Stem Cells Dev 2013;22(3):483–91.
[86] Tang YL, Zhao Q, Qin X, Shen L, Cheng L, Ge J, Phillips MI. Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. Ann Thorac Surg 2005;80(1):229–36.
[87] Angoulvant D, Ivanes F, Ferrera R, et al. Mesenchymal stem cell conditioned media attenuates in vitro and ex vivo myocardial reperfusion injury. J Heart Lung Transplant 2011;30:95–102.
[88] Haider HKh, Ashraf M. Preconditioning and stem cell survival. J Cardiovasc Transl Res 2010;3(2):89–102.
[89] Wallrapp C, Thoenes E, Thürmer F, Jork A, Kassem M, Geigle P. Cell-based delivery of glucagon-like peptide-1 using encapsulated mesenchymal stem cells. J Microencapsul 2013;30:315–324.
[90] Afzal MR, Haider HKh, Idris NM, Jiang S, Ahmed RP, Ashraf M. Preconditioning promotes survival and angiomyogenic potential of mesenchymal stem cells in the infarcted heart via NF-kappaB signaling. Antioxid Redox Signal 2010;12(6):693–702.

[91] Wright EJ, Farrell KA, Malik N, Kassem M, Lewis AL, Wallrapp C, Holt CM. Encapsulated glucagon-like peptide-1-producing mesenchymal stem cells have a beneficial effect on failing pig hearts. Stem Cells Transl Med 2012;1:759–69.
[92] Houtgraaf JH, de Jong R, Monkhorst K, et al. Feasibility of intracoronary GLP-1 eluting cellbead infusion in acute myocardial infarction. Cell Transplant 2013;22:535–543.
[93] Lotfinegad P, Shamsasenjan K, Movassaghpour A, Majidi J, Baradaran B. Immunomodulatory nature and site specific affinity of mesenchymal stem cells: a hope in cell therapy. Adv Pharm Bull 2014;4(1):5–13.
[94] Tasso R, Ilengo C, Quarto R, Cancedda R, Caspi RR, Pennesi G. Mesenchymal stem cells induce functionally active T-regulatory lymphocytes in a paracrine fashion and ameliorate experimental autoimmune uveitis. Invest Ophthalmol Vis Sci 2012;53(2):786–93.
[95] Siegel G, Schäfer R, Dazzi F. The immunosuppressive properties of mesenchymal stem cells. Transplant 2009;87(9 Suppl):S45–9.
[96] Ulivi V, Tasso R, Cancedda R, Descalzi F. Mesenchymal stem cell paracrine activity is modulated by platelet lysate: induction of an inflammatory response and secretion of factors maintaining macrophages in a proinflammatory phenotype. Stem Cells Dev 2014;23(16):1858–69.
[97] Alipour R, Adib M, Hashemi-Beni B, Sadeghi F. The effect of stem cell from human exfoliated deciduous teeth on T lymphocyte proliferation. Adv Biomed Res 2014;3:202.
[98] Melief SM, Schrama E, Brugman MH, et al. Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages. Stem Cells 2013;31(9):1980–91.
[99] Jin HJ, Bae YK, Kim M, et al. Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. Int J Mol Sci 2013;14(9):17986–8001.
[100] Ammar HI, Sequiera GL, Nashed MB, et al. Comparison of adipose tissue- and bone marrow-derived mesenchymal stem cells for alleviating doxorubicin-induced cardiac dysfunction in diabetic rats. Stem Cell Res Ther 2015;6(1):148.
[101] Liu S, Yuan M, Hou K, et al. Immune characterization of mesenchymal stem cells in human umbilical cord Wharton's jelly and derived cartilage cells. Cell Immunol 2012;278(1–2):35–44.
[102] Lee MW, Kim DS, Yoo KH, et al. Human bone marrow-derived mesenchymal stem cell gene expression patterns vary with culture conditions. Blood Res 2013;48(2):107–14.
[103] Yoo KH, Jang IK, Lee MW, et al. Comparison of immunomodulatory properties of mesenchymal stem cells derived from adult human tissues. Cell Immunol 2009;259(2):150–6.
[104] Scruggs BA, Semon JA, Zhang X, et al. Age of the donor reduces the ability of human adipose-derived stem cells to alleviate symptoms in the experimental autoimmune encephalomyelitis mouse model. Stem Cells Transl Med 2013;2(10):797–807.
[105] Du YY, Zhou SH, Zhou T, et al. Immuno-inflammatory regulation effect of mesenchymal stem cell transplantation in a rat model of myocardial infarction. Cytother 2008;10(5):469–78.
[106] Noort WA, Oerlemans MI, Rozemuller H, et al. Human versus porcine mesenchymal stromal cells: phenotype, differentiation potential, immunomodulation and cardiac improvement after transplantation. J Cell Mol Med 2012;16(8):1827–39.
[107] Martinez VG, Ontoria-Oviedo I, Ricardo CP, et al. Overexpression of hypoxia-inducible factor 1 alpha improves immunomodulation by dental mesenchymal stem cells. Stem Cell Res Ther 2017;8:208.
[108] Poncelet AJ, Vercruysse J, Saliez A, Gianello P. Although pig allogeneic mesenchymal stem cells are not immunogenic in vitro, intracardiac injection elicits an immune response in vivo. Transplant 2007;83(6):783–90.
[109] Poncelet AJ, Nizet Y, Vercruysse J, Hiel AL, Saliez A, Gianello P. Inhibition of humoral response to allogeneic porcine mesenchymal stem cell with 12 days of tacrolimus. Transplant 2008;86(11):1586–95.

[110] Aoyagi T, Matsui T. The cardiomyocyte as a source of cytokines in cardiac injury. J Cell Sci Ther 2011;1:2012(S5). pii:003.
[111] Shimano M, Ouchi N, Walsh K. Cardiokines: recent progress in elucidating the cardiac secretome. Circulation 2012;126(21):e327–32.
[112] Ghadge SK, Mühlstedt S, Ozcelik C, Bader M. SDF-1α as a therapeutic stem cell homing factor in myocardial infarction. Pharmacol Ther 2011;129(1):97–108.
[113] Takahashi M. Role of the SDF-1/CXCR4 system in myocardial infarction. Circ J 2010;74(3):418–23.
[114] Grunewald M, Avraham I, Dor Y, et al. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell 2006;124(1):175–189.
[115] Segret A, Rücker-Martin C, Pavoine C, et al. Structural localization and expression of CXCL12 and CXCR4 in rat heart and isolated cardiac myocytes. J Histochem Cytochem 2007;55(2):141–50.
[116] Askari AT, Unzek S, Popovic ZB, et al. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 2003;362(9385):697–703.
[117] Bonaros N, Rauf R, Werner E, et al. Neoangiogenesis after combined transplantation of skeletal myoblasts and angiopoietic progenitors leads to increased cell engraftment and lower apoptosis rates in ischemic heart failure. Interact Cardiovasc Thorac Surg 2008;(2):249–55.
[118] Elmadbouh I, Haider HKh, Jiang S, Idris NM, Lu G, Ashraf M. Ex vivo delivered stromal cell-derived factor-1alpha promotes stem cell homing and induces angiomyogenesis in the infarcted myocardium. J Mol Cell Cardiol 2007;42(4):792–803.
[119] Askari A, Unzek S, Goldman CK, et al. Cellular, but not direct, adenoviral delivery of vascular endothelial growth factor results in improved left ventricular function and neovascularization in dilated ischemic cardiomyopathy. J Am Coll Cardiol 2004;43:1908–14.
[120] Tang J, Wang J, Yang J, et al. Mesenchymal stem cells over-expressing SDF-1 promote angiogenesis and improve heart function in experimental myocardial infarction in rats. Eur J Cardiothorac Surg 2009;36(4):644–50.
[121] Lataillade JJ, Clay D, Bourin P, et al. Stromal cell-derived factor 1 regulates primitive hematopoiesis by suppressing apoptosis and by promoting G(0)/G(1) transition in CD34(+) cells: evidence for an autocrine/paracrine mechanism. Blood 2002;99(4):1117–29.
[122] Haider HKh, Jiang S, Idris NM, Ashraf M. IGF-1-overexpressing mesenchymal stem cells accelerate bone marrow stem cell mobilization via paracrine activation of SDF-1alpha/CXCR4 signaling to promote myocardial repair. Circ Res 2008;103(11):1300–8.
[123] Cai L, Johnstone BH, Cook TG, et al. IFATS collection: human adipose tissue-derived stem cells induce angiogenesis and nerve sprouting following myocardial infarction, in conjunction with potent preservation of cardiac function. Stem Cells 2009;27(1):230–7.
[124] Zhao L, Johnson T, Liu D. Therapeutic angiogenesis of adipose-derived stem cells for ischemic diseases. Stem Cell Res Ther 2017;8:125.
[125] Dabrowski FA, Burdzinska A, Kulesza A, et al. Comparison of the paracrine activity of mesenchymal stem cells derived from human umbilical cord, amniotic membrane and adipose tissue. J Obstet Gynaecol Res 2017;43(11):1758–68.
[126] Nakanishi C, Yamagishi M, Yamahara K, et al. Activation of cardiac progenitor cells through paracrine effects mesenchymal stem cells. Biochem Biophys Res Commun 2008;374:11–6.
[127] Miller-Kasprzak E, Jagodziński PP. Endothelial progenitor cells as a new agent contributing to vascular repair. Arch Immunol Ther Exp (Warsz) 2007;55(4):247–59.
[128] Urbich C, Aicher A, Heeschen C, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 2005;39(5):733–42.

[129] Shafiq M, Lee SH, Jung Y, Kim SH. Strategies for recruitment of stem cells to treat myocardial infarction. Curr Pharm Des 2015;21(12):1584–97.
[130] Potapova IA, Gaudette GR, Brink PR, et al. Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells in vitro. Stem Cells 2007;25(7):1761–8.
[131] Korf-Klingebiel M, Reboll MR, Klede S, et al. Myeloid-derived growth factor (C19orf10) mediates cardiac repair following myocardial infarction. Nat Med 2015;21(2):140–9.
[132] Martin J, Denver R, Bailey M, Krum H. In vitro inhibitory effects of atorvastatin on cardiac fibroblasts: implications for ventricular remodelling. Clin Exp Pharmacol Physiol 2005;32(9):697–701.
[133] Takeda N, Manabe I, Uchino Y, et al. Cardiac fibroblasts are essential for the adaptive response of the murine heart to pressure overload. J Clin Invest 2010;120(1):254–65.
[134] Pedrotty DM, Klinger RY, Kirkton RD, Bursac N. Cardiac fibroblast paracrine factors alter impulse conduction and ion channel expression of neonatal rat cardiomyocytes. Cardiovasc Res 2009;83(4):688–97.
[135] Fredj S, Bescond J, Louault C, Potreau D. Interactions between cardiac cells enhance cardiomyocyte hypertrophy and increase fibroblast proliferation. J Cell Physiol 2005;202(3):891–9.
[136] Fredj S, Bescond J, Louault C, Delwail A, Lecron JC, Potreau D. Role of interleukin-6 in cardiomyocyte/cardiac fibroblast interactions during myocyte hypertrophy and fibroblast proliferation. J Cell Physiol 2005;204(2):428–36.
[137] Manabe R, Tsutsui K, Yamada T, et al. Transcriptome-based systematic identification of extracellular matrix proteins. Proc Natl Acad Sci USA 2008;105(35):12849–54.
[138] Singla DK, Ahmed A, Singla R, Yan B. Embryonic stem cells improve cardiac function in doxorubicin-induced cardiomyopathy mediated through multiple mechanisms. Cell Transplant 2012;21(9):1919–30.
[139] Ohnishi S, Sumiyoshi H, Kitamura S, Nagaya N. Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions. FEBS Lett 2007;581(21):3961–6.
[140] Talman V, Ruskoaho H. Cardiac fibrosis in myocardial infarction – from repair and remodeling to regeneration. Cell Tissue Res 2016;365(3):563–81.
[141] Okayama K, Azuma J, Dosaka N, et al. Hepatocyte growth factor reduces cardiac fibrosis by inhibiting endothelial-mesenchymal transition. Hypertension 2012;59(5):958–65.
[142] Poppe A, Golsong P, Blumenthal B, et al. Hepatocyte growth factor-transfected skeletal myoblasts to limit the development of post-infarction heart failure. Artificial Org 2012;36(3):238–46.
[143] Wang S, Qin X, Sun D, et al. Effects of hepatocyte growth factor overexpressed bone marrow-derived mesenchymal stem cells on prevention from left ventricular remodelling and functional improvement in infarcted rat hearts. Cell Biochem Funct 2012;30:574–81.
[144] Seo J-H, Park B-W, Cho Y-M, et al. Combinational cell therapy with BM-MSC/HGF-eMSC injectable platform can improve cardiac function after myocardial infarction. Circulation 2017;136:A16289.
[145] Burchfield JS, Dimmeler S. Role of paracrine factors in stem and progenitor cell mediated cardiac repair and tissue fibrosis. Fibrogen Tissue Repair 2008;1(1):4.
[146] Yokokawa M, Ohnishi S, Ishibashi-Ueda H, et al. Transplantation of mesenchymal stem cells improves atrioventricular conduction in a rat model of complete atrioventricular block. Cell Transplant 2008;17(10–11):1145–55.
[147] Li L, Shuo Z, Zhang Y, Yan BY, Zhen X, Guan Z. Paracrine action mediate the antifibrotic effect of transplanted mesenchymal stem cells in a rat model of global heart failure. Mol Biol Rep 2009;36(4):725–31.

[148] Li L, Peng C, Zhang M, et al. Mesenchymal stem cells overexpressing adrenomedullin improve heart function through antifibrotic action in rats experiencing heart failure. Mol Med Rep 2018;17:1437–1444.
[149] Mao Q, Lin C-X, Liang X-L, Jian-Shu Gao J-S, Xu B. Mesenchymal stem cells overexpressing integrin-linked kinase attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions. Mol Med Rep 2013;7(5):1617–23.
[150] Dai HY, He T, Li XL, Xu WL, Ge ZM. Urotensin-2 promotes collagen synthesis via ERK1/2-dependent and ERK1/2-independent TGF-β1 in neonatal cardiac fibroblasts. Cell Biol Int 2011;35(2):93–8.
[151] Haider HKh, Ashraf M. Implantation of genetically manipulated BM cells for cardiac repair. Cytother 2005;7(1):74–5.
[152] Lei Y, Haider HKh, Jiang S, Sim EKW. Reversal of myocardial injury using genetically modulated human skeletal myoblasts in a rodent cryoinjured heart model. Eur J Heart Fail 2005;7(6):945–5.
[153] Niagara MI, Haider HKh, Lei Y, Sim EKW. Autologous skeletal myoblasts transduced with a new adenoviral bicistronic vector for treatment of hind limb ischemia J Vasc Surg 2005;40(4):774–85.
[154] Zhang M, Mal N, Kiedrowski M, et al. SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. FASEB J 2007;21:3197–3207.
[155] Kim CK, Haider HKh, Lim SJ. Gene medicine: a new field of molecular medicine. Arch Pharmacol Res 2001;24(1):1–15.
[156] Haider HKh, Elmadbouh I, Jean-Baptiste Michel J-B, Ashraf M. Non-viral vector gene modification of stem cells for myocardial repair. Mol Med 2008;14(1–2):79–86.
[157] Lei Y, Zhang W, Su LP, et al. Nanoparticle based delivery of hypoxia-regulated VEGF transgene system combined with myoblast engraftment for myocardial repair. Biomaterials 2011;32(9):2424–31.
[158] Noiseux N, Gnecchi M, Lopez-Ilasaca M, et al. Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. Mol Ther 2006;14(6):840–50.
[159] He W, Zhang L, Ni A, et al. Exogenously administered secreted frizzled related protein 2 (Sfrp2) reduces fibrosis and improves cardiac function in a rat model of myocardial infarction. Proc Natl Acad Sci USA 2010;107(49):21110–5.
[160] Jiang S, Haider HKh, Niagara MI, Lu G, Ashraf M. Stable therapeutic effects of mesenchymal stem cell-based multiple gene delivery for cardiac repair. Cardiovasc Res 2008;77(3):525–33.
[161] Lai VK, Ashraf M, Jiang S, Haider Kh. MicroRNA-143 is a critical regulator of cell cycle activity in stem cells with co-overexpression of Akt and angiopoietin-1 via transcriptional regulation of Erk5/cyclin-D1. Cell Cycle 2012;11(4):767–77.
[162] Li Y, Guo X, Xue Q, Zhu M, Gao L, Wang Y. Single cell gene profiling revealed heterogeneity of paracrine effects of bone marrow cells in mouse infarcted hearts. PLoS ONE 2013;8(7):e68270.
[163] Yao Y, Huang J, Geng Y, et al. Paracrine action of mesenchymal stem cells revealed by single cell gene profiling in infarcted murine hearts. PLoS One 2015;10(6):e0129164.
[164] Razban V, Lotfi AS, Soleimani M, et al. HIF-1α overexpression induces angiogenesis in mesenchymal stem cells. Biores Open Access 2012;1(4):174–83.
[165] Bonios M, Chang CY, Terrovitis J, et al. Constitutive HIF-1α expression blunts the beneficial effects of cardiosphere-derived cell therapy in the heart by altering paracrine factor balance. J Cardiovasc Transl Res 2011;4(3):363–72.
[166] Won Y-W, Lee M, Kim HA, Nam K, Bull DA, Kim SK. Synergistically combined gene delivery for enhanced VEGF secretion and anti-apoptosis. Mol Pharm 2013;10(10):3676–83.
[167] Won YW, McGinn AN, Lee M, Nam K, Bull DA, Kim SW. Post-translational regulation of a hypoxia-responsive VEGF plasmid for the treatment of myocardial ischemia. Biomaterials 2013;34(26):6229–38.

[168] Troncoso R, Ibarra C, Vicencio JM, Jaimovich E, Lavandero S. New insights into IGF-1 signaling in the heart. Trends Endocrinol Metab 2014;25(3):128–37.
[169] Spiegelstein D, Kim C, Zhang Y, Li G, Weisel RD, Li RK, Yau TM. Combined transmyocardial revascularization and cell-based angiogenic gene therapy increases transplanted cell survival. Am J Physiol Heart Circ Physiol 2007;293(6):H3311–6.
[170] Yau TM, Kim C, Li G, Zhang Y, Weisel RD, Li RK. Maximizing ventricular function with multimodal cell-based gene therapy. Circulation 2005;112(9 Suppl):I123–8.
[171] Jackson R, Tilokee EL, Latham N, et al. Paracrine engineering of human cardiac stem cells with insulin-like growth factor 1 enhances myocardial repair. J Am Heart Assoc 2015;4(9):e002104.
[172] Wang Y, Haider HK, Ahmad N, et al. Combining pharmacological mobilization with intramyocardial delivery of bone marrow cells over-expressing VEGF is more effective for cardiac repair. J Mol Cell Cardiol 2006;40(5):736–45.
[173] Tang Y, Gan X, Cheheltani R, et al. Targeted delivery of vascular endothelial growth factor improves stem cell therapy in a rat myocardial infarction model. Nanomed 2014;10(8):1711–8.
[174] Tang J-M, Wang J-N, Zhang L, et al. VEGF/SDF-1 promotes cardiac stem cell mobilization and myocardial repair in the infarcted heart. Cardiovasc Res 2011;91(3):402–11.
[175] Dong F, Harvey J, Finan A, Weber K, Agarwal U, Penn MS. Myocardial CXCR4 expression is required for mesenchymal stem cell mediated repair following acute myocardial infarction. Circulation 2012;126(3):314–24.
[176] Cho Y-H, Chab M-J, Song B-W, et al. Enhancement of MSC adhesion and therapeutic efficiency in ischemic heart using lentivirus delivery with periostin. Biomaterials 2012;33(5):1376–85.
[177] Wang WE, Yang D, Li L, et al. PHD2 silencing enhances the survival and paracrine function of transplanted adipose-derived stem cells in infarcted myocardium. Circ Res 2013;113(3):288–300.
[178] Herrmann JL, Wang Y, Abarbanell AM, Weil BR, Tan J, Meldrum D. Preconditioning mesenchymal stem cells with transforming growth factor-alpha improves mesenchymal stem cell-mediated cardioprotection. Shock 2010;33(1):24–30.
[179] Khan M, Akhtar S, Mohsin S, N Khan S, Riazuddin S. Growth factor preconditioning increases the function of diabetes-impaired mesenchymal stem cells. Stem Cells Dev 2011;20(1):67–75.
[180] Elmadbouh I, Haider HKh, Ashraf M, Chachques JC. Preconditioning of human skeletal myoblast with stromal cell–derived factor-1α promotes cytoprotective effects against oxidative and anoxic stress. Int J Stem Cells 2011;4(1):50–60.
[181] Ercana E, Baglab AG, Aksoy A, et al. In vitro protection of adipose tissue-derived mesenchymal stem cells by erythropoietin. Acta Histochem 2014;116(1):117–25.
[182] Wang J, Fu X, Yu L, et al. Preconditioning with VEGF enhances angiogenic and neuroprotective effects of bone marrow mononuclear cell transplantation in a rat model of chronic cerebral hypoperfusion. Mol Neurobiol 2016;53(9):6057–68.
[183] Singla DK, Singla RD, Lamm S, Glass C. TGF-β2 treatment enhances cytoprotective factors released from embryonic stem cells and inhibits apoptosis in infarcted myocardium. Am J Physiol Heart Circ Physiol 2011;300(4):H1442–50.
[184] Kim MJ, Kim ZH, Kim SM, Choi YS. Conditioned medium derived from umbilical cord mesenchymal stem cells regenerates atrophied muscles. Tissue Cell 2016;48(5):533–43.
[185] Shen C, Lie P, Miao T, et al. Conditioned medium from umbilical cord mesenchymal stem cells induces migration and angiogenesis. Mol Med Rep 2015;12(1):20–30.
[186] Stern-Straeter J, Bonaterra GA, Birk R, et al. Evaluation of the effects of different culture media on the myogenic differentiation potential of adipose tissue- or bone marrow-derived human mesenchymal stem cells. Int J Mol Med 2014;33:160–70.
[187] Peran M, Ruiz EL, González-Herrera L, Bustamante M, Valenzuela A, Marchal JA. Cellular extracts from post-mortem human cardiac tissue direct cardiomyogenic differentiation of human adipose tissue-derived stem cells. Cytother 2013;15:1541–8.

[188] Alves da Silva ML, Costa-Pinto AR, Martins A, et al. Conditioned medium as a strategy for human stem cells chondrogenic differentiation. J Tissue Eng Regen Med 2015;9(6):2015.
[189] Gutkowska J, Jankowski M. Oxytocin revisited: its role in cardiovascular regulation. J Neuroendocrinol 2012;24(4):599–608.
[190] Zhou S, Zilberman Y, Wassermann K, Bain SD, Sadovsky Y, Gazit D. Estrogen modulates estrogen receptor alpha and beta expression, osteogenic activity, and apoptosis in mesenchymal stem cells (MSCs) of osteoporotic mice. J Cell Biochem 2001;(Suppl 36):144–55.
[191] Hong L, Zhang G, Sultana H, Yu Y, Zen WZ. The effects of 17-β estradiol on enhancing proliferation of human bone marrow mesenchymal stromal cells in vitro. Stem Cells Dev 2011;20(5):925–31.
[192] Erwin GS, Crisostomo PR, Wang Y, et al. Estradiol-treated mesenchymal stem cells improve myocardial recovery after ischemia. J Surg Res 2009;152(2):319–24.
[193] Suzuki Y, Kim HW, Haider HKh, Ashraf M. Novel role of NF-κB-dependent miR-146a induced by diazoxide preconditioning in stem cell survival. FASEB J 2010;24(Suppl 1):1027.6.
[194] Haider HKh, Lee Y-J, Jiang S, Ahmed RPH, Ryon M, Ashraf M. Phosphodiesterase inhibition with tadalafil provides longer and sustained protection of stem cells. Am J Physiol Heart Circ Physiol 2010;299(5):H1395–404.
[195] Wahl EA, Schenck TL, Machens H-G, Balmayor ER. VEGF released by deferoxamine preconditioned mesenchymal stem cells seeded on collagen-GAG substrates enhances neovascularization. Sci Rep 2016;6:Article number: 36879.
[196] Liu GS, Peshavariya HM, Higuchi M, Chan EC, Dusting GJ, Jiang F. Pharmacological priming of adipose-derived stem cells for paracrine VEGF production with deferoxamine. J Tissue Eng Regen Med 2016;10(3):E167–76.
[197] Suzuki Y, Kim HW, Ashraf M, Haider HKh. Diazoxide potentiates mesenchymal stem cell survival via NF-κB-dependent miR-146a expression by targeting Fas. Am J Physiol Heart Circ Physiol 2010;299(4):H1077–82.
[198] Hoke NN, Salloum FN, Kass DA, Das A, Kukreja RC. Preconditioning by phosphodiesterase-5 inhibition improves therapeutic efficacy of adipose-derived stem cells following myocardial infarction in mice. Stem Cells 2012;30(2):326–35.
[199] Durrani S, Kim HW, Ahmed RP, Ashraf M, Haider KhH. Hypoxia inducible factor-1α dependent miR-210 is a critical regulator of paracrine activity in preconditioned mesenchymal stem cells for angiogenic growth factors. Circulation 2010;122(Suppl 21):A17923.
[200] Hung S-P, Ho JH, Y-R V, Lo T, Lee OK. Hypoxia promotes proliferation and osteogenic differentiation potentials of human mesenchymal stem cells. J Orthopedic Res 2012;30:260–6.
[201] Choi JW, Kim KE, Lee CY, et al. Alterations in cardiomyocyte differentiation-related proteins in rat mesenchymal stem cells exposed to hypoxia. Cell Physiol Biochem 2016;39(4):1595–607.
[202] Chang K-C, Hung S-C. Hypoxia-preconditioned allogeneic mesenchymal stem cells can be used for myocardial repair in non-human primates. J Thorac Dis 2016;8(7):E593–5.
[203] Hou J, Wang L, Long H, et al. Hypoxia preconditioning promotes cardiac stem cell survival and cardiogenic differentiation in vitro involving activation of the HIF-1α/apelin/APJ axis. Stem Cell Res Ther 2017;8(1):215.
[204] Speth JM, Hoggatt J, Singh P, Pelus LM. Pharmacologic increase in HIF1α enhances hematopoietic stem and progenitor homing and engraftment. Blood 2014;123(2):203–7.
[205] Yan F, Yao Y, Chen L, Li Y, Sheng Z, Ma G. Hypoxic preconditioning improves survival of cardiac progenitor cells: role of stromal cell derived factor-1α–CXCR4 axis. PLoS One 2012;7(7):e37948.
[206] Hu X, Yu SP, Fraser JL, et al. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. J Thorac Cardiovasc Surg 2008;135(4):799–808.

[207] Jaussaud J, Biais M, Calderon J, et al. Hypoxia-preconditioned mesenchymal stromal cells improve cardiac function in a swine model of chronic myocardial ischaemia. Eur J Cardiothoracic Surg 2013;43(5):1050–7.
[208] Kim HW, Haider HKh, Jiang S, Ashraf M. Ischemic preconditioning augments survival of stem cells via miR-210 expression by targeting caspase-8-associated protein-2. Circulation 2009;120:S755 (Abstract 3177).
[209] Haider HKh, Kim HW, Ashraf M. Hypoxia-inducible factor-1α in stem cell preconditioning: mechanistic role of hypoxia-related micro-RNAs. Circulation 2009;138(1):257.
[210] Kim HW, Haider HKh, Jiang S, Ashraf M. Ischemic preconditioning augments survival of stem cells via miR-210 expression by targeting caspase-8-associated protein 2. J Biol Chem 2009;284:33161–8.
[211] Shivakumar K, Sollott SJ, Sangeetha M, Sapna S, Ziman B, Wang S, Lakatta EG. Paracrine effects of hypoxic fibroblast-derived factors on the MPT-ROS threshold and viability of adult rat cardiac myocytes. Am J Physiol Heart Circ Physiol 2008;294(6):H2653–8.
[212] Tarone G, Brancaccio M. Keep your heart in shape: molecular chaperone networks for treating heart disease. Cardiovasc Res 2014;102(3):346–61.
[213] Byun K, Kim T-K, Oh J, et al. Heat shock instructs hESCs to exit from the self-renewal program through negative regulation of OCT4 by SAPK/JNK and HSF1 pathway. Stem Cell Res 2013;11(3):1323–34.
[214] McGinley LM, McMahon J, Stocca JA, et al. Mesenchymal stem cell survival in the infarcted heart is enhanced by lentivirus vector-mediated heat shock protein 27 expression. Hum Gene Ther 2013;24(10):840–51.
[215] Qiao P-F, Yao L, Zhang X-C, Li G-D, Wu D-Q. Heat shock pre-treatment improves stem cell repair following ischemia-reperfusion injury via autophagy. World J Gastroenterol 2015;21(45):12822–34.
[216] Verdegaal ME, Zegveld ST, van Furth R. Heat shock protein 65 induces CD62e, CD106, and CD54 on cultured human endothelial cells and increases their adhesiveness for monocytes and granulocytes. J Immunol 1996;157(1):369–76.
[217] Clause KC, Liu LJ, Tobita K. Directed stem cell differentiation: the role of physical forces. Cell Commun Adhes 2010;17(2):48–54.
[218] Shafa M, Krawetz R, Zhang Y, et al. Impact of stirred suspension bioreactor culture on the differentiation of murine embryonic stem cells into cardiomyocytes. BMC Cell Biol 2011;12:53.
[219] Ruan J-L, Tulloch NL, Saiget M, et al. Mechanical stress promotes maturation of human myocardium from pluripotent stem cell-derived progenitors. Stem Cells 2015;33(7):2148–57.
[220] Nurzynska D, Meglio FD, Castaldo C, et al. Shock waves activate in vitro cultured progenitors and precursors of cardiac cell lineages from the human heart. Ultrasound in Med Biol 2008;34(2):334–42.
[221] Zhao Y, Wang J, Wang M, et al. Activation of bone marrow-derived mesenchymal stromal cells – a new mechanism of defocused low-energy shock wave in regenerative medicine. Cytother 2013;15(12):1449–57.
[222] Xu L, Zhao Y, Wang M, et al. Defocused low-energy shock wave activates adipose tissue-derived stem cells in vitro via multiple signaling pathways. Cytother 2016;18(12):1503–14.
[223] Tepeköylü C, Wang FS, Kozaryn R, et al. Shock wave treatment induces angiogenesis and mobilizes endogenous CD31/CD34-positive endothelial cells in a hindlimb ischemia model: implications for angiogenesis and vasculogenesis. J Thorac Cardiovasc Surg 2013;146(4):971–8.
[224] Tepeköylü C, Primessnig U, Pölzl L, et al. Shockwaves prevent from heart failure after acute myocardial ischaemia via RNA/protein complexes. J Cell Mol Med 2017;21(4):791–801.
[225] Burrows GG, van't Hof W, Laura FN. Dissection of the human multipotent adult progenitor cell secretome by proteomic analysis. Stem Cells Transl Med 2013;2:745–57.

[226] Seib FP, Prewitz M, Werner C, Bornhäuser M. Matrix elasticity regulates the secretory profile of human bone marrow-derived multipotent mesenchymal stromal cells (MSCs). Biochem Biophys Res Commun 2009;389(4):663–7.
[227] Wehner R, Taubert C, Mende T, et al. Engineered extracellular matrix components do not alter the immunomodulatory properties of mesenchymal stromal cells in vitro. J Tissue Eng Regen Med 2013;7(11):921–4.
[228] Hassan WU, Greiser U, Wang W. Role of adipose-derived stem cells in wound healing. Wound Repair Regen 2014;22(3):313–25.
[229] Alessandria M, Lizzob G, Gualandi C, et al. Influence of biological matrix and artificial electrospun scaffolds on proliferation, differentiation and trophic factor synthesis of rat embryonic stem cells. Matrix Biol 2014;33:68–76.
[230] Weil BR, Markel TA, Herrmann JL, Abarbanell AM, Meldrum DR. Mesenchymal stem cells enhance the viability and proliferation of human fetal intestinal epithelial cells following hypoxic injury via paracrine mechanisms. Surg 2009;146(2):190–7.
[231] Mazhari R, Hare JM. Mechanisms of action of mesenchymal stem cells in cardiac repair: potential influences on the cardiac stem cell niche. Nat Clin Pract Cardiovasc Med 2007;4(Suppl 1):S21–6.
[232] Lai VK, Prindle KS, Sidow B, Jiang S, Haider KH. Treatment with cell lysate from genetically modified stem cells containing pre-formed trophic factors effectively rescues the infarcted heart. Circ Res 2012;111(12):E383–4.
[233] Kupcova Skalnikova H. Proteomic techniques for characterisation of mesenchymal stem cell secretome. Biochimie 2013;95(12):2196–211.

Peter K. Law, Danlin M. Law, Lei Ye, Khawaja Husnain Haider, Shi Jun Song, Ping Lu, Jian-Hua Ma and Jun Ren

4 Myoblasts provide safe and effective treatments for hereditary muscular dystrophies, cardiomyopathies, type 2 diabetes, solid tumors, and aging

Abstract: In the 500 million years of vertebrate evolution, the skeletal muscle, being externally located in the body, has developed specific characteristics for frontline defense against predation, including diseases. Myoblast implantation is a unique, patented technology of muscle regeneration having been tested in phase III clinical trials of muscular dystrophy and ischemic cardiomyopathy, phase II trial of cancer, and phase I trial of type 2 diabetes. Differentiated and committed, myoblasts are not stem cells. They fuse spontaneously and develop to form "new" myofibers, replenishing skeletal myofibers in muscular dystrophy. They develop to become cardiomyocytes in the infracted myocardium. They also fuse with degenerative myofibers of muscular dystrophy or type 2 diabetes, transferring their nuclei containing the normal human genome to provide stable, long-term expression of the missing gene products. When billions of myoblasts are injected into a solid tumor, membrane breakage prior to cell fusion releases high concentration of tumor necrosis factor-α to inhibit cancer growth and metastasis by inducing apoptosis and cell cycle arrest of cancer cells. Newly formed myotubes use up most of the nutrients and oxygen within the tumor and starve the cancer cells. They wrap around cancer cells and prevent them from metastasis. Furthermore, myoblast allograft generates antibodies and triggers localized immune attack on the cancer cells nearby. Myoblasts, because of their small size, spindle shape, and resilience, grow readily within wrinkles and on skin surfaces, thus enhancing the color, luster, and texture of the skin "plated" with them. They can be injected subcutaneously as a cellular filler to reduce wrinkles. Intramuscular injection of myoblasts can augment the size, shape, consistency, tone, and strength of muscle groups, improving the lines, contours, and vitality from the sculpture for a youthful appearance. This highly promising technology has great social economic values in treating hereditary, fatal, and debilitating disease conditions.

Key Words: Human gene therapy, Somatic cell therapy, Myoblasts, Muscular dystrophies, Heart failure, Ischemic cardiomyopathy, Type 2 diabetes, Cancer, Anti-aging, Cosmetology, Muscle regeneration.

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4.1 Introduction

Grandstands over the Human Genome Project (HGP) [1–6] and somatic gene therapies [7–12] in the last two decades fueled enthusiasm that most human diseases will eventually be cured with molecular medicine [13, 14]. The global market for gene therapy is forecasted to reach US$794 million by 2017 and will grow further as driven by the HGP and the increasing incidence of cancer and other critical diseases [15].

In face of debilitation and death of patients without effective remedy, the Food and Drug Administration (FDA), European Medicine Agency, and State Food & Drug Administration have been under pressure since 1990 to approve the initiation of various gene therapy clinical trials. The US National Institutes of Health registers 3253 gene therapy studies worldwide, enrolling tens of thousands of subjects, mostly dying of incurable diseases such as cancer, autoimmune diseases (autoimmune deficiency syndrome), and viral infections.

Despite huge efforts and expenses spanning nearly a generation of molecular genetics research, only four products have gained marketing authorization:

- Gendicine®, authorized in China in 2003: Ad-p53 gene therapy for head and neck squamous cell carcinoma (HNSCC) [16].
- Oncorine®, authorized in China in 2005: recombinant human adenovirus type 5 injection for nasopharyngeal carcinoma combined with chemotherapy [17].
- Glybera®, authorized in Holland in 2013: functional lipoprotein lipase (LPL) gene packaged in an adeno-associated virus, corrects a rare genetic deficiency of LPL, a protein that helps breakdown fats [18].
- Eteplirsen® (EXONDYS 51), authorized in the United States in 2016: an antisense oligonucleotide as the unproven first drug to treat 13% of the Duchenne muscular dystrophy (DMD) population with exon 51 skipping [19].

All these products are based on single gene transduction. Together, they provide therapies to less than 0.01% of the sick population.

4.2 Gene therapy in the United States

Until the approval of Exondys 51, the US FDA has not authorized any gene therapy product because it has not been convinced on the positive benefit/risk ratio of any gene therapy medicinal products. Whereas viral vectors are impregnated with risks [20–24], nonviral transduction efficiency using liposomes or nanoparticles is significantly reduced. Typically, product development is slow because of numerous clinical failures, high compliance standards, and stringent regulatory surveillance.

The viral-related death of Jesse Gelsinger and participants of other trials in 1999 [25] compelled the FDA to reevaluate gene therapy regulations, setting back the development of all gene therapy programs that were then regulated by the Center for

Biologics Evaluation and Research. One of these programs was the human genome therapy using myoblasts to treat X-linked DMD [19, 26, 27]. This Human Myoblast Genome Therapy (HMGT), also called Myoblast Transfer Therapy (MTT), had previously shown significant safety and efficacy to merit the FDA granting of fast-track on a multicenter phase III clinical trial and allowance to charge following annual reviews of progress for four consecutive years of phase II/III clinical trials [28]. HMGT/MTT did not have any viral involvement and had previously demonstrated complete safety record.

4.3 What constitute genetic diseases?

Genetic diseases were formerly believed to have resulted from deoxyribonucleic acid (DNA) defect of a single locus with 100% heritability as exhibited in phenylketonuria, sickle-cell anemia, adenosine deaminase (ADA) deficiency, and others. Although these monolocus genetic ailments constitute less than 2% of all human genetic diseases and affect less than 1% of sick people, far more currently, incurable diseases are the result of multiple gene defects as in type 2 diabetes and/or haphazard interactions between multiple genes as in cancer.

With the advent of diagnostics using molecular genetics, we now consider genetic diseases as those that have a genetic component with or without 100% heritability. Accordingly, the top killers of mankind – cancer, cardiovascular diseases, type 2 diabetes, cystic fibrosis, and aging – fall within the definition of genetic diseases. Together, these fatal and debilitating diseases account for more than 80% of human death. Published reports indicated that the HMGT/MTT platform has applications to treating most, if not all, of these diseases, and it is the goal of this review to provide the essence.

4.4 Two kinds of gene therapies

Gene therapy encompasses interventions that involve deliberate alteration of the genetic material of living cells to prevent or to treat diseases [29]. This FDA definition places two major technologies into the field of gene therapy:

4.4.1 Single gene transduction

In September 1990, a clinical trial was initiated using retroviral-mediated transfer of the ADA gene into the T-cells of a 4-year-old girl with severe combined immunodeficiency disorder [8]. Although integrated vector and ADA gene expression persisted, the subject had to take regular medication throughout the 2 years of gene treatment. It

was concluded that this single gene therapy was safe and effective in treating patients with this rare form of disease [11]. William French Anderson [30] of the National Institutes of Health made a claim of being the first person ever to succeed in gene therapy [11] and became widely acclaimed as the "Father of Gene Therapy" [31].

Central to this technology is the use of viruses as vectors to deliver normal copies of the faulty or missing gene into a particular cell type of a patient, hoping that the therapeutic gene will be expressed to produce a structural or regulatory protein, thereby alleviating the disease symptom(s) [21, 22]. Single gene transduction using adenoviral and retroviral vectors accounted for about one-third of gene therapy studies.

Retroviral vectors exhibit no toxicity. However, they integrate randomly into the host DNA of dividing cells and may cause mutation and cell death. Although they can house larger transgenes than adenoviruses and adeno-associated viruses, the capacity is less than 10 kb. They are unstable in primate complement and cannot be targeted to a specific cell type *in vivo* [24, 32].

Adeno-associated viruses and adenoviruses are widely used. They can accommodate a broad range of genetically modified genes. They are efficiently taken up by nondividing cells *in vivo*. They do not integrate into chromosomal DNA, thus reducing the risk of insertional mutagenesis, and are amenable to redirected tissue targeting [33].

All viruses can cause harm when they revert to wild-type and become replication competent [20, 34, 35]. Dose-dependent inflammation occurred after nasal [36] or lung [20] administration of the cystic fibrosis transmembrane conductance regulator cDNA conjugated with adenoviral vectors. Viruses, even "fully deleted or helper dependent," produce antigens. When exposed to the host immune system, through leakage, secretion, or cell damage, these antigens trigger immune reactions against the transduced cells. Jesse Gelsinger and many others were believed to have died as a result of a severe immune reaction to the adenovirus vector used [24, 31]. Certain viral elements are also toxic. These three inherent problems post almost insurmountable difficulties that prohibit the safe and efficacious clinical use of viral vectors except for terminal cases. To raise caution, the FDA has mandated viral vector validation of every batch to be used on humans.

Nonviral approaches using lipofection, nanoparticles, and plasmids (naked ribonucleic acid [RNA]) account for about 40% of gene therapy studies. Cationic liposome/DNA complexes gain cellular entry via receptor-mediated endocytosis [37, 38]. Assuming that the transgene escapes digestion by lysozyme within endosomes, it has no built-in mechanism to get across the nuclear membrane and is therefore nonintegrative. The minimal and transient expression of the transgene is the result of random targeting, integration, and regulation. Liposomes and nanoparticles have the advantage of being nontoxic and can therefore be used in large quantities and repeatedly [20, 24], especially in large organs such as muscles [24, 38, 39].

Recombinant genes by themselves, plasmids or naked DNA, were shown to have been taken up and expressed in murine skeletal myofibers [40–42] and cardiac

myocytes [43] following intramuscular injections. Gene expression is insignificantly low despite different delivery conditions and methods [44]. This approach lacks basis and evidence of gene integration and regulation.

4.4.2 Single gene transduction deficiencies

There are numerous deficiencies associated with the use of the single gene transduction technology. Much of the hurdles that were present 18 years ago [12, 20, 21] remain unresolved today [18]. These are as follows:

a. In hereditary degenerative diseases, gene defects cause cells to degenerate and die with time. An effective treatment must not only repair degenerating cells but also replace dead cells with live ones. Single gene transduction cannot replenish live cells. There is very limited evidence that it repairs degenerating cells. The technology appears at best applicable to rare diseases involving single regulatory protein deficit but not to diseases involving cell death because it has no provision to replenish live cells.
b. Single gene transduction cannot repair multiple gene defects such as in type 2 diabetes, cancers, and hereditary cardiomyopathies.
c. Viral vectors have limited capacity to accommodate large DNA fragments such as the dystrophin gene, whose anomaly is responsible for Duchenne and Becker muscular dystrophies.
d. Gene expression lasts usually for less than a month and therefore is incapable to treat genetic diseases like cystic fibrosis that need a continual supply of the therapeutic protein throughout the patient's lifetime.
e. The host immune system often mounts a severe reaction against foreign antigens originating from viral vectors, transcriptional regulatory sequences, and transgene products. This is a significant safety issue. Every infected cell is a potential target for immune assault.
f. Plasmids are often digested by lysozymes and rendered ineffective. There are no built-in mechanisms for gene integration, regulation, and expression.
g. For a gene therapy to be effective and efficient, transgene expression requires precise targeting into a specific cell type, integration onto a specific site on a specific chromosome, and regulation by factors that are the products of other genes. This chain of events involves numerous cofactors, many of which are produced transiently during embryonic development but not in adulthood. This is where the approach of single gene transduction is conceptually inadequate because it cannot provide these cofactors. In complex systems, one hardly knows what they are. Only transfer of the whole normal genome can allow the orderly provision of these cofactors necessary for the transgene expression [23].
h. In diseases such as DMD or cystic fibrosis, secondary degenerative changes often accompany the primary protein deficit. Additional structural and/or regulatory

protein(s) are lost. Even if single gene transduction replaces the primary protein deficit, transduced cells still degenerate because of the secondary changes. These latter proteins can only be replaced by retranscribing the complete normal genome inserted [23].

4.4.3 How does single gene transduction fare?

Without resolving the previously mentioned inherent deficiencies, and considering its high risk/benefit ratio for the patients, this kind of gene therapy should only be reserved for terminal cases. It is erroneous to believe that gene therapy is at hand with the discovery of the human genome sequence. Too many people have died believing that the shots of genes into their blood stream would cure them. A revisit of the basics may help.

The cell is the origin of all life. Contained within its nucleus are close to 30,000 genes that determine cell normality and cell characteristics. The genes are composed of DNAs that are spatially and temporally switched on and off during development to produce more than 100,000 different transcripts of RNA. The transcriptional events occur inside the nucleus and require the nuclear matrix and/or the chromatin to operate efficiently. These regulatory events are poorly understood but invariably involve polygenic interactions.

Whereas the basic sequence of the human genome has been determined, exactly how the genome functions will take many decades of further research. Scientists do not know the spatial and temporal interactions of the RNA transcripts within the cell nucleus and know little of their modes of action. Numerous methods have yet to be developed to determine the diverse functions of some 30,000 genes, and more techniques have to be refined to effect gene regulation and expression. It is through this knowledge that molecular genetics may one day provide rational approaches to gene therapy.

Today, analyses of DNA/RNA variations and gene expression are used mainly in diagnostics, while gene therapy success through single gene transduction has been rare. With genetic diagnostics, physicians may identify their patients' genetic predispositions earlier and help patients take steps to minimize damages. Genetic information can also be used by pharmaceutical and biotech companies to develop therapeutics.

4.5 Human genome therapy

An alternative perspective is that a genetically abnormal cell degenerates due to the lack of the normal genome. In hereditary degenerative diseases such as muscular dystrophies, hereditary cardiomyopathies, and type 2 diabetes, the much-needed normal genome can be incorporated into the genetically abnormal myofibers. This

is achieved by taking a muscle biopsy from a normal donor; isolating and proliferating in culture the regenerative satellite cells, now called "myoblasts" as they are in culture; and injecting the normal myoblasts into the genetically abnormal muscles [23, 27]. This cell transplant procedure is called Myoblast Transfer Therapy or Human Myoblast Genome Therapy.

4.5.1 Myoblasts are not stem cells

Ever since the original description of satellite cells by Alexander Mauro in 1961 [45], muscle development [46] and regeneration [47] had been vigorously studied, and the term "myoblasts" has been universally accepted as the most primitively differentiated cells committed to the myogenic lineage. The uses of the terms "muscle progenitors," "muscle stem cells," and "MyoCells" are reinventing the wheel in nomenclature. These latter cells do not differ significantly in structure, function, and their intended purposes from the isolated and cultured myoblasts. For practical purposes, usage with these cells is considered as with myoblasts.

By definition, stem cells exhibit pluripotency, having the potential to *differentiate* into different cell types at one time. Calling muscle stem cells as monopotent stem cells is erroneous because stem cells are never monopotent. Myoblasts are not stem cells and can only *develop* to become muscle, be it striated, cardiac, or smooth. Differentiation and development are different concepts in cell biology. Misunderstanding of these concepts may lead to different outcomes as to how a particular cell therapy will be regulated, a differentiated somatic cell therapy versus a stem cell therapy.

Through natural cell fusion, which is inherent in myogenesis [46] and muscle regeneration [47], donor myoblasts insert their nuclei that contain full complements of normal genes into the genetically abnormal myofibers, forming multinucleated heterokaryons. The donor nuclei integrate spontaneously and transcribe the missing RNA(s) to effect genetic complementation repair [48].

Only transfer of the normal nuclei, carrying the genomic software and the chromosomal hardware, will allow the orderly provision of various cofactors necessary for the regulation and the expression of the transgenes [23].

Natural transcription of the normal genome within the donor nuclei following HMGT/MTT ensures orderly replacement of any protein deficiency resulting from single gene defects or from haphazard polygenic interactions, much of which is unknown. This differs significantly from single gene transduction, effected through viral or nonviral vectors, in that the transgene may find no transcriptional factors/cofactors in the adult environment for its regulation and expression. Many of these cofactors are the products of other genes that are operative only in early development.

HMGT/MTT is a platform technology of cell transplantation, nuclear transfer, gene therapy, and tissue engineering. It is the only human genome therapy in existence and will remain so until another modality is discovered to deliver the human

genome into the defective cells of a genetically ill patient. Myoblast is the only somatic cell type that has the ability of natural cell fusion. HMGT/MTT is uniquely suited to treat hereditary muscle degeneration and weakness through nuclear transfer or genome transfer. Myoblasts cultured from muscle biopsy survive, develop, and function, after transplantation in animal studies and clinical trials, to revitalize degenerative organs in patients with heart failure, ischemic cardiomyopathy, type 2 diabetes, muscular dystrophies, aging dysfunction, and disfigurement. When donor myoblasts fuse among themselves after HMGT/MTT, they form new muscle fibers to repopulate the degenerative organ, depositing contractile filaments to augment its function. Thus, as a cell therapy, HMGT/MTT applies not only to all forms of skeletal muscle degeneration but also to heart muscle degeneration, bodybuilding, antiaging, and soft tissue augmentation [22]. HMGT/MTT replenishes live cells through cell therapy and genetically repairs degenerating myofibers through genome therapy.

4.5.2 World's first human gene therapy

First conducted in February 1990 and published on July 14, 1990, HMGT/MTT truly is the world's first human gene therapy [48]. Through natural cell fusion, which is inherent in myogenesis and muscle regeneration, donor myoblasts insert full complements of normal genes into DMD dystrophic muscle cells to produce dystrophin, a structural protein that is not produced in DMD muscles due to the genetic defect. The transfer of genetic material and information occurs *in vivo*, with the myoblasts serving as the source and the vehicle of gene transfer.

This first case suggests that HMGT/MTT offers a safe and effective means to replenish biochemical deficit(s) in muscles of hereditary diseases [48]. The report stated that "it does not matter which gene is abnormal and which protein is missing, MTT has potential application for many hereditary muscle diseases." If so, one would expect that MTT could replenish the structural and regulatory protein deficits in type 2 diabetes via improvement of genetic transcriptional pathways, as described later in this article. Indeed, HMGT/MTT alters 50 gene expression profiles, mostly upregulation of insulin signaling pathway and mitochondrial biogenesis and function in skeletal muscles of diabetic KK Cg-Ay/J mice [49, 50], and reduces blood glucose in diabetic mice [50] and type 2 diabetes patients [51].

4.5.3 HMGT/MTT corrects gene defects in DMD clinical trials

HMGT/MTT is the first genetic treatment to have produced any functional improvement in humans, through incorporation of normal genes into genetically defective

cells and through incorporation of genetically normal cells into the genetically abnormal organs [19, 25, 48, 50–55]. It provides stable foreign gene expression and its effect is long-lasting [56]. Many DMD subjects treated 16 to 20 years ago in the Cell Therapy Research Foundation are still alive; some are now in their 30s and 40s (Fig. 4.5.1).

Allograft immunogenicity is minimal, as demonstrated in clinical trials with 280 patients having muscular dystrophies [23] and two patients with ischemic cardiomyopathy [57]. Donor myoblasts fused among themselves or with host myofibers within 2 weeks, losing their MHC-1 surface antigens in the fusion process [57].

Daily use of cyclosporine (Cy) at 5 to 7 mg/kg body weight for no more than 1 month was all that was necessary to prevent allograft rejection [58]. This is highly significant for treating genetic diseases such as muscular dystrophies and type 2 diabetes, where allografts are a must, and for genetically predisposed heart failure, where allografts necessitate only 3 weeks of immunosuppressant.

Fig. 4.5.1: Myoblast transfer therapy extended the lifespan of six DMD patients, among others, up to 42 years of age when some of their DMD uncles died at the age of 18 to 20. For the first time in human history has this been achieved and is being reported. It adds tremendous scientific values to the safety and efficacy of the therapy. These patients were treated 16 to 20 years ago in the Cell Therapy Research Foundation, United States. (Reproduced with permission from Law PK. *Open Journal of Regenerative Medicine* 2016; 5: 25–43. ©Scientific Research Publishing.)

The results demonstrated dystrophin restoration, improved histopathology, reduced muscle enzyme leakage, increased maximum contractile force of voluntary muscles, improved pulmonary function, and clinical benefits in motor control, posture, balance, walking, and breathing. These improvements are the basis of improving the quality of life and extending the lifespan of DMD subjects. There was no evidence of serious, permanent adverse reaction [59].

The safety and efficacy of HMGT/MTT were published before the human genome was sequenced. The development of HMGT/MTT is completely independent of the HGP, which has yet to perform its treatment claims. It appears that in treating genetic diseases with muscle defects, HMGT/MTT completely bypassed the necessity of the HGP and Single Gene Transduction. HMGT/MTT is an independent development in biomedical technology.

4.5.4 HMGT/MTT versus Exondys 51

On September 19, 2016, the US FDA approved Exondys 51, developed by Sarepta Therapeutics, as the first DMD drug. FDA approved Exondys 51 on fast track, priority review, and orphan drug designation. The FDA News Release on the same day documented that, "A clinical benefit of Exondys 51, including improved motor function, has not been established" and that "The most common side effects reported by participants taking Exondys 51 in the clinical trials were balance disorder and vomiting" [60]. On the same day, the Associated Press called Exondys 51 "the largely unproven medication" [61]. Tabs. 4.5.1, 4.5.2, and 4.5.3 show a comparison of proven and published results between Exondys 51 and MTT.

Tab. 4.5.1: Efficacy comparison between MTT and Exondys 51.

MTT	Exondys 51
1. 100% dystrophin production	0.28% dystrophin m-RNA increase
2. 70% muscle fiber increase and histology improved	None
3. 123% strength increase in 18 months	None
4. 48.7% decrease in serum CPK in 1 year	None
5. 19 % increase in breathing capacity	None
6. Physical capacity improved	None
7. Life prolonged	None
8. For DMD, BMD, and other MDs	For 13% DMD with Exon 51 skipping

BMD= Becker muscular dystrophy; CPK: phosphocreatine kinase; DMD= Duchenne muscular dystrophy; MD= muscular dystrophy.

Tab. 4.5.2: Safety comparison between MTT and Exondys 51.

MTT	Exondys 51
280 cases published	8 cases + 4 untreated controls, unpublished
100% safe	Balance disorder/vomiting

MTT= Myoblast Transfer Therapy.

Tab. 4.5.3: Logistic comparison between MTT and Exondys 51.

MTT	Exondys 51
One-time intramuscular injection	Daily intravenous injection
Lifelong benefits	No clinical benefit
US$150,000 total, FDA approved	US$300,000 and above per year

FDA= Food and Drug Administration; MTT= Myoblast Transfer Therapy.

FDA exercised a different standard when approving Exondys 51. The arbitrary and capricious behavior, the lack of due process, and the abuse of entrusted authority exhibited by FDA operations constitute a crime against humanity [19].

4.5.5 HMGT/MTT in heart disease clinical trials

Heart muscle degeneration is the leading cause of debilitation and death in humans. Atherosclerosis, ischemic cardiomyopathy, and heart failure are genetic predisposed [62, 63]. These are multifactorial and polygenic diseases with significant polymorphism. It will be an insurmountable task to identify the various gene defects and to design molecular gene therapies toward treatment, not to mention that such designs do not replenish myocardial cells that had degenerated previously, without which the damaged myocardium cannot regain its function.

Cardiomyocytes do not multiply significantly because the human telomeric DNA repeats in these terminally differentiated cells are minimal. Without significant mitotic activity, surviving cardiomyocytes cannot provide enough new cells to generate the contractile filaments necessary to sustain normal heart contractility.

Through endomyocardial injections of cultured skeletal myoblasts, three mechanisms of myogenesis were elucidated [64, 65] as proof-of-concept with 50 human/porcine xenografts using Cy as immunosuppressant [57, 58]. Some myoblasts developed to become cardiomyocytes. Others transferred their nuclei into host cardiomyocytes through natural cell fusion. As yet others formed skeletal myofibers with satellite cells. *De novo* production of contractile filaments augmented heart contractility.

This latter can be translated into the improvement in the quality of life of heart failure and in the prevention of heart attacks [64, 65].

There was a transient elevation of the porcine antihuman-myoblast antibodies at 1 week after the xenograft [58]. The antibody level subsided at the second week after HMGT/MTT, indicating that no more than 2 weeks of Cy immunosuppression would be necessary for human/pig xenografts or for human allografts.

When compared with a heart transplant, myoblast allograft eliminates the use of lifelong immune suppressants, which is the major cause of infection and death of heart transplant patients. Myoblast transplant, either autograft or allograft, is much less invasive, and tissue availability is not an issue. At a fraction of the cost of a heart transplant, it also promises a reduction in health costs.

To date, approximately 300 subjects with chronic myocardial infarction have received essentially autologous myoblasts via catheter delivery or epicardial injection following coronary artery bypass grafting (CABG). Conclusions drawn from phase I, phase II, and early phase III clinical trials from multiple centers over 15 countries [66–83] were as follows:

1) Myoblast implantation with catheter or surgery following CABG is feasible, safe, and efficacious.
2) It improves left ventricular ejection fraction (LVEF), perfusion, viability, kinesis, ventricular wall thickness, diastolic stiffness and stroke volume of the infarcted myocardium, and 6-minute walk distance.
3) It regenerates the scarred myocardium in ischemic cardiomyopathy.
4) It offers a potential treatment for end-stage heart disease.

The 35% to 45% relative increases in LVEF at 1 year after HMGT/MTT reported independently by several teams [66–83] are highly significant. This has never been achieved with any pharmaceutic or therapeutic modality in the treatment of ischemic cardiomyopathy and heart failure. Such significant increases in LVEF would most likely improve the quality of life and extend the lifespan of the patients. Undoubtedly, HMGT/MTT is the most promising treatments for heart diseases in the horizon [74, 76].

Animal experimental data have culminated that vascular endothelial growth factor 165 (VEGF165)-myoblasts, when injected intramyocardially, are potential therapeutic transgene vehicles for concomitant angiogenesis and myogenesis to treat heart failure and ischemic cardiomyopathy [38, 39, 84–89]. Therapeutic angiomyogenesis has potential application to a host of fatal and debilitating diseases and conditions. A nonviral vector gene delivery approach provides a safer alternative to overcome the untoward effects of viral vectors (Tab. 4.5.4). In anticipation of its transitional application into clinical trials in the near future, we envision that nonviral transduced VEGF165-myoblasts will provide better outcome than their nontransduced counterparts.

Tab. 4.5.4: Human VEGF delivery using adenovirus, nanoparticles, and liposome.

	Ad-VEGF165-myoblasts	PEI-VEGF165-myoblasts	CD-VEGF165-myoblasts
Gene delivery efficiency	95%	11%	8%
Peak VEGF	37 ng/ml on 8th day	20.2 ng/ml on 2nd day	13.1 ng/ml on 2nd day
Decrease in VEGF levels	20 ng/ml on 30th day	47 ng/ml on 18th day	41.7 ng/ml on 18th day

2.78 + 0.2 ng/ml VEGF protein efficiently stimulated neovascularization [90]. VEGF= vascular endothelial growth factor.

4.5.6 Autonomous cell injection catheter

The operation of existing catheters used for cell delivery into the infarct boundary zones of the left ventricle is far from optimal. Injection catheters available are handheld devices operated manually through an inner needle and a distal electrode having tip deflection and torque capabilities. Despite a hefty learning curve, interventionists often encounter difficulties in catheter stabilization and infarct detection, resulting in lengthy operation times and nonprecise injections. A design incorporating robotic positional control, feedback signals/images, and an adaptable algorithmic sequence for automation to overcome these problems was examined. The design provides the basis for the construction of a remote cell injection catheter with moments of autonomy to assist the physician to deliver more efficient cell transfer catheterizations [91].

Remote and robotically actuated catheters are the stepping stones toward autonomous catheters, where complex intravascular procedures can be performed with minimal interference from the physician. A concept for the positional, feedforward control of a robotically actuated intramyocardial cell injection catheter was proposed and tested. The prototype for the catheter system was built upon a needle-based catheter with a single degree of deflection, a three-dimensional printed handle combined with stepper motors, and the Arduino microcontroller platform. A bench set-up was used to mimic a left ventricle operation starting from the femoral artery for the injection of committed myogenic cells or undifferentiated stem cells into a myocardial infarct boundary. Using Matlab and an open source video modeling tool, Tracker, the planar coordinates (y, z) of the catheter position were analyzed and a feedforward control system was developed based on empirical models. Using the Student's t-test with a sample size of 26, it was determined that for both the y- and z-axis, the mean discrepancy between the calibrated and theoretical coordinate values had no significant difference compared to the hypothetical value of $\mu = 0$. This proof-of-concept investigation leads to the possibility of further developing a feedforward control system *in vivo* using catheters with omnidirectional deflection. Feedforward positional control allows for more flexibility in the design of an automated catheter system where problems such as systemic time delay may be a hindrance in instances requiring an immediate reaction [92].

4.5.7 HMGT/MTT corrects gene defects in type 2 diabetes

4.5.7.1 Human study

Type 2 diabetes, also called non-insulin-dependent diabetes mellitus, can be traced to the genetic defects of the glucose transporter 4 (GLUT4) and the insulin-regulated aminopeptidase (IRAP) genomes [93–97]. Such a genetic defect is manifested in reduced GLUT4 storage vesicle (GSV) exocytosis and endocytosis trafficking, resulting in a significant reduction in uptake of blood glucose into muscle fibers and adipose tissue, where 75% of the body's glucose metabolism normally occurs. In type 2 diabetics, normal or even elevated levels of plasma insulin would not elicit normal glucose uptake and high blood sugar persists.

Previous studies demonstrated that the injected myoblasts fused spontaneously with host skeletal muscle fibers, transferring their nuclei that carried the complete normal human genome to replenish normal copies of all aberrant genes, presumably in type 2 diabetes, just as it replenished the dystrophin gene in DMD or Becker muscular dystrophy to achieve genetic complementation repair [23, 27]. Other genetically normal donor myoblasts fuse among themselves to form 70% more new myofibers that undoubtedly exhibit normal GLUT-4/IRAP and GSV activities. The two proven mechanisms of HMGT/MTT from the muscular dystrophy studies led us to formulate a feasibility/safety study in two type 2 diabetes human subjects in year 2004 with Institutional Review Board approval [51].

Human myoblasts were manufactured according to in-house standard operation procedures. Cell production was in compliance with current Good Manufacturing Practices and International Organization for Standardization 9001 standards. About 2 g of muscle biopsy was isolated under local anesthesia from a 20-year-old, pathogen-free, male volunteer after he had met muscle donor criteria. Initial dissociation isolated approximately 10,000 satellite cells that constituted the primary culture for myoblasts.

The culture yielded 47.4×10^9 myoblasts that were 100% pure by positive desmin immunostain and 92.8% viable according to vital dye exclusion tests. The cells were potent in myogenicity in that numerous myotubes were observed within 4 days in a fusion medium. Throughout the culture and for the final injectates, the myoblasts were free of endotoxin (<1.0 EU/ml) and mycoplasma and were negative for sterility (14-day test) and gram stain (absence of gram-positive or -negative bacteria) according to certified laboratory analyses.

Patient 1 was 42 years old, was 157 cm tall, and weighed 68 kg. Patient 2 was 36 years old, was 158 cm tall, and also weighed 68 kg. The patients showed about 2-year-history of type 2 diabetes and hypertension but were otherwise normal in heart, lung, kidney, and liver function without obesity. The laboratory report revealed that test results for syphilis, hepatitis B surface antigens, and antibodies to HIV and hepatitis C virus to be negative.

Both patients had previously been enrolled as clinical trial subjects after qualifying for inclusion/exclusion criteria and signing patients' informed consents with institutional approval. The subjects took two oral doses of Cy totaling 5–7 mg/kg body

weight per day, beginning 2 days before grafting, were weaned at half-dosage in the last 2 weeks, and were off Cy at 8 weeks after grafting.

The whole blood trough level of Cy was monitored every 2 weeks. Doses were adjusted in an attempt to maintain the level at about 250 ng/ml.

Myoblasts were harvested and processed under biological safety cabinets (Class 100) inside a cleanroom. Having been washed thoroughly, they were suspended in the injection medium. Quality assurance/quality control processes ensued, and the final quality control release test forms were issued for each of the two subjects. The myoblasts were then carried in syringes within sterile enclosures into two surgical suites for simultaneous implantation into both subjects. These were the world's first cases of allogeneic myoblasts being injected into type 2 diabetes patients.

The patients received 132 injections each and 24/23.4 billion myoblasts, respectively. The 2-hour procedure was performed with the patients under general anesthesia. Cells were injected at 50×10^6/ml and the injections were made under direct vision into 54 major muscle groups of each subject. The patients were transferred to the intensive care unit, where they recovered from the anesthesia and routine monitors were administered. The subjects recovered from the general anesthesia without rash or fever, and both patients were discharged at 48 hours postoperatively.

Most pertinent to the specific goal of this study was that despite Cy discontinuation at 2 months postoperatively, no sign of rejection was observed. Cy is known to increase plasma glucose. The patients appeared to have good general health before and after MTT. Plasma glucose and insulin levels did not show significant difference before versus after 24-billion HMGT.

This was a feasibility and safety study and was not designed to test efficacy. This pioneering feasibility/safety study of myoblast allografts into the skeletal muscles of type 2 diabetes patients led the way in developing a genetic treatment for the disease [51]. The subjects were weaned off Cy. The procedure was shown to be safe for both subjects.

A potential genetic treatment of the disease involves HMGT/MTT similar to the 50-billion myoblast protocol used to treat muscular dystrophy. It consists of culturing genetically normal, immature muscle cells called myoblasts, derived originally from a 2 g skeletal muscle biopsy from a healthy, young, male donor free of blood-borne pathogens, and injecting these allogeneic myoblasts with host serum at approximately 10^8 cells/ml into 80 major muscles of a diabetic patient. A distinct improvement in the technology will be the use of host serum as the carrier solution that enhances the survival and development of the myoblast allograft. Cy will be used for 2 to 3 weeks as an immunosuppressant.

4.5.7.2 Animal studies

Mice with muscle-specific Glut-4 knockout were insulin resistant and glucose intolerant from an early age [96–98]. Muscle-specific LKB1 (a serine/threonine kinase that is a negative regulator of insulin sensitivity) knock-out increased insulin sensitivity and improved glucose homeostasis [99]. These studies indicated that defects in skeletal muscle insulin-stimulated glucose transport were key factors in insulin resistance

and type 2 diabetes mellitus [100]. Ye *et al.* demonstrated that HMGT/MTT attenuated hyperglycaemia and hyperinsulinemia and improved glucose tolerance in a mouse model (KK) of type 2 diabetes mellitus [49] as follows.

KK Cg-Ay/J mice, aged 12–14 weeks, after an initial intraperitoneal glucose tolerance test (GTT) were divided into three groups: KK control group receiving basal medium (M199); KK myoblast group receiving 3×10^7 human myoblasts; and KK fibroblast group receiving 3×10^7 human fibroblasts. Nondiabetic C57BL mice were used as an additional normal control and also had HMGT/MTT. All animals were treated with Cy for 6 weeks.

Immunohistochemistry studies at 12 weeks showed extensive integration of human myoblast nuclei into mouse muscle fibers. Repeat GTT showed a significant decrease in glucose concentrations in the KK myoblast group compared to the KK control and KK fibroblast groups. The KK myoblast group also had reduced mean HbA1c, cholesterol, insulin, and triacylglycerol and increased adiponectin compared with the KK control and KK fibroblast groups. C57BL mice showed no change in glucose homeostasis after HMGT/MTT.

Ma *et al.* demonstrated for the first time that HMGT/MTT resulted in a change in gene transcripts in multiple [50] genes involved in the insulin signaling pathway and in the mitochondrial biogenesis and function of the skeletal muscles of KK mice. Methods of human myoblast implantation and immunosuppression follow that of Ye *et al.* [49]. Hind limb muscles were harvested and used for study of gene expression profiling.

As in Ye *et al.* [49], extensive integration of donor myoblast nuclei into host muscle fibers was demonstrated at 12 weeks after HMGT/MTT. GTT showed a significant decrease in blood glucose in the mice of KK myoblast group compared to the KK control and fibroblast groups. Transcriptional patterns of 23 genes in the insulin signaling pathway showed an upregulation and a downregulation in the KK myoblast group as with the KK control group, the KK fibroblast group, and the C57BL group. In addition, the transcriptional patterns of 27 genes in mitochondrial biogenesis and function also demonstrated alterations in the KK myoblast group as compared with the KK control group, the KK fibroblast group, and the C57BL group. These upregulation and down-regulation in transcriptional pattern levels of the 50 genes after HMGT/MTT represent genetic repair toward attenuating hyperglycaemia and hyperinsulinemia and improving glucose tolerance in the mouse model (KK) of type 2 diabetes mellitus [50].

For the first time, at least in the mouse, type 2 diabetes was shown to be a polygenic disease involving at least 50 gene defects.

Undoubtedly, these studies [49–51] provide strong evidence that HMGT/MTT mediates its beneficial effects through myoblast fusion, not only adding new normal myofibers but also replenishing normal copies of the abnormal genes upon nuclear transfer to effect genetic repair of the dystrophic [23, 48, 52–56], cardiomyopathic [38–40, 57–59, 63–89], or diabetic myofibers [49–51]. Together, they demonstrated the proof-of-concept of HMGT/MTT in developing as a cell therapy and as a

gene therapy for hereditary muscle diseases such as muscular dystrophies, hereditary cardiomyopathies, type 2 diabetes, and others [25, 27, 59].

4.5.8 Myoblast implantation inhibits metastasis and tumor growth and induces cancer apoptosis

Cumulated results from coculture studies, animal experimentation, and clinical trial provided confirmatory evidence to indicate that myoblasts and developing myotubes, either singly or in combination, were potent biologics that inhibited metastasis and tumor growth and induced cancer cell apoptosis (Fig. 4.5.2a, b) [101].

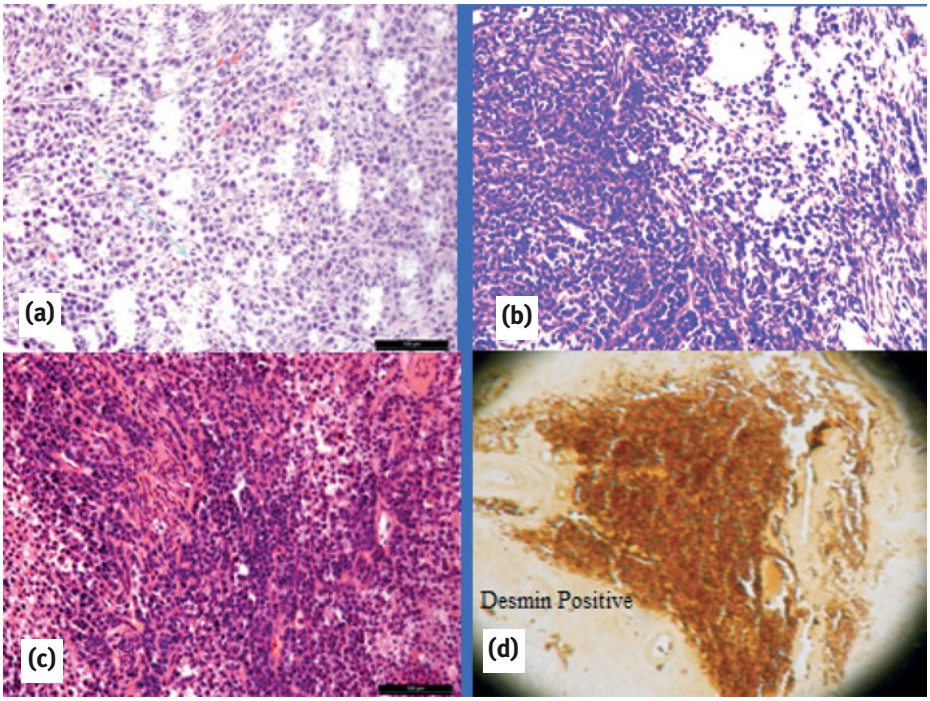

Fig. 4.5.2: Histologic section taken from a shrunken gastrointestinal tumor from the back of a nude mouse at 19 days after intratumor implantation of 12 × 10^6 myoblasts human myoblasts. (a, b) presence of spindle-shaped myoblasts and myotubes amidst round tumor cells in the myoblast-treated tumor, with deaths of cancer cells and nonvascularized myotubes appearing as empty space or vacuoles in the tumor sections. (c) Section of the control tumor compact with cancer cells. Microscope magnification for (a), (b), and (c) is ×200. H&E stain. (d) Myoblasts stained brownish with human desmin immunocytochemistry were observed wrapping around the round cancer cells in sections of the myoblast-treated tumors but not in sections of the control tumor. Microscope magnification for (d) is ×400. (Reproduced with permission from Law et al. *Open Journal of Regenerative Medicine* 2017; 6: 1–16. © Scientific Research Publishing.)

Direct injection of allogeneic (or even autologous) human myoblasts at 100 million per milliliter of host serum into the solid tumor without immunosuppressant was preferred, although myoblast concentration might vary from 75 to 250 million per milliliter.

Exposing the allogeneic myoblasts to 100% host serum primed the myoblasts for proliferation. Implantation of this mixture into the tumor constituted serum restriction, a condition that terminated mitosis and induced cell fusion to occur.

Myoblasts' unique characteristic shared only with cardiac and smooth muscle cells was natural cell fusion, through which myoblasts, at the end of their mitotic cycle, underwent cell membrane breakage, releasing large but natural quantity of cancer-killing tumor necrosis factor-a (TNF-α) and possibly other TNFs into the microenvironment. The second phase of cell fusion was accomplished by massive sarcolemma formation, enclosing 200 to 500 myoblast nuclei into one myotube. Competition for nutrients and oxygen against cancer cells within the tightly encapsulated tumor was fierce, resulting in death of cancer and myogenic cells. Each of the myotube had to be vascularized and innervated to survive, failing which the myotubes would disappear, leaving vacuoles and empty spaces within shrunken tumors (Fig. 4.5.2a, b) as compared to control (Fig. 4.5.2c). Furthermore, allogeneic myoblast implantation triggered inflammation and local immune response, killing myoblasts and cancer cells indiscriminately. Cancer cells also became nonmetastatic as being "wrapped" with myoblasts immuno-stained brownish with desmin (Fig. 4.5.2d) [101]. Stolting et al. previously reported that myoblasts inhibited prostate cancer growth by paracrine secretion of tumor necrosis factor [102].

We postulate four mechanisms of cancer apoptosis: a) myoblasts releasing TNF-α (Fig. 4.5.3a); b) deprivation of nutrients and oxygen (Fig. 4.5.3b); c) local inflammatory and immunologic attacks (Fig. 4.5.3c); and d) prevention from metastasis (Fig. 4.5.2d). These basic and clinical studies demonstrated the preliminary safety and efficacy of intratumor myoblast implantation in the development of prevention and treatment for cancer, now the number one disease killer of mankind.

This is the first report to have documented plausible mechanisms and the use of precision implantation of intratumor myoblast implantation to treat solid tumors in cancer patients. The safety and efficacy that it demonstrated, though preliminary, lead the way to developing a potential new treatment for cancer (Fig. 4.5.4). Lacking graft-versus-host damage, myoblasts and developing myotubes are safe biologics. Obviously, the benefit/risk ratio will favor well-designed clinical trials to be conducted at their earliest, including randomized, double-blind studies.

4.5.9 Anti-aging aesthetica

Beauty is a physical attribute that often enhances one's self-confidence, career, and quality of life. The physical parameters of appearance are size, shape, tone, color,

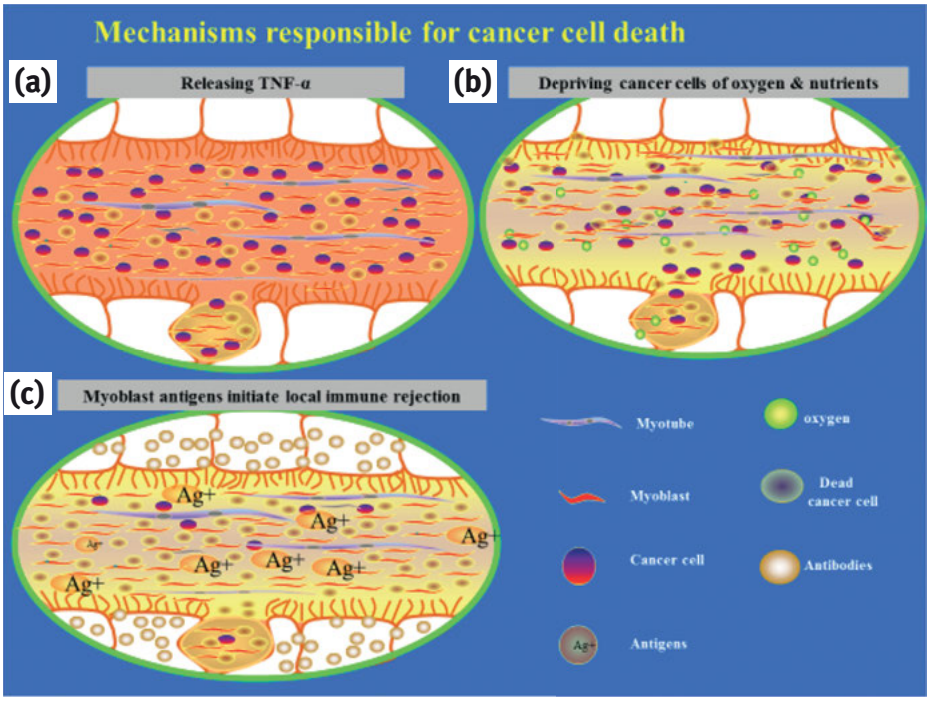

Fig. 4.5.3: Initially, three mechanisms were considered to be responsible for the inhibition of cancer cell proliferation and cancer cell apoptosis: (a) tumor necrosis factor-α (TNF-α) released following cell membrane breakage during myoblast mitosis and cell fusion killed cancer cells; (b) dividing myoblasts and newly developed myotubes competed successfully and had taken away most, if not all, of the nutrients and oxygen inside the tumor from the cancer cells; and (c) injection trauma of allogeneic myoblasts mounted local inflammatory and immunologic attacks on both myoblasts and cancer cells. (Reproduced with permission from Law et al. *Open Journal of Regenerative Medicine* 2017; 6:1–16. ©Scientific Research Publishing.)

luster, texture, consistency, and density. These parameters deteriorate in every organ according to the genetically programmed degeneration of aging. Current technologies favor using live cells to enhance these parameters of appearance. The patented HMGT/MTT is at the forefront of regenerative medicine today (Tab. 4.5.5) [103–107].

4.5.10 Skin cover

The polygonal skin fibroblasts are about 15 times the size of the myoblasts and produce a rough body cover. Cancers are common in skin but rare in muscle. Myoblasts, because of their small size, spindle shape, and resilience, can grow within wrinkles and on skin surfaces, thus enhancing the color, luster, and texture of

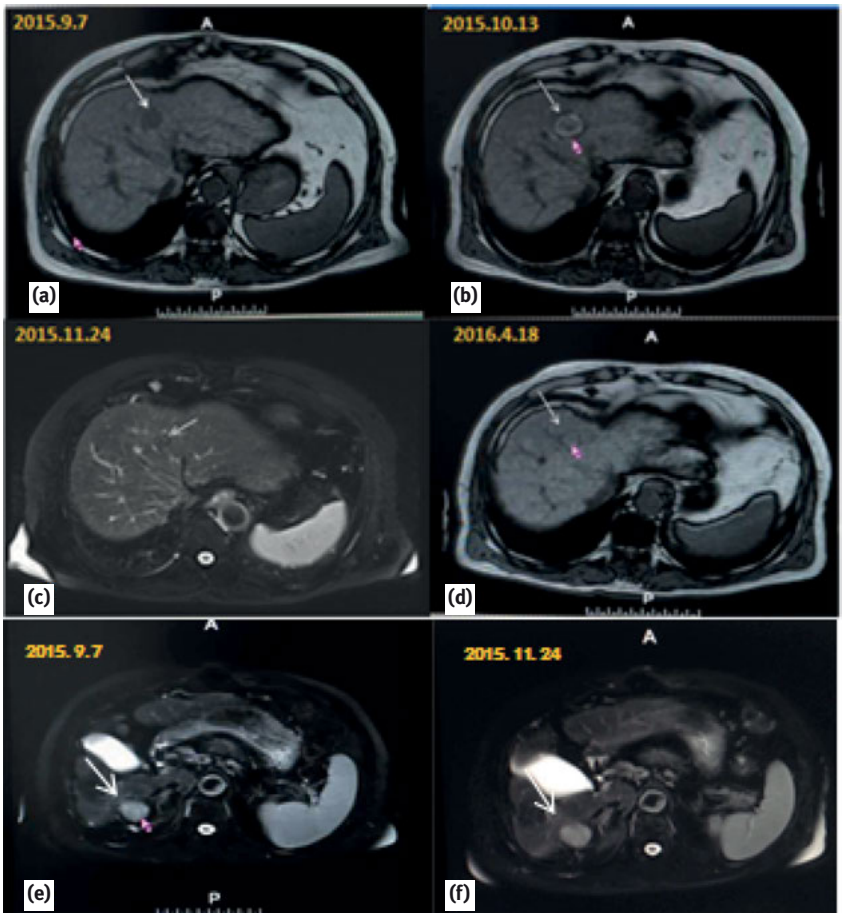

Fig. 4.5.4: Magnetic resonance imaging of liver cancer showing the size and density of the upper tumor (arrow) before myoblast implantation (a) and at (b) 1 month, (c) 2.5 months, and (d) 7 months after implantation. Whereas (e) the noninjected control lower tumor (arrow) in the same liver increased in size in 2 months (f), the myoblast-injected T1 showed a significant decrease in size and density with time, indicating that the myoblasts and myotubes interrupted cancer proliferation and induced cancer apoptosis. (Reproduced with permission from Law et al. *Open Journal of Regenerative Medicine* 2017; 6:1–16. ©Scientific Research Publishing.)

Tab. 4.5.5: Current materials in cosmetology.

Myoblasts	Long-lasting sculpture with live tone
Fibroblasts	Short-lived; easily tumorigenic
Silicone	Bio-non-compatible; leaky; turmorigenic
Saline	No live feeling; leaky
Collagen	Absorbed within 3 to 6 months
Botox	Damage neuromuscular transmission; diminishes facial expression

the skin "plated" with them [27]. Biologic creams are formulated to promote cell survival, growth, and development to enhance the color, luster, density, and texture of the skin. Thus, a new layer of biological skin consisting of pure myoblasts can eliminate skin defects and blemishes.

4.5.11 Body sculpture

Intramuscular injection of myoblasts can augment the size, shape [27], consistency, tone, and strength of muscle groups, improving the lines, contours, and vitality from the sculpture for a youthful appearance. Myoblast technology can be used for cosmetic enhancement such as bodybuilding and in tissue implants for breast, buttock, or facial augmentation (Fig. 4.5.5).

The myoblasts can be injected intramuscularly to grow muscles or subcutaneously as a filler. Unlike the noncellular collagen, which is absorbed in 3 to 6 months after injection, injected myoblasts are cells that will survive and last for tens of years within the host. Myoblasts are endogenous to the human body and have been proven

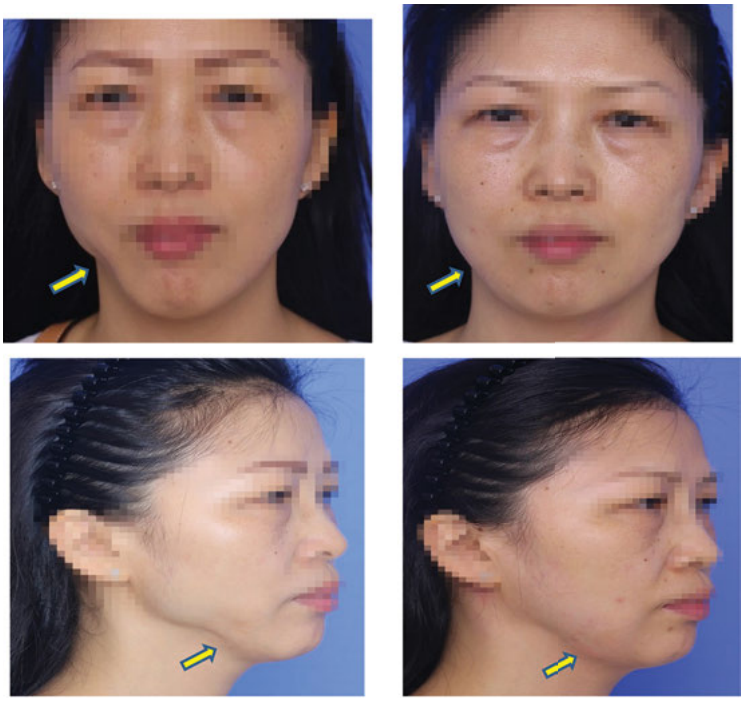

Fig. 4.5.5: Facial restoration with implantation of 10 billion myoblasts. Ultrasound examination demonstrated that subcutaneous soft tissue thickness increased from 0.30 cm before implantation to 1.35 cm 3 months after.

safe in clinical trials involving over 280 muscular dystrophy patients and 300 heart failure worldwide. Myoblasts will not cause cancer like silicone or burst and absorbed like saline or collagen implants.

4.5.12 Anti-aging angiomyogenesis

The distribution of oxygen and nutrients to the peripheral organs is significantly reduced for people aged over 45. In developing treatment for human myocardial infarction, we have grown five times more blood capillaries and muscle simultaneously using human myoblasts transduced with angiogenic factors [84, 89]. In addition to their application to treat heart diseases, these cells can potentially be used to treat male/female impotency and baldness and to produce redder lips and pinker face because of the higher density of capillaries within layers of myogenic cells after myoblast treatment. The latter serves as a fertile ground to seed new hair follicle cells on the bald head or other body parts to give the desirable hair color, density, and consistency.

4.6 Conclusion

HMGT/MTT mediates its effect through transfer of the normal myoblast nuclei that supply the complete human genome, in addition to just replenishing the missing gene(s) or normal copies of the aberrant gene(s). The replacement genes then transcribe to produce the necessary proteins or factors for genetic repair. Donor myoblasts also develop to supply significantly large numbers of normal myofibers to combat muscle degeneration and weakness.

One can envision a variety of uses of this discovery, including that for disease treatment, disease prevention, drug discovery, and selection of superior cells and clones for therapy. It is through continual research and development that we can fully harness HMGT/MTT to relieve human suffering, to improve quality of life, and to prolong the life expectancy of mankind.

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Conflict of interest: As originator of the MTT/HMGT technology, the first author holds the pioneering patent and many others related to the compositions, methods, and medical devices.

4.7 References

[1] DeLisi C. The Human Genome Project. Am Sci 1988;76:488.
[2] Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. Science 2001;291:1304-51. doi: 10.1126/science.1058040. Bibcode: 2001Sci...291.1304V. PMID: 11181995.
[3] Venter D. A part of the human genome sequence. Science 2003;299(5610):1183–4. doi: 10.1126/science.299.5610.1183. PMID: 12595674.
[4] White House Press Release. Retrieved July 22, 2006.
[5] DeLisi C. Meetings that changed the world: Santa Fe 1986: human genome baby-steps. Nature 2008;455(7215):876.
[6] Collins F. The Language of God: A Scientist Presents Evidence for Belief. Free Press, 2006. ISBN 0-7432-8639-1. OCLC 65978711.
[7] Beardsley T. Profile: Gene doctor. W. French Anderson pioneers gene therapy. Sci Am 1990;263:33–4.
[8] Anderson WF. Editorial. The beginning. Hum Gene Ther 1990;1:371–2.
[9] Culver KW, Osborne WR, Miller AD, et al. Correction of ADA deficiency in human T lymphocytes using retroviral-mediated gene transfer. Transplant Proc 1991;23:170–1.
[10] Karlsson S. Treatment of genetic defects in hematopoietic cell function by gene transfer. Blood 1991;78:2481–92.
[11] Anderson WF. Human gene therapy. Science 1992;256:808–13.
[12] Culver KW. The first human gene therapy experiment. In: Culver KW, ed. Gene Therapy. A Primer for Physicians. New York: Mary Ann Liebert Inc., 1996, 47–53.
[13] Gonzaga-Jauregui C, Lupski JR, Gibbs RA. Human genome sequencing in health and disease. Ann Rev Med 2012;63:35–61.
[14] Snyder M, Du J, Gerstein M. Personal genome sequencing: current approaches and challenges. Genes Dev 2012;24:423–31.
[15] GIA announcement, San Jose, California (PRWEB), February 28, 2012.
[16] Pearson S, Jia H, Kandachi K. China approves first gene therapy. Nat Biotech. 2004; 22:3–4. doi: 10.1038/nbt0104-3.
[17] Liang M. Clinical development of oncolytic viruses in China. Curr Pharm Biotechnol. 2012;13:1852–7.
[18] Ylä-Herttuala S. Endgame: Glybera finally recommended for approval as the first gene therapy drug in the European Union. Mol Ther 2012;20:1831–2. doi:10.1038/mt.2012.194.
[19] Law PK. Editorial: crime against humanity. Open J Regen Med 2017;6:35–45. https://doi.org/10.4236/ojrm.2017.64004
[20] Brenner MK. Human somatic gene therapy progress and problems. Int Med 1995;22:2379.
[21] Crystal RG. Transfer of genes to humans: early lesions and obstacles to success. Science 1997;270:404–10.
[22] Verma I, Somia N. Gene therapy – promises, problems and prospects. Nature 1997; 389:239–42.
[23] Law PK, Goodwin TG, Fang Q, et al. Myoblast transfer as a platform technology of gene therapy. Gene Ther Molec Biol 1998;1:345–63.
[24] Thomas CE, Ehrhardt A, Kay, MA. Progress and problems with the use of viral vectors for gene therapy. Nat Rev Genet 2003;4:346–58. doi:10.1038/nrg1066.
[25] Law PK. Myoblast transplantation. Science 1992;257:1329. doi:10.1126/science.1529326.
[26] Furlong TE. Myoblast transplantation. Science 1992;257:1329. doi:10.1126/science.1529326.
[27] Law PK, Law DM. Human myoblast genome therapies and devices in regenerative medicine. Recent Patents Regen Med 2011;1:88–117.

[28] Kessler DA, Siegel JP, Noguchi PD, Zoon KC, Feiden KL, Woodcock J. Regulation of somatic cell-therapy and gene therapy by the Food and Drug Administration. N Engl J Med 1993;329:1169–73.
[29] William French Anderson. Wikipedia, the free encyclopedia, 2013. en.wikipedia.org/wiki/William_French_Anderson.
[30] Anderson WF. Gene Therapy. Sci Am 1995;273:96–8B.
[31] Stolberg SG. The biotech death of Jesse Gelsinger. New York Times Magazine, 1999. Available from: naberbiology.com
[32] Morsy MA, Caskey CT. Safe gene vectors made simpler. Nature Biotech 199;15:17.
[33] Coutelle C, Caplen N, Hart S, et al. Towards gene therapy for cystic fibrosis. In: Dodge JA. Brock JH, Widdicombe JH, eds. Cystic Fibrosis Current Topics. New York: John Wiley and Sons, 1994, 2, 33–54.
[34] Curiel DT, Pilewski JM, Albelda S. Gene therapy approaches for inherited and acquired lung diseases. Am J Respir Cell Mol Biol 1996;14:1–18.
[35] Knowles MR, Hohneker K, Zhou ZQ, et al. A double blind vehicle-controlled study of adenoviral mediated gene transfer in the nasal epithelium of patients with cystic fibrosis. N Eng J Med 1995;333:823–31.
[36] Stewart MJ, Plautz GE, Del Buono L, et at. Gene transfer in vivo with DNA-liposome complexes safety and acute toxicity in mice. Hum Gene Ther 1992;3:267–75.
[37] Trubetskoy VS, Torchilin VP, Kennel SJ, et al. Cationic liposomes enhance targeted delivery and expression of exogenous DNA mediated by N-terminal modified poly-L-lysine-antibody conjugate in mouse lung endothelial cells. Biochem Biophys Acta 1992;1131:311–3.
[38] Ye L, Haider HKH, Tan RS, et al. Transplantation of nanoparticle transfected skeletal myoblasts overexpressing vascular endothelial growth factor-165 for cardiac repair. Circulation 2007;116: I-113–20.
[39] Ye L, Haider HKH, Tan R, et al. Angiomyogenesis using liposome based vascular endothelial growth factor-165 transfection with skeletal myoblast for cardiac repair. Biomaterials 2008;29:2125–37.
[40] Wolff IA, Malone RW, Williams P, et al. Direct gene transfer into mouse muscle in vivo. Science 1990;247:1465–8.
[41] Ascadi G, Dickson G, Loves DR, et al. Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs. Nature 1991;352:815–8.
[42] Davis HL, Whalen RG, Demeneix BA. Direct gene transfer into skeletal muscles in vivo, factors affecting efficiency of transfer and stability of expression. Hum Gene Ther 1993;4:151–9.
[43] Lin H, Parmacek MS, Morle G, Bolling S, Leiden JM. Expression of recombinant genes in myocardium in vivo after direct injection of DNA. Circulation 1991;82:2217–21.
[44] Wolff IA, Williams P, Ascadi G. et al. Conditions affecting direct gene transfer into rodent muscle in vivo. Biotech 1991;11:474–85.
[45] Mauro A. Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 1961;9:493.
[46] Banker BQ, Przybylski RJ, Van Der Meulen JP, Victor M, eds. Research in Muscle Development and the Muscle Spindle. Amsterdam: Excerpta Medica, 1972, 3, 226.
[47] Criggs RC, Karpati G, eds. Myoblast Transfer Therapy. New York: Plenum Press, 1990, 1–316.
[48] Law PK, Bertorini T, Goodwin TG, et al. Dystrophin production induced by Myoblast Transfer Therapy in Duchenne muscular dystrophy. Lancet 1990;336:114–5.
[49] Ye L; Lee KO; Su LP, et al. Skeletal myoblast transplantation for attenuation of hyperglycaemia, hyperinsulinaemia and glucose intolerance in a mouse model of type 2 diabetes mellitus. Diabetologia 2009;52:1925–34.
[50] Ma JH, Su LP, Zhu J, et al. Skeletal myoblast transplantation on gene expression profiles of insulin signaling pathway and mitochondrial biogenesis and function in skeletal muscle. Diabetes Res Clin Pract 2013;102:43–52.

[51] Law PK, Law DLM, Lu P, et al. The world's first myoblast study of type II diabetic patients. Business Briefing: North American Pharmacotherapy, 2004, Issue 2.
[52] Law PK, Goodwin TG, Fang Q, et al. Feasibility, safety, and efficacy of Myoblast Transfer Therapy on Duchenne muscular dystrophy boys. Cell Transplant 1992;1:235–44.
[53] Law PK, Goodwin TG, Fang Q, et al. Pioneering development of myoblast transfer therapy. In: Angelini C, Danieli GA, Fontanari D, eds. Muscular Dystrophy Research: From Molecular Diagnosis toward Therapy. New York: Elsevier Science Publishers, 1991, 109–16.
[54] Law PK, Goodwin TG, Fang Q, et al. Long-term improvement in muscle function, structure and biochemistry following myoblast transfer in DMD. Acta Cardiol 1991;1:281–301.
[55] Law PK, Goodwin TG, Fang Q, et al. Cell transplantation as an experimental treatment for Duchenne muscular dystrophy. Cell Transplant 1993;2:485–505.
[56] Law PK, Goodwin TG, Fang Q, et al. First human Myoblast Transfer Therapy continues to show dystrophin after 6 years. Cell Transplant 1997;6:95–100.
[57] Law PK, Sim EKW, Haider KhH, et al. Myoblast genome therapy and the regenerative heart. In: Kipshidze NN, Serruys PW, eds. Handbook of Cardiovascular Cell Transplantation. London: Dunitz Ltd., 2004, 241–57.
[58] Haider HKh, Jiang SJ, Ye L, Aziz S, Law PK, Sim EK. Effectiveness of transient immunosuppression using cyclosporine for xenomyoblast transplantation for cardiac repair. Transplant Proc 2004;36:232–5.
[59] Law PK. Disease prevention and alleviation by human myoblast transplantation. Open J Regen Med 2016;5:25–43.
[60] US Food & Drug Association. FDA grants accelerated approval to first drug for Duchenne muscular dystrophy [Press release]. 2016. Retrieved from: https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm521263.htm
[61] Perrone M. FDA Oks 1st muscular dystrophy drug; awaits proof it works. Associated Press. September 19, 2016. Retrieved from https://apnews.com/9c4e469479ea438eb42957ba6ecd4710/fda-tentatively-approves-first-drug-muscular-dystrophy
[62] Beutner F, Teupser D, Gielen S, et al. Rationale and design of the Leipzig (LIFE) Heart Study: phenotyping and cardiovascular characteristics of patients with coronary artery disease. PLoS One 2011;6(12):e29070.
[63] Alavantic D, Djuric T. Risk factors of atherosclerosis: a review of genetic epidemiology data from a Serbian population. Exp Clin Cardiol 2006;11:78–82.
[64] Law PK, Haider K, Fang G, et al. Mechanisms of myoblast transfer in treating heart failure. In: Kimchi A, ed. Advances in Heart Failure. New York: Medimont, 2002, 43–48.
[65] Law PK. The regenerative heart. Business Briefing, Pharmtech 2002, 65–71.
[66] Law PK, Leo AB, Lu P, et al. Human myoblast genome therapy. J Geriat Cardiol 2006;3:135–51.
[67] Hagege AA, Carrion C, Menasché P, et al. Viability and differentiation of autologous skeletal myoblast grafts in ischemic cardiomyopathy. Lancet 2003;361:491–2.
[68] Pagani FD, DerSimonian H, Zawadzka A, et al. Autologous skeletal myoblasts transplanted toischemia damaged myocardium in humans: histological analysis ofcell survival and differentiation. J Am Coll Cardiol 2003;41:879–88.
[69] Menasche P, Vilquin JT, Desnos M. Early results of autologous skeletal myoblast transplantation in patients with severe ischemic heart failure. Circulation 2001;104(Suppl 1):213–7.
[70] Menasché P, Hagège AA, Scorsin M, et al. Myoblast transplantation for heart failure. Lancet 2001;357:279–80.
[71] Menasche P, Hagege AA, Vilquin JT, et al. Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. J Am Coll Cardiol. 2003;41:1078–83.
[72] Menasché P, Alfieri O, Janssens S, et al. The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial. Circulation 2008;117:1189–200.

[73] Dib N, Michler RE, Pagani FD, et al. Safety and feasibility of autologous myoblasttransplantation in patients with ischemic cardiomyopathy. Four years follow-up. Circulation 2005;112:1748–55.
[74] Dib N, Dinsmore J, Lababidi Z, et al. One-year follow-up of feasibility and safety of the first US randomized, controlled study using 3-dimensional guided catheter-based delivery of autologous skeletal myoblasts for ischemic cardiomyopathy (CAuSMIC Study). J Am Coll Cardiol 2009;2:9–16.
[75] Veltman CE, Soliman OI, Geleijnse ML, et al. Four-year follow-up of treatment within tramyocardial skeletal myoblasts injection in patients with ischemic cardiomyopathy. Eur Heart J 2008;29:1386–96.
[76] Thomas JP, O'Connor CM, Henry T, et al. A double-blind, randomized, controlled, multicenter study to assess the safety and cardiovascular effects of skeletal myoblast implantation by catheter delivery in patients with chronic heart failure after myocardial infarction. Am Heart J 2011;162:654–62.
[77] Smits PC, van Geuns RJ, Poldermans D, et al. Catheter-based intramyocardial injection of autologous skeletal myoblasts as a primary treatment of ischemic heart failure: clinical experience with six-month follow-up. J Am Coll Cardiol 2003;42:2063–9.
[78] Gavira JJ, Herreros J, Perez A, et al. Autologous skeletal myoblast transplantation in patients with non-acute myocardial infarction: 1-year follow-up. J Thorac Cardiovasc Surg 2006;131:799–804.
[79] Herrerosa J, Prósperb F, Perezb A, et al. Autologous intramyocardial injection of cultured skeletal muscle-derived stem cells in patients with non-acute myocardial infarction. Eur Heart J 2003;24(22):2012–20.
[80] Siminiak T, Fiszer D, Jerzykowska O, et al. Percutaneous trans-coronary-venous transplantation of autologous skeletal myoblasts in the treatment of post-infarction myocardial contractility impairment: the POZNAN trial. Eur Heart J 2005;26:1188–95.
[81] Ince H, Petzsch M, Rehders TC, Chatterjee T, Nienaber CA. Transcatheter transplantation of autologous skeletal myoblasts in postinfarction patients with severe left ventricular dysfunction. J Endovasc Ther 2004;11:695–704.
[82] Law PK, Fang G, Chua F, et al. First-in-man myoblast allografts for heart degeneration. Int J Med Implants Devices 2003;1:100–55.
[83] Rosinberg A, Rana JS, Laham RJ. Skeletal myoblast transplantation for cardiac repair. In: Laham RJ, ed. Direct Myocardial Revascularization Contemporary Cardiology 2005. New York: Springer, 2005, 311–28.
[84] Law PK, Haider K, Fang G, et al. Human VEGF165-myoblasts produce concomitant angiogenesis/ myogenesis in the regenerative heart. Mol Cell Biochem 2004;263:173–8.
[85] Ye L, Haider HKh, Jiang S, Ge R, Law PK, Sim EK. In vitro functional assessment of human skeletal myoblasts after transduction with adenoviral bi-cistronic vector carrying human VEGF165 and angiopoietin-1. J Heart Lung Transplant 2005;24:1393–402.
[86] Ye, L., Husnain, H., Jiang, S. and Eugene, S. Therapeutic angiogenesis. Basic Res Cardiol 2004;99:121–32.
[87] Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. Am J Physiol 2001;280:C1358–66.
[88] Haider HKH, Ye L, Jiang S, et al. Angiomyogenesis for cardiac re pair using human myoblasts as carriers of human vascular endothelial growth factor. J Mol Med 2004;82:539–49.
[89] Ye L, Haider HKH, Jiang SJ. Reversal of myocardial injury using genetically modulated human skeletal myoblasts in a rodent cryoinjured heart model. Eur J Heart Fail 2005;7:945–52.
[90] Suzuki K, Murtuza B, Smolenski RT, et al. Cell transplantation for the treatment of acute myocardial infarction using vascular endothelial growth factor expressing skeletal myoblasts. Circulation 2001;104:I207–12.

[91] Cheng W, Law PK. Conceptual design and procedure for an autonomous robotic intramyocardial injection catheter. Cell Transplant 2017;26:735–51.
[92] Cheng W, Law PK. Feedforward coordinate control of a robotically actuated injection catheter. Cell Transplant 2017;26:1319–30.
[93] James DE, Brown R, Navarro J, Pilch PF. Insulin-regulatable tissues express a unique insulin-sensitive glucose transport protein. Nature 1988;333:183–5.
[94] James DE, Strube M, Mueckler M. Molecular cloning and characterization of an insulin-regulatable glucose transporter. Nature 1989;338:83–7.
[95] Cushman SW, Wardzala LJ. Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. Apparent translocation of intracellular transport systems to the plasma membrane. J Biol Chem 1980;255:4758–62.
[96] Stenbit AE, Tsao TS, Li J, et al. GLUT4 heterozygous knockout mice develop muscle insulin resistance and diabetes. Nat Med 1997;3:1096–101.
[97] Morgan BJ, Chai SY, Albiston AL. GLUT4 associated proteins as therapeutic targets for diabetes. Recent Patents Endocr Metabolic Immune Drug Discov 2011;5:25–32.
[98] Zisman A, Peroni OD, Abel ED, et al. Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. Nat Med 2000;6:924–8.
[99] Koh HJ, Arnolds DE, Fujii N, et al. Skeletal muscle-selective knockout of LKB1 increases insulin sensitivity, improves glucose homeostasis, and decreases TRB3. Mol Cell Biol 2006;26:8217–27.
[100] Saltiel AR. New perspectives into the molecular pathogenesis and treatment of type 2 diabetes. Cell 2001;104:517–29.
[101] Law PK, Song SJ, Lu P, et al. World's first myoblast treatment of human acncer found safe and efficacious. Open J Regen Med 2017;6:1–16.
[102] Stölting MNL, Ferrari S, Handschin C, et al. Myoblasts inhibit prostate cancer growth by paracrine secretion of tumor necrosis factor. J Urol 2013;189:1952–9.
[103] Law PK. Myoblast therapy for cosmetic treatment. US7341719 (2008), SI99279 (2004), CN03101588 (2003).
[104] Law PK. Biologic skin repair and enhancement. WO017972 (2004), CN03819963 (2008), SI110581 (2007).
[105] Masato I, Satoshi A. Fibroblast proliferators. EP2110123 (2009) [German] [French].
[106] Alexander MH, Ross HI, Anthony JK, et al. Soft tissue repair and regeneration using postpartum-derived cells. US2005058629 (2005).
[107] Law PK. Pioneering Human Myoblast Genome Therapy. New York: Nova Science, 2009.

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5 Stem cells in ophthalmology

Abstract: Stem cells are unspecialized cells with the potential to differentiate into a variety of specialized cells that develop into different types of tissue in the body. Depending on the source of the stem cells, they can be categorized as embryonic or adult stem cells. Pluripotent stem cells, including both embryonic stem cells and induced pluripotent stem cells have the ability to differentiate into any of the three germ layers as well as tissue-specific adult stem cells. Multiple studies have reported on the differentiation of pluripotent stem cells into various ocular cell types, including cornea-, lens-, and retina-specific stem cells. Adult stem cells can differentiate into a particular cell lineage and have been identified in multiple tissues, including the eye, hematopoietic system, skin, intestinal epithelia, and nervous system. The ocular stem cells, which reside in different components of the eye, include cornea, conjunctival, iris, ciliary body, scleral, limbal, choroidal, trabecular meshwork, orbital, lens, and retinal stem cells. Ocular stem cells can differentiate into multiple ocular cell lineages and have different functional aspects. In conclusion, pluripotent stem cells and ocular stem/progenitor cells residing in different regions of the eye are capable of differentiation that enables cell repopulation and tissue regeneration, which are capabilities with enormous potential in the field of ophthalmology.

Key Words: Conjunctiva, Cornea, Eye, Embryonic, iPSCs, Limbal stem cells, Ophthalmology, Stem cells.

5.1 Introduction

Stem cells are precursor cells with the capabilities of self-renewal and generating multiple mature cell types. Pluripotency, or the potential to differentiate into multiple cell lineages, and proliferation are the two hallmark characteristics of stem cells [1]. According to the extent of their pluripotent ability, stem cells can be categorized as totipotent (capable of producing the entire organism), pluripotent (capable of differentiating into three germ layers), multipotent (able to differentiate into multiple cell lineages), oligopotent (able to differentiate into a few cell lineages), and unipotent (able to differentiate into a single cell lineage) stem cells [1–3]. The discovery of pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), opened new avenues for cell-based therapy and brought to light an instrumental tool for the research of inherited human diseases [1–3].

The human eye has a complex, unique structure. Its potential as an immunologically privileged organ and comparative accessibility for therapeutic intervention make the eye an ideal tissue for cell-based therapies [4–6]. Stem-cell-based ocular therapies

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fall into two broad classes: nonocular, pluripotent-stem-cell-derived therapies and endogenous ocular-adult-stem-cell-based therapies, such as the treatments derived from ciliary pigment epithelial (CPE) stem cells [7, 8], retinal pigment epithelial (RPE) stem cells [9], and retinal Müller stem cells [10, 11].

Several studies have reported the differentiation of ESCs and iPSCs into various specialized ocular cells, including corneal epithelial cells [12, 13], RPE cells [14, 15], photoreceptors, and retinal ganglion cells [16–21]. Adult stem cells have been identified in the hematopoietic system [22, 23], skin [24], intestinal epithelia [25], and nervous system [26, 27]. Among the ocular adult stem cells, multipotent retinal progenitor cells have been identified in CPE, which showed the ability to differentiate into rhodopsin$^+$ photoreceptors [28–31]. RPE cells have been reported to be capable of partial regeneration in chick retina [32]. Retinal Müller cells have stem/progenitor-cell-like properties, including the ability to differentiate into multiple retinal lineages [33, 34]. In parallel with pluripotent-stem-cell-based ocular therapies, endogenous ocular-stem-cell-based therapies offer a unique and powerful potential to treat ocular diseases, especially degenerative retinal diseases. Several advancements are currently being studied and implemented in regenerative treatments for corneal dystrophies. Corneal epithelial stem cells, corneal stromal stem cells, and corneal endothelial cell (CEC) progenitors have been reported to have self-renewal capacity [35, 36]. The self-renewing property of corneal epithelium is attributed to the limbal stem cells (LSCs) located in the basal region of the limbus [35, 36]. Du and colleagues highlighted the potential of human corneal stromal stem cells (hCSSCs) in a cell-based therapeutic approach to treat stromal scarring [37]. To date, few studies have been conducted to identify and isolate putative progenitor cells from the ocular lens [38–42]. Different groups have reported the *in vitro* generation of lens progenitor cells and lentoid bodies from human ESCs (hESCs) and iPSCs [43–47]. In this chapter, we discuss ocular resident stem cell populations and pluripotent-stem-cell-derived ocular stem cells, with a focus on their functional applications.

5.2 Cornea

The cornea, a transparent, avascular tissue, is the outermost layer of the eyeball. It consists of three cellular layers, the epithelium, the stroma, and the endothelium, and two noncellular layers, Bowman's layer and Descemet's membrane [48]. The cornea serves as a physical barrier to debris and infection but also accounts for approximately two-thirds of the total refractive power of the human eye [48].

5.2.1 Corneal epithelium

The corneal epithelium is a stratified, nonkeratinizing layer and covers the corneal surface and plays a major role in protection and transparency [36, 49]. The self-renewing

property of corneal epithelium is attributed to the LSCs located in the basal region of the limbus [35, 36]. LSCs are localized to a well-protected microenvironment, the limbal-stem-cell niche, which is protected from ultraviolet radiation by melanocytes [50–52] and regulates the cell cycle to keep LSCs in an undifferentiated state [53, 54]. The basement membrane protects LSCs from sheer force, and the limbal stromal blood vessels and mesenchymal cells supply oxygen, cytokines, growth factors, and nutrients [53, 55, 56]. Upon proliferation, LSCs give rise two daughter cells, an oligopotent LSC and a transient, fast-dividing amplifying cell that subsequently transforms into terminally differentiated cell [57–62]. A deficiency of LSCs results in malfunction of limbus barrier function, replacement of corneal epithelium with conjunctival epithelial cells, neovascularization, and loss of corneal transparency leading to vision loss [63]. The etiology of LSC deficiency can be broadly divided into four causes, including 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 [58, 63–66].

5.2.2 Corneal stroma

The corneal stroma is a collagenous intermediate connective tissue that constitutes approximately 80–90% of the total corneal thickness [67, 68]. The stroma is made of highly organized collagens, proteoglycans, glycoproteins, and quiescent mesenchymal cells called keratocytes, responsible for collagen production and turnover [69–71]. The precise alignment and consistent diameter of stromal collagen fibers are crucial to maintaining the transparency and strength of the cornea [71].

5.2.3 Corneal stromal stem cells

Stromal scarring due to ocular trauma, surgery, or infection can cause opacities that obscure vision and constitute a significant portion of corneal blindness worldwide [72, 73]. 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 (ECM) [74–77]. Primary keratocytes can be differentiated into the fibroblast phenotype under *ex vivo* culturing in serum-supplemented and serum-free growth media [75, 78–80]. Corneal transplantation, or keratoplasty, using cadaveric human stromal tissue is currently the only available treatment for corneal blindness [81]. The limited availability of cadaveric human stromal tissue has driven researchers to develop alternative sources, such as biosynthetic corneas [82], and alternative treatments, such as the regeneration of stromal tissue with various cell types and hCSSCs [83–95]. Funderburgh and colleagues identified cells from bovine corneal stroma that exhibit gene expression associated with mesenchymal stem cells [96].

Subsequently, Du *et al.* first identified and isolated keratocyte progenitor cells from the human corneal stroma, which exhibit stem-cell-like characteristics such as *in vitro* clonal growth and self-renewal capability [97]. The expression of the adult stem cell marker *ABCG2*, an ATP-binding cassette, was identified in keratocyte progenitor stromal cells [97]. The *ABCG2*-expressing cells exhibited a higher multipotent differentiation potential compared to *ABCG2*$^{(-)}$ cells isolated from the same region [97]. In another study, hCSSCs cultured in serum-free media were found to have up-regulated expression of a keratocyte-specific marker, including a corneal-stroma-specific proteoglycan and the deposition of tissue-like ECM [98]. Additionally, several studies reported that stromal cells from the cornea exhibit characteristics typical of mesenchymal stem cells [99–104]. Du and colleagues reported the stromal transparency by injecting hCSSCs in the stroma of Lumincan-null mice [86]. These findings highlight the potential of hCSSCs in cell-based therapeutic approaches to the treatment of stromal scarring [37].

5.2.4 Corneal endothelium

The corneal endothelium (CE) is a monolayer of hexagonal cells that is critical in maintaining corneal clarity, which is largely dependent on the regulation of osmosis by CE barriers and pump functions [105]. The CEC density is approximately 2500 cells/mm^2 in a normal adult human CE [81]. Hereditary corneal endothelial dystrophies and surgical trauma are the most common factors contributing to the loss of CECs and subsequent decrease in CEC density [106]. The physiological functioning of the CE is substantially compromised when the cell density decreases below 500 cells/mm^2; the loss of function results in corneal edema and loss of vision [81]. Although keratoplasty has largely been a successful mode of visual rehabilitation, graft rejection and the scarcity of suitable donor tissue continue to impede the further reduction of worldwide corneal blindness [81]. In an effort to discover an alternative to donor-cornea transplantation, multiple therapeutic options, including culturing CECs, isolating and characterizing corneal endothelial progenitor cells, and culturing ESC- and iPSC-derived CECs, have been investigated extensively to determine their efficacy in treating corneal endothelial dystrophies.

5.2.5 Corneal endothelial stem cells

To identify corneal endothelial stem cells, several research teams isolated human corneal endothelial stem cell precursors with the sphere-forming assay [100, 107]. The precursor cells lacked any evidence of the expression of stem cell markers; however, they did exhibit clonogenic potential, proliferative capacity, and the ability to form a hexagonal monolayer of cells with pump function, all of which are characteristics suggesting that the identified cells may be corneal endothelial progenitor cells [100, 107].

Further characterization of the sphere-forming precursor cells revealed that peripheral cells have a much higher tendency to form spheres than the cells located at the center of the endothelium [108, 109]. In another study, potential progenitor-like cells were identified in the transition zone between the peripheral endothelium/Schwalbe's line and the anterior portion of the trabecular meshwork (TM) [110–113]. The identification of progenitor-like cells was further strengthened by the high proliferative potential and density of CECs located at the periphery of the cornea, compared with the central region of the cornea [110–113].

Stem-cell-specific markers were used to identify stem/progenitor-like cells in the human CE [114, 115]. 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 located at the peripheral border of the CE [114]. They observed a higher proliferation capacity of *LGR5* $^{(+)}$ endothelial cells compared to *LGR5* $^{(-)}$ cells [114]. They also showed that persistent *LGR5* expression not only maintains endothelial phenotype but also inhibits mesenchymal transformation [114]. Neural crest markers were used to isolate and characterize the progenitor-like cells from human CE [115, 116]. The progenitor-like endothelial cells expressed neural-crest markers such as *p75*, *SOX9*, and *FOXC2* [115]. However, progenitor cells from young corneas exhibited higher proliferative potential than those isolated from older corneas [116]. Based on the origin of human CECs in the neural crest, a recent study investigated the neural crest lineage of progenitor-like cells that had been isolated from normal CE tissue and from corneas of subjects with Fuchs corneal endothelial dystrophy [116]. These cells exhibited characteristic features of neural-crest-derived progenitor cells, including an absence of senescence, a tendency to form spheres, increased colony-forming ability, and the expression profile of transcription factors regulating multipotency (*SOX2*, *OCT4*, *LGR5*, and *TP63*) and neural-crest progenitor (*PSIP1*, *PAX3*, *SOX9*, *AP2B1*, and *NES*) characteristics [116].

5.2.6 Conjunctiva

The conjunctiva is the transparent membrane covering the anterior surface of the eye, except for the cornea, and the posterior surface of the eyelids [117, 118]. For classification, the conjunctiva is divided into three regions: bulbar (covering the surface of the eye), palpebral (lining the eyelids), and forniceal (forms the junction between the bulbar and the palpebral regions). The conjunctival epithelium contains goblet cells, which produce mucins for the tear film [117, 118].

5.2.7 Conjunctival stem cells

The localization of corneal epithelial stem cells, known as limbal stem cells, to the limbus of corneal epithelium, has been conclusively established; however, the precise

location of conjunctival stem cells (CJSCs) is not yet well defined. Several studies have identified putative CJSC populations in the conjunctiva [119–122]. Nagasaki and colleagues reported the uniform distribution of CJSCs in the conjunctiva using an assay of bromodeoxyuridine label retention (a property of quiescent stem cells) in GFP-labeled mice [123, 124]. In another study, Pellegrini *et al.* suggested the localization of CJSCs in the bulbar conjunctiva and the fornices [120]. In a recent study, Stewart and colleagues reported that their experiments, measuring clonogenic ability and the expression of putative stem cell markers, indicated the medial canthal and the inferior forniceal areas are the preferred areas for the human CJSCs [123]. In other studies, typical stem cell characteristics such as slow cell-cycling and high proliferative potential were explored to identify a possible stem cell niche in the rabbit conjunctiva [119, 125]. The forniceal region exhibited more cells with increased proliferative potential and slow cycling than the bulbar and palpebral conjunctiva [119, 125].

As yet, CJSC-associated markers are not definitively known; however, ATP-binding cassette transporter G2 (*ABCG2*) and *p63*, a transcription factor, are considered to be the most reliable markers [126–128]. Budak and colleagues identified *ABCG2*-positive cells in the bulbar region of the human conjunctiva that exhibit an epithelial-stem-cell-like phenotype [129]. Other immunohistological studies also suggest the presence of *p63* and *ABCG2*-positive cells in the bulbar conjunctival epithelium [130].

5.3 Iris

The iris divides the space between the cornea and lens and is composed of four layers: the anterior border layer, the stroma, the dilator muscle layer, and the posterior epithelium [131]. Iris pigment epithelial (IPE) cells are arranged in two parallel monolayers of cells on the posterior part of the retina, and these two layers contact each other at their apices via specialized junctional complexes [132].

5.3.1 IPE stem cells

IPE stem cells can grow in spheres and express neural-stem/progenitor-cell markers [133, 134]. In two independent studies, the results revealed that IPE cells can differentiate into neuronal lineages as well as glial lineages [133, 134]. The IPE and stroma develop from the anterior region of the optic cup; the inner layer of the optic cup gives rise to posterior IPE and the neural retina, and an outer layer of the optic cup gives rise to the anterior IPE and RPE layer [135]. In an adult newt, IPE cells have the ability to regenerate a structurally and functionally complete lens [136, 137]. Similarly, Kosaka and colleagues reported that IPE cells from a one-day-old chicken not only maintain a differentiated state *in vitro* but also show higher trans-differentiation efficiency in RPE and lentoid bodies [138]. In another study, Sun and colleagues isolated

chicken IPE cells and observed sphere-forming ability exhibiting retinal progenitor marker expression in nonadherent culture [139]. They showed that postnatal chicken IPE cells can efficiently proliferate as neural stem/progenitor cells with a remarkable plasticity, generating multiple cell types, including retinal-specific neurons, glia, and the lens [139].

5.4 Ciliary body

The ciliary body is mainly composed of ciliary muscle and ciliary epithelium, which is responsible for aqueous humor production [140]. The ciliary body is involved in the regulation of aqueous flow, blood flow, intraocular pressure, and the maintenance of the immune-privileged status of the ocular anterior chamber [140].

5.4.1 Ciliary epithelial stem cells

The ciliary body has a neuro-ectodermal origin, like the retina, and multiple studies have confirmed the presence of multipotent stem cells in CPE cells, using neurospheres cultured from adult mice, rats, rabbits, pigs, and humans [7, 8, 141–144]. Researchers measured the proliferative capacity in adult ciliary body cells isolated from mice and rats and estimated that ~1-in-100 to 1-in-500 ciliary body cells have proliferative potential [8, 145, 146]. The expression of progenitor and/or neural retinal markers was used to identify and isolate CPE-derived progenitor cells [28, 144]. Recently, Xu and colleagues isolated progenitor-like cells from the ciliary bodies of adult mice, rats, and human cadaver eyes [28]. They showed that CPE-derived progenitor cells express neuronal/retinal markers and *in vitro* culturing results in neurosphere formation [28].

5.5 Trabecular meshwork

The TM is a triangular-shaped tissue located between the cornea and iris that regulates majority of the aqueous humor flow between the chambers of the eye [147]. The characteristic features of TM cells include the secretion of TM-specific enzymes and ECM and the phagocytosis of debris in the aqueous humor [147, 148].

5.5.1 TM stem cells

The decrease in TM cellularity, which occurs with age and with glaucoma, correlates directly with an increased resistance to aqueous humor outflow and elevated intraocular

pressure [149–151]. Recently, the characterization of TM cells revealed a subpopulation of cells in human primary TM cells that escape replicative senescence, express mesenchymal stem-cell-associated markers, and retain the ability to differentiate into a different lineage [152, 153]. Additionally, Du and colleagues reported multipotent stem cells exhibiting phagocytic activity in human TM tissue [152]. Gene expression analysis showed the expression of mesenchymal stem cell markers (*ABCG2*, *CD73*, *CD90*, *CD166*, and *Bmi1*) and pluripotent-stem-cell markers (*Notch1* and *OCT-3/4*) in multipotent stem cells isolated from the TM [152–154]. These cells exhibited the ability to differentiate into TM-like cells with phagocytic function and to express TM-associated markers such as *AQP1*, *MGP*, *CHI3L1*, and *TIMP3* [152, 154]. Similarly, multiple studies suggested stem-cell-like cells in primary TM cultures, capable of forming free-floating neurospheres [155–159].

5.6 Lens

The lens is an avascular and transparent biconvex structure that helps to focus light on the retina [160, 161]. The lens arises from the head ectoderm, which thickens to form the lens placode [160, 161]. The lens placode invaginates, together with the optic vesicle, to form the lens pit and the optic cup, respectively [160, 161]. Subsequently, the lens pit separates from the ectoderm to form the lens vesicle, which gives rise to a single anterior and a single posterior layer of cells [160, 161]. Cells of the former layer (anterior) differentiate into the epithelium, while cells constituting the posterior layer differentiate into primary fiber cells, which form the lens nucleus [160, 161]. The lens grows rapidly during the late embryonic and early postnatal stages, due to the proliferation of epithelial cells that elongate and differentiate into secondary fiber cells, continuously adding to the fiber cell mass [160, 161]. The tightly packed nature of the lens fiber cells, the high concentration of crystallin proteins, and the absence of organelles (lost during lens fiber cell differentiation) all function to minimize light scattering and to provide refractive index required for transparency and focusing of lens [162, 163].

5.6.1 Lens stem cells

To date, few studies have been successful in identifying and isolating putative progenitor cells from the ocular lens [38–42]. These studies investigated the locations of fast- and slow-cycling cells using DNA-labeling techniques in mouse, rat, and chick ocular lenses [38–42]. The studies revealed high proliferative activity in a transition zone located at the peripheral region of the anterior lens epithelium, whereas mitotically inactive cells were observed in the central region of the anterior lens epithelium [38–42]. Several studies have reported the *in vitro* generation of lens progenitor cells and lentoid bodies from hESCs and iPSCs [43–47].

5.7 Retina

The retina is an intricate structure composed of three layers of neural cells (photoreceptor cells, bipolar cells, and ganglion cells) and the RPE, a cellular monolayer between the retina and the choroid [164]. The human retina has a poor regeneration ability, and the damage caused by various retinal degenerative diseases, such as retinitis pigmentosa, age-related macular degeneration, Leber congenital amaurosis, glaucoma, and diabetic retinopathy, results in permanent visual impairment [165].

5.7.1 Retinal stem cells

The mature human retina has shown limited regenerative ability; however, lower vertebrates have the ability to regenerate neural retina [164, 166]. In fish and amphibians, retinal injury can induce proliferation activity of RPE cells [167, 168]. In another study, authors reported the ability of the postnatal chicken retina to generate neurons [167]. They identified the Müller glial cells, which reenter the cell cycle, dedifferentiate, acquire progenitor-like phenotypes, and produce new neurons and glia in response to damage and which express transcription factors (*Pax6* and *Chx10*) associated with embryonic retinal progenitors [167]. Multipotent stem cells have been identified in the retina of adult birds [169, 170] and in mammals [8, 26, 171].

5.7.2 Retinal pigment epithelial stem cells

During development, the outer layer of optic vesicles contributes to the RPE layer formation, which differentiates early in humans and becomes a polarized, pigmented, single-layered epithelium that remains nonproliferative throughout life [172]. The RPE supports the metabolic and cellular processes of retinal photoreceptors, including phagocytosis of the photoreceptors' outer segments [173–177].

Several studies have reported the proliferative potential of RPE cells isolated from both fetal and adult human retinas [178–180]. Recently, Salero and colleagues identified a subpopulation of RPE cells that can be induced into a stem cell state (RPESC), which enables extensive self-renewal and multipotency, producing either stable RPE progeny or neural, osteo-, chondro-, or adipo-lineage mesenchymal progeny [9]. Blenkinsop *et al*. reported that RPESCs divide robustly and have the ability to differentiate into a highly polarized cobblestone RPE monolayer [180].

5.7.3 Retinal Müller stem cells

During retinal development, Müller glial cells emerge from multipotent retinal progenitor cells [181, 182] and constitute ~4–5% of the total cells of mature mouse

retina [183, 184]. Retinal Müller cells have progenitor-cell-like properties, including the ability to differentiate into multiple retinal lineages such as photoreceptors and inner retinal neurons [185, 186]. Studies have reported the limited regeneration ability of Müller cells in chickens [167], rats [187, 188], mice [10, 189], and humans [190]. It has been previously shown that Müller cells can reenter the cell cycle and regenerate the inner nuclear layer and ganglion cells in an injured zebrafish retina [191, 192]. Müller glia cells also showed the potential to regenerate into several types of neurons after N-methyl-d-aspartate (NMDA) damage to the retinas of rodents [10]. Wan and colleagues reported the preferential regeneration ability of Müller glia to the photoreceptor in an NMDA-mediated retinal photoreceptor degeneration in adult rats [193]. Other studies have shown the regeneration ability of Müller glia into ganglion cells under defined conditions [194–196].

5.8 References

[1] Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–7.
[2] Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–72.
[3] Mimeault M, Hauke R, Batra SK. Stem cells: a revolution in therapeutics – recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. Clin Pharmacol Ther 2007;82:252–64.
[4] Jones MK, Lu B, Girman S, Wang S. Cell-based therapeutic strategies for replacement and preservation in retinal degenerative diseases. Prog Retin Eye Res 2017;58:1–27.
[5] Medawar PB. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. Br J Exp Pathol 1948;29:58–69.
[6] Streilein JW, Ma N, Wenkel H, Ng TF, Zamiri P. Immunobiology and privilege of neuronal retina and pigment epithelium transplants. Vision Res 2002;42:487–95.
[7] Ahmad I, Tang L, Pham H. Identification of neural progenitors in the adult mammalian eye. Biochem Biophys Res Commun 2000;270:517–21.
[8] Tropepe V, Coles BL, Chiasson BJ, et al. Retinal stem cells in the adult mammalian eye. Science 2000;287:2032–6.
[9] Salero E, Blenkinsop TA, Corneo B, et al. Adult human RPE can be activated into a multipotent stem cell that produces mesenchymal derivatives. Cell Stem Cell 2012;10:88–95.
[10] Ooto S, Akagi T, Kageyama R, et al. Potential for neural regeneration after neurotoxic injury in the adult mammalian retina. Proc Natl Acad Sci USA 2004;101:13654–9.
[11] Reichenbach A, Bringmann A. New functions of Muller cells. Glia 2013;61:651–78.
[12] Homma R, Yoshikawa H, Takeno M, et al. Induction of epithelial progenitors in vitro from mouse embryonic stem cells and application for reconstruction of damaged cornea in mice. Invest Ophthalmol Vis Sci 2004;45:4320–6.
[13] Hayashi R, Ishikawa Y, Ito M, et al. Generation of corneal epithelial cells from induced pluripotent stem cells derived from human dermal fibroblast and corneal limbal epithelium. PLoS One 2012;7:e45435.
[14] Hirami Y, Osakada F, Takahashi K, et al. Generation of retinal cells from mouse and human induced pluripotent stem cells. Neurosci Lett 2009;458:126–31.

[15] Buchholz DE, Hikita ST, Rowland TJ, et al. Derivation of functional retinal pigmented epithelium from induced pluripotent stem cells. Stem Cells 2009;27:2427–34.
[16] Lamba DA, Gust J, Reh TA. Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice. Cell Stem Cell 2009;4:73–9.
[17] Osakada F, Ikeda H, Mandai M, et al. Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. Nat Biotechnol 2008;26:215–24.
[18] Homma K, Okamoto S, Mandai M, et al. Developing rods transplanted into the degenerating retina of Crx-knockout mice exhibit neural activity similar to native photoreceptors. Stem Cells 2013;31:1149–59.
[19] Eiraku M, Takata N, Ishibashi H, et al. Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 2011;472:51–6.
[20] Maekawa Y, Onishi A, Matsushita K, et al. Optimized culture system to induce neurite outgrowth from retinal ganglion cells in three-dimensional retinal aggregates differentiated from mouse and human embryonic stem cells. Curr Eye Res 2016;41:558–68.
[21] Zhong X, Gutierrez C, Xue T, et al. Generation of three-dimensional retinal tissue with functional photoreceptors from human iPSCs. Nat Commun 2014;5:4047.
[22] Mikkola HK, Orkin SH. The journey of developing hematopoietic stem cells. Development 2006;133:3733–44.
[23] Moore KA, Lemischka IR. Stem cells and their niches. Science 2006;311:1880–5.
[24] Ghazizadeh S, Taichman LB. Multiple classes of stem cells in cutaneous epithelium: a lineage analysis of adult mouse skin. EMBO J 2001;20:1215–22.
[25] Bjerknes M, Cheng H. Multipotential stem cells in adult mouse gastric epithelium. Am J Physiol Gastrointest Liver Physiol 2002;283:G767–7.
[26] Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 1992;255:1707–10.
[27] Clarke DL, Johansson CB, Wilbertz J, et al. Generalized potential of adult neural stem cells. Science 2000;288:1660–3.
[28] Xu H, Sta Iglesia DD, Kielczewski JL, et al. Characteristics of progenitor cells derived from adult ciliary body in mouse, rat, and human eyes. Invest Ophthalmol Vis Sci 2007;48:1674–82.
[29] Ballios BG, Clarke L, Coles BL, Shoichet MS, van der Kooy D. The adult retinal stem cell is a rare cell in the ciliary epithelium whose progeny can differentiate into photoreceptors. Biol Open 2012;1:237–46.
[30] Clarke L, Ballios BG, van der Kooy D. Generation and clonal isolation of retinal stem cells from human embryonic stem cells. Eur J Neurosci 2012;36:1951–9.
[31] Del Debbio CB, Peng X, Xiong H, Ahmad I. Adult ciliary epithelial stem cells generate functional neurons and differentiate into both early and late born retinal neurons under non-cell autonomous influences. BMC Neurosci 2013;14:130.
[32] Fischer AJ. Neural regeneration in the chick retina. Prog Retin Eye Res 2005;24:161–82.
[33] Osakada F, Ooto S, Akagi T, Mandai M, Akaike A, Takahashi M. Wnt signaling promotes regeneration in the retina of adult mammals. J Neurosci 2007;27:4210–9.
[34] Lu B, Morgans CW, Girman S, et al. Neural stem cells derived by small molecules preserve vision. Transl Vis Sci Technol 2013;2:1.
[35] Hall PA, Watt FM. Stem cells: the generation and maintenance of cellular diversity. Development 1989;106:619–33.
[36] Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell 1989;57:201–9.
[37] Pinnamaneni N, Funderburgh JL. Concise review: stem cells in the corneal stroma. Stem Cells 2012;30:1059–63.

[38] Hanna C, O'Brien JE. Cell production and migration in the epithelial layer of the lens. Arch Ophthalmol 1961;66:103–7.
[39] Persons BJ, Modak SP. The pattern of DNA synthesis in the lens epithelium and the annular pad during development and growth of the chick lens. Exp Eye Res 1970;9:144–51.
[40] Rafferty NS, Rafferty KA, Jr. Cell population kinetics of the mouse lens epithelium. J Cell Physiol 1981;107:309–15.
[41] Zhou M, Leiberman J, Xu J, Lavker RM. A hierarchy of proliferative cells exists in mouse lens epithelium: implications for lens maintenance. Invest Ophthalmol Vis Sci 2006;47:2997–3003.
[42] Yamamoto N, Majima K, Marunouchi T. A study of the proliferating activity in lens epithelium and the identification of tissue-type stem cells. Med Mol Morphol 2008;41:83–91.
[43] Yang C, Yang Y, Brennan L, Bouhassira EE, Kantorow M, Cvekl A. Efficient generation of lens progenitor cells and lentoid bodies from human embryonic stem cells in chemically defined conditions. FASEB J 2010;24:3274–83.
[44] Qiu X, Yang J, Liu T, Jiang Y, Le Q, Lu Y. Efficient generation of lens progenitor cells from cataract patient-specific induced pluripotent stem cells. PLoS One 2012;7:e32612.
[45] O'Connor MD, McAvoy JW. In vitro generation of functional lens-like structures with relevance to age-related nuclear cataract. Invest Ophthalmol Vis Sci 2007;48:1245–52.
[46] Li D, Qiu X, Yang J, Liu T, Luo Y, Lu Y. Generation of human lens epithelial-like cells from patient-specific induced pluripotent stem cells. J Cell Physiol 2016;231:2555–62.
[47] Fu Q, Qin Z, Jin X, et al. Generation of functional lentoid bodies from human induced pluripotent stem cells derived from urinary cells. Invest Ophthalmol Vis Sci 2017;58:517–27.
[48] DelMonte DW, Kim T. Anatomy and physiology of the cornea. J Cataract Refract Surg 2011;37:588–98.
[49] Dua HS, Joseph A, Shanmuganathan VA, Jones RE. Stem cell differentiation and the effects of deficiency. Eye (Lond) 2003;17:877–85.
[50] Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature 1971;229:560–1.
[51] Higa K, Shimmura S, Miyashita H, Shimazaki J, Tsubota K. Melanocytes in the corneal limbus interact with K19-positive basal epithelial cells. Exp Eye Res 2005;81:218–23.
[52] Chee KY, Kicic A, Wiffen SJ. Limbal stem cells: the search for a marker. Clin Exp Ophthalmol 2006;34:64–73.
[53] Dziasko MA, Armer HE, Levis HJ, Shortt AJ, Tuft S, Daniels JT. Localisation of epithelial cells capable of holoclone formation in vitro and direct interaction with stromal cells in the native human limbal crypt. PLoS One 2014;9:e94283.
[54] Shortt AJ, Secker GA, Munro PM, Khaw PT, Tuft SJ, Daniels JT. Characterization of the limbal epithelial stem cell niche: novel imaging techniques permit in vivo observation and targeted biopsy of limbal epithelial stem cells. Stem Cells 2007;25:1402–9.
[55] Schofield R. The stem cell system. Biomed Pharmacother 1983;37:375–80.
[56] Gipson IK. The epithelial basement membrane zone of the limbus. Eye (Lond) 1989;3(Pt 2):132–40.
[57] Kruse FE, Tseng SC. Growth factors modulate clonal growth and differentiation of cultured rabbit limbal and corneal epithelium. Invest Ophthalmol Vis Sci 1993;34:1963–76.
[58] Tseng SC. Concept and application of limbal stem cells. Eye (Lond) 1989;3(Pt 2):141–57.
[59] Kruse FE. Stem cells and corneal epithelial regeneration. Eye (Lond) 1994;8(Pt 2):170–83.
[60] Zieske JD. Perpetuation of stem cells in the eye. Eye (Lond) 1994;8(Pt 2):163–9.
[61] Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J Cell Biol 1986;103:49–62.
[62] Pellegrini G, Rama P, Mavilio F, De LM. Epithelial stem cells in corneal regeneration and epidermal gene therapy. J Pathol 2009;217:217–28.

[63] Dua HS, Saini JS, Azuara-Blanco A, Gupta P. Limbal stem cell deficiency: concept, aetiology, clinical presentation, diagnosis and management. Indian J Ophthalmol 2000;48:83–92.
[64] Nishida K, Kinoshita S, Ohashi Y, Kuwayama Y, Yamamoto S. Ocular surface abnormalities in aniridia. Am J Ophthalmol 1995;120:368–75.
[65] Ramaesh K, Ramaesh T, Dutton GN, Dhillon B. Evolving concepts on the pathogenic mechanisms of aniridia related keratopathy. Int J Biochem Cell Biol 2005;37:547–57.
[66] Hughes WF, Jr. Alkali burns of the eye; review of the literature and summary of present knowledge. Arch Ophthal 1946;35:423–49.
[67] Patel S, McLaren J, Hodge D, Bourne W. Normal human keratocyte density and corneal thickness measurement by using confocal microscopy in vivo. Invest Ophthalmol Vis Sci 2001;42:333–9.
[68] Patel SV, McLaren JW, Hodge DO, Bourne WM. Confocal microscopy in vivo in corneas of long-term contact lens wearers. Invest Ophthalmol Vis Sci 2002;43:995–1003.
[69] Maurice DM. The structure and transparency of the cornea. J Physiol 1957;136:263–86.
[70] Freegard TJ. The physical basis of transparency of the normal cornea. Eye (Lond) 1997;11 (Pt 4):465–71.
[71] Knupp C, Pinali C, Lewis PN, et al. The architecture of the cornea and structural basis of its transparency. Adv Protein Chem Struct Biol 2009;78:25–49.
[72] Oliva MS, Schottman T, Gulati M. Turning the tide of corneal blindness. Indian J Ophthalmol 2012;60:423–7.
[73] Shortt AJ, Tuft SJ, Daniels JT. Ex vivo cultured limbal epithelial transplantation. A clinical perspective. Ocul Surf 2010;8:80–90.
[74] Funderburgh JL, Funderburgh ML, Mann MM, Corpuz L, Roth MR. Proteoglycan expression during transforming growth factor beta–induced keratocyte-myofibroblast transdifferentiation. J Biol Chem 2001;276:44173–8.
[75] Funderburgh JL, Mann MM, Funderburgh ML. Keratocyte phenotype mediates proteoglycan structure: a role for fibroblasts in corneal fibrosis. J Biol Chem 2003;278:45629–37.
[76] Fini ME. Keratocyte and fibroblast phenotypes in the repairing cornea. Prog Retin Eye Res 1999;18:529–51.
[77] Cintron C, Schneider H, Kublin C. Corneal scar formation. Exp Eye Res 1973;17:251–9.
[78] Long CJ, Roth MR, Tasheva ES, et al. Fibroblast growth factor-2 promotes keratan sulfate proteoglycan expression by keratocytes in vitro. J Biol Chem 2000;275:13918–23.
[79] Beales MP, Funderburgh JL, Jester JV, Hassell JR. Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: maintenance of the keratocyte phenotype in culture. Invest Ophthalmol Vis Sci 1999;40:1658–63.
[80] Karamichos D, Funderburgh ML, Hutcheon AE, et al. A role for topographic cues in the organization of collagenous matrix by corneal fibroblasts and stem cells. PLoS One 2014;9:e86260.
[81] Tan DT, Dart JK, Holland EJ, Kinoshita S. Corneal transplantation. Lancet 2012;379:1749–61.
[82] Lagali N, Fagerholm P, Griffith M. Biosynthetic corneas: prospects for supplementing the human donor cornea supply. Expert Rev Med Devices 2011;8:127–30.
[83] Boulze PM, Goyer B, Zaguia F, et al. Biocompatibility and functionality of a tissue-engineered living corneal stroma transplanted in the feline eye. Invest Ophthalmol Vis Sci 2014;55:6908–20.
[84] Espandar L, Bunnell B, Wang GY, Gregory P, McBride C, Moshirfar M. Adipose-derived stem cells on hyaluronic acid-derived scaffold: a new horizon in bioengineered cornea. Arch Ophthalmol 2012;130:202–8.
[85] Giasson CJ, Deschambeault A, Carrier P, Germain L. Adherens junction proteins are expressed in collagen corneal equivalents produced in vitro with human cells. Mol Vis 2014;20:386–94.
[86] Du Y, Carlson EC, Funderburgh ML, et al. Stem cell therapy restores transparency to defective murine corneas. Stem Cells 2009;27:1635–42.

[87] Basu S, Hertsenberg AJ, Funderburgh ML, et al. Human limbal biopsy-derived stromal stem cells prevent corneal scarring. Sci Transl Med 2014;6:266ra172.
[88] Wu J, Du Y, Mann MM, Yang E, Funderburgh JL, Wagner WR. Bioengineering organized, multilamellar human corneal stromal tissue by growth factor supplementation on highly aligned synthetic substrates. Tissue Eng Part A 2013;19:2063–75.
[89] Arjamaa O. Corneal reconstruction by stem cells and bioengineering. Clin Ophthalmol 2012;6:1407–9.
[90] Katikireddy KR, Dana R, Jurkunas UV. Differentiation potential of limbal fibroblasts and bone marrow mesenchymal stem cells to corneal epithelial cells. Stem Cells 2014;32:717–29.
[91] Du L, Wu X. Development and characterization of a full-thickness acellular porcine cornea matrix for tissue engineering. Artif Organs 2011;35:691–705.
[92] Wu J, Du Y, Watkins SC, Funderburgh JL, Wagner WR. The engineering of organized human corneal tissue through the spatial guidance of corneal stromal stem cells. Biomaterials 2012;33:1343–52.
[93] Ren R, Hutcheon AE, Guo XQ, et al. Human primary corneal fibroblasts synthesize and deposit proteoglycans in long-term 3-D cultures. Dev Dyn 2008;237:2705–15.
[94] Nakatsu MN, Gonzalez S, Mei H, Deng SX. Human limbal mesenchymal cells support the growth of human corneal epithelial stem/progenitor cells. Invest Ophthalmol Vis Sci 2014;55:6953–9.
[95] Yoeruek E, Bayyoud T, Maurus C, et al. Reconstruction of corneal stroma with decellularized porcine xenografts in a rabbit model. Acta Ophthalmol 2012;90:e206–10.
[96] Funderburgh ML, Du Y, Mann MM, SundarRaj N, Funderburgh JL. PAX6 expression identifies progenitor cells for corneal keratocytes. FASEB J 2005;19:1371–3.
[97] Du Y, Funderburgh ML, Mann MM, SundarRaj N, Funderburgh JL. Multipotent stem cells in human corneal stroma. Stem Cells 2005;23:1266–75.
[98] Du Y, SundarRaj N, Funderburgh ML, Harvey SA, Birk DE, Funderburgh JL. Secretion and organization of a cornea-like tissue in vitro by stem cells from human corneal stroma. Invest Ophthalmol Vis Sci 2007;48:5038–45.
[99] Yoshida S, Shimmura S, Nagoshi N, et al. Isolation of multipotent neural crest-derived stem cells from the adult mouse cornea. Stem Cells 2006;24:2714–22.
[100] Amano S, Yamagami S, Mimura T, Uchida S, Yokoo S. Corneal stromal and endothelial cell precursors. Cornea 2006;25:S73–7.
[101] Polisetty N, Fatima A, Madhira SL, Sangwan VS, Vemuganti GK. Mesenchymal cells from limbal stroma of human eye. Mol Vis 2008;14:431–442.
[102] Branch MJ, Hashmani K, Dhillon P, Jones DR, Dua HS, Hopkinson A. Mesenchymal stem cells in the human corneal limbal stroma. Invest Ophthalmol Vis Sci 2012;53:5109–16.
[103] Li GG, Zhu YT, Xie HT, Chen SY, Tseng SC. Mesenchymal stem cells derived from human limbal niche cells. Invest Ophthalmol Vis Sci 2012;53:5686–97.
[104] Garfias Y, Nieves-Hernandez J, Garcia-Mejia M, Estrada-Reyes C, Jimenez-Martinez MC. Stem cells isolated from the human stromal limbus possess immunosuppressant properties. Mol Vis 2012;18:2087–95.
[105] Bonanno JA. Molecular mechanisms underlying the corneal endothelial pump. Exp Eye Res 2012;95:2–7.
[106] Lorenzetti DW, Uotila MH, Parikh N, Kaufman HE. Central cornea guttata. Incidence in the general population. Am J Ophthalmol 1967;64:1155–8.
[107] Yokoo S, Yamagami S, Yanagi Y, et al. Human corneal endothelial cell precursors isolated by sphere-forming assay. Invest Ophthalmol Vis Sci 2005;46:1626–31.
[108] Mimura T, Yamagami S, Yokoo S, Araie M, Amano S. Comparison of rabbit corneal endothelial cell precursors in the central and peripheral cornea. Invest Ophthalmol Vis Sci 2005;46:3645–8.

[109] Yamagami S, Yokoo S, Mimura T, Takato T, Araie M, Amano S. Distribution of precursors in human corneal stromal cells and endothelial cells. Ophthalmology 2007;114:433–9.
[110] He Z, Campolmi N, Gain P, et al. Revisited microanatomy of the corneal endothelial periphery: new evidence for continuous centripetal migration of endothelial cells in humans. Stem Cells 2012;30:2523–34.
[111] Whikehart DR, Parikh CH, Vaughn AV, Mishler K, Edelhauser HF. Evidence suggesting the existence of stem cells for the human corneal endothelium. Mol Vis 2005;11:816–24.
[112] Bednarz J, Rodokanaki-von SA, Engelmann K. Different characteristics of endothelial cells from central and peripheral human cornea in primary culture and after subculture. In Vitro Cell Dev Biol Anim 1998;34:149–53.
[113] Mimura T, Joyce NC. Replication competence and senescence in central and peripheral human corneal endothelium. Invest Ophthalmol Vis Sci 2006;47:1387–96.
[114] Hirata-Tominaga K, Nakamura T, Okumura N, et al. Corneal endothelial cell fate is maintained by LGR5 through the regulation of hedgehog and Wnt pathway. Stem Cells 2013;31:1396–407.
[115] Hara S, Hayashi R, Soma T, et al. Identification and potential application of human corneal endothelial progenitor cells. Stem Cells Dev 2014;23:2190–201.
[116] Katikireddy KR, Schmedt T, Price MO, Price FW, Jurkunas UV. Existence of neural crest-derived progenitor cells in normal and Fuchs endothelial dystrophy corneal endothelium. Am J Pathol 2016;186:2736–50.
[117] Di GN. Stem cells of the human cornea. Br Med Bull 2011;100:191–207.
[118] Inatomi T, Spurr-Michaud S, Tisdale AS, Zhan Q, Feldman ST, Gipson IK. Expression of secretory mucin genes by human conjunctival epithelia. Invest Ophthalmol Vis Sci 1996;37:1684–92.
[119] Wei ZG, Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells are preferentially located in fornical epithelium: implications on conjunctival epithelial homeostasis. Invest Ophthalmol Vis Sci 1995;36:236–46.
[120] Pellegrini G, Golisano O, Paterna P, et al. Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. J Cell Biol 1999;145:769–82.
[121] Chen W, Ishikawa M, Yamaki K, Sakuragi S. Wistar rat palpebral conjunctiva contains more slow-cycling stem cells that have larger proliferative capacity: implication for conjunctival epithelial homeostasis. Jpn J Ophthalmol 2003;47:119–28.
[122] Wirtschafter JD, Ketcham JM, Weinstock RJ, Tabesh T, McLoon LK. Mucocutaneous junction as the major source of replacement palpebral conjunctival epithelial cells. Invest Ophthalmol Vis Sci 1999;40:3138–46.
[123] Stewart RM, Sheridan CM, Hiscott PS, Czanner G, Kaye SB. Human conjunctival stem cells are predominantly located in the medial canthal and inferior forniceal areas. Invest Ophthalmol Vis Sci 2015;56:2021–30.
[124] Nagasaki T, Zhao J. Uniform distribution of epithelial stem cells in the bulbar conjunctiva. Invest Ophthalmol Vis Sci 2005;46:126–32.
[125] Wei ZG, Wu RL, Lavker RM, Sun TT. In vitro growth and differentiation of rabbit bulbar, fornix, and palpebral conjunctival epithelia. Implications on conjunctival epithelial transdifferentiation and stem cells. Invest Ophthalmol Vis Sci 1993;34:1814–28.
[126] Watanabe K, Nishida K, Yamato M, et al. Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. FEBS Lett 2004;565:6–10.
[127] de Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li DQ. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. Stem Cells 2005;23:63–73.
[128] Kawasaki S, Tanioka H, Yamasaki K, Connon CJ, Kinoshita S. Expression and tissue distribution of p63 isoforms in human ocular surface epithelia. Exp Eye Res 2006;82:293–9.
[129] Budak MT, Alpdogan OS, Zhou M, Lavker RM, Akinci MA, Wolosin JM. Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. J Cell Sci 2005;118:1715–24.

[130] Vascotto SG, Griffith M. Localization of candidate stem and progenitor cell markers within the human cornea, limbus, and bulbar conjunctiva in vivo and in cell culture. Anat Rec A Discov Mol Cell Evol Biol 2006;288:921–31.
[131] Grierson I, Pfeiffer N, Cracknell KP, Appleton P. Histology and fine structure of the iris and outflow system following latanoprost therapy. Surv Ophthalmol 2002;47(Suppl 1):S176–84.
[132] Freddo TF. Intercellular junctions of the iris epithelia in *Macaca mulatta*. Invest Ophthalmol Vis Sci 1984;25:1094–104.
[133] Arnhold S, Semkova I, Andressen C, et al. Iris pigment epithelial cells: a possible cell source for the future treatment of neurodegenerative diseases. Exp Neurol 2004;187:410–7.
[134] Seko Y, Azuma N, Kaneda M, et al. Derivation of human differential photoreceptor-like cells from the iris by defined combinations of CRX, RX and NEUROD. PLoS One 2012;7:e35611.
[135] Herbert J, Cavallaro T, Martone R. The distribution of retinol-binding protein and its mRNA in the rat eye. Invest Ophthalmol Vis Sci 1991;32:302–9.
[136] Eguchi G. Cellular and molecular background of wolffian lens regeneration. Cell Differ Dev 1988;25(Suppl):147–58.
[137] Tsonis PA, Del Rio-Tsonis K. Lens and retina regeneration: transdifferentiation, stem cells and clinical applications. Exp Eye Res 2004;78:161–72.
[138] Kosaka M, Kodama R, Eguchi G. In vitro culture system for iris-pigmented epithelial cells for molecular analysis of transdifferentiation. Exp Cell Res 1998;245:245–51.
[139] Sun G, Asami M, Ohta H, Kosaka J, Kosaka M. Retinal stem/progenitor properties of iris pigment epithelial cells. Dev Biol 2006;289:243–52.
[140] Napier HR, Kidson SH. Molecular events in early development of the ciliary body: a question of folding. Exp Eye Res 2007;84:615–25.
[141] Abdouh M, Bernier G. In vivo reactivation of a quiescent cell population located in the ocular ciliary body of adult mammals. Exp Eye Res 2006;83:153–64.
[142] Inoue Y, Yanagi Y, Tamaki Y, et al. Clonogenic analysis of ciliary epithelial derived retinal progenitor cells in rabbits. Exp Eye Res 2005;81:437–45.
[143] MacNeil A, Pearson RA, MacLaren RE, Smith AJ, Sowden JC, Ali RR. Comparative analysis of progenitor cells isolated from the iris, pars plana, and ciliary body of the adult porcine eye. Stem Cells 2007;25:2430–8.
[144] Moe MC, Kolberg RS, Sandberg C, et al. A comparison of epithelial and neural properties in progenitor cells derived from the adult human ciliary body and brain. Exp Eye Res 2009;88:30–8.
[145] Kohno R, Ikeda Y, Yonemitsu Y, et al. Sphere formation of ocular epithelial cells in the ciliary body is a reprogramming system for neural differentiation. Brain Res 2006;1093:54–70.
[146] Das AV, James J, Zhao X, Rahnenfuhrer J, Ahmad I. Identification of c-Kit receptor as a regulator of adult neural stem cells in the mammalian eye: interactions with Notch signaling. Dev Biol 2004;273:87–105.
[147] Buller C, Johnson DH, Tschumper RC. Human trabecular meshwork phagocytosis. Observations in an organ culture system. Invest Ophthalmol Vis Sci 1990;31:2156–63.
[148] Stamer WD, Seftor RE, Snyder RW, Regan JW. Cultured human trabecular meshwork cells express aquaporin-1 water channels. Curr Eye Res 1995;14:1095–100.
[149] Alvarado J, Murphy C, Polansky J, Juster R. Age-related changes in trabecular meshwork cellularity. Invest Ophthalmol Vis Sci 1981;21:714–27.
[150] He Y, Leung KW, Zhang YH, et al. Mitochondrial complex I defect induces ROS release and degeneration in trabecular meshwork cells of POAG patients: protection by antioxidants. Invest Ophthalmol Vis Sci 2008;49:1447–58.
[151] Alvarado J, Murphy C, Juster R. Trabecular meshwork cellularity in primary open-angle glaucoma and nonglaucomatous normals. Ophthalmology 1984;91:564–79.

[152] Du Y, Roh DS, Mann MM, Funderburgh ML, Funderburgh JL, Schuman JS. Multipotent stem cells from trabecular meshwork become phagocytic TM cells. Invest Ophthalmol Vis Sci 2012;53:1566–75.
[153] Tay CY, Sathiyanathan P, Chu SW, Stanton LW, Wong TT. Identification and characterization of mesenchymal stem cells derived from the trabecular meshwork of the human eye. Stem Cells Dev 2012;21:1381–90.
[154] Du Y, Yun H, Yang E, Schuman JS. Stem cells from trabecular meshwork home to TM tissue in vivo. Invest Ophthalmol Vis Sci 2013;54:1450–9.
[155] Gonzalez P, Epstein DL, Luna C, Liton PB. Characterization of free-floating spheres from human trabecular meshwork (HTM) cell culture in vitro. Exp Eye Res 2006;82:959–67.
[156] McGowan SL, Edelhauser HF, Pfister RR, Whikehart DR. Stem cell markers in the human posterior limbus and corneal endothelium of unwounded and wounded corneas. Mol Vis 2007;13:1984–2000.
[157] Kelley MJ, Rose AY, Keller KE, Hessle H, Samples JR, Acott TS. Stem cells in the trabecular meshwork: present and future promises. Exp Eye Res 2009;88:747–51.
[158] Xue W, Comes N, Borras T. Presence of an established calcification marker in trabecular meshwork tissue of glaucoma donors. Invest Ophthalmol Vis Sci 2007;48:3184–94.
[159] Vittitow J, Borras T. Genes expressed in the human trabecular meshwork during pressure-induced homeostatic response. J Cell Physiol 2004;201:126–37.
[160] McAvoy JW, Chamberlain CG, de Iongh RU, Hales AM, Lovicu FJ. Lens development. Eye (Lond) 1999;13 Pt 3b):425–37.
[161] Cvekl A, Ashery-Padan R. The cellular and molecular mechanisms of vertebrate lens development. Development 2014;141:4432–47.
[162] Bloemendal H, de Jong W, Jaenicke R, Lubsen NH, Slingsby C, Tardieu A. Ageing and vision: structure, stability and function of lens crystallins. Prog Biophys Mol Biol 2004;86:407–485.
[163] Michael R, van MJ, Vrensen GF, van den Berg TJ. Changes in the refractive index of lens fibre membranes during maturation – impact on lens transparency. Exp Eye Res 2003;77:93–9.
[164] Raymond PA, Hitchcock PF. Retinal regeneration: common principles but a diversity of mechanisms. Adv Neurol 1997;72:171–84.
[165] Alonso-Alonso ML, Srivastava GK. Current focus of stem cell application in retinal repair. World J Stem Cells 2015;7:641–8.
[166] Reh TA, Levine EM. Multipotential stem cells and progenitors in the vertebrate retina. J Neurobiol 1998;36:206–20.
[167] Fischer AJ, Reh TA. Muller glia are a potential source of neural regeneration in the postnatal chicken retina. Nat Neurosci 2001;4:247–52.
[168] Mitashov VI. Retinal regeneration in amphibians. Int J Dev Biol 1997;41:893–905.
[169] Klein LR, MacLeish PR, Wiesel TN. Immunolabelling by a newt retinal pigment epithelium antibody during retinal development and regeneration. J Comp Neurol 1990;293:331–9.
[170] Goldman SA, Nottebohm F. Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci USA 1983;80:2390–4.
[171] Fischer AJ, Reh TA. Identification of a proliferating marginal zone of retinal progenitors in postnatal chickens. Dev Biol 2000;220:197–210.
[172] Rapaport DH, Rakic P, Yasamura D, LaVail MM. Genesis of the retinal pigment epithelium in the macaque monkey. J Comp Neurol 1995;363:359–76.
[173] Strauss O. The retinal pigment epithelium in visual function. Physiol Rev 2005;85:845–81.
[174] Marmor MF. Mechanisms of fluid accumulation in retinal edema. Doc Ophthalmol 1999;97:239–49.
[175] Miller SS, Edelman JL. Active ion transport pathways in the bovine retinal pigment epithelium. J Physiol 1990;424:283–300.

[176] Steinberg RH. Interactions between the retinal pigment epithelium and the neural retina. Doc Ophthalmol 1985;60:327–346.
[177] Bok D. The retinal pigment epithelium: a versatile partner in vision. J Cell Sci Suppl 1993;17:189–95.
[178] Hu J, Bok D. A cell culture medium that supports the differentiation of human retinal pigment epithelium into functionally polarized monolayers. Mol Vis 2001;7:14–9.
[179] Maminishkis A, Chen S, Jalickee S, et al. Confluent monolayers of cultured human fetal retinal pigment epithelium exhibit morphology and physiology of native tissue. Invest Ophthalmol Vis Sci 2006;47:3612–24.
[180] Blenkinsop TA, Salero E, Stern JH, Temple S. The culture and maintenance of functional retinal pigment epithelial monolayers from adult human eye. Methods Mol Biol 2013;945:45–65.
[181] Martin PR, Grunert U. Spatial density and immunoreactivity of bipolar cells in the macaque monkey retina. J Comp Neurol 1992;323:269–87.
[182] Strettoi E, Masland RH. The organization of the inner nuclear layer of the rabbit retina. J Neurosci 1995;15:875–88.
[183] Jeon CJ, Strettoi E, Masland RH. The major cell populations of the mouse retina. J Neurosci 1998;18:8936–46.
[184] Young RW. Cell proliferation during postnatal development of the retina in the mouse. Brain Res 1985;353:229–39.
[185] Jadhav AP, Roesch K, Cepko CL. Development and neurogenic potential of Muller glial cells in the vertebrate retina. Prog Retin Eye Res 2009;28:249–62.
[186] Bernardos RL, Barthel LK, Meyers JR, Raymond PA. Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J Neurosci 2007;27:7028–40.
[187] Ferraro S, Gomez-Montalvo AI, Olmos R, Ramirez M, Lamas M. Primary cilia in rat mature Muller glia: downregulation of IFT20 expression reduces sonic hedgehog-mediated proliferation and dedifferentiation potential of Muller glia primary cultures. Cell Mol Neurobiol 2015;35:533–42.
[188] Reyes-Aguirre LI, Ferraro S, Quintero H, Sanchez-Serrano SL, Gomez-Montalvo A, Lamas M. Glutamate-induced epigenetic and morphological changes allow rat Muller cell dedifferentiation but not further acquisition of a photoreceptor phenotype. Neuroscience 2013;254:347–60.
[189] Karl MO, Hayes S, Nelson BR, Tan K, Buckingham B, Reh TA. Stimulation of neural regeneration in the mouse retina. Proc Natl Acad Sci USA 2008;105:19508–13.
[190] Lawrence JM, Singhal S, Bhatia B, et al. MIO-M1 cells and similar Muller glial cell lines derived from adult human retina exhibit neural stem cell characteristics. Stem Cells 2007;25:2033–43.
[191] Raymond PA, Barthel LK, Bernardos RL, Perkowski JJ. Molecular characterization of retinal stem cells and their niches in adult zebrafish. BMC Dev Biol 2006;6:36.
[192] Fimbel SM, Montgomery JE, Burket CT, Hyde DR. Regeneration of inner retinal neurons after intravitreal injection of ouabain in zebrafish. J Neurosci 2007;27:1712–24.
[193] Wan J, Zheng H, Chen ZL, Xiao HL, Shen ZJ, Zhou GM. Preferential regeneration of photoreceptor from Muller glia after retinal degeneration in adult rat. Vision Res 2008;48:223–4.
[194] Song WT, Zhang XY, Xia XB. Atoh7 promotes the differentiation of Muller cells-derived retinal stem cells into retinal ganglion cells in a rat model of glaucoma. Exp Biol Med (Maywood) 2015;240:682–90.
[195] Song WT, Zeng Q, Xia XB, Xia K, Pan Q. Atoh7 promotes retinal Muller cell differentiation into retinal ganglion cells. Cytotechnology 2016;68:267–77.
[196] Zhao JJ, Ouyang H, Luo J, et al. Induction of retinal progenitors and neurons from mammalian Muller glia under defined conditions. J Biol Chem 2014;289:11945–51.

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6 Stem cells for ocular therapies

Abstract: The ability to rehabilitate and restore visual function is the ultimate aspiration of the science of ophthalmology. Conventional ocular treatment strategies have focused on protecting against vision impairment with the administration of laser treatments or drugs or by surgical intervention. The current standard medical treatment modalities for the most frequent causes of blindness are very effective. However, ophthalmology has yet been unable to discover a cure for many common ocular dystrophies or a comprehensive strategy to halt the progression of blindness. It is therefore necessary to continue to formulate new treatment strategies to cure the causes of blindness and to achieve the best standard of visual acuity. To these ends, stem cells have immense potential to replenish the degenerated cells in our body, including damaged cells in the eye. The mammalian eye, with its intricate structure and unique characteristics, offers various advantages for stem-cell-based therapies, including its immunologically privileged status and easy accessibility for therapeutic intervention. Already, several stem-cell-based preclinical and clinical trials are in progress, investigating treatments for a wide range of ocular diseases in various animal models, as well as in humans. Stem cells, including pluripotent stem cells (both embryonic stem cells and induced pluripotent stem cells) and adult stem cells, have already been successfully manipulated to generate specialized ocular cells and tissues. Several laboratories have reported the generation of cornea-specific cells, such as corneal epithelial and endothelial cells, retina-specific cells such as retinal pigment epithelial cells, photoreceptors, and retinal ganglion cells, lens, conjunctival, limbal, and trabecular-meshwork-specific cells. In conclusion, although various concerns still need to be addressed, the use of bioengineered stem cells appears to be a promising therapeutic strategy for future replacement and regeneration of ocular tissues in cases of formerly incurable ocular diseases.

Key Words: Conjunctiva, Cornea, Eye, Embryonic, iPSCs, Limbal stem cells, Ophthalmology, Stem cells.

6.1 Introduction

Stem cells are unspecialized cells that have the potential to renew themselves and differentiate into a wide variety of specialized cells. Stem cells are capable of replacing impaired or diseased cells and can comprise different types of tissues in the human body, including the tissues of the eye. The mammalian eye has an intricate structure and harbors various advantages for stem-cell-based therapies [1–3]. Stem-cell-based

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therapy, or regenerative medicine, has been employed to regenerate diseased tissue residing in different components of the eye in animal models and humans. Retinal degenerative diseases, such as glaucoma, age-related macular degeneration (AMD), and retinitis pigmentosa (RP), are characterized by the early loss of specific cell types: retinal ganglion cells (RGCs), photoreceptors, and retinal pigment epithelial (RPE) cells, respectively [4–6]. Each of these pathological conditions ultimately causes the loss of photoreceptors and result in permanent vision loss [5, 7, 8]. Unfortunately, the limited regenerative potential of mammalian retina hampers the therapeutic measures currently available to treat retinal degeneration [9].

Since the identification of pluripotent stem cells, including embryonic stem cells (ESCs) and, more recently, induced pluripotent stem cell (iPSCs) have been used extensively in the generation of multiple ocular cell types, such as corneal epithelial cells [10, 11], RPE cells [12, 13], photoreceptors, and RGCs [14–19]. In parallel, multipotent adult ocular stem/progenitor cells have been employed in the regeneration of RPE and photoreceptors cells. Among the rapidly expanding repertoire of ocular stem-cell-based therapies, limbal stem cells have been tested for the treatment of degenerated corneal epithelium [10, 11], human pluripotent stem-cell-derived trabecular meshwork (TM) cells [20–22] and RGCs [23–25] and have been utilized for treating glaucoma, and stem-cell-derived RPE, retinal Müller, and amacrine cells have been engaged in the treatment of retinal degenerative diseases like RP and AMD [26–31]. In this chapter, we will discuss stem-cell-based therapies for the replacement and regeneration of the predominant ocular tissues.

6.2 Cornea

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) [32]. The functions of the cornea include acting as a barrier to debris and infection, as well as accounting for approximately two-thirds of the total refractive power of the human eye [32].

6.2.1 Stem cell-based therapies for corneal epithelium

The corneal epithelium is composed of small basal cells, flattened middle cells (wing cells), and polygonal flattened superficial cells [32]. The corneal epithelial stem cells, also known as limbal stem cells, are located in a basal layer of the limbus (the transitional zone between the cornea and the conjunctiva) [32]. A loss or dysfunction of limbal epithelial cells can result in limbal stem cell deficiency (LSCD) [33].

Several investigations have highlighted the ability of limbal stem cells to treat LSCD by replacing the degenerated corneal epithelium [34–37]. Pellegrini *et al.* were

the first to report autologous transplantation of *ex vivo* cultivated limbal stem cells for the treatment of LSCD [38]. The autologous and allogeneic transplantation of limbal stem cells has been reported [35]. However, these are hampered by both donor shortage and postoperative complications, including immunological rejection and bacterial or fungal keratitis [36, 37, 39, 40]. Although the transplantation of autologous limbal stem cells 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 [41]. In parallel, Homma and colleagues studied the therapeutic efficacy of epithelial progenitors derived from mouse ESCs (mESCs) when transplanted into a damaged cornea [42]. Another study, by Hayashi *et al.*, was the first to demonstrate the differentiation of human iPSCs into corneal-epithelium-like cells [43]. More recently, the generation of corneal organoids using human iPSCs has been reported [44, 45].

6.2.2 Therapeutic applications of stem cells to corneal stroma

The stroma is made of organized collagens, proteoglycans, glycoproteins, and quiescent mesenchymal cells called keratocytes, that are responsible for collagen production and turnover [46–48]. Stromal scarring caused by ocular trauma, surgery, or infection can result in stromal opacities, which are a significant contributor to corneal blindness worldwide [49, 50].

Deep anterior lamellar keratoplasty is currently the only option to treat corneal stromal disease [51]. However, this strategy has some limitations, including immune rejection and the scarcity of donor corneas [51]. Griffith and colleagues reported the generation of functional human corneal equivalents developed from immortalized human corneal cells [52]. Buznyk *et al.* implanted bioengineered corneal grafts into three patients and reported the subsequent restoration of corneal integrity, from the promotion of endogenous regeneration of corneal tissues, and improved vision in two out of three patients [53]. Basu and colleagues reported the regeneration of damaged stromal tissue by grafting human limbal biopsy-derived stromal cells into the corneal wounds of mice [54]. Furthermore, the generation of stromal keratocytes by differentiating adult dental pulp stem cells, human ESCs (hESCs), and mesenchymal stem cells (MSCs) has been reported [55–57].

6.2.3 Stem cells and corneal endothelium treatment

The corneal endothelium is a monolayer of hexagonal cells critical to maintaining corneal clarity through the mediation of hydration, specifically by barrier and

pump functions [58]. Corneal hereditary endothelial dystrophies and surgical trauma are the two major factors that contribute to the loss of corneal endothelial cells (CECs) and a decrease in CEC density [59].

Keratoplasty has been a successful method of visual rehabilitation; however, as with other applications of transplantation, graft rejection and the lack of suitable donor tissue continue to be impediments to further reducing worldwide corneal blindness [51]. As alternatives to donor cornea transplantation, multiple therapeutic options, including cultured CECs, corneal endothelial progenitor cells, and hESC- and iPSC-derived CECs, have been investigated extensively. The injection of cultured corneal endothelial cells in combination with Rho kinase (ROCK) inhibitor resulted in the regeneration of corneal endothelium in macaque corneal endothelium dysfunction model [60]. Multiple studies reported that the ROCK inhibitor enhances the adhesion potential of cultured CECs [61, 62].

CECs have previously been generated from human pluripotent stem cells [63–67]. Fukuta *et al.* differentiated human pluripotent stem cells into CECs under chemically defined conditions [63]. 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 [64].

6.3 TM and stem cell therapy

The TM regulates the majority of the aqueous humor outflow pathway in the eye [68], and a decrease in TM cellularity, which is a complication frequently observed with glaucoma, as well as with normal aging, correlates with increased outflow resistance and elevated intraocular pressure (IOP) [69, 70]. Current treatments for glaucoma involve pharmacological reduction of aqueous humor production and surgical enhancement of outflow to restore a normal IOP [71, 72]. Stem-cell-based therapies are an attractive prospect for the restoration of TM cellularity in glaucoma and aging [73]. The first indication of a successful restoration of TM function was reported in a study of laser trabeculoplasty, a technique used for the management of elevated IOP in patients with primary open angle glaucoma [74]. Treatment with laser trabeculoplasty resulted in increased TM cell division activity, suggesting the presence of "stem-cell-like cells" in the anterior nonfiltering region of the TM [75, 76]. Du and colleagues reported the isolation of stem cells from human TM and investigated their therapeutic potential by transplantation into mouse TM tissue [77]. In another study, human embryoid bodies were generated using iPSCs, followed by differentiation into TM-like cells [78]. In an alternative approach, MSCs were used to repair the TM in laser-induced glaucoma animal models [79]. In a recent study, Ding *et al.* successfully demonstrated the generation of TM-like cells by culturing mouse iPSCs in primary

human TM cells preconditioned culture media [80]. In two successive studies, Zhu and colleagues performed the iPSC-derived TM cell transplantation in a mouse model of glaucoma [21, 22]. The transplanted iPSC-derived TM cells rescued the glaucoma phenotype *in vivo* by restoring aqueous humor outflow, lowering IOP, preventing the loss of RGCs, and enhancing the proliferative response of endogenous TM cells [21, 22].

6.4 Ocular lens and stem cell treatment

Congenital and age-related cataracts are the leading cause of blindness world wide [81]. The most common and successful procedure for cataract treatment is the surgical removal of the cataractous lens, followed by implantation of an artificial intraocular lens [82]. Cataract surgery is effective in restoring sight; however, it is associated with postoperative complications such as posterior capsule opacification [83, 84]. The study of stem cells has facilitated the discovery of lens regeneration, reported in lower vertebrates [85, 86]. Surgical removal of the ocular lens in a rabbit resulted in the proliferation of residual lens epithelial stem/progenitor cells and the generation of a limited amount of lens fiber cells [87, 88]. In a recent study, Lin and colleagues reported a novel surgical method for cataractous lens removal that preserves endogenous lens epithelial cells and achieved a functional regeneration of the lens in rabbits and macaques, as well as in human infants with cataracts [89]. O'Connor and McAvoy reported, the *in vitro* generation of functional (light-focusing) lens-like structures using rat lens epithelial cells [90]. They observed that regenerated rat lenses closely resembled those of newborn rats and that the prolonged culturing of these regenerated rat lenses eventuated in cataract formation [90]. Previously, lens progenitor cells and functional lentoid bodies had been successfully generated from hESCs and iPSCs [91–94]. Yang and colleagues differentiated hESCs into lens progenitor cells to form lentoid bodies, directed by chemically defined conditions [91]. Although the lentoid bodies generated by Yang and colleagues expressed lens-specific makers, they lacked functional optical properties [91]. Fu and colleagues generated functional lentoid bodies from human urinary-epithelial-cell-derived iPSCs, employing the "fried egg" differentiation method [94]. These lentoid bodies expressed lens-specific markers and exhibited a transparent structure – like the human lens [94]. Recently, Murphy and colleagues reported the generation of light-focusing human micro-lenses from pluripotent stem cells [95].

6.5 Stem cell-based retinal therapy

Stem-cell-based retinal transplantation therapies are an attractive therapeutic approach because of the limitless self-renewal ability of pluripotent stem cells. hESCs and iPSCs have been used extensively in the generation and transplantation of RPE

and photoreceptors cells. As yet, few studies have investigated the therapeutic potential of adult stem/progenitor cells in the treatment of retinal degenerative diseases.

Salero and colleagues reported a subpopulation of adult human RPE cells that can be induced *in vitro* to become self-renewing, and they demonstrated that the RPE stem cells (RPESC) that lose the markers of RPE can proliferate extensively and form stable cobblestone monolayers [96]. Blenkinsop and colleagues reported a new culture model and demonstrated that RPE cells from an adult human eye are capable of generating extremely pure cultures of RPE that exhibit characteristics of native RPE [97]. Subsequently, Davis and colleagues reported the prevention of photoreceptor loss by subretinal transplantation of RPESC-derived RPE cells in the Royal College of Surgeons (RCS) rats [98, 99]. In another study, Davis *et al.* demonstrated the successful preservation of vision in a rat model of RPE cell dysfunction through the subretinal transplantation of RPESC-derived RPE cells [99].

Müller glial cells exhibit stem cell-like characteristics and have been reported to aid in the regeneration of the injured retinas in amphibians [100]. Recently, Jayaram and colleagues demonstrated the therapeutic potential of Müller glial cells in P23H rats, a photoreceptor degeneration model [101]. They transplanted Müller-cell-derived photoreceptor cells into the retinas of 3-week-old P23H rats and reported the migration and integration of transplanted cells into the outer nuclear layer of degenerated retinas, leading to improvement in rod photoreceptor function [101].

Multiple studies have reported the generation of hESC-derived human neural retinal progenitor cells and functional RPE cells [12, 15, 102–105]. Mouse pluripotent stem cells were used to create three-dimensional organoids resembling the optic cup and the neural retina [17, 19, 106]. Recently, DiStefano and colleagues reported an accelerated and improved differentiation of retinal organoids from pluripotent stem cells in rotating-wall vessel bioreactors [107]. Gonzalez-Cordero and colleagues investigated the integration potential of rod precursors into the degenerated retinas of adult mice [108]. Assawachananont and colleagues reported the transplantation of mESCs or iPSC-derived three-dimensional retinal sheets in a retinal degeneration model that lacked the outer nuclear layer [109]. In two independent studies, the intraocular transplantation of hESC-derived neural precursor cells and donor cells from developing retinas showed integration and subsequent differentiation into different cell types in the subretinal microenvironment [110, 111].

As an alternative, iPSC-based therapies represent a promising approach for the treatment of degenerative retinal diseases. iPSCs hold an edge over hESCs in stem-cell-based therapies because the patient-specific approach, using the same genetic background, reduces the risk of immune rejection while also excluding the various ethical issues associated with ESCs. In addition, gene-corrected patient-specific iPSCs generated using different genome editing tools, such as clustered regularly interspaced short palindromic repeats (CRISPR/Cas9), zinc finger nucleases, and transcription activator-like effector nucleases, offer a unique approach for the treatment of retinal degenerative diseases [112–114]. Recently, Bassuk and colleagues

reported a CRISPR/Cas9-mediated mutation correction in an RP GTPase regulator gene responsible for X-linked RP in patient-specific iPSCs [115].

The RPE cells derived from iPSCs and hESCs exhibited similar gene expression profiles and phenotypic features [13, 116, 117]. Moreover, neural retinal progenitor cells, including photoreceptor cells, were also successfully differentiated from human iPSCs [19, 116–119]. Multiple investigations reported improved visual function after transplantation of iPSC-derived RPE cells in different RP mouse models [120–125]. Recently, Tucker and colleagues reported the differentiation of adult mouse fibroblast-originated iPSCs into retinal precursor cells expressing retinal- and photoreceptor-specific markers, and the subsequent transplantation restored retinal structure and function in rhodopsin-null mice [121, 126]. Two independent studies reported the ability of native RPE transplantation to improve electroretinogram function in RCS rats [120, 122]. In another study, Carr and colleagues differentiated human iPSCs into functional RPE layers, and subsequent transplantation in RCS rats facilitated short-term maintenance of the photoreceptor-mediated phagocytosis of photoreceptor outer segments [123]. Sun and colleagues investigated the protective effects of human iPSC-derived RPE cells in comparison with human mesenchymal stromal cells and neural stem cells in the retina of rd1 mice [125]. Their findings suggest that iPSC-derived RPE cells can delay photoreceptor degeneration in the retina of rd1 mice [125]. In another study, Li and colleagues injected iPSC-derived RPE cells into the subretinal space of the *Rpe65* mouse model and observed the integration of transplanted cells into the native RPE and improved visual function [124].

Due to the similar developmental origin of iris pigment epithelium (IPE) and RPE cells, multiple studies were performed to transplant freshly isolated or cultured autologous IPE cells in rabbits, monkeys, rats, and humans for the treatment of retinal degenerative diseases [127–138]. The transplantation of fresh autologous IPE cells into the subretinal space in rabbits results in no immune rejection, and transplanted IPE cells revealed specific phagocytosis activity [127]. Similar findings were observed after transplantation of cultured autologous IPE cells in monkeys [129]. In addition multiple studies used autologous IPE cells to treat AMD and subretinal neovascularization in humans [133–136]. Recently, Thumann and colleagues reported the stabilization of visual acuity, without any increased complications and posttransplantation rejection of freshly isolated, autologous IPE cells into the subretinal space of 20 patients with advanced AMD and neovascularization [136]. Similarly, Abe *et al.* reported similar results by transplantation of cultured autologous IPE cells into the subretinal space of eight patients with AMD [133, 134], whereas, Lappas *et al.* performed IPE cell transplantation in 12 patients with wet AMD and reported that preoperative visual acuity was preserved but not improved after a follow-up of 6 months [137].

Previously, hESC-derived RPE cells have been used in a number of human clinical studies [139–143], whereas only one human clinical trial was performed using iPSC-derived RPE cells [144]. Recently, Mandai and colleagues performed the first human clinical trial using iPSC-derived RPE cells in an AMD patient [144]. iPSCs

were generated from skin fibroblasts obtained from two patients with AMD and were differentiated into RPE cells [144]. The iPSC-based autologous transplantation in one patient showed no adverse effects such as immune-mediated rejection and tumorigenicity, while the transplanted RPE sheet remained intact with no improvement for best corrected visual acuity [144]. However, iPSC-derived RPE cells were not transplanted in the second patient due to the identification of three deletions in patient-specific iPSCs, absent in patient fibroblast cells [144].

6.6 References

[1] Jones MK, Lu B, Girman S, Wang S. Cell-based therapeutic strategies for replacement and preservation in retinal degenerative diseases. Prog Retin Eye Res 2017;58:1–27.
[2] Medawar PB. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. Br J Exp Pathol 1948;29:58–69.
[3] Streilein JW, Ma N, Wenkel H, Ng TF, Zamiri P. Immunobiology and privilege of neuronal retina and pigment epithelium transplants. Vision Res 2002;42:487–95.
[4] Davis BM, Crawley L, Pahlitzsch M, Javaid F, Cordeiro MF. Glaucoma: the retina and beyond. Acta Neuropathol 2016;132:807–26.
[5] de Jong PT. Age-related macular degeneration. N Engl J Med 2006;355:1474–85.
[6] Ferrari S, Di IE, Barbaro V, Ponzin D, Sorrentino FS, Parmeggiani F. Retinitis pigmentosa: genes and disease mechanisms. Curr Genomics 2011;12:238–49.
[7] Berson EL. Retinitis pigmentosa. The Friedenwald lecture. Invest Ophthalmol Vis Sci 1993;34:1659–76.
[8] Quigley HA. Open-angle glaucoma. N Engl J Med 1993;328:1097–106.
[9] Klassen H. Stem cells in clinical trials for treatment of retinal degeneration. Expert Opin Biol Ther 2016;16:7–14.
[10] Daniels JT, Notara M, Shortt AJ, Secker G, Harris A, Tuft SJ. Limbal epithelial stem cell therapy. Expert Opin Biol Ther 2007;7:1–3.
[11] O'Sullivan F, Clynes M. Limbal stem cells, a review of their identification and culture for clinical use. Cytotechnology 2007;53:101–6.
[12] Hirami Y, Osakada F, Takahashi K, et al. Generation of retinal cells from mouse and human induced pluripotent stem cells. Neurosci Lett 2009;458:126–31.
[13] Buchholz DE, Hikita ST, Rowland TJ, et al. Derivation of functional retinal pigmented epithelium from induced pluripotent stem cells. Stem Cells 2009;27:2427–34.
[14] Lamba DA, Gust J, Reh TA. Transplantation of human embryonic stem cell–derived photoreceptors restores some visual function in Crx-deficient mice. Cell Stem Cell 2009;4:73–9.
[15] Osakada F, Ikeda H, Mandai M, et al. Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. Nat Biotechnol 2008;26:215–24.
[16] Homma K, Okamoto S, Mandai M, et al. Developing rods transplanted into the degenerating retina of Crx-knockout mice exhibit neural activity similar to native photoreceptors. Stem Cells 2013;31:1149–59.
[17] Eiraku M, Takata N, Ishibashi H, et al. Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 2011;472:51–6.
[18] Maekawa Y, Onishi A, Matsushita K, et al. Optimized culture system to induce neurite outgrowth from retinal ganglion cells in three-dimensional retinal aggregates differentiated from mouse and human embryonic stem cells. Curr Eye Res 2016;41:558–68.

[19] Zhong X, Gutierrez C, Xue T, et al. Generation of three-dimensional retinal tissue with functional photoreceptors from human iPSCs. Nat Commun 2014;5:4047.
[20] Du Y, Roh DS, Mann MM, Funderburgh ML, Funderburgh JL, Schuman JS. Multipotent stem cells from trabecular meshwork become phagocytic TM cells. Invest Ophthalmol Vis Sci 2012;53:1566–75.
[21] Zhu W, Jain A, Gramlich OW, Tucker BA, Sheffield VC, Kuehn MH. Restoration of aqueous humor outflow following transplantation of iPSC-derived trabecular meshwork cells in a transgenic mouse model of glaucoma. Invest Ophthalmol Vis Sci 2017;58:2054–62.
[22] Zhu W, Gramlich OW, Laboissonniere L, et al. Transplantation of iPSC-derived TM cells rescues glaucoma phenotypes in vivo. Proc Natl Acad Sci USA 2016;113:E3492–500.
[23] Kador KE, Montero RB, Venugopalan P, et al. Tissue engineering the retinal ganglion cell nerve fiber layer. Biomaterials 2013;34:4242–50.
[24] Venugopalan P, Wang Y, Nguyen T, Huang A, Muller KJ, Goldberg JL. Transplanted neurons integrate into adult retinas and respond to light. Nat Commun 2016;7:10472.
[25] Sluch VM, Davis CH, Ranganathan V, et al. Differentiation of human ESCs to retinal ganglion cells using a CRISPR engineered reporter cell line. Sci Rep 2015;5:16595.
[26] Fischer AJ, Reh TA. Muller glia are a potential source of neural regeneration in the postnatal chicken retina. Nat Neurosci 2001;4:247–52.
[27] Dyer MA, Cepko CL. Control of Muller glial cell proliferation and activation following retinal injury. Nat Neurosci 2000;3:873–80.
[28] Chen M, Tian S, Glasgow NG, et al. Lgr5(+) amacrine cells possess regenerative potential in the retina of adult mice. Aging Cell 2015;14:635–43.
[29] Lenkowski JR, Raymond PA. Muller glia: Stem cells for generation and regeneration of retinal neurons in teleost fish. Prog Retin Eye Res 2014;40:94–123.
[30] Fausett BV, Goldman D. A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina. J Neurosci 2006;26:6303–13.
[31] Bernardos RL, Barthel LK, Meyers JR, Raymond PA. Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J Neurosci 2007;27:7028–40.
[32] DelMonte DW, Kim T. Anatomy and physiology of the cornea. J Cataract Refract Surg 2011;37:588–98.
[33] Dua HS, Joseph A, Shanmuganathan VA, Jones RE. Stem cell differentiation and the effects of deficiency. Eye (Lond) 2003;17:877–85.
[34] Utheim TP. Limbal epithelial cell therapy: past, present, and future. Methods Mol Biol 2013;1014:3–43.
[35] Kenyon KR, Tseng SC. Limbal autograft transplantation for ocular surface disorders. Ophthalmology 1989;96:709–22.
[36] Gomes JA, Santos MS, Ventura AS, Donato WB, Cunha MC, Hofling-Lima AL. Amniotic membrane with living related corneal limbal/conjunctival allograft for ocular surface reconstruction in Stevens-Johnson syndrome. Arch Ophthalmol 2003;121:1369–74.
[37] Ilari L, Daya SM. Long-term outcomes of keratolimbal allograft for the treatment of severe ocular surface disorders. Ophthalmology 2002;109:1278–84.
[38] Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De LM. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet 1997;349:990–3.
[39] Samson CM, Nduaguba C, Baltatzis S, Foster CS. Limbal stem cell transplantation in chronic inflammatory eye disease. Ophthalmology 2002;109:862–8.
[40] Shimazaki J, Shimmura S, Fujishima H, Tsubota K. Association of preoperative tear function with surgical outcome in severe Stevens-Johnson syndrome. Ophthalmology 2000;107:1518–23.
[41] Priya CG, Arpitha P, Vaishali S, et al. Adult human buccal epithelial stem cells: identification, ex-vivo expansion, and transplantation for corneal surface reconstruction. Eye (Lond) 2011;25:1641–9.

[42] Homma R, Yoshikawa H, Takeno M, et al. Induction of epithelial progenitors in vitro from mouse embryonic stem cells and application for reconstruction of damaged cornea in mice. Invest Ophthalmol Vis Sci 2004;45:4320–6.
[43] Hayashi R, Ishikawa Y, Ito M, et al. Generation of corneal epithelial cells from induced pluripotent stem cells derived from human dermal fibroblast and corneal limbal epithelium. PLoS One 2012;7:e45435.
[44] Foster JW, Wahlin K, Adams SM, Birk DE, Zack DJ, Chakravarti S. Cornea organoids from human induced pluripotent stem cells. Sci Rep 2017;7:41286.
[45] Susaimanickam PJ, Maddileti S, Pulimamidi VK, et al. Generating minicorneal organoids from human induced pluripotent stem cells. Development 2017;144:2338–51.
[46] Maurice DM. The structure and transparency of the cornea. J Physiol 1957;136:263–86.
[47] Freegard TJ. The physical basis of transparency of the normal cornea. Eye (Lond) 1997; 11(Pt 4):465–71.
[48] Knupp C, Pinali C, Lewis PN, et al. The architecture of the cornea and structural basis of its transparency. Adv Protein Chem Struct Biol 2009;78:25–49.
[49] Oliva MS, Schottman T, Gulati M. Turning the tide of corneal blindness. Indian J Ophthalmol 2012;60:423–7.
[50] Shortt AJ, Tuft SJ, Daniels JT. Ex vivo cultured limbal epithelial transplantation. A clinical perspective. Ocul Surf 2010;8:80–90.
[51] Tan DT, Dart JK, Holland EJ, Kinoshita S. Corneal transplantation. Lancet 2012;379:1749–61.
[52] Griffith M, Osborne R, Munger R, et al. Functional human corneal equivalents constructed from cell lines. Science 1999;286:2169–72.
[53] Buznyk O, Pasyechnikova N, Islam MM, Iakymenko S, Fagerholm P, Griffith M. Bioengineered corneas grafted as alternatives to human donor corneas in three high-risk patients. Clin Transl Sci 2015;8:558–62.
[54] Basu S, Hertsenberg AJ, Funderburgh ML, et al. Human limbal biopsy-derived stromal stem cells prevent corneal scarring. Sci Transl Med 2014;6:266ra172.
[55] Syed-Picard FN, Du Y, Lathrop KL, Mann MM, Funderburgh ML, Funderburgh JL. Dental pulp stem cells: a new cellular resource for corneal stromal regeneration. Stem Cells Transl Med 2015;4:276–85.
[56] Hertsenberg AJ, Funderburgh JL. Generation of corneal keratocytes from human embryonic stem cells. Methods Mol Biol 2016;1341:285–94.
[57] Zhang L, Coulson-Thomas VJ, Ferreira TG, Kao WW. Mesenchymal stem cells for treating ocular surface diseases. BMC Ophthalmol 2015;15(Suppl 1):155.
[58] Bonanno JA. Molecular mechanisms underlying the corneal endothelial pump. Exp Eye Res 2012;95:2–7.
[59] Lorenzetti DW, Uotila MH, Parikh N, Kaufman HE. Central cornea guttata. Incidence in the general population. Am J Ophthalmol 1967;64:1155–8.
[60] Okumura N, Sakamoto Y, Fujii K, et al. Rho kinase inhibitor enables cell-based therapy for corneal endothelial dysfunction. Sci Rep 2016;6:26113.
[61] Okumura N, Ueno M, Koizumi N, et al. Enhancement on primate corneal endothelial cell survival in vitro by a ROCK inhibitor. Invest Ophthalmol Vis Sci 2009;50:3680–7.
[62] Peh GS, Adnan K, George BL, et al. The effects of Rho-associated kinase inhibitor Y-27632 on primary human corneal endothelial cells propagated using a dual media approach. Sci Rep 2015;5:9167.
[63] Fukuta M, Nakai Y, Kirino K, et al. Derivation of mesenchymal stromal cells from pluripotent stem cells through a neural crest lineage using small molecule compounds with defined media. PLoS One 2014;9:e112291.
[64] Zhang K, Pang K, Wu X. Isolation and transplantation of corneal endothelial cell-like cells derived from in-vitro-differentiated human embryonic stem cells. Stem Cells Dev 2014;23:1340–54.

[65] McCabe KL, Kunzevitzky NJ, Chiswell BP, Xia X, Goldberg JL, Lanza R. Efficient generation of human embryonic stem cell-derived corneal endothelial cells by directed differentiation. PLoS One 2015;10:e0145266.
[66] Song Q, Yuan S, An Q, et al. Directed differentiation of human embryonic stem cells to corneal endothelial cell-like cells: a transcriptomic analysis. Exp Eye Res 2016;151:107–14.
[67] Zhao JJ, Afshari NA. Generation of human corneal endothelial cells via in vitro ocular lineage restriction of pluripotent stem cells. Invest Ophthalmol Vis Sci 2016;57:6878–84.
[68] Buller C, Johnson DH, Tschumper RC. Human trabecular meshwork phagocytosis. Observations in an organ culture system. Invest Ophthalmol Vis Sci 1990;31:2156–63.
[69] Alvarado J, Murphy C, Polansky J, Juster R. Age-related changes in trabecular meshwork cellularity. Invest Ophthalmol Vis Sci 1981;21:714–27.
[70] Alvarado J, Murphy C, Juster R. Trabecular meshwork cellularity in primary open-angle glaucoma and nonglaucomatous normals. Ophthalmology 1984;91:564–79.
[71] Kaufman PL, Rasmussen CA. Advances in glaucoma treatment and management: outflow drugs. Invest Ophthalmol Vis Sci 2012;53:2495–500.
[72] Serle JB. Pharmacological advances in the treatment of glaucoma. Drugs Aging 1994;5:156–70.
[73] Pearson C, Martin K. Stem cell approaches to glaucoma: from aqueous outflow modulation to retinal neuroprotection. Prog Brain Res 2015;220:241–56.
[74] Alexander RA, Grierson I. Morphological effects of argon laser trabeculoplasty upon the glaucomatous human meshwork. Eye (Lond) 1989;3(Pt 6):719–26.
[75] Acott TS, Samples JR, Bradley JM, Bacon DR, Bylsma SS, Van Buskirk EM. Trabecular repopulation by anterior trabecular meshwork cells after laser trabeculoplasty. Am J Ophthalmol 1989;107:1–6.
[76] Dueker DK, Norberg M, Johnson DH, Tschumper RC, Feeney-Burns L. Stimulation of cell division by argon and Nd:YAG laser trabeculoplasty in cynomolgus monkeys. Invest Ophthalmol Vis Sci 1990;31:115–24.
[77] Du Y, Yun H, Yang E, Schuman JS. Stem cells from trabecular meshwork home to TM tissue in vivo. Invest Ophthalmol Vis Sci 2013;54:1450–9.
[78] Abu-Hassan DW, Li X, Ryan EI, Acott TS, Kelley MJ. Induced pluripotent stem cells restore function in a human cell loss model of open-angle glaucoma. Stem Cells 2015;33:751–61.
[79] Manuguerra-Gagne R, Boulos PR, Ammar A, et al. Transplantation of mesenchymal stem cells promotes tissue regeneration in a glaucoma model through laser-induced paracrine factor secretion and progenitor cell recruitment. Stem Cells 2013;31:1136–48.
[80] Ding QJ, Zhu W, Cook AC, Anfinson KR, Tucker BA, Kuehn MH. Induction of trabecular meshwork cells from induced pluripotent stem cells. Invest Ophthalmol Vis Sci 2014;55:7065–72.
[81] Stevens GA, White RA, Flaxman SR, et al. Global prevalence of vision impairment and blindness: magnitude and temporal trends, 1990–2010. Ophthalmology 2013;120:2377–84.
[82] Visser N, Bauer NJ, Nuijts RM. Toric intraocular lenses: historical overview, patient selection, IOL calculation, surgical techniques, clinical outcomes, and complications. J Cataract Refract Surg 2013;39:624–37.
[83] Lois N, Taylor J, McKinnon AD, Forrester JV. Posterior capsule opacification in mice. Arch Ophthalmol 2005;123:71–7.
[84] Wormstone IM. Posterior capsule opacification: a cell biological perspective. Exp Eye Res 2002;74:337–47.
[85] Barbosa-Sabanero K, Hoffmann A, Judge C, Lightcap N, Tsonis PA, Del Rio-Tsonis K. Lens and retina regeneration: new perspectives from model organisms. Biochem J 2012;447:321–34.
[86] Tsonis PA, Del Rio-Tsonis K. Lens and retina regeneration: transdifferentiation, stem cells and clinical applications. Exp Eye Res 2004;78:161–72.
[87] Gwon AE, Gruber LJ, Mundwiler KE. A histologic study of lens regeneration in aphakic rabbits. Invest Ophthalmol Vis Sci 1990;31:540–7.
[88] Gwon A. Lens regeneration in mammals: a review. Surv Ophthalmol 2006;51:51–62.

[89] Lin H, Ouyang H, Zhu J, et al. Lens regeneration using endogenous stem cells with gain of visual function. Nature 2016;531:323–8.
[90] O'Connor MD, McAvoy JW. In vitro generation of functional lens-like structures with relevance to age-related nuclear cataract. Invest Ophthalmol Vis Sci 2007;48:1245–52.
[91] Yang C, Yang Y, Brennan L, Bouhassira EE, Kantorow M, Cvekl A. Efficient generation of lens progenitor cells and lentoid bodies from human embryonic stem cells in chemically defined conditions. FASEB J 2010;24:3274–83.
[92] Qiu X, Yang J, Liu T, Jiang Y, Le Q, Lu Y. Efficient generation of lens progenitor cells from cataract patient-specific induced pluripotent stem cells. PLoS One 2012;7:e32612.
[93] Li D, Qiu X, Yang J, Liu T, Luo Y, Lu Y. Generation of human lens epithelial-like cells from patient-specific induced pluripotent stem cells. J Cell Physiol 2016;231:2555–62.
[94] Fu Q, Qin Z, Jin X, et al. Generation of functional lentoid bodies from human induced pluripotent stem cells derived from urinary cells. Invest Ophthalmol Vis Sci 2017;58:517–27.
[95] Murphy P, Kabir MH, Srivastava T, et al. Light-focusing human micro-lenses generated from pluripotent stem cells model lens development and drug-induced cataract in vitro. Development 2018;145.
[96] Salero E, Blenkinsop TA, Corneo B, et al. Adult human RPE can be activated into a multipotent stem cell that produces mesenchymal derivatives. Cell Stem Cell 2012;10:88–95.
[97] Blenkinsop TA, Saini JS, Maminishkis A, et al. Human adult retinal pigment epithelial stem cell-derived RPE monolayers exhibit key physiological characteristics of native tissue. Invest Ophthalmol Vis Sci 2015;56:7085099.
[98] Davis RJ, Blenkinsop TA, Campbell M, et al. Human RPE stem cell–derived RPE preserves photoreceptors in the Royal College of Surgeons rat: method for quantifying the area of photoreceptor sparing. J Ocul Pharmacol Ther 2016;32:304–9.
[99] Davis RJ, Alam NM, Zhao C, et al. The developmental stage of adult human stem cell-derived retinal pigment epithelium cells influences transplant efficacy for vision rescue. Stem Cell Reports 2017;9:42–9.
[100] Mitashov VI. Mechanisms of retina regeneration in urodeles. Int J Dev Biol 1996;40:833–44.
[101] Jayaram H, Jones MF, Eastlake K, et al. Transplantation of photoreceptors derived from human Muller glia restore rod function in the P23H rat. Stem Cells Transl Med 2014;3:323–33.
[102] Lamba DA, Karl MO, Ware CB, Reh TA. Efficient generation of retinal progenitor cells from human embryonic stem cells. Proc Natl Acad Sci USA 2006;103:12769–74.
[103] Klimanskaya I, Hipp J, Rezai KA, West M, Atala A, Lanza R. Derivation and comparative assessment of retinal pigment epithelium from human embryonic stem cells using transcriptomics. Cloning Stem Cells 2004;6:217–45.
[104] Lund RD, Wang S, Klimanskaya I, et al. Human embryonic stem cell-derived cells rescue visual function in dystrophic RCS rats. Cloning Stem Cells 2006;8:189–99.
[105] Idelson M, Alper R, Obolensky A, et al. Directed differentiation of human embryonic stem cells into functional retinal pigment epithelium cells. Cell Stem Cell 2009;5:396–408.
[106] Nakano T, Ando S, Takata N, et al. Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell 2012;10:771–85.
[107] DiStefano T, Chen HY, Panebianco C, et al. Accelerated and improved differentiation of retinal organoids from pluripotent stem cells in rotating-wall vessel bioreactors. Stem Cell Rep 2018;10:300–13.
[108] Gonzalez-Cordero A, West EL, Pearson RA, et al. Photoreceptor precursors derived from three-dimensional embryonic stem cell cultures integrate and mature within adult degenerate retina. Nat Biotechnol 2013;31:741–7.
[109] Assawachananont J, Mandai M, Okamoto S, et al. Transplantation of embryonic and induced pluripotent stem cell-derived 3D retinal sheets into retinal degenerative mice. Stem Cell Rep 2014;2:662–74.

[110] Banin E, Obolensky A, Idelson M, et al. Retinal incorporation and differentiation of neural precursors derived from human embryonic stem cells. Stem Cells 2006;24:246257.
[111] MacLaren RE, Pearson RA, MacNeil A, et al. Retinal repair by transplantation of photoreceptor precursors. Nature 2006;444:203–7.
[112] Chuang K, Fields MA, Del Priore LV. Potential of gene editing and induced pluripotent stem cells (iPSCs) in treatment of retinal diseases. Yale J Biol Med 2017;90:635–42.
[113] Peng YQ, Tang LS, Yoshida S, Zhou YD. Applications of CRISPR/Cas9 in retinal degenerative diseases. Int J Ophthalmol 2017;10:646–51.
[114] Hung SSC, McCaughey T, Swann O, Pebay A, Hewitt AW. Genome engineering in ophthalmology: application of CRISPR/Cas to the treatment of eye disease. Prog Retin Eye Res 2016;53:1–20.
[115] Bassuk AG, Zheng A, Li Y, Tsang SH, Mahajan VB. Precision medicine: genetic repair of retinitis pigmentosa in patient-derived stem cells. Sci Rep 2016;6:19969.
[116] Meyer JS, Shearer RL, Capowski EE, et al. Modeling early retinal development with human embryonic and induced pluripotent stem cells. Proc Natl Acad Sci USA 2009;106:16698–703.
[117] Osakada F, Jin ZB, Hirami Y, et al. In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. J Cell Sci 2009;122:3169–79.
[118] Lamba DA, McUsic A, Hirata RK, Wang PR, Russell D, Reh TA. Generation, purification and transplantation of photoreceptors derived from human induced pluripotent stem cells. PLoS One 2010;5:e8763.
[119] Mellough CB, Sernagor E, Moreno-Gimeno I, Steel DH, Lako M. Efficient stage-specific differentiation of human pluripotent stem cells toward retinal photoreceptor cells. Stem Cells 2012;30:673–86.
[120] Lopez R, Gouras P, Kjeldbye H, et al. Transplanted retinal pigment epithelium modifies the retinal degeneration in the RCS rat. Invest Ophthalmol Vis Sci 1989;30:586–8.
[121] Tucker BA, Park IH, Qi SD, et al. Transplantation of adult mouse iPS cell-derived photoreceptor precursors restores retinal structure and function in degenerative mice. PLoS One 2011;6:e18992.
[122] Yamamoto S, Du J, Gouras P, Kjeldbye H. Retinal pigment epithelial transplants and retinal function in RCS rats. Invest Ophthalmol Vis Sci 1993;34:3068–75.
[123] Carr AJ, Vugler AA, Hikita ST, et al. Protective effects of human iPS-derived retinal pigment epithelium cell transplantation in the retinal dystrophic rat. PLoS One 2009;4:e8152.
[124] Li Y, Tsai YT, Hsu CW, et al. Long-term safety and efficacy of human-induced pluripotent stem cell (iPS) grafts in a preclinical model of retinitis pigmentosa. Mol Med 2012;18:1312–9.
[125] Sun J, Mandai M, Kamao H, et al. Protective effects of human iPS-derived retinal pigmented epithelial cells in comparison with human mesenchymal stromal cells and human neural stem cells on the degenerating retina in rd1 mice. Stem Cells 2015;33:1543–53.
[126] Tucker BA, Mullins RF, Streb LM, et al. Patient-specific iPSC-derived photoreceptor precursor cells as a means to investigate retinitis pigmentosa. Elife 2013;2:e00824.
[127] Thumann G, Bartz-Schmidt KU, El Bakri H, et al. Transplantation of autologous iris pigment epithelium to the subretinal space in rabbits. Transplantation 1999;68:195–201.
[128] Crafoord S, Geng L, Seregard S, Algvere PV. Photoreceptor survival in transplantation of autologous iris pigment epithelial cells to the subretinal space. Acta Ophthalmol Scand 2002;80:387–94.
[129] Abe T, Tomita H, Kano T, et al. Autologous iris pigment epithelial cell transplantation in monkey subretinal region. Curr Eye Res 2000;20:268–75.
[130] Rezai KA, Kohen L, Wiedemann P, Heimann K. Iris pigment epithelium transplantation. Graefes Arch Clin Exp Ophthalmol 1997;235:558–62.
[131] Schraermeyer U, Kociok N, Heimann K. Rescue effects of IPE transplants in RCS rats: short-term results. Invest Ophthalmol Vis Sci 1999;40:1545–56.

[132] Sheedlo HJ, Li L, Turner JE. Photoreceptor cell rescue at early and late RPE-cell transplantation periods during retinal disease in RCS dystrophic rats. J Neural Transplant Plast 1991;2:55–63.
[133] Abe T, Yoshida M, Tomita H, et al. Functional analysis after auto iris pigment epithelial cell transplantation in patients with age-related macular degeneration. Tohoku J Exp Med 1999;189:295–305.
[134] Abe T, Yoshida M, Tomita H, et al. Auto iris pigment epithelial cell transplantation in patients with age-related macular degeneration: short-term results. Tohoku J Exp Med 2000;191:7–20.
[135] Lappas A, Weinberger AW, Foerster AM, Kube T, Rezai KA, Kirchhof B. Iris pigment epithelial cell translocation in exudative age-related macular degeneration. A pilot study in patients. Graefes Arch Clin Exp Ophthalmol 2000;238:631–41.
[136] Thumann G, Aisenbrey S, Schraermeyer U, et al. Transplantation of autologous iris pigment epithelium after removal of choroidal neovascular membranes. Arch Ophthalmol 2000;118:1350–5.
[137] Lappas A, Foerster AM, Weinberger AW, Coburger S, Schrage NF, Kirchhof B. Translocation of iris pigment epithelium in patients with exudative age-related macular degeneration: long-term results. Graefes Arch Clin Exp Ophthalmol 2004;242:638–47.
[138] Aisenbrey S, Lafaut BA, Szurman P, et al. Iris pigment epithelial translocation in the treatment of exudative macular degeneration: a 3-year follow-up. Arch Ophthalmol 2006;124:183–8.
[139] Reardon S, Cyranoski D. Japan stem-cell trial stirs envy. Nature 2014;513:287–8.
[140] Kimbrel EA, Lanza R. Current status of pluripotent stem cells: moving the first therapies to the clinic. Nat Rev Drug Discov 2015;14:681–92.
[141] Schwartz SD, Regillo CD, Lam BL, et al. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet 2015;385:509–16.
[142] Schwartz SD, Tan G, Hosseini H, Nagiel A. Subretinal transplantation of embryonic stem cell-derived retinal pigment epithelium for the treatment of macular degeneration: an assessment at 4 years. Invest Ophthalmol Vis Sci 2016;57:ORSFc1-ORSFc9.
[143] Song WK, Park KM, Kim HJ, et al. Treatment of macular degeneration using embryonic stem cell-derived retinal pigment epithelium: preliminary results in Asian patients. Stem Cell Reports 2015;4:860–72.
[144] Mandai M, Watanabe A, Kurimoto Y, et al. Autologous induced stem-cell-derived retinal cells for macular degeneration. N Engl J Med 2017;376:1038–46.

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7 Cell therapy for liver regeneration

Abstract: Successful clinical applications of cellular therapies depend on a number of factors. First, there should be an efficient way of producing high-quality cells for transplantation. Second, the differentiation of stem cells into mature hepatocytes should be accomplished without the need for viral vectors or alterations in cell cycle regulators to evade the risk of tumorigenicity. Finally stem cell techniques developed in laboratories should be tested on larger animal models like primates as they can better predict the responses in humans as compared to rodents. This chapter will present information on liver structure, function, associated diseases, and their prevalence worldwide and especially in Pakistan. Furthermore, this chapter will address the conventional therapies used to treat the prevalent liver diseases along with their limitations. The chapter will discuss potential of various cell types such as hepatocytes, hepatic progenitor cells, mesenchymal stem cells, and embryonic stem cells for liver regeneration. It will further review studies related to liver bioengineering and scaffolds-assisted liver regeneration. Finally, this chapter will discuss the future prospects of stem cells for liver regeneration.

Key Words: Cellular, Cirrhosis, Clinical, Fibrosis, Liver, Stem cells, Therapy, Transplantation.

7.1 Introduction

Regenerative medicine has come about as a new revolutionary clinical practice. The initial target application of this new clinical invention is liver disorders. There are two primary reasons for placing priority on liver disorders. First, this vital human organ exhibits a miraculous regenerative ability. Second, an effective therapeutic treatment for liver diseases has not yet been identified, especially for cases identified in later stages; consequently, the amount of liver patients is steadily increasing.

7.1.1 Worldwide prevalence of liver diseases

In the United States, liver diseases are considered the second highest cause of death among all digestive disorders [1]. According to National statistics of the UK (http://www.statistics.gov.uk/), diseases of the liver are ranked the fifth most common cause of death. In 2010, it alone caused 31 million disability-adjusted life years globally, resulting from equal proportions of hepatitis C, hepatitis B and alcohol consumption [2]. In Asia, Hepatitis B and hepatitis C are the leading cause

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of liver cirrhosis [3]. In Pakistan and China chronic hepatitis is the principal cause of mortality. According to World Health Organization figures [4], Pakistan ranks second highest in the world with regard to the prevalence of Hepatitis C and about ten million people are affected. In the People's Republic of China, liver diseases affect approximately 300 million people yearly [5].

7.2 Structure and function of the liver

The liver is the largest glandular organ of the human body and carries out several very important physiological functions. It regulates chemical levels in the blood and also excretes bile that takes away the waste products from the liver. Other crucial functions of the liver include regulation of blood clotting, transformation of glucose into glycogen, conversion of harmful ammonia into urea, regulation of levels of amino acids in the blood, clearance of bilirubin and processing of hemoglobin, and production of plasma proteins and cholesterol.

Like all other organs, the liver is composed of several different types of cells, which perform different functions. The major cell types are Kupffer cells, hepatocytes, and cholangiocytes. Kupffer cells destroy old red blood cells. Hepatocytes are epithelial cells that perform most of the metabolic functions of the liver. Cholangiocytes are epithelial cells that constitute mainly the lining of the intrahepatic and extrahepatic ducts of the biliary tree. Their main function is to modify primary bile derived from hepatocytes and formation of ductal bile. In addition to these major types, there are stellate cells, macrophages, endothelial cells, and dendritic cells that conjointly perform various other complex functions of the liver.

7.3 Liver diseases

Major diseases of the liver are generally caused by a lack of autoimmunity, congenital infection, viral infections, and metabolic liver disorders. The most critical liver disease is cirrhosis, caused by progression of liver fibrosis.

7.3.1 Progression of liver fibrosis to liver cirrhosis

Liver fibrosis is caused by the disruption of liver architecture due to an accumulation of extracellular matrix (ECM) rich in collagens [6]. It directly affects hepatic stellate cells and fibroblasts and is activated by soluble factors released by Kupffer cells during the course of chronic liver disorder [7]. Liver fibrosis progresses into liver cirrhosis, characterized by disruption of liver vasculature and manifested by the formation of nodules within the fibrous tissue [8, 9], ultimately leading to hemodynamic

instability and portal hypertension. The vascular disruption results in the propulsion of the blood supply from the artery directly into the portal vein and distorts the exchange between liver parenchyma and hepatic sinusoids [7].

7.3.2 Liver failure

Liver failure is a clinical condition marked by the appearance of jaundice, coagulopathy, formation of ascites, and hepatic encephalopathy with extremely high rates of morbidity and mortality. There are two types of pathological changes, namely, progressive damage to liver cells and severe necrosis of liver tissues. The major causes of liver failure include excessive use of alcohol consumption, drug abuse, and viral infections [10].

7.3.3 Liver failure causes malfunctioning of other organs

Because the liver plays a central role in many physiological functions, its malfunctioning affects the functioning of lungs, kidneys, brain, and other key organs [11]. Hence, liver failure induces a variety of other disorders, including hepato-renal syndrome and hepatic encephalopathy [11, 12].

7.4 Conventional therapies for liver diseases

Liver transplantation is the standard course of treatment in a majority of clinical settings because it is the only therapy shown to alter the state of mortality. However, its usage is limited due to the acute shortage of organ donors. There is also the option of partial liver transplants, which takes advantage of the exceptional regenerative ability of the liver; however, vascular and biliary complications cause major constraints in the use of these clinical procedures [13].

7.5 Cellular therapies for liver diseases

Cellular therapies for liver diseases began with the introduction of primary hepatocytes, via the portal vein, into patients diagnosed with end-stage liver disease (ESLD) or certain genetic and metabolic liver disorders [14, 15]. Multiple reports have been published that account for the beneficial effects of the introduction of hepatocytes leading to improvements in the liver function for variable durations. The observed variability is attributable to the fact that (i) liver hepatocytes are difficult to multiply *in vitro*, prior to introduction into the damaged liver, and (ii) the introduced hepatocytes repopulate

the liver in only selected liver disorders such as Wilson disease, hereditary tyrosinemia, and progressive familial intrahepatic cholestasis [16]. Furthermore, the duration in which the foreign hepatocytes remain viable and interact with native hepatocytes is uncertain. All these factors make primary hepatocyte intervention a poor therapeutic option even among patients with ESLD or metabolic/genetic disorders [17].

7.6 Stem cell therapy for liver diseases

Stem cell therapy is an attractive alternative to overcome the shortage of donors for liver transplantation as well as the drawbacks in the use of hepatocyte interventions. Stem cells are undifferentiated cells that possess inherent abilities for self-renewal and differentiate into cell types of other lineages such as heart, liver, kidney, pancreas, etc. A single cell embryo is basically a totipotent stem cell as it can give rise to a whole organism while other stem cells may be pluripotent or multipotent.

From a clinical perspective, the most popular stem cell types are the adult and infant mesenchymal stem cells (MSCs) because these cells exhibit low immunogenicity in comparison to the progenitor or hematopoietic stem cells when used from allogenic sources. MSCs do not express major histocompatibility complex (MHC) class I and specifically express MHC class II at very low levels. Additionally, MSCs may be obtained from numerous sources; the most frequently studied variety is derived from either bone marrow, adipose tissue, umbilical cord tissue, umbilical cord blood, or synovial fluid. The identity of isolated stem cells can be ascertained by its specificity for a panel of approved MSC markers. Bone-marrow- and adipose-tissue-derived MSCs (BM-MSCs and ADSCs, respectively) present the most suitable option for autologous therapies, but their use is restricted because of the invasiveness of procedures required for deriving adipose and bone marrow samples.

During the last decade, different types of stem cells such as liver stem/progenitor cells (LSPCs) and MSCs have been used, with varying degrees of success in the treatment of various liver diseases [18, 19]. The results of these studies firmly support the notion that stem cell therapy is a revolutionary option in the treatment of liver diseases and is a superior option than liver transplantation. Furthermore, recent successes with the induction of somatic cells into pluripotent stem cells and accompanying successes in gene editing have given birth to yet another revolutionary candidate for stem cell therapy [20]. Needless to mention, the innovation will have the added advantage of being devoid of immune and ethical concerns.

7.6.1 Liver stem/progenitor cells

LSPCs represent progenitors of liver parenchyma cells and can transdifferentiate into hepatocytes or cholangiocytes; however, their efficacy in the repair of different

liver diseases is uncertain. On the other hand, embryonic LSPCs, identified as Dlk-1$^+$, CD13$^+$, E-cadherin$^+$, and Liv-2$^+$ cells in midgestation, differentiate satisfactorily into hepatocytes and cholangiocytes during development. LSPCs residing in terminal bile ductules (canals of Herring) can be activated to proliferate and differentiate when hepatocyte replication is severely compromised [21]. Adult liver progenitor cells, enriched in cellular fractions positive for epithelial cell adhesion molecule, CD13, CD133, and MIC1–1C3, are isolated from healthy and injured livers [22].

The transplantation of LSPCs can be accomplished via intrasplenic injections or infusion into the portal and peripheral veins [23]. Although in the case of fibrosis engraftment, repopulation of the liver has been observed [24], a regenerative stimulus such as a retrosine injection or partial hepatectomy is quite beneficial for the success of the engraftment. Even though some studies have shown a reduction in fibrosis [24], there are serious concerns in the wake of reports of severe fibrogenic response due to the activation of hepatic progenitor compartment [25]. Furthermore, ethical concerns have been raised on the use of human fetal progenitor cells within clinical settings [17].

7.6.2 MSCs in the therapy of liver diseases

MSCs are nonhematopoietic, fibroblast-like, undifferentiated and multipotent progenitor cells. These cells have the capability to differentiate into various lineages such as adipocytes, chondrocytes, and osteoblasts [26, 27]. MSCs can be isolated from bone marrow, adipose tissues, umbilical cord, muscle, cartilage, trabecular bone, and peripheral blood [28–30]. MSCs can be isolated in large quantities and easily expanded through several passages. Moreover, MSCs are an ethically acceptable and ideal source for the treatment of various liver disorders.

7.6.2.1 Bone marrow-derived MSCs

Bone marrow is the most commonly studied source of MSCs. However, the painful procedure of harvesting bone marrow has prevented its liberal clinical use. Moreover, stem cells so isolated show a decline in differentiation potential, number, and functionality with growing age [31]. Nonetheless, it has been shown that BM-MSCs are able to alleviate liver fibrosis in CCL_4-induced liver model. BM-MSCs also demonstrate antifibrotic and anti-inflammatory effects *in vivo* [32]. It has been further demonstrated in unequivocal terms that BM-MSCs improve the overall architecture of the liver and liver functions in a rat bile duct ligation model. The transplanted stem cells mediate their beneficial effect by down-regulation of CK19 mRNA and upregulation of hepatocyte growth factor (HGF) mRNA, which aids in the restoration of deteriorated liver and induce liver regeneration [33]. It has been further shown that an injection of granulocyte colony stimulating factor (G-CSF) into rats with liver failure improved their survival, attributable to the mobilization of BM-MSCs [34, 35].

In agreement with the results of these studies, we observed an improvement in the function and architecture of fibrotic liver through an injection of BM-MSCs in carbon tetrachloride (CCl_4)-induced fibrosis in mice [36–38]. We further demonstrated that the differentiation of MSCs was enhanced as a result of pretreatment with injured liver tissue [39]. We also observed enhanced survival of CCl_4 injured hepatocytes when cocultured with BM-MSCs and treated with interleukin (IL)-6 (Fig. 7.6.1). Furthermore, pretreatment of fibrotic liver with IL-6 resulted in enhanced reduction of liver injury (Fig. 7.6.2). Other studies have reported that infusion of bone marrow into humans with liver cirrhosis resulted in raising the levels of G-CSF and IL-1β, with accompanying improvement in liver function [40, 41]. It is evident from these studies that BM-MSCs offer a preferable therapeutic option in attenuation of liver apoptosis and fibrosis accompanied by improved liver function [37]. On the basis of these results, BM-MSCs

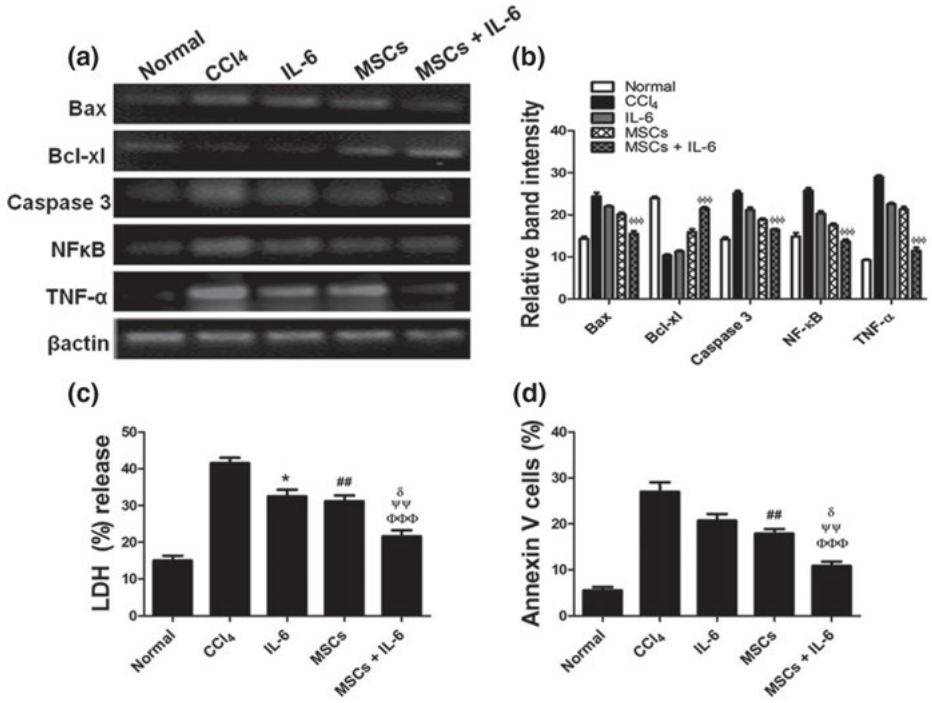

Fig. 7.6.1: Enhanced hepatocyte survival subsequent to co-culture with MSCs + IL-6. (a) Gene expression of apoptotic (Bax, caspase-3, NF-κB, tumor necrosis factor α [TNF-α]) and antiapoptotic (Bcl-xl) markers in hepatocytes cocultured with MSCs together with IL-6. (b) Gel band quantification of gene expressions through Image J (n = 3). CCl_4 vs. MSCs + IL-6: $^{\Phi}P < 0.05$, $^{\Phi\Phi}P < 0.01$, and $^{\Phi\Phi\Phi}P < 0.001$. (c) LDH (%) release levels in the control and treatment groups (n = 4). (d) Analysis of apoptotic cells through annexin V staining (n = 4). CCl_4 vs. IL-6: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; CCl_4 vs. MSCs: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$; CCl_4 vs. MSCs + IL-6: $^{\Phi}P < 0.05$, $^{\Phi\Phi}P < 0.01$, and $^{\Phi\Phi\Phi}P < 0.001$; IL-6 vs. MSCs + IL-6: $^{\Psi}P < 0.05$, $^{\Psi\Psi}P < 0.01$, and $^{\Psi\Psi\Psi}P < 0.001$; MSCs vs. MSCs + IL-6: $^{\delta}P < 0.05$, $^{\delta\delta}P < 0.01$, and $^{\delta\delta\delta}P < 0.001$ [37].

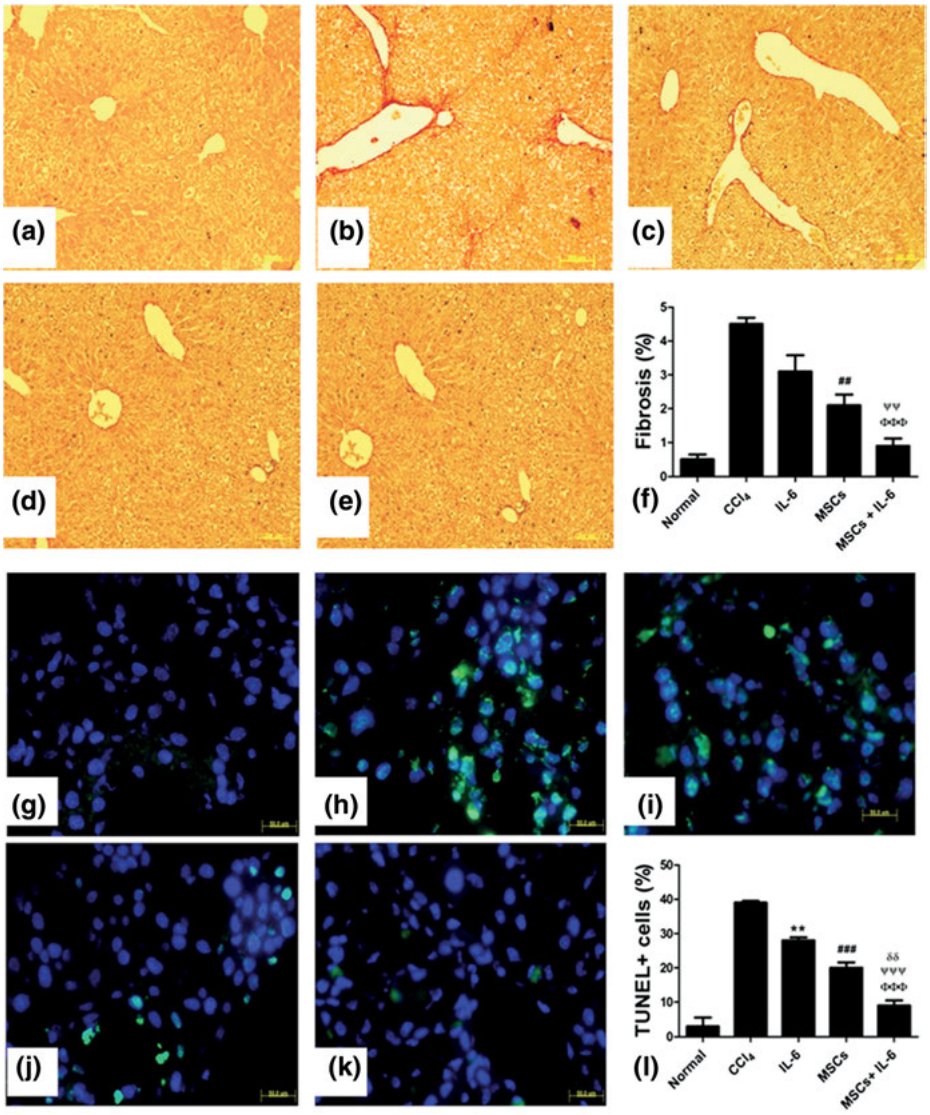

Fig. 7.6.2: Reduced fibrosis and apoptosis in injured liver treated with MSCs + IL-6. (a) Normal, (b) CCl_4 injury, (c) IL-6 treated, (d) MSC transplanted, (e) IL-6 + MSC transplanted, (f) Graphical presentation of collagen levels (n=). CCl_4 vs. IL-6: NS; CCl_4 vs. MSCs: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$; CCl_4 vs. MSCs + IL-6: $^{\phi}P < 0.05$, $^{\phi\phi}P < 0.01$, and $^{\phi\phi\phi}P < 0.001$; IL-6 vs. MSCs + IL-6: $^{\psi}P < 0.05$, $^{\psi\psi}P < 0.01$, and $^{\psi\psi\psi}P < 0.001$; MSCs vs. MSCs + IL-6: $^{\delta}P < 0.05$, $^{\delta\delta}P < 0.01$, and $^{\delta\delta\delta}P < 0.001$. (g–l) Estimation of apoptosis in control fibrotic liver and treatment groups through TUNEL assay. (g) Normal, (h) CCl_4 injury, (i) IL-6 treated, (j) MSC transplanted, (k) IL-6 + MSC transplanted, (l) graphical presentation of apoptotic positive cells in different treatment groups (n=). CCl_4 vs. IL-6: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; CCl_4 vs. MSCs: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$; CCl_4 vs. MSCs + IL-6: $^{\phi}P < 0.05$, $^{\phi\phi}P < 0.01$, and $^{\phi\phi\phi}P < 0.001$; IL-6 vs. MSCs + IL-6: $^{\psi}P < 0.05$, $^{\psi\psi}P < 0.01$, and $^{\psi\psi\psi}P < 0.001$; MSCs vs. MSCs + IL-6: $^{\delta}P < 0.05$, $^{\delta\delta}P < 0.01$, and $^{\delta\delta\delta}P < 0.001$ [37].

appear a suitable therapeutic material; however, difficulties in obtaining bone marrow as the source material for the isolation of MSCs remain the primary constraint.

7.6.2.2 Adipose (tissue)-derived MSCs

Adipose tissue is another promising source of MSCs [42]. In comparison to bone marrow, adipose tissue is conveniently obtainable through simple lipo-aspiration. Furthermore, ADSCs exhibit high proliferation capacity, can be isolated in abundance [31], and possess immunosuppressive properties. When cocultured with hepatic stellate cells, ADSCs exhibit retarded growth and *in vitro* proliferation. When infused *in vivo*, ADSCs were able to alleviate liver fibrosis in a CCL_4-induced rat liver model. Furthermore, ADSCs exhibit antifibrotic and anti-inflammatory effects *in vivo* [32] and differentiate into hepatocyte-like cells both morphologically and functionally. When transplanted, they were engrafted in liver parenchyma and improved liver fibrosis [18]. In our studies, we also observed increased differentiation of ADSCs into hepatocytes *in vitro* (Fig. 7.6.3a, b) and improved liver function *in vivo* (Fig. 7.6.3c–e) subsequent to transplantation of ADSCs, pretreated with serum from liver fibrosis. It seems that the serum from rats with acute liver injury may contain stimulatory cytokines and growth factors [18].

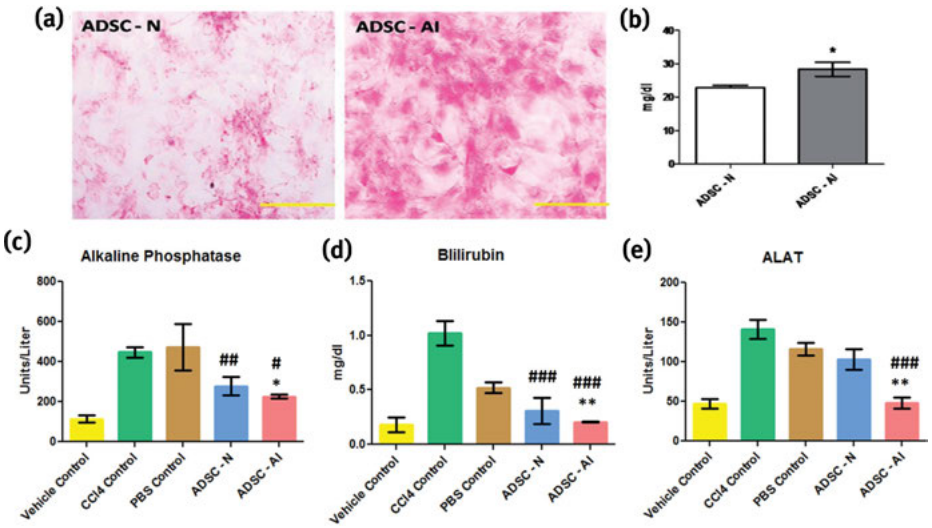

Fig. 7.6.3: (a, b) Glycogen storage and urea production. (a) Periodic acid–Schiff staining (PAS) for glycogen storage ability of serum pre conditioned ADSCs after 7 days. Scale bar indicates magnification at 200×. (b) Urea production assay after 7-day serum preconditioning of ADSCs. Values are expressed as mean ± SEM. $^{*}P < 0.05$ vs. ADSC-N. (c–e) Functional recovery after transplantation by alkaline phosphatase, ALAT, and bilirubin assays. Values are expressed as mean ± SEM. $^{***}P < 0.001$ (ADSC-AI vs. PBS group), $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (ADSC-AI/Serum-N vs. CCl_4 group) [18].

7.6.2.3 Umbilical-cord-tissue-derived MSCs

Umbilical cord tissue, i.e., Wharton jelly and umbilical cord blood, obtainable through a literally noninvasive method, offer a more promising source of MSCs [30]. Wharton jelly MSCs (WJMSCs) are easily obtainable and remain viable even after lengthy cryopreservation. The source material is conveniently obtainable from gynecological wards in hospitals as a discard organ. WJMSCs are capable of differentiation into hepatocyte-like cells and exhibit normal functions such as secretion of albumin and storage of glycogen. The WJMSCs secrete multiple cellular factors that stimulate the proliferation of host hepatocytes, presumably through a paracrine mechanism and promote recovery of damaged liver [43]. In another study, WJMSCs were transplanted into rats and it alleviated liver fibrosis, with a concomitant increase in the levels of IL-4 and IL-10. Both IL-4 and IL-10 are involved in injury repair [44]. In yet another study, WJMSCs were infused in a rat model of liver cirrhosis and led to a significant increase in levels of serumalanine aminotransferase and it led to a significant decrease in the levels of serum albumin. Hepatic CK-18 and albumin mRNA and protein expression levels were all increased as a consequence of transplantation [45]. These studies prove that WJMSCs provide a promising clinical material for the repair of damaged livers.

7.6.2.4 Umbilical cord blood stem cells

Umbilical cord blood stem cells (UCB-MSCs) possess high plasticity and thus act as another promising source of clinical therapeutic material for the repair of damaged livers [46]. However, these stem cells have not been studied extensively. Some recent studies suggest that engraftment of stem cells in the recipient's liver is mediated through cell fusion in most cells [47], while some other studies have reported no cell fusion [48]. These later reports claim that human UCB-MSCs differentiate into hepatocytes subsequent to transplantation into NOD/SCID mice [48]. UCB-MSCs were shown to differentiate into hepatocytes after infusion into a pre-immune fetal sheep model [49, 50]. Furthermore, it substantially improved liver architecture and overall function in liver cirrhotic mice, presumably due to the upregulation of albumin synthesis and inhibition of profibrotic genes [51].

7.6.3 A comparison of the various sources of MSCs

MSCs isolated from various sources such as bone marrow, adipose tissues, and umbilical cord have been shown to differentiate into hepatocyte-like cells in rats [52], mice [53], and humans [54]. Subsequent to introduction in various model animals, MSCs produce a number of cytokines and other substances that have a beneficial effect at the site of liver injuries, including antiapoptotic, anti-inflammatory, immune-modulatory, and proliferative effects and differentiate into hepatic lineage [55, 56]. A comparison between the three kinds of MSCs revealed no significant differences in regard to

morphology and immune phenotype. Nevertheless, slight differences exist in the ultimate outcome from the use of different types of MSCs in treating liver diseases [31, 57].

7.6.4 Clinical promises of MSCs

MSCs possess unique properties that make them ideal for use in clinical therapies. These properties include immunomodulation, easy access and expansion, abundance in number, paracrine secretion, engraftment in target tissue, and transdifferentiation. Furthermore, use of MSCs does not cause any ethical or tumorigenic concern as is the case with the use of embryonic stem cells. It has been reported that usage of autologous BM-MSCs in the repair of liver cirrhosis resulted in improvement of the liver function [58]. It has been further reported that introduction of autologous BM-MSCs through hepatic artery for treatment of liver cirrhosis resulted in histological and functional improvement of the liver [59, 60]. Similarly, improvement in liver function was observed subsequent to injection of autologous BM-MSCs for the treatment of hepatitis-B-virus-associated cirrhosis [61]. When allogeneic UCB-MSCs were used to treat patients with chronic hepatitis B, liver function was improved, as evidenced by the reduction in ascites and recovery of biliary enzymes [62, 63]. These results are in agreement with earlier studies [64] that demonstrated that MSC transplantation increased the survival rate of patients with hepatitis-B-virus-associated acute or chronic liver failure.

Although these reports confirm that clinical usage of MSCs improved liver function, additional studies are needed to explain the underlying mechanism of their therapeutic benefits [27]. Despite the fact that MSCs pose a low risk of tumorigenesis, concerns exist about the possibility to develop tumors *in vivo* [65]. Therefore, it is suggested that as a safety measure, all MSCs should be screened for gene expression signature prior to infusion [65, 66]. Furthermore, it should be kept in mind that in a few cases, no significant beneficial effects were observed. For example, when BM-MSCs were injected into patients with liver cirrhosis, it did not cause any therapeutic effects [67, 68].

7.6.5 Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are embryonic-like stem cells generated by the induction of somatic cells by transient expression of key transcription factors such as OCT4 (O), SOX2 (S), KLF4 (K), and c-MYC (M) (OSKM cocktail) by permanently integrating retroviral vectors [69, 70]. iPSCs are pluripotent cells that make them ideal tools for cellular therapies. They have the capacity for unlimited self-renewal *in vitro* and therefore can be maintained indefinitely as cell lines in cell culture. They are also being used in *in vitro* disease models [71] and in drug screening [72].

It has been convincingly proven that iPSCs efficiently differentiate into hepatocytes [73–76]. When transplanted in a hepatic failure model animal, these cells lead to reversal of liver failure, boost regeneration, and enhance the health status of diseased mice, affected by CCl_4 injury [77], NOD/SCID [76], and fumarylacetoacetate hydrolase deficiency [78]. When injected intrahepatically along with carboxymethyl-hexanoyl chitosan hydrogel with sustained HGF release, the injected complex rescued the liver function of the recipient animal, presumably due to increased antiapoptotic and antioxidant activity that reduced hepatic necrotic areas [79]. It appears that iPSCs provide engineered donor grafts, including recellularized biomatrix [80] and liver buds [81], that might act as "autologous" organs for liver transplantation. Evidently, therefore, IPSCs offer enormous repair potential for the treatment of liver failure [65]. By combining *ex vivo* gene correction and cell transplantation, iPSCs offer tremendous potential to correct inherited liver disorders [82, 83] in addition to acquired liver diseases.

7.7 Liver bioengineering/scaffold-assisted liver regeneration

Besides cell therapies, extracorporeal devices and scaffolds are being studied in the quest of attaining increased therapeutic outcome. Scaffolds support cell engraftment and differentiation in diseased tissue microenvironment (invaded with reactive oxygen species), nonconducive for survival of transplanted cells. Hence, multiple liver tissue engineering strategies employing scaffold materials, synthetic polymers, nanotechnology-based microchip devices, and ultimately whole organ engineering approaches are being used in pursuit of beneficial effects [84].

Bioactive scaffolds are superior as they mimic the natural ECM environment of the liver. These include collagen, hyaluronic acid, chitosan, alginate, silk, and Matrigel. Hydrogels containing hyaluronic acid increase the adhesion of hepatocytes and, in turn, their viability. Mixtures of various scaffold materials are also being studied with the aim of attaining improved therapeutic outcome of cell therapies. Hydrogels constituted by alginate and Matrigel also improve hepatocyte adhesion. However, Matrigel may have toxicogenic and tumorigenic effects. In addition to promoting cell-to-cell and cell-to-matrix interactions, the variable mechanical and physicochemical properties of natural polymers prevent their clinical uses. To overcome these drawbacks, the possibility of using synthetic polymers such as polylacticacid, polyglycolic acid, polyurethanes, polyanhydrides, poly-fumarates, poly-ortho-esters, polycaprolactones, and polycarbonates is being investigated.

Tissue engineering is being applied to build functional liver organ in order to overcome the shortage of donors for liver transplantation. In this context, porcine/murine-based scaffolds are the most widely studied scaffolds. Complete decellularization of the liver while maintaining the integrity of ECM is performed, followed by recellularization of xenogenic scaffold with highly functional hepatocytes. In this

regard, the use of human bone marrow cells gave promising results *in vitro*. However, further research to determine ideal cell type, optimal cell seeding techniques, and cell volume is needed [84, 85].

7.8 Future prospects of cellular therapies for the repair of damaged livers

The use of different types of MSCs offers various associated advantages and disadvantages in treating a variety of diseased tissues. BM-MSCs have been more thoroughly studied and used in stem cell therapy as opposed to ADSCs and WJMSCs. However, both BM-MSCs and ADSCs are obtained through invasive surgical procedures, making WJMSCs the most favorable for clinical therapies. The umbilical cord is just a discarded tissue; therefore, it is the most conveniently obtainable source material. Needless to mention, WJMSCs also overcome the difficulty of procuring the high number of stem cells that is required, more so if additional transplantations are required, based on the patient's condition. Furthermore, MSCs obtained from umbilical cords possess additional advantageous characteristics, such as having low immunogenicity, which renders them the most desirable and feasible therapeutic clinical option.

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7.9 References

[1] Everhart JE, Ruhl CE. Burden of digestive diseases in the United States. Part III: liver, biliary tract, and pancreas. Gastroenterology 2009;136:1134–44.
[2] Murray CJ, Vos T, Lozano R, et al. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2012;380:2197–223.
[3] Mokdad AA, Lopez AD, Shahraz S, et al. Liver cirrhosis mortality in 187 countries between 1980 and 2010: a systematic analysis. BMC Med 2014;12:145.
[4] Memon MS, Zaki M. Burden of chronic liver disease and liver transplantation in Sindh. JLUMHS 2013;12:1.
[5] Wang FS, Fan JG, Zhang Z, Gao B, Wang HY. The global burden of liver disease: the major impact of China. Hepatology 2014;60:2099–108.
[6] Germani G, Hytiroglou P, Fotiadu A, Burroughs AK, Dhillon AP. Assessment of fibrosis and cirrhosis in liver biopsies: an update. Semin Liver Dis 2011;31:82–90.
[7] Suk KT, Kim DJ. Staging of liver fibrosis or cirrhosis: The role of hepatic venous pressure gradient measurement. World J Hepatol 2015;7:607.
[8] Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. Lancet 2001;357:1069-75.
[9] Brenner DA. Reversibility of liver fibrosis. Gastroenterol Hepatol 2013;9:737.

[10] Sarin SK, Kumar A, Almeida JA, et al. Acute-on-chronic liver failure: consensus recommendations of the Asian Pacific Association for the Study of the Liver (APASL). Hepatol Int 2009;3:269.
[11] Xing TJ. Clinical classification of liver failure: consensus, contradictions and new recommendations. J Clin Gastroenterol 2017;1:2.
[12] Wang Y. New concept in nomenclature, classification and diagnosis of liver failure. Zhonghua gan zang bing za zhi= Zhonghua ganzangbing zazhi= Chin J Hepatol 2010;18:803.
[13] Gerken G, Paul A. Challenges in liver transplantation. Visc Med 2016;32:232–3.
[14] Weber A, Groyer-Picard MT, Franco D, Dagher I. Hepatocyte transplantation in animal models. Liver Transpl 2009;15:7–14.
[15] Dhawan A, Puppi J, Hughes RD, Mitry RR. Human hepatocyte transplantation: current experience and future challenges. Nat Rev Gastroenterol Hepatol 2010;7:288.
[16] Rountree CB, Mishra L, Willenbring H. Stem cells in liver diseases and cancer: recent advances on the path to new therapies. Hepatology 2012;55:298–306.
[17] Huebert RC, Rakela J. Cellular therapy for liver disease. Mayo Clin Proc 2014;89:414–24.
[18] Baig MT, Ali G, Awan SJ, et al. Serum from CCl4-induced acute rat injury model induces differentiation of ADSCs towards hepatic cells and reduces liver fibrosis. Growth Factors 2017;35:144–60.
[19] Irudayaswamy A, Muthiah M, Zhou L, et al. Long-term fate of human fetal liver progenitor cells transplanted in injured mouse livers. Stem Cells 2018;36:103–13.
[20] Orqueda AJ, Giménez CA, Pereyra BF. iPSCs: a minireview from bench to bed, including organoids and the CRISPR system. Stem Cells Int 2016;2016:5934782.
[21] Lanthier N, Rubbia-Brandt L, Spahr L. Liver progenitor cells and therapeutic potential of stem cells in human chronic liver diseases. Acta Gastroenterol Belg 2013;76:3–9.
[22] Tanimizu N, Ichinohe N, Yamamoto M, Akiyama H, Nishikawa Y, Mitaka T. Progressive induction of hepatocyte progenitor cells in chronically injured liver. Sci Rep 2017;7:39990.
[23] Yovchev MI, Dabeva MD, Oertel M. Isolation, characterization, and transplantation of adult liver progenitor cells. Methods Mol Biol 2013;976:37–51.
[24] Yovchev MI, Xue Y, Shafritz DA, Locker J, Oertel M. Repopulation of the fibrotic/cirrhotic rat liver by transplanted hepatic stem/progenitor cells and mature hepatocytes. Hepatology 2014;59:284–95.
[25] Kuramitsu K, Sverdlov DY, Liu SB, et al. Failure of fibrotic liver regeneration in mice is linked to a severe fibrogenic response driven by hepatic progenitor cell activation. Am J Pathol 2013;183:182–94.
[26] Elahi KC, Klein G, Avci AM, Sievert KD, MacNeil S, Aicher WK. Human mesenchymal stromal cells from different sources diverge in their expression of cell surface proteins and display distinct differentiation patterns. Stem Cells Int 2016;2016:1–9.
[27] Tsuchiya A, Kojima Y, Ikarashi S, et al. Clinical trials using mesenchymal stem cells in liver diseases and inflammatory bowel diseases. Inflamm Regen 2017;37:16.
[28] Álvarez-Viejo M, Menéndez-Menéndez Y, Otero-Hernández J. CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture. World J Stem Cells 2015;7:470.
[29] Zarrabi M, Mousavi SH, Abroun S, Sadeghi B. Potential uses for cord blood mesenchymal stem cells. Cell J 2014;15:274.
[30] Secunda R, Vennila R, Mohanashankar A, Rajasundari M, Jeswanth S, Surendran R. Isolation, expansion and characterisation of mesenchymal stem cells from human bone marrow, adipose tissue, umbilical cord blood and matrix: a comparative study. Cytotechnology 2015;67:793–807.
[31] Liu Wh, Song Fq, Ren Ln, et al. The multiple functional roles of mesenchymal stem cells in participating in treating liver diseases. J Cell Mol Med 2015;19:511–20.
[32] Hao T, Chen J, Zhi S, Zhang Q, Chen G, Yu F. Comparison of bone marrow– vs. adipose tissue–derived mesenchymal stem cells for attenuating liver fibrosis. Exp Ther Med 2017;14:5956–64.

[33] Mohamed HE, Elswefy SE, Rashed LA, Younis NN, Shaheen MA, Ghanim AM. Bone marrow–derived mesenchymal stem cells effectively regenerate fibrotic liver in bile duct ligation rat model. Exp Biol Med 2016;241:581–91.
[34] Zhang L, Kang W, Lei Y, et al. Granulocyte colony-stimulating factor treatment ameliorates liver injury and improves survival in rats with d-galactosamine-induced acute liver failure. Toxicol Lett 2011;204:92–9.
[35] Qujeq D, Abassi R, Faeizi F, et al. Effect of granulocyte colony-stimulating factor administration on tissue regeneration due to carbon tetrachloride–induced liver damage in experimental model. Toxicol Ind Health 2013;29:498–503.
[36] Ali G, Mohsin S, Khan M, et al. Nitric oxide augments mesenchymal stem cell ability to repair liver fibrosis. J Transl Med 2012;10:75.
[37] Nasir GA, Mohsin S, Khan M, et al. Mesenchymal stem cells and Interleukin-6 attenuate liver fibrosis in mice. J Transl Med 2013;11:78.
[38] Shams S, Mohsin S, Nasir GA, Khan M, Khan SN. Mesenchymal stem cells pretreated with HGF and FGF4 can reduce liver fibrosis in mice. Stem Cells Int 2015;2015:747245.
[39] Mohsin S, Shams S, Nasir GA, et al. Enhanced hepatic differentiation of mesenchymal stem cells after pretreatment with injured liver tissue. Differentiation 2011;81:42–8.
[40] Mizunaga Y, Terai S, Yamamoto N, et al. Granulocyte colony-stimulating factor and interleukin-1β are important cytokines in repair of the cirrhotic liver after bone marrow cell infusion: comparison of humans and model mice. Cell Transplant 2012;21:2363-75.
[41] Saramipoor BI, Keating A, Peter GR. Concise review: bone marrow autotransplants for liver disease? Stem Cells 2013;31:2313–29.
[42] Taha MF, Hedayati V. Isolation, identification and multipotential differentiation of mouse adipose tissue-derived stem cells. Tissue Cell 2010;42:211–6.
[43] Zhou R, Li Z, He C, et al. Human umbilical cord mesenchymal stem cells and derived hepatocyte-like cells exhibit similar therapeutic effects on an acute liver failure mouse model. PLoS One 2014;9:e104392.
[44] Chai NL, Zhang XB, Chen SW, Fan KX, Linghu EQ. Umbilical cord–derived mesenchymal stem cells alleviate liver fibrosis in rats. World J Gastroenterol 2016;22:6036.
[45] Lihua Y, Yun W, Xiaohua W, Yanqing L. Effect of allogeneic umbilical cord mesenchymal stem cell transplantation in a rat model of hepatic cirrhosis. J Tradit Chin Med 2015;35:63–8.
[46] Di CC, Piscaglia AC, Pierelli L, et al. A human umbilical cord stem cell rescue therapy in a murine model of toxic liver injury. Dig Liver Dis 2004;36:603–13.
[47] Fujino H, Hiramatsu H, Tsuchiya A, et al. Human cord blood CD34+ cells develop into hepatocytes in the livers of NOD/SCID/γcnull mice through cell fusion. FASEB J 2007;21:3499–510.
[48] Newsome PN, Johannessen I, Boyle S, et al. Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. Gastroenterol 2003;124:1891–900.
[49] Kögler G, Sensken S, Airey JA, et al. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 2004;200:123–35.
[50] Esrefoglu M. Role of stem cells in repair of liver injury: experimental and clinical benefit of transferred stem cells on liver failure. World J Gastroenterol 2013;19:6757.
[51] Le VT, Nguyen NH, Do HQ, Le HM, Truong NH. Transplantation of umbilical cord blood-derived mesenchymal stem cells to treat liver cirrhosis in mice: a comparison of tail and portal vein injection. Prog Stem Cell 2017;4:201–16.
[52] Yan C, Xue G, Wu L, Liu J, Hou Y. Differentiation of human umbilical cord mesenchymal stem cells into hepatocytes induced by rat fibrotic liver tissue extracts. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 2015;29:878–83.

[53] Nagaishi K, Ataka K, Echizen E, Arimura Y, Fujimiya M. Mesenchymal stem cell therapy ameliorates diabetic hepatocyte damage in mice by inhibiting infiltration of bone marrow–derived cells. Hepatology 2014;59:1816–29.
[54] Hasan MH, Botros KG, El-Shahat MA, Abdallah HA, Sobh MA. In vitro differentiation of human umbilical cord blood mesenchymal stem cells into functioning hepatocytes. Alexandria J Med 2017;53:167–73.
[55] Kyurkchiev D, Bochev I, Ivanova TE, et al. Secretion of immunoregulatory cytokines by mesenchymal stem cells. World J Stem Cells 2014;6:552.
[56] Madrigal M, Rao KS, Riordan NH. A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. J Transl Med 2014;12:260.
[57] Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006;24:1294–301.
[58] Kharaziha P, Hellström PM, Noorinayer B, et al. Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I–II clinical trial. Eur J Gastroenterol Hepatol 2009;21:1199–205.
[59] Jang YO, Kim YJ, Baik SK, et al. Histological improvement following administration of autologous bone marrow-derived mesenchymal stem cells for alcoholic cirrhosis: a pilot study. Liver Int 2014;34:33–41.
[60] Suk KT, Yoon JH, Kim MY, et al. Transplantation with autologous bone marrow-derived mesenchymal stem cells for alcoholic cirrhosis: phase 2 trial. Hepatology 2016;64:2185–97.
[61] Xu L, Gong Y, Wang B, et al. Randomized trial of autologous bone marrow mesenchymal stem cells transplantation for hepatitis B virus cirrhosis: regulation of Treg/Th17 cells. J Gastroenterol Hepatol 2014;29:1620–8.
[62] Zhang Z, Lin H, Shi M, et al. Human umbilical cord mesenchymal stem cells improve liver function and ascites in decompensated liver cirrhosis patients. J Gastroenterol Hepatol 2012;27:112–20.
[63] Wang L, Li J, Liu H, et al. A pilot study of umbilical cord-derived mesenchymal stem cell transfusion in patients with primary biliary cirrhosis. J Gastroenterol Hepatol 2013;28:85–92.
[64] Shi M, Zhang Z, Xu R, et al. Human mesenchymal stem cell transfusion is safe and improves liver function in acute-on-chronic liver failure patients. Stem Cells Transl Med 2012;1:725–31.
[65] Tsolaki E, Yannaki E. Stem cell-based regenerative opportunities for the liver: state of the art and beyond. World J Gastroenterol 2015;21:12334.
[66] Pan Q, Fouraschen SM, de Ruiter PE, et al. Detection of spontaneous tumorigenic transformation during culture expansion of human mesenchymal stromal cells. Exp Biol Med 2014;239:105–15.
[67] Kantarcıoğlu M, Demirci H, Avcu F, et al. Efficacy of autologous mesenchymal stem cell transplantation in patients with liver cirrhosis. Turk J Gastroenterol 2015;26:244–50.
[68] Mohamadnejad M, Alimoghaddam K, Bagheri M, et al. Randomized placebo-controlled trial of mesenchymal stem cell transplantation in decompensated cirrhosis. Liver Int 2013;33:1490–6.
[69] Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–72.
[70] Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature 2007;448:313.
[71] Bahmad H, Hadadeh O, Chamaa F, et al. Modeling human neurological and neurodegenerative diseases: from induced pluripotent stem cells to neuronal differentiation and its applications in neurotrauma. Front Mol Neurosci 2017;10:50.
[72] Cao L, Tan L, Jiang T, Zhu XC, Yu JT. Induced pluripotent stem cells for disease modeling and drug discovery in neurodegenerative diseases. Mol Neurobiol 2015;52:244–55.

[73] Palakkan AA, Nanda J, Ross JA. Pluripotent stem cells to hepatocytes, the journey so far. Biomed Rep 2017;6:367–73.
[74] Wang B, Jakus AE, Baptista PM, et al. Functional maturation of induced pluripotent stem cell hepatocytes in extracellular matrix – a comparative analysis of bioartificial liver microenvironments. Stem Cells Transl Med 2016;5:1257–67.
[75] Takayama K, Inamura M, Kawabata K, et al. Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4α transduction. Mol Ther 2012;20:127–37.
[76] Chen YF, Tseng CY, Wang HW, Kuo HC, Yang VW, Lee OK. Rapid generation of mature hepatocyte-like cells from human induced pluripotent stem cells by an efficient three-step protocol. Hepatology 2012;55:1193–203.
[77] Asgari S, Moslem M, Bagheri LK, Pournasr B, Miryounesi M, Baharvand H. Differentiation and transplantation of human induced pluripotent stem cell–derived hepatocyte-like cells. Stem Cell Rev 2013;9:493–504.
[78] Espejel S, Roll GR, McLaughlin KJ, et al. Induced pluripotent stem cell–derived hepatocytes have the functional and proliferative capabilities needed for liver regeneration in mice. J Clin Invest 2010;120:3120–6.
[79] Chiang CH, Wu WW, Li HY, et al. Enhanced antioxidant capacity of dental pulp-derived iPSC-differentiated hepatocytes and liver regeneration by injectable HGF-releasing hydrogel in fulminant hepatic failure. Cell Transplant 2015;24:541–59.
[80] Uygun BE, Yarmush ML, Uygun K. Application of whole-organ tissue engineering in hepatology. Nat Rev Gastroenterol Hepatol 2012;9:738.
[81] Takebe T, Sekine K, Enomura M, et al. Vascularized and functional human liver from an iPSC-derived organ bud transplant. Nature 2013;481:4.
[82] Pournasr B, Duncan SA. Modeling inborn errors of hepatic metabolism using induced pluripotent stem cells. Arterioscler Thromb Vasc Biol 2017;37:1994–9.
[83] Garate Z, Davis BR, Quintana-Bustamante O, Segovia JC. New frontier in regenerative medicine: site-specific gene correction in patient-specific induced pluripotent stem cells. Hum Gene Ther 2013;24:571–83.
[84] Tayyeb A, Azam F, Nisar R, Nawaz R, Qaisar U, Ali G. Liver Cirrhosis-Update and Current Challenges. In: Tsoulfas G, ed. Regenerative Medicine in Liver Cirrhosis: Promises and Pitfalls. Liver Cirrhosis – Update and Current Challenges. London: InTech, 2017, 233–56.
[85] Mazza G, Al-Akkad W, Rombouts K, Pinzani M. Liver tissue engineering: From implantable tissue to whole organ engineering. Hepatol Commun 2018;2:131–41.

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8 Patient-specific induced pluripotent stem cells for cardiac disease modeling

Abstract: Reprogramming of human somatic cells to induced pluripotent stem cells (iPSCs) via induction of pluripotency genes is one of the most influential scientific breakthroughs during the last decade. Behind this breakthrough is the capacity of iPSCs to self-renew and differentiate into derivatives of all the three germ layers, similar to human embryonic stem cells. Importantly, iPSCs can be generated using somatic cells from healthy donors or patients retaining the genetic and epigenetic make-up of the donor and can be used for regenerative applications without provoking immune rejection. Given their potential use in basic and translational research, iPSCs have become an attractive cell type to create "disease-on-a-dish" models to investigate disease phenotype *in vitro*, to assess drug response and evaluate cardiac toxicity for drug discovery, and to develop personalized cell therapy for various diseases. Among these diseases, inherited or acquired forms of cardiovascular diseases are the most common reason of mortality worldwide. Cardiac arrhythmias and channelopathies are a distinct group of disorders caused by abnormal ion homeostasis and action potential of cardiomyocytes, accounting for a large subset of hospitalization and sudden cardiac death. Pharmacological, catheter, or medical device implants and surgical approaches have been largely applied in the clinical perspective for symptomatic treatment and to improve the quality of life for patients with arrhythmias and other heart diseases. Recently, stem-cell-based regenerative approaches have been vigorously assessed in clinical trials, and novel stem-cell-based treatments are being evaluated for their potential use to provide lasting recovery. In this book chapter, we focus on the recent progress in the application of iPSC-related research in selected channelopathies and cardiac arrhythmia modeling *in vitro* and their potential application in the clinical perspective.

Key Words: Action potential, Cardiac, Cardiomyocytes, Channelopathy, Electrophysiology, Heart, iPSCs, Models.

8.1 Introduction

Understanding the genetic, molecular, and electrophysiological basis underlying cardiac pathologies is essential for the diagnosis, treatment, and prevention of cardiovascular disease. Nevertheless, it is difficult to obtain patient-derived primary cells for research due to the invasive surgical procedures involved and limited availability of the

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cell source. Hence, murine, guinea pig, and non-human-primate-based experimental animal models have been employed as the most popular and relevant models to study the pathological and molecular mechanisms underlying the cardiac pathologies. Despite the advantages associated with the development of transgenic experimental models in mice due to their considerable genetic similarity with humans, the developmental and physiological differences between the two in terms of heart size, beating rate, and hormonal regulation should be taken into account. Although a variety of transgenic mice carrying mutations in disease-associated genes show disease-like phenotype, for arrhythmias and some other relevant channelopathies, murine models have shown limited disease phenotype. Given these limitations, noninvasive derivation of patient-derived stem cells *in vitro,* human embryonic stem cells (hESCs), and studies in "disease-on-a-dish" models have gained popularity due to ease, convenience, and substantial source availability to study disease mechanisms at the developmental, cellular, and molecular levels.

The first hESC lines were derived and established in 1998, providing a novel approach to investigating cardiac lineage specification and disease process [1]. However, due to the inefficient gene targeting technologies in hESCs, the associated ethical concerns, and the risk of immune rejection in designing embryonic stem cell (ESC)-based therapy, scientists have explored ways to artificially generate surrogate ESCs that share ESC-like properties. The pioneering work of Takahashi and Yamanaka led to the successful reprogramming of adult mouse and human fibroblasts, respectively, to induced pluripotent stem cells (iPSCs) by using lentiviral delivery of the quartet of pluripotency transcription factors including Sox2, Klf4, Oct4, and c-Myc for providing proliferative advantage [2, 3]. Since the publication of these studies, there has been immense progress in the field of somatic cell reprogramming [4]. Subsequently, researchers generated different reprogramming strategies to induce iPSCs from various somatic cells, i.e., skeletal myoblasts, bone marrow cells, neural stem cells, etc. [5–7], that were transduced by variety of transcription factors [5, 8] and by using nonintegrating viral or nonviral vectors, i.e., episomal vectors, small molecules, adenoviruses, synthetic mRNAs, and proteins [9–12]. Human iPSCs (hiPSCs) show close resemblance to hESCs in terms of pluripotency, self-renewal capacity, gene expression profile, and developmental potential. Since iPSCs can be derived from different somatic cell types obtained directly from the patients, the derivative cells carry genetic and epigenetic memory resembling the donor-derived parent cells. Therefore, differentiation of patient-derived iPSCs to mature cell types of the tissues that display abnormal physiology may provide valuable and continuous source of cells for *in vitro* disease models. Consequently, iPSC technology offers opportunities to dissect and understand disease mechanisms, to screen drugs in healthy or patient-derived cell types displaying disease phenotype, and to develop novel treatment strategies.

By mimicking the *in vivo* developmental processes *in vitro*, various mature and functional cell types, including cardiomyocytes (CMs), could be generated from hESCs and iPSCs. Recently, direct reprogramming strategies have been developed for direct conversion of somatic cells to cardiac phenotype by the delivery of specific

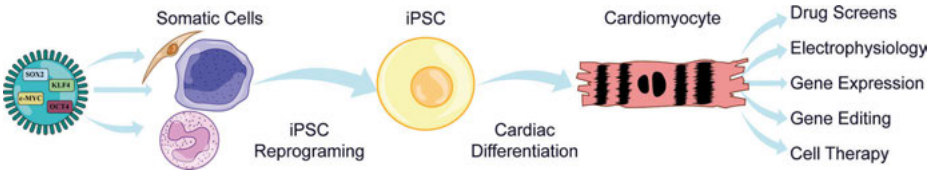

Fig. 8.1.1: Schematic representation of the derivation of cardiomyocytes from iPSCs and their potential applications.

cardiac transcription factors, either alone or in combination with small molecules to enhance the efficiency of reprogramming. Thus, hiPSCs provide an optimal source for derivation of CMs and other cardiac cell types for basic research and translational purposes (Fig. 8.1.1). Conventionally, iPSCs can be differentiated into CMs by different methodologies that either implement embryoid body formation or a monolayer of stem cells to induce cardiac lineage differentiation by manipulating Wingless related integration site (Wnt), Bone morphogenetic protein (BMP), and Hedgehog pathways. Functionally, hiPSC-CMs show strong cellular, molecular, and electrophysiological similarities to human primary CMs by contraction, sarcomeric structures, and action potential (AP) kinetics [13, 14].

Heart diseases may be classified under coronary heart disease, arrhythmia, heart failure, heart valve disease, cardiomyopathy, pericarditis, aortic diseases, etc. Here, we focus on iPSC-derived models of cardiac channelopathies in general and arrhythmias in particular at the structural, morphological, electrophysiological, and molecular levels. Throughout the chapter, iPSC-derived CMs will be abbreviated as iPSC-CMs.

8.2 Methods for electrophysiological analysis

The rhythmicity and strength of cardiac contractions are controlled by the electrical conduction system of the heart, which includes the sinoatrial node (SAN), the atrioventricular node, and His-purkinje fibers. The sympathetic and parasympathetic nerves innervate the cardiac plexus and initiate heart beat by stimulating the SAN. The impulse then propagates through the atrioventricular node, reaching the ventricles, and dissipates *via* His-Purkinje fibers. The electrical activity in the heart is analyzed by electrocardiogram (ECG) *in vivo* (Fig. 8.2.1a).

Atrial and ventricular myocytes have a resting membrane potential around –85 mV. To exceed the threshold required to generate AP, myocardial cells need an impulse from an electrical conduction system [15]. As the impulse reaches the myocardial cells, voltage-gated Na^+ channels (Na_V) open, and a fast positive rise in the resting potential occurs [15, 16]. This rapid and short increase is called fast AP (phase 0, Fig. 8.2.1b). After exceeding the threshold, transient potassium channels allow K^+ flow out of the cell, making the AP to rise to –15 mV (phase 1). Depolarization of the

membrane activates L-type Ca^{2+} channels and slows the delayed rectifier K^+ channels conducting influx of Ca^{2+} and outflux of K^+, respectively. The opposite flow of the positively charged ions creates a plateau in the AP, called phase 2. Inward Ca^{2+} current induces the Ca^{2+} activated channels located in the sarcoplasmic reticulum (SR) to release internal Ca^{2+} stores. During phase 3, rapid rectifier K^+ channels open and conduct K^+ ions into the cell repolarizing the membrane. The Na^+/K^+ pump, sarcolemmal Na^+/Ca^{2+} exchanger, and SR Ca^{2+}/ATPase restore intracellular ion concentrations to the resting state at phase 4. The interval between the onset of the resting point and the end-point of the AP is named as action potential duration (APD), which is a critical evaluation criteria for rhythm disorders *in vitro* (Fig. 8.2.1b) [16].

Pacemaker cells at the SAN produce spontaneous APs and regulate the heart beat. Different from atrial and ventricular CMs, pacemaker cells produce AP at only three stages, i.e., phase 0, 3, and 4. Pacemaker cells have around –60 mV resting membrane potential at phase 4. Hyperpolarization activated cyclic nucleotide-gated channels located along the pacemaker cell membrane open in response to the hyperpolarization of the previous AP and regulate Na^+/K^+ ion concentrations in the nodal CMs. The Na^+/K^+ discharge slowly depolarizes the pacemaker membrane potential, reaching –40 mV, resulting in voltage-gated Ca^{2+} channels (Ca_v) to open and the membrane potential rising up to –10 mV (phase 0). Then, voltage-gated Ca^{2+} channels close and voltage-gated K^+ channels (K_v) open (phase 3), enabling the membrane to return to the resting stage.

The electrical activity in the heart is recorded and analyzed *in vivo* by ECG and *in vitro* by several tools such as multielectrode array (MEA), impedance and field potential (FP) recorders, patch clamp, and voltage-sensitive or Ca^{2+} sensitive dyes. These tools are briefly described here with their relevance to cardiac disease phenotype characterization.

8.2.1 Electrocardiogram

ECG is a technique used for monitoring and diagnosing heart disease in the clinical practice. The electrical signals of the heart are recorded from the skin *via* surface electrodes during ECG [15]. The signals obtained from various leads in ECG characteristically show P-, QRS-, and T-waves (Fig. 8.2.1a). The P-wave represents atrial depolarization, the QRS complex represents ventricular depolarization during systole, and the lasting T-wave indicates ventricular repolarization at diastole [15]. Normal QT interval, which is defined as the time between the onset of QRS-complex and the end point of T-wave, is below 440 ms for men and below 460 ms for women in healthy individuals. The PQRST wave complex is measured to diagnose several cardiac abnormalities from myocardial infarcts to arrhythmias. In particular, the QT interval is measured, and prolongation of the QT interval above 500 ms in ECG signifies a risk of long QT syndrome (LQTS) (Fig. 8.2.1a).

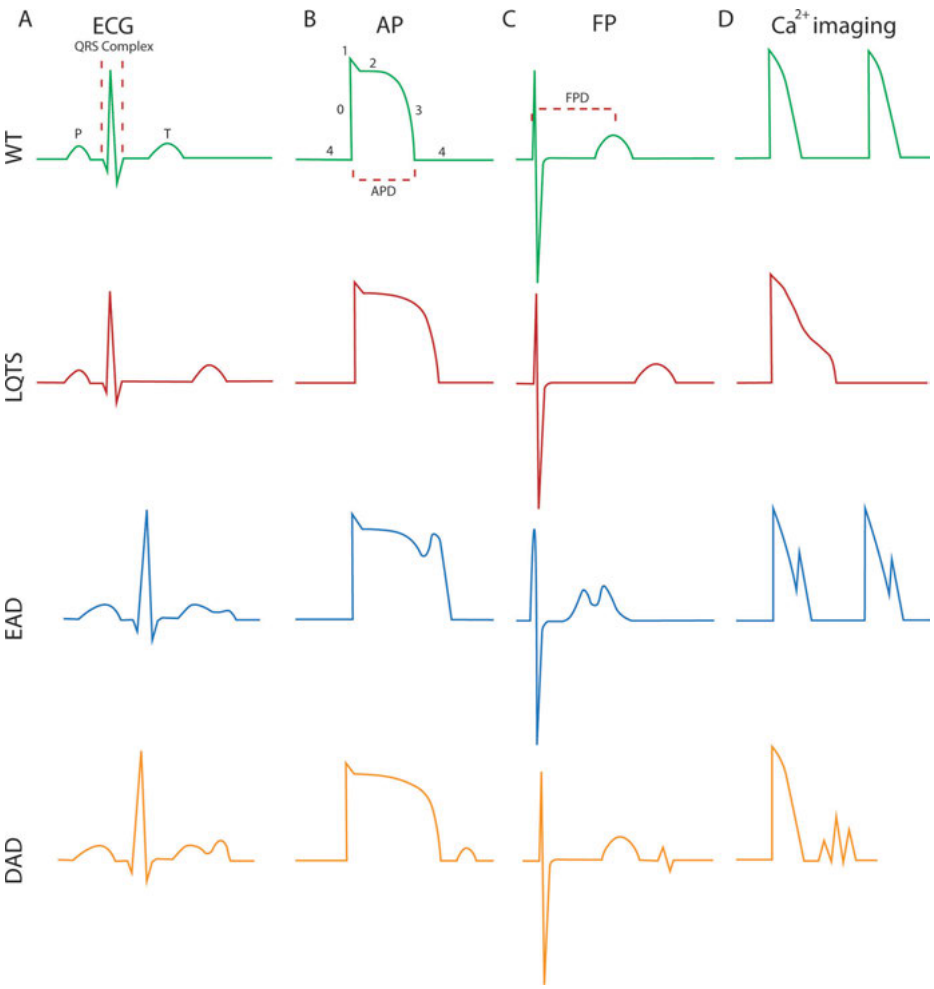

Fig. 8.2.1: Schematic illustration of (a) QRS complex from the heart recorded by ECG, (b) representative action potential, (c) FP, and (d) calcium transients in the cardiac cells. WT= wild type, LQTS= long QT syndrome, EAD= early afterdepolarization, DAD= delayed afterdepolarization.

8.2.2 Patch clamp analysis

Patch clamp electrophysiology is a technique for measuring voltage and current changes on the cell membrane due to ion channel activity in a single cell (Fig. 8.2.1b). Various CM subtypes originated from the ventricle, atrium, and SAN, and the conduction system can be defined by electrical characteristics recorded by using the patch clamp technique. For instance, ventricular-like CMs have a higher AP amplitude, while the AP of nodal CMs have a higher frequency compared to other CM subtypes [17]. In addition to examining voltage changes on the cell membrane,

channel behavior, conductance to ions, or response to drugs can be explored with patch clamp analysis. In case of channelopathy chracterization, the patch clamp method could reveal an abnormality in conductance with the associated ion channel current and voltage ratio that would otherwise be challenging or impossible to study in depth (Fig. 8.2.1b).

8.2.3 Multielectrode array (MEA)

The electrophysiological characteristics of CMs can be measured by MEA platforms *in vitro* [18]. In the MEA system, field potential (FP) produced by CM clusters, that are positioned over metal or conductive electrodes can be recorded (Fig. 8.2.1c). The interval between the start and the end-point of FP is defined as FP duration (FPD). Similar to the electrophysiological pattern observed in ECG, CMs carrying ion channel mutations could show changes in the duration and/or the amplitude of FP, as well as emergence of early afterdepolarization (EAD) or delayed afterdepolarization (DAD) [18]. Hence, the MEA system could be used to monitor CM electrical activity carrying the LQTS disease phenotype, which distinctively demonstrates longer FPD compared to healthy ones in the MEA platform (Fig. 8.2.1c).

8.2.4 Impedance and field potential (FP) recorders

Recording devices that enable simultaneous examination of both CM contractility by impedance and extracellular potential by FP measurements are becoming widely used for characterization of cardiac electrophysiology. Cardio xCELLigence ExtraCellular Recording (ECR) or CardioExcyte96 are such tools to record cardiac FP (Fig. 8.2.1c) [19, 20]. These systems work similar to MEA but with some functional divergences. First, the main difference between the two techniques is that these hybrid systems are integrated with impedance electrodes that detect cell attachment and mechanical changes that are observed during contraction of the CMs. Generally, 32 or 64 electrodes are located over the MEA surface, whereas xCELLigence ECR has two FP electrodes to capture cardiac electrical activity. The electrophysiological features of cardiac arrhythmias could be evaluated by beating irregularity, beating rate, and FP parameters in patient-derived iPSC-CMs under physiological conditions or in response to drug candidates [19, 20].

8.2.5 Voltage-sensitive and Ca^{2+}-sensitive dyes

Commercially available voltage-sensitive dyes are preferred to detect changes in the membrane potential of electrically active CMs. Bis-(1, 3-Dibutylbarbituric Acid) Trimethine Oxonol ($DiBAC_4(3)$) and aminonaphthylethenylpyridinium (ANEP) dyes reside on

the cell membrane and show differing fluorescence intensities in response to voltage changes in the membrane potential. On the other hand, intracellular Ca^{2+} transients in CMs can be detected by using Ca^{2+}-sensitive dyes. Rhod-2-, Fura-2-, Fluo-4-, and similar Ca^{2+}-sensitive ratiometric dyes show altered fluorescence intensity in response to an increase or a decrease in intracellular Ca^{2+}. Besides the synthetic dyes, genetically encoded Ca^{2+} indicators such Genetically Ca^{2+} Modified Protein (GCaMP) GCaMP2, GCaMP3, GCaMP6, and GCaMP8 are also used commonly to monitor Ca^{2+} changes in the cell (Fig. 8.2.1d). The genetically encoded indicators constitute a fusion product of green fluorescence protein, calmodulin (CaM), and myosin light chain kinase peptide M13, which can be used in living organisms for Ca^{2+} relevant cardiac studies [21].

8.3 Channelopathies

Channelopathies are a heterogeneous group of disorders that are caused by inherited genetic changes in the ion channel coding genes. Channelopathies generally show clinically abnormal physiological symptoms in the nervous system, cardiovascular system, endocrine system, urinary system, and/or immune system. As described earlier, ion channel activity is responsible for generating APs in the cardiac muscle cells. Any genetic abnormality affecting the ion channels may have life-threatening consequences in terms of arrhythmias and sudden cardiac death that accounts for approximately 1 million deaths worldwide every year. Various genetic mutations in the ion channel encoding genes could result in the loss-of-function or gain-of-function of channel activity. The genetic mutations associated with ion channels and the mutation hot spots have been largely identified by complex genome analysis. With the recent advancements in the genome analysis techniques, genetic testing has turned out to be a routine diagnostic tool in the clinic to identify cardiac channelopathies such as LQTS, short QT syndrome, catecholaminergic polymorphic ventricular tachycardia (CPVT), and Brugada syndrome (BrS) [15]. To this end, generation of patient-specific and disease-specific iPSC-derived cellular models and platforms with genetically inherited forms of cardiac arrhythmias serve as valuable tools to model these diseases *in vitro*, study the molecular mechanisms underlying the pathogenesis of the disease process, elucidate new drug targets, accelerate drug screening, and pave the way for regenerative therapies as part of the personalized medicine (Tab. 8.3.1).

8.3.1 Long QT Syndrome Type-1 (LQTS-1)

LQTS has 15 clinical subtypes, most of which stem from monogenic mutations. LQTS-1 accounts for 40–50% of all the reported LQTS cases in the clinic and is genetically inherited by mutations in the voltage-gated K^+ channel gene encoding KCNQ1 protein (Fig. 8.3.1a). To date, more than 300 missense mutations have been reported in *KCNQ1* gene,

Tab. 8.3.1: Patient-specific iPSC models of selected channelopathies and a summary of their genetic, elelctrophysiological, and pharmacological *in vitro* analysis.

Subtypes	Genes (synonyms)	Mutations	Electrophysiological analysis	Pharmacological test	References
LQTS-1	KCNQ1 LQT, RWS, WRS, LQT1, SQT2, ATFB1, ATFB3, JLNS1, KCNA8, KCNA9, $K_v1.9$, $K_v7.1$, K_vLQT1	p.R190Q/c.569G>A	– Prolonged APD – I_{Ks} channel dysfunction – Prolonged FPD – I_{Kr} blockade	Chromanol 293B, E4031, isoproterenol, adrenaline	Moretti 2010
		1893delC (P631fs/33)		E4031, chromanol 293B, barium, isoproterenol, propranolol	Egashira 2012
		p.G269S/c.805G>A		Nifedipine, pinacidil	Wang 2014
		p.G589D/c.1766G>A		Isoprenaline, cisapride monohydrate, erythromycin, sotalol hydrochloride, quinidine and E4031	Kuusela 2016b
		p.G589D		Bisoprolol, JNJ303, ML277	Kuusela 2016a
		G589D, ivs7-2A>G		E4031, JNJ303	Kiviaho 2015
		Exon 7 Deletion		ML277	Ma 2015
LQTS-2	KCNH2, ERG1, HERG, LQT2, SQT1, ERG-1, H-ERG, HERG1, $K_v11.1$	A614V	– Prolonged APD and FPD – EAD – Higher sensitivity to arrhythmogenic drugs	E4031, cisapride, nifedipine, ranolazine	Itzhaki 2011
		R176W/c.526C>T		Isoprenaline, cisapride monohydrate, erythromycin, sotalol hydrochloride, quinidine and E4031	Kuusela 2016b
		p.A561T/c.G1681A		Isoprenaline, nadolol, propranolol, E4031, nicorandil, PD-118057	Matsa 2011
		R176W		E4031, sotalol, erythromycin, cisapride	Lahti 2012
		N996I/c.A2987T		E4031, JNJ303, nifedipine	Bellin 2013
		p.A422T/c.1264G>A		Nifedipine, TTX, caffeine, E4031, cisapride	Spencer 2014
		c.G1681A		Nicorandil; PD-118057, E4031	Matsa 2014
		p.A561V/c.1682C>T		ALLN, E4031, nimodilpine	Mehta 2014
		p.A561P/c.1681G>C		Nifedipine, E4031	Jouni 2015
		N996I/c.A2987T		Astemizole; tritiumlabelled defetilide, LUF7346, NS164, rottlerin	Sala, L. 2016
		IVS9-28A/G		ALLN	Mura 2017
		K897T		TTX, E4031, chromanol 293B, mexiletine	Terrenoire 2012

Tab. 8.3.1 (continued)

Subtypes	Genes (synonyms)	Mutations	Electrophysiological analysis	Pharmacological test	References
LQTS-3	SCN5A, HB1, HB2, HH1, IVF, VF1, HBBD, ICCD, LQT3, SSS1, CDCD2, CMD1E, CMPD2, PFHB1, Nav1.5	p.N406K/c.1218C>A	– Na^+ channel defect – Prolonged APD	Nifedipine, tetradotoxin, caffeine, E-4031, cisapride, LiCl, cyclopiazonic acid	Spencer 2014
		p.V240M/c.718G>A, p.R535Q/c.1604G>A		Not tested	Fatima 2013
		p.V1763M/c.5287G>A		Mexiletine, TTX	Ma 2013
		p.R1664H/c.4991G>A		Mexiletine, ranolazine, and phenytoin	Malan 2016
		p.F1473C		TTX, E4031, chromanol 293B, mexiletine	Terrenoire 2012
Brugada syndrome	SCN5A, HB1, HB2, HH1, IVF, VF1, HBBD, ICCD, LQT3, SSS1, CDCD2, CMD1E, CMPD2, PFHB1, Nav1.5	179insD/+, overlap syndrome	– Loss of function in Na^+ channel mutation	TTX	David 2012
		179insD/+		Not tested	Veerman 2016
		R620H, R811H, Δ1397		Not tested	Liang 2016
LQTS-8 Timothy syndrome (TS)	CACNA1C, TS, LQT8, CACH2, CACN2, CaV1.2, CCHL1A1, CACNL1A1	p.G406R/c.1216G>A	– Prolonged myocardial AP – Abnormalities in Ca^{2+} exchange – Ca^{2+} overload – T wave variance – Increased incidence of DADs	PHA-793887, DRF053	Song 2017
		p.G406R/c.1216G>A		Roscovitine (Ros)	Yazawa 2011
LQTS-14	CALM1, CaM, CAMI, PHKD, CPVT4, DD132, LQT14, CALML2	p.F142L/c.426C>G	– Loss of ICaL inactivation – Abnormal APD	Verapamil, amlodipine	Rocchetti 2017

Tab. 8.3.1 (continued)

Subtypes	Genes (synonyms)	Mutations	Electrophysiological analysis	Pharmacological test	References
LQTS-15	CALM2, CaM, PHKD, CAMII, LQT15, PHKD2	p.D130G/c.389A>G	– Prolongation of the APD – Disruption of Ca^{2+}/CaM-dependent inactivation of L-type Ca^{2+} channels	Not tested	Limpitikul 2017
CVPT	RYR2, RyR, ARVC2, ARVD2, VTSIP	p.D307H/c.919G>C	– Elevated diastolic Ca^{2+} concentrations – Abnormal Ca^{2+} release – DAD and EAD	Isoproterenol	Novak 2012
		p.L3741P/c.11222T>C		Flecainide, Isoproterenol, Nadolol	Preininger 2016
		p.P2328S/c.6982C>T		Adrenaline	Kujala 2012
		p.S406L/c.1217C>T		Dantrolone	Jung 2012
		p.M4109R/c.12056T>G		Forskolin, isoproterenol, flecainide, thapsigargin	Itzhaki 2012
		p.I4587V/c.13759A>G		S107, isoproterenol	Sasaki 2016
		p.F2483I/c.7447T>A		Caffeine, forskolin	Zhang 2013, Fatima 2011
		p.E2311D/c.6933G>T		KN-93, isoproterenol, nadolol	Di Pasquale 2013

iPSC= induced pluripotent stem cells; LQTS= long QT syndrome; APD= action potential duration; FPD= field potential duration; EAD= early afterdepolarization; CMs= cardiomyocytes; DAD= delayed after depolarization; JNJ303= 2-(4-chlorophenoxy)-2-methyl-N-[5 [(methylsulfonyl)amino]tricyclo [3.3.1.13,7]dec-2-yl]-propanamide; ML277= R)-N-(4-(4-methoxyphenyl)thiazol-2-yl)-1-tosylpiperidine-2-carboxamide; TTX= tetrodotoxin; PD-118057= 2-[[4-[2-(3,4-Dichlorophenyl) ethyl]phenyl]amino]benzoic acid; ALLN= (N-[N-(N-acetyl-L-leucyl)-L-leucyl]-L-norleucine); LUF7346= 2-[4-(2-Bromo-benzoyl)-phenoxy]-N-pyridin-3-yl-acetamide; LiCI= lithium chloride; KN-93= N-[2-[[[3-(4-Chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulphonamide; S107= semicarbazide hydrochloride; PHA-793887= 3-Methyl-N-[1,4,5,6-tetrahydro-6,6-dimethyl-5-[(1-methyl-4-piperidinyl)carbonyl]pyrrolo[3,4-c]pyrazol-3-yl]butanamide; DRF053= 2-[[9-(1-Methylethyl)-6-[[3-(2-pyridinyl)phenyl]amino]- 9H-purin-2-yl]amino]-1-butanol hydrochloride hydrate.

resulting in an LQTS-1 phenotype that follows a dominant inheritance pattern. Among the various relevant K^+ channel genes, *KCNQ1* encodes for six transmembrane helixes and acts as a slow rectifier channel (K_s) (Fig. 8.3.1a). Four of the KCNQ1 proteins form a complex with two KCNE1 and yotiao proteins and assemble together to form the K_s channel on the plasma membrane. The KCNQ1 protein forms the pore acting as a selective filter for K^+ ions. Moreover, the KCNQ1 protein also has an intracellular carboxyl terminus associated with scaffolding, assembly, and trafficking proteins as well as signaling molecules. In the channel complex, the KCNE1 protein is responsible for slow-activating kinetics of K_s channel by modulating the opening of the K_s channel (Fig. 8.3.1a). Yotiao protein, on the other hand, regulates K_s channel activity by anchoring to phosphatidylinositol 4,5-biphosphate (PIP_2) and protein kinase A at the intracellular domain. While PIP_2 stabilizes the open state of the channel, protein kinase A phosphorylates the channel, thus changing its electrical charge and increasing APD. KCNQ1 activity is responsible for CM repolarization (Fig. 8.2.1b). As the channel conducts K^+ ions outside of the cell, Ca^{2+} channels open in response to Ca^{2+} influx. Thus, the channel plays a role not only during the resting phase but also during the plateau phase of AP. Consequently, dysregulation of K_s may lead to APD and QT interval prolongation in LQTS-1 patients [15].

The first report on an iPSC model derived from LQTS-1 patients was conducted by Moretti and colleagues [22]. In this study, patient-specific iPSC-CMs carrying a dominant missense mutation at R190Q in the *KCNQ1* gene showed longer APD and a slower repolarization velocity as compared to the iPSC-CMs of healthy individuals (Tab. 8.3.1). Electrophysiological characterization of patient-derived iPSC-CMs was conducted by administration of the specific inhibitors of I_{Ks} and I_{Kr}, chromanol 293B and E4031, respectively. Chromanol 293B specifically affected only the iPSC-CMs derived from healthy individuals and did not affect the iPSC-CMs derived from LQTS-1 patients. These data provide functional evidence that LQTS-1 derived CMs possessed dysfunctional I_{Ks}. To further characterize the LQTS-1 phenotype *in vitro*, administration of a common catecholamine and beta-adrenergic agonist, isoproterenol or adrenalin, in CM cultures resulted in an increase in the arrhythmic events and EAD, respectively. Similar to clinical practice, administration of a nonselective beta-blocker, propranolol, to patient-derived CMs attenuated catecholamine-induced arrhythmias and ameliorated the disease phenotype. Immunolabeling of KCNQ1 receptor in the LQTS-1 patient-specific iPSC-CMs illustrated that this mutation did not alter protein expression but abrogated its cellular trafficking from endoplasmic reticulum to the cell membrane *via* a dominant-negative effect. Through this report, the LQTS-1 patient-specific iPSC-CMs were shown to represent disease phenotype in a petri dish [22].

To curtail the differences in the genetic background to the minimum, isogenic iPSC lines were generated with *KCNQ1* gene mutations using genome-editing technology [23]. Using zinc finger nucleases, iPSC lines with LQTS-1 phenotype were created by overexpression of the *KCNQ1* gene carrying dominant negative G269S mutation. Comparison of the genome edited and patient-derived iPSC-CMs showed that both

have similar electrophysiological characteristics such as prolonged APD or EAD. These abnormal electrophysiological phenotypes could be ameliorated by using Ca^{2+} channel blocker, nifedipine, or pinacidil, a K_{ATP} channel opener, resulting in shortened APD in LQTS-1 derived CMs.

A novel heterozygous deletion mutation (c.922~1,032 del; p.308~344 del) at exon 7, which encodes a major part of the S6 transmembrane-spanning segment and the loop connecting S5 and S6 of the KCNQ1 protein (Fig. 8.3.1a), was reported to cause LQTS-1 [24]. In this study, immunocytochemical analysis of LQTS-1 derived iPSC-CMs revealed that the truncated KCNQ1 protein was localized in the perinuclear region instead of the perimembrane location, indicating haplo-insufficiency and trafficking defects. This genetic abnormality leads to reduced I_{Ks}, thus resulting in prolonged APD in LQTS-1 iPSC-derived CMs. Administration of I_{Ks} activator ML277 to the LQTS-1 derived iPSC-CM cultures resulted in an increase in amplitude and ion conduction, suggestive of partial restoration of the disease phenotype [24].

In several studies, the iPSC-CMs derived from LQTS-1 patients have been used to screen novel drug candidate molecules or to investigate the mechanism of approved drugs whose effect on channelopathy not yet evaluated. In a similar study, iPSC-CMs were derived from clinically symptomatic and asymptomatic LQTS-1 patients carrying the G589D mutation in the *KCNQ1* gene [25, 26]. The electrophysiological properties of wild-type and patient-derived iPSC-CMs were studied by using the MEA system. Detrended fluctuation analysis was also used to investigate electrophysiological characteristics of cardiomyocytes at baseline and their responses to various drugs, including beta-blockers (bisoprolol and sotalol), IKs blocker (JNJ303), and IKs activator (ML277). At baseline, CMs modeling LQTS-1 showed significant prolongation of FPD without any difference in their beating rate as compared to healthy-donor-derived CMs. Moreover, both healthy-donor- and patient-derived CMs showed similar phenotypes after drug administration. Healthy-donor-derived CMs demonstrated higher sensitivity to JNJ303, indicating that the I_{Ks} effect was diminished in the LQTS-1 derived CMs due to missense mutations. On the other hand, ML277 shortened FPD in all CM types without any significant electrophysiological changes after treatment with bisoprolol. Detrended fluctuation analysis revealed acute beta-blocker effect on both healthy and LQTS-1 derived CMs [25].

Mechanical beating behavior of the iPSC-CMs was also analyzed to reveal the effect of *KCNQ1* mutations. In one of the first reports related to contraction analysis, mechanical beating behavior and disease phenotypes were correlated using iPSC-CMs carrying *KCNQ1* G589D (trafficking mutation) or ivs7-2ANG mutation (loss-of-function mutation) [27]. Patch clamp recordings illustrated prolonged duration in both mutations and EAD in only G589D mutation. In addition, imaging of a Ca^{2+} transient fluorescence indicator demonstrated an arrhythmic pattern in the mutant CMs. Moreover, iPSC-CMs carrying G589D mutation showed prolonged contractions, whereas iPSC-CMs with ivs7-2ANG mutation had abnormally extended relaxation period. This study showed that mechanical analysis of the CMs derived from LQTS patients or healthy individuals correlated directly with their genetic background given the

Ca^{2+} transients and electrophysiological findings. In another study, a novel mutation at 1893delC (e631fs/33) in the *KCNQ1* gene causing LQTS-1 was characterized [28]. Treatment of iPSC-CMs with the K^+ channel blocker E4031 induced prolongation in FPD of both control and LQTS CMs, whereas EAD was observed specifically in the LQTS CMs, similar to the clinical phenotype. On the same note, chromanol 293B administration caused FPD prolongation in the control CMs but not in the LQTS CMs, thus indicating that mutation in the *KCNQ1* gene caused K_s channel dysfunction. Electrophysiological data further revealed that in the event of functional deficiency of K_s, K_r channels substituted the K_s channels in LQTS-1 patient CMs. For further validation, immunocytochemistry analysis of 1893delC mutation in *KCNQ1* showed a dominant-negative effect *via* trafficking deficiency. Moreover, isoproterenol-induced arrhythmogenic phenotype in LQTS-1 CMs was reversed by propranolol [28].

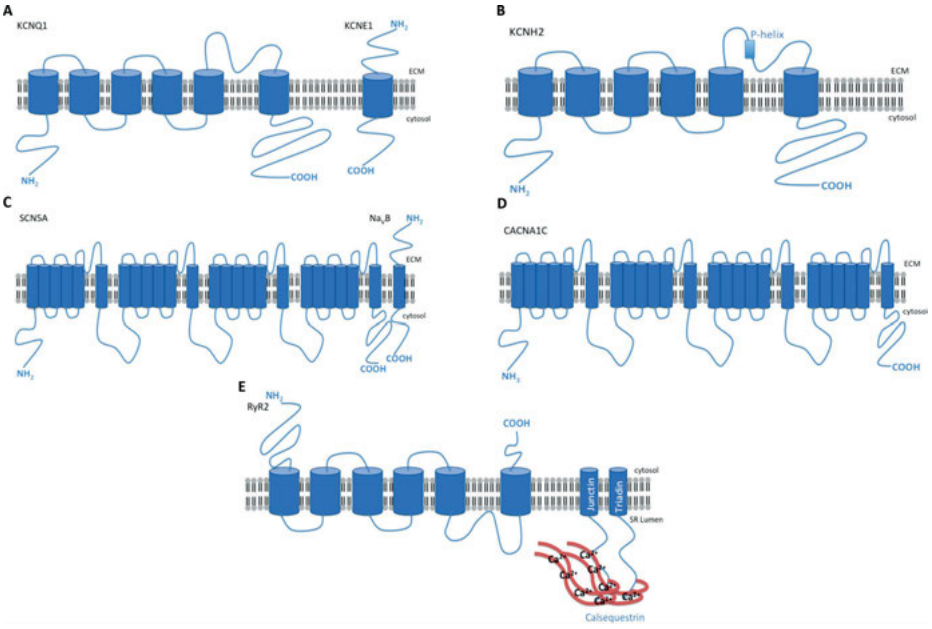

Fig. 8.3.1: Schematic illustration of (a) KCNQ1 and KCNE1, (b) KCNH2, (c) SCN5A, (d) CACNA1C, and (e) RyR2 and Calsequestrin channels. ECM= extracellular matrix.

8.3.2 LQTS-2

Malfunctioning of rapid rectifier potassium channel (K_r) expressed by *KCNH2* gene causes LQTS-2. The human *ether-a-go-go*-related gene (hERG, also known as *KCNH2*) encodes for $K_v11.1$, which constitutes the alpha subunit of the K^+ channel (Fig. 8.3.1b), playing a significant role in the plateau (phase 2) and repolarization (phase 3) of CMs

(Fig. 8.2.1b). K_r is called rapid rectifier since its inactivation and recovery from inactivation occur more rapidly than its opening and closing states. KCNH2 is the most widely studied K^+ channel due to its significant role in cardiac function and off-target effects on this channel associated with various pharmacological agents.

The first study illustrating the LQTS-2 phenotype in the iPSC-CM system was derived from patients carrying A614V missense mutation in the *KCNH2* gene and was reported to represent a clinically relevant electrophysiological phenotype *in vitro* [29] (Tab. 8.3.1). Since this mutation site was associated with K_r channel pore formation, iPSC-CMs with LQTS-2 showed prolonged APD and EAD as compared to the control cells from healthy individuals. After recapitulating LQTS-2 phenotype in a culture platform, the cells were treated with I_{Kr} channel blockers: E4031 or cisapride. The pharmacological treatment with either drug resulted in an increased APD and emergence of EAD. Since EADs are thought to result from late membrane-inward currents, treatment with nifedipine, an L-type Ca^{2+} blocker, abrogated APD and eliminated EAD. Another novel drug tested in this study was ranolazine, a known Na^+ channel blocker, which eliminated EAD without any change in APD [29].

In an interesting study, patient-derived iPSC-CMs were generated from a female symptomatic patient with G1681A mutation in the *KCNH2* gene and her mother carrying the same mutation without a clinical phenotype in ECG as an asymptomatic control [30]. It was observed that the CMs derived from asymptomatic LQTS-2 patients had no abnormality *in vitro* in line with the clinical examination. From MEA and patch clamp recordings, only patch clamping revealed APD prolongation in iPSC-CMs derived from symptomatic patients. These data implied that the patch clamp technique was more sensitive in the detection of electrophysiological differences as compared to the MEA system. Treatment of iPSC-CMs with isoprenaline, which is generally used to pharmacologically treat LQTS patients in the clinic, alleviated APD in CMs from both patient-derived iPSCs, while EAD was observed only in the symptomatic LQTS-2 derived CMs. Treatment of LQTS-2 derived iPSC-CMs with nadolol and propranolol ameliorated EAD symptoms. Importantly, administration of E4031 induced prolongation of APD in all iPSC-CMs cultures; however, EAD changes were observed only in the CMs derived from LQTS-2 patients. Furthermore, treatment of the cells with K^+-channel enhancers, including nicorandil (I_{KATP} channel opener) and PD-118057 (K^+-channel activator), reversed the effects of E4031 on patient-derived CMs [30].

Bellin *et al.* (2013) highlighted the importance of the use of isogenic cell lines to eliminate the limitations of individual variability among independent controls [31]. They used iPSC-CMs with the *KCNH2* gene mutated at N996I, c.A2987T T/A, and the corresponding A/T correction was carried out by gene editing with homologous recombination technique. MEA analysis showed that E4031 prolonged APD in the mutated cells as compared to the control healthy cells. Immunocytochemical analysis revealed that proteasomes have a role in the degradation of N996I hERG protein, causing a KCNH2 trafficking defect. These findings demonstrated that the N996I hERG protein is haplo-insufficient rather than being a dominant negative form [31].

Several LQTS-2 disease modeling studies have been conducted by using patient-derived iPSC-CMs to compare CMs derived from symptomatic and asymptomatic patients (Tab. 8.3.1). In one report, iPSCs derived from asymptomatic LQTS-2 patients were differentiated into CMs to explore whether the same medical treatment for symptomatic patients could be used for asymptomatic patients [32]. In contrast to the clinical phenotype, iPSC-CMs from asymptomatic LQTS-2 patients carrying R176W mutation in the *KCNH2* gene showed prolonged APD at the baseline relative to the control CMs. The magnitude and density of I_{Kr} in LQTS-2 CMs decreased after administration of E4031, suggesting that a patient carrying LQTS-2 mutation without severe symptoms could exhibit arrhythmogenic characteristics *in vitro* [26]. In another study, membrane potential and intracellular Ca^{2+} currents of iPSC-CMs derived from patients inheriting A422T mutation in the *KCNH2* gene or N406K mutation in the *SCN5A* gene were evaluated [33]. The data from this study showed a significant correlation between Ca^{2+} changes in the microcluster and single cell electrical activity independent of LQTS types. Therefore, the authors proposed a common Ca^{2+}-dependent arrhythmogenic mechanism in both LQTS-2 and LQTS-3 [33]. Moreover, N-[N-(N-acetyl-L-leucyl)-L-leucyl]-L-norleucine) (ALLN), a pharmacological agent with protease inhibitory properties, was conducted to ameliorate LQTS-2 phenotype [34]. The iPSC-CMs used in this study carried A561V missense mutation in the *KCNH2* gene, resulting in reduced membrane localization of glycosylated and mature hERG protein. ALLN is an inhibitor of calpain and proteasomes and successfully corrected the hERG trafficking defect.

RNA interference technology has also been employed in iPSC-CMs carrying c.G1681A mutation in the *KCNH2* gene to improve the LQTS-2 disease phenotype [35]. This mutation was localized in the I_{Kr} ion-channel pore regions and impaired glycosylation and channel transport to the plasma membrane. While the mutated KCNH2 mRNA was allele-specifically targeted and knocked down, the wild-type mRNA expression remained unaffected. Subsequent to adrenergic stimulation or K^+ blockage, the mutant allele targeted iPSC-CMs showed normal APD without EAD as compared to iPSC-CMs with LQTS-2 mutated allele. In addition to genetic manipulation, treatment of corrected iPSC-CMs with K^+ channel regulator nicorandil or PD-118057 shortened APD. This study illustrated that knockdown of mutated KCNH2 mRNA would attenuate dominant-negative effect and partially correct LQTS-2 phenotype [35].

In addition to blood and skin biopsy samples, urine has also been used as an alternative noninvasive source to generate iPSCs lines [36]. Patient urine derived iPSC-CMs demonstrated that A561P mutation in hERG protein causes a hERG trafficking defect. The urine-derived iPSC-CMs showed LQTS-2 phenotype, including prolonged APD and drug-induced abnormal electrophysiology similar to blood- or fibroblast-derived iPSC-CMs [36].

Sala and colleagues (2016) used LUF7346, a hERG allosteric modulator to rescue not only the congenital (N996I, c.A2987T) but also the Astemizole or ^3H-labeled defetilide (hERG blockers) drug-induced LQTS-2. MEA and patch clamp analysis revealed that LUF7346 ameliorated the pathological phenotype of the congenital as well as

drug-induced LQTS-2 in a dose-dependent manner by slowing I_{Kr} closing. In this study, LUF7346 was found to be a more effective hERG activator than NS1643 and rottlerin [37]. A recent study has shown that a novel mutation at *KCNH2*-IVS9-28A/G caused a significant reduction in the full-length ERG1a while upregulating the ERG_{1aUSO} isoform [38]. This shift in selective expression of ERG_{1aUSO} higher than ERG1a expression may result in impairment of hERG trafficking, causing prolongation in FPD. Moreover, the KCNH2 trafficking defect was reversed by using ALLN, thus making the cell regain the electrical properties akin to the wild-type CMs and reducing perinuclear accumulation while increasing membrane localization of KCNH2 protein [38]. Jones and colleagues (2014) were the first to report the significance and function of hERG-1b in cardiac function. In human CMs, hERG is composed of both hERG-1a and hERG-1b. Knockdown of hERG-1a expression using short hairpin RNA or exogenous expression of PAS domain in the iPSC-CMs resulted in significant reduction in hERG-1b levels, lowering I_{Kr} amplitude, prolongation of APD, and appearance of EAD [39].

8.3.3 LQTS-3 (Gain-of-Function of Na^+ Channel)

Mutations in the *SCN5A* gene expressing the alpha subunit of $Na_v1.5$ have been associated with LQTS-3 [15]. $Na_v1.5$ has four homologous asymmetric domains on the plasma membrane. Each domain has six helixes, in which S1 to S4 are responsible for voltage sensing and S5 and S6 form the pore (Fig. 8.3.1c). The isoleucine, phenylalaline, and methionine motif located between the third and the fourth domains plays a significant role in fast inactivation; hence, many mutations in this region impair $Na_v1.5$ channel inactivation. In addition, carboxylic terminal of $Na_v1.5$ channel, where most mutations occur, stabilizes the inactivation state.

The voltage-gated $Na_v1.5$ channels open at –50 mV membrane potential and permits the diffusion of Na^+ into the cytoplasm. The following rapid increase in Na^+ ion concentration in the cytoplasm activates Ca^{2+} and K^+ channels, resulting in cardiac muscle contraction. Various gain-of-function mutations in the *SCN5A* gene result in the LQTS-3 phenotype in the clinic. Based on pathophysiological differences, the clinical phenotype of LQTS-3 is distinct from the previously described LQTS-1 and LQTS-2. While sudden cardiac arrest can occur in LQTS-3 patients during resting state, exercise- and adrenergic-stimulus-triggered fatal arrhythmias are often observed in LQTS-1 and LQTS-2 patients [15].

iPSC-CMs derived from LQTS-3 patients carrying mutations at two different amino acids, V240M and R535Q, in the *SCN5A* gene showed longer APD duration compared to the healthy control CMs [40] (Tab. 8.3.1). These iPSC-CMs with LQTS-3 phenotype showed low Na^+ current density and slow inactivation tendency, resulting in persistent Na^+ current. In another study, iPSC-CMs were derived from a patient carrying both F1473C mutation in the *SCN5A* gene and a polymorphism K897T in the *KCNH2*

gene. The iPSC-CMs showed LQTS-3 phenotype without any effects from *KCNH2* polymorphism. Moreover, administration of mexiletine inhibited the Na^+ rapid recovery inactivation of iPSC-CMs derived from this LQTS-3 patients [41].

iPSC-CM derived from LQTS-3 patients carrying R1664H mutation at the *SCN5A* gene illustrated faster Na^+ recovery from inactivation and EAD compared to healthy control cell lines [42]. The authors used several clinically relevant Na^+ channel blockers, i.e., mexiletine, ranolazine, and phenytoin, to ameliorate LQTS-3 electrophysiological phenotype. MEA and patch clamp analysis showed that treatment with Na^+ channel blockers abolished EAD and reduced APD [42]. In another study, iPSC-CMs derived from an LQTS-3 patient carrying V1763M at the *SCN5A* gene and the patient's healthy sibling showed similar levels of the *SCN5A* gene expression as well as similar $Na_v1.5$ cellular distribution. Further characterization showed that the proportion of mutant SCN5A allele expression was higher and fast recovery from inactivation was also observed in the patient-specific iPSC-CMs. Treatment with mexiletine of iPSC-CMs derived from LQTS-3 patients shortened the open state of the Na^+ channel and thus reduced the late Na^+ current and shortened APD, consistent with its clinical use in pharmacological treatment of LQTS-3 patients [43]. In addition, the effect of lidocaine, a class I antiarrythmogenic drug, was evaluated on LQTS-3 derived iPSC-CMs, and it was found to inhibit Na^+ currents *in vitro*, implying a potentially therapeutic effect on LQTS-3 phenotype [44].

8.3.4 Brugada syndrome

BrS has an autosomal dominant inheritance patterns characterized by the loss of function of Na^+ channels, resulting in ventricular fibrillations and sudden cardiac death. BrS is diagnosed by a negative T-wave and ST-segment elevation in ECG recordings. Although the majority of mutations leading to BrS is associated with *SCN5A*, several genes, i.e., *SCN1B*, *SCN3B*, *CACNA1c*, *CACNB2b*, *KCNE3*, *KCNE5*, and *KCND3*, have been implicated in BrS pathology (Fig. 8.3.1). Class I antiarrhythmic drugs, such as Flecainide, procainamide, disopyramide, ajmaline, propafenone, and pilsicainide, have been used to pharmacologically treat BrS symptoms in the clinic as well as in *in vitro* BrS disease models [45].

The first iPSC models of BrS were derived from three unrelated BrS patients whose genetic analysis did not show a mutation in any BrS associated gene (Tab. 8.3.1) [46]. In this study, iPSC-CMs derived from BrS patients showed similar Na^+ channel characteristics compared to the CMs generated from iPSCs of healthy individuals. In order to understand whether other mechanisms were involved in the BrS phenotype in these patients, BrS-related channels such as L-type Ca^{2+} channels and K^+ channels were analyzed using patch clamp technique. Electrophysiological analysis of cation channels showed normal activity in the CMs derived from BrS patients. Based on these findings, fibrosis in the right ventricle was hypothesized to decrease

CM coupling, and unknown epigenetic factors may play roles in BrS pathology. Additionally, the immature nature of hiPSC-CMs was speculated to potentially mask the BrS electrophysiological abnormalities *in vitro* [46].

BrS and LQTS-3 are considered to be associated with *SCN5A* gene mutations, and a distinct subclass of disorder termed as the overlap syndrome may show symptoms of both BrS and LQTS-3. It was reported that 179insD/+ mutation in the *SCN5A* gene caused the overlap syndrome [47]. Human- and mouse-derived iPSCs and ESC lines carrying 179insD/+ mutation in the *SCN5A* gene showed persistent I_{Na}, reduced upstroke velocity, and a prolonged APD, while channel opening and inactivation kinetics remained unaffected [47]. Moreover, a recent study has investigated BrS using patient-specific iPSC-CMs derived from two patients at the single cell level [48]. The patients had two different Na^+ gated channel alpha subunit variants showing a reduction in Na^+ current density and AP upstroke velocity. While one patient had two missense mutations at R620H and R811H in the *SCN5A* gene, the other patient had a frame-shift mutation in the *SCNA5* gene, leading to a nonfunctional truncated form. Both BrS patient-derived iPSC-CMs showed abnormal Ca^{2+} transients, variations in beating interval, and triggered activity in comparison with healthy controls. CRISPR/Cas9-mediated genome correction of the BrS-associated genetic variant in iPSC-CMs rescued the abnormal Ca^{2+} transients and channel activity observed in the patient CMs. Interestingly, iPSC-CMs from BrS patients showed downregulation of not only the SCN5A gene expression but also of the KCNB3 related to $K_V4.3$ and outward K^+-channel and the KCNJ2 associated with $K_V2.1$ and inward K^+ current. This study signifies the role of CRISPR/Cas9 gene editing approach to ameliorate the BrS phenotype similar to the healthy-donor-derived iPSC-CM phenotype and possible compensatory mechanisms in ion channel expression and activity in the diseased state [48].

8.3.5 Timothy syndrome (LQTS-8)

Timothy syndrome (TS), also known as LQTS-8, is a rare genetic disorder due to gain-of-function mutations in the L-type cardiac voltage-gated Ca^{2+} channel (CACNA1C, $Ca_V1.2$) encoding gene (Fig. 8.3.1d). Among the different LQTS subtypes, TS patients show the longest prolongation of the QT interval in the clinical examination. The CACNA1C channels regulate Ca^{2+} influx into the cytoplasm, and the resultant elevated intracellular Ca^{2+} in turn triggers the inactivation of Ca^{2+} channels in the CMs. These ionic changes lead to membrane depolarization via voltage-dependent inactivation and Ca^{2+}/CaM-dependent inactivation mechanisms [49]. Mutations in *CACNA1C* may cause abnormalities in Ca^{2+} exchange, prolonged APD, calcium overload, T-wave variance, polymorphic ventricular tachycardias, and an increased incidence of DADs. The first case of TS reported heart defects and syndactyly [50]. The best characterized mutation at *CACNA1C* is a *de novo* missense mutation at the position G406R

(Tab. 8.3.1) [51]. Most children affected by this mutation die early, around 2–3 years of age. G406R *CACNA1C* mutation causes a continuous open state of the channel, an increase in Ca^{2+} influx, and an impaired voltage-dependent inactivation, collectively resulting in myocardial APD prolongation [51]. In another report, a rare variant of TS was genetically associated with two point mutations at the G406R and G402S positions in the *CACNA1C* gene. The patients carrying these two mutations showed more severe cardiac defects, with longer QT intervals compared to single missense mutation carriers [52, 53].

Verapamil and other L-type Ca^{2+} channel blocker analogs are useful in pharmacological treatment of frequent ventricular tachyarrhythmias observed in TS patients with mutations in the *CACNA1C* gene at G402S [54]. In this regard, ECG analysis of TS patients revealed an effective reduction in tachyarrhythmias after administration of verapamil, possibly by reducing the Ca^{2+} influx and shortening the QT interval [54]. The more complex form of TS associated with A1473G mutation was shown to result in the long QT interval, cortical blindness, and stroke [52]. Consistent with clinical case studies, iPSC-CMs generated from patients carrying G406R mutation demonstrated abnormal Ca^{2+} exchange and prolonged APD, which could be reversed by treatment with roscovitine, a compound that increases the VDI of CACNA1C [55]. In later studies, cell cycle regulators and other roscovitine analogs have been examined on iPSC-CMs derived from TS patients for their therapeutic potential based on mechanisms that have yet to be revealed [56].

8.3.6 LQTS-14

CaM is a Ca^{2+}-dependent regulatory protein that is encoded by *CALM1*, *CALM2*, and *CALM3* genes. CaM activity is essential for homeostasis of the heart function, immune system, and nervous system. Mutations in the *CALM1* gene have been recently associated with LQTS-14 [57]. In this study, hiPSC-CMs derived from patients carrying F142L mutation in the *CALM1* gene were shown to prolong CM repolarization, which could be recovered to normal phenotype by isoproterenol treatment (Tab. 8.3.1) [57]. Moreover, in CMs carrying the mutant *CALM1* gene, Ca^{2+}/CaM-dependent inactivation of I_{CaL} was severely impaired, resulting in elevated and extended inward current potentially associated with the prolonged CM repolarization. Interestingly, Ca^{2+} channel blocker verapamil and amlodipine treatment abolished mutation-induced electrical abnormality and impaired Ca^{2+}/CaM-dependent inactivation observed in LQTS-14 derived iPSC-CMs.

8.3.7 LQTS-15

In a recent report, patient-specific iPSC-CMs carrying the D130G mutation in the *CALM2* gene was associated with a newly defined subtype of LQTS-15 [58].

Electrophysiological analysis of iPSC-CMs derived from D130G-*CALM2* patients showed prolonged APD and Ca^{2+}/CaM-dependent inactivation of L-type Ca^{2+} channels (Tab. 8.3.1). By using CRISPR/Cas9 genome interference strategy, the mutated *CALM2* gene was silenced through allele-specific suppression. Suppressed mutant *CALM2* gene expression functionally rescued the consequent effect of the mutation by normalizing the APD and recovering the Ca^{2+}/CaM-dependent inactivation [58].

8.4 Catecholaminergic polymorphic ventricular tachycardia

CPVT is a form of genetic arrhythmogenic disorder that could be triggered by adrenergic stimulus increasing heart rate due to physical activity and emotional or metabolic stress. CPVT has been categorized into two groups, CPVT-1 and CPVT-2. CPVT-1 has an autosomal dominant inheritance and has a higher incidence than CPVT-2 does in the population. CPVT-1 is associated with mutations in the Ca^{2+} channel ryanodine receptor 2 (*RyR2*) gene, causing an abnormal flux of Ca^{2+} from the SR to the cytosol during muscle relaxation [59]. RyR2 mutations exert their effect as hyperactive channels, producing gain-of-function phenotype [60, 61], or hypoactive channels, resulting in loss-of-function phenotype [62, 63], resulting in abnormal channel sensitivity to Ca^{2+}.

CPVT-2 has an autosomal recessive inheritance pattern and is associated with the cardiac calsequestrin 2 (*CASQ2*) and triadin (*TRDN*) gene mutations (Fig. 8.3.1e) [64]. CASQ2 is a Ca^{2+} binding protein that has high capacity but low affinity to Ca^{2+} in the SR [65, 66]. RyR2, triadin, junctin, and CASQ2 form a complex at the SR perimembrane region (Fig. 8.3.1e); thus, mutations in triadin or cytoplasmic CaM could affect RyR2 activity in the SR. Clinical investigation by ECG analysis implied that CASQ2-mutation-induced DADs trigger arrhythmias in CPVT patients [67, 68]. It is pertinent to mention that both the *RYR2* and *CASQ2* genes encode for the proteins that are responsible for the maintenance of normal heartbeat under physiological conditions. In almost 25% of CPVT patients, the commonly administered beta-blockers for therapy are inefficient [69, 70]; therefore, iPSC-CMs derived from CPVT patients provide a valuable tool to investigate CPVT mechanism and more effective drug targets for therapy.

In one of the first *in vitro* disease models of CPVT-1, iPSCs were generated from patients carrying M4109R heterozygous mutation in the *RyR2* gene (Tab. 8.3.1) [71]. Electrophysiological analysis of iPSC-CMs derived from CPVT-1 patients by whole-cell patch clamp and MEA recordings showed DADs in patient CMs compared to the hiPSC-CMs derived from healthy individuals. To mimic the emergence of arrhythmias in CPVT patients during exercise or emotional stress, iPSC-CMs derived from CPVT patients were stimulated with forskolin, a known direct activator of adenylate cyclase, and isoproterenol [71]. Both forskolin and isoproterenol increased the frequency of DADs in *in vitro* disease models [71]. In order to investigate further the

CPVT disease mechanism, an SR Ca^{2+} ATPase pump inhibitor, thapsigargin, was used to deplete the intracellular Ca^{2+} stores that decreased the DAD incidences [71]. Moreover, administration of the Na^{+} channel blocker flecainide to iPSC-CMs derived from CPVT patients completely abolished DADs, implying flecainide as an effective secondary agent for CPVT treatment [71]. These findings collectively indicate the important balance between Na^{+} and Ca^{2+} during cardiac muscle contraction and relaxation preserved by various ion pumps and channels on the plasma membrane and SR membrane.

Another CPVT-1 iPSC-CM model was established from patients inheriting S406L missense mutation in the *RyR2* gene [72]. Treatment of CPVT-1-specific iPSC-CMs with dantrolene, a muscle relaxant that binds to the N-terminal of the cardiac RyR channels, showed relaxation of the cardiac cells and restored rhythmic behavior by abolishing DADs [72]. Moreover, in hiPSC-CMs generated from a CPVT patient with F2483I mutation in the *RyR2* gene, forskolin treatment abolished intracellular Ca^{2+} release by elevating cytosolic cAMP levels similar to previous studies [73]. Further Ca^{2+} current and Na^{+}/Ca^{2+} exchanger current studies of *RyR2* F2483I mutant iPSC-CMs showed higher I_{Ca}-gated Ca^{2+} release and sensitized adrenergic regulation correlating with the clinical phenotype of patients [74]. *In vitro* modeling CPVT-1 associated with *RyR2* gene mutation P2328S not only showed similar electrophysiological phenotype but also showed EADs similar to the clinical features [75].

In CPVT-1 hiPSC lines generated from I4587V mutation in the *RyR2* gene, mRNA expression analysis revealed increased production of two Ca^{2+} homeostasis regulators, inositol 1,4,5 triphosphate receptor-2 and calreticulin, and decreased expression of CASQ2 in patient-derived iPSC-CMs compared to adult human CMs, indicating immature Ca^{2+} handling in patient cells. Treatment of RyR2-deficient iPSC-CMs with S107, which stabilizes the closed state of the RyR2 receptor, significantly decreased DADs. These findings highlight the therapeutic potential of S107 and similar RyR2 stabilizer CPVT-1 treatment [76].

A CPVT-1 model created from a 12-year-old individual inheriting a missense gene mutation at L3741P in *RyR2* responded to a beta-blocker nadolol and flecainide [77]. Flecainide treatment was shown to decrease ventricular arrhythmias in the CPVT patient following the *in vitro* characterization. This study emphasizes the use of patient-specific iPSC-CMs for custom tailoring drugs in personalized medicine [77].

Various CPVT-2 disease models have been established by hiPSC-CMs (Tab. 8.3.1). In one such study, delivery of the wild-type *CASQ2* gene using adeno-associated virus vectors to iPSC-CMs derived from CPVT-2 patients was shown to be effective in restoring the normal CASQ2 function in cardiac cells and abolished the disease-associated DADs [78]. In another report, iPSC-CMs derived from CPVT-2 patients carrying the E2311D mutation in the *RyR2* gene were obtained and the arrhythmic phenotype was induced by isoproterenol treatment [79]. Incubation of iPSC-CMs derived from CPVT-2 patients with an antiarrhythmic drug, KN-93, reduced DADs by inhibiting Ca^{2+}/CaM-dependent serine-threonine protein kinase II [79]. These two studies underline both

gene therapy and pharmacological approaches to be effective in ameliorating CPVT-2 phenotype *in vitro*.

In-depth electron microsocopy analysis of hiPSC-CMs derived from patients carrying missense mutation D307H in the *CASQ2* gene revealed unorganized sarcomeric structure reminiscent of immature fetal-like CM morphology [80]. In the same study, isoproterenol stimulation induced DADs were associated with a diastolic intracellular Ca^{2+} increase in CASQ2 mutation-dependent CPVT phenotype.

8.5 Conclusion

The emerging progress in iPSC technology offers opportunities to dissect the underlying molecular mechanisms propagating the cardiovascular disease process and the efficient screening of drug libraries to characterize novel drug candidates and to develop modern treatment strategies. IPSCs also provide a continuous and replenishable source of cells to establish disease-on-a-dish models to investigate channelopathy disease phenotype *in vitro* to appraise drug response and evaluate the cardiac toxicity of novel compounds during drug discovery and development. Moreover, the strategy permits personalized cell therapy options for various cardiovascular pathologies. Patient-derived iPSC models are valuable tools to investigate molecular characterization of cardiac arrhythmias, are fast and reliable screens for drugs, electrophysiologically mimic disease phenotype *in vitro*, and can be examined by using various techniques such as patch clamp, MEA, and Ca^{2+} imaging. Through reprogramming technologies in combination with CRISPR/Cas9 genome editing and allele-specific suppression tools, the generation of isogenic cell lines became possible in order to eliminate the limitations of individual variability among independent controls and to minimize the differences in the genetic background. Although the various selected channelopathies discussed here have a mostly monogenic basis associated with a single ion channel mutation, recent reports drew attention to the intricate balance between different ions in CMs, indicating a necessity for high-throughput drug screening strategies for various ion channel targets to explore therapeutic intervention. In one such example, effective functional recovery of the CPVT phenotype, that is, primarily emerging from Ca^{2+} channel mutations, could be achieved by treatment with flecainide to block accompanying Na^{+} channels. These findings collectively indicate the important balance of ion homeostasis in healthy and diseased CMs. In-depth molecular, electrophysiological, and pharmacological evaluation of arrhythmia-associated phenotype *in vitro* became feasible with hiPSC disease-on-a-dish models, which would otherwise be challenging or even impossible.

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8.6 References

[1] Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282(5391):1145–7.
[2] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126(4):663–76.
[3] Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131(5):861–72.
[4] Ibrahim AY, Mehdi Q, Abbas AO, Alashkar A, Haider HKh. Induced pluripotent stem cells: next generation cells for tissue regeneration. J Biomed Sci Eng (JBiSE) 2016;9(4):226–44.
[5] Kim JB, Zaehres H, Wu G, et al. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature 2008;454(7204):646–50.
[6] Ahmed RPH, Haider HK, Buccini S, Li L, Jiang S, Ashraf M. Reprogramming of skeletal myoblasts for induction of pluripotency for tumor-free cardiomyogenesis in the infarcted heart. Cir Res 2011;9(1):60–70.
[7] Buccini S, Haider KH, Ahmed RPH, Jiang S, Ashraf M. Cardiac progenitors derived from reprogrammed mesenchymal stem cells contribute to angiomyogenic repair of the infarcted heart. Basic Res Cardiol 2012;107(6):301–310.
[8] Nemajerova A, Kim SY, Petrenko O, Moll UM. Two-factor reprogramming of somatic cells to pluripotent stem cells reveals partial functional redundancy of Sox2 and Klf4. Cell Death Differ 2012;19(8):1268–76.
[9] Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. Science 2008;322(5903):949–53.
[10] Fontes A, Macarthur CC, Lieu PT, Vemuri MC. Generation of human-induced pluripotent stem cells (hiPSCs) using episomal vectors on defined Essential 8™ Medium conditions. Methods Mol Biol Clifton NJ 2013;997:57–72.
[11] Hu K, Slukvin I. Generation of transgene-free iPSC lines from human normal and neoplastic blood cells using episomal vectors. Methods Mol Biol Clifton NJ 2013;997:163–76.
[12] Meraviglia V, Zanon A, Lavdas AA, et al. Generation of induced pluripotent stem cells from frozen buffy coats using non-integrating episomal plasmids. J Vis Exp JoVE 2015;(100): e52885.
[13] Yang X, Pabon L, Murry CE. Engineering adolescence: maturation of human pluripotent stem cell-derived cardiomyocytes. Circ Res 2014;114(3):511–23.
[14] Mummery CL, Zhang J, Ng ES, et al. Differentiation of human ES and iPS cells to cardiomyocytes: a methods overview. Circ Res 2012;111(3):344–58.
[15] Bohnen MS, Peng G, Robey SH, Terrenoire C, Iyer V, Sampson KJ, Kass RS. Molecular pathophysiology of congenital long QT syndrome. Physiol Rev 2017;97(1):89–134.
[16] Nattel S, Carlsson L. Innovative approaches to anti-arrhythmic drug therapy. Nature Rev Drug Discov 2006;5(12):1034–49.
[17] Schram G, Pourrier M, Melnyk P, Nattel S. Differential distribution of cardiac ion channel expression as a basis for regional specialization in electrical function. Circ Res 2002;90(9):939–50.
[18] Spira E M, Hai A. Multi-electrode array technologies for neuroscience and cardiology. Nat Nanotechnol 2013;8(2):83–94.

[19] Xi B, Wang T, Li N, et al. Functional cardiotoxicity profiling and screening using the xCELLigence RTCA Cardio System. J Lab Autom 2011,16(6):415–21.
[20] Doerr L, Thomas U, Guinot DR, et al. New easy-to-use hybrid system for extracellular potential and impedance recordings. J Lab Autom 2015;20(2):175–88.
[21] Chen TW, Wardill TJ, Sun Y. Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 2013;499(7458):295–300.
[22] Moretti A, Bellin, M, Welling A, et al. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. N Engl J Med 2010;363(15):1397–409.
[23] Wang Y, Liang P, Lan F, et al. Genome editing of isogenic human induced pluripotent stem cells recapitulates long QT phenotype for drug testing. J Am Coll Cardiol 2014;64(5):451–9.
[24] Ma D, Wei H, Lu J, et al. Characterization of a novel KCNQ1 mutation for type 1 long QT syndrome and assessment of the therapeutic potential of a novel IKs activator using patient-specific induced pluripotent stem cell-derived cardiomyocytes. Stem Cell Res Ther 2015;19;6:39.
[25] Kuusela J, Kim J, Rasanen E, Aalto-Setala K. The effects of pharmacological compounds on beat rate variations in human long QT-syndrome cardiomyocytes. Stem Cell Rev 2016a;12(6):698–707.
[26] Kuusela J, Kujala VJ, Kiviaho A, et al. Effects of cardioactive drugs on human induced pluripotent stem cell derived long QT syndrome cardiomyocytes. Springerplus 2016b;5:234.
[27] Kiviaho AL, Ahola A, Larsson K, et al. Distinct electrophysiological and mechanical beating phenotypes of long QT syndrome type 1-specific cardiomyocytes carrying different mutations. Int J Cardiol Heart Vasc 2015;8:19–31.
[28] Egashira T, Yuasa S, Suzuki T, et al. Disease characterization using LQTS-specific induced pluripotent stem cells. Cardiovasc Res 2012;95(4):419–29.
[29] Itzhaki I, Maizels L, Huber I, Zwi-Dantsis L, et al. Modelling the long QT syndrome with induced pluripotent stem cells. Nature 2011;471(7337):225–9.
[30] Matsa E, Rajamohan D, Dick E, et al. Drug evaluation in cardiomyocytes derived from human induced pluripotent stem cells carrying a long QT syndrome type 2 mutation. Eur Heart J 201;32(8):952–62.
[31] Bellini M, Casini S, Davis RP, et al. Isogenic human pluripotent stem cell pairs reveal the role of a KCNH2 mutation in long-QT syndrome. EMBO J 2013;32(24):3161–75.
[32] Lathi AL, Kujala VJ, Chapman H, et al. Model for long QT syndrome type 2 using human iPS cells demonstrates arrhythmogenic characteristics in cell culture. Dis Model Mech 2012;5(2):220–30.
[33] Spencer CI, Baba S, Nakamura K, et al. Calcium transients closely reflect prolonged action potentials in iPSC models of inherited cardiac arrhythmia. Stem Cell Rep 2014;3(2):269–81.
[34] Mehta A, Sequiera GL, Ramachandra CJ, Re-trafficking of hERG reverses long QT syndrome 2 phenotype in human iPS-derived cardiomyocytes. Cardiovasc Res 2014;102(3):497–506.
[35] Matsa E, Dixon JE, Medway C, et al. Allele-specific RNA interference rescues the long-QT syndrome phenotype in human-induced pluripotency stem cell cardiomyocytes. Eur Heart J 2014;35(16):1078–87.
[36] Jouni M, Si-Tayeb K, Es-Salah-Lamoureux Z, et al. Toward personalized medicine: using cardiomyocytes differentiated from urine-derived pluripotent stem cells to recapitulate electrophysiological characteristics of type 2 long QT syndrome. J Am Heart Assoc 2015;4(9):e002159.
[37] Sala L, Yu Z, Ward-van Oostwaard D, van Veldhoven JP, et al. A new hERG allosteric modulator rescues genetic and drug-induced long-QT syndrome phenotypes in cardiomyocytes from isogenic pairs of patient induced pluripotent stem cells. EMBO Mol Med 2016;8(9):1065–81.
[38] Mura M, Mehta A, Ramachandra CJ, et al. The KCNH2-IVS9-28A/G mutation causes aberrant isoform expression and hERG trafficking defect in cardiomyocytes derived from patients affected by long QT syndrome type 2. Int J Cardiol 2017;240:367–71.

[39] Jones DK, Liu F, Vaidyanathan R, Eckhardt LL, Trudeau MC, Robertson GA. hERG 1b is critical for human cardiac repolarization. Proc Natl Acad Sci USA 2014;111(50):18073–7.
[40] Fatima A, Kaifeng S, Dittmann S, et al. The disease-specific phenotype in cardiomyocytes derived from induced pluripotent stem cells of two long QT syndrome type 3 patients. PLoS One 2013;8(12):e83005.
[41] Terrenoire C, Wang K, Tung KW, et al. Induced pluripotent stem cells used to reveal drug actions in a long QT syndrome family with complex genetics. J Gen Physiol 2013;141(1):61–72.
[42] Malan D, Zhang M, Stallmeyer B, et al. Human iPS cell model of type 3 long QT syndrome recapitulates drug-based phenotype correction. Basic Res Cardiol 2016;111(2):14.
[43] Ma D, Wei H, Zhao Y, et al. Modeling type 3 long QT syndrome with cardiomyocytes derived from patient-specific induced pluripotent stem cells. Int J Cardiol 2013;168(6): 5277–86.
[44] Moreau A, Mercier A, Theriault O, et al. Biophysical, molecular, and pharmacological characterization of voltage-dependent sodium channels from induced pluripotent stem cell-derived cardiomyocytes. Can J Cardiol 2017;33(2):269–78.
[45] Berne P, Brugada J. Brugada syndrome spectrum and prevalence of mutations involving BrS1- through BrS12-susceptibility genes in a cohort of unrelated patients referred for Brugada syndrome genetic testing – implications for genetic testing. Circ J 2012;76(7): 1563–71.
[46] Veerman CC, Mengarelli I, Guan K, et al. hiPSC-derived cardiomyocytes from Brugada syndrome patients without identified mutations do not exhibit clear cellular electrophysiological abnormalities. Sci Rep 2016;6:30967.
[47] Davis RP, Casini S, van den Berg CW, et al. Cardiomyocytes derived from pluripotent stem cells recapitulate electrophysiological characteristics of an overlap syndrome of cardiac sodium channel disease. Circulation 2012;125(25):3079–91.
[48] Liang P, Sallam K, Wu H, Li Y, et al. Patient-specific and genome-edited induced pluripotent stem cell-derived cardiomyocytes elucidate single-cell phenotype of Brugada syndrome. J Am Coll Cardiol 2016;68(19):2086–96.
[49] Morotti S, Grandi E, Summa A, Ginsburg KS, Bers DM. Theoretical study of L-type Ca^{2+} current inactivation kinetics during action potential repolarization and early afterdepolarizations. J Physiol 2012;590(18):4465–81.
[50] Reichenbach H, Meister EM, Theile H. The Heart hand syndrome: a new cariante with conduction disturbances on the heart and syndactyls inexhaustible ossuary changes on hands and feet. Kinderarztl Prax.1992;60:54–56.
[51] Splawski I, Timothy KW, Sharpe LM, et al. $Ca_v1.2$ calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. Cell 2004;119(1):19–31.
[52] Gillis J, Burashnikov E, Antzelevitch C, et al. Long QT, syndactyly, joint contractures, stroke and novel CACNA1C mutation: expanding the spectrum of Timothy syndrome. Am J Med Genet A 2012;158A(1):182–7.
[53] Splawski I, Timothy KW, Decher N, et al. Severe arrhythmia disorder caused by cardiac L-type calcium channel mutations. Proc Natl Acad Sci USA 2005;102(23):8089–96.
[54] Jacobs A, Knight BP, McDonald KT, et al. Verapamil decreases ventricular tachyarrhythmias in a patient with Timothy syndrome (LQT8). Heart Rhythm 2006;3(8):967–70.
[55] Yazawa M, Hsueh B, Jia X, et al. Using induced pluripotent stem cells to investigate cardiac phenotypes in Timothy syndrome. Nature 2011;471(7337):230–4.
[56] Song L, Park SE, Isseroff Y, et al. Inhibition of CDK5 alleviates the cardiac phenotypes in Timothy syndrome. Stem Cell reports 2017;9(1):50–7.
[57] Rocchetti M, Sala L, Dreizehnter L, et al. Elucidating arrhythmogenic mechanisms of long-QT syndrome CALM1-F142L mutation in patient-specific induced pluripotent stem cell-derived cardiomyocytes. Cardiovasc Res 2017;113(5):531–41.

[58] Limpitikul WB, Dick IE, Tester DJ, et al. A precision medicine approach to the rescue of function on malignant calmodulinopathic long-QT syndrome. Circ Res 2017;120(1):39–48.
[59] Priori SG, Napolitano C, Tiso N, et al. Mutations in the cardiac ryanodine receptor gene (hRyR2) underlie catecholaminergic polymorphic ventricular tachycardia. Circulation 2011;103(2):196–200.
[60] Jiang MT, Lokuta AJ, Farrell EF, et al. Abnormal Ca^{2+} release, but normal ryanodine receptors, in canine and human heart failure. Circ Res 2002;91(11):1015–22.
[61] Jiang D, Wang R, Xiao B, et al. Enhanced store overload-induced Ca^{2+} release and channel sensitivity to luminal Ca^{2+} activation are common defects of RyR2 mutations linked to ventricular tachycardia and sudden death. Circ Res 2005;97(11):1173–81.
[62] Jiang D, Chen W, Wang R, et al. Loss of luminal Ca^{2+} activation in the cardiac ryanodine receptor is associated with ventricular fibrillation and sudden death. Proc Natl Acad Sci USA 2007;104(46):18309–14.
[63] Zhao Y-T, Valdivia CR, Gurrola GB, Hernández JJ, Valdivia HH. Arrhythmogenic mechanisms in ryanodine receptor channelopathies. Sci China Life Sci 2015;58(1):54.
[64] Lahat H, Pras E, Olender T, et al. A missense mutation in a highly conserved region of CASQ2 is associated with autosomal recessive catecholamine-induced polymorphic ventricular tachycardia in Bedouin families from Israel. Am J Hum Genet 2001;69(6):1378–84.
[65] Kontula K, Laitinen PJ, Lehtonen A, Toivonen L, Viitasalo M, Swan H. Catecholaminergic polymorphic ventricular tachycardia: recent mechanistic insights. Cardiovasc Res 2005;67(3):379–87.
[66] Liu HN, Ohya S, Wang J, et al. Involvement of ryanodine receptors in pacemaker Ca^{2+} oscillation in murine gastric ICC. Biochem Biophys Res Commun 2005;328(2):640–6.
[67] Priori SG, Chen SR. Inherited dysfunction of sarcoplasmic reticulum Ca^{2+} handling and arrhythmogenesis. Circ Res 2011;108(7):871–83.
[68] Paavola J, Viitasalo M, Pä ivi JL, et al. Mutant ryanodine receptors in catecholaminergic polymorphic ventricular tachycardia generate delayed after depolarizations due to increased propensity to Ca^{2+} waves. Eur Heart J 2007;28(9):1135–42.
[69] Roston TM, Vinocur JM, Maginot KR, et al. Catecholaminergic polymorphic ventricular tachycardia in children analysis of therapeutic strategies and outcomes from an international multicenter registry. Circ Arrhythm Electrophysiol 2015;8:633–42.
[70] Smith GL, MacQuaide N. The direct actions of flecainide on the human cardiac ryanodine receptor keeping open the debate on the mechanism of action of local anesthetics in CPVT. Circulation Res 2015;116:1284–6.
[71] Itzhaki I, Maizels L, Huber I, et al. Modeling of catecholaminergic polymorphic ventricular tachycardia with patient-specific human-induced pluripotent stem cells. J Am Coll Cardiol 2012;60(11):990–1000.
[72] Jung CB, Moretti A, Mederos Y, et al. Dantrolene rescues arrhythmogenic RYR2 defect in a patient-specific stem cell model of catecholaminergic polymorphic ventricular tachycardia. EMBO Mol Med 2012;4(3):180–91.
[73] Fatima A, Xu G, Shao K, et al. In vitro modeling of ryanodine receptor 2 dysfunction using human induced pluripotent stem cells. Cell Physiol Biochem 2011;28(4):579–92.
[74] Zhang XH, Haviland S, Wei H, et al. Ca^{2+} signaling in human induced pluripotent stem cell-derived cardiomyocytes (iPS-CM) from normal and catecholaminergic polymorphic ventricular tachycardia (CPVT)-afflicted subjects. Cell Calcium 2013;54(2):57–70.
[75] Kujala K, Paavola J, Lathi A, et al. Cell model of catecholaminergic polymorphic ventricular tachycardia reveals early and delayed after depolarizations. PLoS One 2012;7(9):e44660.
[76] Sasaki K, Makiyama T, Yoshida Y, et al. Patient-specific human induced pluripotent stem cell model assessed with electrical pacing validates S107 as a potential therapeutic agent for catecholaminergic polymorphic ventricular tachycardia. PLoS One 2016;11(10):e0164795.

[77] Preininger MK, Jha R, Joshua T A human pluripotent stem cell model of catecholaminergic polymorphic ventricular tachycardia recapitulates patient-specific drug responses. Dis Models Mechanisms 2016;9(9):927–39.
[78] Lodola F, Morone D, Denegri M, et al. Adeno-associated virus-mediated CASQ2 delivery rescues phenotypic alterations in a patient-specific model of recessive catecholaminergic polymorphic ventricular tachycardia. Cell Death Dis 2016;7(10):e2393.
[79] Di Pasquale E, Lodola F, Miragoli M, et al. CaMKII inhibition rectifies arrhythmic phenotype in a patient-specific model of catecholaminergic polymorphic ventricular tachycardia. Cell Death Dis 2013;4:e843.
[80] Novak A, Barad Lili, Zeevi-Levin N, et al. Cardiomyocytes generated from CPVTD307H patients are arrhythmogenic in response to beta-adrenergic stimulation. J Cell Mol Med 2012;16(3):468–482.

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9 Role of stem cells on evolution: a hypothesis

Abstract: Life evolution is a fascinating theme. According to Lamarck, "creation" is in a constant state of advancement, a result of cumulative changes of unclear etiology, although the potential role of stem cells has not been analyzed. Hierarchical and somatotopic development suggests that the whole organism's cells are linked through complex hierarchical clustering network systems that provide several advantages to the organism cells, including cell feedback controls, which allow somatic cells to promote and/or repress gene behaviors of anybody's cell, including germ stem cells (GSCs). Gene flexibility for accepting duplicated genetic elements is mandatory for evolution; thus, the updated selected epigenetic information of SC is transmitted to GSCs and consequent generations. However, we should consider the competition between the two parental chromosomes. This dual parental occurrence of an epigenetic modification could create an "optimum selective peak"; therefore, life "sexualization" was the first primordial step of evolution. The second primordial step was brain emergence, which was initiated by the development of motor, sensing, and sensorial functions. This has contributed, by successive cascades, to brain evolution across species. In mammals and primates, comport functions are imprinted as "fixed brain engrams" because genes have been fixed at the level of genomic organization, whereas in humans, the brain genome has escaped "fixation." Consequently, the plasticity of brain gene regulatory mechanisms has reduced the genetically predetermined gene expression of "brain engrams," leading to the development of complex brain regions and brain functional diversification, with their complex interaction.

Key Words: Evolution, Stem cells, Life sexualization, Brain, Human.

9.1 Introduction

The aim of any hypothesis is not to give an exact picture of reality but rather to give rise to more accurate concepts that could lead to reality [1, 2]. Consequently the principles of any theory must be based on coherent sets of ideas to construct a fictive and provisional model of reality sustained by a large class of observations; no matter how much time results agree as any theory should evolve with our knowledge. Therefore, a theory is built to help solve problems, explain indefinite events, or guide further researches. One of the most fascinating themes of theory is life evolution, because several puzzling topics or set of concepts must be assembled to explain the origin of life and either species emergence or evolution. The universal thought, although

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disputed [3], is that all life arise from a common ancestor, from which three primary lines of descents developed, including Archea, Bacteria, and Eucarya, with each line generating different subgroups of genetic organisms [3, 4]. The genetic system depends on genes used to transmit information and the epigenetic system that comprises the transmission of the on/off switching of genes during development. Although it is well admitted that the main driving force of evolution is competition and the main tool is genetic and epigenetic modifications, how such genetic phenomena is evolutionary is still matter of debate and several theories have been suggested [4–9].

According to Lamarck, "creation" is in a constant state of advancement; it was an innate characteristic of nature that organisms are constantly "improved" by successive generations, so an organism adapts to its environment at each stage of its evolution and large changes in population are the result of cumulative changes [10–12]. Although It has been stated that mutations within promoter regions constitute the most "relevant or important" source of genetic variations [13, 14], we agree that regulatory gene changes rather than gene mutations are responsible for the vast majority of observed biological differences [15], because spontaneous mutations, instead of gaining new functions, most often lead to deleterious functions [16–20].

9.2 Stem cells and evolution

Despite various theories, the potential role of stem cells in life evolution has not been considered. First, it is recognized that all cells are related to each other, following symmetrical or asymmetrical division, through a somatic cell (SC) tree, which starts from an embryonic toti-potential stem cell to adult differentiated SCs, with pluripotential stem cells (PSCs) being progenitors or common ancestors of several clusters of multipotential stem cells (MSCs) and differentiated somatic cells (SCs). Second, although it is recognized that cell-cell communication is crucial to large multicellular organisms [21], little attention has been accorded to the possibility that stem cells may represent a component of "a biologically interconnected cellular complex system" even if the knowledge-based approach to constructing biological network models is recognized as one of the most promising advances in computational biology [22]. Biological systems can be modeled as complex hierarchical clustering network systems, with many interactions between the components [23]. This may help in understanding the organization within organism cells and their dynamics as information from the cell state and the outside environment is translated into a correctly timed expression of genes [24]. Due to the high reliability of each relation, many network models, even those with large, complex structures, have been constructed for various biological phenomena by a knowledge-based approach [25]. However, the complexity of biological systems does not reside solely in the number of components and interactions or in their associated structural and physicochemical properties, but in the hierarchical network connections across space and time scales from gene level to cell

level to tissue level to organism level and finally to population level [26]. Although difficult to materialize, it could be deduced that organism cells, including the human cell pool, are linked by a complex dynamic physiological tree with several pathways including stem cell-SC interrelations in both directions and stem cell interconnections. These pathway trees have a spatial and temporal organization, and the same "cluster" of cells may have a privileged or facilitated way to communicate each other, allowing identical cells to coordinate their physiological behavior(s), create a cooperative whole, perceive and correctly respond to their microenvironment, and regulate their metabolism; this communication type is the basis of development, tissue repair, and normal tissue homeostasis [21]. These communications provide several other advantages to the whole organs or organism cells, including stem cells:

1. They provide opportunities for cell feedback control.
2. Intercell communications allow stem cells to remain quiescent until they are activated by disease or tissue injury to repair the dependent tissue or to self-renew. The balance of self-renewal and differentiation is not likely being governed by single or few stem cell factors but rather by the integration of many interacting signal inputs [27] from somatic, multipotential, and pluripotential stem cells.
3. They allow PSCs to modify their epigenetic registry in response to environmental "spike stress," which could lead to the emergence of epigenetic "mutational" event [6]. PSCs may restructure the genome expression of the next generation of stem cells in response to environmental stressful conditions, by transposition of DNA elements or a change in heterochromatin, because the programmed pathway(s) by which inductive signals have controlled the identity, proliferation, and timing of differentiation of progenitor cells (in establishing spatial pattern of developing tissue) remains in stem cell memory and accessibility to the genome is not restricted to PSCs [28].
4. Cell communications allow PSCs to promote and/or repress the gene behaviors of any body cell, including germ stem cells (GSCs) [29–32]. Even if the mechanisms of gene regulation and evolution are complex and blurred, they do not occur without a definite and specific order. The rules that govern gene regulation and evolution are the primary controls "on what it is allowed to happen in the evolution" of the overall genotype-phenotype system, suggesting strong controls on the categories of developmental changes that might be associated with macroevolution [33]. Therefore, the short-term, clock-dependent, temporo-spatial embryonic network communication should be distinguished from the stable, dynamic, regulatory, stem cell network it has induced in achieved organism. Communications between PSCs (of the whole organism) and GSCs lead to GSCs' epigenetic registry update, according to PSCs' "acquired experience." The epigenetic changes in a whole stem cell, induced by the environment, occur through a long period, by successive steps, which require a key period where the environmental stimulus persists and produces an effect on the organism before it becomes imprinted in the PSC genome and transmitted to GSCs. PSCs may act as an on-off molecular

switch to update epigenetic registry of GSCs, which is transmitted to gametocytes. Therefore, the updated embryological system gives the offspring organism new anatomical [34] and physiological [35] survival strategies and aids the new organism to develop the most adequate functions, according to its environment and/or constraints. This genetic transmission allows short-term adaptation of each generation to its environment. This concept may explain intraspecies and interspecies evolution.

9.3 Evolution stages

One should differentiate between the phenomena occurring at the beginning of life and those occurring at following stages when the core and fundamental components of life have led to species emergence, with each specific genome. Therefore, we should distinguish the epigenetic innovation of the phenotype and/or genotype (novelty) that was probably predominant at the initiating conditions of life from epigenetic and/or genetic variation (or improvement). Although they could share the same mechanisms, the line between them has moved from innovation toward variation with time and species emergence. While the morphological and functional effects of innovation are major, the effects of improvement (due to environment shift) are finite when considering the individual and should rather be quantified across generations and the whole life of the species because it represents the sum of epigenetic adaptations to the environment by the species. Environmental epigenetic modifications induce minor morphological and functional variations on which selection then acts; once a process of gradual genetic modification begins, it becomes directional and leads to organism (morphological and functional) adaptation over successive generations. The epigenetically modified gene should generate an advantage (substantiated after a positive period) before the selection become fixed and maintained in the population, thus confirming the emergence of improved morphological and/or functional diversification, which could be related to environmental change. Adaptive evolution operates only at a few sites; thus, epigenetic selection is likely to represent a fine balance between positive and negative influences rather than a global and sudden change.

9.4 Evolution and update of genetic database

9.4.1 Evolution of genetic database

We believe that genes, loci, or nucleotide sequences, incriminated in organ structures or functions, can be duplicated and used to reproduce the same or similar “cellular mechanistic system” to achieve another organ or function. Furthermore, duplication and insertion of genes, loci, and nucleotide sequences are under the control of stem

cell nuclear machinery, which may duplicate those genetic elements that should be activated but which are localized in a timed or timeless silenced gene(s). It is well known that in a living organism, the entire phenotype diversity of specialized SCs obey the rule that only the set of expressing genes unique to a particular cell type is expressed [36]. Therefore, genes, loci, and nucleotide sequences necessary to develop or improve a new organ or function, but which cannot be activated without peril, are duplicated. The control of transcription mechanisms by PSCs allows them to activate different dynamic processes that analyze the cis-acting regulatory elements of a genome and identify the nature of genes that should be silenced, activated, or replicated according to organism needs. This requires nucleus chromatin from PSCs to be folded into higher-ordered structures [37], permitting PSC nuclear machinery to interact with those genes having an "additive effect" to duplicate elements localized within silenced genes and to insert them (where and when necessary) on flexible genomic regions, which leads to positive changes.

Consequently, gene flexibility for accepting duplicated genetic elements is mandatory for evolution and suggests a progressive evolutionary model rather than mutational events. It is most likely that the use of duplicated genetic elements in the early phases of evolution was more frequent and related to structural and functional states to improve organism structures and functions, while in highly advanced stages, they probably involve a functional state rather than structural. The duplicated genes encode plausibly proteins of fundamentally important biological functions such as signal transduction, RNA metabolism, intercellular communication, transcription factors, etc., which are essential common parts of intricate and complex molecular circuits and mechanisms, which can be useful in different organs. In addition, evolution has also been improved by evolution of gene functionality starting from individual gene function at early phases of evolution toward gene coexpression and clustering at advanced stages. The grouping of genes into clusters allows coevolution, functional similitude, and interaction between genes.

These assumptions are sustained by several lines of evidence. It has been pointed out that the primary molecular mechanisms underlying genome evolution are single-nucleotide pleomorphism, gene segmental duplication, genome rearrangement [38], and perhaps loss of genetic material [39, 40]. Eukaryotic genome frequently includes repeat sequences that are scattered through it. It is well known that the function of nucleotide sequences in the DNA depends on the manner they are combined and a simple repeat may change the protein function by difference in length and base composition [41]. The human genome has undergone numerous segmental duplications [42], some of which contain genes and others contain no genes, and the extent of duplication shows great variation among chromosomal regions, suggesting that several duplications containing genes were maintained by selection after duplication [43]. Finally, coexpressions of neighboring genes is common in many eukaryotic species, which in humans [44] have preferential arrangements of specific subsets of genes in specific "gene networks" [44, 45], and the molecular machinery that regulates

gene expression can generate long-distance interchromosomal associations [45, 46] and formations of complex arrays of protein coding regulatory sequences that give rise to new combinations within the same genome [47]. Consequently, a single initiating step may lead, gradually, to remarkable effects in the long run in the same species or can give rise to new genes in the evolutionary lineage leading to new species.

9.4.2 Role of GSCs in genetic database update

GSCs are a unique lineage with many unusual features. They are the only cells in the normal life cycle of an organism that carry the genome onto the next generation and they are the only cells that are able to undergo the reductive meiotic division, which is fundamental for gametogenesis. Furthermore, GSCs play a primordial role in heredity and evolution because the epigenetic determination of gene expression can be reversed or reprogrammed in the germ line [48] and the newly established pattern of *de novo* methylation can be transmitted to the offspring and may remain stable for many generations [49]. Although there are extreme differences between instability (of switches) in the gene expression of PSCs and GSCs in contrast to the stable gene pattern of SCs [48, 50], the remarkable stability and proliferative activity of GSCs suggest that they have a unique machinery to prevent the transmission of genetic and epigenetic damages to the offspring because stable genetic transmission to future generations is essential for life [51]. The updated selected epigenetic information of PSCs is transmitted to GSCs and consequent generations. GSC line passage is needed for the initiation of some specific epigenetic modifications by erasing existing imprints that are inherited from the previous generation and establish the imprint of the updated imprint for the next generation. The RNA factors' repression ensures that the existing "silenced" chromatin is not expressed in meiosis, and dynamic modification by different mechanisms (i.e., transposition of some histones variants) induces epigenetic changes in nuclear gene expression, and consequently, the updated information is incorporated in the genome of gametocytes. Therefore, as something new is added, something old is slipped away from the gene database, to compensate for the gain. The segmentation of the genome in topographically (in)dependent domains [37] suggests the possibility of "one-to-one correspondence" between genotype and phenotype, which might ensure the fidelity of epigenetic transmission of the chromatin structure and the control of epigenetic modifications of gene expression. Therefore, during evolution, new functions are acquired as a consequence of an accumulation of epigenetic substitutions that modify the coding of regulatory sequences of genes. In addition, we should consider the competition between the two parental chromosomes and their epigenetic marks during fecundation. Since the epigenetic change is not random, a meeting of male and female with the same epigenetic change is an event of sufficiently high probability, and therefore, this dual-parental occurrence of an epigenetic modification could create an "optimum selective peak," which can

provoke its selection in offspring. While occurring in only one parental gene, the modification could not reach the "optimum peak" for its selection. Therefore, GSCs are able to add, to each germinal generation, some epigenetic information linked to the adaptive changes experienced by the previous SCs or organs and transmitted through the PSCs of both parents. It could be stated that life "sexualization" was the first primordial step of evolution.

9.4.3 Memory box and update of genetic database

The development network that derives from one cell is finely orchestrated and governed by "embryonic central clocks" [52], which ensure organism homeostasis and dynamical changes with regulatory effects on chromatin structures and gene expression, allowing a chronological and a highly dynamic spatial and temporal development from embryo-organogenesis up to final behavior. The plan of organism formation and function of each individual is imprinted in its PSC genome, and the whole organism PSC genome could be considered as a "memory box" (MB), with each specimen of any species having its own MB. It could be speculated that gene evolution is continuously occurring by selective genetic element substitutions or additions involving those genes regulating the molecular features of organism functionality. However, some restrictions related to such replications and insertions merit emphasis; they are, in all probability, less frequent with advanced stages of evolution or when the species has reached the "summit" of its organization and functionality, except those modifications related to its environmental adaptation and survival. Therefore, each MB type could be considered as a genomic record of the positive events underlying species emergence and development, and genetic-epigenetic modifications accumulated during the individual life are recorded in the MB and transmitted to offspring via updated germ cells. This implies that any species offspring has a genotype and a phenotype summarizing the entire positive epigenetic environmental and genotype changes experienced by its previous lineage ancestors. This may explain intraspecies differences. As humans were the latest species to differentiate, human stem cell genome embraces the entire selected common genetic and epigenetic phenomena shared by all species, deriving from its lineage through evolution, including its particular and specific genes.

9.5 Evolution and human emergence

Human evolution is the part of the theory by which human beings have emerged as a distinct species. The validity of evolution and the origins of humanity have often been a subject of great controversies through scientific disciplines, although most authors believe that the brain is the cause of human emergence [47, 53–56]. This obvious

difference is believed to be both anatomical and physiological, although more recently, this view has moved toward genomic domain [57–59].

9.5.1 Brain evolution

It is not the content that mostly counts, but rather the functional organization. Brain evolution involves more than brain expansion; it involves reorganization [55] and an increased number of intercommunicating areas to support the specific human cognitive functions.

It could be suggested that brain emergence-evolution was the second primordial step in evolution and it was initiated by the development of motor, sensing, and sensorial functions (vision, smell, hear, taste, and tactile sensing). The progressive combinations of these individual subsystems induced the development of new signaling pathways or mechanisms that have set the stage for new brain functions. New afferent inputs may serve to configure functional circuits through cell-cell and interarea connections and assign new functions and new cell signaling categories to the brain. These new brain connections may initiate interrelated functional gene connections through genetic and/or epigenetic modifications of PSC genome, which are transmitted to the offspring. The neural network is subject to the process of tissue patterning, which is influenced by genetically encoded boundary conditions that determine which, where, how neurones take place in the course of brain development and how axonal guidance should occur [47]. This has contributed, through successive cascades, to brain evolution across species directing brain development in offspring in that manner that less functional regions are reduced and the most functional regions are developed; such anatomical and functional organizations are genetically regulated, and changes occur slowly over time. However, it is difficult to speculate if the complex network of neurons has induced specific modification in gene function from "individual gene function" to "gene cluster function," with more and more complicated functional relationships, or the inverse.

If such statement is true, therefore, the marks of genetic variation in gene expression and gene expression clustering may exist within the brain and within the same functional region and should reflect functional or structural specialization by particular brain regions. Several reports have provided elegant analysis of gene expression in different brain regions [60–64], and it has been shown that the variation between brain regions appears to be greater than originally shown by anatomical and histological studies. Furthermore, transcriptome analyses have identified a large number of mRNAs expressed predominantly in different brain regions, of which some displayed a very specific expression profile, which provide a molecular confirmation for the anatomical boundaries between the subregions [65]. It has been suggested that a specific gene expression may traduce a very specific region and behavior [60–62, 66, 67], while a differential gene expression may reflect associative regions or mixture

with gene groups of basic cellular maintenance and survival, as genes from diverse functional categories are represented in the nervous system [64].

9.5.2 Differences between human and mammal brains

It is now considered that brain development is one of the most significant biological bases for human emergence, and there is a considerable amount of literature on the subject. Recently, discussion on brain evolution has moved from the traditional realms of anatomy, physiology, and behavior to the domain of genes and genome organization [58, 59, 68, 69]. However, as the genetic difference between humans and chimpanzees amounts to no more than about 1–6% of their genomes [70, 71], there is no reason to suppose that a gene important for brain evolution should exert its influence only in the human branch. This rather favors the possibility that positive modification was actually directed toward the brain activity and organization rather than morphology.

In mammals and primates, the "comport functions" are probably imprinted as "fixed genetic engrams" in their brain through gene transcription with potential biological themes of survival associated with specific and particular brain regions. These engrams, genetically formed, contribute to the conditioned network response that results from the activation of related stimulus and could be activated under minim specific signals. They have an immediate effect on brain functions owing to the "facilitated" neural processes and result in specific stereotypical responses according to the nature of the signal and the species. This allows mammals to walk immediately after birth, to migrate, and to survive. These engrams have steady interconnections; although their brain pathways may evolve, their genetic simultaneous coevolution is less probable because genes have been fixed at the level of genomic organization or are under gene control, with a "restrictive regulatory function" that blocks brain gene reduplication and brain gene cluster reorganization or innovation. The plasticity of noncoding gene and regulatory mechanisms affect the dynamics of gene expression and are the major driving force in establishing species-specific gene expression patterns [72].

Although little is known about the key events that made our brain unique related to other primates, it is likely that a new gene family or new functional gene is associated with this human behavior. It has been hypothesized that the large diversity within and between species, including humans, has been driven mainly by changes in gene regulation rather than altered protein-coding gene sequences [15, 73]. Empirically, it could be deduced that the human branch of such new gene should show a high genomic acceleration, and its noncoding sequences should evolve significantly faster and exclusively in humans, owing to their rapid evolution. This new gene or function may occur as a result of loss of gene with a restrictive regulatory function or positive selection of gene with unrestricted regulatory function. It is well known that

there are multiple biological processes responsible for the creation of new gene families [74–78], and several human genes show this fast genomic acceleration [79–83].

It could be suggested that in humans, the brain genome has escaped "fixation," allowing brain gene duplication, reorganization, gene clustering, innovation, etc.; this has led to the breakdown of brain engrams and the development of new signaling mechanisms due to the availability of new elements or pathways that have set the development of brain plasticity. Brain plasticity has, in turn, increased gene plasticity and so on. Pollard *et al.* identified brain genomic regions that are highly conserved over a large evolutionary distance between chicken and chimpanzee but have undergone accelerated evolution specifically in the human lineage [81]. Consequently, the plasticity of brain gene regulatory mechanisms has reduced the genetically predetermined gene expression of brain engrams, leading to the development of complex brain regions and brain functional diversification, with their complex interaction. This has allowed more development of the human brain in comparison to mammals and primates. Evolution encompasses several different network interplays between the genotype and phenotype determined by gain and loss of gene network components [84, 85]. Furthermore, in the evolutionary process, the conserved functional physiology may improve the anatomy by successive gene reorganization. The new functional noncoding gene may disrupt binding of transcription factors to their cognate DNA attachment regions by modifying DNA-binding affinity [86, 87] and an improved physiological function with different structures will develop [88]. Therefore, genes incriminated in organism anatomy move toward another equilibrium so that they may be more adaptive to environmental changes in the evolutionary process and new genotype and phenotypes are more easily generated in order to be more adaptive to the new environment, including bipedalism in humans, consequent of a breakdown in brain motor engrams, which are the first to react in infants during their brain maturity before they acquire bipedalism.

The origin of humans was accompanied by the emergence of new physical behavior and cognitive functions, including bipedalism, structured language, abstract representations, symbolic though, etc. [57]. The picture of the primary essence of human language was a combination of sounds uttered to show an object or animal, while the tone of the voice, the body movement, and the expression of the face expressed the state of the mind. The association of movement with sound, image, and mind established, in the brain, association pathways and areas between words, things, and primary minds and allowed the progressive competence and ability of the human brain to store, retrieve, and transform present experiences into future performance, because the elementary universal language, the roots of the different languages, has evolved. It is well known that in humans, recent functionality and ancestral ones have a hierarchical dependence. The recent cognitive functions control ancestral ones such as primary comport reactions in front of danger, fear, and pleasure, allowing humans to respond in qualitatively novel habits to such stimuli, although the control of their visceral component may escape such control. The emergence of hierarchy in the control and regulation layouts of brain areas allowed the most effective way to

transfer information and coordinate processes to break down “engrams codes” into pieces and reuse certain functions – functions that are called and used by many other brain areas – thus, it can reuse its tools more often and progressively require fewer new tools and pathways for novel tasks, including bipedalism and structured language. This may explain the focus of language and that of most cognitive functions in one hemisphere according to the handedness of the individual, as most connections between cortical areas are between adjacent or nearly adjacent areas.

9.6 Conclusions

The complexity of the numerous active genetic information on species, including humans, shares several basic structures and mechanisms, some of which have a precise and steady determinism, while others may evolve. The core and fundamental components of life exist in every genome, and once a species has emerged, it usually escapes the (anatomical) evolutionary process or evolves very slowly with a low contributory impact. In opposition, the functional noncoding DNA segments, including gene regulatory regions, are vital to specie (functional) evolution and adaptive changes. The plasticity of brain gene remodeling and brain gene regulatory mechanisms, which has occurred extensively in the human genome, is the major driving force of its emergence-evolution. Brain evolution is the main difference betwen human and others species. The human brain is charactrised by the lack of “engrams” and “blocked circuitries” relatively to other species. However, if mammals and primates brains may integrate minor “functional” skills improvements, Human brain has more ability and capacity to integrate more and complexes functional improvements by creating new brain neuronal circuitries. This possibility is lesser in other species in which the new skill is integrated in a fixed engram circuitry. Life evolution is a global, dynamic, and complex temporo-spatial interaction of each specie whole genome and various environmental factors, *via* the complex SC-GSCs, suggesting that evolution is not random but curved by environment.

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9.7 References

[1] Popper K. Conjectures and Refutations. London: Routledge and Kegan Paul, 1963.
[2] Schick T. Readings in the Philosophy of Science. Mountain View, CA: Mayfield Publishing Company, 2000.
[3] Woese C. The universal ancestor. Proc Natl Acad Sci USA 1998;95:6854–9.
[4] Wachterhauser G. From volcanic origins of chemoautotrophic life to Bacteria, Archea and Eukarya. Phylos Trans R Soc Lond B Biol Sci 2006;361:1787–808.

[5] Harris EE, Meyer D. The molecular signature of selection underlying human adaptation. Am J Antrhopol 2006;43:89–130.
[6] Maresca B, Schwartz JH. Sudden origins: a general mechanism of evolution based on stress protein concentration and rapid environment change. Anat Rec B New Anat 2006;289: 38–46.
[7] Ohnishi K, Oshima M, Furuichi N. Evolution from possible primitive tRna viroids to early poly-tRNA-derived mRNAs: a new approach from the poly-tRNA theory. Genome Inform 2005;6:94–103.
[8] Poole AM, Penny D. Evaluating hypotheses for the origin of eukaryotes. Bioessays 2007;29:74–84.
[9] Yi SV. Non adaptive evolution of genome complexity. Bioessays 2006;28:979–82.
[10] Burkhardt RW. Lamarck, evolution and the politics of science. J Hist Biol 1970;3:275–96.
[11] Burkhardt RW. The inspiration of Lamarck's belief in evolution. J Hist Biol 1972;5:413–38.
[12] Jablonka E, Lamb MJ. Epigenetic Inheritance and Evolution: The Lamarckian Dimension. Oxford: Oxford University Press, 1995.
[13] Caroll SB. Endless forms: the evolution of gene regulation and morphological diversity. Cell 2000;101:577–80.
[14] Stern DL. Perspective: evolutionary developmental biology and the problem of variation. Evolution 2000;54:1079–91.
[15] King MC, Wilson AC. Evolution at two levels in humans and chimpanzees. Science 1975;188:107–16.
[16] Lahiry P, Torkamani A, Schork NJ, Hegele RA. Kinase mutations in human disease: interpreting gentotype-phenotype relationships. Nat Rev Genet 2010;11:60–74.
[17] Mues G, Kapadia H, Wang Y, D'Souza RN. Genetics and human malformations. J Craniofac Surg 2009;20(Suppl 2):1652–4.
[18] Northcott PA, Rutka JT, Taylor MD. Genomics of medulloblastoma: from Giemsa-banding to next-generation sequencing in 20 years. Neurosurg Focus 2010;28(1):E6.
[19] Palmero EI, Achatz MI, Ashton-Prolla P, Olivier M, Hainaut P. Tumor protein 53 mutations and inherited cancer: beyond Li-Fraumeni syndrome. Curr Opin Oncol 2010;22:64–9.
[20] Rodin SN, Riggs AD. Epigenetic silencing may aid evolution by gene duplication. J Mol Evol 2003;56:718–29.
[21] Cooper GM. The Cell: A Molecular Approach. Sinauer Associates, Inc., US 1997.
[22] Bonetta L. Bioinformatics-from genes to pathways. Nat Methods 2004;1:169–76.
[23] Mete M, Tang F, Xu X, Yuruk N. A structural approach for finding functional modules from large biological networks. BMC Bioinformatics 2008;9(Suppl 9):S19.
[24] Crombach A, Hogeweg P. Evolution of evolvability in gene regulatory networks. PLoS Comput Biol 2008;4(7):e1000112.
[25] Saito S, Aburatani S, Horimoto K. Network evaluation from the consistency of the graph structure with the measured data. BMC Systems Biol 2008;2:84.
[26] Chen BS, Wu WS. Underlying principles of natural selection in network evolution: systems biology approach. Evol Bioinform Online 2007;3:245–62.
[27] Hackney JA, Charbord P, Brunk BP, Stoeckert CJ, Lemischka IR, Moore KA. A molecular profile of hematopoietic stem cell niche. Proc Natl Acad Sci USA 2002;99:13061–6.
[28] Szutorisz H, Canzonetta C, Georgiou A, Chow CM, Tora L, Dillon N. Formation of an active tissue-specific chromatin domain initiated by epigenetic marking at the embryonic stem cell stage. Mol Cell Biol 2005;25:1804–20.
[29] Poulsom R, Alison MR, Cook T, et al. Bone marrow stem cells contribute to healing of the kidney. J Am Soc Nephrol 2003;14:S48–54.
[30] Rizvi AZ, Wong MH. Epithelial stem cells and their niche: there's no place like home. Stem Cells 2005;23:150–65.

[31] Donovan PJ, De Miguel MP, Hirano MP, Parsons MS, Lincoln J. Germ cell biology – from generation to generation. Int J Dev Biol 2001;l45:523–31.
[32] Mann JR. Imprinting in the germ line. Stem Cells 2001;19:287–94.
[33] Budd GE. On the origin and evolution of major morphological characters. Biol Rev Camb Philos Soc 2006;81:609–28.
[34] Burke AC, Nelson CE, Morgan BA, Tabin C. Hox gene and the evolution of vertebrate axial morphology. Development 1995;121:333–46.
[35] Segal JA, Barnet JL, Crawford DL. Functional analysis of natural variation in Sp1 binding sites of TATA-less promoter. J Mol Evol 1999;49:736–49.
[36] Serov O, Serova I. Genes and chromosomes: control of development. An Acad Bras Cienc 2004;76:529–40.
[37] Robinson PJ, Rhodes D. Structure of the '30nm' chromatin fibre: a key role for the linker histone. Curr Opin Struct Biol 2006;16:336–43.
[38] Samonte RV, Eichler EE. Segmental duplication and the evolution of the primate genome. Nat Rev Genet 2002;3:65–72.
[39] Olson MV. When less is more: gene loss as an engine of evolutionary change. Am J Hum Genet 1999;64:18–23.
[40] Wang X, Grus WE, Zhang J. Genes losses during human origins. PLoS Biol 2006;4:e52.
[41] Schönbach C. From masking repeats to identifying functional repeats in the mouse transcriptome. Brief Bioinform 2004;5:107–17.
[42] Hattori M, Fujiyama A, Taylor TD, et al. The DNA sequence of human chromosome 21. Nature. 2000;405:311–9.
[43] Zhang L, Lu HHS, Chung WY, Yang J, Li WH. Patterns of segmental duplication in the human genome. Mol Biol Evol 2005;22:135–41.
[44] Fukuoka Y, Inaoka H, Kohane IS. Inter-species differences of co-expression of neighboring genes in eukaryotic genomes. BMC Genomics 2004;5:4.
[45] Dekker J, Rippe K, Dekker M, Kleckner N. Capturing chromosome conformation. Science 2002;295:1306–11.
[46] Grimaud C, Bantignies F, Pal-Bhadra M, Ghana P, Bhadra U, Cavalli G. RNAi components are required for nuclear clustering of polycomb group response elements. Cell 2006;124:957–71.
[47] Gierer A. Human brain evolution, theories of innovation and lessons from the history of technology. J Biosci 2004;29:235–44.
[48] Holliday R. DNA methylation and epigenetic inheritance. Philos Trans R Soc Lond B Biol Sci 1990;326:329–38.
[49] Schumacher A, Koetsier PA, Hertz J, Doerfler W. Epigenetic and genotype-specific effects on the stability of de novo imposed methylation patters in transgenic mice. J Biol Chem 2000;275:37915–21.
[50] Holliday R. Dual inheritance. Curr Top Microbiol Immunol 2006;301:243–56.
[51] Kanatsu-Shinohara M, Ogonuki N, Iwano T, et al. Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. Development 2005;132:4155–63.
[52] Nothias JY, Majumber S, Kaneko KJ, DePamphilis ML. Regulation of gene expression at the beginning of mammalian development. J Biol Chem 1995;270:22077–80.
[53] Finlay BL, Darlington RB. Linked regularities in the development and evolution of mammalian brains. Science 1995;268:1578–84.
[54] Northcutt RG, Kaas JH. The emergence and evolution of mammalian cortex. Trends Neurosci 1995;18:373–9.
[55] Weaver AH. Reciprocal evolution of the cerebellum and neocortex in fossil humans. Proc Natl Acad Sci USA 2005;102:3576–80.
[56] Kaas JH. From mice to men: the evolution of the large, complex human brain. J Biosci 2005;30:155–65.

[57] Caceres M, Lachuer J, Zapala MA, et al. Elevated gene expression levels distinguish human from non human primates brains. Proc Natl Acad Sci USA 2003;100:13030–5.
[58] Evans PD, Anderson JR, Vallender EJ, Choi SS, Lahn BT. Reconstructing the evolutionary history of microcephalin, a gene controlling brain size Hum Mol Genet 2004;13:1139–45.
[59] Zhang J. Evolution of the human ASPM gene a major determinant of brain size. Genetics 2003;165:2063–70.
[60] Hackam AS, Qian J, Liu D, et al. Comparative gene expression analysis of murine retina and brain. Mol Vis 2004;10:637–49.
[61] Letwin NE, Kafkafi N, Benjamini Y, et al. Combined application of behaviour genetics and microarray analysis to identify regional expression themes and genes-behavior associations. J Neurosci 2006;26:5277–87.
[62] Pavlidis P, Noble WS. Analysis of strain and regional variation in gene expression in mouse brain. Genome Biol 2001;2:RESEARCH0042. Available from: http://genomebiology.com/2001/2/10/research/0042
[63] Stead JDH, Neal C, Meng F, et al. Transcriptional profiling of the developing rat brain reveals that the most dramatic regional differentiation in gene expression occurs postpartum. J Neurosci 2006;26:345–53.
[64] Zhang J, Moseley A, Jegga AG, et al. Neural system-enriched gene expression: relationship to biological pathways and neurological diseases. Physiol Genom 2004;18:167–83.
[65] De Chaldée M, Gaillard MC, Bizat N, et al. Quantitative assessment of transcriptome differences between brain territories. Genome Res 2003;13:1646–53.
[66] Enard W, Przeworski M, Fisher SE, et al. Molecular evolution of FOXP2, a gene involved in speech and language. Nature 2002;418:869–72.
[67] SLI Consortium (SLIC). A genomewide scan identifies two novel loci involved is specific language impairment. Am J Hum Genet 2002;70:384–98.
[68] Olson MV, Varki A. Sequencing the chimpanzee genome: insights into human evolution and disease. Nat Rev Genet 2003;4:20–8.
[69] Carroll SB. Genetics and the making of Homo sapiens. Nature 2003;422:849–57.
[70] Demuth JP, Bie TD, Stajich JE, Cristianini N, Hahn MW. The evolution of mammalian gene families. PLoS ONE 2006;1:e85.
[71] Varki A, Altheide TK. Comparing the human and chimpanzee genomes: searching for needles in a haystack. Genome Res 2005;15:1746–58.
[72] Wilson MD, Barbosa-Morais NL, Schmidt D, et al. Species specific transcription in mice carrying human chromosome-21. Science 2008;322:434–8.
[73] Wray GA, Hahn MW, Abouheif E, et al. The evolution of transcriptional regulation in eukaryotes. Mol Biol Evol 2003;20:1377–419.
[74] Bergthorsson U, Adams KL, Thomason B, Palmer JD. Widespread horizontal transfer of mitochondrial genes in flowering plants. Nature 2003;424:197–201.
[75] Copley RR, Goodstadt L, Ponting C. Eukaryotic domain evolution inferred from genome comparisons. Curr Opin Genet Dev 2003;13:623–8.
[76] Long M, Betran E, Thornton K, Wang W. The origin of new genes: glimpses from the young and old. Nat Rev Genetics 2003;4:865–75.
[77] McLysaght A, Baldi PF, Gaut BS. Extensive gene gain associated with adaptive evolution of poxviruses. Proc Natl Acad Sci USA 2003;100:15655–60.
[78] Perry GH, Yang F, Marques-Bonet, T et al. Copy number variation and evolution in humans and chimpanzees. Genome Res 2008;18:1698–710.
[79] Bird CP, Stranger BE, Liu M, et al. Fast-evolving noncoding sequences in the human genome. Genome Biol 2007;8:R118.
[80] Prabhakar S, Noonan JP, Paabo S, Rubin EM. Accelerated evolution of conserved noncoding sequences in humans. Science 2006;314:786.

[81] Pollard KS, Salama SR, Lambert N, et al. An RNA gene expressed during cortical development evolved rapidly in humans. Nature 2006;443:167–72.
[82] Wassink TH, Piven J, Vieland VJ, et al. Evaluation of the chromosome 2q37.3 gene CENTG2 as an autism susceptibility gene. Am J Med Genet B Neuropsychiatr Genet 2005;136B:36–44.
[83] Zhang J, Webb DM, Podlaha O. Accelerated protein evolution and origins of human-specific features: FOXP2 as an example. Genetics 2002;162:1825–35.
[84] Keightley PD, Lercher MJ, Eyre-Walker A. Evidence for widespread degradation of gene control regions in hominid genomes. PLoS Biol 2005;3(2):e42.
[85] Woolfe A, Goodson M, Goode DK, et al. Highly conserved non-coding sequences are associated with vertebrate development. PLoS Biol 2005;3:e7.
[86] Lapidot M, Mizrahi-Man O, Pilpel Y. Functional characterization of variations on regulatory motifs. PLoS Genet 2008;4:e1000018.
[87] Rahimov F, Marazita ML, Visel A, et al. Disruption of an AP-2alpha binding site in an IRF6 enhancer is associated with cleft lip. Nat Genet 2008;40:1341–7.
[88] Freeman M. Feedback control of intercellular signalling in development. Nature 2000;408:313–9.

Index

3-D cell culture 56

ABCG2 101, 103, 105
ABCG2-positive cells 103
aberrant 84, 92
abnormal 76, 77, 78, 79, 86
abrogated 45, 51, 54
Action Potential Duration 149
acute MI 22
ADA 73
adeno-associated 72, 74
adenovirally 48
adenovirus 72, 74, 147
adenylate cyclase 165
adhesion 134, 140
adhesion molecule 134
adhesion 46
Adipocytes 36, 134
adipokines 36
adipose 137
adrenergic 160, 161, 165, 166
adrenomedullin 47
adverse events 25
age-related macular degeneration 117
aging 89, 119
Akt signaling 41, 52
allogeneic 85, 88, 89
allogenic 23
allograft 79
allotransplanted 43
amplitude 150, 151, 157, 161
anesthesia 84, 85
angiocrine 39, 40, 52, 55
angiogenesis 39, 40, 45, 48
angiogenic 35, 36, 39, 40, 41, 45, 47, 48, 52, 53, 54
angiogenin 45, 54
angiomyogenesis 82, 92
angiomyogenic 39, 44, 50
angiotropic 39
animal model 23, 26, 27, 116, 117, 119
aniridia 100
anoikis 24
anoxia-reoxygenation 54
antiangiogenic 36, 39
Antiapoptotic 41
antiarrhythmic 11
antifibrotic 134
anti-inflammatory 134, 137, 138
apoptosis 35, 40, 41, 44, 45, 49, 50, 51, 53, 135, 136
aqueous 104
aqueous humor 119
arrhythmia 6, 7, 11, 28, 29, 146, 147, 148, 149, 151, 152, 156, 161, 165, 166, 167
arrhythmogenic 7
arrhythmogenicity 23
asymptomatic 157, 159, 160
attenuates 50
augmentation 78, 91
autoimmunity 131
autologous 82, 88, 118, 122, 123
automated 83

Barth syndrome 9
Bcl-2 54
beating 147, 151, 157, 163
beta-adrenergic 156
beta-blocker 156, 157, 166
bFGF 39, 45, 46, 51, 53, 57
bioactive 35, 44, 45, 52, 56
biocompatibility 25
biodegradable 57
bioengineered 116, 118
biogenesis 78, 86
biological 85, 91
biologically 174
Biologics 73, 87, 88
biomaterials 24
biomatrix 140
biopharmaceutical 57
bioplotting 25
bioprinting 25
bioreactors 121
biosynthetic 100
biowires 9
bipedalism 182
bisoprolol 157
blebbing 41
blindness 100, 101
blocker 157, 158, 159, 164, 166
BMMNCs 21

https://doi.org/10.1515/9783110587043-010

BM-MSCs 134, 135, 139, 141
bone morphogenic protein 4 2
Bowman's layer 99, 117
brain expansion 180
Brugada 7
Brugada Syndrome 162

Ca^{2+} channels 149, 156, 162, 163, 165
Ca^{2+} influx 156, 163, 164
Ca^{2+} transients 152, 158, 163
Ca^{2+}/CaM dependent 163, 164, 165
CABG 82
CADUCEUS 22
calcium 4, 9, 11
calmodulin 152
calsequestrin 165
cancer 71, 72, 73, 87, 88, 89, 90, 92
capillaries 92
cardiac 2, 7, 9, 20, 21, 22, 23, 24, 25, 26, 29, 34, 35, 40, 43, 44, 45, 46, 49, 50, 54, 57, 74, 77, 88, 146, 147, 148, 149, 150, 151, 152, 159, 161, 162, 163, 165, 166, 167
cardiac function 23, 35, 40, 45, 49
cardiac stem cells 34
cardiac stem/progenitor cells 22
cardiogenesis 1, 2
cardiogenic 20, 29
cardiokines 44
cardiomyocyte 1, 20, 34, 35, 39, 40, 41, 44, 45, 46, 48, 50, 51, 52, 53, 55, 81, 146, 147, 148
cardiomyofibroblasts 46
cardiomyopathy 7, 40, 71, 75, 76, 87
cardiospheres 7, 9
cardiovascular 20, 26, 29, 52, 146, 152, 167
cataract 120
Catecholaminergic 165
catheter 82, 83
catheterizations 83
cation channels 162
cell-based 34
cellular 147, 148, 152, 156, 162
cellularity 119
channel behavior 151
channelopathies 146, 147, 148, 151, 152, 167
channels 148, 149, 152, 158, 161, 162, 163, 165, 166, 167
characterization 162, 166, 167
chemotaxis 45
cholangiocytes 131, 133
cholesterol 131
chondriogenic 35
chondrocytes 134
choroid 106
chromanol 293B 156, 158
chromatin 177, 178, 179
chromosomal 177
chromosome 75, 173, 178
chronological 179
cicatricial 100
ciliary 98, 99, 104
cirrhosis 130, 131
clinical trial 20, 22, 29, 71, 72, 73, 78, 79, 80, 82, 88, 92, 122
clonogenic 101, 103
CMs 1
cobblestone 106
coculture 35, 41, 46, 51, 55
cognitive 180, 182
collagen 91, 140
collagen fibers 100
collaterals 27
combinatorial 48, 51
compensatory 163
complex 173, 174, 175, 177, 178, 180, 182, 183
compliance 72, 84
complications 118, 120, 122
computer 13
concomitant 40, 44, 46, 53, 55
conduction system 148, 150
congenital 160
conjunctiva 102, 103
conjunctival 98, 100, 102, 103
continuous 147, 164, 167
CorMatrix 24
cornea transplantation 119
corneal 98, 99, 100, 101, 102, 116, 117, 118, 119
corneal endothelial cells 119
coronary 26, 27, 28
costimulatory 43
CPVT phenotype 167
CSCs 34, 41, 44, 45, 46
$CXCR4^{+}$ 44
cyclosporine 79
cytokine 7, 34, 36, 40, 52, 100, 137, 138
cytoprotective 35, 40, 44, 50, 51

DADs 163, 165, 166, 167
dantrolene 166
decellularized 24, 25
defects 73, 75, 77, 80, 81, 84, 85, 86, 91
defroxamine 53
degeneration 78, 81, 89, 92, 117, 121, 122
degenerative 117, 121, 122
delivery 39, 44, 48, 50, 57
Depolarization 148, 149, 151, 163
Descemet's membrane 99, 117
desmin 84, 87, 88
diabetes 71, 73, 75, 76, 78, 79, 84, 85, 86, 87
diagnostic tool 152
diagnostics 73, 76
diastolic 82
diazoxide 53
$DiBAC_4$ 151
differentiate 98, 99, 100, 103, 105, 106, 107, 133, 134, 137, 138, 140
differentiation 2, 4, 13, 22, 23, 25, 34, 35, 36, 39, 42, 45, 48, 51, 52, 53, 55, 56, 57, 118, 119, 120, 121, 122, 130, 134, 135, 137, 138, 139, 140, 175
Dilated cardiomyopathy 26
dilator muscle 103
disease-on-a-dish 146, 147, 167
disopyramide 162
DMD 72, 73, 75, 78, 79, 80, 84
dobutamine 29
donor 34, 36, 40, 41, 43, 45, 49, 53
doxorubicin 9, 27, 35
drug screening 139
Drug testing 9
Duchenne 72, 75
dynamic 175, 177, 178, 179, 183
dysfunction 21, 27, 28, 117, 119, 121
dystrophic 78, 86
dystrophies 99, 101
dystrophin 75, 78, 80, 84

ectoderm 105
effectiveness 35, 39, 47, 48
efficacy 73, 79, 80, 85, 88
ejection fraction 27, 29
elasticity 56
electrical 6, 7, 13, 23, 24
electrical integration 23
electromechanical 23
electromechanically 21
electrophysiological 146, 148, 151, 157, 158, 159, 162, 163, 166, 167
electroretinogram 122
eliminate 91
embryoid 119
embryoid bodies 22
embryonic 2, 21, 24
embryonic central clocks 179
embryonic stem cells 98, 116, 117, 130, 139
emigrational 46, 56
encapsulated 88
encephalopathy 132
endocardium 4
endocrine 152
endocytosis 74, 84
endogenous 99
endomyocardial 81
endoplasmic 156
endothelial 36, 47, 49, 99, 101, 102
endothelial cells 13
endothelial progenitor cells 21
endotoxin 84
engineered 40, 42, 56
engineered heart tissue 9
environmental 175, 176, 179, 182, 183
EPCs 39, 40, 45, 55
epigenetic 146, 147, 163, 173, 174, 175, 176, 178, 179, 180
episomal 147
epithelial cells 116, 117, 120
epithelial stem cells 117, 118
epithelium 99, 102, 103, 104, 105, 106, 117, 122
erythropoietin 54
ESC-CMs 21, 22
ESCs 98, 99
Eukaryotic 177
evolution 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183
evolutionary 174, 177, 178, 182, 183
exocytosis 84
Exondys 51 72, 80, 81
exosomes 21
experimental 147
expression 36, 39, 42, 44, 46, 48, 50, 51, 52, 53, 55
expression profiles 78
extracellular matrix 24, 39, 131
extravasation 44

FDA 72, 73, 74, 80, 81
feasibility 84, 85
feedback 83, 173, 175
fetal 106
fibrillations 162
fibroblast 24, 39, 46, 86, 122, 123, 160
fibroblast growth factor 2
fibrogenic 134
fibronectin-1 54
fibrosis 40, 46, 49
field potential 149, 151
flecainide 166, 167
fluorescence 152, 157
forniceal 102, 103
functional 22, 23, 26, 29, 78, 118, 120, 121, 122, 147, 151, 156, 158, 163, 167
fusion 71, 77, 78, 79, 81, 84, 86, 88, 89

G589D mutation 157
gain-of-function 152, 161, 163, 165
gametogenesis 178
ganglion cells 116, 117
gap-junction 7
G-CSF 134, 135
Gendicine 72
Gene 105
gene database 178
gene expression 173, 178, 179, 180, 181, 182
gene network 182
gene regulation 76, 175, 181
gene therapy 71, 72, 73, 74, 75, 76, 77, 78, 87, 167
generation 21, 22, 23, 99, 105, 116, 117, 118, 119, 120, 121, 175, 176, 178
genes 173, 174, 176, 177, 178, 179, 180, 181, 182
genetic 146, 147, 152, 156, 157, 160, 162, 163, 165, 167, 173, 174, 176, 177, 178, 179, 180, 181, 183
genetic diseases 73, 75, 79, 80
genetically 35, 40, 41, 44, 47, 48, 50, 173, 180, 181, 182
genome 152, 156, 163, 165, 167, 173, 175, 176, 177, 178, 179, 180, 181, 182, 183
Genome Project 72
Genome Therapy 73, 77
genomes 181
GFP-labeled 103
glaucoma 104, 106, 117, 119
global 40, 44, 45, 49, 50
Glybera 72
glycoproteins 100
glycosylated 160
glycosylation 160
GMP 57
goblet cells 102
grafts 21, 22, 23, 24
growth factor 2, 4, 36, 39, 40, 42, 100, 137
guinea pig 147

haplo-insufficiency 157
haplo-insufficient 159
heart 20, 21, 22, 24, 25, 26, 27, 28, 29, 78, 79, 81, 82, 84, 92
Heart failure 20
Heat shock 55
hematopoietic 41, 51, 98, 99
hemodynamic 27, 29
hepatectomy 134
hepatic sinusoids 132
hepatitis B 84
Hepatitis C 131
hepatocyte growth factor 134
hepatocytes 130, 131, 132, 133, 135, 137, 138, 140
Hereditary 101
hERG 6, 11, 158, 159, 160
hERG trafficking 160, 161
hESC 119, 121, 122, 147
heterochromatin 175
heterokaryons 77
HGF-1, 39, 40, 43, 47, 51, 53, 57
hiPSC-CMs 2, 6
Hirata-Tominaga 102
histopathologic 29
HLA-G 42, 43
home in 35, 44
homeostasis 22, 146, 164, 166, 167
homologous 159, 161
HSP-20 55
HSP-65 55
HSPs 52, 55
human 1, 6, 9, 11, 13, 98, 99, 100, 101, 102, 103, 104, 105, 106
human stem cell 179
human studies 26
Hydrogels 140
hyperglycaemia 86
hyperglycemia 47

hyperinsulinemia 86
Hyperpolarization 149
hypertrophy 22, 27, 28, 46
hypothesis 173
hypothetical 83
hypoxia 49, 54
hypoxic 24

I_{Ks} activator 157
image 182
imaging 157, 167
immune 71, 74, 75, 82, 88, 146, 147, 152, 164
immune isolation 42
immunocytochemical 157
immunogenicity 25
immunomodulation 139
immunomodulators 39
immunomodulatory 42
immunostain 84
immunosuppressant 79, 81, 85, 88
immunosuppression 13
immunosuppressive 25, 137
impedance 149, 151
implantation 21, 23, 71, 82, 85, 86, 87, 88, 90, 91
impulse 148
In vitro 7
inclusion/exclusion 84
Induction 47
infarct 83
infarct size 42, 50
infarcted 39, 40, 41, 43, 44, 47, 48, 50, 51, 53, 54, 55, 56, 82
infarction 21, 28
inflammatory 42, 43, 56
injection 71, 72, 82, 83, 85, 88, 89, 91
insulin 78, 84, 85, 86
insulin-like growth factor-1 36
integrate 74, 77
integration 74, 75, 86
integrins 50
interleukin-6 135
intracellular 149, 152, 156, 160, 163, 166, 167
intracoronary 42, 47, 57
intrahepatic 133
intrahepatically 140
intramyocardial 13
intramyocardially 82
intraocular 104
intraperitoneal 86
ion channel mutation 167
IPE cells 103
iPSC 146, 148, 151, 152, 153, 156, 157, 159, 160, 161, 162, 163, 164, 165, 166, 167
iPSC-CMs 21, 22, 23, 25
iPSCs 1, 2, 4, 7, 13, 22, 98, 99, 105, 116, 117, 118, 119, 120, 121, 122, 146, 147, 148, 159, 160, 163, 164, 167
ischemia 36, 54, 56
ischemia-reperfusion 28
ischemic 26, 27, 71, 78, 79, 81, 82
isoproterenol 156, 158, 164, 165, 166, 167

juxtacrine 35

K^+ channel 152, 158, 160
K^+-channel 159, 163
KCNB3 163
KCNE1 156, 158
KCNH2 158, 159, 160, 161
KCNQ1 gene 152, 156, 157, 158
keratocytes 118
keratoplasty 100, 101
Ki67 55
KK mice 86
KLF5 46
K_S channel 156
Kupffer cells 131
$K_V4.3$ 163

laminin 7
large-animal models 20, 23, 27, 28, 29
LCM 49
lens 98, 99, 103, 105
ligation 27, 28
limbal 116, 117, 118
limbal stem cells 98, 99, 102
lineage 147, 148
lipofection 74
Liposomes 74
liver 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140
liver vasculature 131
long QT syndrome 149
long-QT syndrome 7
long-term 20, 24, 29
long-term culture 57
LQTS-1 152, 156, 157, 158, 161

LQTS-1 patient 156, 158
LQTS-14 164
LQTS-2 158, 159, 160, 161
LQTS-3 160, 161, 162, 163
LQTS-3 patients 161, 162
LQTS-3 phenotype 162
LQTS CMs 158
L-type Ca^{2+} 149, 159, 162, 164, 165
Lumincan-null mice 101
lusitropic 27
LVEF 82

macrophages 42, 56
mammalian 116
Matlab 83
Matrigel 7, 24, 140
maturation 23, 24
MEA system 151, 157, 159
mechanical 6, 7, 13
mechanically 23
mechanisms 173, 175, 177, 178, 180, 181, 182, 183
Mechanotransduction 9
melanocytes 100
membrane potential 148, 149, 151, 160, 161
mESCs 118
mesenchymal 21, 22, 100, 102, 105, 106, 118, 119, 122
mesenchymal stem cells 35, 100, 118, 130, 133
mesodermal cells 4
metabolism 177
metalloproteinases 46
metastasis 71, 87, 88
methylation 178
mexiletine 162
MHC-II 43
microcluster 160
microenvironment 34, 35, 36, 45, 54, 56, 57, 88, 121, 140
micro-lenses 120
microRNAs 34, 36
microsocopy 167
microtissue 9
microvascular 28
microvesicles 21
migrate 181
migration 39, 41, 43, 44, 45, 52, 53
miR-1 6
missense 152, 156, 157, 159, 160, 163, 166, 167
mitochondria 11
mitochondrial 78, 86
mitral 26, 28
mobilization 134
model 1, 26, 27, 28, 146, 147, 148, 152, 162, 165, 166, 167, 173, 174, 177
modulation 43
molecular 146, 148, 152, 167
monokine 41, 45
monolayer 101, 106
monolocus 73
monopotent 77
morphofunctional 45
morphological 56, 176
mouse models 122
MSC 21, 22, 133, 139
Müller glial cells 106, 121
Müller stem cells 99, 106
multielectrode array 149
multifunctional 4, 6
multipotency 102, 106
Multipotent 106, 117, 133, 134
multipotential 174, 175
multipotential stem cells 174
murine 147
muscles 74, 77, 78, 80, 85, 86, 91
muscular 71, 72, 75, 76, 78, 79, 84, 85, 87, 92
mutant 157, 160, 162, 164, 165, 166
mutations 147, 151, 152, 156, 157, 161, 162, 163, 164, 165, 167, 174
Myoblast Transfer Therapy 73, 77
myoblasts 71, 73, 77, 78, 79, 81, 82, 84, 85, 86, 87, 88, 89, 90, 91, 92
myocardial 6, 11, 21, 23, 28, 29, 34, 41, 44, 45, 47, 49, 55, 56
myocardin 47
myocardium 20, 23, 24, 27, 28, 34, 39, 41, 44, 45, 46, 54, 57, 71, 81, 82
MyoCells 77
myocytes 34, 46
myofibers 71, 74, 76, 77, 78, 79, 81, 84, 86, 92
myosin heavy chain 6
myotubes 71, 84, 87, 88, 89, 90

Na^+ gated channel 163
nadolol 159, 166
nanoparticle 41, 72, 74

native 121, 122
neoangiogenesis 40
neovascular 35
neovascularization 45, 53, 54, 100
neural crest 102
neuregulin 35
neurohormonal 26
neurospheres 104, 105
Nifedipine 157
noncontractile 46
non-hematopoietic 134
non-viral 147
nuclear matrix 76

ocular lens 120
ocular therapies 98, 99
ocular trauma 100
oligopotent 98, 100
oncogene 47
Oncorine®, 72
optic cup 103, 105
optimum peak 179
organism 173, 174, 175, 176, 177, 178, 179, 182
organization 173, 174, 180, 181
organoids 118, 121
overexpression 41, 43, 44, 47, 48, 50, 55
Oxytocin 52

pacemaker 149
pacemaker cells 23
palindromic 121
palpebral 102, 103
paracrine 34, 35, 36, 40, 41, 42, 44, 45, 46, 48, 50, 51, 52, 53, 55, 56, 57
parameters 88
patch clamp 149, 150, 157
patch clamping 159
patches 21, 25
pathogenesis 152
pathogens 85
pathological 147, 160
patient-derived 146, 147, 156, 157, 159
patients 72, 74, 76, 78, 79, 82, 84, 85, 88, 92
patient-specific 121, 123
PCR 49
percutaneous 21
perfusion 27
peri-infarct 41, 42, 43, 53
perinatal 22
periocular 119
periostin 50
peripheral blood 45
personalized cell therapy 167
personalized medicine 2, 7, 152, 166
phagocytosis 104, 106
pharmaceutic 82
pharmacological 39, 51, 52, 53, 57
pharmacological agents 159
Phase II/III 73
PHD-2 50
phenotype 23, 27, 100, 102, 103, 146, 147, 149, 151, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 175, 176, 177, 178, 179, 182
phenotypic 122
photoreceptor 99, 106, 107, 116, 117, 121
physiological 175, 176, 180, 182
pigmented 106
pigmentosa 117
pinacidil 157
plasticity 104, 173, 181, 182, 183
plateau 149, 156, 158
platform 1, 6, 9, 13, 73, 77, 83
pleiotropic 50, 52
pleomorphism 177
pluripotency 77, 146, 147
pluripotent 98, 99, 105, 116, 117, 119, 120, 121, 133, 139
pluripotential 174, 175
polyanhydrides 140
polycaprolactones 140
polygenic 76, 77, 81, 86
polypeptide 51
porcine 81, 82
postengraftment 42, 48
post-transplantation 56
PPARs 47
preconditioning 24, 28, 41, 51, 52, 53, 54, 57
precursor 98, 101
primate 23, 26, 147
procainamide 162
profile 36, 42, 43, 45, 47, 48, 50, 51, 53, 56, 57
progenitor cells 2, 98, 99, 101, 102, 104, 105, 106, 117, 119, 120, 121, 122, 130, 133, 134
programmed 24
progression 25, 27
proinflammatory 43

proliferation 35, 39, 45, 46, 51, 52, 53, 56, 88, 89, 90, 98, 100, 102, 105, 106
proliferative 101, 102, 103, 104, 105, 106
prolongation 149, 156, 157, 158, 159, 161, 163
propagation 23
Prophylactic 28
propranolol 156, 158, 159
prosurvival 35, 45, 48, 53, 54
protection 35, 53
protein, thereby 74
Proteomic analysis 41
protocol 85
PSCs 174, 175, 177, 178, 179

QRS complex 149, 150
quiescent 100, 103

randomized 88
ranolazine 159, 162
Recombinant 72, 74
regenerates 82
regeneration 71, 77, 78, 98, 99, 100, 106, 107, 116, 117, 118, 119, 120, 121
regenerative 21, 34, 35, 117, 130
regulatory function 181
rejection 79, 85, 118, 119, 121, 122, 123
relaxation 157, 165, 166
remodeling 20, 21, 24, 26, 27, 28
reorganization 180, 182
repair 34, 39, 41, 43, 44, 45, 46, 50, 54, 57
repolarization 6, 11, 149, 156, 158, 164
reprograming 147
reprogrammed 22
reprogramming 1, 2, 4, 7, 147, 148, 167
resident stem cell 99
responsiveness 34, 49, 54
retinal 98, 99, 104, 106
retinitis 117
retinopathy 106
Retroviral 74
revascularization 21, 28
revolutionary 130, 133
rhesus monkeys 23
Rho kinase 119
rhythmicity 148
robotically 83
roscovitine 164
RyR2, 158, 165, 166
RyR2 activity 165

safety 73, 75, 79, 80, 84, 85, 88
sarcomeres 9, 11
sarcomeric 148, 167
sarcoplasmic 149
satellite cells 77, 81, 84
Scaffold 24, 140
scar tissue 20, 28
Schwalbe's line 102
severe combined immunodeficiency disorder 73
sclerocornea 100
SCN5A gene 160, 161, 162, 163
secretable 40, 47, 48, 52
secretions 34, 36, 40, 45, 48, 49, 50, 55
secretome 34, 35, 36, 39, 45, 48, 49, 50, 51, 52, 53, 56, 57
secretome profile 36, 45, 49, 51, 53, 56
self-renewal 98, 99, 101, 106, 175
senescence 102, 105
sequence 76
sfrp-2 49
signaling 78, 86, 180, 182
signaling pathways 35, 41, 53
signals 35
sildenafil 53
silenced gene 177
Single Gene Transduction 80
skeletal 71, 74, 78, 81, 84, 85, 86
skeletal myoblasts 21
skin 71, 89
skin fibroblasts 123
SkMs 40, 45
SMCs 39, 40, 46, 55
soluble factor 42
somatic 174, 175
somatic cells 174
sotalol 157
species 173, 176, 177, 179, 180, 181, 183
specific 71, 74, 75, 85
sphere-forming 101, 104
spheres 102, 103
spheroids 45
stem cell 21, 22, 34, 35, 36, 39, 40, 41, 44, 45, 47, 48, 50, 51, 52, 53, 55, 56, 57, 71, 77, 83, 98, 133, 174, 175
stemness 56
stereolithography 25
stereotypical 181
Stevens-Johnson syndrome 100
Stromal 118

stromal stem 99, 100
structural 22, 23, 24, 26, 27
subcellular 51
subcutaneously 71, 91
subretinal 121, 122
surrogate 147
survival 24, 34, 35, 39, 40, 41, 44, 45, 48, 50, 51, 52, 53, 56, 85, 91, 134, 135, 139, 140, 176, 179, 181
Syndromes 21
synergistic 46, 48, 52
synthetic 21, 24

tachyarrhythmias 164
tachycardia 27
TACTICS 21
Teratoma 25
theory 173, 179
therapeutic 21, 34, 39, 40, 42, 46, 47, 48, 50, 51, 52, 55, 57, 76, 98, 99, 101, 130, 133, 135, 138, 139, 140, 141, 162, 164, 166
therapy 116, 117, 119, 120, 121, 132, 133, 134, 141
thickness 82, 91
tissue engineering 77
tolerance 86
torsades de pointes 11
trabecular 116, 117
trabecular meshwork 98, 102, 104
transcription 147, 148, 177, 181, 182
transcriptional 75, 76, 77, 78, 86
transcriptome 180
transdifferentiate 133
transdifferentiated 4, 9, 11
trans-differentiation 103
transduction 72, 73, 74, 75, 76, 77
transfer 73, 75, 77, 78, 79, 83, 86, 92
transformation 102
transforming growth factor beta 2
transgene 44, 48, 50, 51, 74, 77
transgenic 147
transient 74, 82
transient expression 139
transitional 82
transmembrane 156, 157
Transnational 21
transparency 99, 100, 101, 105
transplantation 6, 11, 34, 35, 40, 41, 43, 44, 45, 47, 48, 49, 50, 54, 56, 57, 77, 100, 101, 118, 119, 120, 121, 122, 123, 130, 132, 133, 134, 137, 138, 139, 140
transplanted cells 34, 49, 53
treatment 73, 75, 78, 80, 81, 82, 85, 88, 92
trophic factors 34, 35, 36, 39, 45, 46, 48
tubulogenesis 55
tubulogenic 36
TNF-1α 55
tumorigenesis 139
tumorigenicity 123
TUNEL 42, 50

umbilical cord 42, 52, 133, 134, 138, 141
undifferentiated 35, 43, 83, 133, 134
unipotent 98
unspecialized 116

vascular 132
vasculogenesis 51
vasculogenic 39, 48
vector 73, 74, 82
VEGF 36, 39, 40, 41, 43, 44, 45, 46, 49, 50, 51, 52, 53, 54, 55, 57
VEGF165 82
ventricles 148
ventricular 20, 22, 23, 26, 27, 28, 29
ventricular tachycardia 152, 163, 165
verapamil 164
viral 72, 74, 75, 77, 82, 147

wall thickness 26
Wharton's Jelly 42, 138
WJMSCs 141
Wnt 2, 4
Wnt signaling 49

xCELLigence 151
xenogenic 140