Stem Cells International

Stem/Progenitor Cells in Cardiopulmonary Health, Disease, and Treatment

Lead Guest Editor: Fatemeh Sharifpanah
Guest Editors: Jahar Bhattacharya, Hossein A. Ghofrani, Suk Ying TSANG, and Heinrich Sauer

Stem/Progenitor Cells in Cardiopulmonary Health, Disease, and Treatment

Stem Cells International

Stem/Progenitor Cells in Cardiopulmonary Health, Disease, and Treatment

Lead Guest Editor: Fatemeh Sharifpanah
Guest Editors: Jahar Bhattacharya, Hossein A. Ghofrani, Suk Ying TSANG, and Heinrich Sauer

Editorial Board

Contents

Hindawi
Stem Cells International
Volume 2019, Article ID 9861403, 4 pages
https://doi.org/10.1155/2019/9861403

Editorial

Stem/Progenitor Cells in Cardiopulmonary Health, Disease, and Treatment

Fatemeh Sharifpanah,[1] **Hossein A. Ghofrani**,[2,3] **Suk Ying Tsang**,[4,5,6,7] **and Heinrich Sauer**[1]

[1]*Department of Physiology, Faculty of Medicine, Justus Liebig University, Giessen, Germany*
[2]*Department of Internal Medicine, Justus Liebig University Giessen, Universities of Giessen and Marburg Lung Center (UGMLC), German Center for Lung Research (DZL), Giessen, Germany*
[3]*Department of Medicine, Imperial College London, London, UK*
[4]*School of Life Sciences, The Chinese University of Hong Kong, Hong Kong*
[5]*Key Laboratory for Regenerative Medicine, Ministry of Education, The Chinese University of Hong Kong, Hong Kong*
[6]*State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Hong Kong*
[7]*Centre for Novel Biomaterials, The Chinese University of Hong Kong, Hong Kong*

Correspondence should be addressed to Fatemeh Sharifpanah; fatemeh.sharifpanah@med.uni-giessen.de

Received 23 October 2018; Accepted 23 October 2018; Published 6 January 2019

The cardiopulmonary system comprises various organs, structures, and substances from both the heart and lung systems. Since the cardiopulmonary system interacts intimately with every other system in the body and our health is closely related to the function of the cardiopulmonary system, its function and maintenance were always the center of attention by scientists and clinicians. Despite plentiful research in this area, cardiopulmonary diseases still remain widely prevalent and are known as a significant and devastating cause of morbidity and mortality on the globe. The most common cardiopulmonary diseases are hypertension, chronic obstructive pulmonary disease, coronary heart disease, and rheumatic heart fever. Generally, cardiopulmonary disorders have a poor prognosis and current treatments only offer a modest improvement of symptoms without repairing the damaged tissues in the heart or lung system. But recent progress in the field of stem cell science, regenerative medicine, and tissue engineering offered a new perspective in the treatment of cardiopulmonary disorders.

This great achievement provided tremendous potential to develop disease- and patient-specific induced pluripotent stem (iPS) cells as well as organoid cultures for gathering detailed insight into the pathomechanisms of various cardiopulmonary disorders which is important in drug discovery and treatment of patients. Furthermore, the revolutionized progress in the stem cell field has opened a great opportunity for novel personalized regenerative therapeutic approaches to treat cardiopulmonary disorders. There is abundant and even growing number of evidence in stem/progenitor cell therapy of various cardiopulmonary disorders of which some generated controversial information. To summarize and incorporate these scattered data and develop a more comprehensive understanding on the impact of stem/progenitor cells in the cardiopulmonary system, the *Stem Cells International* journal sets out to publish a special issue focused on "Stem/Progenitor Cells in Cardiopulmonary Health, Disease, and Treatment" which covers research from diverse disciplines related to cardiopulmonary health. This special issue sums up recent findings concerning the essential role of stem/progenitor cells in the cardiac as well as pulmonary system. It includes selected reviews and original articles discussing the role and properties of stem/progenitor cells not only from the perspective of basic science but also from the perspective of therapeutic strategies.

As known, the human adult heart lacks a robust endogenous repair mechanism and is not able to regenerate and repair itself after injuries. Therefore, developing and establishing approaches to regenerate and repair the injured heart

are a top priority in treating heart failure. One of the most promising therapeutic strategies for cardiac injury treatment is cell therapy. But before using cell therapy for treating patients, it is necessary to be able to efficiently generate a sufficient number of functional cardiomyocytes which are capable to functionally and safely integrate into the injured area of the heart. The recently developed "direct cardiac reprogramming" technique is a promising approach for generating functional cardiomyocytes. J. L. Engel and R. Ardehali on their review article entitled "Direct Cardiac Reprogramming: Progress and Promise" have comprehensively reviewed the key transcription factors and cardiogenic genes as well as various biological molecules (e.g., epigenetic modifiers, noncoding RNAs, and small molecules) which improve the efficiency of cardiac reprogramming to develop safer reprogramming approaches for future clinical applications. In this context, Y. Zhou et al. have examined the function of various epigenetic factors involved in chromatin remodeling and RNA splicing to identify the inhibitory or facilitating effects of these factors in direct cardiac reprogramming. As they have reported on their interesting research article entitled "A Loss of Function Screen of Epigenetic Modifiers and Splicing Factors during Early Stage of Cardiac Reprogramming," the splicing factors Sf3a1 and Sf3b1 are essential regulators for direct reprogramming, while the splicing factor Zrsr2 as well as the epigenetic modulators Bcor and Stag2 functions as inhibitory barriers for direct cardiac reprogramming. They have finally highlighted the impact of epigenetic regulation and the RNA splicing process during cell fate conversion. As it is also discussed by J. L. Engel and R. Ardehali, small molecules can be used to improve the efficiency of cardiac regeneration. Various small molecules are intensively investigated by different groups for more profound understanding of the cardiac regeneration process which can be potentially used for the development of new therapeutic strategies for heart failure treatment. In this regard, E. H. Ali et al. have investigated the translational potential of the small molecule silibinin in cardiomyogenesis of embryonic stem cells. The small molecule silibinin is a natural compound isolated from milk thistle seed extracts and is traditionally used as a hepatoprotectant. In research article "The Milk Thistle (*Silybum marianum*) Compound Silibinin Inhibits Cardiomyogenesis of Embryonic Stem Cells by Interfering with Angiotensin II Signaling" authored by E. H. Ali et al., it has been demonstrated that small molecule silibinin as an inhibitor of the angiotensin II type 1 (AT1) receptor inhibits the cardiomyogenesis process of embryonic stem cells by interfering with angiotensin II signaling downstream of the AT1 receptor.

Amongst multiple therapeutic approaches and various pharmacological agents which have shown an improvement in patient care and decrease of mortality rate, stem/progenitor cell-based therapeutic approaches present the most promising approach to treat the cardiopulmonary disorders in the future. S. Bardelli and M. Moccetti on their article entitled "Stem and Progenitor Cells in Human Cardiopulmonary Development and Regeneration" have briefly reviewed the contribution of stem/progenitor cells in the development of the cardiopulmonary system as well as the cellular plasticity potential of stem/progenitor cells in the regeneration of the injured heart and lung organs and their current therapeutic applications in the treatment of cardiopulmonary diseases. In this regard, N. Witman and M. Sahara in the review article entitled "Cardiac Progenitor Cells in Basic Biology and Regenerative Medicine" have comprehensively discussed the biology of embryonic and adult cardiac progenitor cells and their regenerative capabilities as well as their current clinical applications in cardiac repair. Cardiac progenitor cells are a heterogeneous cell population distributed throughout the heart which are quiescent under physiological conditions and become activated after injury and may differentiate into new myocytes and vascular cells [1]. J. L. Engel and R. Ardehali review a novel technique which is generating expandable cardiac progenitor cells by direct reprogramming in culture for transplantation in their abovementioned review article.

Mesenchymal stem cells (MSCs), the major stem cells for regenerative cell-based therapy, have been characterized by Dr. Alexander Friedenstein over 40 years ago and are widely used for treating a variety of diseases. Along with the immunomodulatory and differentiation potential of MSCs, it has been shown that they express various essential cytokines which stimulate local tissue repair [2]. The review made by J. Gronbach et al. entitled "The Potentials and Caveats of Mesenchymal Stromal Cell-Based Therapies in the Preterm Infant" has essentially described the role of stem cells in lung development as well as pulmonary diseases. They have further discussed the potential and challenges of mesenchymal stem cell-based therapies in preterm infants and explained the positive effects of exogenous MSC therapy in the diseased lung with the special focus on the bronchopulmonary dysplasia, a chronic lung disease of preterm infants. Although MSC-based therapies are a highly promising approach for treating patients, the authors explained that still a lot of research is needed to bring optimized MSC products into upcoming clinical trials and their outcomes have to be critically evaluated before a broad introduction of MSC-based therapies into the clinics can be achieved. One of the most important points for optimizing MSC products is maintenance and expansion of MSCs in culture before transplantation. With a well optimized protocol, it will be possible to generate a sufficient cell number with the best quality needed for transplantation. Despite regulatory issues about using animal-derived cell culture supplements in clinic, most clinical trials of MSC-based cell therapies are still dependent on fetal bovine serum for cell expansion before transplantation. In order to solve this problem, T. Z. Nazari-Shafti et al. have investigated the effect of human serum from heart failure patients and also healthy donors on cord blood MSCs during regular short-term cultivation as well as stimulated acute and chronic stress conditions and compared their results with the findings from cells treated with fetal bovine serum in the same conditions on their research article entitled "Mesenchymal Stromal Cells Cultured in Serum from Heart Failure Patients Are More Resistant to Simulated Chronic and Acute Stress." They have demonstrated that autologous human serum will be a valid alternative to fetal bovine serum in cell-based therapies addressing severe heart disease.

Cell-based therapies using MSCs are widely used for treating various pathological conditions. One of the recent usages of MSCs is in sepsis. G. M. Galstian et al. have recently shown that treatment of neutropenic patients with MSCs during the first hours of septic shock might improve their short-term survival, but cannot prevent decease due to long-term sepsis-related organ dysfunctions [3]. In the present special issue, J. Horák et al. have explained in detail the properties of MSCs and their mechanisms of action in physiological conditions and in sepsis on their article entitled "Mesenchymal Stem Cells in Sepsis and Associated Organ Dysfunction: A Promising Future or Blind Alley?" They have further discussed the beneficial effect of MSCs on the cardiovascular system as well in sepsis-associated organ dysfunction with a special focus on acute kidney injury which is the most frequent complication of sepsis. Based on recent literature, they have suggested that MSCs with their anti-inflammatory actions are able to reverse detrimental effects of endotoxemia in the heart. They have finally recommended continued evaluation of the safety of MSCs in treatment of sepsis for further translational research in the form of multicenter projects in several world renowned laboratories for additional justification and assurance.

Another useful stem/progenitor cell type which is also commonly used in cell therapy approaches is bone marrow-derived CD34-positive cells. CD34-positive cells are a well-characterized population of stem cells which are traditionally used to reconstitute hematopoietic cells after radiation or chemotherapy. The capability of CD34-positive cells in the induction of therapeutic angiogenesis is reported in a variety of animal models and includes pathological conditions like myocardial, peripheral, and cerebral ischemia as well as acute lung injury [4, 5]. Furthermore, different studies have shown promising results from treatment with CD34-positive cells in various clinical trials on patients with ischemic diseases. Despite increasing numbers of successful clinical trials which confirm the clinical significance of autologous CD34-positive cell therapy and introduce them as potent contributors in vascular, cardiovascular, and pulmonary repair, the selection of optimal time points, cell dosage, and route of administration still remains unclear. An optimized and common evaluation technique on therapeutic efficacy of cell therapy can help to solve these problems. In an interesting in vivo study, T.-H. Huang et al. on their article "Correlation between Therapeutic Efficacy of $CD34^+$ Cell Treatment and Directed *In Vivo* Angiogenesis in Patients with End-Stage Diffuse Coronary Artery Disease" have investigated the therapeutic efficacy of CD34-positive cell treatment in patients with end-stage diffuse coronary artery disease as reflected in the rate of angiogenesis/neovascularization pre- and posttreatment with autologous CD34-positive cells. This method is known as "angiographic grading". T.-H. Huang et al. have carefully compared the results of the angiogenic grading method with findings of the "directed in vivo angiogenesis assay (DIVAA)," which is a known quantitative assessment technique for angiogenic responses. They have finally suggested the DIVAA method as a better and reliable technique than angiographic grading for assessing coronary vascularization after CD34-positive cell treatment. DIVAA has a potential to become routine for assessment of angiogenic responses in the clinical setting.

Exosomes are cell-derived extracellular vesicles which can be found in perhaps all eukaryotic fluids and provide a means of intercellular communication. They correspond to intraluminal vesicles (ILVs) which are formed within intracellular multivesicular bodies (MVBs). ILVs are generated by invagination of the endosomal membrane via a dependent and/or independent manner to the endosomal sorting complexes required for transport. ILVs and exosomes as well will release from the cell into the extracellular space upon fusion of MVBs with plasma membrane. The specific contents of exosomes (e.g., RNAs, lipids, and proteins) which only originate from the cell cytosol or endosomal compartments appear to be as diverse as cell types and are highly dependent to the state of the cell and its changes. Exosomes can be potentially used as biomarkers for prognosis of different diseases and also for therapeutic purposes [6, 7]. J. A. Maring et al. contributed with a review entitled "Myocardial Regeneration via Progenitor Cell-Derived Exosomes" where they have broadly described the nature of exosomes in their review article and also explained the current state of progenitor cell-derived exosomes in experimental therapy of heart diseases and their translational potential in myocardial regeneration.

In agreement with the results of increasing numbers of clinical trials and various experimental reports, adult stem cell-based therapy is a promising novel approach for the treatment of various diseases. Biomaterial scaffolds can support the transplantation of stem cell by providing not only a physical support for transplanted cells but also chemical and physiological cues for the cells to facilitate tissue regeneration. The recent development of tissue and biomaterial engineering during last years resulted in a revolution in the field of regenerative medicine and gives a promising future in the field of organ regeneration and transplantation which can secure a better life quality for the patients. Despite various valuable studies in the field of tissue engineering, there is still a long way to find the best and desired biomaterial scaffolds which should be seeded with sufficient numbers of suitable stem cell types to assemble functional engineered constructs that preserve, maintain, and improve damaged tissue or whole organs. Within this special issue, A. O. J. Fakoya et al. published the review article entitled "Current Trends in Biomaterial Utilization for Cardiopulmonary System Regeneration" in which they have broadly discussed the current natural and synthetic biomaterial scaffolds which are utilized to regenerate cardiac and pulmonary organs. They have further highlighted advantages and disadvantages of each utilized biomaterial scaffold for cardiopulmonary system regeneration. This comprehensive review can be an insight to find out the best choice of biomaterial scaffolds to generate functional heart and lung tissue batched for transplantation.

In summary, this special issue offers a compact overview of the major findings concerning the impact of stem/progenitor cells in health, disease, and treatment of cardiopulmonary diseases. The recent developments should lead to new scientific insights into the function of stem/progenitor cells in the

context of potential therapeutic applications and next generation stem cell therapies for cardiopulmonary disorders. Combined and continued endeavor between basic, translational, and clinical research will hopefully generate an innovative and efficient means for the treatment or even cure of devastating cardiopulmonary diseases.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this editorial letter in the SPCCH special issue of *Stem Cells International* journal.

Fatemeh Sharifpanah
Hossein A. Ghofrani
Suk Ying Tsang
Heinrich Sauer

References

[1] T. Le and J. J. H. Chong, "Cardiac progenitor cells for heart repair," *Cell Death Discovery*, vol. 2, no. 1, article 16052, 2016.

[2] L. Bagno, K. E. Hatzistergos, W. Balkan, and J. M. Hare, "Mesenchymal stem cell-based therapy for cardiovascular disease: progress and challenges," *Molecular Therapy*, vol. 26, no. 7, pp. 1610–1623, 2018.

[3] G. M. Galstian et al., "The results of the Russian clinical trial of mesenchymal stromal cells (MSCs) in severe neutropenic patients (pts) with septic shock (SS) (RUMCESS trial)," *Blood*, vol. 126, p. 2220, 2015.

[4] B. C. Lo, M. J. Gold, S. Scheer et al., "Loss of vascular CD34 results in increased sensitivity to lung injury," *American Journal of Respiratory Cell and Molecular Biology*, vol. 57, no. 6, 2017.

[5] A. R. Mackie and D. W. Losordo, "CD34-positive stem cells: in the treatment of heart and vascular disease in human beings," *Texas Heart Institute Journal*, vol. 38, no. 5, pp. 474–485, 2011.

[6] C. Théry, L. Zitvogel, and S. Amigorena, "Exosomes: composition, biogenesis and function," *Nature Reviews Immunology*, vol. 2, no. 8, pp. 569–579, 2002.

[7] E. Van der Pol, A. N. Boing, P. Harrison, A. Sturk, and R. Nieuwland, "Classification, functions, and clinical relevance of extracellular vesicles," *Pharmacological Reviews*, vol. 64, no. 3, pp. 676–705, 2012.

Hindawi
Stem Cells International
Volume 2018, Article ID 9215792, 10 pages
https://doi.org/10.1155/2018/9215792

Research Article

The Milk Thistle (*Silybum marianum*) Compound Silibinin Inhibits Cardiomyogenesis of Embryonic Stem Cells by Interfering with Angiotensin II Signaling

Enas Hussein Ali,[1] Fatemeh Sharifpanah,[1] Amer Taha,[1] Suk Ying Tsang,[2,3] Maria Wartenberg,[4] and Heinrich Sauer[1]

[1]*Department of Physiology, Faculty of Medicine, Justus Liebig University Gießen, Germany*
[2]*School of Life Sciences, The Chinese University of Hong Kong, Hong Kong*
[3]*Key Laboratory for Regenerative Medicine, Ministry of Education, The Chinese University of Hong Kong, Hong Kong*
[4]*Department of Internal Medicine I, Division of Cardiology, Angiology, Pneumology, and Intensive Medical Care, University Hospital Jena, Friedrich Schiller University Jena, Germany*

Correspondence should be addressed to Heinrich Sauer; heinrich.sauer@physiologie.med.uni-giessen.de

Received 15 January 2018; Revised 29 June 2018; Accepted 6 September 2018; Published 13 December 2018

Academic Editor: Krisztian Nemeth

The milk thistle (*Silybum marianum* (L.) Gaertn.) compound silibinin may be an inhibitor of the angiotensin II type 1 (AT_1) receptor which is expressed in differentiating embryonic stem (ES) cells and is involved in the regulation of cardiomyogenesis. In the present study, it was demonstrated that silibinin treatment decreased the number of spontaneously contracting cardiac foci and cardiac cell areas differentiated from ES cells as well as contraction frequency and frequency of calcium (Ca^{2+}) spiking. In contrast, angiotensin II (Ang II) treatment stimulated cardiomyogenesis as well as contraction and Ca^{2+} spiking frequency, which were abolished in the presence of silibinin. Intracellular Ca^{2+} transients elicited by Ang II in rat smooth muscle cells were not impaired upon silibinin treatment, excluding the possibility that the compound acted on the AT_1 receptor. Ang II treatment activated extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH_2-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways in embryoid bodies which were abolished upon silibinin pretreatment. In summary, our data suggest that silibinin inhibits cardiomyogenesis of ES cells by interfering with Ang II signaling downstream of the AT_1 receptor.

1. Introduction

Silibinin is the pharmacologically most important compound of silymarin which contains different flavonolignans and is an extract from milk thistle (*Silybum marianum* (L.) Gaertn., Asteraceae) [1]. The pharmacologic actions of silibinin have been mainly attributed to its hepatoprotective and anticancer properties [2]. However, silibinin has been also shown to be pharmacologically active in the cardiovascular system. In this respect, it has been demonstrated to exert cardioprotective properties, e.g., following isoproterenol-induced cardiac myocyte injury [3, 4] or doxorubinin-mediated cardiotoxicity [5]. Moreover, silibinin reduced blood pressure and the incidence of postocclusion arrhythmias in spontaneously hypertensive rats, and it was suggested that this compound may be beneficial when used in hypertensive patients who develop acute myocardial infarction [6]. Silymarin exhibited significant antihypertensive activity in a DOCA salt model of hypertension [7]. In anesthetized open chest cats, silibinin lowered the amplitude and duration of diastolic blood pressure and produced a marked depression of cardiac contractility [8], suggesting that silibinin affects the hemodynamic properties of the heart.

The mechanism by which silibinin is pharmacologically active in the heart is so far not known. Recently, it was suggested that silibinin may act as an antagonist of angiotensin receptor 1 (AT_1) since it inhibited Ang II-mediated Ca^{2+} signals in Chinese hamster ovary (CHO) cells overexpressing

the AT_1 receptor [9]. The physiological impact of Ang II in the adult heart is so far not sufficiently investigated. Cardiomyocytes express the AT_1 as well as the AT_2 receptor [10]. In cultured cardiomyocytes, AT_1 receptors have been demonstrated to mediate apoptosis [11] or to promote hypertrophy [12, 13], depending on the experimental conditions and the expression pattern of AT receptor subtypes.

The renin-angiotensin aldosteron system (RAAS) is likely crucial for proper embryogenesis. Components of the RAAS are highly expressed in many tissues during embryonic development. AT_1 receptor expression is downregulated shortly after birth, whereas the AT_2 receptor is upregulated, suggesting a potential role of AT_1 in cell/tissue differentiation processes during embryogenesis and a potential role of AT_2 in adult organ function [14]. In fetal ovine cardiomyocytes, Ang II stimulates hyperplastic growth [15], indicating that Ang II is involved in fetal heart growth. In ES cells, Ang II has been shown to regulate glucose uptake [16], supporting the notion that Ang II may play a role in energy metabolism during embryogenesis. Notably, Ang II has been demonstrated to stimulate cardiomyogenesis [17] and smooth muscle differentiation [18] of ES cells. In differentiating ES cell-derived embryoid bodies, the AT_1 receptor is expressed already at very early stages of cardiac cell commitment. Moreover, besides insulin-like growth factor (IGF) receptors, AT_1 receptor expression has been shown to be present in human cardiac stem cells [19], thus outlining an impact of Ang II signaling in differentiation and/or cardiac progenitor cell proliferation.

In the present study, we investigated the effect of silibinin on cardiomyogenesis of ES cells. Our data demonstrate that silibinin inhibited cardiac cell differentiation and contraction frequency. Notably, silibinin abolished Ang II-mediated procardiogenic effects and decreased Ca^{2+} spiking frequency without interfering with Ang II receptor function. In conclusion, our data suggest that silibinin interferes with Ang II-mediated signaling pathways by inhibition of mitogen-activated protein kinases (MAPKs) downstream of the AT_1 receptor.

2. Materials and Methods

2.1. Materials. Silibinin-C-2′,3-dihydrogen succinate, disodium salt (Legalon SIL) was a generous gift from MEDA Pharma GmbH & Co. KG (Bad Homburg, Germany). Drug substance was as follows: silibinin-C-2′3-dihydrogen succinate, 528.5 mg (corresponding to 476 mg mono-, dihydrogensuccinate sodium salts (HPLC)) equivalent to 350 mg of silibinin. The drug substance contained 70 mg inulin (USP) as excipient. Ang II, FGF-2, L-NAME, and LY294002 were purchased from Sigma-Aldrich (Munich, Germany). Eicosapentanoic acid (EPA) was from Tocris Bioscience (Wiesbaden, Germany).

2.2. Cell Culture of ES Cells and Embryoid Body Formation. Mouse ES cells (line CCE) were grown on mitotically inactivated feeder layers of primary mouse embryonic fibroblasts (purchased from Amsbio, Abingdon, UK) in Iscove's basal medium (Biochrom, Berlin, Germany) supplemented with 15% heat-inactivated (56°C, 30 min) foetal calf serum (FCS) (Sigma-Aldrich), 2 mM glutamine, (PAA, Cölbe, Germany), 100 μM 2-mercaptoethanol (Sigma-Aldrich), 1% (v/v) NEA nonessential amino acid stock solution (Biochrom), 1 mM Na^+-pyruvate (Biochrom), 0.4% penicillin/streptomycin (Biochrom), and 1000 U/ml leukemia inhibitory factor (LIF) (Merck Millipore, Darmstadt, Germany) in a humidified environment containing 5% CO_2 at 37°C, and passaged every 2-3 days. Adherent cells were enzymatically dissociated using 0.05% trypsin-EDTA in phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA, USA) and seeded at a density of $3 \cdot 10^6$ cells/ml in 250 ml siliconized spinner flasks (CellSpin, Integra Biosciences, Fernwald, Germany) containing 125 ml Iscove's medium supplemented as described above, but devoid of LIF. Following 24 h, 125 ml medium was added to give a final volume of 250 ml. The spinner flask medium was stirred at 20 r.p.m. using a stirrer system (Integra Biosciences). The spinning direction was changed every 1440°. 125 ml cell culture medium was exchanged every day.

2.3. Immunohistochemistry. As the primary antibody, a mouse monoclonal anti-α-actinin antibody (Abcam, Cambridge, UK) (dilution 1 : 100) was used. The embryoid bodies were fixed in ice-cold methanol for 20 min at −20°C and washed with phosphate-buffered saline (PBS) containing 0.01% Triton X-100 (PBST). Blocking against unspecific binding was performed for 60 min at room temperature with 10% heat-inactivated FCS (AppliChem, Darmstadt, Germany) dissolved in PBST. Embryoid bodies were subsequently incubated overnight at 4°C with primary antibody (dilution 1 : 100) dissolved in PBST supplemented with 10% FCS. The embryoid bodies were thereafter washed three times with PBST and reincubated for 1 h at room temperature in the dark with Alexa Fluor 647 sheep anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1 : 100 in PBST containing 10% FCS. After washing three times with PBST, the tissues were stored in PBST until inspection.

2.4. Western Blot Analysis. Protein extraction was carried out after washing the embryoid bodies in PBS and lysing in RIPA lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM glycerophosphate, 0.1% SDS, and 1% Nonidet P-40) supplemented with protease inhibitor cocktail (PXBioVisioN, Hannover, Germany) and phosphatase inhibitor cocktail (Sigma-Aldrich) for 20 min on ice. Samples were centrifuged at 24,700*g* for 10 min at 4°C to pellet the debris. After determination of the protein concentration using a Lowry protein assay, 20 μg of protein samples was boiled for 10 min at 70°C, separated in PAGE Ex Precast gels (4–12%) (Lonza, Cologne, Germany), and transferred to PVDF membranes by the XCell SureLock Mini-Cell Blot Module (Invitrogen) at 180 mA for 90 min. Membranes were blocked with 5% (wt/vol) dry fat-free milk powder in Tris-buffered saline with 0.1% Tween (TBST) for 60 min at room temperature. Incubation with the primary antibody was performed at 4°C overnight. Used primary antibodies were phospho-ERK (Thr202/Tyr204), phospho-38 (Thr180/

Tyr182), phospho-JNK (Thr183/Tyr185), and cleaved caspase 3 (Asp 175) (Cell Signaling Technology Europe, Frankfurt, Germany). Primary antibodies against either vinculin or β-actin (Sigma-Aldrich), which are housekeeping proteins, were used for the standardization of blotting. After washing with 0.1% TBST, the membrane was incubated with a horseradish peroxidase- (HRP-) conjugated secondary antibody (dilution 1 : 1000) (Abcam, Cambridge, UK) for 60 min at room temperature. The blot was developed using an enhanced chemiluminescence (ECL) solution to produce a chemiluminescence signal. For quantification, the density of the protein bands on the western blot image, which was acquired using the Peqlab gel documentation system (VWR International, Darmstadt, Germany), was assessed by ImageJ [20]. The final quantification reflects the relative amounts of protein as a ratio of each target protein band to the respective housekeeping protein.

2.5. Recording of Intracellular Ca^{2+} Concentrations. Intracellular Ca^{2+} was recorded in single cardiac contracting cells. Single cell preparations were obtained by enzymatic digestion of 7-day-old embryoid bodies for 30 min at 37°C in PBS containing 2 mg/ml Collagenase B (Roche, Mannheim, Germany). Dissociated single cells were plated onto gelatin-coated cover slips in 24-well cell culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany), and cultivated in Iscove's medium supplemented with 15% FCS. Following 24 h of culture, cells were loaded in serum-free medium with 1 μM Fluo-4/AM (Life Technologies) for 30 min. Subsequently, the cover slips were transferred in fresh serum-free cell culture medium to the incubation chamber of a confocal laser scanning microscope (Leica SP2, AOBS, Leica, Bensheim, Germany). Fluorescence excitation was performed at 488 nm, and emission was recorded at 500–550 nm. Sampling rate was 2 frames/s. The fluorescence emission of single cells was assessed by using the image analysis software of the confocal setup.

2.6. Statistical Analysis. For statistical analysis, GraphPad InStat statistics software (GraphPad Software Inc., La Jolla, CA) was used. Data are given as mean values ± standard deviation (S.D.), with n denoting the number of experiments performed with independent ES cell cultures. In each experiment, at least 20 culture objects were analyzed unless otherwise indicated. Student's t-test for unpaired data and one-way ANOVA was applied as appropriate for statistical analysis. A value of $P \leq 0.05$ was considered significant.

3. Results

3.1. Inhibition of Cardiomyogenesis of ES Cells and Contractility of Differentiated Cardiac Cells. To examine the effects of silibinin on the differentiation of cardiomyocytes, embryoid bodies were treated from day 3 until day 10 with different concentrations of silibinin (1 μM, 10 μM, 20 μM, and 50 μM). From day 7 to day 10, the number of contracting cardiac foci, the size of the α-actinin-positive cardiac area, and the contraction frequency were assessed. It was observed that silibinin treatment dose-dependently decreased the number of contracting foci (Figure 1(a)), the size of α-actinin-positive cell areas (Figure 1(b)), and the contraction frequency (Figure 1(c)). To investigate in more detail the time course of decline in the contraction frequency of cardiac areas, 10-day-old embryoid bodies were incubated with 20 μM silibinin and the decrease in contraction frequency was assessed over time. It was observed that within 1 h of incubation a significant slowdown of contraction frequency occurred, which decreased further over 8 h (Figure 1(d)). To exclude the possibility that silibinin induced apoptosis in embryoid bodies, cleaved caspase-3 was assessed after 7 days of incubation with either 20 or 50 μM silibinin. It was evidenced that silibinin did not induce apoptosis under the experimental conditions of the present study (supplemental Figure 1).

3.2. Effect of Silibinin on Angiotensin II-Induced Cardiomyogenesis and Contraction Frequency. Previous studies have shown that Ang II stimulated the cardiomyogenesis of ES cells [17]. Moreover, a recent study demonstrated that silibinin may act as an Ang II receptor 1 (AT_1) antagonist [9]. Since the data of the present study evidenced that silibinin decreased the cardiomyogenesis of ES cells and the frequency of contractions, we investigated whether silibinin would interfere with Ang II-induced cardiomyogenesis and contraction frequency. To characterize the effect of silibinin on Ang II-mediated cardiomyogenesis of ES cells, the number of contracting cardiac foci was counted from day 7 to day 14 of the cell culture either in the absence or presence of silibinin. It was apparent that Ang II (1 μM) increased the number of cardiac foci, which was completely blunted upon coincubation with silibinin (20 μM) (Figure 2(a)). Moreover, we assessed the size of α-actinin-positive cell areas on day 14 and demonstrated that Ang II significantly increased cardiac cell areas, which was completely abolished in the presence of silibinin (Figure 2(b)). To investigate whether the contraction frequency was affected by silibinin and Ang II treatment, we calculated the frequency of contractions per minute. We found that the contraction frequency was significantly increased upon Ang II treatment compared to the untreated control, whereas silibinin (20 μM) alone significantly decreased contraction frequency. Preincubation with silibinin abolished the increase in contraction frequency achieved with Ang II (Figure 2(c)).

3.3. Effect of Silibinin on Ca^{2+} Oscillations upon Angiotensin II Treatment. Spontaneous contractions and action potentials in cardiac cells are associated to rhythmic Ca^{2+} oscillations. Since our data demonstrated that Ang II treatment stimulated the cardiomyogenesis of ES cells, we investigated whether Ang II treatment would have an impact on cardiac cell function. To achieve this aim, contracting embryoid bodies (day 7 of cell culture) were enzymatically dissociated, labeled with the Ca^{2+}-sensitive fluorescence dye Fluo-4, AM on day 8, and intracellular Ca^{2+} oscillations were recorded in single cardiac cells after different times of incubation (200 s, 600 s, and 1500 s) with either Ang II (1 μM), silibinin (20 μM), or a combination of both. It was evident that

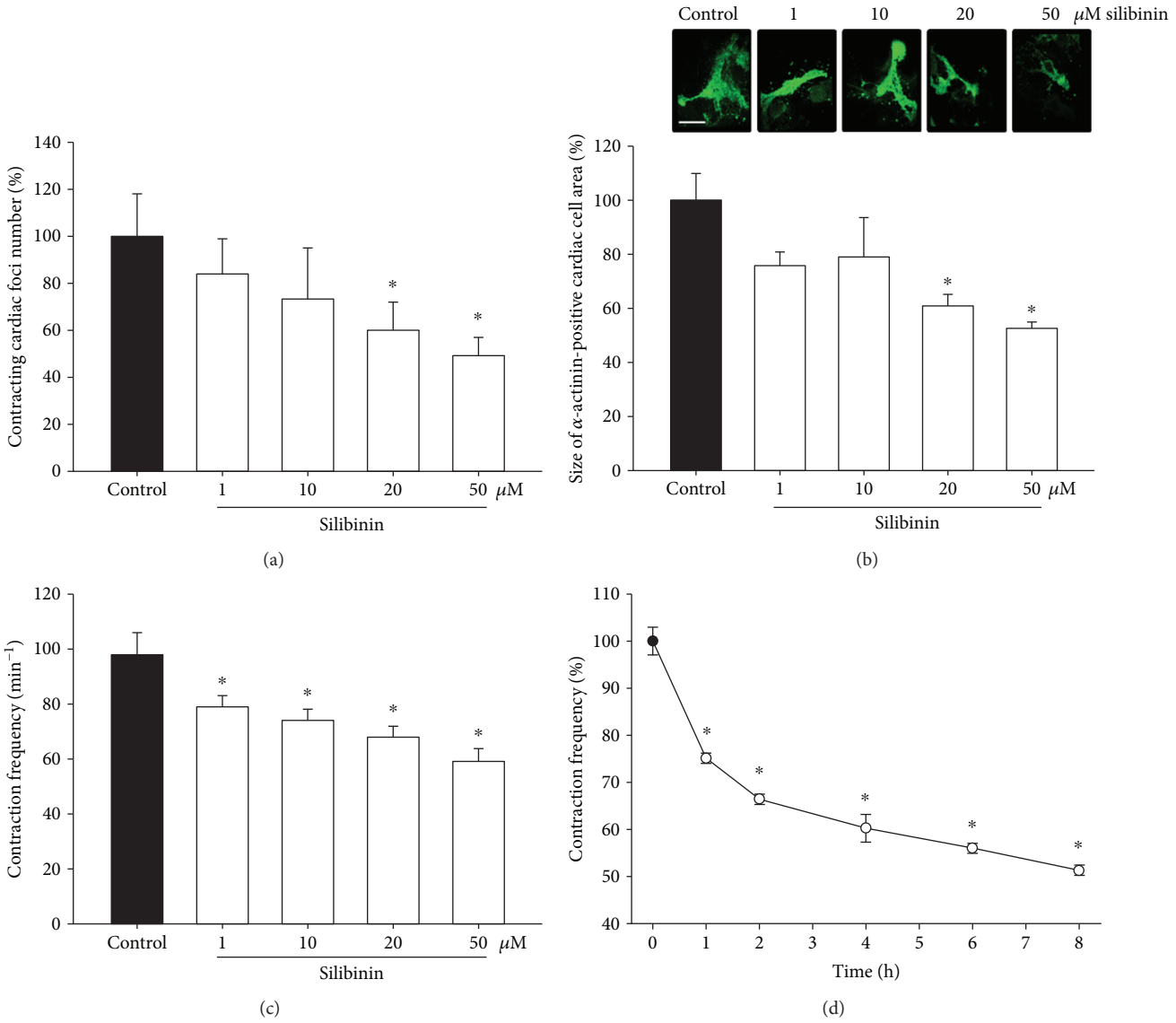


Figure 1: Effect of increasing doses of silibinin on (a) the number of contracting cardiac foci differentiated from ES cells ($n = 4$), (b) the size of cardiac foci ($n = 3$), and (c) the contraction frequency ($n = 4$). (d) Image shows the decay in contraction frequency over time following treatment with 20 μM silibinin ($n = 3$). The images in (b) show representative cardiac cell areas immunolabeled with an antibody against α-actinin. The bar represents 300 μm. $^*P \leq 0.05$, significantly different to the untreated control.

silibinin treatment decreased the frequency of Ca^{2+} spikes (Figures 3(b) and 3(e)) as compared to the untreated control (Figures 3(a) and 3(e)). In contrast, an increase in spiking frequency was observed upon Ang II treatment (Figures 3(c) and 3(e)). However, when Ang II was applied with silibinin, the stimulation of Ca^{2+} spiking frequency was abolished, which indicates that silibinin interferes with Ang II-mediated signaling pathways (Figures 3(d) and 3(e)).

3.4. Effect of Silibinin on Ang II-Induced Ca^{2+} Responses in Rat Smooth Muscle Cells. A previous report on CHO cells overexpressing the AT_1 receptor suggested that silibinin may be an AT_1 receptor antagonist that inhibited the Ang II-mediated Ca^{2+} response [9]. To examine this assumption, rat smooth muscle cells, which are well known to express the AT_1 receptor [21], were exposed to Ang II (1 μM) in the absence or presence of silibinin (20 μM) (Figure 3(f)). It was observed that Ang II raised Ca^{2+} even in the presence of silibinin, which suggests that silibinin does not affect AT_1 receptor function, but may interfere with Ang II-mediated signaling pathways downstream of the AT_1 receptor. The Ang II-induced Ca^{2+} response could not be inhibited at silibinin concentrations up to 100 μM (supplemental Figure 2).

3.5. Silibinin Inhibits Ang II-Mediated Activation of ERK1/2, JNK, and p38. Since silibinin did not affect the Ang II-mediated Ca^{2+} response, we assumed that it may interfere with downstream signaling cascades. Since it has been previously shown that Ang II activates ERK1/2, JNK, and p38 in differentiating ES cells [17], we investigated whether silibinin (20 μM) would abolish MAPK activation upon treatment of

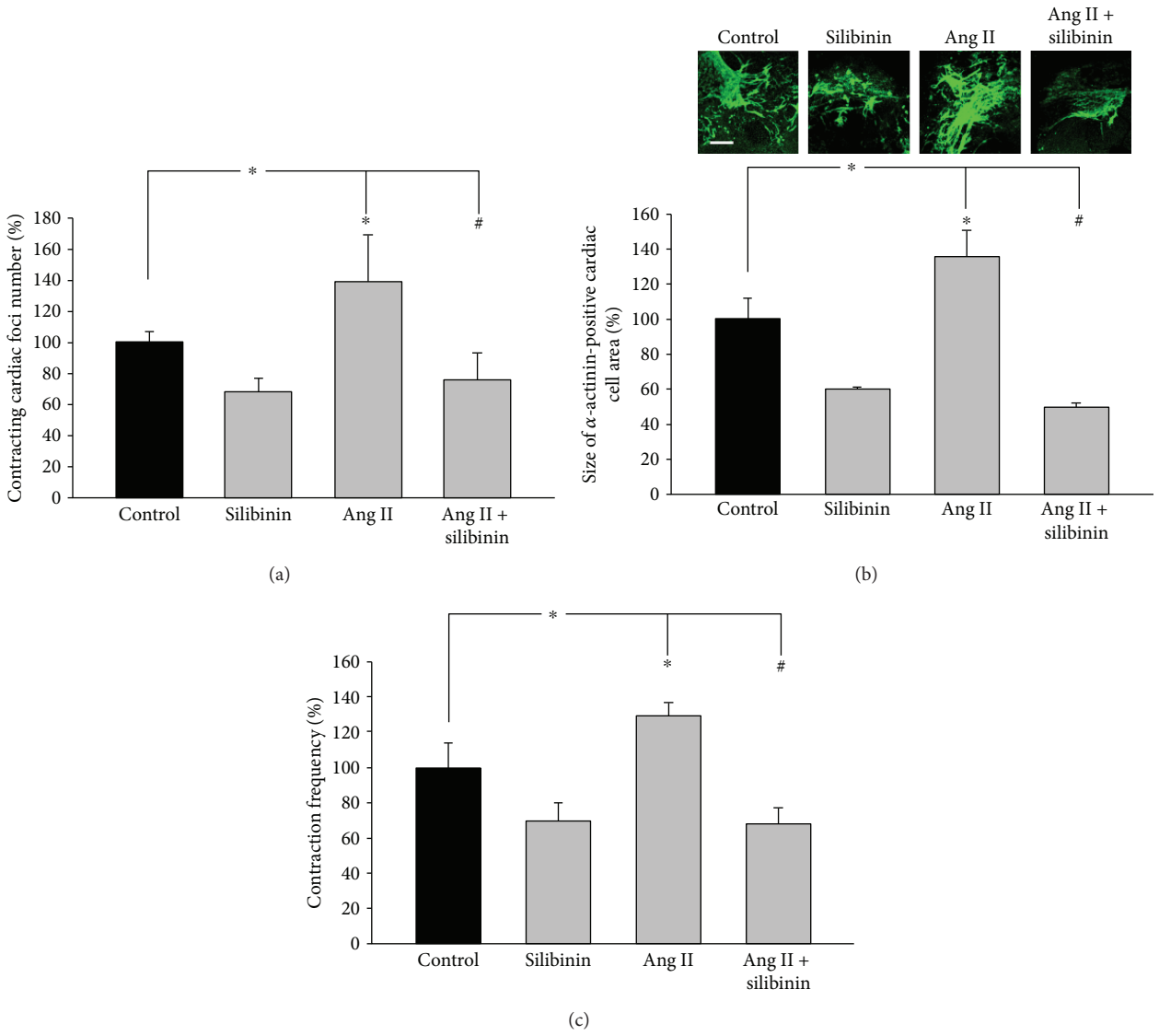


Figure 2: Inhibitory effect of silibinin on Ang II-induced cardiomyogenesis and contraction frequency. (a) Number of contracting cardiac foci differentiated from ES cells ($n = 5$), (b) size of cardiac cell areas ($n = 4$), and (c) contraction frequency ($n = 5$). The images in (b) show representative cardiac cell areas immunolabeled with an antibody against α-actinin. The bar represents 300 μm. Embryoid bodies were treated from day 3 to day 14 of cell culture with either silibinin (20 μM), Ang II (1 μM), or a combination of both. $^{*}P \leq 0.05$, significantly different to the untreated control. $^{\#}P \leq 0.05$, significantly different to the Ang II-treated sample.

embryoid bodies with Ang II (1 μM). Indeed, silibinin treatment of 6-day-old embryoid bodies efficiently abolished the Ang II-mediated activation of ERK1/2 (Figure 4(a)), p38 (Figure 4(b)), and JNK (Figure 4(c)) as evaluated using phosphospecific antibodies. These data corroborated our assumption that silibinin interfered with Ang II signaling downstream of the AT_1 receptor.

3.6. Effect of Silibinin on FGF-2 and EPA-Induced Cardiomyogenesis of ES Cells. Treatment of differentiating ES cells with either FGF-2 [22] or the omega-3 polyunsaturated fatty acid EPA [23] has been previously demonstrated to stimulate cardiomyogenesis. To investigate whether silibinin would block the stimulatory effect of other agents on the cardiomyogenesis of ES cells, embryoid bodies were treated from day 3 to day 10 of differentiation with either FGF-2 (10 ng/ml) or EPA (50 μM) in the absence or presence of silibinin (20 μM), and the number of spontaneously contracting cardiac foci was assessed (Figures 5(a) and 5(b)). Our data demonstrated that silibinin completely inhibited the stimulation of cardiomyogenesis by FGF-2 (Figure 5(a)). In contrast, the stimulation of cardiomyogenesis by EPA could not be blocked upon coincubation with silibinin (Figure 5(b)). In further experiments, we investigated whether interference with signaling pathways, i.e., phosphoinositide 3-kinase PI3-K or nitric oxide (NO) which have been previously shown to be important in silibinin-driven cellular changes [24], would block the inhibitory effect of silibinin on cardiomyogenesis. The results of these experiments (Figure 5(c)) showed, that indeed inhibition of PI3-K by LY294002 (5 μM) abolished the anticardiomyogenic effect of silibinin and even stimulated cardiomyogenesis above the level of

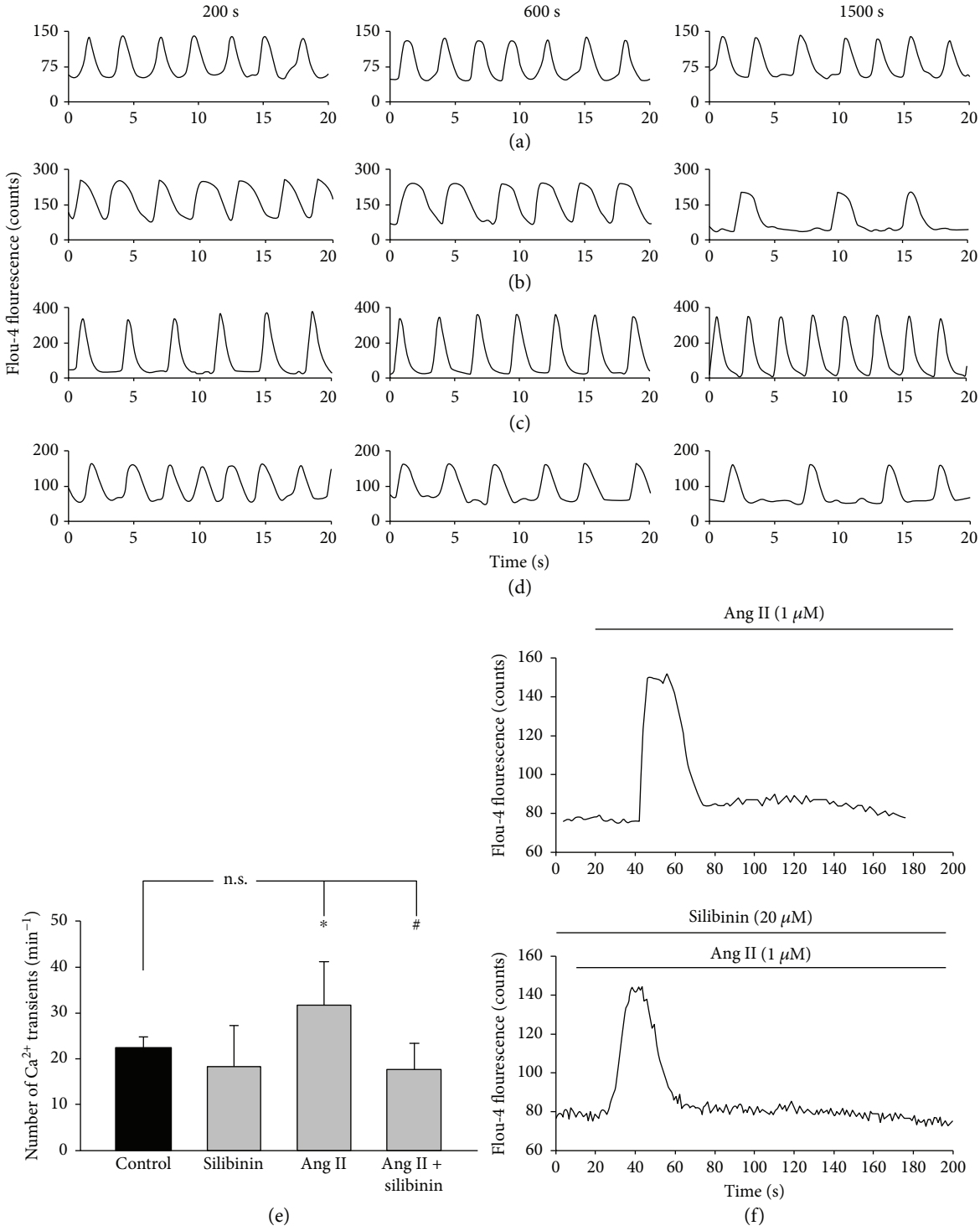


Figure 3: Effects of Ang II and silibinin on the frequency of Ca^{2+} transients in cardiac cells differentiated from ES cells. Cardiac cells were enzymatically dissociated from 7-day-old embryoid bodies and labeled on day 8 with the Ca^{2+}-sensitive fluorescence dye Fluo-4. Ca^{2+} spiking was evaluated in 3 different time windows, i.e., 200 s, 600 s, and 1500 s. Shown are representative traces of individual cells. (a) Untreated controls, (b) silibinin- (20 μM) treated cells, (c) Ang II- (1 μM) treated cells, and (d) cells treated with a combination of Ang II (1 μM) and silibinin (20 μM). The bar chart in (e) shows the means ± S.D. of 10 experiments. $^{*}P \leq 0.05$, significantly different to the untreated control. $^{\#}P \leq 0.05$, significantly different to the Ang II-treated sample. (f) Representative Ca^{2+} transients in rat smooth muscle cells. Upper panel: cells were treated with Ang II (1 μM) and changes in Fluo-4 fluorescence were recorded. Bottom panel: cells were preincubated for 60 min with silibinin (20 μM) and subsequently treated with Ang II (1 μM) ($n = 3$).

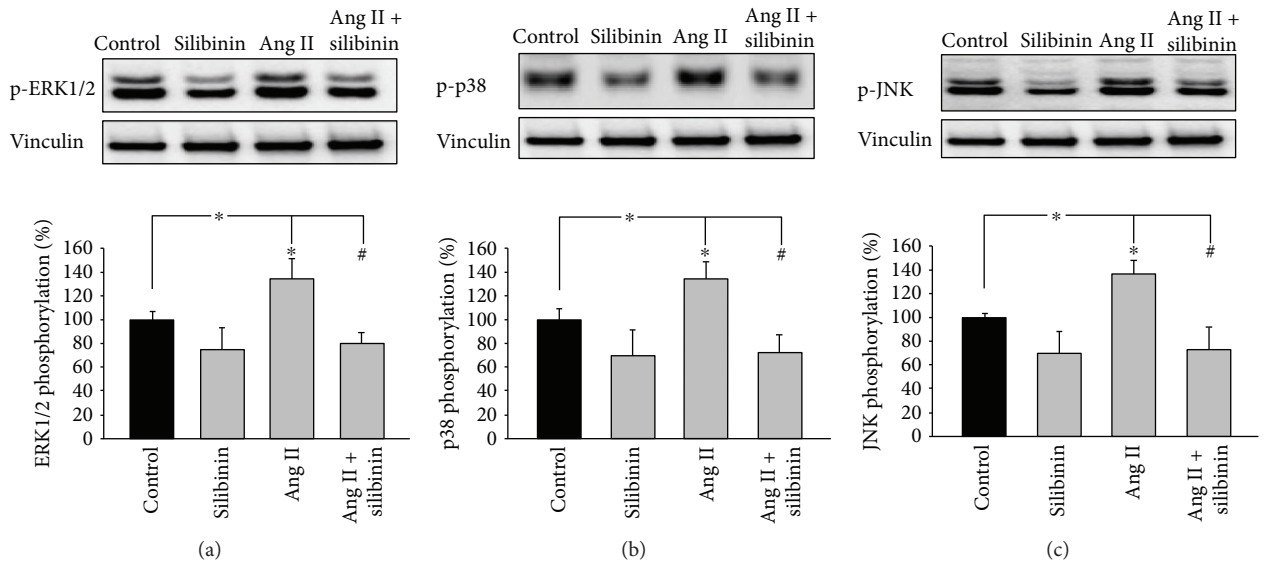


Figure 4: Effects of Ang II and silibinin on the activation of (a) ERK1/2 ($n = 6$), (b) p38 ($n = 5$), and (c) JNK ($n = 5$). 6-Day-old embryoid bodies remained either untreated or were treated with Ang II (1 μM), silibinin (20 μM), or a combination of both. MAPK activation was monitored after 15 min of incubation with Ang II by western blot analysis using phosphospecific antibodies. Shown are representative western blots. The bar charts show the means ± S.D. of ($n = 6$) experiments for ERK1/2 and ($n = 5$) experiments for p38 and JNK, respectively. $^{*}P \leq 0.05$, significantly different to the untreated control. $^{\#}P \leq 0.05$, significantly different to the Ang II-treated sample.

the untreated control. Moreover, inhibition of endothelial NO synthase (eNOS) partially abolished the adverse action of silibinin on the cardiomyogenesis of ES cells.

4. Discussion

Previous studies have shown that the vasoactive hormone Ang II stimulated the cardiomyogenesis [17] as well as smooth muscle cell differentiation [18] of ES cells. Moreover, a recent study evidenced that silibinin may act as an AT_1 receptor antagonist [9].

The data of the present study demonstrated that silibinin dose-dependently inhibited cardiomyogenesis of ES cells. Moreover, silibinin decelerated the frequency of Ca^{2+} spikes in differentiated cardiac cells. To investigate whether the effects of silibinin on cardiomyogenesis and cardiac cell function were due to the inhibition of Ang II-mediated signaling pathways, we investigated whether silibinin treatment would abolish the stimulation of cardiomyogenesis achieved upon Ang II treatment of differentiating ES cells. In corroboration with the data of Wu et al. [17], we observed the stimulation of cardiomyogenesis following incubation with Ang II. Moreover, Ang II treatment increased the contraction frequency of cardiac areas differentiated from ES cells and the frequency of Ca^{2+} spikes in differentiated cardiac cells. The stimulation of cardiomyogenesis as well as the increase in Ca^{2+} spiking frequency achieved with Ang II was completely abolished upon cotreatment with silibinin, supporting the notion that silibinin is interfering with Ang II signaling. Previous studies of Wu et al. [17] and Zheng et al. [18] suggested that the effects of Ang II on cardiac and smooth muscle cell differentiation were mediated via the AT_1 receptor, since the specific AT_1 receptor antagonist losartan abolished the observed effects. Notably, silibinin has been discussed to act as an AT_1 receptor antagonist in CHO cells which were stably transfected with the human AT_1 receptor [9]. We therefore investigated whether silibinin would abolish the Ang II-mediated Ca^{2+} response in smooth muscle cells which are well known to express the AT_1 receptor [25]. Interestingly, it was observed that silibinin was not able to inhibit the Ca^{2+} response elicited by Ang II even at high (100 μM) concentrations. Thus, the data of the present study argue against an involvement of AT_1 receptor inhibition by silibinin at least in the physiological Ang II concentrations (1 μM) used in our experiments.

If the AT_1 receptor activity and Ca^{2+} signaling are not affected, silibinin could possibly interfere with the signaling cascade further downstream. It has been previously described in rat neonatal cardiomyocytes that Ang II activates ERK1/2, p38, and JNK, whereby the phosphorylation of p38 and JNK is dependent on reactive oxygen species (ROS) generation [26]. In the experiments of the present study, silibinin significantly inhibited ERK1/2, p38, and JNK activity as compared to the untreated control, whereas MAPK stimulation was observed upon Ang II treatment. According to our assumptions, silibinin totally abolished the stimulation of all members of the MAPK family by Ang II, which indicates that the compound interferes with the MAPK signaling cascade downstream of the AT_1 receptor. Moreover, silibinin blunted the procardiomyogenic effect of FGF-2, which should be expected since FGF-2 signaling has several crossovers with Ang II signaling, including the activation of MAPK pathways. In contrast, silibinin failed to abolish the EPA-induced stimulation of cardiomyogenesis, suggesting that EPA activates signaling pathways which are distinct from Ang II signaling and MAPK activation. Recent data from our group [24] demonstrated that silibinin increases nitric oxide (NO) generation and activates endothelial NO

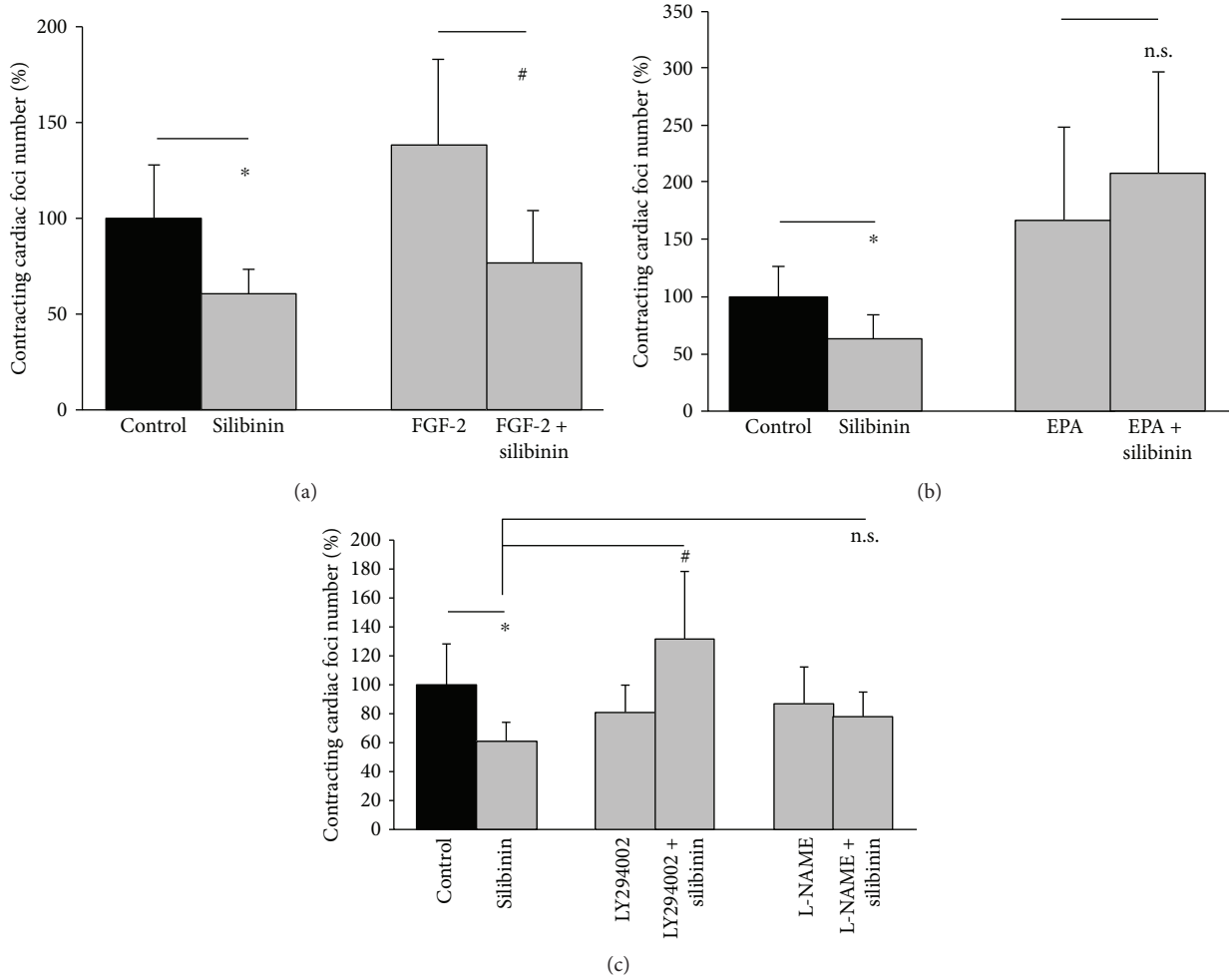


Figure 5: Effect of the procardiogenic agents FGF-2 (a) and EPA (b) on the inhibition of cardiomyogenesis by silibinin. Embryoid bodies were treated from day 3 to day 10 of differentiation with silibinin (20 μM) alone or with either FGF-2 (10 ng/ml) ($n = 5$) or EPA (50 μM) ($n = 5$) in the absence or presence of silibinin. On day 10, the number of contracting cardiac foci was counted. (c) Interference with PI3-K and NO signaling and its impact on the inhibition of cardiomyogenesis by silibinin. Embryoid bodies were treated from day 3 to day 10 with either silibinin (20 μM) alone or with either the PI3-K inhibitor LY294002 (5 μM) ($n = 5$) or L-NAME (100 μM) ($n = 5$) and the number of contracting cardiac foci was counted. $^{*}P \leq 0.05$, significantly different to the untreated control. $^{\#}P \leq 0.05$, significantly different to the FGF-2 or LY294002-treated sample. n.s., not significant.

synthase. Notably, inhibition of NO generation by L-NAME partially reversed the adverse effect of silibinin on cardiomyogenesis. Moreover, inhibition of PI3-K reversed the silibinin effect and even stimulated cardiomyogenesis above the level of the untreated control. PI3-K is well known to activate eNOS which generates NO [27]. Since NO is well known as a free radical scavenger for ROS [28], it may be assumed that the inhibitory effect of silibinin on MAPK activity may be due to its capacity to raise NO concentration in the tissue. Previous data from others who showed that silibinin protects H9c2 cardiac cells from oxidative stress and inhibits phenylephrine-induced hypertrophy, presumably by repression of the phenylephrine-induced phosphorylation of ERK1/2 kinases [29], are pointing to the same direction. Moreover, the property of silibinin to act as a free radical scavenger has been validated in several studies [24, 30, 31].

The RAAS has been shown to exert a deep impact on cardiac development [32]. In humans, all components of RAAS are expressed at very early stages of embryogenesis (30–35 days of gestation) in different organs, suggesting that Ang II likely plays a role in the growth and differentiation of various organotypic cells [33]. Although triple knockouts of the AT_{1a}, AT_{1b}, and AT_2 receptors are viable and fertile, the lack of both AT_1 subtypes was associated with atrophic changes in the myocardium, a reduced coronary flow, and a reduced left ventricular systolic pressure [34, 35]. Recently, it has been outlined that antihypertensive medication of pregnant women is associated with an increased risk for congenital heart defects. This was the case for the treatment with β-blockers as well as with the use of renin-angiotensin system blockers [36]. Milk thistle seeds as well as their pharmacologically active ingredients are frequently used as dietary herbal supplements mainly to detoxify the liver. Since the data of the

present study demonstrate that silibinin inhibits cardiac differentiation of ES cells and affects Ang II-mediated signaling cascades, its use should be avoided in pregnant women.

Abbreviations

Ang II: Angiotensin II
AT_1: Angiotensin II type 1
Ca^{2+}: Calcium
CHO: Chinese hamster ovary
DOCA: Deoxycorticosterone acetate
ECL: Enhanced chemiluminescence
eNOS: Endothelial NO synthase
EPA: Eicosapentanoic acid
ERK1/2: Extracellular signal-regulated kinase 1/2
FCS: Foetal calf serum
FGF-2: Fibroblast growth factor-2
HRP: Horseradish peroxidase
JNK: C-Jun NH_2-terminal kinase
IGF: Insulin-like growth factor
MAPK: Mitogen-activated protein kinase
NO: Nitric oxide
PBS: Phosphate-buffered saline
RAAS: Renin-angiotensin aldosteron system
ROS: Reactive oxygen species.

Data Availability

There are no data in the present study which have been published previously.

Conflicts of Interest

The authors have no competing interests to declare.

Acknowledgments

This work was supported by a scholarship for E. Hussein Ali from the Ministry of Higher Education & Scientific Research, Kufa University, Iraq as well as by the Excellence Cluster Cardiopulmonary System (ECCPS) (German Research Foundation).

Supplementary Materials

Supplemental Figure 1: effect of silibinin on apoptosis induction in differentiating ES cells. Embryoid bodies were treated from day 3 to 10 of differentiation with silibinin (20, 50 μM) and cleaved caspase 3 expression was evaluated by western blot ($n = 5$). β-Actin was used as loading control. n.s., not significant. Supplemental Figure 2: effect of preincubation (60 min) with silibinin (100 μM) on Ang II-induced Ca^{2+} transients in adult rat smooth muscle cells. *(Supplementary Materials)*

References

[1] P. Surai, "Silymarin as a natural antioxidant: an overview of the current evidence and perspectives," *Antioxidants*, vol. 4, no. 1, pp. 204–247, 2015.

[2] Neha, A. S. Jaggi, and N. Singh, "Silymarin and its role in chronic diseases," in *Drug Discovery from Mother Nature*, vol. 929 of Advances in Experimental Medicine and Biology, , pp. 25–44, Springer, 2016.

[3] B. Zhou, L. J. Wu, S. Tashiro, S. Onodera, F. Uchiumi, and T. Ikejima, "Silibinin protects rat cardiac myocyte from isoproterenol-induced DNA damage independent on regulation of cell cycle," *Biological and Pharmaceutical Bulletin*, vol. 29, no. 9, pp. 1900–1905, 2006.

[4] B. Zhou, L. J. Wu, L. H. Li et al., "Silibinin protects against isoproterenol-induced rat cardiac myocyte injury through mitochondrial pathway after up-regulation of SIRT1," *Journal of Pharmacological Sciences*, vol. 102, no. 4, pp. 387–395, 2006.

[5] A. Raskovic, N. Stilinovic, J. Kolarovic, V. Vasovic, S. Vukmirovic, and M. Mikov, "The protective effects of silymarin against doxorubicin-induced cardiotoxicity and hepatotoxicity in rats," *Molecules*, vol. 16, no. 10, pp. 8601–8613, 2011.

[6] H. Chen, S. C. Chen, T. H. Zhang, H. C. Tian, Y. Guan, and D. F. Su, "Protective effects of silybin and tetrandrine on the outcome of spontaneously hypertensive rats subjected to acute coronary artery occlusion," *International Journal of Cardiology*, vol. 41, no. 2, pp. 103–108, 1993.

[7] G. B. Jadhav and C. D. Upasani, "Antihypertensive effect of silymarin on DOCA salt induced hypertension in unilateral nephrectomized rats," *Oriental Pharmacy and Experimental Medicine*, vol. 11, no. 2, pp. 101–106, 2011.

[8] Y. C. Rui, X. S. Chen, A. H. Guan, and J. S. Han, "Effects of silybin on hemodynamics in anesthetized open-chest cats," *Zhongguo Yao Li Xue Bao*, vol. 7, no. 1, pp. 34–36, 1986.

[9] R. Bahem, A. Hoffmann, A. Azonpi, C. Caballero-George, and P. Vanderheyden, "Modulation of calcium signaling of angiotensin AT_1, endothelin ET_A, and ET_B receptors by silibinin, quercetin, crocin, diallyl sulfides, and ginsenoside Rb1," *Planta Medica*, vol. 81, no. 8, pp. 670–678, 2015.

[10] S. Busche, S. Gallinat, R. M. Bohle et al., "Expression of angiotensin AT_1 and AT_2 receptors in adult rat cardiomyocytes after myocardial infarction: a single-cell reverse transcriptase-polymerase chain reaction study," *The American Journal of Pathology*, vol. 157, no. 2, pp. 605–611, 2000.

[11] J. Kajstura, E. Cigola, A. Malhotra et al., "Angiotensin II induces apoptosis of adult ventricular myocytes in vitro," *Journal of Molecular and Cellular Cardiology*, vol. 29, no. 3, pp. 859–870, 1997.

[12] Y. Liu, A. Leri, B. Li et al., "Angiotensin II stimulation in vitro induces hypertrophy of normal and postinfarcted ventricular myocytes," *Circulation Research*, vol. 82, no. 11, pp. 1145–1159, 1998.

[13] L. Chen, L. Zhao, A. Samanta et al., "STAT3 balances myocyte hypertrophy vis-à-vis autophagy in response to angiotensin II by modulating the AMPKα/mTOR axis," *PLoS One*, vol. 12, no. 7, article e0179835, 2017.

[14] J. Gao, J. Chao, K. J. K. Parbhu et al., "Ontogeny of angiotensin type 2 and type 1 receptor expression in mice," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 13, no. 3, pp. 341–352, 2012.

[15] N. C. Sundgren, G. D. Giraud, P. J. S. Stork, J. G. Maylie, and K. L. Thornburg, "Angiotensin II stimulates hyperplasia but not hypertrophy in immature ovine cardiomyocytes," *The Journal of Physiology*, vol. 548, no. 3, pp. 881–891, 2003.

[16] H. J. Han, J. S. Heo, and Y. J. Lee, "ANG II increases 2-deoxyglucose uptake in mouse embryonic stem cells," *Life Sciences*, vol. 77, no. 15, pp. 1916–1933, 2005.

[17] L. Wu, Z. Jia, L. Yan et al., "Angiotensin II promotes cardiac differentiation of embryonic stem cells via angiotensin type 1 receptor," *Differentiation*, vol. 86, no. 1-2, pp. 23–29, 2013.

[18] X. Zheng, Y. Wu, L. Zhu et al., "Angiotensin II promotes differentiation of mouse embryonic stem cells to smooth muscle cells through PI3-kinase signaling pathway and NF-κB," *Differentiation*, vol. 85, no. 1-2, pp. 41–54, 2013.

[19] D. D'Amario, M. C. Cabral-Da-Silva, H. Zheng et al., "Insulin-like growth factor-1 receptor identifies a pool of human cardiac stem cells with superior therapeutic potential for myocardial regeneration," *Circulation Research*, vol. 108, no. 12, pp. 1467–1481, 2011.

[20] J. Schindelin, I. Arganda-Carreras, E. Frise et al., "Fiji: an open-source platform for biological-image analysis," *Nature Methods*, vol. 9, no. 7, pp. 676–682, 2012.

[21] K. Hong, G. Zhao, Z. Hong et al., "Mechanical activation of angiotensin II type 1 receptors causes actin remodelling and myogenic responsiveness in skeletal muscle arterioles," *The Journal of Physiology*, vol. 594, no. 23, pp. 7027–7047, 2016.

[22] T. Kawai, T. Takahashi, M. Esaki et al., "Efficient cardiomyogenic differentiation of embryonic stem cell by fibroblast growth factor 2 and bone morphogenetic protein 2," *Circulation Journal*, vol. 68, no. 7, pp. 691–702, 2004.

[23] P. Shabani, Z. Ghazizadeh, S. Pahlavan et al., "Exogenous treatment with eicosapentaenoic acid supports maturation of cardiomyocytes derived from embryonic stem cells," *Biochemical and Biophysical Research Communications*, vol. 461, no. 2, pp. 281–286, 2015.

[24] E. Ali, F. Sharifpanah, M. Wartenberg, and H. Sauer, "Silibinin from *Silybum marianum* stimulates embryonic stem cell vascular differentiation via the STAT3/PI3-K/AKT axis and nitric oxide," *Planta Medica*, vol. 84, no. 11, pp. 768–778, 2018.

[25] T. J. Murphy, K. Takeuchi, and R. W. Alexander, "Molecular cloning of AT1 angiotensin receptors," *American Journal of Hypertension*, vol. 5, 12, Part 2, pp. 236S–242S, 1992.

[26] M. Nishida, S. Tanabe, Y. Maruyama et al., "Gα12/13- and reactive oxygen species-dependent activation of c-Jun NH_2-terminal kinase and p38 mitogen-activated protein kinase by angiotensin receptor stimulation in rat neonatal cardiomyocytes," *Journal of Biological Chemistry*, vol. 280, no. 18, pp. 18434–18441, 2005.

[27] B. R. Everaert, E. M. van Craenenbroeck, V. Y. Hoymans et al., "Current perspective of pathophysiological and interventional effects on endothelial progenitor cell biology: focus on PI3K/AKT/eNOS pathway," *International Journal of Cardiology*, vol. 144, no. 3, pp. 350–366, 2010.

[28] S. Kumar, R. K. Singh, and T. R. Bhardwaj, "Therapeutic role of nitric oxide as emerging molecule," *Biomedicine & Pharmacotherapy*, vol. 85, pp. 182–201, 2017.

[29] I. Anestopoulos, A. Kavo, I. Tentes et al., "Silibinin protects H9c2 cardiac cells from oxidative stress and inhibits phenylephrine-induced hypertrophy: potential mechanisms," *The Journal of Nutritional Biochemistry*, vol. 24, no. 3, pp. 586–594, 2013.

[30] L. Mira, M. Silva, and C. F. Manso, "Scavenging of reactive oxygen species by silibinin dihemisuccinate," *Biochemical Pharmacology*, vol. 48, no. 4, pp. 753–759, 1994.

[31] C. Dehmlow, N. Murawski, and H. de Groot, "Scavenging of reactive oxygen species and inhibition of arachidonic acid metabolism by silibinin in human cells," *Life Sciences*, vol. 58, no. 18, pp. 1591–1600, 1996.

[32] R. L. Price, W. Carver, D. G. Simpson et al., "The effects of angiotensin II and specific angiotensin receptor blockers on embryonic cardiac development and looping patterns," *Developmental Biology*, vol. 192, no. 2, pp. 572–584, 1997.

[33] S. Schutz, J. M. Le Moullec, P. Corvol, and J. M. Gasc, "Early expression of all the components of the renin-angiotensin-system in human development," *The American Journal of Pathology*, vol. 149, no. 6, pp. 2067–2079, 1996.

[34] F. Gembardt, S. Heringer-Walther, J. H. M. van Esch et al., "Cardiovascular phenotype of mice lacking all three subtypes of angiotensin II receptors," *The FASEB Journal*, vol. 22, no. 8, pp. 3068–3077, 2008.

[35] J. H. M. van Esch, F. Gembardt, A. Sterner-Kock et al., "Cardiac phenotype and angiotensin II levels in AT_{1a}, AT_{1b}, and AT_2 receptor single, double, and triple knockouts," *Cardiovascular Research*, vol. 86, no. 3, pp. 401–409, 2010.

[36] S. C. Fisher, A. van Zutphen, M. M. Werler et al., "Maternal antihypertensive medication use and congenital heart defects: updated results from the National Birth Defects Prevention Study," *Hypertension*, vol. 69, no. 5, pp. 798–805, 2017.

Hindawi
Stem Cells International
Volume 2018, Article ID 3123961, 32 pages
https://doi.org/10.1155/2018/3123961

Review Article

Current Trends in Biomaterial Utilization for Cardiopulmonary System Regeneration

Adegbenro Omotuyi John Fakoya,[1] **David Adeiza Otohinoyi**,[2] **and Joshua Yusuf**[2,3]

[1]*Department of Anatomical Sciences, All Saints University School of Medicine, Roseau, Dominica*
[2]*All Saints University School of Medicine, Roseau, Dominica*
[3]*All Saints University School of Medicine, Kingstown, Saint Vincent and the Grenadines*

Correspondence should be addressed to Adegbenro Omotuyi John Fakoya; gbenrofakoya@gmail.com

Received 31 July 2017; Revised 15 November 2017; Accepted 1 March 2018; Published 29 April 2018

Academic Editor: Fatemeh Sharifpanah

The cardiopulmonary system is made up of the heart and the lungs, with the core function of one complementing the other. The unimpeded and optimal cycling of blood between these two systems is pivotal to the overall function of the entire human body. Although the function of the cardiopulmonary system appears uncomplicated, the tissues that make up this system are undoubtedly complex. Hence, damage to this system is undesirable as its capacity to self-regenerate is quite limited. The surge in the incidence and prevalence of cardiopulmonary diseases has reached a critical state for a top-notch response as it currently tops the mortality table. Several therapies currently being utilized can only sustain chronically ailing patients for a short period while they are awaiting a possible transplant, which is also not devoid of complications. Regenerative therapeutic techniques now appear to be a potential approach to solve this conundrum posed by these poorly self-regenerating tissues. Stem cell therapy alone appears not to be sufficient to provide the desired tissue regeneration and hence the drive for biomaterials that can support its transplantation and translation, providing not only physical support to seeded cells but also chemical and physiological cues to the cells to facilitate tissue regeneration. The cardiac and pulmonary systems, although literarily seen as just being functionally and spatially cooperative, as shown by their diverse and dissimilar adult cellular and tissue composition has been proven to share some common embryological codevelopment. However, necessitating their consideration for separate review is the immense adult architectural difference in these systems. This review also looks at details on new biological and synthetic biomaterials, tissue engineering, nanotechnology, and organ decellularization for cardiopulmonary regenerative therapies.

1. Introduction

Cardiopulmonary disease refers to diverse forms of diseases affecting the heart and lungs. Some of these diseases might result in considerable damage to the tissues of these organs and occasionally might cause irreparable damage to parts of these organs, thus impairing their overall function, consequently resulting in the reduction in the quality of life of the affected individual. The duties of these two systems are so integral, such that a chronically diseased state in one will invariably affect the efficient functioning of the other [1].

Stem cells have been explored in regenerative therapies of both the heart and the lungs, and sections below will briefly consider this. However, the survival of these cells is largely dependent on the environment in which they are placed [2], hence the search for the suitable biomaterials that can potentiate survival, proliferation, differentiation, and engraftment of the transplanted cells to enhance tissue regeneration. Biomaterial scaffolds should provide not only physical support but also the chemical and biological clues needed in forming functional tissues in either the heart or the lungs [3].

In this review, we shall distinctly be considering the biomaterials that have been used in heart and pulmonary regenerative therapies. Also, this review will reveal a skew towards cardiovascular research over pulmonary research. This is an anticipated skew as the cardiovascular system

occupies a critical central role in the overall functioning of the body. Thus, the restoration of a healthy heart will translate into increased quality of life universally, reducing morbidity and mortality. This fundamental knowledge is the driver for more research into possible ways of restoring structure and function to a damaged heart which is at immense risk by modern-day lifestyle.

2. Biomaterials for Cardiac Regeneration

The need for new therapeutic inventions for cardiovascular diseases (CVDs) has been consistently indicated by the increased rate of associated diseases [1]. Statistics estimate a total annual expense of 1.2 trillion US dollars by 2030 in the United States if the current therapeutic interventions for CVD are maintained [2]. Among various CVDs, the most common is myocardial infarction (MI), which is the leading cause of morbidity and death in developing and developed nations [4]. MI involves the pathogenesis of anaerobic respiration, the accumulation of reactive oxygen species, and the death of cardiomyocytes (CM), thus affecting the normal physiological process of the heart [5]. Post myocardial infarction, the CM extracellular matrix (ECM) undergoes inflammatory, proliferation, and maturation stages of tissue remodeling to support other healthy CM [6, 7]. However, the scar tissue or collagen formed by the remodeling of the ECM at the maturation stage does not participate in the concomitant beating of the heart due to loss of organized architecture [8], which eventually leads to cardiomegaly and, ultimately, heart failure [6]. Present-day remedies like surgical, pharmacological, and endovascular interventions only have soothing purposes and do not address the fundamental flaw, which is the loss of functional CM [9]. Though heart transplant remains effective, the availability of donors and the occurrence of immune rejection pose a serious disadvantage. The recent discovery of cardiomyogenesis in humans has brought to light the role of cardiac regeneration from stem cells [10]. Cell varieties such as embryonic stem cells, cardiac stem cells, endothelial progenitor cells, skeletal myoblasts, and bone marrow mononuclear cells have been recognized to have regenerative properties in cardiomyogenesis. The use of these stem cells still has drawbacks like poor cell delivery and integration, low survival rate, and long-term toxicity; however, it is believed that modified biomaterials could limit these hindrances [11].

CMs were previously thought to be postmitotic, but recent studies have shown that cardiac tissue possesses some intrinsic mitotic activity with some regenerative potential as it contains a diversity of stem cells with regenerative potential in various niches in the heart [12]. These cardiac stem cells (CSCs) can be categorized into the following groups: ckit cells [13], $Sca1^+$ cells [14], $IsI1^+$ cells [15], cardiosphere-derived cells ($ckit^+/Sca1^+/flh1^+$) [16], cardiac mesoangioblasts [17], side population cells (expressing Abcg2/Mdr1) [18], and epicardial progenitors [19]. However, these cells alone are unable to regenerate the heart in the face of an MI, hence necessitating collaborative research into other solutions such as the use of biomaterials. Before an ideal biomaterial can be developed for suitable stem cell integration, a good knowledge of the heart's extracellular matrix (ECM) is required. Below, we briefly consider the cardiac ECM.

2.1. The Extracellular Matrix of the Heart. The cardiac ECM is a complex mesh of structural and nonstructural components for support and good cellular remodeling [20]. The structural component of the cardiac ECM consists mainly of cardiofibroblasts (Cfs) and collagen fibrils while the nonstructural element is made up of glycosylated proteins such as glycoproteins (GPs), glycosaminoglycan (GAG), and proteoglycans (PGs) [20, 21]. Other ECM components include cytokines and enzymes with their inhibitory factors [22]. Receptors and signaling proteins also serve as a vital makeup of the ECM.

During cardiac injury, cardiac fibroblasts (Cf) play a critical role in tissue repair and remodeling, during which they usually undergo phenotypic modulation to become myofibroblasts after TGFβ1 and fibronectin variant activation. In tissue repair, Cf function to synthesize the ECM components such as collagen and alpha-smooth muscle actin among other components [22].

Biomaterials in cardiomyogenesis are designed to model the natural cardiac ECM without its setbacks [22]. The healing process associated with MI involves the replacement of myocytes with granulation tissue despite the presence of CSCs within the heart tissue [1]. This occurs due to inadequate differentiation potential, not enough CSCs, and poor stimulation of stem cells within the myocardium, thus restricting cardiomyogenesis to the borders of the infarction, which is shown by maintained perfusion and signaling of CSCs by factors in the extracellular matrix around the borders of the infarct [1, 23]. It is stipulated that the introduction of stem cells with differentiation and growth signals (or biomaterials) could solve the setback in cardiomyogenesis [24]. Biomaterials, which are medical tools for grafting stem cells, are typically expected to be biodegradable and biocompatible, have minimal autoimmune stimulation, and possess a long half-life with sufficient reservoir capacity for bioactive molecules [11, 24]; however, when administered alone, biomaterials have a temporary remedy [23]. Biomaterials recognized for cardiomyogenesis can be broadly classified as either nature-based or synthetic; however, there are other categories that can serve as biomaterials in cardiovascular regeneration as discussed in this review (Figure 1).

2.2. Natural Biomaterials. Natural polymers are biodegradable matrices consisting of complex components found in native tissues. They can be easily manipulated, thus increasing or decreasing their half-life in vivo [25]. Natural biomaterials could either be protein, polysaccharide, or decellularized tissue-derived [26]. The ability of natural polymers to be biodegradable, biocompatible, and remoldable gives them an advantage over the synthetic polymers [24]. Protein and polysaccharide-based biomaterials are made by treating organic components with solvents and enzymes till interested components are separated from their biological source, whereas decellularized tissues involve the exclusion of cells from organic tissue, thereby maintaining the architectural and structural composition of the tissue [26].

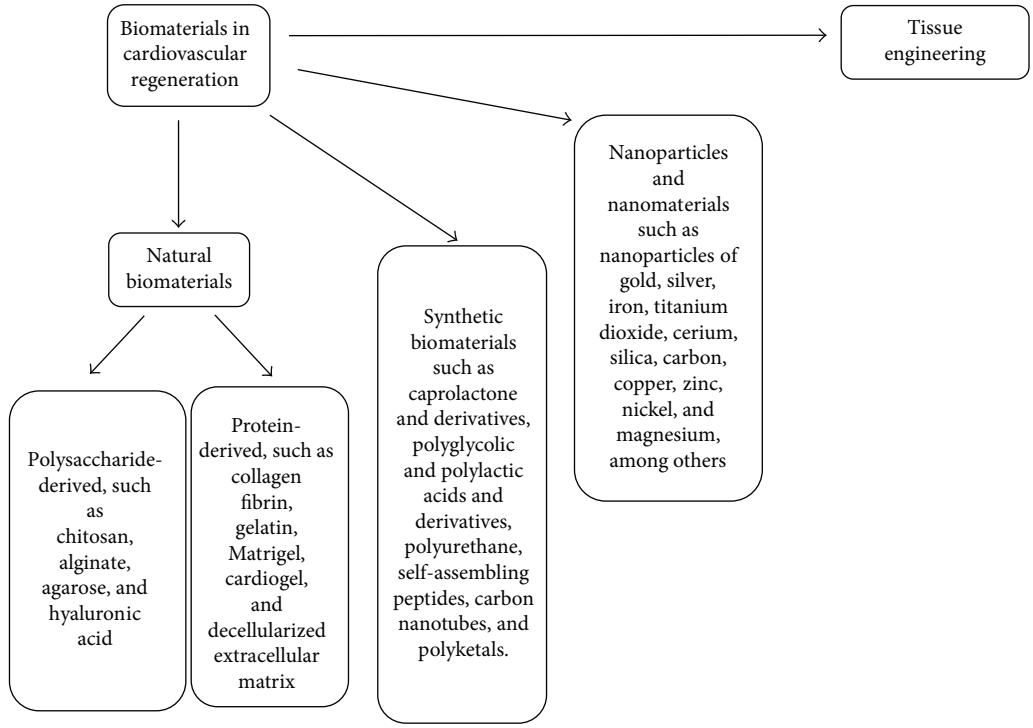


Figure 1: Classification of current biomaterials in cardiovascular regeneration.

2.2.1. Polysaccharide-Derived Biomaterials in Cardiomyogenesis. Polysaccharides are described as polymeric carbohydrate molecules that are made up of monosaccharide units linked by glycosidic bonds [27]. The use of polysaccharides such as chitosan, alginate, agarose, and hyaluronic acid has been indicated in cardiomyogenesis [28, 29].

(1) Chitosan. Chitosan is the partial alkaline deacetylation of chitin, which is the second largest natural polymer after cellulose, and is readily found in the exoskeleton of insects and fungi [27]. Chitosan is a linear copolymer of β-(1–4)-D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) [27]. Foster et al. suggested that deacetylation should be above 75% to ensure optimal stem cell activity [28]. Chitosan supports cardiomyogenesis as it is biodegradable, biocompatible, marginally immunogenic, hydrophilic, hemostatic, nontoxic, and cohesive in character [30, 31]. Chitosan is usually combined with other composites to form complexes via electrostatic force or physical/chemical cross-linking due to its poor stability and low electrical conduction when used alone [30]. Studies done by Martins et al. showed that the coupling of carbon nanofibers with porous chitosan scaffolds improved the growth of neonatal rat heart cells in vitro. The supposed reason was that carbon nanofibers boosted electrical signaling transmissions between the cells [32]. Chitosan has also been observed to boost silk fibroin (SF) potential by improving the differentiation of rat mesenchymal stem cells (MSC) to CM in vitro, thus making the hybrid of chitosan and SF indicated in cardiomyogenesis [29]. Liu et al. also suggested that adipose-derived stem cells (ADSC) on chitosan enhanced the formation of transplantable spheroids with cardiac markers such as Gata4, Nkx2-5, Myh6, and Tnnt2 due to increased calcium signaling [33]. Thermosensitive conductive hydrogel generated from chitosan was also observed to support cardiomyogenic differentiation of MSC in the presence of gold nanoparticles (AuNPs) [34]. Hydrogels synthesized from chitosan also showed good potential in cardiomyogenesis after having been made electrically conductive by aniline oligomers or paratoluenesulfonate electrodeposition [35–37]. When tested for the effect of chitosan on the differentiation of brown adipose-derived stem cell (BASC) to CM, the expression of cardiac markers such as GATA-4, Nkx2.5, Myl7, Myh6, cTnI, and Cacna1a was noted [38]. The behavior of chitosan in vivo has also been monitored, with reports showing that BASC on chitosan increased cardiac function, neovascularization, and left ventricular pressure and reduced infarct size in rat models [39]. Chi et al. also reported that using cardiac patches consisting mainly of chitosan and without stem cells on myocardial infarcted rats led to increased wall thickness and reduced left ventricular dilation. However, there was no significant increase in neovascularization [39]. Simultaneous injection of the basic fibroblast growth factor (bFGF) with a temperature-sensitive chitosan hydrogel also enhanced the role of bFGF on neovascularization and cardiac function [40]. A common factor among researchers concerning the use of chitosan in cardiomyogenesis is in improving the potential of chitosan by measuring the synergistic effect combined with other factors, thus improving the integration of stem cells into cardiac tissues. This is because chitosan has a low mechanical resistance and is also susceptible to proteolytic enzymes when poorly acetylated [41].

(2) Alginate. Alginate, or alginic acid, is an anionic linear polysaccharide found in algae and bacteria. It is commercially harvested from the cell walls of brown algae (Phaeophyceae), like *Laminaria hyperborea*, *Laminaria lessonia*, *Macrocystis pyrifera*, and *Ascophyllum nodosum* [27]. Alginate forms hydrogel by ionic cross-linking with divalent ions like calcium and zinc, thus ensuring the retention of cells and proteins within the hydrogel by 90% [42]. Similar to chitosan, purified alginates have a negligible immune response in vivo [43]. They are also biocompatible and nonthrombogenic [44]. Alginate-based hydrogels can also be modified to suit the host myocardium by molecular weight dispensation or by cross-linkage changes [43]. Alginate scaffolds alone had a substantial effect on the cardiac function of MI heart in rat, swine, and dog models, with no arrhythmia or thrombus formation. However, similarly to chitosan, the seeding of alginate with stem cells/fetal cardiac cells has been observed to have an enhanced effect on the ischemic heart of animal models [42, 45–47]. An investigation after 65 days post treatment with rat fetal cardiac cells (RFCC) in alginate scaffolds found that rat MI models had improved neovascularization, persistent fractional shortening, and end systolic and diastolic internal diameters and encouraged the formation of myofibers and cardiac gap junctions [48]. Correspondingly, when the RFCC was replaced by human embryonic stem cells (hESC) with an inhibited p38 mitogen-activated protein kinase, there was a significant improvement, and no immune response was noted [49]. Alginate is commonly modified by ECM-derived peptides like the arginine-glycine-aspartate (RGD peptide) sequence [42]. The RGD sequence is a signaling domain of fibronectin and laminin and thus assists the scaffolding in cell adhesion and signaling by binding ECM proteins to integrin receptors [43]. When compared with unmodified alginate, RGD-alginate showed a significant increase in the level of angiogenesis [50]. A comparative study, however, revealed that unmodified alginate shows a lower left ventricle expansion index, reduced left ventricle fractional shortening, and more scar thickness than RGD-alginate does [51]. The addition of a heparin-binding peptide to RGD-alginate, seeded with RFCC, stimulated a striated fiber organization similar to native tissue in vivo, but this was negative with RGD-alginate [52]. Aside from seeding alginate scaffold pores with cells, the use of a 3D nanocomposite of gold nanowires has also been indicated as it improves electrical communication between adjacent cardiac cells, leading to better cell organization, synchronous contractions, and higher levels of sarcomeric α-actinin and Cx-43 [53]. Other studies have shown that the use of a magnetic field of 5 Hz to stimulate alginate scaffolds filled with magnetically responsive nanoparticles (NPs) leads to increased troponin-T levels and a greater activation rate of AKT protein kinase [54]. Alginate was also identified as a structural complement of chitosan and combined for possible synergistic effect; the chitosan-alginate beads produced similar results with and without cell incorporation. It was also observed that alginate alone showed better results than did a chitosan-alginate combination [55]. Clinical trials involving alginate showed no significant improvement in ejection fraction and left ventricular end-systolic and end-diastolic volumes, though it did not deteriorate the status of the participants [56]. Some limitations have also been reported with the use of alginate as a biomaterial. Some authors describe cross-linked alginate to have poor long-term stability due to the ability of the gel to dissolve as a result of released divalent ions and exchange reactions. It is also speculated that the small pore size of alginate hydrogels (approximately 5 nm) could limit the number of regenerative mediators released [57].

(3) Agarose. Agarose is a repeating unit of D-galactose and 3,6-anhydro-L-galactopyranose, refined from algae. Agarose is well recognized as an enzyme stabilizer and a good culture medium for cells [58]. It possesses the ability to aggregate stem cells such as marine induced pluripotent stem cell (iPSC) and hESC due to its noncell-adhesive, transparent, and moldability characteristics [58]. The culture also showed potential for cardiac cell differentiation [58]. The application of agarose in cardiomyogenesis has not been fully elucidated upon as compared to other polysaccharides, though its role has been mentioned in the differentiation of stem cells to chondrocytes and dopaminergic neurons [59, 60].

(4) Hyaluronic Acid. Hyaluronic acid (HA), or hyaluronan, is a nonsulfated, high-molecular-weight GAG that is abundant in the ECM. It consists of repeating polymeric glucuronic acid and N-acetyl-glucosamine disaccharide conjugated by a glucuronidic β $(1 \rightarrow 3)$ bond and hexosaminidic β $(1 \rightarrow 4)$ bonds [61]. Without modification, HA has poor mechanical properties that limit its use as a biomaterial [62]. The most common modification of HA involves cross-linking, mediated by cross-linkers like cysteine derivatives, adipic dihydrazide, glutaraldehyde, carbodiimides, and divinylsulfone [63]. Additionally, comparative research indicated that the potential of HA in cardiomyogenesis depends on the molecular weight of HA and the evolution of MI [63]. The use of HA modified with polyethylene glycol-thiol injected into MI rat models showed a decrease in the size of the infarcted area and the rate of apoptosis, with a considerable increase in the number of arterioles and capillaries [64]. When combined with SF and seeded with rat MSCs, HA enhanced the expression of cardiac genes including Gata4, Nkx2.5, Tnnt2, and Actc1 in vitro. It was noted that the CD44 surface markers influenced the differentiation [65]. Combining HA and gelatin together with other chemical modifiers like activin-a, BMP-4, insulin, valproic acid, and 5-azacytidine in various combinations leads to the differentiation of human ADSC to CM with the expression of GATA4, TBX5, and cTnI [66].

2.2.2. Protein-Derived Biomaterials in Cardiomyogenesis. They constitute as one of the major biomaterials used in cardiomyogenesis. These protein isolates retain their innate biological function, such as aiding differentiation, providing support, and assisting cell proliferation [9, 67]. They are one of the major scaffolds employed in cardiomyogenesis because of their role in cell relocation, multiplication, and differentiation of both CSC and other body stem cells to CM [68], as observed in studies showing the role of trade

names Matrigel and Geltrex protein isolates in the reprogramming of mouse fibroblasts to CM [69]. Extracellular matrix protein isolates (ECMPi) were also observed to facilitate sarcomere alignment in human iPSC [70]. The remodeling of ECMPi, such as collagens into fibrils, has been linked with its effect in assisting the conversion of MSC to CM [71]. An in vivo investigation also showed that collagen and fibrin could increase cardiac function when implanted into the epicardium of rat models suffering from MI. Also, a percutaneous approach in larger animals has also been found to be supportive in MI [72].

(1) Collagen. Collagen is the most widely used natural polymer due to its ability to support an infarcted heart and improve cardiac function by inhibiting fibrosis, enhancing vascularization, and improving cell migration [73]. It is the most abundant ECM protein, and it provides structural scaffolding and tensile integrity and guides biological processes in tissue repair [43]. It contributes to the integrin interactions within the ECM, thus having a part in cell migration, cell differentiation, and tissue restoration [74]. It is classified as a nontoxic, nonimmunogenic, and biodegradable compound that is available commercially at a low cost [23]. Its viscosity also helps maintain MSC in the infarcted regions [72]. The native environment of CM has demonstrated collagen types I and III to be the main support for optimal cardiac functioning by providing structural sustenance and maintaining flexibility for contractile elements, respectively [75]. Nonfibrillar collagens, including collagen IV, V, and VI, have also been indicated in cardiac repair [76]. However, it has been reported that the collagen type I content in MI drastically reduced from 80% to 40% [77]. Studies prompted by this observation showed that acellular type I collagen in the form of a cardiac patch on MI murine hearts preserved contractility, prevented remodeling, and improved heart function, by reducing infarcted region fibrosis and supporting blood vessel formation [73]. Similar to other biomaterials, studies have described that the inclusion of other complexes to collagen could attenuate its potential [1]. However, studies done by Dawson et al. showed that the addition of the RGD peptide to type I/III collagen, seeded with mouse ESC-derived embryoid bodies, had no significant input in the potentials of collagen in CM formation [78]. The addition of carbon nanotubes to type I collagen hydrogels showed better results with increased cardiac cell functions compared to using pure collagen hydrogels. The authors suggested that the addition of carbon nanotubes to collagen hydrogels is recommended for future studies involving collagen to avoid mismatches in the mechanics, conductivity, and submicrometer structure of the matrix [79]. Similarly, Sun et al. attempted the use of collagen hydrogel combined with single-walled carbon nanotubes on neonatal rat ventricular myocytes and noticed a better cell alignment, stronger contraction potential, no CM toxicity, and enhanced cardiac constructs [80]. Carbon nanotubes aside, other studies have revealed that a collagen-gold nanocomposite (43.5 ppm)-coated catheter with MSCs enhances cellular migration and thus leads to improved neovascularization [81]. Likewise, vitronectin-collagen scaffolds have been shown to support neovasculogenesis and boost ventricular function [82]. The incorporation of chitosan with collagen has shown a decrease in tensile modulus (1.82 to 0.33 MPa) and an increase in compressive modulus (23.50 to 55.25 kPa). The action of collagenase was also reduced in the presence of chitosan [83]. When tested for its role in cardiomyogenesis, chitosan-collagen hydrogel incorporated with prosurvival angiopoietin-1-derived protein improved the left ventricular ejection fraction and left ventricular fractional shortening and decreased the systolic dimension and volume although it did not affect diastolic parameters. Also, the chitosan-collagen hydrogel did not slow the rate of apoptosis; as compared to nonmodified collagen hydrogel, however, it was able to enhance the survival of CM in the rat MI model [84, 85]. A comparative review on the role of cross-linking of collagen on cardiomyogenesis showed that noncross-linked scaffold obtained a higher biocompatibility and complete adhesion to the heart with a mild inflammatory reaction [43]. The role of collagen in cardiomyogenesis keeps on unfolding with the current attention it gets from researchers, and it remains one of the most studied biomaterials concerned with stem cell differentiation.

(2) Fibrin. Fibrin is a self-assembling peptide associated with clot formation in the endothelium. It functions as a biomaterial by having good seeding competence, efficient biocompatibility and biodegradability, uniform cellular migration, and efficient adhesion [86]. It is formed by the reaction of thrombin and fibrinogen. Thus, it could be harvested from the patient's blood, therefore minimizing immune rejection [74]. Fibrin can be manipulated to form hydrogels, microbeads, and gels [74]. It can also be manipulated to incorporate biological molecules like fibroblast growth factor and transforming growth factor-β1 to enhance the presence of growth factors and to resemble the native ECM of the heart [87, 88]. Its intrinsic regenerative properties give it the ability to be a stand-alone therapy [43]. A meta-analysis by other authors pointed out that fibrin glue, administered alone, reduced the infarct size and stimulated neovascularization more than when administered with neonatal skeletal myoblasts in rat MI models [43]. However, fibrin scaffolds incorporated with thymosin β4 (encapsulated in gelatin microspheres) significantly increased vascular growth and sustained the survival of swine MSC, thus producing a synergistic effect [89]. In vivo experiments showing the potential of human embryonic stem cell-derived cardiac progenitors (hESC-CPC) in nonhuman primate models when seeded on a fibrin patch led to the commencement of clinical trials accessing the possibilities of fibrin patch with hESC-CPC for individuals with heart failure. The clinical study is titled "Transplantation of Human Embryonic Stem Cell-Derived CD15+ Isl-1+ Progenitors in Severe Heart Failure" and can be located at https://clinicaltrials.gov/ct2/show/record/NCT02057900. It is expected to be completed in 2018 [90]. Fibrin gel has also been described to be an efficient sealant after intramyocardial injection, thus avoiding displacement of injected materials [91].

Advanced studies using fibrin hydrogel have also illustrated that synchronous electromechanical cell conditioning (2-millisecond pulses of 50 mV/cm at 1 Hz and 10%

stretching for seven days) of cardiac ADSC before implanting into a murine heart might be an ideal strategy for future research [92]. Tao et al. also suggested seeding fibrin gels with 3 to 4 million neonatal cardiac cells from Sprague-Dawley rats to achieve optimal results for in vivo cardiac patch implantation. They speculated that seeding with quantities outside the given range may affect cell density on spontaneous contraction rates, contraction forces, and paced response frequencies [86]. Ichihara et al. recommended epicardial placement to intramyocardial injection when delivering fibrin glue incorporated with male bone marrow MSC, as this led to better initial retention and long-term presence of MSC. There was also the quicker recovery of cardiac function and structure as measured by echocardiography and catheterization in female MI rats [93]. Further studies have also shown that fibrin gel could be used in tissue engineering to form aortic valves with bioinspired textile reinforcement [94].

(3) Gelatin. Gelatin is a product of collagen hydrolysis. It involves the loss of the triple helical assemblies of collagen. It considered safe by the US Food and Drug Administration [95]. It is biocompatible and biodegradable and has a low antigenic level [43]. Gelatin hydrogels have flexible elastic moduli and can be cross-linked with transglutaminase to be thermostable [96]. Gelatin hydrogels have also been indicated to be preferred to alginate and fibronectin-patterned polydimethylsiloxane due to their performance in sustaining neonatal rat cardiac myocyte tissue in vitro for three weeks at maintained spontaneous beating, higher spare respiratory capacity, and consistent levels of contractile stresses [97]. Gelatin hydrogel-muscular thin films also supported the growth of human iPSC-derived cardiac myocytes [97]. Results of the Autologous Human Cardiac-Derived Stem Cell to Treat Ischemic Cardiomyopathy (ALCADIA) experimentation illustrated that 200 μg of bFGF in a biodegradable gelatin hydrogel sheet implanted on the epicardium of human patients with ischemic cardiomyopathy and heart failure leads to the continuous release of bFGF, thus suggesting the effectiveness of the methodology [98, 99]. Ultraviolet cross-linkable gold nanorod-incorporated gelatin ethacrylate hybrid hydrogels seeded with neonatal rat ventricular CM exhibited excellent cell retention, viability, and metabolic activity [100]. There was also a synchronous beating of the CM [100]. Comparative studies involving a pig MI model also demonstrated that gelatin scaffold + bFGF + human cardiosphere-derived cells had a higher ejection fraction, lower infarct volume, and more cellular differentiation to CM, than when compared to the use of gelatin scaffold + bFGF and gelatin scaffold + bFGF + human bone marrow-derived MSCs [98]. The use of gelatin as a scaffold for cardiomyogenesis has been adopted as a standard protocol for further investigations. For example, Kudová et al. demonstrated that mouse ESC on gelatin-coated dishes had poor CM differentiation when hypoxia-inducible factor-1α was deficient in the medium in vitro [101]. Bioartificial constructs constituting polylactic-co-glycolic acid and gelatin to resemble the anisotropic structure and mechanical characteristics of the myocardium indicated that the combination leads to good involvement of human MSCs to form CM due to the presence of markers, namely, Gata4 and Mef2c [102].

(4) Matrigel. Matrigel is a protein isolate from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma cells [103]. It is predominantly composed of laminin with other components including heparin sulfate proteoglycan, entactin, and collagen type IV. Studies revealing Matrigel's robustness in containing growth factors such as bFGF, epidermal growth factor (EGF), IGF-1, PDGF, nerve growth factor (NGF), TGFβ1, and others have prompted researchers to investigate its potential in cardiomyogenesis. However, information on its mechanical and chemical behavior has not been fully reported [38, 43, 104]. Comparative and descriptive reviews have already illustrated the role of Matrigel in the treatment of MI by enhancing the recruitment of $CD34^+$ and c-kit^+ stem cells in mouse models, enhancing CM function in vitro, and serving as an efficient scaffold for pluripotent stem cells and embryonic stem cell delivery and integration [9, 37, 43, 74]. Current studies using Matrigel have included an advanced investigation. Zhang et al. demonstrated that seeding Matrigel with CSC from embryonic heart tubes could differentiate into cardiac peacemaking cells after endothelin-1 treatment, thus forming a tissue-engineered cardiac pacemaker. When combined with endothelial stem cells and introduced in vivo in rat models, there was enhanced vascularization and electrical activity [105]. Matrigel also enhanced the type I collagen matrix due to its type IV collagen and sulfated proteoglycan content, thus enhancing the mechanical culture of heart valve interstitial cells in vitro [106]. However, comparative studies show that graphene promoted the differentiation of hESC to CM than Matrigel did in vitro [107]. The comparative studies were prompted by different opinions on the use of Matrigel or a "mouse tumor" derivative in human infarcted tissue [108]. Since there is also poor control on the cells that secrete the Matrigel components, matrigels from various Engelbreth-Holm-Swarm mouse sarcoma cells may also lead to matrigels with varying qualitative and quantitative differences, thus altering their role in cardiomyogenesis.

(5) Cardiogel. Cardiogel is an ECM matrix, synthesized by cardiac fibroblasts. It contains laminin, fibronectin, types I and III collagen, growth factors, and proteoglycans [109]. The synergistic effect of these components has been reported to influence the growth of CM, enhance spontaneous contractile activity, and stimulate stem cell differentiation [109, 110]. The debate concerning whether the components of cardiogel support cardiomyogenesis, more than the complex cardiogel itself, led to comparative studies which suggested that simple matrices will be ideal for structural and biochemical investigation; however, native complex matrices like cardiogel overwhelm cell polarity faster than do simple mediums and will also produce favorable results [111]. Similarly, culturing MSCs from rat models on a 3D matrix, cardiogel was better in cellular expansion and adhesion than were MSCs cultured on plastic and fibronectin-coated plates [112]. Previous reviews also pointed out that cardiogel protects murine bone marrow-derived mesenchymal stem cells

(BMSC) from oxidative stress better than does Matrigel [113]. Another study also reported BMSC differentiating to cardiogenic components without induction with 5-aza, thus emphasizing the potential of cardiogel. However, the authors could not provide a good explanation for their result [114]. Cardiogel seeded with ASCs has also been shown to support angiogenesis in vivo [115]. A proper explanation of the biochemical and mechanical role of cardiogel is still needed for proper direction of how cardiogel could be manipulated for optimal results.

(6) Decellularized Extracellular Matrix. Decellularization involves the removal of cells and nuclear materials from organic tissues while making possible efforts at not tampering with the structural integrity or native components of the ECM [74]. Similarly to ECMPi, they are biodegradable and safe byproducts. The major reason to decellularize is to minimize immune reaction when used [9]. The idea to use decellularized matrices (DECM) was also due to the lack of a native architectural framework in other biomaterials [24]. DECM could be in the form of an intact whole organ, small tissue sections, thin sheets, hydrogels, or coating [116]. Although the potential for the use of DECM in cardiomyogenesis is undisputable, several authors have initiated discussions concerning its uses. Kim et al. described that DECMs might possess remnant molecules ranging from microRNA to cellular proteins, and thus, they suggested that DECMs should have less than 50 ng of dsDNA per mg dry weight and less than a 200-base pair DNA fragment length before use [117]. Decullarization methods have also been reported to affect the architecture of tissues. For example, decellularization by freeze-thawing could affect the ultrastructure of the ECM; pressure techniques could affect their mechanical properties; solutions with high or low pH could degrade some ECM components; alcohol could cross-link collagen, making it stiff; and ionic detergents could also disrupt covalent bonds [118–122]. Thus, some reviews have suggested that the type of tissue (such as heart, kidney, or liver) and the need for decellularization should be considered before selecting a decellularization protocol [116]. Another study reported that the current decellularizing protocols have also led to residual fragments of Triton X-100, sodium deoxycholate, and sodium dodecyl sulfate (SDS) in decellularized scaffolds, thus affecting their functionality [123]. This illustrates that proper clearance of the reagents used in protocols is necessary for optimal results. Several protocols for the decellularization of the human myocardium have also been tested, and suitable protocols have been derived [124]. Other studies have shown that CPCs isolated as cardiosphere-derived cells thrive better on a healthy DECM than on a pathologic DECM [125]. Opinions on decellularization can be found in very detailed reviews [116, 117, 123, 124, 126–128].

While the subject of decellularization has been elucidated upon, studies on the role of DECM in cardiomyogenesis have been going on concurrently. Over the past few decades, several studies have been conducted and mentioned in reviews [9, 24, 43, 74]. Recent studies include descriptions of how DECM sheets produced from thin, cardiac sections from rat neonatal ventricles efficiently preserved and improved phenotypic characteristics and cell proliferation and viability rates of CM in vitro [129]. MSC and iPSC cultured on DECM from the left ventricle of humans were shown to have good adhesion, proliferation, and viability compared to cells cultured in a purchased cardiac myocyte medium [130]. Repopulating decellularized mouse hearts with human iPSC-derived multipotential cardiovascular progenitor cells showed signs of CM differentiation, proliferation, and myofilament formation [131]. Nonseeded decellularized homografts, derived from donated human heart valves, were also able to reduce complications associated with bovine jugular vein conduits and cryopreserved conventional homografts [132]. The addition of fibronectin to enhance the adhesion of human cardiovascular cells to a decellularized porcine heart scaffold for proper proliferation and integration was also reported [133]. Recellularization of decellularized rat heart with isolated rat CM showed the expression of CD31, α-actinin, troponin-T, connexin, Nkx-2.5, c-kit, and GATA4. The electric potential was detected on the scaffolds, and the cardiac pacing was also observed on the scaffold [134]. Further studies also showed that surface heparin treatment tends to reduce tissue calcification of the decellularized porcine heart valve in a rabbit intramuscular implantation model [135]. Hodgson et al. also developed a protocol to ensure 98% decellularization of the whole porcine heart with reduced time of exposure to detergent [136].

2.3. Synthetic Biomaterials. The use of synthetic biomaterials in cardiomyogenesis was prompted because of the need to develop polymers that are easy to fabricate and manipulate, thus enabling the production of biomaterials for specific stem cell response [9]. The possibility of having polymers with a steady manufacturing process and the opportunity to construct their physical properties also attracted researchers to the potential of synthetic materials. The ability to also influence their molecular weight, heterogeneity index, and copolymerization ratio to control their degradation speed has also caused researchers to develop greater interest [74]. Since the involvement of synthetic biomaterials in cardiomyogenesis, their prospects have been climbing. However, their use is limited by poor bioactivity, potential toxicity, and low interaction with cells and signaling proteins. Thus, their ability to sustain cells has not reached the levels attained by natural biomaterials [137]. This setback has led to combining synthetic biomaterials with natural biomaterials so that the resulting scaffold interacts properly with cells [138].

Some of the synthetic biomaterials used in cardiomyogenesis include caprolactone and derivatives, polyglycolic and polylactic acids and derivatives, polyurethane, self-assembling peptides, carbon nanotubes, and polyketals [24].

Caprolactone is considered nontoxic and tissue compatible with good pH sensitivity. However, it is difficult to synthesize and degrades slowly. Polyglycolic and polylactic acids and derivatives have good biocompatibility and degrade easily, although they acidify their environment when degrading, which could lead to erosion. Polyurethane is biocompatible but nonbiodegradable unless copolymerized; it also lacks conductivity. Self-assembling peptides are bioreabsorbable and can be used to design

3D microenvironments. Their toxicity profile and side effects still require more elucidation. Carbon nanotubes provide good conductivity and mechanical support, but they are hydrophobic, toxic, and expensive. Polyketals are cheaper, nonimmunogenic, inert degradation products and are sensitive to low pH. Their use is limited by complexity in the synthesis and quick macrophage uptake and degradation [139–144].

Poly(ε-caprolactone) combined with poly(L-lactic acid) and collagen to form a nanostructured matrix supported the isolated rabbit CM with results similar to those expected in the native myocardium [145]. Conductive polymers like polypyrrole have been combined with poly(ε-caprolactone) and gelatin to form nanofibrous membranes, and these membranes supported human CM attachment, proliferation, and interaction [146]. The biodegradable patch composed of poly(l-lactic-co-ε-caprolactone) and polyglycolic acid also supported human-induced pluripotent stem cell-derived CM in regenerating a host myocardium in the athymic rat [147]. The ultrafine fiber scaffold made by combining the additive manufacturing of poly(hydroxymethyl glycolide-co-ε-caprolactone) with melt electrospinning writing showed that it aligned the growth of cardiac progenitor cells in the direction of the melt electrospun and had better results than using electrospun poly(ε-caprolactone)-based scaffolds alone [148]. The rapamycin-loaded polylactic-polyglycolic acid NPs delivered locally in minipigs considerably reduced the MMP-2/TIMP-2 ratio and proliferating cell nuclear antigen expression, increased the p27 (kip1) mRNA expression, thus relieving the degree of stenosis, and showed excellent acute procedural results in the interventional coronary artery-oversized balloon injury model [149]. The electroactive polyurethane/siloxane films containing aniline tetramer moieties (EPUSF) supported the proliferation and differentiation of C2C12 myoblasts with the expression of cardiac-specific genes of HL-1 cells involved in muscle contraction and electrical couplings such as Cx43, TrpT-2, and SERCA genes. The EPUSF were nontoxic and did not alter the intrinsic electrical characteristics of HL-1 cells [150]. 3D biomimetic scaffolds using a polymer blend of polyurethane and cellulose were also reported to have good biocompatibility, provided good mechanical support, and housed frequent contraction cycles of cardiac tissue [151]. Soft polyurethane-urea scaffolds with regular tubular pores were also observed to withstand tensile stresses associated with diastole without opposing tissue contraction. Also, they supported the growth of cardiac myocytes than did tissue culture plastic. Enhanced seeding efficiency was further noted [152]. A comparative study also showed that poly(L-lactic acid) and polyurethane nanofibrous mats fabricated by solution blow spinning were better substrates for cardiac cell culture than polystyrene was [153]. The role of carbon nanotubes has been described in this review [32, 79, 81]. Other studies have gone on to demonstrate that embedding carbon nanotubes in mouse embryoid bodies to control mechanical and electrical activity in stem cell niches led to cardiac differentiation and beating activity [154]. Polyketals have been employed due to their efficient role as a vehicle for the delivery of molecules. Studies on polyketals showed that they serve as good vehicles for delivering siRNA to an MI heart, thus posing as a technique in targeting oxidative stress [155].

It will be interesting to note that synthetic biomaterials are currently being well utilized in the field of nanotechnology. The use of nanotechnology in cardiomyogenesis is reviewed in the next section. The current natural and synthetic biomaterials in cardiac regeneration field which are discussed in this review are summarized in the Table 1. Furthermore, they are classified based on the type of reported experimental studies.

2.4. Nanotechnology in Cardiomyogenesis. The use of nanotechnology has advanced significantly in the medical field in recent years. Nanotechnology can generally be described as the manipulation of matter on an atomic, molecular, or supramolecular scale, and it can also be described as the understanding and manipulation of processes in structures having sizes ranging between 1 and 100 nanometers (nm) [156, 157].

NPs can be produced by using different composite materials, thus altering their physical properties. Some of the materials used in the production of NPs include gold, silver, iron, titanium dioxide, cerium, silica, carbon, copper, zinc, nickel, and magnesium, among others [156]. Among all these materials, gold, because of its hydrophobic nature, has been shown to be most favorable in the construction of NPs. De la Fuente et al., in 2001, reported the first water-soluble AuNPs [158]. Various factors are considered in application of NPs, such as their physical properties, biocompatibility, and cytotoxic effects [156]. Based on these properties, NPs have various applications in the cardiomyogenesis filed which are summarized in the Figure 2.

Nanotechnology incorporates diverse novel, powerful tissue engineering techniques such as 3D bioprinting, and studying these techniques has shown great prospects [159]. In 3D bioprinting, biomaterials, cells, drugs, growth factors, and genes are deployed in a layered manner to produce a 3D construct regarded as a "bioink" [160–163]. Technology has also evolved to enable the creation of scaffold-free or scaffold-based tissues and organ constructs [164–168].

Three main modalities are used in bioprinting: laser, droplet, and extrusion-based bioprinting, of which droplet-based bioprinting (DBB) provides several advantages because of its simplicity, versatility, and agility and the enormous level of control over the pattern of deposition [169]. Current DBB methods include inkjet, acoustic, electrohydrodynamic, and microvalve bioprinting [169]. Regardless of its vast benefits, this technology still faces challenges, such as bioprinting-triggered cell damage at significant levels, limited bioink materials, bioprinted constructs with limited structural and mechanical integrity, and size restrictions of constructs due to lack of porosity and vascularization [169].

3D bioprinting in the cardiovascular field has been reviewed with respect to biomaterial dependence or independence. Moldovan et al. concluded that biomaterial or scaffold-dependent bioprinting was appropriate for tasks needing faster, larger, anatomically correct, matrix-rich, and

Table 1: Classifications of natural and synthetic biomaterials used in cardiac regeneration.

Biomaterials Classification	Subclassification	Experimental studies In vitro	Experimental studies In vivo	Clinical trials	References
Natural biomaterials: polysaccharide-derived	Chitosan	Yang et al. (2009): chitosan improved silk fibroin effect on rat MSC. Liu et al. (2013): chitosan improved the differentiation of ADSC.	Chi et al. (2013): BASC on chitosan improved overall cardiac function in MI rat models. Wang et al. (2010): chitosan improved the function of bFGF on cardiac function.	None reported.	[29, 33, 39, 40]
	Alginate	Wang et al. (2012): hydrogels from alginate can enhance the growth of stem cells.	Leor et al. (2000): RFCC in alginate scaffolds supported neovascularization in rat models. Yeghiazarians et al. (2012): hESC with inhibited p38 mitogen-activated protein kinase on alginate scaffolds improved cardiac function with no immune response.	None reported.	[42, 47, 48]
	Agarose	Dahlmann et al. (2013): agarose microwells supported the differentiation of pluripotent stem cells to cardiomyocytes.		None reported.	[58]
	Hyaluronic acid	Yang et al. (2010): HA combined with SF seeded with rat MSCs enhanced cardiac gene expression. Göv et al. (2016): HA and gelatin enhanced the differentiation of human ADSC to CM.	Yoon et al. (2016): HA modified with polyethylene glycol-thiol reduced infarct size and promoted neovascularization in a rat model.	None reported.	[64–66]
Natural biomaterials: protein-derived	Collagen	Yu et al. (2017): type I collagen with carbon nanotubes boosted cardiac cell function. Sun et al. (2017): collagen hydrogels and carbon nanotubes improved cell alignment.	Frederick et al. (2010): collagen-gold nanocomposite coated with MSCs improved neovascularization. Hsieh et al. (2016): vitronectin-collagen improved ventricular function in rat models.	None reported.	[79–82]
	Fibrin	Ye et al. (2013): fibrin scaffolds with thymosin $\beta 4$ sustained swine MSC. Nie et al. (2010) and Yang et al. (2012): fibrin scaffold manipulated by growth factors resembled native ECM of the human heart.	Ichihara et al. (2017): epicardial placement of bone marrow MSC in fibrin scaffold should have better retention of the MSC.	Menasché et al. (2014): trials in observing the prospects of fibrin patch with hESC-CPC on individuals with heart failure. To be completed in 2018	[87–90, 93]
	Gelatin	Navaei et al. (2016): Ultraviolet cross-linkable gold nanorod-incorporated gelatin ethacrylate hybrid hydrogels improved cell metabolic activity.	Takehara et al. (2008): gelatin scaffold + bFGF + human cardiosphere-derived cells had a higher ejection fraction in pig MI models.	Yacoub et al. (2013) illustrated that bFGF in biodegradable gelatin hydrogel sheet implanted on the epicardium of human patients with ischemic cardiomyopathy and heart failure leads to the continuous release of bFGF.	[98–100]

Table 1: Continued.

Biomaterials Classification	Subclassification	Experimental studies In vitro	Experimental studies In vivo	Clinical trials	References
	Matrigel	Lam et al. (2017): matrigel enhanced the type I collagen matrix.	Zhang et al. (2017): matrigel and endothelial stem cells improved vascularization and electrical activity.	None reported.	[105, 106]
	Cardiogel	Chang et al. (2007): MSCs on cardiogel had better cellular expansion.	Matsuda et al. (2013): ASCs on cardiogel supported angiogenesis.	None reported.	[112, 115]
	Decellularized extracellular matrix	Pagano et al. (2017): CPCs thrived on healthy DECM. Lee et al. (2015): DECM from rat preserved and improved the survival of CM.	Söylen et al. (2017): nonseeded decellularized homografts from human donors reduced complications with bovine jugular vein conduits.		[125, 129, 132]
Synthetic		Mukherjee et al. (2011): poly(ε-caprolactone) combined with poly(L-lactic acid) and collagen supported rabbit CM. Castilho et al. (2017): poly(hydroxymethyl glycolide-co-ε-caprolactone) with melt electrospinning writing aligned the growth of cardiac progenitor cells.	Sugiura et al (2016): poly(L-lactic-co-ε-caprolactone) and polyglycolic acid supported human-induced pluripotent stem cell-derived CM in athymic rat. Somasuntharam et al. (2013): polyketals serve as good vehicles for delivering siRNA to the MI heart.	None reported.	[145, 147, 148]

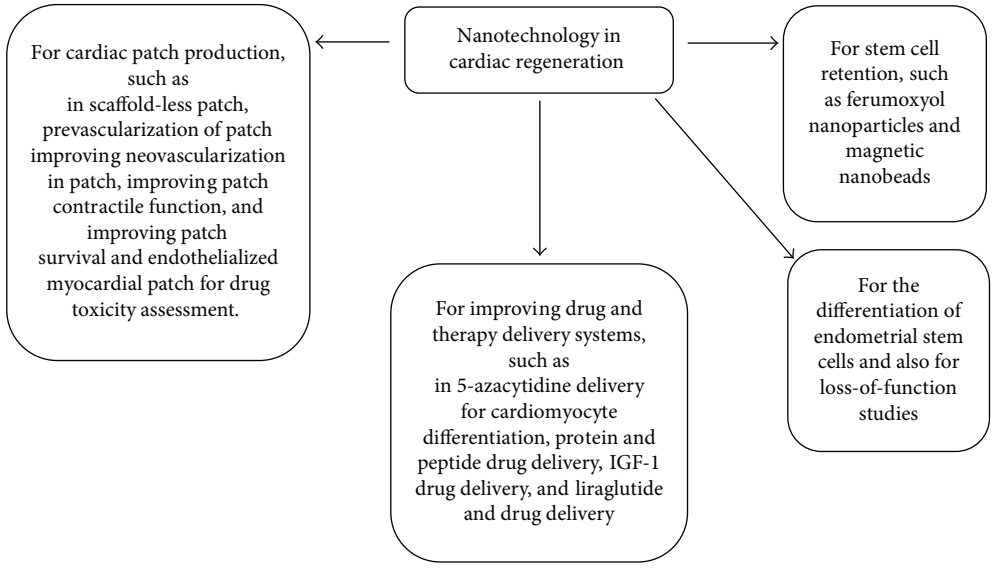


Figure 2: Application of nanotechnology in cardiomyogenesis.

cell-homogenous constructs while scaffold-free bioprinting is suited for complex and smaller constructs with poor matrices, longer time preparation, and cell-heterogeneous components [159]. Currently, the interface of scaffold-free and scaffold-dependent bioprinting is the utilization of a new generation of bioinks exclusively prepared from natural materials, like collagen, fibrin, and other organ-specific extracellular matrices [170].

The diverse potentials and applications of nanotechnology in cardioregeneration have been shown by several studies which shall be briefly considered in this review.

2.4.1. Cardiac Patch Production

(1) Scaffold-Less Patch. Native cardiac tissue simulation requires that the CMs are closely packed with adequate

electrical connections between cells maintained by the nexus (gap junctions). However, cardiac tissue construction with scaffolds attenuates these cell-to-cell-nexus interactions, and also the scaffold biodegradation can also result in inflammation [171]. Several methods have been considered for the production of scaffold-free cardiac tissue, and this includes grafting poly(N-isopropylacrylamide) to a culture surface that is thermoresponsive [172]. Another is the spheroids, which is a scaffold-free, tissue-like aggregation of cells [173].

Shimizu et al. in 2007 developed a technology which they termed "Magnetic force-based tissue engineering (Mag-TE)," which was used to construct contiguous sheets of CM [171]. Original magnetite cationic liposomes (MCLs) with a magnetite NP (Fe_3O_4) content of 10 nm were used in the study. The CM construct was shown to be a magnetically aligned functional cluster, the presence of the gap junction protein connexin 43 was demonstrated, and no toxicity was demonstrated in the construct after 24 hours of incubation [171]. However, the study did not consider the required uptake of MCLs, as well as the impact of the magnetic force.

(2) Prevascularization of Patch. Although the cell patch platform has shown successful results in cardiomyogenesis, key barriers such as low biophysical integration and a lack of organized vascular plexus still need to be overcome to achieve the high level of functional repair needed for treating myocardial injury [174]. Having this background challenge in mind, Jang et al. designed a "3D printed prevascularized stem cell patch," which they proposed would enhance tissue regeneration and repair by promoting speedy vascularization post patch transplantation [175]. The study utilized dual stem cells (CSC and MSC) spatially patterned on a decellularized extracellular matrix. The printed structure was shown to improve cell-to-cell interactions and differentiation capabilities. The patch also enhanced cardiac functions by reducing cardiac hypertrophy and fibrosis. Migration from the patch to the area of infarct was enhanced, and cardiomyogenesis and neovascularization were demonstrated at the injured myocardium [175]. However, the study did not look into the effects of structural parameters like the line width of the construct, the number of required cells and ratio of cells, and the in vitro conditioning of the prevascularized stem cell patch.

(3) Improving Neovascularization in Patch. As previously mentioned, neovascularization and organized vascular networks remain a challenge to the clinical application of the cardiac patch. The impaired nutrient supply and oxygenation perfusion post myocardial infarction will cause the regenerated cardiac tissue to be restricted to a particular zone with only a marginal improvement in function. Thus, Gaebel and colleagues designed a cardiac patch by using the laser-induced forward transfer (LIFT) cell printing method [176].

In the study, they prepared a polyester urethane urea (PEUU) cardiac patch which was seeded with hMSC and human umbilical vein endothelial cells (HUVEC) in a defined pattern. The LIFT-fabricated and controlled patches (where an equal quantity of cells was randomly seeded without LIFT) were transplanted into an infarcted rat heart, and cardiac performance was measured eight weeks post infarction. The study demonstrated that the LIFT-derived patches increased vessel formation, enhanced the capillary density, and thus significantly improved the function of the infarcted hearts [176].

(4) Improving Patch Contractile Function. We also considered the expected weakness in the contraction force of myocardial cells in the region of the heart that has been injured. As such, Fleischer et al. sought for ways to improve the performance of the cardiac patches that were transplanted for therapy [177]. It has accordingly been shown that there is a unique subpopulation of coiled perimysial fibers within the natural heart matrix [178]. These fibers have been said to provide the heart with the unique mechanical properties needed for efficient and continuous contractions as these fibers stretch and recoil with the CM [178, 179].

Fleischer et al created a nanocomposite coiled fiber scaffold, which was incorporated into the coiled fiber scaffold for heart tissue engineering [177]. The study utilized poly(ε-caprolactone), dichloromethane, and dimethylformamide which were electrospun to fabricate the coiled fibers, after which gold was deposited on their surface to create the nanocomposite. The electrospun fiber structure resembled the previously described perimysial fibers [177].

The study demonstrated that the addition of AuNPs to the scaffolds caused a rapid fabrication of elongated and aligned heart tissues with a similar morphology to that of cardiac cell bundles in vivo [177]. The study further demonstrated that the coiled fiber scaffolds demonstrated a stronger force of contraction, increased beating rates, and reduced excitation thresholds when compared with tissues grown within straight fiber scaffolds [177]. Hence, the conclusion is that this construct can be used to engineer cardiac tissues with superior functionality within diverse types of biomaterial scaffolds [177].

Likewise, in the bid to create a more functional cardiac patch that is capable of stronger contractile functions, Ravichandran et al. proposed a "gold nanoparticle-loaded hybrid nanofiber" for myocardium tissue regeneration [180]. The study sought to create a hybrid scaffold that can couple mechanical, electrical, and biological properties desired for cardiomyogenesis [180]. In the fabrication of the scaffold, the study used BSA/PVA scaffolds embedded with AuNPs by electrospinning (BSA is a water-soluble transporter protein of important physiological ligands, while PVA is polyvinyl alcohol, which is a water-soluble synthetic polymer). The study showed that differentiated cells on AuNP-loaded nanofibers expressed the cardiac proteins troponin-T, actinin, and connexin 43 and also exhibited the characteristic multinucleated morphology.

(5) Improving Patch Survival. Gaetani et al., while considering poor cell engraftment and significant cell death post transplantation, designed a cell patch targeted at increasing cell retention and survival [181]. The study looked into the therapeutic possibilities of a 3D-printed cardiac patch fabricated from human cardiac-derived progenitor cells in a matrix base of HA/gelatin. The 3D-printed biocomplex was transplanted

into a myocardial infarcted mouse model, leading to cardiac performance preservation and remarkable reduction in adverse cardiac remodeling [181]. Over the 4-week follow-up period, the matrix also showed a prolonged in vivo cell survival and engraftment with a demonstrable temporal increase in heart and vascular differentiation markers.

(6) Endothelialized Myocardial Patch for Drug Toxicity Assessment. Zhang et al. also proposed a 3D bioprinting-based novel hybrid strategy in the fabrication of an endothelialized myocardium [182]. In the study, endothelial cells were directly printed within hydrogel scaffolds, and the 3D endothelial bed was thereafter seeded with cardiac cells to create an aligned myocardium, capable of spontaneous and synchronous contractions [182]. The CM greatly expressed proteins obligatory for proper cardiac contractile function. Also, the CM expressed well-aligned sarcomeric banding with a large number of gap junctions, thus providing the histologic basis for the synchronous contraction of the cardiac construct [182].

They further went on to create an "endothelialized-myocardium-on-chip platform" by embedding the construct into a specially designed bioreactor; this was to assess for cardiovascular toxicity. The chip construct was exposed to doxorubicin, a common anticancer drug. This resulted in the reduction in the beating rate of the cardiac cells, while the control maintained a relatively high beating rate. Likewise, there was a reduction in the levels of the Von Willebrand factor secreted by the endothelial cells relative to the control [182].

2.4.2. Improving Drug and Therapy Delivery Systems

(1) 5-Azacytidine Delivery for Cardiomyocyte Differentiation. A variety of nanomaterials have been employed as nanocarriers and have been used successfully in drug delivery, and this includes gold nanorods [183], graphene NPs [184], quantum dots (GQs) [185], mesoporous silica nanoparticles (MSNs) [186], and graphene NPs [184]. The safest of the investigated nanomaterials are the MSNs because of their low toxicity, tunable particle size and pore diameter, high loading potentials, incomparable biocompatibility, and multifunctional surface properties [187, 188].

Cheng et al. utilized the MSNs to deliver the drug 5-azacytidine to regulate the differentiation of P19 cells into CM. P19 cells are teratocarcinoma-derived and have been extensively used to model cardiac cell development and CM differentiation for cardiac repair [189]. Drugs like dimethyl sulfoxide (DMSO), retinoic acid, butyrate, and 5-azacytidine can cause P19 cells to differentiate into CM; however, a major limitation to the use of P19 cells is its low efficiency of differentiation [189].

They further went on to investigate if there would be any significant difference in P19 cell differentiation into CM if 5-azacytidine were delivered differently. In the study, 5-azacytidine was delivered using fluorescein isothiocyanate isomer I mesoporous nanoparticles (FMNs) and poly(allylamine hydrochloride) (PAH); the construct was coined FMNs + 5-azacytidine + PAH nanocomplex [189]. It was reported that 5-azacytidine delivered by FMNSs demonstrated a high induction efficiency than did 5-azacytidine alone [189].

(2) Protein and Peptide Drug Delivery. The growing attention and demand on various protein and peptide drugs for treatment purposes is serving as a driver for an increasing need for efficient delivery carriers of these drugs. Currently, polymeric NPs are being considered the most preferred and suitable means of sustained delivery of protein and peptide drugs [190]. Studies have shown that polyesters such as poly(lactide-co-glycolide) possess some intrinsic shortcomings as they are said to be more hydrophobic than the majority of the protein drugs deemed for encapsulation; also, a lot of stability problems have been associated with the protein drugs during storage and release [191]. Also, various liposomal formulations have been developed for the delivery of protein drugs and considered for clinical applications [192]. However, the application of liposomes clinically is not without disadvantages, including instability [193] and a short half-life due to rapid uptake by the reticuloendothelial system [194, 195]. A number of studies subsequently looked into the characterization of "polymer-supported liposomal systems," with attention given to triblock copolymers (pluronic), which are copolymers of poly(ethylene oxide)-poly (propylene oxide)-poly(ethylene) (PEO-PPO-PEO) [196, 197]. Subsequently, a protein delivery system combining pluronic-based micelle and liposomal systems was developed and designed as a core/shell NP with a lecithin core loaded with a growth factor and a pluronic shell [198, 199].

Oh et al. reported the fabrication of a temperature-induced gel made up of core/shell NPs [190]. The construct was made of a core of lecithin loaded with a vascular endothelial growth factor (VEGF) and a shell made of a pluronic F-127 (PEO-PPO-PEO) triblock copolymer. The addition of capryol 90 (propylene glycol monocaprylate) to the core/shell NP aqueous solution resulted in the formation of a temperature-induced gel of core/shell NPs at body temperature [190]. This construct was produced to enhance stable localization and sustained release of therapeutics by core/shell NPs at the site of the ischemia. The construct was injected into a myocardial infarcted rat model, and a functional analysis of the heart was performed after four weeks. The result showed that the VEGF-loaded core/shell NP with a gel construct improved cardiac functions, especially concerning cardiac output and ejection fraction.

(3) IGF-1 Drug Delivery. Insulin-like growth factor-1 (IGF-1) has been shown to induce Akt phosphorylation in cultured CM, thus preventing apoptosis of CM [200]. Although it has been proven that IGF-1 can be used in the treatment of both acute and chronic MI, it has equally been reported that prolonged overexpression of IGF-1 results in a reduction of the functional recovery of the heart [201], hence the need for a mechanism that can accurately control the release of IGF-1. This led to the study by Chang et al., in which they complexed IGF-1 with poly(D,L-lactide-co-glycolide) (PLGA) NPs (PLGA-IGF-1 NPs) [200]. The study reported that the PLGA-IGF-1 NP complex showed increased IGF-1

retention, induced the phosphorylation of Akt, and provided early cardioprotection post myocardial infarction when compared to IGF-1 alone [200].

(4) Liraglutide Drug Delivery. A recent study by Qi et al. evaluated the long-term retention and therapeutic effects of Liraglutide, a drug developed for type 2 diabetes treatment, on cardiac regeneration [202]. In the study, liraglutide was loaded in poly(lactic-co-glycolic acid)-poly(ethylene glycol) (PLGA-PEG), and this resulted in an efficiently loading and sustained release of bioactive liraglutide. The reported therapeutic effects of liraglutide on the heart include improved cardiac performance [202] and enhanced myocardial blood flow [203], inhibition of CM apoptosis [204], attenuation of infarct size [205], and myocardial signaling pathway activation [206]. The cardioprotective effect of liraglutide has been ascribed to several factors. These include the promotion of glucose metabolism over the metabolism of fatty acid which results in decreased oxygen demand [207], increased vascularization [208], and reduction of myocardial apoptosis [205]. However, the short half-life (13 hours) of liraglutide limits its clinical applicability, thus necessitating repeated subcutaneous injections [209, 210].

The biomaterial PLGA has been demonstrated to be a potential vehicular candidate for maintaining the local concentrations of proteins by sustained release for the treatment of cardiac diseases [211, 212]. Meanwhile, PEG prevents the phagocytosis of NPs by allowing them to evade the immune system [213, 214]. From the study, liraglutide thus benefitted from the advantages offered by the combined PLGA-PEG delivery system.

In all, Qi et al. demonstrated that the intramyocardial injection of NP-liraglutide in a rat model of myocardial infarction sufficiently improved cardiac function, attenuated the infarct size, preserved myocardial wall thickness, prevented myocardial apoptosis, and promoted angiogenesis when compared with the control that had an intramyocardial injection of saline [212]. Another positive side is that the glucose levels were not altered in the rat model [212].

2.4.3. Nanoparticles and Magnets: Role in Stem Cell Retention

(1) Ferumoxytol Nanoparticles. A major limitation to the therapeutic effect of stem cell transplantation is the prevalence of low retention and engraftment rates [113]. Some studies have sought for ways to enhance cell retention and engraftment using NPs. One of those few studies includes the work done by Vandergriff et al. where ferumoxytol NPs in the presence of heparin and protamine was used to label human cardiosphere-derived stem cells [215]. The cardiosphere-derived stem cells labeled FHP were infused into syngeneic rats through the coronary vessel with magnetic targeting and without magnetic targeting as the control. This technique of augmenting acute cell retention by magnetic targeting resulted in attenuation of left ventricular remodeling and better therapeutic benefit such as improved ejection fraction three weeks after therapy. Histological sections showed enhanced engraftment of cells and angiogenesis in the cardiac tissues of the magnet-targeted group [215]. The study also demonstrated that FHP-magnetic targeting did not cause any iron overload or exacerbate cardiac inflammation and hence was said to be safe to cardiac stem cells [215]. However, further studies need to be done to determine the time window when stem cells can be reliably tracked using ferumoxytol labeling [215].

(2) Magnetic Nanobeads. A previous study using magnetic NPs is the work done by Zhang et al. where the human VEGF (hVEGF) gene was encoded in adenoviral vector (Ad)/magnetic nanobeads (MNBs), and the control of an external magnetic field was used to investigate its regenerative function on the hearts of rat models with acute myocardial infarction [216]. The complex, termed MNB/AdhVEGF, was injected intravenously with a magnet applied epicardially serving as an attractant for the circulating magnetic nanobead complex. The MNB/AdhVEGF complex when compared with the control resulted in a 50-fold higher therapeutic gene expression in the ischemic area of the heart. Also, over the control group, the MNBs/AdhVEGF complex group showed significant improvement in left ventricular function and also demonstrated higher arteriolar and capillary density with reduced collagen deposition [216].

2.5. Differentiation of Endometrial Stem Cells. Two important challenges with myocardial tissue engineering include the selection of a suitable cell source and the induction of angiogenesis [217]. Bioactive glass has been reported to affect angiogenesis, but the knowledge of its effect on soft tissue is not sufficient [218, 219]. The human endometrial stromal cells have been put forward as a rich and readily available resource in regenerative medicine. Barabadi and colleagues investigated the capacity of the endometrial stem cells to differentiate into the CM lineage in vitro. The study also evaluated the capability of bioactive glass NPs on hydrogel scaffolds to induce the differentiation of endometrial stromal cells into the endothelial lineage and to induce angiogenesis [217]. The report suggested that endometrial stem cells can be conveniently programmed into CM and are a suitable candidate for myocardial regeneration. Also, the study demonstrated improved angiogenesis [217].

2.6. Loss-of-Function Studies. The zebrafish has become an important model for studying myocardial regeneration because of its remarkable regenerative capacity. However, for the adult heart, loss-of-function studies are limited by effective gene knockdown and conditional knockout techniques [220]. Kikuchi et al. demonstrated the activation of Aldh1a2 in the endocardium and epicardium and showed that retinoic acid signaling is crucial for CM proliferation during zebrafish cardiac regeneration [221]. Also actively involved in the myocardial regeneration in zebrafish are the Gata4-GFP myocytes [221]. Diao et al. reported a novel siRNA knockdown technique using NPs of poly(ethylene glycol)-b-poly(D,L-lactide) (PEG-PLA) [220]. The siRNA-encapsulated NPs were delivered intrapleurally, and afterward, they transferred the siRNA into the cardiac tissue while avoiding the endosomes. This resulted in significant gene-

specific knockdown effects in the adult heart exhibited by downregulation of the Aldh1a2 and Dusp6 proteins [220]. This downregulation sufficiently inhibited myocardial proliferation and reduced the number of Gata4-positive cardiac cells when compared with the control, suggesting that retinoic acid signaling was compromised by siAldh1a2 therapy [220].

2.7. Tissue Engineering in Cardiovascular Regeneration. The introduction of tissue engineering in cardiovascular regeneration has led to better understanding of the cardiac ECM and its constituting cells like cardiomyocytes and fibroblasts. It has also illustrated the problem with the association of functional active biomaterials with mathematical models [222]. Engineered 3D cardiac tissue constructs (ECTCs) possess the ability to imitate a complex cardiac physiology under optimal and pathological conditions [223]. ECTCs have also been gaining more attention than traditional 2D cell cultures have by being more cost-effective, by supporting vessel formation, and by accurately duplicating in vivo cell and tissue functions of cardiovascular structures [224]. Despite its promising role in cardiac regeneration, there are few ECTCs translated to the clinic, and the major reason has been linked to inconsistency in result performed in clinical trials [225]. Another setback is the difficulty in providing a metabolically appropriate environment in ECTCs since diffusion alone cannot sustain healthy cells. The current introduction of bioreactors to create a good environment for ECTCs has also improved some of the setbacks associated with ECTCs. However, these bioreactors are complex to use, unreliable, not cost-effective, and limited in functions [226]. Currently, studies are still done on ECTCs to ensure a stable and efficient transition from bench to humans that are stable for use. Some of these studies have identified the potentials of the "I-wire" platform in controlling the applied force on ECTCs while cross-examining their inactive and active mechanical and electrical characteristics, which can be vital in ECTC production [223]. The I-wire platform in ECTCs has also proved vital in examining cardiomyocyte mechanics during auxotonic contraction [222]. Other current reports have also shown new modifiable production processes for rapid fabrication of fibrous, semilunar heart valve scaffolds by varied parameters for biomimetic heart valve replacement which lasted for 15 hours in an ovine model [227]. The current application of nanotechnology and tissue engineering in cardiac regeneration which are discussed in this review are summarized in the Table 2. In addition, they are classified based on the type of reported experimental studies.

3. Pulmonary System and Diseases

The respiratory system is a highly complex operation which is comprised of the airways (categorized into the conduction sector and the respiratory sector) and the respiratory muscles such as the diaphragm. The conducting portion transports and humidifies the air and includes the trachea, bronchi, and bronchioles up to the terminal bronchioles. At the same time, the respiratory portion is involved in the actual exchange of gas, which includes the respiratory bronchioles, alveolar ducts, and the alveolar sacs.

There are over 40 pathological conditions identifiable with the airways [228]. However, of primary concern are end-stage pulmonary problems such as lung cancers, cystic fibrosis, pulmonary hypertension, and fibrosis and the increase in the use of tobacco. Especially in developing countries, this behavior is resulting in a surge of chronic, obstructive pulmonary diseases (COPD) [229]. Treatment options that are available for acute end-stage pulmonary failures are currently limited to mechanical ventilation and extracorporeal membrane oxygenation (ECMO) [229]. However, these treatments can only temporarily sustain the patient until it is possible to perform a lung transplant. We know, unfortunately, that lung transplants are limited by a person's chances of having a suitable and matching organ donor coupled with lifelong immunosuppressive therapy. About 1000–1500 lung transplants are performed every year in the United States, and they are accompanied by diverse challenges. Recent developments in the field of regenerative medicine and tissue engineering are suggesting possible alternative therapies [230].

3.1. Strategies for Pulmonary Regenerative Therapy. The success of lung regenerative medicine will help overcome complications associated with other currently available treatments such as anastomosis failure, material failure, stenosis, and the need for a lifetime of immunosuppression [228]. Pulmonary regeneration is a daunting process, considering its highly specialized cells and complex ECM which shall be subsequently discussed. The regenerative strategies that have been applied to the lungs are broadly categorized as either cellular or a combination of cells and ECM. Although this study is mainly focussed on biomaterials which are essentially the materials that simulate the actual ECM environment, we shall briefly consider the stem cells of the lungs.

3.2. Pulmonary Cells with Regenerating Potentials. The lungs, unlike other organs, are comprised of more than 40 different types of highly specialized cells with an equally specialized extracellular matrix; hence, regenerative approaches have been challenging [228]. The lung possesses some intrinsic epithelial regenerative potentials performed by the type II alveolar pneumocytes (epithelial cells) which can proliferate and differentiate into either type I alveolar pneumocytes or more of type II pneumocytes. Other precursor cells in the lungs include the bronchiolar Clara cells and the basal cells of the pseudostratified epithelium of the human airway [228].

Studies have been carried out on the regenerative potential of human airway basal cells [231, 232], where the highly proliferative basal cell population was identified by their expression of KRT5b TP63b [225]. It is also assumed that an additional subset of basal stem cells may exist that include the reported lineage-negative epithelial progenitor (LNEP) cells in the distal part of a healthy lung, which can specifically proliferate after an injury [233]. Schilders et al. characterized basal cells by the expression of Trp63, podoplanin (Pdpn or T1α), Ngfr, GSIβ4, lectin, and cytokeratin 5 (Krt5). They also

Table 2: The current applications of nanotechnology and tissue engineering in cardiac regeneration.

Biomaterials		Experimental studies			
Classification	Subclassification	In vitro	In vivo	Clinical trials	References
Nanotechnology	Cardiac patch production	Yamato and Okano (2004): grafting poly(*N*-isopropylacrylamide) to a culture surface that is thermoresponsive in order to produce scaffold-free cardiac tissue. Fleischer et al. (2014): poly(ε-caprolactone), dichloromethane, and dimethylformamide which was electrospun resembled perimysial fibers.	Jang et al. (2017): 3D-printed prevascularized stem cell patch with CSC and MSC improved cardiomyogenesis and neovascularization. Gaebel et al. (2011): PEUU seeded with hMSC and HUVEC enhanced capillary density.	None reported.	[172, 175–177]
	Improving drug and therapy delivery systems	Cheng et al. (2016): 5-azacytidine delivered by FMNSs induced the differentiation of P19 cells to CM. Oh et al. (2006); Lee and Yuk (2007): a pluronic-based micelle and liposomal system was developed and designed as a core/shell NP with a lecithin core loaded with a growth factor and a pluronic shell and showed prospect in drug delivery.	Change et al. (2013): the PLGA-IGF-1 NP complex showed increased IGF-1 retention, induced the phosphorylation of Akt, and provided early cardioprotection postmyocardial infarction. Pascual-Gil et al. (2015): intramyocardial injection of NP-liraglutide in a rat model of myocardial infarction sufficiently improved cardiac function.	None reported.	[189, 198–200, 212]
	Nanoparticles and magnets: role in stem cell retention	None reported.	Vadergriff et al. (2014): ferumoxytol NPs in the presence of heparin and protamine were used to label stem cells. Zhang et al. (2012): MNBs/AdhVEGF complex showed significant improvement in left ventricular function.	None reported.	[215, 216]
	Differentiation of endometrial stem cells	Barabadi et al. (2016) showed that endometrial stem cells can be conveniently programmed into CM.	None reported.	None reported.	[217, 219]
	Loss-of-function studies	None reported.	Diao et al. (2015): on a zebrafish model, retinoic acid signaling was compromised by siAldh1a2 therapy.	None reported.	[220]
Tissue engineering		Sidorov et al. (2017): identified the potentials of the "I-wire" platform in controlling the applied force on ECTCs while cross-examining their inactive and active mechanical and electrical characteristics.	Emmert et al. (2017): rapid fabrication of fibrous, semilunar heart valve scaffolds for the ovine model.		[223, 226]

reported two distinct groups of basal cells: basal stem cells (BSCs) and basal, luminal precursor cells (BLPCs), both of which are also $Krt5^+$ and $Trp63^+$ [232]. The BSCs asymmetrically divide to give rise to a BSC and one BLPC, which can then differentiate into a secretory cell of a neuroendocrine cell. The BLPCs have a low rate of self-amplification and differentiation, and their expression of Krt8 makes them distinct from BSCs [232]. A small subset of basal cells (<1/5) expressing Krt14 has been shown to have a couple of functions, including maintenance of the $Krt5^+$ population of basal cells, regeneration of the ciliated and secretory cells, and rapid upregulation post injury. They may be used as markers for the identification of activated stem cells in the regenerating epithelium [232]. It has been shown that despite sharing similar markers ($Trp63^+/Krt5^+$), the distal alveolar stem cells and the tracheal basal stem cells have different fates in in vivo transplantation and culture. Other multipotent stem cells in the lungs include variant club cells, positive for secretoglobin family 1a member 1 (Scgb1a1) and Cyp2f2-negative. Another subset of $Scgb1a1^+$ cells coexpressing the

surfactant protein C (Sftpc) are the bronchoalveolar stem cells (BASCs) [232].

3.3. The Extracellular Matrix of the Lungs. The lung ECM can authoritatively be called the driver for pulmonary regeneration as it plays diverse functions under the tutelage of cellular behavior, developmental biology, and tissue mechanics [234]. It is the material in the immediate environment of the cells within a tissue, and it actively stimulates the cells. This material includes fibers (such as collagen and reticular and elastic fibers), ground substances (such as glycosaminoglycans, glycoproteins, and proteoglycans), and tissue fluids. The ECM can be said to provide the mechanical and biochemical cues that control fundamental cellular processes such as cell shape and function, cell signaling, cytoskeletal organization and differentiation, changes in proliferation and migration, gene expression and stimulation of polarity, metastatic activity induction, growth factor responses, and formation of stress fibers and focal adhesions, among others [235].

The success or failure of a regenerative construct largely depends on the quality of the underlying extracellular matrix scaffold. The breakdown of the ECM has been reported to contribute to the progression of many lung pathologies [234]. The goal of regenerative medicine, particularly tissue engineering, is to create a natural tissue to replace a damaged body part. Currently, nanotechnology is being used to see how the spatiotemporal profile of the ECM that regulates cellular behaviors can be adequately controlled [236]. Most human cells have their sizes in the microscale range (10–100 μm); however, the ECM that plays the crucial role in almost all cellular functions is in the order of the nanoscales [236]. Recreating the ECM of the lung tissue at the nanoscale has been a daunting task due to the highly complex ECM of the lung tissue, hence the move towards the decellularization of donor human or animal lung tissue as a scaffold and then recellularizing it with the required (stem) cells.

3.4. Natural/Biological and Synthetic Biomaterials in Pulmonary Regeneration. Before the whole organ decellularization technique, there has been researching into how the ECM can be reproduced or replicated to support lung regeneration using biomaterials. Biomaterials to be used for lung tissue regeneration must be biocompatible, biodegradable, and porous, and the mechanical integrity should to a large extent be equal that of the native, healthy lung tissue [237].

The biomaterials that have been studied in lung regeneration can be broadly categorized as (a) natural or protein-derived or (b) synthetic [238]. Some biomaterials could also consist of both natural and synthetic as shown in this review (Figure 3).

Natural scaffolds include collagen, fibrin, hyaluronic acid, glycosaminoglycans, elastin, alginate, chitosan, gelatin, silk, fibronectin, vitronectin laminin, casein, zein, albumin, and growth factors.

Synthetics include poly(ε-caprolactone), poly(ethylene glycol), poly(acrylic acid), poly(glycolic acid), poly(lactic acid), poly(lactic-co-glycolic acid), and poly(vinyl alcohol).

Older studies that investigated the use of either biological or synthetic biomaterials for lung tissue regeneration include elastin-based fibrous scaffolds with conducting polyaniline polymers or with a mixture of polyglycolic acid/polylactic acid [238], gel foam sponges [239], and commercial benzyl ester of hyaluronic acid and laboratory cross-linked Hylan [240]. Tracheal scaffolds have also been successfully closely replicated using polyethylene terephthalate (PET) as well as nanocomposites of PET and polyurethane (PU) fibers. However, to reproduce the more complex native lung architecture, moldable synthetic hydrogels like polyvinyl alcohol (PVA), poly(ethylene glycol), and synthetic elastomers [241] like poly(glycerol sebacate) (PGS) have been instrumental [242]. Furthermore, alveolus-like structures have reportedly been formed in collagen hydrogel [243]. Similarly, alveolar-like structures have been produced using a collagen-glycosaminoglycan structure [244]. Polyester biomaterials have also been studied for use in lung regeneration. Lung progenitor cells have also been grown both in vivo and in vitro using polyglycolic acid [245].

These studies have invariably corroborated that a single biomaterial whether biological or synthetic lacks the potential to recapitulate the complex ECM of the lung tissue. This is understandable as the ECM of any tissue is almost never made up of one substance and more so a complex ECM like that of the lungs. Hence, more recent studies have looked into harnessing and combining the individual properties of these biomaterials to create a material that might be similar or able to mimic the actual extracellular matrix of the lungs and also have the ability to facilitate adhesion and support the growth of pulmonary epithelial cells. This review has closely considered the more recent feasible uses of biomaterials for lung tissue regeneration. Hence, this section considers the natural, combined use of natural and synthetic biomaterials, while finalizing with the decellularization technique for lung tissue regeneration.

3.4.1. Natural Biomaterials

(1) Protein Scaffold: Albumin. Albumin is the most abundant serum protein in humans and has been shown to influence the attachment of cells to diverse scaffolds like collagen and fibronectin [246]. Albumin can comfortably serve as an interface between cells and scaffolds, hence enhancing the integration of one with the other. Therefore, albumin can be utilized before recellularization of a decellularized lung scaffold to facilitate cellular engraftment to the scaffold. A lot of effort has been garnered into the use of protein as a biomaterial [247, 248] because protein scaffolds like albumin are readily biodegradable and cheap and can be produced in large quantities [249, 250].

The source of albumin is not only the serum but also egg white, milk, and a host of other plant and animal tissues [246]. However, the most commonly used albumin types for tissue engineering scaffold include (a) human serum albumin, (b) porcine serum albumin, and (c) bovine serum albumin [246]. Several techniques can be employed in the synthesis of albumin scaffold; these include freeze-drying methods, chemical/enzymatic cross-linking, solution evaporation, templating and leaching, and 3D printing,

among others [251–253]. Overall, for albumin, freeze-drying, cross-linking, heat aggregation, and electrospinning techniques have been shown to be efficient in the production of their scaffolds [246].

Though the application of albumin-based biomaterials has been well established in cardiac, bone, and neural tissue engineering, albumin, to the best of our knowledge, has only been proposed for use as a biomaterial in lung regenerative therapy by Aiyelabegan and colleagues [246]. Therefore, further studies will still be required to establish the use of this material.

(2) Fibrin Gel. Angiogenesis plays vital roles in the regenerative alveolarization of adult lungs [254–256]. It has been shown that angiogenic deregulation contributes to the development of chronic lung diseases such as COPD [257], pulmonary fibrosis [258], and bronchopulmonary dysplasia [259, 260]. Hence, understanding the fundamental mechanisms for lung-specific angiogenesis is essential to the development of more efficient methods for lung tissue engineering and regenerative therapy [261]. Polymer fibrils of fibrin gels, produced from thrombin-cleaved fibrinogen, have been shown to trap angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) to facilitate angiogenesis in vivo [262]. Subcutaneous implantation of hydrogel has been extensively utilized for research in angiogenesis [263–265]. However, those methods do not summarize organ-specific angiogenesis [261]. Hence, the purpose of one study was to have fibrin gel (hydrogel) implanted directly on the surface of the lung. It was proposed that this system would allow researchers to explore the specific roles that the lung environment plays in angiogenesis and alveolar regeneration [261]. In the study, it was hypothesized that the mechanisms involved in angiogenesis and lung regeneration might have been recapitulated by manipulating the fibrin hydrogel with angiogenic factors similar to the ECM and ECM stiffness [261]. It has indeed been shown that the fibrinogen concentration alters the stiffness of fibrin gel [262]. Thus, manipulating the concentration of fibrinogen may facilitate angiogenesis through either chemical or physical signals [266]. As a result, there is a need to carefully optimize the physicochemical properties of the fibrin gels to recapitulate angiogenesis in an organ-specific manner [261].

(3) Fibrinogen/Thrombin-Based Collagen Fleece (Tachocombo). Besides angiogenic problems associated with chronic lung diseases, we could also have a case of major bleeding from a severe injury of the pulmonary artery. Tachocombo (TC) has previously been used to arrest small bleeding vessels and has been played down with regard to massive vascular injuries [267]. However, Okada et al. demonstrated in their study how TC could be used to secure hemostasis in a large defect created in the pulmonary artery of a canine since the canine's mean pulmonary arterial pressure and wall composition are known to be similar to a human's [268]. The pulmonary artery is a low-pressure apparatus with thin walls, and this makes it a suitable material for injury site compression and TC attachment [267]. TC has been shown to have an advantage over suturing as it prevents vessel stricture [267]. However, it was reported that the most critical aspect of securing hemostasis with TC is the ability to ensure complete adherence and attachment of the TC to the wall of the vessel in a field that is relatively dry [267]. Furthermore, complications associated with such procedures, such as rebleeding, thrombi, stenosis, and pseudoaneurysm, were not observed in the study at 2, 4, and 8 weeks post surgery. Also, the study showed a complete reconstruction of the defect, with minimal scarring, resembling the native vessel at 8 weeks post surgery [267]. However, it appears that this application might be limited to the pulmonary vessels because of the low pressure, as preliminary studies showed rebleeding on the aorta with the same defect size (3×3 mm) post TC application [267].

(4) Collagen-Elastic Fiber Hydrogel. Collagen is the predominant fiber content of the ECM of human tissue, including lung tissue. Lung parenchymal ECM is mainly composed of collagen types I and III, and these provide the required structural integrity [269]. However, hyaline cartilage support for the lung is essentially collagen type II. The ECM fiber content of the interalveolar septum is mainly reticular collagen and elastic fibers.

Collagen has been utilized in a variety of tissue engineering techniques, but unless further modifications are made, its use is limited to nonload-bearing applications due to its low mechanical properties. Dunphy et al. investigated the effect of adding soluble elastin to collagen hydrogel, and this addition increased the stiffness of the biomaterial [237]. The stiffness of a biomaterial has been demonstrated to influence critical cellular functions such as proliferation and differentiation [270, 271]. The combination of collagen with elastin yielded a biomaterial with high mechanical properties. Also, lung fibroblasts were introduced to the construct and resulted in Young's modulus equaling the theoretical measure of a single alveolar [237]. However, further work will still be required to explore in-depth the viscoelastic properties of this biomaterial in a time-dependent manner, diffusion through the material, and specific lung cell types, and to perform a detailed analysis of the effect of the material on cell phenotypes and behaviors [237].

3.4.2. Combination of Natural and Synthetic

(1) Gelatin-Modified Poly(ε-caprolactone) Film. Poly(ε-caprolactone)/PCL has been widely utilized in the field of regenerative medicine and tissue engineering. Kosmala et al. performed some modifications on PCL by immobilizing gelatin on the PCL surface using the amine groups. The modification of PCL increased both the strength and the biocompatibility of the material but also resulted in a loss of flexibility [272]. PCL showed no cytotoxic effects, and the cells spread properly. Furthermore, there was increased cellular proliferation on the modified PCL relative to control [272]. In this study, PCL/gelatin modification did not inhibit the spread of the human epithelial cell line NCI-H292 cells. Also, the cellular metabolic activities increased at 24 hours post seeding to 103% to control [272].

(2) Electrospun Nanofibers of Poly(ε-caprolactone) (PCL)/Depolymerized Chitosan. A recent study by Mahoney et al. prepared nanofibers of PCL/chitosan by using water-soluble chitosan. This technique was utilized because acid usually hydrolyzes PCL while it is being prepared for electrospinning and, thus, later weakens the strength of the nanofibers [273]. Trifluoroethanol (TFE), a water-miscible fluorinated alcohol, was used to dissolve PCL, and the TFE also helped to stabilize the PCL/chitosan complexes through hydrogen bonding. Favorable PCL/chitosan molecular interaction is essential to maintaining mechanical and structural integrity for use as a biomaterial in tracheal tissue regeneration therapy. However, owing to its immiscibility, the maximum PCL/chitosan ratio that could be achieved was 70 : 30, and it was virtually impossible to attain greater than 30% chitosan nanofibers [273]. The 80/20 and 70/30 ratios demonstrate minor differences in the nanofibers' morphology concerning cell-to-fiber interaction [273]. The tensile strength of PCL/chitosan has been shown to be much higher than PCL-based composite scaffolds for trachea bioengineering [274]. These scaffolds have also been shown to be nontoxic as the cytotoxic levels of PCL/chitosan nanofibers were shown to be nearly equivalent to PCL and a collagen-coated control [273]. However, additional reinforcing material might be required to achieve a better tensile strength as the PCL/chitosan is expected to degrade and be replaced with regenerated tissue in the long term.

(3) Hyaluronic Acid-g-Poly(2-hydroxyethyl methacrylate) (HEMA) Copolymer. Hyaluronic acid (HA) is a component of the ECM which promotes growth as well as the proliferation of cells. Despite its diverse applicability, it has some inherent pitfalls. These include its poor biochemical properties, and its coiled structure gives it an enormous water affinity as it can trap about 1000 times its weight in water, and this ultimately affects its applicability to the field of regenerative medicine [275]. However, several reports have shown that modified HA still appears a suitable material for tissue engineering [276–278]. Radhakumary et al. reported a copolymer of HA and poly(HEMA) [275]. The biomaterial poly(HEMA) is considered one of the most important hydrogels, and its advantages outnumber the other hydrogels [279]. Poly(HEMA) is inert to biological processes, contains water content close to living tissues, is permeable to metabolites, resists degradation, and resists absorption by the body [275]. The copolymer was proven to be an excellent choice for a "natural-synthetic polymer hybrid matrix" and demonstrated the synergistic properties of both materials, such as biocompatibility and water stability [275]. Additionally, in contrast to virgin HA, the copolymer films were found to be stable in water at both neutral and acidic pH. Other advantages also include noncytotoxicity, and most importantly, the copolymer was shown to support multiple cell types, such as alveolar cell adhesion and growth [275].

(4) 3D Macroporous Hydroxyethyl Methacrylate-Alginate-Gelatin (HAG) Cryogel. Singh et al. considered a combination of hydroxyethyl methacrylate (HEMA), alginate, and gelatin in lung tissue regeneration [280]. Hydroxyethyl methacrylate (HEMA) is a known highly biocompatible polymer that has been used extensively in tissue engineering [281, 282]. Alginate is also famous for tissue engineering, and it is nontoxic and biodegradable [283, 284]. Gelatin has well-known cell-adhesive properties (arginlyglycylaspartic acid), hence the choice of these three materials [282].

Singh et al. reported that the HAG cryogel does not require surgical intervention to be removed from the system, as the alginate and gelatin components are quickly degraded into smaller fragments that are below the renal threshold [282]. HAG cryogel was shown to be highly elastic and possessed a quick hydration, which indicated that the porous network of polymers is interconnected. It also maintained a good flow rate, and no back pressure was reported [282]. In the study, the implantation of the scaffold was done without cells; however, the scaffold was shown to recruit cells from the surrounding tissue, and the scaffold was completely integrated into the tissue at five weeks. Interestingly, the infiltrated cells were not the expected first-line defense cells, such as the mast cells and dendritic cells that are responsible for graft rejection; hence, biocompatibility of this combination was demonstrated. The HAG cryogel seeded with lung cells showed collagen deposition which is an obligate fiber of the ECM of the lung; as already stated, the cell matrix interaction is an ultimate determinant of the fate of tissue regeneration. However, the study reported a small amount of infiltrating mast cells which were said to have diminished over a few weeks [282].

3.4.3. Whole Lung Decellularization. Decellularization involves the utilization of radiation, detergents, enzymes like nucleases and trypsin, and chemical treatments like acid/alkaline or salt solutions, among others [76]. Although several approaches to decellularization are being used, the optimal decellularization technique is yet to be defined [285]. The breakthrough in organ bioengineering in regenerative medicine where intact lung scaffolding can be decellularized and recellularized is carried out in a bioreactor. The bioreactor is a sterile, closed system with a well-designed mechanism for perfusion and ventilation [286]. The recellularization of the lung scaffold has been performed using the following: autologous, allogeneic, and xenogeneic cell sources; adult, amniotic, differentiated, embryonic, and induced pluripotent cell types; and mixing, perfusion, priming, surface seeding, and microinjection seeding techniques. On the other hand, culture has been done using static approaches such as air exposure, submerging, and, as mentioned earlier, the use of bioreactors [76]. One study proposed a possible alternative approach to deliver cells to the scaffold after implantation, whereby the body's repair mechanism would be harnessed to deliver (stem) cells with their correct spatial organization [73]. This is because during lung injury, fibroblasts and endothelial and endothelial progenitor cells (circulating bone marrow-derived cells) are home to injured sites from the blood. However, Lemon et al. thought it would be speculative to assume this will succeed as it is not clear that the bone marrow contains sufficient amount of precursor cells to facilitate regeneration [73]. However, in a previous study, Lim et al. demonstrated that the decellularized pig's trachea

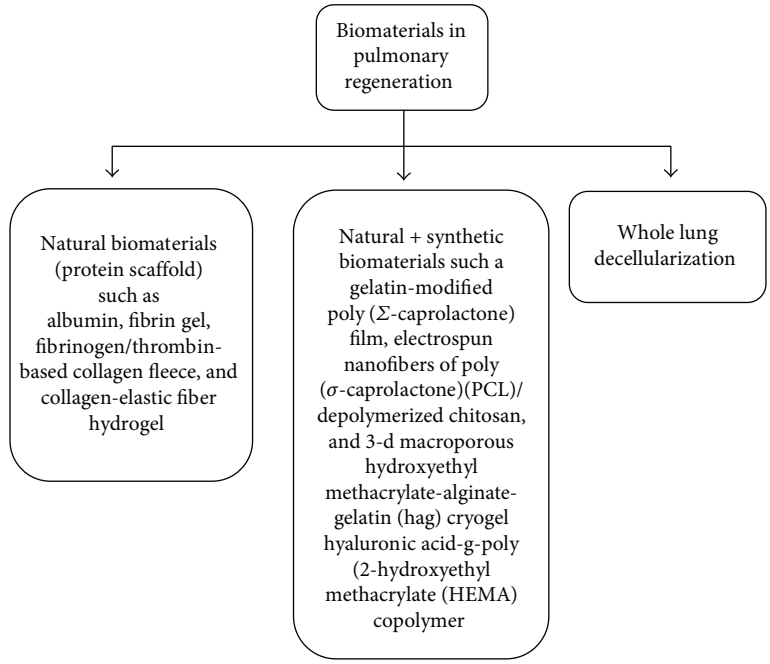


Figure 3: Biomaterials in pulmonary regeneration.

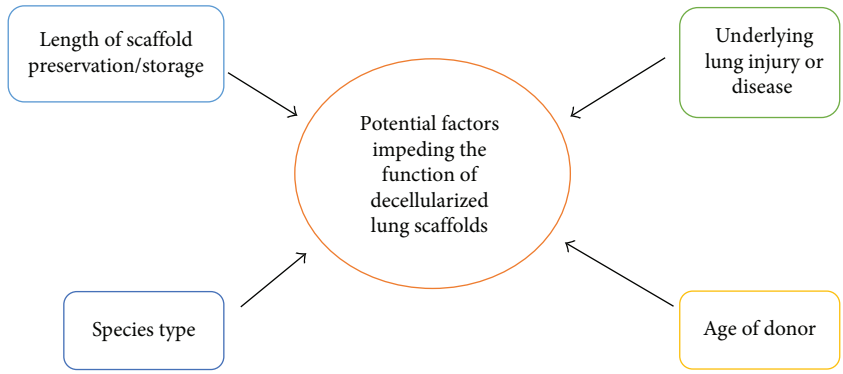


Figure 4: Limitations to the use of decellularized scaffold.

regenerated in vivo without it being recellularized before it was transplanted. They showed the possibility of the body as a bioreactor which, of course, will reduce engineering cost, contamination, and processing time. In the study, they boosted the in vivo regeneration by administering growth factors erythropoietin (EPO) and the granulocyte colony-stimulating factor (GCSF) to enhance the mobilization and differentiation of stem cells and progenitors as an additional therapeutic concept [285]. The limitation, however, was that the mechanisms and pathways were poorly understood and thus further recommendation for a routine clinical application cannot be made.

3.4.4. The Possible Limitations to the Use of Decellularized Scaffold for Regenerative Therapy. There are a couple of factors that have been reported by literatures to be possible drawbacks to the utilization of some decellularized lung scaffolds as a material for regenerative medicine. Some of these factors may occur in isolation or combination. The factors which may possibly impede the functionality of decellularized lung scaffolds are summarized in Figure 4.

(1) Age. The source of the potential donor lung may be an older adult. Thus, research into the effect of age on this ECM biomaterial is necessary to understand its suitability for regenerative therapy. Sokocevic et al. suggested that although organs from a donor of advanced age might appear unsuitable for utilization, the ECM structures required for the initial binding of cells with subsequent growth and proliferation were mostly preserved [73]. Therefore, decellularized aged lungs might be considered for bioengineering approaches, but other varieties of stem cells need to be studied aside from the stromal cell line mesenchymal stem cells (MSCs) used in this study. Furthermore, to the best of our knowledge, no study has been done to determine the age range beyond which a donor's lung becomes unsuitable for bioengineering.

Table 3: Classifications of biomaterials used in lung tissue regeneration.

Biomaterials		Experimental studies			
Classification	Subclassification	In vitro	In vivo	Clinical trials	References
Natural biomaterials	Albumin	Aiyelabegan et al. (2016): albumin enhanced the integration of cells and scaffolds with one another.	None reported.	None reported.	[246]
	Fibrin gel	None reported.	Mammoto et al. (2013): polymer fibrils of fibrin gels trapped VEGF and bFGF and enhanced angiogenesis in a rat model	None reported.	[260]
	Fibrinogen/ thrombin-based collagen fleece	None reported.	Ikeda et al. (2011): TC is better than suturing because it prevents vessel stricture in a canine model.	None reported.	[267]
	Collagen-elastic fiber hydrogel	Hadjipanayi et al. (2009): influenced cellular proliferation and differentiation	None reported.	None reported.	[270]
Combination of natural and synthetic biomaterials	Gelatin-modified poly(ε-caprolactone) film	Kosmala et al. (2016): PCL/gelatin modification did not stop human epithelial cell line NCI-H292 cells to proliferate.	None reported.	None reported.	[272]
	Electrospun nanofibers of poly(ε-caprolactone)(pcl)/ depolymerized chitosan	Mahoney et al. (2016): PCL/chitosan molecular interaction helped maintain the architecture of tracheal tissue regeneration therapy.	None reported.	None reported.	[273]
	Hyaluronic acid-g-poly (2-hydroxyethyl methacrylate (hema) copolymer	Radhakumary et al. (2011): copolymer of HA and poly(HEMA) was observed as the best choice for the "natural-synthetic polymer hybrid matrix"	None reported.	None reported.	[275]
	3D macroporous hydroxyethyl methacrylate-alginate-gelatin (hag) cryogel	Singh et al. (2011): combining HEMA, alginate, and gelatin improved lung tissue regeneration.	None reported.	None reported.	[280]

(2) Underlying Lung Injury or Disease. The effects of pulmonary diseases like emphysema and fibrosis on the ECM have been reported in some studies. Sokocevic et al. reported that severely diseased lungs would not be considered suitable for decellularization-recellularization, but mild or moderately injured lungs could be regarded as suitable [286]. They showed that despite changes as a result of injury to the lungs (emphysematous changes and fibrosis), ECM structures were appropriately preserved and good initial engraftment was reported. However, despite this initial engraftment, the survival of the MSCs was reduced in the emphysematous lungs. On the other hand, comparable binding, proliferation, and survival were reported in the bleomycin-induced fibrotic lungs, but the more fibrotic zones demonstrated no initial stem cell engraftment and growth [286]. Similarly, another study showed that despite the initial binding of the various stem cells (bronchial epithelial cells, bone marrow-derived MSCs, endothelial cells, and lung fibroblasts) inoculated into decellularized emphysematous lungs, they did not survive for more than one week as opposed to the healthy lungs that survived for about one month [287]. This study also demonstrated that there were no significant differences in the ECM of normal and emphysematous lungs. Wagner et al., in another study, showed the difficulty involved in generating a uniformly decellularized scaffold from a human lung with an underlying interstitial pulmonary fibrosis [287].

(3) Length of Scaffold Preservation or Storage. Biological scaffolds, such as those obtained from bone and cartilage, among others, can be stored for a relatively prolonged period before use, especially when treated for instance with low levels of irradiation [288]. However, despite maintaining sterile conditions, it was shown that decellularized lungs should not be stored for more than three months. Also, although the lung scaffold was irradiated at a dose lower than recommended, it showed significant lung ECM distortion and only partly responded to subsequent lung inflation [288]. Baiguera et al. similarly showed that the histoarchitecture of a decellularized trachea became fragmented and less organized after one year, with a relatively loose ECM accompanied by some structural alterations [289]. It was also reported that the angiogenic properties of a one-year decellularized scaffold declined relative to the fresh samples [290]. In this study, they considered a possible restoration of the ECM structure of one-year decellularized tissue by attempting to

stabilize the triple-helix structure of the collagen with a natural cross-linking agent known as genipin (1% concentration). This resulted in a more compact, organized matrix that was more resistant to collagenase relative to control and also expressed increased angiogenic potential [291]. However, genipin did not significantly increase the mechanical properties of the one-year stored sample, nor did it affect the organization of its vascular network. This suggests that some long-term ECM deterioration may be irreversible. Genipin has been shown to be a relatively safe cross-linker [292], and a high concentration of genipin might make the scaffold more stable. However, increasing its concentration has reportedly exerted some distinct effects of cell type varieties [293, 294].

(4) Species Types. A recent study compared the decellularized scaffolds of different kinds of species: rat, pig, primate, and human. Residual DNA, mechanical property, and fundamental matrix proteins—such as collagen, elastin, and glycosaminoglycans—were assessed in these scaffolds [234]. The study revealed that despite similar levels of collagen among the different species after decellularization, primate and human lung scaffolds were stiffer and possessed more elastin but retained fewer glycosaminoglycans than did rat or pig scaffolds [234]. Moreover, the adhesion of seeded endothelial cells was remarkably enhanced on both the primate and human scaffolds. In all, the decellularized human lung scaffold possessed the ECM profile that closely resembled that of native lung tissue. The primate had modest ECM changes while the rat and pig showed significant losses of ECM [234]. On the other hand, this work was limited by differences in age among the selected species. It can then be inferred from this work that in the search for a scaffold for lung regeneration, the human decellularized scaffold provides an unparalleled ECM. The current biomaterial scaffolds which are used for the lung tissue regeneration are summarized in the Table 3.

4. Reflection and Speculations

This review has revealed how complex the cardiopulmonary system is as defined by the individual systemic architectural diversity at the levels of the cells and the extracellular matrix. Hence, the design of any biomaterial for regeneration or therapeutic purposes must be largely based on the natural tissue composition of the damaged site for the efficiency of integration and optimal functionality.

Regeneration of the heart and lungs probably might have been less cumbersome assuming they were an offshoot of the same embryological region. However, the heart is carved out mainly from the lateral mesoderm [113] and the lungs being more of an anterior endodermal offshoot [295], thus accounting for the observed diversity in cellular and tissue composition and thus necessitating research into diverse biomaterials able to efficiently simulate these systems.

Current medical and surgical techniques can only manage cardiopulmonary diseases conservatively and sometimes temporarily but hopefully in the nearest future; regenerative medicine might provide the much needed long-lasting therapy with improvement in the quality of life.

Understanding that the histology of the lungs vastly varies from the conducting zone to the respiratory zone, which also largely differs from that of the heart, should direct the therapeutic approach to be employed especially when biomaterials have to be utilized. The biomaterials have to be tailored to suit the damaged tissue area for it to be properly integrated into the host tissue. However, this is currently a daunting task.

Interestingly in 2013, Peng et al. made an important discovery describing the codevelopment of the cardiopulmonary system. The team demonstrated multipotent population of cardiopulmonary mesodermal progenitors (CPPs) at the posterior cardiac pole expressing Wnt2, Isll, and Glil. Regulating the CPPs was the sonic hedgehog (Shh) expressed from the foregut endodermal origin of the primitive tracheal diverticulum (lung bud). This facilitates the connection of the heart to the pulmonary vasculature [295]. These CPPs produce the mesodermal lines of the cardiac inflow tract and lungs, also including the cardiac cells, pulmonary vascular and smooth muscles of the airways, proximal vascular epithelial cell, and cells resembling pericytes.

The excitement here, although still premature, is the possibility of having some parts of the cardiopulmonary system especially the pulmonary vascular tree being regenerated in concert with the mesodermal derivatives of the lungs. Probably, these multipotent progenitor cells in combination with other progenitors of epithelial and endothelial cells, incorporated with the right signals or growth factors, and transplanted on the right biomaterial platform might just make the right concoction.

In speculating for a pathology like COPD, especially chronic bronchitis, there is irreversible damage to the wall of the organ. These CPPs, if translated with the right biomaterial, might just help in the regeneration of the mesodermal derivatives. However, this might not apply to emphysema, another COPD, which is largely a problem with elastic fibers at the distal alveoli as the CPPs do not extend this far embryologically [289]. However, stem cell therapy is currently not advocated for COPDs (https://www.copdfoundation.org).

Another speculation might be for the decellularized scaffolds for which a couple of negative factors have been identified. Decellularization takes out not only the epithelial cells but also cells of mesodermal origin. Hence, these scaffolds might benefit from incubation in these CPPs alongside other cells as they might help with regenerating the core of the airway such as the cartilages, smooth muscles, connective tissue, and blood vessels, among others.

Although this discovery and its potential therapeutic applications are still in its infancy, it certainly has helped to demystify the gray area in cardiopulmonary embryologic development.

Hence, the CPP-biomaterial combination might be an area for future research considerations for cardiopulmonary regeneration and therapies.

5. Conclusion

Several therapies have been trialed in the treatment of cardiopulmonary diseases, but they have yet to provide a desired

quality of life. Likewise, organ transplants are not a readily available therapy and, when available, only provide a temporary palliation as they are not devoid of complications. These issues have fueled the exploration of stem cells and regenerative medicine to find ways to repair damaged cardiac tissue, using not only stem cells but biomaterials that could replace the damaged environment in which the cells reside. This review has shown that it will probably become a necessity to combine biomaterials, either biological or synthetic, to stimulate the damaged ECM while, equally, incorporating more recent nanotechnology techniques. However, owing to the complexities involved in recapitulating this ECM, decellularizing and recellularizing of donor tissues or organs appear to be a reprieve, but this also is not free of its challenges. Thus, further research is still required to explore the synergy of biomaterials and improve decellularization-recellularization methods. This review also speculated on the possible regenerative potentials of CPPs which is currently at its infancy.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Adegbenro Omotuyi John Fakoya and David Adeiza Otohinoyi wrote the manuscript; Adegbenro Omotuyi John Fakoya and Joshua Yusuf reviewed it; and Adegbenro Omotuyi John Fakoya, David Adeiza Otohinoyi, and Joshua Yusuf approved the manuscript for publication.

Acknowledgments

The authors would like to thank Professor Paulo Madeddu, professor of Experimental Cardiovascular Medicine, University of Bristol, Bristol, UK, for his constructive comments that enhanced this work.

References

[1] Z. Cui, B. Yang, and R.-K. Li, "Application of biomaterials in cardiac repair and regeneration," *Engineering*, vol. 2, no. 1, pp. 141–148, 2016.

[2] Writing Group Members, D. Mozaffarian, E. J. Benjamin et al., "Heart Disease and Stroke Statistics—2016 update," *Circulation*, vol. 133, no. 4, pp. e38–e360, 2015.

[3] M. Arnal-Pastor, J. C. Chachques, M. Monleón Pradas, and A. Vallés-Lluch, "Biomaterials for cardiac tissue engineering," in *Regenerative Medicine and Tissue Engineering*, J. A. Andrades, Ed., 2013, InTech, https://mts.intechopen.com/books/regenerative-medicine-and-tissue-engineering/biomaterials-for-cardiac-tissue-engineering.

[4] M. Kharaziha, A. Memic, M. Akbari, D. A. Brafman, and M. Nikkhah, "Nano-enabled approaches for stem cell-based cardiac tissue engineering," *Advanced Healthcare Materials*, vol. 5, no. 13, pp. 1533–1553, 2016.

[5] C. Jourdan-LeSaux, J. Zhang, and M. L. Lindsey, "Extracellular matrix roles during cardiac repair," *Life Sciences*, vol. 87, no. 13-14, pp. 391–400, 2010.

[6] Z. Lister, K. J. Rayner, and E. J. Suuronen, "How biomaterials can influence various cell types in the repair and regeneration of the heart after myocardial infarction," *Frontiers in Bioengineering and Biotechnology*, vol. 4, 2016.

[7] L. Gaucherand, B. A. Falk, S. P. Evanko, G. Workman, C. K. Chan, and T. N. Wight, "Crosstalk between T lymphocytes and lung fibroblasts: generation of a Hyaluronan-enriched extracellular matrix adhesive for monocytes," *Journal of Cellular Biochemistry*, vol. 118, no. 8, pp. 2118–2130, 2017.

[8] T. Kempf, A. Zarbock, D. Vestweber, and K. C. Wollert, "Anti-inflammatory mechanisms and therapeutic opportunities in myocardial infarct healing," *Journal of Molecular Medicine*, vol. 90, no. 4, pp. 361–369, 2012.

[9] J. Cutts, M. Nikkhah, and D. A. Brafman, "Biomaterial approaches for stem cell-based myocardial tissue engineering," *Biomarker Insights*, vol. 10s1, article BMI.S20313, 2015.

[10] L. Saludas, S. Pascual-Gil, F. Prósper, E. Garbayo, and M. Blanco-Prieto, "Hydrogel based approaches for cardiac tissue engineering," *International Journal of Pharmaceutics*, vol. 523, no. 2, pp. 454–475, 2017.

[11] D. Seliktar, "Designing cell-compatible hydrogels for biomedical applications," *Science*, vol. 336, no. 6085, pp. 1124–1128, 2012.

[12] A. P. Beltrami, L. Barlucchi, D. Torella et al., "Adult cardiac stem cells are multipotent and support myocardial regeneration," *Cell*, vol. 114, no. 6, pp. 763–776, 2003.

[13] R. Bolli, A. R. Chugh, D. D'Amario et al., "Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial," *The Lancet*, vol. 378, no. 9806, pp. 1847–1857, 2011.

[14] K. Matsuura, T. Nagai, N. Nishigaki et al., "Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes," *Journal of Biological Chemistry*, vol. 279, no. 12, pp. 11384–11391, 2004.

[15] L. Bu, X. Jiang, S. Martin-Puig et al., "Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages," *Nature*, vol. 460, no. 7251, pp. 113–117, 2009.

[16] E. Messina, L. De Angelis, G. Frati et al., "Isolation and expansion of adult cardiac stem cells from human and murine heart," *Circulation Research*, vol. 95, no. 9, pp. 911–921, 2004.

[17] C.-L. Cai, J. C. Martin, Y. Sun et al., "A myocardial lineage derives from Tbx18 epicardial cells," *Nature*, vol. 454, no. 7200, pp. 104–108, 2008.

[18] O. Pfister, F. Mouquet, M. Jain et al., "CD31- but not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation," *Circulation Research*, vol. 97, no. 1, pp. 52–61, 2005.

[19] B. G. Galvez, M. Sampaolesi, A. Barbuti et al., "Cardiac mesoangioblasts are committed, self-renewable progenitors, associated with small vessels of juvenile mouse ventricle," *Cell Death and Differentiation*, vol. 15, no. 9, pp. 1417–1428, 2008.

[20] M. Rienks, A.-P. Papageorgiou, N. G. Frangogiannis, and S. Heymans, "Myocardial extracellular matrix: an ever-changing and diverse entity," *Circulation Research*, vol. 114, no. 5, pp. 872–888, 2014.

[21] L. Brown, "Cardiac extracellular matrix: a dynamic entity," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 289, no. 3, pp. H973–H974, 2005.

[22] D. Fan, A. Takawale, J. Lee, and Z. Kassiri, "Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease," *Fibrogenesis & Tissue Repair*, vol. 5, no. 1, p. 15, 2012.

[23] V. F. M. Segers and R. T. Lee, "Biomaterials to enhance stem cell function in the heart," *Circulation Research*, vol. 109, no. 8, pp. 910–922, 2011.

[24] M. Cheraghi, M. Namdari, B. Negahdari, and A. Eatemadi, "RETRACTED: recent advances in cardiac regeneration: stem cell, biomaterial and growth factors," *Biomedicine & Pharmacotherapy*, vol. 87, pp. 37–45, 2017.

[25] D. M. Nelson, Z. Ma, K. L. Fujimoto, R. Hashizume, and W. R. Wagner, "Intra-myocardial biomaterial injection therapy in the treatment of heart failure: materials, outcomes and challenges," *Acta Biomaterialia*, vol. 7, no. 1, pp. 1–15, 2011.

[26] T. L. B. Ha, T. M. Quan, D. N. Vu, and D. M. Si, "Naturally derived biomaterials: preparation and application," in *Regenerative Medicine and Tissue Engineering*, J. A. Andrades, Ed., 2013, InTech, https://mts.intechopen.com/books/regenerative-medicine-and-tissue-engineering/naturally-derived-biomaterials-preparation-and-application.

[27] I. F. Wahab and S. I. A. Razak, "Polysaccharides as composite biomaterials," in *Composites from Renewable and Sustainable Materials*, M. Poletto, Ed., 2016, InTech, https://mts.intechopen.com/books/composites-from-renewable-and-sustainable-materials/polysaccharides-as-composite-biomaterials.

[28] L. J. R. Foster, S. Ho, J. Hook, M. Basuki, and H. Marçal, "Chitosan as a biomaterial: influence of degree of deacetylation on Its physiochemical, material and biological properties," *PLoS One*, vol. 10, no. 8, article e0135153, 2015.

[29] M.-C. Yang, S.-S. Wang, N.-K. Chou et al., "The cardiomyogenic differentiation of rat mesenchymal stem cells on silk fibroin–polysaccharide cardiac patches in vitro," *Biomaterials*, vol. 30, no. 22, pp. 3757–3765, 2009.

[30] T. Wu, Y. Li, and D. S. Lee, "Chitosan-based composite hydrogels for biomedical applications," *Macromolecular Research*, vol. 25, no. 6, pp. 480–488, 2017.

[31] E. Khor, "Chitin: a biomaterial in waiting," *Current Opinion in Solid State and Materials Science*, vol. 6, no. 4, pp. 313–317, 2002.

[32] A. M. Martins, G. Eng, S. G. Caridade, J. F. Mano, R. L. Reis, and G. Vunjak-Novakovic, "Electrically conductive chitosan/carbon scaffolds for cardiac tissue engineering," *Biomacromolecules*, vol. 15, no. 2, pp. 635–643, 2014.

[33] B.-H. Liu, H.-Y. Yeh, Y.-C. Lin et al., "Spheroid formation and enhanced cardiomyogenic potential of adipose-derived stem cells grown on chitosan," *BioResearch Open Access*, vol. 2, no. 1, pp. 28–39, 2013.

[34] P. Baei, S. Jalili-Firoozinezhad, S. Rajabi-Zeleti, M. Tafazzoli-Shadpour, H. Baharvand, and N. Aghdami, "Electrically conductive gold nanoparticle-chitosan thermosensitive hydrogels for cardiac tissue engineering," *Materials Science and Engineering: C*, vol. 63, pp. 131–141, 2016.

[35] B. Guo, A. Finne-Wistrand, and A.-C. Albertsson, "Facile synthesis of degradable and electrically conductive polysaccharide hydrogels," *Biomacromolecules*, vol. 12, no. 7, pp. 2601–2609, 2011.

[36] R. A. Green, R. T. Hassarati, J. A. Goding et al., "Conductive hydrogels: mechanically robust hybrids for use as biomaterials," *Macromolecular Bioscience*, vol. 12, no. 4, pp. 494–501, 2012.

[37] G. Camci-Unal, N. Annabi, M. R. Dokmeci, R. Liao, and A. Khademhosseini, "Hydrogels for cardiac tissue engineering," *NPG Asia Materials*, vol. 6, no. 5, article e99, 2014.

[38] H. Wang, J. Shi, Y. Wang et al., "Promotion of cardiac differentiation of brown adipose derived stem cells by chitosan hydrogel for repair after myocardial infarction," *Biomaterials*, vol. 35, no. 13, pp. 3986–3998, 2014.

[39] N.-H. Chi, M.-C. Yang, T.-W. Chung, N.-K. Chou, and S.-S. Wang, "Cardiac repair using chitosan-hyaluronan/silk fibroin patches in a rat heart model with myocardial infarction," *Carbohydrate Polymers*, vol. 92, no. 1, pp. 591–597, 2013.

[40] H. Wang, X. Zhang, Y. Li et al., "Improved myocardial performance in infarcted rat heart by co-injection of basic fibroblast growth factor with temperature-responsive chitosan hydrogel," *The Journal of Heart and Lung Transplantation*, vol. 29, no. 8, pp. 881–887, 2010.

[41] S. Chopra, S. Mahdi, J. Kaur, Z. Iqbal, S. Talegaonkar, and F. J. Ahmad, "Advances and potential applications of chitosan derivatives as mucoadhesive biomaterials in modern drug delivery," *Journal of Pharmacy and Pharmacology*, vol. 58, no. 8, pp. 1021–1032, 2006.

[42] L. Wang, J. Shansky, C. Borselli, D. Mooney, and H. Vandenburgh, "Design and fabrication of a biodegradable, covalently crosslinked shape-memory alginate scaffold for cell and growth factor delivery," *Tissue Engineering Part A*, vol. 18, no. 19-20, pp. 2000–2007, 2012.

[43] I. Perea-Gil, C. Prat-Vidal, and A. Bayes-Genis, "In vivo experience with natural scaffolds for myocardial infarction: the times they are a-changin," *Stem Cell Research & Therapy*, vol. 6, no. 1, p. 248, 2015.

[44] E. Ruvinov and S. Cohen, "Alginate biomaterial for the treatment of myocardial infarction: progress, translational strategies, and clinical outlook," *Advanced Drug Delivery Reviews*, vol. 96, pp. 54–76, 2016.

[45] N. Landa, L. Miller, M. S. Feinberg et al., "Effect of injectable alginate implant on cardiac remodeling and function after recent and old infarcts in rat," *Circulation*, vol. 117, no. 11, pp. 1388–1396, 2008.

[46] J. Leor, S. Tuvia, V. Guetta et al., "Intracoronary injection of in situ forming alginate hydrogel reverses left ventricular remodeling after myocardial infarction in swine," *Journal of the American College of Cardiology*, vol. 54, no. 11, pp. 1014–1023, 2009.

[47] H. Sabbah, M. Wang, R. Gupta et al., "Augmentation of left ventricular wall thickness with alginate hydrogel implants improves left ventricular function and prevents progressive remodeling in dogs with chronic heart failure," *JACC: Heart Failure*, vol. 1, no. 3, pp. 252–258, 2013.

[48] J. Leor, S. Aboulafia-Etzion, A. Dar et al., "Bioengineered cardiac grafts : a new approach to repair the infarcted myocardium?," *Circulation*, vol. 102, Supplement 3, pp. III-56–III-61, 2000.

[49] Y. Yeghiazarians, M. Gaur, Y. Zhang et al., "Myocardial improvement with human embryonic stem cell-derived cardiomyocytes enriched by p38MAPK inhibition," *Cytotherapy*, vol. 14, no. 2, pp. 223–231, 2012.

[50] J. Yu, K. T. Du, Q. Fang et al., "The use of human mesenchymal stem cells encapsulated in RGD modified alginate microspheres in the repair of myocardial infarction in the rat," *Biomaterials*, vol. 31, no. 27, pp. 7012–7020, 2010.

[51] O. Tsur-Gang, E. Ruvinov, N. Landa et al., "The effects of peptide-based modification of alginate on left ventricular remodeling and function after myocardial infarction," *Biomaterials*, vol. 30, no. 2, pp. 189–195, 2009.

[52] Y. Sapir, O. Kryukov, and S. Cohen, "Integration of multiple cell-matrix interactions into alginate scaffolds for promoting cardiac tissue regeneration," *Biomaterials*, vol. 32, no. 7, pp. 1838–1847, 2011.

[53] T. Dvir, B. P. Timko, M. D. Brigham et al., "Nanowired three-dimensional cardiac patches," *Nature Nanotechnology*, vol. 6, no. 11, pp. 720–725, 2011.

[54] Y. Sapir, B. Polyak, and S. Cohen, "Cardiac tissue engineering in magnetically actuated scaffolds," *Nanotechnology*, vol. 25, no. 1, article 014009, 2014.

[55] B. Deng, L. Shen, Y. Wu et al., "Delivery of alginate-chitosan hydrogel promotes endogenous repair and preserves cardiac function in rats with myocardial infarction," *Journal of Biomedical Materials Research Part A*, vol. 103, no. 3, pp. 907–918, 2015.

[56] R. J. Lee, A. Hinson, S. Helgerson, R. Bauernschmitt, and H. N. Sabbah, "Polymer-based restoration of left ventricular mechanics," *Cell Transplantation*, vol. 22, no. 3, pp. 529–533, 2013.

[57] K. Y. Lee and D. J. Mooney, "Alginate: properties and biomedical applications," *Progress in Polymer Science*, vol. 37, no. 1, pp. 106–126, 2012.

[58] J. Dahlmann, G. Kensah, H. Kempf et al., "The use of agarose microwells for scalable embryoid body formation and cardiac differentiation of human and murine pluripotent stem cells," *Biomaterials*, vol. 34, no. 10, pp. 2463–2471, 2013.

[59] F. N. Alaribe, S. L. Manoto, and S. C. K. M. Motaung, "Scaffolds from biomaterials: advantages and limitations in bone and tissue engineering," *Biologia*, vol. 71, no. 4, 2016.

[60] A. D. Cigan, B. L. Roach, R. J. Nims et al., "High seeding density of human chondrocytes in agarose produces tissue-engineered cartilage approaching native mechanical and biochemical properties," *Journal of Biomechanics*, vol. 49, no. 9, pp. 1909–1917, 2016.

[61] N. Itano, "Simple primary structure, complex turnover regulation and multiple roles of hyaluronan," *Journal of Biochemistry*, vol. 144, no. 2, pp. 131–137, 2008.

[62] P. Bulpitt and D. Aeschlimann, "New strategy for chemical modification of hyaluronic acid: preparation of functionalized derivatives and their use in the formation of novel biocompatible hydrogels," *Journal of Biomedical Materials Research*, vol. 47, no. 2, pp. 152–169, 1999.

[63] F. Bonafè, M. Govoni, E. Giordano, C. M. Caldarera, C. Guarnieri, and C. Muscari, "Hyaluronan and cardiac regeneration," *Journal of Biomedical Science*, vol. 21, no. 1, p. 100, 2014.

[64] S. J. Yoon, Y. H. Fang, C. H. Lim et al., "Regeneration of ischemic heart using hyaluronic acid-based injectable hydrogel," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 91B, no. 1, pp. 163–171, 2009.

[65] M.-C. Yang, N.-H. Chi, N.-K. Chou et al., "The influence of rat mesenchymal stem cell CD44 surface markers on cell growth, fibronectin expression, and cardiomyogenic differentiation on silk fibroin - hyaluronic acid cardiac patches," *Biomaterials*, vol. 31, no. 5, pp. 854–862, 2010.

[66] E. Göv, H. Kenar, Z. S. Halbutoğullari, K. Y. Arğa, and E. Karaöz, "Cardiomyogenic differentiation potential of human lipoaspirate-derivedstem cells on hyaluronic acid/gelatin plasma gels," *Turkish Journal of Biology*, vol. 40, pp. 369–379, 2016.

[67] J. M. Aamodt and D. W. Grainger, "Extracellular matrix-based biomaterial scaffolds and the host response," *Biomaterials*, vol. 86, pp. 68–82, 2016.

[68] N. Bildyug, "On the problem of cardiogenic differentiation: extracellular matrix as an emerging clue," *International Journal of Cardiovascular Research*, vol. 06, no. 02, 2017.

[69] J. A. Efe, S. Hilcove, J. Kim et al., "Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy," *Nature Cell Biology*, vol. 13, no. 3, pp. 215–222, 2011.

[70] F. B. Bedada, S. S.-K. Chan, S. K. Metzger et al., "Acquisition of a quantitative, stoichiometrically conserved ratiometric marker of maturation status in stem cell-derived cardiac myocytes," *Stem Cell Reports*, vol. 3, no. 4, pp. 594–605, 2014.

[71] J. A. Santiago, R. Pogemiller, and B. M. Ogle, "Heterogeneous differentiation of human mesenchymal stem cells in response to extended culture in extracellular matrices," *Tissue Engineering Part A*, vol. 15, no. 12, pp. 3911–3922, 2009.

[72] T. Kofidis, D. R. Lebl, E. C. Martinez, G. Hoyt, M. Tanaka, and R. C. Robbins, "Novel injectable bioartificial tissue facilitates targeted, less invasive, large-scale tissue restoration on the beating heart after myocardial injury," *Circulation*, vol. 112, 9 Supplement, pp. I173–I177, 2005.

[73] V. Serpooshan, M. Zhao, S. A. Metzler et al., "The effect of bioengineered acellular collagen patch on cardiac remodeling and ventricular function post myocardial infarction," *Biomaterials*, vol. 34, no. 36, pp. 9048–9055, 2013.

[74] K. Maeda, R. Tiwari-Pandey, M. Ruel, and E. Suuronen, "Matrix therapies for cell support and cardiac repair," in *Biomaterials for Cardiac Regeneration*, E. Suuronen and M. Ruel, Eds., pp. 117–146, Springer, London, 2015.

[75] N. S. Dhalla, H. K. Saini-Chohan, D. Rodriguez-Leyva, V. Elimban, M. R. Dent, and P. S. Tappia, "Subcellular remodelling may induce cardiac dysfunction in congestive heart failure," *Cardiovascular Research*, vol. 81, no. 3, pp. 429–438, 2008.

[76] C. Gil-Cayuela, E. Roselló-LLetí, A. Ortega et al., "New altered non-fibrillar collagens in human dilated cardiomyopathy: role in the remodeling process," *PLoS One*, vol. 11, no. 12, article e0168130, 2016.

[77] E. Herpel, M. Pritsch, A. Koch, T. J. Dengler, P. Schirmacher, and P. A. Schnabel, "Interstitial fibrosis in the heart: differences in extracellular matrix proteins and matrix metalloproteinases in end-stage dilated, ischaemic and valvular cardiomyopathy," *Histopathology*, vol. 48, no. 6, pp. 736–747, 2006.

[78] J. Dawson, O. Schussler, A. Al-Madhoun, C. Menard, M. Ruel, and I. S. Skerjanc, "Collagen scaffolds with or without the addition of RGD peptides support cardiomyogenesis after aggregation of mouse embryonic stem cells," *In Vitro Cellular & Developmental Biology - Animal*, vol. 47, no. 9, pp. 653–664, 2011.

[79] H. Yu, H. Zhao, C. Huang, and Y. Du, "Mechanically and electrically enhanced CNT–collagen hydrogels as potential scaffolds for engineered cardiac constructs," *ACS Biomaterials Science & Engineering*, vol. 3, no. 11, pp. 3017–3021, 2017.

[80] H. Sun, J. Zhou, Z. Huang et al., "Carbon nanotube-incorporated collagen hydrogels improve cell alignment and the performance of cardiac constructs," *International Journal of Nanomedicine*, vol. 12, pp. 3109–3120, 2017.

[81] J. R. Frederick, J. R. Fitzpatrick, R. C. McCormick et al., "Stromal cell-derived factor-1 activation of tissue-engineered endothelial progenitor cell matrix enhances ventricular function after myocardial infarction by inducing neovasculogenesis," *Circulation*, vol. 122, 11_Supplement_1, pp. S107–S117, 2010.

[82] S.-C. Hsieh, H.-J. Chen, S.-h. Hsu et al., "Prominent vascularization capacity of mesenchymal stem cells in collagen–gold nanocomposites," *ACS Applied Materials & Interfaces*, vol. 8, no. 42, pp. 28982–29000, 2016.

[83] X. Wu, L. Black, G. Santacana-Laffitte, and C. W. Patrick, "Preparation and assessment of glutaraldehyde-crosslinked collagen–chitosan hydrogels for adipose tissue engineering," *Journal of Biomedical Materials Research Part A*, vol. 81A, no. 1, pp. 59–65, 2007.

[84] L. A. Reis, L. L. Y. Chiu, J. Wu et al., "Hydrogels with integrin-binding angiopoietin-1-derived peptide, QHREDGS, for treatment of acute myocardial infarction," *Circulation: Heart Failure*, vol. 8, no. 2, pp. 333–341, 2015.

[85] D. G. D. Rocca, B. J. Willenberg, Y. Qi et al., "An injectable capillary-like microstructured alginate hydrogel improves left ventricular function after myocardial infarction in rats," *International Journal of Cardiology*, vol. 220, pp. 149–154, 2016.

[86] Z.-W. Tao, M. Mohamed, M. Hogan, L. Gutierrez, and R. K. Birla, "Optimizing a spontaneously contracting heart tissue patch with rat neonatal cardiac cells on fibrin gel," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 11, no. 1, pp. 153–163, 2017.

[87] S. Nie, W. Xiao, Q. Shi-bin et al., "Improved myocardial perfusion and cardiac function by controlled-release basic fibroblast growth factor using fibrin glue in a canine infarct model," *Journal of Zhejiang University SCIENCE B*, vol. 11, no. 12, pp. 895–904, 2010.

[88] H. S. Yang, S. H. Bhang, I.-K. Kim et al., "In situ cardiomyogenic differentiation of implanted bone marrow mononuclear cells by local delivery of transforming growth factor-β1," *Cell Transplantation*, vol. 21, no. 1, pp. 299–312, 2012.

[89] L. Ye, P. Zhang, S. Duval, L. Su, Q. Xiong, and J. Zhang, "Thymosin 4 increases the potency of transplanted mesenchymal stem cells for myocardial repair," *Circulation*, vol. 128, 11_Supplement_1, pp. S32–S41, 2013.

[90] P. Menasché, V. Vanneaux, J.-R. Fabreguettes et al., "Towards a clinical use of human embryonic stem cell-derived cardiac progenitors: a translational experience," *European Heart Journal*, vol. 36, no. 12, pp. 743–750, 2015.

[91] J. Terrovitis, R. Lautamäki, M. Bonios et al., "Noninvasive quantification and optimization of acute cell retention by in vivo positron emission tomography after intramyocardial cardiac-derived stem cell delivery," *Journal of the American College of Cardiology*, vol. 54, no. 17, pp. 1619–1626, 2009.

[92] A. Llucià-Valldeperas, C. Soler-Botija, C. Gálvez-Montón et al., "Electromechanical conditioning of adult progenitor cells improves recovery of cardiac function after myocardial infarction," *Stem Cells Translational Medicine*, vol. 6, no. 3, pp. 970–981, 2017.

[93] Y. Ichihara, N. Tano, L. Fields et al., "Epicardial placement of instantly produced fibrin glue-incorporating mesenchymal stromal cells for the treatment of heart failure," *Circulation*, vol. 134, Supplement 1, article A14735, 2016.

[94] R. Moreira, C. Neusser, M. Kruse et al., "Tissue-engineered fibrin-based heart valve with bio-inspired textile reinforcement," *Advanced Healthcare Materials*, vol. 5, no. 16, pp. 2113–2121, 2016.

[95] R. Lopez-Cebral, M. Martin-Pastor, J. Evelin Parraga, G. Konat Zorzi, B. Seijo, and A. Sanchez, "Chemically modified gelatin as biomaterial in the design of new nanomedicines," *Medicinal Chemistry*, vol. 7, no. 3, pp. 145–154, 2011.

[96] C. W. Yung, L. Q. Wu, J. A. Tullman, G. F. Payne, W. E. Bentley, and T. A. Barbari, "Transglutaminase crosslinked gelatin as a tissue engineering scaffold," *Journal of Biomedical Materials Research Part A*, vol. 83A, no. 4, pp. 1039–1046, 2007.

[97] M. L. McCain, A. Agarwal, H. W. Nesmith, A. P. Nesmith, and K. K. Parker, "Micromolded gelatin hydrogels for extended culture of engineered cardiac tissues," *Biomaterials*, vol. 35, no. 21, pp. 5462–5471, 2014.

[98] N. Takehara, Y. Tsutsumi, K. Tateishi et al., "Controlled delivery of basic fibroblast growth factor promotes human cardiosphere-derived cell engraftment to enhance cardiac repair for chronic myocardial infarction," *Journal of the American College of Cardiology*, vol. 52, no. 23, pp. 1858–1865, 2008.

[99] M. H. Yacoub and J. Terrovitis, "CADUCEUS, SCIPIO, ALCADIA: cell therapy trials using cardiac-derived cells for patients with post myocardial infarction LV dysfunction, still evolving," *Global Cardiology Science and Practice*, vol. 2013, no. 1, 8 pages, 2013.

[100] A. Navaei, H. Saini, W. Christenson, R. T. Sullivan, R. Ros, and M. Nikkhah, "Gold nanorod-incorporated gelatin-based conductive hydrogels for engineering cardiac tissue constructs," *Acta Biomaterialia*, vol. 41, pp. 133–146, 2016.

[101] J. Kudová, J. Procházková, O. Vašiček et al., "HIF-1alpha deficiency attenuates the cardiomyogenesis of mouse embryonic stem cells," *PLoS One*, vol. 11, no. 6, article e0158358, 2016.

[102] C. Cristallini, E. Cibrario Rocchietti, M. Gagliardi et al., "Micro- and macrostructured PLGA/gelatin scaffolds promote early cardiogenic commitment of human mesenchymal stem cells in vitro," *Stem Cells International*, vol. 2016, Article ID 7176154, 16 pages, 2016.

[103] H. K. Kleinman, M. L. McGarvey, L. A. Liotta, P. G. Robey, K. Tryggvason, and G. R. Martin, "Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma," *Biochemistry*, vol. 21, no. 24, pp. 6188–6193, 2002.

[104] R. Chaudhuri, M. Ramachandran, P. Moharil, M. Harumalani, and A. K. Jaiswal, "Biomaterials and cells for cardiac tissue engineering: current choices," *Materials Science and Engineering: C*, vol. 79, pp. 950–957, 2017.

[105] L. Zhang, X. Li, X. Yu et al., "Construction of vascularized pacemaker tissues by seeding cardiac progenitor cells and

endothelial progenitor cells into Matrigel," *Life Sciences*, vol. 179, pp. 139–146, 2017.

[106] N. T. Lam, H. Lam, N. M. Sturdivant, and K. Balachandran, "Fabrication of a matrigel–collagen semi-interpenetrating scaffold for use in dynamic valve interstitial cell culture," *Biomedical Materials*, vol. 12, no. 4, article 045013, 2017.

[107] T.-J. Lee, S. Park, S. H. Bhang et al., "Graphene enhances the cardiomyogenic differentiation of human embryonic stem cells," *Biochemical and Biophysical Research Communications*, vol. 452, no. 1, pp. 174–180, 2014.

[108] Y.-K. (. A.). Wu and J. Yu, "The role of tissue engineering in cellular therapies for myocardial infarction: a review," *Journal of Materials Chemistry B*, vol. 3, no. 31, pp. 6401–6410, 2015.

[109] W. B. Vanwinkle, M. B. Snuggs, and L. M. Buja, "Cardiogel: a biosynthetic extracellular matrix for cardiomyocyte culture," *In Vitro Cellular & Developmental Biology - Animal*, vol. 32, no. 8, pp. 478–485, 1996.

[110] R. J. Bick, M. B. Snuggs, B. J. Poindexter, L. M. Buja, and W. B. V. Winkle, "Physical, contractile and calcium handling properties of neonatal cardiac myocytes cultured on different matrices," *Cell Adhesion and Communication*, vol. 6, no. 4, pp. 301–310, 2009.

[111] R. A. Davis, W. B. van Winkle, L. M. Buja, B. J. Poindexter, and R. J. Bick, "Effect of a simple versus a complex matrix on the polarity of cardiomyocytes in culture," *Journal of Burns and Wounds*, vol. 5, 2006.

[112] W. Chang, S. Lim, H. Song et al., "In vitro expansion of mesenchymal stem cells using 3-D matrix derived from cardiac fibroblast," *Tissue Engineering and Regenerative Medicine*, vol. 4, no. 3, pp. 370–375, 2007.

[113] A. O. J. Fakoya, "New delivery systems of stem cells for vascular regeneration in ischemia," *Frontiers in Cardiovascular Medicine*, vol. 4, 2017.

[114] P. Sreejit and R. S. Verma, "Enhanced cardiomyogenic lineage differentiation of adult bone-marrow-derived stem cells grown on cardiogel," *Cell and Tissue Research*, vol. 353, no. 3, pp. 443–456, 2013.

[115] K. Matsuda, K. J. Falkenberg, A. A. Woods, Y. S. Choi, W. A. Morrison, and R. J. Dilley, "Adipose-derived stem cells promote angiogenesis and tissue formation for in vivo tissue engineering," *Tissue Engineering Part A*, vol. 19, no. 11-12, pp. 1327–1335, 2013.

[116] G. Agmon and K. L. Christman, "Controlling stem cell behavior with decellularized extracellular matrix scaffolds," *Current Opinion in Solid State and Materials Science*, vol. 20, no. 4, pp. 193–201, 2016.

[117] B. S. Kim, H. Kim, G. Gao, J. Jang, and D.-W. Cho, "Decellularized extracellular matrix: a step towards the next generation source for bioink manufacturing," *Biofabrication*, vol. 9, no. 3, article 034104, 2017.

[118] R.-H. Fu, Y.-C. Wang, S.-P. Liu et al., "Decellularization and recellularization technologies in tissue engineering," *Cell Transplantation*, vol. 23, no. 4-5, pp. 621–630, 2014.

[119] J. E. Arenas-Herrera, I. K. Ko, A. Atala, and J. J. Yoo, "Decellularization for whole organ bioengineering," *Biomedical Materials*, vol. 8, no. 1, article 014106, 2013.

[120] Q. Zhou, L. Li, and J. Li, "Stem cells with decellularized liver scaffolds in liver regeneration and their potential clinical applications," *Liver International*, vol. 35, no. 3, pp. 687–694, 2015.

[121] T. J. Keane, I. T. Swinehart, and S. F. Badylak, "Methods of tissue decellularization used for preparation of biologic scaffolds and in vivo relevance," *Methods*, vol. 84, pp. 25–34, 2015.

[122] D. M. Faulk, S. A. Johnson, L. Zhang, and S. F. Badylak, "Role of the extracellular matrix in whole organ engineering," *Journal of Cellular Physiology*, vol. 229, no. 8, pp. 984–989, 2014.

[123] M. P. Francis, E. Breathwaite, A. A. Bulysheva et al., "Human placenta hydrogel reduces scarring in a rat model of cardiac ischemia and enhances cardiomyocyte and stem cell cultures," *Acta Biomaterialia*, vol. 52, pp. 92–104, 2017.

[124] F. Di Meglio, D. Nurzynska, V. Romano et al., "Optimization of human myocardium decellularization method for the construction of implantable patches," *Tissue Engineering Part C: Methods*, vol. 23, no. 9, pp. 525–539, 2017.

[125] F. Pagano, F. Angelini, C. Castaldo et al., "Normal versus pathological cardiac fibroblast-derived extracellular matrix differentially modulates cardiosphere-derived cell paracrine properties and commitment," *Stem Cells International*, vol. 2017, Article ID 7396462, 9 pages, 2017.

[126] Y. Li and D. Zhang, "Artificial cardiac muscle with or without the use of scaffolds," *BioMed Research International*, vol. 2017, Article ID 8473465, 15 pages, 2017.

[127] L. T. Saldin, M. C. Cramer, S. S. Velankar, L. J. White, and S. F. Badylak, "Extracellular matrix hydrogels from decellularized tissues: structure and function," *Acta Biomaterialia*, vol. 49, pp. 1–15, 2017.

[128] T. Hoshiba, G. Chen, C. Endo et al., "Decellularized extracellular matrix as an in vitro model to study the comprehensive roles of the ECM in stem cell differentiation," *Stem Cells International*, vol. 2016, Article ID 6397820, 10 pages, 2016.

[129] K. M. Lee, H. Kim, J. G. Nemeno et al., "Natural cardiac extracellular matrix sheet as a biomaterial for cardiomyocyte transplantation," *Transplantation Proceedings*, vol. 47, no. 3, pp. 751–756, 2015.

[130] B. Oberwallner, A. Brodarac, Y.-H. Choi et al., "Preparation of cardiac extracellular matrix scaffolds by decellularization of human myocardium," *Journal of Biomedical Materials Research Part A*, vol. 102, no. 9, pp. 3263–3272, 2014.

[131] T.-Y. Lu, B. Lin, J. Kim et al., "Repopulation of decellularized mouse heart with human induced pluripotent stem cell-derived cardiovascular progenitor cells," *Nature Communications*, vol. 4, 2013.

[132] B. Söylen, S. Sarikouch, and A. Horke, "Improved results using decellularized human valves for children with congenital heart defects," *Journal of Nursing & Health Sciences*, vol. 3, no. 1, pp. 1–4, 2017.

[133] H. Bäcker, L. Polgár, P. Soós et al., "Impedimetric analysis of the effect of decellularized porcine heart scaffold on human fibrosarcoma, endothelial, and cardiomyocyte cell lines," *Medical Science Monitor*, vol. 23, pp. 2232–2240, 2017.

[134] M. Kasai, H. Yagi, K. Okamoto, Y. Kitagawa, and H. Shimizu, "Novel method of creating three-dimensional myocardial sheet using heart scaffold and isolated cardiomyocytes," *The Journal of Heart and Lung Transplantation*, vol. 36, no. 4, p. S381, 2017.

[135] M. Yang, Y.-H. Lin, W.-P. Shi, H.-C. Shi, Y. J. Gu, and Y.-S. Shu, "Surface heparin treatment of the decellularized porcine heart valve: effect on tissue calcification," *Journal of*

Biomedical Materials Research Part B: Applied Biomaterials, vol. 105, no. 2, pp. 400–405, 2017.

[136] M. J. Hodgson, C. C. Knutson, N. Momtahan, and A. D. Cook, "Extracellular matrix from whole porcine heart decellularization for cardiac tissue engineering," in *Methods in Molecular Biology*, pp. 1–8, Humana Press, New York, NY, USA, 2017.

[137] S. Pascual-Gil, E. Garbayo, P. Díaz-Herráez, F. Prosper, and M. J. Blanco-Prieto, "Heart regeneration after myocardial infarction using synthetic biomaterials," *Journal of Controlled Release*, vol. 203, pp. 23–38, 2015.

[138] R. Ravichandran, J. R. Venugopal, S. Sundarrajan, S. Mukherjee, and S. Ramakrishna, "Minimally invasive cell-seeded biomaterial systems for injectable/epicardial implantation in ischemic heart disease," *International Journal of Nanomedicine*, vol. 7, pp. 5969–5994, 2012.

[139] R. Lakshmanan, U. M. Krishnan, and S. Sethuraman, "Polymeric scaffold aided stem cell therapeutics for cardiac muscle repair and regeneration," *Macromolecular Bioscience*, vol. 13, no. 9, pp. 1119–1134, 2013.

[140] T. K. Dash and V. B. Konkimalla, "Poly-ϵ-caprolactone based formulations for drug delivery and tissue engineering: a review," *Journal of Controlled Release*, vol. 158, no. 1, pp. 15–33, 2012.

[141] K. Mi, G. Wang, Z. Liu, Z. Feng, B. Huang, and X. Zhao, "Influence of a self-assembling peptide, RADA16, compared with collagen I and Matrigel on the malignant phenotype of human breast-cancer cells in 3D cultures and in vivo," *Macromolecular Bioscience*, vol. 9, no. 5, pp. 437–443, 2009.

[142] S. Zhang, F. Gelain, and X. Zhao, "Designer self-assembling peptide nanofiber scaffolds for 3D tissue cell cultures," *Seminars in Cancer Biology*, vol. 15, no. 5, pp. 413–420, 2005.

[143] B. D. Ulery, L. S. Nair, and C. T. Laurencin, "Biomedical applications of biodegradable polymers," *Journal of Polymer Science Part B: Polymer Physics*, vol. 49, no. 12, pp. 832–864, 2011.

[144] J. Du, S. Wang, H. You, and X. Zhao, "Understanding the toxicity of carbon nanotubes in the environment is crucial to the control of nanomaterials in producing and processing and the assessment of health risk for human: a review," *Environmental Toxicology and Pharmacology*, vol. 36, no. 2, pp. 451–462, 2013.

[145] S. Mukherjee, J. Reddy Venugopal, R. Ravichandran, S. Ramakrishna, and M. Raghunath, "Evaluation of the biocompatibility of PLACL/collagen nanostructured matrices with cardiomyocytes as a model for the regeneration of infarcted myocardium," *Advanced Functional Materials*, vol. 21, no. 12, pp. 2291–2300, 2011.

[146] D. Kai, M. P. Prabhakaran, G. Jin, and S. Ramakrishna, "Polypyrrole-contained electrospun conductive nanofibrous membranes for cardiac tissue engineering," *Journal of Biomedical Materials Research Part A*, vol. 99A, no. 3, pp. 376–385, 2011.

[147] T. Sugiura, N. Hibino, C. K. Breuer, and T. Shinoka, "Tissue-engineered cardiac patch seeded with human induced pluripotent stem cell derived cardiomyocytes promoted the regeneration of host cardiomyocytes in a rat model," *Journal of Cardiothoracic Surgery*, vol. 11, no. 1, p. 163, 2016.

[148] M. Castilho, D. Feyen, M. Flandes-Iparraguirre et al., "Melt electrospinning writing of poly-hydroxymethylglycolide-co-ε-caprolactone-based scaffolds for cardiac tissue engineering," *Advanced Healthcare Materials*, vol. 6, no. 18, article 1700311, 2017.

[149] L. F. Miao, Y. P. Yin, Y. L. Cui et al., "Efficacy and mechanism of local delivery of rapamycin and rapamycin-loaded poly (lactic-co-glycolic) acid nanoparticles on coronary restenosis of injury-stenosis model of minipigs," *National Medical Journal of China*, vol. 96, no. 1, pp. 36–42, 2016.

[150] N. Baheiraei, R. Gharibi, H. Yeganeh et al., "Electroactive polyurethane/siloxane derived from castor oil as a versatile cardiac patch, part I: synthesis, characterization, and myoblast proliferation and differentiation," *Journal of Biomedical Materials Research Part A*, vol. 104, no. 3, pp. 775–787, 2016.

[151] W.-F. Su, C.-C. Ho, T.-H. Shih, C.-H. Wang, and C.-H. Yeh, "Exceptional biocompatibility of 3D fibrous scaffold for cardiac tissue engineering fabricated from biodegradable polyurethane blended with cellulose," *International Journal of Polymeric Materials and Polymeric Biomaterials*, vol. 65, no. 14, pp. 703–711, 2016.

[152] R. Hernández-Córdova, D. A. Mathew, R. Balint et al., "Indirect three-dimensional printing: a method for fabricating polyurethane-urea based cardiac scaffolds," *Journal of Biomedical Materials Research Part A*, vol. 104, no. 8, pp. 1912–1921, 2016.

[153] E. Tomecka, M. Wojasinski, E. Jastrzebska, M. Chudy, T. Ciach, and Z. Brzozka, "Poly(l-lactic acid) and polyurethane nanofibers fabricated by solution blow spinning as potential substrates for cardiac cell culture," *Materials Science and Engineering: C*, vol. 75, pp. 305–316, 2017.

[154] S. Ahadian, S. Yamada, M. Estili et al., "Carbon nanotubes embedded in embryoid bodies direct cardiac differentiation," *Biomedical Microdevices*, vol. 19, no. 3, p. 57, 2017.

[155] I. Somasuntharam, A. V. Boopathy, R. S. Khan et al., "Delivery of Nox2-NADPH oxidase siRNA with polyketal nanoparticles for improving cardiac function following myocardial infarction," *Biomaterials*, vol. 34, no. 31, pp. 7790–7798, 2013.

[156] R. Bayford, T. Rademacher, I. Roitt, and S. X. Wang, "Emerging applications of nanotechnology for diagnosis and therapy of disease: a review," *Physiological Measurement*, vol. 38, no. 8, pp. R183–R203, 2017.

[157] C. Kumara, X. Zuo, D. A. Cullen, and A. Dass, "Faradaurate-940: synthesis, mass spectrometry, electron microscopy, high-energy X-ray diffraction, and X-ray scattering study of Au~940±20(SR)~160±4 nanocrystals," *ACS Nano*, vol. 8, no. 6, pp. 6431–6439, 2014.

[158] J. M. de la Fuente, A. G. Barrientos, T. C. Rojas et al., "Gold glyconanoparticles as water-soluble polyvalent models to study carbohydrate interactions," *Angewandte Chemie International Edition*, vol. 40, no. 12, pp. 2257–2261, 2001.

[159] L. Moldovan, C. M. Babbey, M. P. Murphy, and N. I. Moldovan, "Comparison of biomaterial-dependent and -independent bioprinting methods for cardiovascular medicine," *Current Opinion in Biomedical Engineering*, vol. 2, pp. 124–131, 2017.

[160] V. Mironov, R. P. Visconti, V. Kasyanov, G. Forgacs, C. J. Drake, and R. R. Markwald, "Organ printing: tissue spheroids as building blocks," *Biomaterials*, vol. 30, no. 12, pp. 2164–2174, 2009.

[161] B. Derby, "Printing and prototyping of tissues and scaffolds," *Science*, vol. 338, no. 6109, pp. 921–926, 2012.

[162] S. V. Murphy and A. Atala, "3D bioprinting of tissues and organs," *Nature Biotechnology*, vol. 32, no. 8, pp. 773–785, 2014.

[163] I. T. Ozbolat, "Bioprinting scale-up tissue and organ constructs for transplantation," *Trends in Biotechnology*, vol. 33, no. 7, pp. 395–400, 2015.

[164] I. T. Ozbolat, "Scaffold-based or scaffold-free bioprinting: competing or complementing approaches?," *Journal of Nanotechnology in Engineering and Medicine*, vol. 6, no. 2, article 024701, 2015.

[165] W. Wu, A. Deconinck, and J. A. Lewis, "Omnidirectional printing of 3D microvascular networks," *Advanced Materials*, vol. 23, no. 24, pp. H178–H183, 2011.

[166] D. Huh, D. C. Leslie, B. D. Matthews et al., "A human disease model of drug toxicity-induced pulmonary edema in a lung-on-a-chip microdevice," *Science Translational Medicine*, vol. 4, no. 159, p. 159ra147, 2012.

[167] A. K. Capulli, K. Tian, N. Mehandru et al., "Approaching the in vitro clinical trial: engineering organs on chips," *Lab on a Chip*, vol. 14, no. 17, pp. 3181–3186, 2014.

[168] M. Matsusaki, K. Sakaue, K. Kadowaki, and M. Akashi, "Three-dimensional human tissue chips fabricated by rapid and automatic inkjet cell printing," *Advanced Healthcare Materials*, vol. 2, no. 4, pp. 534–539, 2013.

[169] H. Gudapati, M. Dey, and I. Ozbolat, "A comprehensive review on droplet-based bioprinting: past, present and future," *Biomaterials*, vol. 102, pp. 20–42, 2016.

[170] F. Pati, J. Jang, D.-H. Ha et al., "Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink," *Nature Communications*, vol. 5, 2014.

[171] K. Shimizu, A. Ito, J.-K. Lee et al., "Construction of multi-layered cardiomyocyte sheets using magnetite nanoparticles and magnetic force," *Biotechnology and Bioengineering*, vol. 96, no. 4, pp. 803–809, 2007.

[172] M. Yamato and T. Okano, "Cell sheet engineering," *Materials Today*, vol. 7, no. 5, pp. 42–47, 2004.

[173] J. M. Kelm and M. Fussenegger, "Microscale tissue engineering using gravity-enforced cell assembly," *Trends in Biotechnology*, vol. 22, no. 4, pp. 195–202, 2004.

[174] K. L. K. Coulombe, V. K. Bajpai, S. T. Andreadis, and C. E. Murry, "Heart regeneration with engineered myocardial tissue," *Annual Review of Biomedical Engineering*, vol. 16, no. 1, pp. 1–28, 2014.

[175] J. Jang, H.-J. Park, S.-W. Kim et al., "3D printed complex tissue construct using stem cell-laden decellularized extracellular matrix bioinks for cardiac repair," *Biomaterials*, vol. 112, pp. 264–274, 2017.

[176] R. Gaebel, N. Ma, J. Liu et al., "Patterning human stem cells and endothelial cells with laser printing for cardiac regeneration," *Biomaterials*, vol. 32, no. 35, pp. 9218–9230, 2011.

[177] S. Fleischer, M. Shevach, R. Feiner, and T. Dvir, "Coiled fiber scaffolds embedded with gold nanoparticles improve the performance of engineered cardiac tissues," *Nanoscale*, vol. 6, no. 16, pp. 9410–9414, 2014.

[178] T. F. Robinson, M. A. Geraci, E. H. Sonnenblick, and S. M. Factor, "Coiled perimysial fibers of papillary muscle in rat heart: morphology, distribution, and changes in configuration," *Circulation Research*, vol. 63, no. 3, pp. 577–592, 1988.

[179] P. J. Hanley, A. A. Young, I. J. Legrice, S. G. Edgar, and D. S. Loiselle, "3-Dimensional configuration of perimysial collagen fibres in rat cardiac muscle at resting and extended sarcomere lengths," *The Journal of Physiology*, vol. 517, no. 3, pp. 831–837, 1999.

[180] R. Ravichandran, R. Sridhar, J. R. Venugopal, S. Sundarrajan, S. Mukherjee, and S. Ramakrishna, "Gold nanoparticle loaded hybrid nanofibers for cardiogenic differentiation of stem cells for infarcted myocardium regeneration," *Macromolecular Bioscience*, vol. 14, no. 4, pp. 515–525, 2014.

[181] R. Gaetani, D. A. M. Feyen, V. Verhage et al., "Epicardial application of cardiac progenitor cells in a 3D-printed gelatin/hyaluronic acid patch preserves cardiac function after myocardial infarction," *Biomaterials*, vol. 61, pp. 339–348, 2015.

[182] Y. S. Zhang, A. Arneri, S. Bersini et al., "Bioprinting 3D microfibrous scaffolds for engineering endothelialized myocardium and heart-on-a-chip," *Biomaterials*, vol. 110, pp. 45–59, 2016.

[183] F. Yin, C. Yang, Q. Wang et al., "A light-driven therapy of pancreatic adenocarcinoma using gold nanorods-based nanocarriers for co-delivery of doxorubicin and siRNA," *Theranostics*, vol. 5, no. 8, pp. 818–833, 2015.

[184] P. Nigam, S. Waghmode, M. Louis, S. Wangnoo, P. Chavan, and D. Sarkar, "Graphene quantum dots conjugated albumin nanoparticles for targeted drug delivery and imaging of pancreatic cancer," *Journal of Materials Chemistry B*, vol. 2, no. 21, pp. 3190–3195, 2014.

[185] C. E. Probst, P. Zrazhevskiy, V. Bagalkot, and X. Gao, "Quantum dots as a platform for nanoparticle drug delivery vehicle design," *Advanced Drug Delivery Reviews*, vol. 65, no. 5, pp. 703–718, 2013.

[186] Q. He and J. Shi, "Mesoporous silica nanoparticle based nano drug delivery systems: synthesis, controlled drug release and delivery, pharmacokinetics and biocompatibility," *Journal of Materials Chemistry*, vol. 21, no. 16, pp. 5845–5855, 2011.

[187] F. Tang, L. Li, and D. Chen, "Mesoporous silica nanoparticles: synthesis, biocompatibility and drug delivery," *Advanced Materials*, vol. 24, no. 12, pp. 1504–1534, 2012.

[188] C. Argyo, V. Weiss, C. Bräuchle, and T. Bein, "Multifunctional mesoporous silica nanoparticles as a universal platform for drug delivery," *Chemistry of Materials*, vol. 26, no. 1, pp. 435–451, 2013.

[189] J. Cheng, Q. Ding, J. Wang et al., "5-Azacytidine delivered by mesoporous silica nanoparticles regulates the differentiation of P19 cells into cardiomyocytes," *Nanoscale*, vol. 8, no. 4, pp. 2011–2021, 2016.

[190] K. S. Oh, J. Y. Song, S. J. Yoon, Y. Park, D. Kim, and S. H. Yuk, "Temperature-induced gel formation of core/shell nanoparticles for the regeneration of ischemic heart," *Journal of Controlled Release*, vol. 146, no. 2, pp. 207–211, 2010.

[191] C. Dai, B. Wang, and H. Zhao, "Microencapsulation peptide and protein drugs delivery system," *Colloids and Surfaces B: Biointerfaces*, vol. 41, no. 2-3, pp. 117–120, 2005.

[192] J. Meyer, L. Whitcomb, and D. Collins, "Efficient encapsulation of proteins within liposomes for slow release in vivo," *Biochemical and Biophysical Research Communications*, vol. 199, no. 2, pp. 433–438, 1994.

[193] M. JoséFonseca, M. Asunción Alsina, and F. Reig, "Coating liposomes with collagen (Mr 50 000) increases uptake into liver," *Biochimica et Biophysica Acta (BBA) - Biomembranes*, vol. 1279, no. 2, pp. 259–265, 1996.

[194] I. J. Fidler, A. Raz, W. E. Fogler, R. Kirsh, P. Bugelski, and G. Poste, "Design of liposomes to improve delivery of macrophage-augmenting agents to alveolar macrophages," *Cancer Research*, vol. 40, no. 12, pp. 4460–4466, 1980.

[195] G. Poste, C. Bucana, A. Raz, P. Bugelski, R. Kirsh, and I. J. Fidler, "Analysis of the fate of systemically administered liposomes and implications for their use in drug delivery," *Cancer Research*, vol. 42, no. 4, pp. 1412–1422, 1982.

[196] P. R. Desai, N. J. Jain, R. K. Sharma, and P. Bahadur, "Effect of additives on the micellization of PEO/PPO/PEO block copolymer F127 in aqueous solution," *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 178, no. 1–3, pp. 57–69, 2001.

[197] M. A. Firestone, A. C. Wolf, and S. Seifert, "Small-angle X-ray scattering study of the interaction of poly(ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide) triblock copolymers with lipid bilayers," *Biomacromolecules*, vol. 4, no. 6, pp. 1539–1549, 2003.

[198] K. S. Oh, S. K. Han, H. S. Lee et al., "Core/shell nanoparticles with lecithin lipid cores for protein delivery," *Biomacromolecules*, vol. 7, no. 8, pp. 2362–2367, 2006.

[199] K. Y. Lee and S. H. Yuk, "Polymeric protein delivery systems," *Progress in Polymer Science*, vol. 32, no. 7, pp. 669–697, 2007.

[200] M.-Y. Chang, Y.-J. Yang, C.-H. Chang et al., "Functionalized nanoparticles provide early cardioprotection after acute myocardial infarction," *Journal of Controlled Release*, vol. 170, no. 2, pp. 287–294, 2013.

[201] C. M. Prêle, M. E. Reichelt, S. E. Mutsaers et al., "Insulin-like growth factor-1 overexpression in cardiomyocytes diminishes ex vivo heart functional recovery after acute ischemia," *Cardiovascular Pathology*, vol. 21, no. 1, pp. 17–27, 2012.

[202] Q. Qi, L. Lu, H. Li et al., "Spatiotemporal delivery of nanoformulated liraglutide for cardiac regeneration after myocardial infarction," *International Journal of Nanomedicine*, vol. 12, pp. 4835–4848, 2017.

[203] L. A. Nikolaidis, S. Mankad, G. G. Sokos et al., "Effects of glucagon-like peptide-1 in patients with acute myocardial infarction and left ventricular dysfunction after successful reperfusion," *Circulation*, vol. 109, no. 8, pp. 962–965, 2004.

[204] P. Richards, H. E. Parker, A. E. Adriaenssens et al., "Identification and characterization of GLP-1 receptor-expressing cells using a new transgenic mouse model," *Diabetes*, vol. 63, no. 4, pp. 1224–1233, 2014.

[205] T. Inoue, T. Inoguchi, N. Sonoda et al., "GLP-1 analog liraglutide protects against cardiac steatosis, oxidative stress and apoptosis in streptozotocin-induced diabetic rats," *Atherosclerosis*, vol. 240, no. 1, pp. 250–259, 2015.

[206] M. H. Noyan-Ashraf, M. A. Momen, K. Ban et al., "GLP-1R agonist liraglutide activates cytoprotective pathways and improves outcomes after experimental myocardial infarction in mice," *Diabetes*, vol. 58, no. 4, pp. 975–983, 2009.

[207] H. Bugger and E. D. Abel, "Molecular mechanisms of diabetic cardiomyopathy," *Diabetologia*, vol. 57, no. 4, pp. 660–671, 2014.

[208] M. Kavianipour, M. R. Ehlers, K. Malmberg et al., "Glucagon-like peptide-1 (7–36) amide prevents the accumulation of pyruvate and lactate in the ischemic and non-ischemic porcine myocardium," *Peptides*, vol. 24, no. 4, pp. 569–578, 2003.

[209] K. Sato, M. Kameda, T. Yasuhara et al., "Neuroprotective effects of liraglutide for stroke model of rats," *International Journal of Molecular Sciences*, vol. 14, no. 12, pp. 21513–21524, 2013.

[210] M. Marre, J. Shaw, M. Brändle et al., "Liraglutide, a once-daily human GLP-1 analogue, added to a sulphonylurea over 26 weeks produces greater improvements in glycaemic and weight control compared with adding rosiglitazone or placebo in subjects with type 2 diabetes (LEAD-1 SU)," *Diabetic Medicine*, vol. 26, no. 3, pp. 268–278, 2009.

[211] F. R. Formiga, B. Pelacho, E. Garbayo et al., "Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia–reperfusion model," *Journal of Controlled Release*, vol. 147, no. 1, pp. 30–37, 2010.

[212] S. Pascual-Gil, T. Simón-Yarza, E. Garbayo, F. Prosper, and M. J. Blanco-Prieto, "Tracking the in vivo release of bioactive NRG from PLGA and PEG–PLGA microparticles in infarcted hearts," *Journal of Controlled Release*, vol. 220, no. - Part A, pp. 388–396, 2015.

[213] C. Bi, A. Wang, Y. Chu et al., "Intranasal delivery of rotigotine to the brain with lactoferrin-modified PEG-PLGA nanoparticles for Parkinson's disease treatment," *International Journal of Nanomedicine*, vol. 11, pp. 6547–6559, 2016.

[214] T. Simón-Yarza, F. R. Formiga, E. Tamayo, B. Pelacho, F. Prosper, and M. J. Blanco-Prieto, "PEGylated-PLGA microparticles containing VEGF for long term drug delivery," *International Journal of Pharmaceutics*, vol. 440, no. 1, pp. 13–18, 2013.

[215] A. C. Vandergriff, T. M. Hensley, E. T. Henry et al., "Magnetic targeting of cardiosphere-derived stem cells with ferumoxytol nanoparticles for treating rats with myocardial infarction," *Biomaterials*, vol. 35, no. 30, pp. 8528–8539, 2014.

[216] Y. Zhang, W. Li, L. Ou et al., "Targeted delivery of human VEGF gene via complexes of magnetic nanoparticle-adenoviral vectors enhanced cardiac regeneration," *PLoS One*, vol. 7, no. 7, p. e39490, 2012.

[217] Z. Barabadi, M. Azami, E. Sharifi et al., "Fabrication of hydrogel based nanocomposite scaffold containing bioactive glass nanoparticles for myocardial tissue engineering," *Materials Science and Engineering: C*, vol. 69, pp. 1137–1146, 2016.

[218] R. M. Day, A. R. Boccaccini, S. Shurey et al., "Assessment of polyglycolic acid mesh and bioactive glass for soft-tissue engineering scaffolds," *Biomaterials*, vol. 25, no. 27, pp. 5857–5866, 2004.

[219] H. Keshaw, A. Forbes, and R. M. Day, "Release of angiogenic growth factors from cells encapsulated in alginate beads with bioactive glass," *Biomaterials*, vol. 26, no. 19, pp. 4171–4179, 2005.

[220] J. Diao, H. Wang, N. Chang et al., "PEG–PLA nanoparticles facilitate siRNA knockdown in adult zebrafish heart," *Developmental Biology*, vol. 406, no. 2, pp. 196–202, 2015.

[221] K. Kikuchi, J. E. Holdway, R. J. Major et al., "Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration," *Developmental Cell*, vol. 20, no. 3, pp. 397–404, 2011.

[222] A. K. Schroer, M. S. Shotwell, V. Y. Sidorov, J. P. Wikswo, and W. D. Merryman, "I-Wire Heart-on-a-Chip II: biomechanical analysis of contractile, three-dimensional cardiomyocyte tissue constructs," *Acta Biomaterialia*, vol. 48, pp. 79–87, 2017.

[223] V. Y. Sidorov, P. C. Samson, T. N. Sidorova, J. M. Davidson, C. C. Lim, and J. P. Wikswo, "I-Wire Heart-on-a-Chip I: three-dimensional cardiac tissue constructs for physiology and pharmacology," *Acta Biomaterialia*, vol. 48, pp. 68–78, 2017.

[224] A. V. Borovjagin, B. M. Ogle, J. L. Berry, and J. Zhang, "From microscale devices to 3D printing advances in fabrication of 3D cardiovascular tissues," *Circulation Research*, vol. 120, no. 1, pp. 150–165, 2017.

[225] A. Bayés-Genís, C. Gálvez-Montón, and S. Roura, "Cardiac tissue engineering: lost in translation or ready for translation?," *Journal of the American College of Cardiology*, vol. 68, no. 7, pp. 724–726, 2016.

[226] M. Y. Emmert, R. W. Hitchcock, and S. P. Hoerstrup, "Cell therapy, 3D culture systems and tissue engineering for cardiac regeneration," *Advanced Drug Delivery Reviews*, vol. 69-70, pp. 254–269, 2014.

[227] A. K. Capulli, M. Y. Emmert, F. S. Pasqualini et al., "JetValve: rapid manufacturing of biohybrid scaffolds for biomimetic heart valve replacement," *Biomaterials*, vol. 133, pp. 229–241, 2017.

[228] K. M. Brouwer, H. R. Hoogenkamp, W. F. Daamen, and T. H. van Kuppevelt, "Regenerative medicine for the respiratory system," *American Journal of Respiratory and Critical Care Medicine*, vol. 187, no. 5, pp. 468–475, 2013.

[229] G. Lemon, M. L. Lim, F. Ajalloueian, and P. Macchiarini, "The development of the bioartificial lung," *British Medical Bulletin*, vol. 110, no. 1, pp. 35–45, 2014.

[230] D. Sokocevic, N. R. Bonenfant, D. E. Wagner et al., "The effect of age and emphysematous and fibrotic injury on the recellularization of de-cellularized lungs," *Biomaterials*, vol. 34, no. 13, pp. 3256–3269, 2013.

[231] D. N. Kotton and E. E. Morrisey, "Lung regeneration: mechanisms, applications and emerging stem cell populations," *Nature Medicine*, vol. 20, no. 8, pp. 822–832, 2014.

[232] K. A. A. Schilders, E. Eenjes, S. van Riet et al., "Regeneration of the lung: lung stem cells and the development of lung mimicking devices," *Respiratory Research*, vol. 17, no. 1, p. 44, 2016.

[233] A. E. Vaughan, A. N. Brumwell, Y. Xi et al., "Lineage-negative progenitors mobilize to regenerate lung epithelium after major injury," *Nature*, vol. 517, no. 7536, pp. 621–625, 2014.

[234] J. L. Balestrini, A. L. Gard, K. A. Gerhold et al., "Comparative biology of decellularized lung matrix: implications of species mismatch in regenerative medicine," *Biomaterials*, vol. 102, pp. 220–230, 2016.

[235] J. L. Balestrini and L. E. Niklason, "Extracellular matrix as a driver for lung regeneration," *Annals of Biomedical Engineering*, vol. 43, no. 3, pp. 568–576, 2015.

[236] Y. W. Chun, S. W. Crowder, S. C. Mehl, X. Wang, H. Bae, and H.-J. Sung, "Therapeutic application of nanotechnology in cardiovascular and pulmonary regeneration," *Computational and Structural Biotechnology Journal*, vol. 7, no. 8, article e201304005, 2013.

[237] S. E. Dunphy, J. A. J. Bratt, K. M. Akram, N. R. Forsyth, and A. J. El Haj, "Hydrogels for lung tissue engineering: biomechanical properties of thin collagen–elastin constructs," *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 38, pp. 251–259, 2014.

[238] P. I. Lelkes, M. Li, A. Perets et al., "Designing intelligent polymeric scaffolds for tissue engineering: blending and co-electrospinning synthetic and natural polymers," in *Experimental Analysis of Nano and Engineering Materials and Structures*, E. E. Gdoutos, Ed., pp. 831-832, Springer, Dordrecht, 2007.

[239] C. F. Andrade, A. P. Wong, T. K. Waddell, S. Keshavjee, and M. Liu, "Cell-based tissue engineering for lung regeneration," *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 292, no. 2, pp. L510–L518, 2007.

[240] A. Fakhari and C. Berkland, "Applications and emerging trends of hyaluronic acid in tissue engineering, as a dermal filler and in osteoarthritis treatment," *Acta Biomaterialia*, vol. 9, no. 7, pp. 7081–7092, 2013.

[241] Q. Chen, S. Liang, and G. A. Thouas, "Elastomeric biomaterials for tissue engineering," *Progress in Polymer Science*, vol. 38, no. 3-4, pp. 584–671, 2013.

[242] Y. Wang, G. A. Ameer, B. J. Sheppard, and R. Langer, "A tough biodegradable elastomer," *Nature Biotechnology*, vol. 20, no. 6, pp. 602–606, 2002.

[243] H. Sugihara, S. Toda, S. Miyabara, C. Fujiyama, and N. Yonemitsu, "Reconstruction of alveolus-like structure from alveolar type II epithelial cells in three-dimensional collagen gel matrix culture," *American Journal of Pathology*, vol. 142, no. 3, pp. 783–792, 1993.

[244] P. Chen, E. Marsilio, R. H. Goldstein, I. V. Yannas, and M. Spector, "Formation of lung alveolar-like structures in collagen–glycosaminoglycan scaffolds in vitro," *Tissue Engineering*, vol. 11, no. 9-10, pp. 1436–1448, 2005.

[245] J. Cortiella, J. E. Nichols, K. Kojima et al., "Tissue-engineered lung: an in vivo and in vitro comparison of polyglycolic acid and pluronic F-127 hydrogel/somatic lung progenitor cell constructs to support tissue growth," *Tissue Engineering*, vol. 12, no. 5, pp. 1213–1225, 2006.

[246] H. T. Aiyelabegan, S. S. Z. Zaidi, S. Fanuel, A. Eatemadi, M. T. K. Ebadi, and E. Sadroddiny, "Albumin-based biomaterial for lung tissue engineering applications," *International Journal of Polymeric Materials and Polymeric Biomaterials*, vol. 65, no. 16, pp. 853–861, 2016.

[247] F. Quaglia, "Bioinspired tissue engineering: the great promise of protein delivery technologies," *International Journal of Pharmaceutics*, vol. 364, no. 2, pp. 281–297, 2008.

[248] X. Wang, E. Wenk, X. Hu et al., "Silk coatings on PLGA and alginate microspheres for protein delivery," *Biomaterials*, vol. 28, no. 28, pp. 4161–4169, 2007.

[249] X. Wang, H. J. Kim, C. Wong, C. Vepari, A. Matsumoto, and D. L. Kaplan, "Fibrous proteins and tissue engineering," *Materials Today*, vol. 9, no. 12, pp. 44–53, 2006.

[250] E. Hannachi Imen, M. Nakamura, M. Mie, and E. Kobatake, "Construction of multifunctional proteins for tissue engineering: epidermal growth factor with collagen binding and cell adhesive activities," *Journal of Biotechnology*, vol. 139, no. 1, pp. 19–25, 2009.

[251] J. J. Song, S. S. Kim, Z. Liu et al., "Enhanced in vivo function of bioartificial lungs in rats," *The Annals of Thoracic Surgery*, vol. 92, no. 3, pp. 998–1006, 2011.

[252] H. Liu and T. J. Webster, "Nanomedicine for implants: a review of studies and necessary experimental tools," *Biomaterials*, vol. 28, no. 2, pp. 354–369, 2007.

[253] C. Z. Liu, Z. D. Xia, Z. W. Han, P. A. Hulley, J. T. Triffitt, and J. T. Czernuszka, "Novel 3D collagen scaffolds fabricated by indirect printing technique for tissue engineering," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 85B, no. 2, pp. 519–528, 2008.

[254] B.-S. Ding, D. . J. Nolan, P. Guo et al., "Endothelial-derived angiocrine signals induce and sustain regenerative lung alveolarization," *Cell*, vol. 147, no. 3, pp. 539–553, 2011.

[255] E. Crivellato, "The role of angiogenic growth factors in organogenesis," *The International Journal of Developmental Biology*, vol. 55, no. 4-5, pp. 365–375, 2011.

[256] D. Panigrahy, B. T. Kalish, S. Huang et al., "Epoxyeicosanoids promote organ and tissue regeneration," *Proceedings of the National Academy of Sciences*, vol. 110, no. 33, pp. 13528–13533, 2013.

[257] N. F. Voelkel, I. S. Douglas, and M. Nicolls, "Angiogenesis in chronic lung disease," *Chest*, vol. 131, no. 3, pp. 874–879, 2007.

[258] C. Hanumegowda, L. Farkas, and M. Kolb, "Angiogenesis in pulmonary fibrosis," *Chest*, vol. 142, no. 1, pp. 200–207, 2012.

[259] K. R. Stenmark and S. H. Abman, "Lung vascular development: implications for the pathogenesis of bronchopulmonary dysplasia," *Annual Review of Physiology*, vol. 67, no. 1, pp. 623–661, 2005.

[260] T. Mammoto, E. Jiang, A. Jiang, and A. Mammoto, "Extracellular matrix structure and tissue stiffness control postnatal lung development through the lipoprotein receptor–related protein 5/Tie2 signaling system," *American Journal of Respiratory Cell and Molecular Biology*, vol. 49, no. 6, pp. 1009–1018, 2013.

[261] T. Mammoto and A. Mammoto, "Implantation of fibrin gel on mouse lung to study lung-specific angiogenesis," *Journal of Visualized Experiments*, no. 94, 2014.

[262] T. Mammoto, A. Jiang, E. Jiang, and A. Mammoto, "Platelet rich plasma extract promotes angiogenesis through the angiopoietin1-Tie2 pathway," *Microvascular Research*, vol. 89, pp. 15–24, 2013.

[263] K. M. Malinda, "In vivo Matrigel migration and angiogenesis assay," *Methods in Molecular Biology*, vol. 467, pp. 287–294, 2009.

[264] K. Norrby, "In vivo models of angiogenesis," *Journal of Cellular and Molecular Medicine*, vol. 10, no. 3, pp. 588–612, 2006.

[265] A. Stolzing, H. Colley, and A. Scutt, "Effect of age and diabetes on the response of mesenchymal progenitor cells to fibrin matrices," *International Journal of Biomaterials*, vol. 2011, 9 pages, 2011.

[266] E. Kniazeva, S. Kachgal, and A. J. Putnam, "Effects of extracellular matrix density and mesenchymal stem cells on neovascularization in vivo," *Tissue Engineering Part A*, vol. 17, no. 7-8, pp. 905–914, 2011.

[267] T. Ikeda, Y. Miyata, Y. Tsutani, K. Misumi, K. Arihiro, and M. Okada, "Fibrinogen/thrombin-based collagen fleece (TachoComb(R)) promotes regeneration in pulmonary arterial injury," *European Journal of Cardio-Thoracic Surgery*, vol. 41, no. 4, pp. 926–932, 2012.

[268] M. Okada, C. Yamashita, M. Okada, and K. Okada, "Role of endothelin-1 in beagles with dehydromonocrotaline-induced pulmonary hypertension," *Circulation*, vol. 92, no. 1, pp. 114–119, 1995.

[269] B. Suki, S. Ito, D. Stamenović, K. R. Lutchen, and E. P. Ingenito, "Biomechanics of the lung parenchyma: critical roles of collagen and mechanical forces," *Journal of Applied Physiology*, vol. 98, no. 5, pp. 1892–1899, 2005.

[270] E. Hadjipanayi, V. Mudera, and R. A. Brown, "Close dependence of fibroblast proliferation on collagen scaffold matrix stiffness," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 3, no. 2, pp. 77–84, 2009.

[271] J. L. Young and A. J. Engler, "Hydrogels with time-dependent material properties enhance cardiomyocyte differentiation in vitro," *Biomaterials*, vol. 32, no. 4, pp. 1002–1009, 2011.

[272] A. Kosmala, M. Fitzgerald, E. Moore, and F. Stam, "Evaluation of a gelatin-modified poly(ε-caprolactone) film as a scaffold for lung disease," *Analytical Letters*, vol. 50, no. 1, pp. 219–232, 2016.

[273] C. Mahoney, D. Conklin, J. Waterman, J. Sankar, and N. Bhattarai, "Electrospun nanofibers of poly(ε-caprolactone)/depolymerized chitosan for respiratory tissue engineering applications," *Journal of Biomaterials Science, Polymer Edition*, vol. 27, no. 7, pp. 611–625, 2016.

[274] C.-H. Lin, J.-M. Su, and S.-H. Hsu, "Evaluation of type II collagen scaffolds reinforced by poly(ε-caprolactone) as tissue-engineered trachea," *Tissue Engineering Part C: Methods*, vol. 14, no. 1, pp. 69–77, 2008.

[275] C. Radhakumary, A. M. Nandkumar, and P. D. Nair, "Hyaluronic acid-g-poly(HEMA) copolymer with potential implications for lung tissue engineering," *Carbohydrate Polymers*, vol. 85, no. 2, pp. 439–445, 2011.

[276] B. Demirdögen, A. E. Elçin, and Y. M. Elçin, "Neovascularization by bFGF releasing hyaluronic acid–gelatin microspheres:in vitroandin vivostudies," *Growth Factors*, vol. 28, no. 6, pp. 426–436, 2010.

[277] H. Fan, H. Tao, Y. Wu, Y. Hu, Y. Yan, and Z. Luo, "TGF-β3 immobilized PLGA-gelatin/chondroitin sulfate/hyaluronic acid hybrid scaffold for cartilage regeneration," *Journal of Biomedical Materials Research Part A*, vol. 95A, no. 4, pp. 982–992, 2010.

[278] M. Rampichová, E. Filová, F. Varga et al., "Fibrin/hyaluronic acid composite hydrogels as appropriate scaffolds for in vivo artificial cartilage implantation," *ASAIO Journal*, vol. 56, no. 6, pp. 563–568, 2010.

[279] L. Pescosolido, T. Vermonden, J. Malda et al., "In situ forming IPN hydrogels of calcium alginate and dextran-HEMA for biomedical applications," *Acta Biomaterialia*, vol. 7, no. 4, pp. 1627–1633, 2011.

[280] D. Singh, S. M. Zo, A. Kumar, and S. S. Han, "Engineering three-dimensional macroporous hydroxyethyl methacrylate-alginate-gelatin cryogel for growth and proliferation of lung epithelial cells," *Journal of Biomaterials Science, Polymer Edition*, vol. 24, no. 11, pp. 1343–1359, 2013.

[281] F. Fornasiero, F. Krull, C. J. Radke, and J. M. Prausnitz, "Diffusivity of water through a HEMA-based soft contact lens," *Fluid Phase Equilibria*, vol. 228-229, pp. 269–273, 2005.

[282] D. Singh, A. Tripathi, V. Nayak, and A. Kumar, "Proliferation of chondrocytes on a 3-D modelled macroporous poly(hydroxyethyl methacrylate)–gelatin cryogel," *Journal of Biomaterials Science, Polymer Edition*, vol. 22, no. 13, pp. 1733–1751, 2011.

[283] A. Tripathi and A. Kumar, "Multi-featured macroporous agarose-alginate cryogel: synthesis and characterization for bioengineering applications," *Macromolecular Bioscience*, vol. 11, no. 1, pp. 22–35, 2011.

[284] A. D. Augst, H. J. Kong, and D. J. Mooney, "Alginate hydrogels as biomaterials," *Macromolecular Bioscience*, vol. 6, no. 8, pp. 623–633, 2006.

[285] A. Panoskaltsis-Mortari, "Bioreactor development for lung tissue engineering," *Current Transplantation Reports*, vol. 2, no. 1, pp. 90–97, 2015.

[286] P. Jungebluth, A. Bader, S. Baiguera et al., "The concept of in vivo airway tissue engineering," *Biomaterials*, vol. 33, no. 17, pp. 4319–4326, 2012.

[287] D. E. Wagner, N. R. Bonenfant, C. S. Parsons et al., "Comparative decellularization and recellularization of normal versus emphysematous human lungs," *Biomaterials*, vol. 35, no. 10, pp. 3281–3297, 2014.

[288] D. E. Wagner, N. R. Bonenfant, D. Sokocevic et al., "Three-dimensional scaffolds of acellular human and porcine lungs for high throughput studies of lung disease and regeneration," *Biomaterials*, vol. 35, no. 9, pp. 2664–2679, 2014.

[289] N. R. Bonenfant, D. Sokocevic, D. E. Wagner et al., "The effects of storage and sterilization on de-cellularized and re-cellularized whole lung," *Biomaterials*, vol. 34, no. 13, pp. 3231–3245, 2013.

[290] S. Baiguera, C. Del Gaudio, M. O. Jaus et al., "Long-term changes to in vitro preserved bioengineered human trachea and their implications for decellularized tissues," *Biomaterials*, vol. 33, no. 14, pp. 3662–3672, 2012.

[291] H. Nagaoka, H. Nagaoka, R. Walter et al., "Characterization of genipin-modified dentin collagen," *BioMed Research International*, vol. 2014, Article ID 702821, 7 pages, 2014.

[292] J. Haag, S. Baiguera, P. Jungebluth et al., "Biomechanical and angiogenic properties of tissue-engineered rat trachea using genipin cross-linked decellularized tissue," *Biomaterials*, vol. 33, no. 3, pp. 780–789, 2012.

[293] C.-Y. Zhang, L. E. Parton, C. P. Ye et al., "Genipin inhibits UCP2-mediated proton leak and acutely reverses obesity- and high glucose-induced β cell dysfunction in isolated pancreatic islets," *Cell Metabolism*, vol. 3, no. 6, pp. 417–427, 2006.

[294] G. Wang, S. Wu, J. Rao et al., "Genipin inhibits endothelial exocytosis via nitric oxide in cultured human umbilical vein endothelial cells," *Acta Pharmacologica Sinica*, vol. 30, no. 5, pp. 589–596, 2009.

[295] T. Peng, Y. Tian, C. J. Boogerd et al., "Coordination of heart and lung co-development by a multipotent cardiopulmonary progenitor," *Nature*, vol. 500, no. 7464, pp. 589–592, 2013.

Hindawi
Stem Cells International
Volume 2018, Article ID 9591421, 8 pages
https://doi.org/10.1155/2018/9591421

Research Article

Correlation between Therapeutic Efficacy of $CD34^+$ Cell Treatment and Directed *In Vivo* Angiogenesis in Patients with End-Stage Diffuse Coronary Artery Disease

Tien-Hung Huang,[1,2] Cheuk-Kwan Sun,[3] Yi-Ling Chen,[1,2] Pei-Hsun Sung,[1] Chi-Hsiang Chu,[4] Mel S. Lee,[5] Yuan-Ping Lin,[6] Hon-Kan Yip,[1,2,7,8,9] and Fan-Yen Lee[10,11]

[1]*Division of Cardiology, Department of Internal Medicine, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung 83301, Taiwan*
[2]*Institute for Translational Research in Biomedicine, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung 83301, Taiwan*
[3]*Department of Emergency Medicine, E-Da Hospital, I-Shou University School of Medicine for International Students, Kaohsiung 82445, Taiwan*
[4]*Clinical Trial Center, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung 83301, Taiwan*
[5]*Department of Orthopedic Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung 83301, Taiwan*
[6]*Department of Health and Beauty, Shu-Zen Junior College of Medicine and Management, Kaohsiung 82144, Taiwan*
[7]*Center for Shockwave Medicine and Tissue Engineering, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung 83301, Taiwan*
[8]*Department of Medical Research, China Medical University Hospital, China Medical University, Taichung 40402, Taiwan*
[9]*Department of Nursing, Asia University, Taichung 41354, Taiwan*
[10]*Division of Thoracic and Cardiovascular Surgery, Department of Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung 83301, Taiwan*
[11]*Tri-Service General Hospital, National Defense Medical Center, Taipei 11490, Taiwan*

Correspondence should be addressed to Hon-Kan Yip; han.gung@msa.hinet.net and Fan-Yen Lee; fanyenlee2015@gmail.com

Received 3 October 2017; Revised 6 February 2018; Accepted 4 March 2018; Published 15 April 2018

Academic Editor: Heinrich Sauer

Background. This study was aimed at testing the association between the therapeutic efficacy of $CD34^+$ cell treatment in patients with end-stage diffuse coronary artery disease as reflected in angiographic grading and results of directed *in vivo* angiogenesis assay (DIVAA) on their isolated peripheral blood mononuclear cell- (PBMC-) derived endothelial progenitor cells (EPCs). *Methods*. Angiographic grades (0: <5%; 1: 5–35%; 2: 35–75%; 3: >75%) which presented the improvement of vessel density pre- and post-$CD34^+$ treatment were given to 30 patients with end-stage diffuse coronary artery disease having received $CD34^+$ cell treatment. The patients were categorized into low-score group (angiographic grade 0 or 1, $n = 12$) and high-score group (angiographic grade 2 or 3, $n = 18$). The percentages of circulating EPCs with $KDR^+/CD34^+/CD45^-$, $CD133^+/CD34^+/CD45^-$, and $CD34^+$ were determined in each patient using flow cytometry. PBMC-derived EPCs from all patients were subjected to DIVAA through a 14-day implantation in nude mice. The DIVAA ratio (i.e., mean fluorescent units in angioreactors with EPCs/mean fluorescent units in angioreactors without EPCs) was obtained for each animal with implanted EPCs from each patient. *Results and Conclusions*. The number of EPCs showed no significant difference among the two groups. The DIVAA ratio in the high-score group was significantly higher than that in the low-score group ($p = 0.0178$). Logistic regression revealed a significant association between the DIVAA ratio and angiographic grading (OR 3.12, 95% CI: 1.14–8.55, $p = 0.027$). The area under the ROC curve (AUC) was 0.8519 ($p = 0.0013$). We proposed that DIVAA may be a reliable tool for assessing coronary vascularization after $CD34^+$ cell treatment.

1. Introduction

The capability of bone marrow-derived $CD34^+$ cells of vascular tissue repair and regeneration has been widely reported in a variety of animal disease models, including myocardial, peripheral, and cerebral ischemia [1, 2]. Besides, the clinical significance of $CD34^+$ cell treatment has been highlighted in the promising results of a number of clinical trials enrolling patients with ischemic diseases unsuitable for surgery or refractory to medications [3–8]. Although the increasing number of successful bench-to-bedside (i.e., phase 1) studies has provided enough evidence to support the clinical safety of autologous stem cell treatment, the selection of optimal time points, cell dosage, and route of administration remains unclear [9, 10].

In our previous clinical trial [6], patients with severe diffuse coronary artery disease (CAD) receiving intracoronary transfusion of circulating-derived $CD34^+$ cells directly without enrichment for endothelial progenitor cells (EPCs) were divided into angiographic low- and high-score groups after a 9-month follow-up. The results of cardiac MRI and 3D echocardiography demonstrated a significant improvement of the left ventricular ejection fraction (LVEF) in the high-score subjects compared to that in their low-score counterparts. The findings, therefore, indicated that angiographic grading could reliably reflect the long-term outcome of patients after $CD34^+$ cell treatment. On the other hand, since up to 31.6% of patients (12 of 38) responded unsatisfactorily to cell treatment in that study, prediction of treatment outcome with angiographic grading may be of clinical importance for selection of patients for advanced stem cell treatment, such as enrichment for EPC purity and quantity in Good Tissue Practice (GTP) facilities.

The present study attempted to investigate the association of angiographic grading with the results of directed *in vivo* angiogenesis assay (DIVAA) in patients with end-stage diffuse coronary artery disease having received $CD34^+$ cell treatment.

2. Subjects and Methods

2.1. Study Populations and Study Protocol. Between February 2015 and March 2016, all patients of age between 20 and 80 having received $CD34^+$ cell treatment (phase I clinical trial) for obstructive coronary disease (including stable and unstable angina and ≥3 months of myocardial infarction ≤ Killip-3) with coronary angiographic findings of severe diffuse CAD, noncandidates for percutaneous coronary intervention or coronary artery bypass surgery (CABG) (i.e., when vessel involvement was too diffuse and the diameter was too small for intervention), those who had Canadian Cardiovascular Society class II–IV angina, and those whose thallium (Tl-201) scan showed reversible myocardial ischemia as well as those who had previously received CABG with venous or arterial graft failure that could not be revascularized by the catheter-based approach for either the native vessel or the graft were prospectively enrolled. Patients who were carriers of hepatitis B or C; those with history of surgery, trauma, or myocardial infarction within the preceding 3 months; those with liver cirrhosis, hematology disorders, renal insufficiency (defined as creatinine clearance [Ccr] < 20 mL/min), malignancy, febrile disorders, acute or chronic inflammatory disease at study entry, severe mitral or aortic regurgitation, congestive heart failure (NYHA Fc 4), expected life expectancy less than 2.0 years, and age younger than 20 years or 80 years or older; or pregnant women were excluded from the present study. A total of 38 patients were enrolled in our previous phase I clinical trial, and results have been published [6]. However, three patients expired due to cardiovascular death and five patients expired due to noncardiovascular death in the period of long-term follow-up. As a result, only 30 of the 38 patients participated in the present study.

All eligible patients having received $CD34^+$ cell treatment for over one year underwent peripheral blood sampling in the outpatient clinic of a tertiary referral center (Kaohsiung Chang Gung Memorial Hospital) for the culturing of endothelial progenitor cells (EPCs). Quantitative assessment of angiogenic responses in the cultured EPCs was performed by directed *in vivo* angiogenesis assay (DIVAA) using nude mice. Patients with incomplete clinical follow-up were excluded. An angiographic study of the coronary arteries was performed nine months after cell therapy. The degree of angiogenesis on angiography was compared to that of DIVAA for each patient. An overview of the study protocol is shown in Figure 1. This study was approved by the Institutional Review Committee on Human Research at Chang Gung Memorial Hospital (IRB No.: 103–7569A3) and conducted at Kaohsiung Chang Gung Memorial Hospital.

2.2. Definition of Angiographic Grading. Improved angiogenesis/neovascularization for patients pre- and post-autologous $CD34^+$ cell treatment was assessed angiographically using the scoring system of vessel density previously described [3]. Briefly, four cardiologists blinded to the treatment had reached a consensus for grading with scores being defined as follows: grade 0: less than 5.0%; grade 1: 5.0% to 35.0%; grade 2: greater than 35.0% to less than or equal to 75.0%; and grade 3: greater than 75.0%. Because of the limited number of patients for each angiographic grade (i.e., 0, 1, 2, and 3), the patients were divided into low- and high-score groups. High-score was defined as grade 2 and grade 3, while low-score was defined as grade 0 and grade 1.

2.3. Flow Cytometry Analysis. For identification of EPCs derived from peripheral blood, flow cytometry was applied for the assessment of EPC surface markers, including sets of $KDR^+/CD34^+/CD45^-$, $CD133^+/CD34^+/CD45^-$, and $CD34^+$, as described in our recent reports [6, 8, 11]. Briefly, mononuclear cells were isolated by density-gradient centrifugation of Ficoll-Paque Plus™ (GE Healthcare Biosciences, Uppsala, Sweden) and further incubated with fluorescent dye-conjugated mononuclear antibodies. After incubation, the mononuclear cells were fixed in 1% of paraformaldehyde for flow cytometry analysis by using a fluorescence-activated cell sorter (FACSCalibur system; Beckman Coulter, CA, USA). The assays for circulating EPCs in each sample were performed in duplicate, and the mean levels were reported.

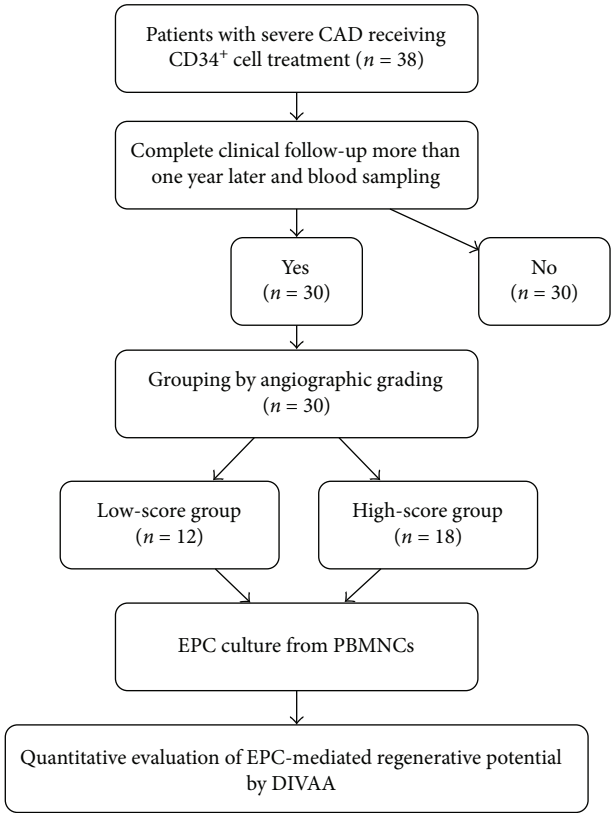


Figure 1: Protocol of follow-up, grouping, and analysis in the present study. Patients with severe coronary artery disease (CAD) having received $CD34^+$ cell treatment with complete follow-up, divided into three groups based on different angiographic grades. Application of directed *in vivo* angiogenesis assay (DIVAA) further delineating the correlation between angiographic grading and endothelial progenitor cell- (EPC-) mediated regenerative potential.

2.4. Endothelial Progenitor Cell Culture. Ficoll gradient was used to isolate peripheral blood mononuclear cells (PBMCs) from blood collection in heparin tubes for the culture of endothelial progenitor cells (EPCs), and the protocol of human EPC culture has been described in our previous studies [6, 8]. In brief, whole blood was diluted (1 : 1) with PBS containing 2% fetal bovine serum (FBS; Gibco BRL, CA, USA), and 7 mL of diluted blood was then carefully overlaid onto 5 mL of Ficoll-Paque Plus (GE Healthcare Biosciences, Uppsala, Sweden). The PBMC layer was isolated by centrifugation without a brake and washed with PBS. Isolated PBMCs were cultured with the endothelial growth medium-2 (EGM-2; Clonetics®, CA, USA) on fibronectin-coated dish at 37°C with 5% CO_2.

2.5. Directed In Vivo Angiogenesis Assay (DIVAA). Angiogenic responses of EPCs of each patient were quantitatively assessed by directed *in vivo* angiogenesis assay (DIVAA) in a nude mouse according to the protocol of the manufacturer (Trevigen, MD, USA). Briefly, female nude mice, 6 to 8 weeks of age, were anesthetized with 2% isoflurane for the implantation of the angioreactor. Two incisions were made on the dorsal-lateral skin of a nude mouse, one on each side, approximately 1 cm in length. Two angioreactors containing growth factors with (i.e., right side) and two without (i.e., left side) cultured EPCs (4×10^5 EPCs per angioreactor) from one patient were subcutaneously implanted under the surgical incisions in one nude mouse which were closed with interrupted 4-0 nylon sutures (Figures 2(a) and 2(b)). After 14 days, the 30 nude mice carrying EPCs from 30 patients were anesthetized with 2% isoflurane before careful removal of the implanted angioreactors. Cells in the angioreactor were collected for incubation with fluorescein isothiocyanate- (FITC-) conjugated lectin at 4°C overnight. Vascularization in the angioreactor was assessed with a spectrophotometer (Thermo Scientific™ Fluoroskan Ascent™ FL Microplate Fluorometer, Vantaa, Finland) at 485 nm based on the degree of specific binding between lectin and endothelial cells as reflected in the intensity of FITC-lectin fluorescence signals (i.e., relative fluorescent units, RFU). After a 14-day implantation, the level of angiogenesis and neovascularization as reflected in the mean fluorescence intensity in the two angioreactors on the right side (i.e., EPCs) to that in the other two on the left side (i.e., controls) was compared using the DIVAA ratio (i.e., mean fluorescent units in angioreactors with EPCs/mean fluorescent units in angioreactors without EPCs) for each animal. All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at Kaohsiung Chang Gung Memorial Hospital (Affidavit of Approval of Animal Use Protocol No. 2014121814) and performed in accordance with the National Research Council of the National Academies Guide for the Care and Use of Laboratory Animals (Eighth Edition, 2011).

2.6. Statistical Analysis. All values are expressed as the mean ± SD, number, or percentage where appropriate. Differences in continuous variables between two groups were analyzed by the independent *t*-test. The means of more than three independent groups were analyzed by ANOVA. Continuous variables were compared with a paired *t*-test for matched-paired samples. The Pearson correlation test was used to assess the relation between two quantitative variables. A binary logistic regression model was further used to evaluate the major factors related to angiogenesis grading. Statistical analysis was performed using SPSS statistical software for Windows version 13 (SPSS for Windows, version 13; SPSS, Chicago, IL). A value of p less than 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of Study Patients. Of the 38 patients with severe diffuse CAD having undergone $CD34^+$ cell treatment, eight of the patients expired in the long-term follow-up. As a result, only 30 of the 38 patients participated in the present study (Figure 1). According to follow-up angiography grading, CAD patients were categorized into the low-score group (angiographic grade 0 or 1, $n = 12$) and high-score group (angiographic grade 2 or 3, $n = 18$). The characteristics of the 30 patients are summarized in Table 1. The age, gender, body height, body weight, body mass index, CAD risk factors, and incidences of previous stroke, old myocardial infarction, and coronary artery bypass surgery did not differ

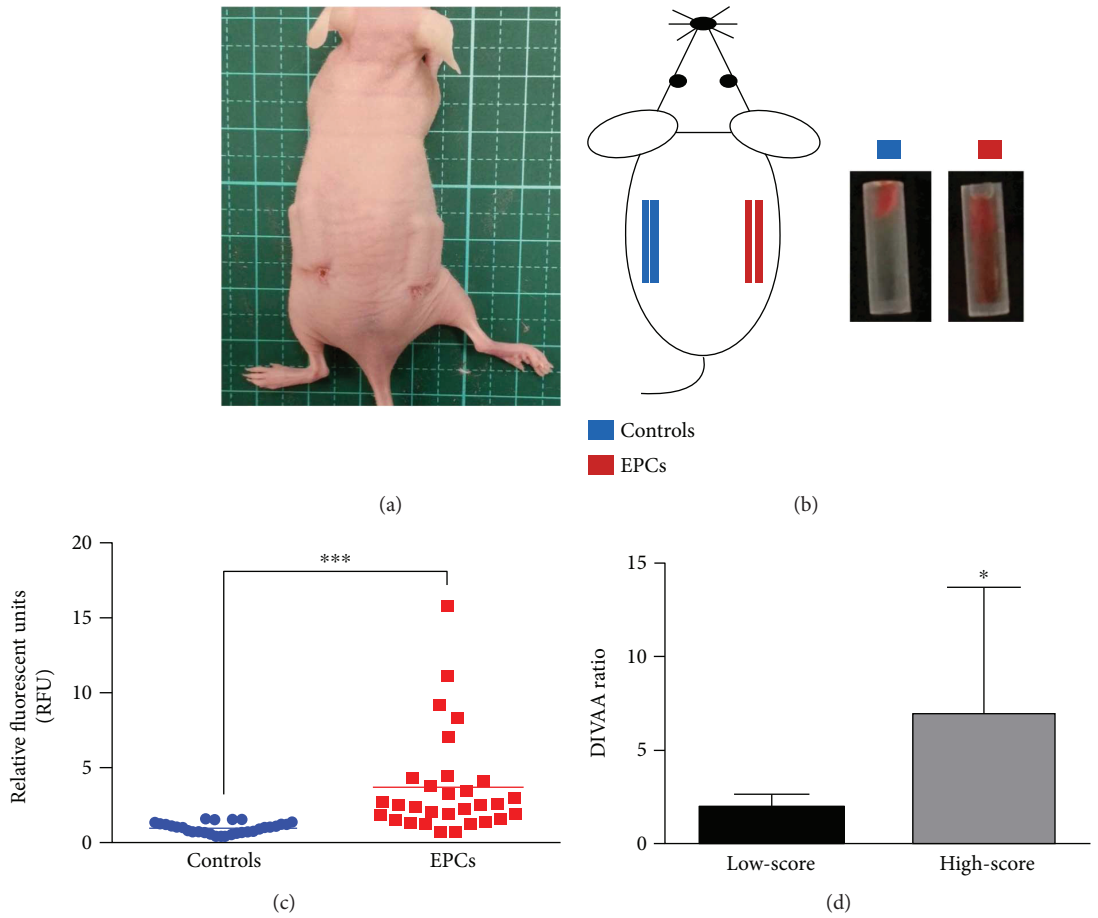


Figure 2: Evaluation of endothelial progenitor cell- (EPC-) mediated angiogenesis/neovascularization by directed *in vivo* angiogenesis assay (DIVAA) in nude mice. (a) Angioreactors implanted subcutaneously on each flank of a nude mouse. (b) Two angioreactors containing growth factors with (i.e., right side, EPCs) and two without (i.e., left side, controls) cultured EPCs (4×10^5 EPCs/angioreactor) from one patient subcutaneously implanted under the surgical incisions. After a 14-day implantation, retrieved angioreactors displayed on the right panel. (c) Comparison of relative fluorescent units (RFUs) between controls and EPCs with the paired *t*-test. (d) Comparison of the DIVAA ratio between the low-score group (grades 0-1) and the high-score group (grades 2-3), using the independent *t*-test; each error bar represents the mean with SD, $^{***}p < 0.0001$ and $^{*}p < 0.05$.

between low-score and high-score group patients. Additionally, findings of vessel diseases and use of medication did not show a significant difference among these groups. The laboratory findings are also summarized. The serum creatinine level, creatinine clearance rate, and incidence of moderate and severe chronic kidney disease (CKD, stage III, and stage IV) did not differ among the three groups. For the percentages of circulating cells with $KDR^+/CD34^+/CD45^-$, $CD133^+/CD34^+/CD45^-$, and $CD34^+$, the three stem cell marker sets showed no difference among the three groups.

3.2. In Vivo Angiogenic Potential of EPCs Determined by DIVAA. Comparison of the fluorescence intensities of the 30 mice on both sides revealed significantly higher relative fluorescent units (RFU) on the right side (i.e., EPCs) compared to that on the left side (i.e., controls) ($p < 0.0001$, Figure 2(c)). To clarify whether capacities of angiogenesis and neovascularization of EPCs isolated from patients varied with their angiographic grades, EPCs were divided into low-score group (angiographic grade = 0 or 1, $n = 12$) and high-score group (angiographic grade = 2 or 3, $n = 18$) for comparison. Comparison of the DIVAA between the two groups demonstrated that the DIVAA ratio of the high-score group was significantly higher than that in the low-score group ($p = 0.0178$) (Figure 2(d)).

3.3. Association of the DIVAA Ratio with Angiographic Grading. To study the association between the DIVAA ratio and angiographic grading, both univariate and multivariate logistic regression models were used to find the relationships between the important variables and angiographic findings. The univariate logistic regression analysis was applied to select the significant variable (p value < 0.05) (Table 2). When the DIVAA ratio was used as the predictive tool for the angiographic findings after receiving autologous $CD34^+$ cell treatment, patients with a higher DIVAA ratio had a higher angiogenesis/neovasculature score (OR 3.12, 95% CI: 1.14–8.55, $p = 0.027$).

3.4. Predictive Power of the DIVAA Ratio. To clarify the power of DIVAA in predicting angiographic outcomes of patients having received autologous $CD34^+$ cell treatment,

Table 1: Clinical and laboratory findings of 30 study patients.

Variables	Low-score ($n = 12$)	High-score ($n = 18$)	p value
Age (years)	65.3 ± 9.8	66.0 ± 6.3	0.799
Male gender	58.3% (7)	72.2% (13)	0.429
Body height (cm)	158.8 ± 8.1	161.9 ± 6.2	0.243
Body weight (kg)	67.4 ± 14.4	70.0 ± 10.2	0.200
Body mass index	26.1 ± 4.7	26.7 ± 3.7	0.692
Hypertension	91.7% (11)	83.3% (15)	0.632
Diabetes mellitus	75.0% (9)	83.3% (15)	0.660
History or current smoking	33.3% (4)	50.0% (9)	0.367
Total cholesterol (mg/dL)	182.1 ± 36.3	164.4 ± 42.1	0.246
Low density lipoprotein	102.9 ± 25.5	106.0 ± 40.1	0.815
High density lipoprotein	47.6 ± 13.1	42.8 ± 8.5	0.238
Previous stroke	16.7% (2)	22.2% (4)	1.000
Old myocardial infarction	16.7% (2)	16.7% (3)	1.000
History of CABG	41.7% (5)	33.3% (6)	0.643
Diffuse multivessel disease	100% (12)	100% (18)	1.000
Diffuse triple vessel disease	83.3% (10)	83.3% (15)	1.000
Previous PCI	58.3% (7)	44.4% (8)	0.456
Aspirin/clopidogrel therapy	100% (12)	100% (18)	1.000
β-Blocker therapy	91.7% (11)	94.4% (17)	1.000
Stain therapy	58.3% (7)	77.8% (14)	0.255
ARB/ACEI therapy	75.0% (9)	77.8% (14)	1.000
Creatinine level (mg/dL)	1.08 ± 0.31	1.26 ± 0.42	0.391
Creatinine clearance rate (mL/min)	64.8 ± 17.7	59.9 ± 23.1	0.539
Stage III–IV CKD	58.3% (7)	55.6% (10)	0.880
Flow cytometry (before G-CSF treatment)			
$KDR^+/CD34^+/CD45^-$ (%)	0.22 ± 0.19	0.24 ± 0.14	0.430
$CD133^+/CD34^+/CD45^-$ (%)	0.12 ± 0.11	0.08 ± 0.05	0.458
$CD34^+$ (%)	1.19 ± 0.91	1.05 ± 1.02	0.711
Flow cytometry (after G-CSF treatment)			
$KDR^+/CD34^+/CD45^-$ (%)	0.52 ± 0.67	0.34 ± 0.55	0.325
$CD133^+/CD34^+/CD45^-$ (%)	1.10 ± 0.87	0.75 ± 0.52	0.347
$CD34^+$ (%)	2.42 ± 2.08	1.88 ± 1.65	0.879

Data are expressed as mean ± SD or % (n). CABG = coronary artery bypass grafting; PCI = percutaneous coronary intervention; ARB = angiotensin II type I receptor blocker, ACEI = angiotensin-converting enzyme inhibitor; CKD = chronic kidney disease.

receiver operator characteristic (ROC) curve analysis was performed (Figure 3). When comparison was made between low and high angiographic scores, the area under the ROC curve (AUC) was 0.8519 (95% CI: 0.7132–0.9905, $p = 0.0013$). An optimal cut-off point at 3.07 was determined using the ROC curve. When patients were divided according to the cut-off point, sensitivity and specificity of the high score were 72.22% and 100%, respectively. Furthermore, if we want to correctly identify the high score of patients (more than 85%), an appropriate value of the cut-off point was considered to be 1.965 with sensitivity 88.9% and specificity 50.0%, respectively.

4. Discussion

This study, which attempted to investigate the correlation between angiogenesis/neovascularization of EPCs from patients with severe diffuse coronary artery disease having received autologous $CD34^+$ cell treatment and their angiographic grading, demonstrated that higher DIVAA ratios were associated with higher angiographic grading. The present investigation represents the first study to address the possibility of using a DIVAA murine model to correlate with clinical outcomes.

$CD34^+$ is the most commonly used marker of endothelial progenitor cells (EPCs) in clinical hematology and stem cell trials [4, 6, 12]. Although the capacity of transdifferentiation in hematopoietic stem cells and EPCs to cardiac myocytes has been widely debated [13, 14], neovascularization is still the most important outcome after receiving $CD34^+$ cell treatment through direct incorporation of transfused cells into newly formed vessels and release of angiogenic cytokines in a paracrine manner [1, 15]. Since we found that many baseline characteristics and routine laboratory findings cannot

Table 2: Univariate logistic regression models for the association between significant variables and improved angiographic findings.

Variables	Univariate logistic regression model		
	OR	95% CI	p value
Age	1.01	(0.92–1.12)	0.791
Gender	1.86	(0.40–8.69)	0.432
Body height	1.07	(0.96–1.20)	0.237
Body weight	1.02	(0.96–1.09)	0.557
Body mass index	1.04	(0.86–1.25)	0.706
Hypertension	0.46	(0.04–4.98)	0.518
Diabetes mellitus	1.67	(0.28–10.09)	0.578
History or current smoking	2.00	(0.44–9.10)	0.370
Total cholesterol	0.99	(0.97–1.01)	0.242
Low-density lipoprotein	1.00	(0.98–1.03)	0.808
High-density lipoprotein	0.96	(0.89–1.03)	0.236
Previous stroke	1.43	(0.22–9.38)	0.710
Old myocardial infarction	1.00	(0.14–7.10)	1.000
History of CABG	0.70	(0.16–3.17)	0.643
Diffuse multivessel disease	—	—	—
Diffuse triple vessel disease	1.00	(0.14–7.10)	1.000
Previous PCI	0.57	(0.13–2.50)	0.458
Aspirin/clopidogrel therapy	—	—	—
β-Blocker therapy	0.77	(0.09–27.36)	0.767
Stain therapy	2.50	(0.51–12.35)	0.261
ARB/ACEI therapy	1.17	(0.21–6.48)	0.860
Creatinine level	3.79	(0.43–33.72)	0.232
Creatinine clearance rate	0.99	(0.95–1.02)	0.525
Stage III–IV CKD	0.89	(0.20–3.91)	0.880
Before G-CSF treatment			
$KDR^+/CD34^+/CD45^-$	2.26	(0.02–319.53)	0.747
$CD133^+/CD34^+/CD45^-$	0.001	(0.00–118.39)	0.240
$CD34^+$	0.85	(0.39–1.89)	0.696
After G-CSF treatment			
$KDR^+/CD34^+/CD45^-$	0.58	(0.16–2.13)	0.409
$CD133^+/CD34^+/CD45^-$	0.46	(0.14–1.49)	0.197
$CD34^+$	0.85	(0.56–1.28)	0.430
DIVAA ratio	3.12	(1.14–8.55)	0.027*

*Defined significant at $\alpha = 0.05$.

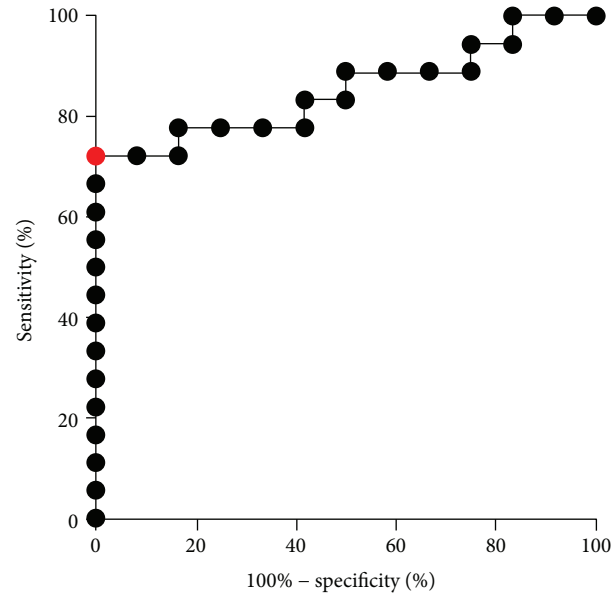


Figure 3: Association between angiographic findings and the DIVAA ratio. Assessment of the receiver operator characteristic (ROC) curve for the predictive power of the directed *in vivo* angiogenesis assay (DIVAA) ratio in patients having received autologous $CD34^+$ cell treatment. The area under the ROC curve (AUC) was 0.8519 ($p = 0.0013$). The optimal cut-off point is labeled by the red solid circle.

clearly explain the changes in angiographic grading in advance, we focused on the investigation of the regenerative potential of isolated $CD34^+$ cells that may reflect the neovascularization of coronary vasculature in the present study.

Tube formation assay is a regular angiogenesis assay widely applied to determine the angiogenic potential of EPCs *in vitro*. However, our previous study has demonstrated that there is no significant difference in EPC-mediated angiogenesis between low-score and high-score groups before G-CSF (granulocyte colony-stimulating factor) stimulation by tube formation assay [6]. There are three merits of using DIVAA to replace the conventional tube formation assay to determine EPC-mediated angiogenesis/neovascularization in this study. First, DIVAA more closely mimics the actual physiological environment of the human body. Second, DIVAA allows long-term observation for 9 to 15 days to enable a more accurate assessment of vascularization. In contrast, the capillary-like structure in tube formation assay, which is usually observed after a 6- to 12-hour incubation, invariably disappears on prolonged incubation so that long-term observation of vascularization is impossible. Third, the outcome of conventional tube formation assay that depends on cell density and cellular migration capacity [16, 17] cannot reliably assess EPC-mediated neovascularization, which is mainly attributable to the differentiation from EPCs to mature endothelial cells [18]. Therefore, not only does DIVAA provide a physiological environment for EPC differentiation to mature endothelial cells, but it also allows long-term observation of angiogenesis/neovascularization.

The therapeutic benefits of administering $CD34^+$ cells in patients with ischemic heart disease are believed to be attributable to the angiogenic capacity of EPCs. The discrepancy in treatment outcome of using $CD34^+$ cells in previous studies may be attributable to the fact that EPCs comprise only a minor proportion of $CD34^+$ cells that also include several subpopulations of hematopoietic stem cells [19–21]. Indeed, it has been demonstrated that EPCs account for merely 1% of all circulating mononuclear cells [22]. Therefore, direct transfusion of isolated $CD34^+$ cells might not guarantee sufficient purity of EPCs to achieve an optimal therapeutic outcome. On the other hand, a sufficient quantity of EPCs may not attain the expected treatment goal since the angiogenic capacity of EPCs, including proliferation, differentiation, and mobilization in bone marrow, may be tarnished by the presence of chronic diseases like diabetes, hypertension,

and renal dysfunction that often cause systemic oxidative stress [23, 24]. Hence, the quality of stem cells for clinical application should be taken into consideration [21]. For this purpose, a long-term evaluation of *in vivo* angiogenic potential by DIVAA might help in differentiating the capacity of EPC-mediated angiogenesis/neovascularization in patients with different underlying diseases.

As stem cell therapy has been gaining popularity as a standard treatment option, the concept of improving such a "stem cell drug" product, such as safety, reproduction, and efficiency, has been developed [25]. The results of the present study demonstrated a significant correlation between the DIVAA ratio and clinical angiographic grading, suggesting the possibility of using this ratio as guidance for choosing the subsequent treatment strategy. For example, patients with a high DIVAA ratio may be suggested to receive autologous $CD34^+$ cells directly with minimum manipulation, whereas patients with a low DIVAA ratio may receive EPCs enriched in good tissue practice (GTP) facilities before intracoronary transfusion. Although most of the stem cell-based clinical trials focused on the investigation of the optimal routes, dosage, and timing for stem cell administration, the results of the current study suggest that determination of the quality and quantity of stem cells is necessary to achieve an optimal therapeutic outcome for an individual based on the concept of precision medicine that emphasizes on tailoring medical care to cater for individual needs [26].

5. Limitations

This study has limitations. First, the sample size was relatively small in the present study. As a result, patients from our phase II and III clinical trials will be enrolled in the future to reinforce our findings. Second, since EPCs were not isolated from the study subjects before $CD34^+$ cell treatment, the value of DIVAA in predicting the subsequent angiographic grading remains unclear. Third, it is hard to distinguish whether EPC-directed angiogenesis contributed by paracrine effect of proangiogenic factors or direct incorporation into new vessels in our DIVAA model.

6. Conclusions

The results of the present study demonstrated a significant correlation between EPC angiogenic capacity from patients with end-stage diffuse coronary artery disease in an *in vivo* murine model and their angiographic grading, suggesting the possible use of this model in the prediction of clinical outcome after $CD34^+$ cell treatment.

Conflicts of Interest

The authors report no relationships that could be construed as a conflict of interest.

Authors' Contributions

Tien-Hung Huang, Cheuk-Kwan Sun, Hon-Kan Yip, and Fan-Yen Lee conceived the study and participated in the design of the study, data acquisition, and analysis as well as drafting of the manuscript. Yi-Ling Chen, Pei-Hsun Sung, and Chi-Hsiang Chu were responsible for the laboratory assay and troubleshooting. Mel S. Lee and Yuan-Ping Lin participated in the data acquisition, analysis, and interpretation. All authors read and approved the final manuscript. Tien-Hung Huang, Cheuk-Kwan Sun, Fan-Yuan Lee, and Hon-Kan Yip contributed equally to this paper.

Acknowledgments

This study was supported by program grants from Chang Gung Memorial Hospital, Chang Gung University (no. CMRPG8E0941), and Ministry of Science and Technology, Taiwan (no. 102-2314-B-650-004).

References

[1] A. R. Mackie and D. W. Losordo, "CD34-positive stem cells: in the treatment of heart and vascular disease in human beings," *Texas Heart Institute Journal*, vol. 38, no. 5, pp. 474–485, 2011.

[2] W. C. Shyu, S. Z. Lin, M. F. Chiang, C. Y. Su, and H. Li, "Intracerebral peripheral blood stem cell ($CD34^+$) implantation induces neuroplasticity by enhancing β1 integrin-mediated angiogenesis in chronic stroke rats," *The Journal of Neuroscience*, vol. 26, no. 13, pp. 3444–3453, 2006.

[3] T. J. Povsic, T. D. Henry, J. H. Traverse et al., "The RENEW Trial: Efficacy and Safety of Intramyocardial Autologous $CD34^+$ Cell Administration in Patients With Refractory Angina," *JACC: Cardiovascular Interventions*, vol. 9, no. 15, pp. 1576–1585, 2016.

[4] D. W. Losordo, T. D. Henry, C. Davidson et al., "Intramyocardial, autologous CD34+ cell therapy for refractory angina," *Circulation Research*, vol. 109, no. 4, pp. 428–436, 2011.

[5] D. W. Losordo, R. A. Schatz, C. J. White et al., "Intramyocardial transplantation of autologous $CD34^+$ stem cells for intractable angina: a phase I/IIa double-blind, randomized controlled trial," *Circulation*, vol. 115, no. 25, pp. 3165–3172, 2007.

[6] F. Y. Lee, Y. L. Chen, P. H. Sung et al., "Intracoronary transfusion of circulation-derived CD34+ cells improves left ventricular function in patients with end-stage diffuse coronary artery disease unsuitable for coronary intervention," *Critical Care Medicine*, vol. 43, no. 10, pp. 2117–2132, 2015.

[7] T. D. Henry, G. L. Schaer, J. H. Traverse et al., "Autologous $CD34^+$ cell therapy for refractory angina: 2-year outcomes from the ACT34-CMI study," *Cell Transplantation*, vol. 25, no. 9, pp. 1701–1711, 2016.

[8] M. S. Lee, F. Y. Lee, Y. L. Chen et al., "Investigated the safety of intra-renal arterial transfusion of autologous CD34+ cells and time courses of creatinine levels, endothelial dysfunction biomarkers and micro-RNAs in chronic kidney disease patients-phase I clinical trial," *Oncotarget*, vol. 8, no. 11, pp. 17750–17762, 2017.

[9] A. Trounson and C. McDonald, "Stem cell therapies in clinical trials: progress and challenges," *Cell Stem Cell*, vol. 17, no. 1, pp. 11–22, 2015.

[10] S. Golpanian, I. H. Schulman, R. F. Ebert et al., "Concise review: review and perspective of cell dosage and routes of administration from preclinical and clinical studies of stem cell therapy for heart disease," *Stem Cells Translational Medicine*, vol. 5, no. 2, pp. 186–191, 2016.

[11] H. K. Yip, L. T. Chang, W. N. Chang et al., "Level and value of circulating endothelial progenitor cells in patients after acute ischemic stroke," *Stroke*, vol. 39, no. 1, pp. 69–74, 2008.

[12] S. Wang, J. Cui, W. Peng, and M. Lu, "Intracoronary autologous CD34+ stem cell therapy for intractable angina," *Cardiology*, vol. 117, no. 2, pp. 140–147, 2010.

[13] C. E. Murry, M. H. Soonpaa, H. Reinecke et al., "Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts," *Nature*, vol. 428, no. 6983, pp. 664–668, 2004.

[14] I. Gruh, J. Beilner, U. Blomer et al., "No evidence of transdifferentiation of human endothelial progenitor cells into cardiomyocytes after coculture with neonatal rat cardiomyocytes," *Circulation*, vol. 113, no. 10, pp. 1326–1334, 2006.

[15] A. H. S. Kumar and N. M. Caplice, "Clinical potential of adult vascular progenitor cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 6, pp. 1080–1087, 2010.

[16] R. L. Saunders and D. A. Hammer, "Assembly of human umbilical vein endothelial cells on compliant hydrogels," *Cellular and Molecular Bioengineering*, vol. 3, no. 1, pp. 60–67, 2010.

[17] Y. Lu, Y. Gong, J. Lian et al., "Expansion of endothelial progenitor cells in high density dot culture of rat bone marrow cells," *PLoS One*, vol. 9, no. 9, article e107127, 2014.

[18] S. Balaji, A. King, T. M. Crombleholme, and S. G. Keswani, "The role of endothelial progenitor cells in postnatal vasculogenesis: implications for therapeutic neovascularization and wound healing," *Advances in Wound Care*, vol. 2, no. 6, pp. 283–295, 2013.

[19] M. C. Yoder, "Human endothelial progenitor cells," *Cold Spring Harbor Perspectives in Medicine*, vol. 2, no. 7, 2012.

[20] L. E. Sidney, M. J. Branch, S. E. Dunphy, H. S. Dua, and A. Hopkinson, "Concise review: evidence for CD34 as a common marker for diverse progenitors," *Stem Cells*, vol. 32, no. 6, pp. 1380–1389, 2014.

[21] C. E. Muller-Sieburg, H. B. Sieburg, J. M. Bernitz, and G. Cattarossi, "Stem cell heterogeneity: implications for aging and regenerative medicine," *Blood*, vol. 119, no. 17, pp. 3900–3907, 2012.

[22] D. A. Ingram, N. M. Caplice, and M. C. Yoder, "Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells," *Blood*, vol. 106, no. 5, pp. 1525–1531, 2005.

[23] T. Imanishi, H. Tsujioka, and T. Akasaka, "Endothelial progenitor cells dysfunction and senescence: contribution to oxidative stress," *Current Cardiology Reviews*, vol. 4, no. 4, pp. 275–286, 2008.

[24] G. Krenning, P. Y. W. Dankers, J. W. Drouven et al., "Endothelial progenitor cell dysfunction in patients with progressive chronic kidney disease," *American Journal of Physiology-Renal Physiology*, vol. 296, no. 6, pp. F1314–F1322, 2009.

[25] F. Gao, S. M. Chiu, D. A. L. Motan et al., "Mesenchymal stem cells and immunomodulation: current status and future prospects," *Cell Death & Disease*, vol. 7, no. 1, article e2062, 2016.

[26] F. S. Collins and H. Varmus, "A new initiative on precision medicine," *The New England Journal of Medicine*, vol. 372, no. 9, pp. 793–795, 2015.

Hindawi
Stem Cells International
Volume 2018, Article ID 9652897, 15 pages
https://doi.org/10.1155/2018/9652897

Review Article

The Potentials and Caveats of Mesenchymal Stromal Cell-Based Therapies in the Preterm Infant

Judith Gronbach,[1,2] **Tayyab Shahzad**,[1,2] **Sarah Radajewski**,[1,2] **Cho-Ming Chao**,[1,2,3] **Saverio Bellusci**,[3] **Rory E. Morty**,[4] **Tobias Reicherzer**,[5] **and Harald Ehrhardt**[1,2]

[1]*Department of General Pediatrics and Neonatology, Justus-Liebig-University, Feulgenstrasse 12, 35392 Gießen, Germany*
[2]*German Center for Lung Research (DZL), Universities of Giessen and Marburg Lung Center (UGMLC), Feulgenstrasse 12, 35392 Gießen, Germany*
[3]*Excellence Cluster Cardio-Pulmonary System (ECCPS), German Center for Lung Research (DZL), Department of Internal Medicine II, Universities of Giessen and Marburg Lung Center (UGMLC), Aulweg 130, 35392 Giessen, Germany*
[4]*Department of Lung Development and Remodeling, Max Planck Institute for Heart and Lung Research, German Center Lung Research (DZL), 61231 Bad Nauheim, Germany*
[5]*Division of Neonatology, Perinatal Center, University Children's Hospital, Ludwig-Maximilians-University, Campus Grosshadern, Marchioninistr. 15, 81377 Munich, Germany*

Correspondence should be addressed to Harald Ehrhardt; harald.ehrhardt@paediat.med.uni-giessen.de

Received 9 August 2017; Accepted 4 March 2018; Published 8 April 2018

Academic Editor: Suk Ying TSANG

Preponderance of proinflammatory signals is a characteristic feature of all acute and resulting long-term morbidities of the preterm infant. The proinflammatory actions are best characterized for bronchopulmonary dysplasia (BPD) which is the chronic lung disease of the preterm infant with lifelong restrictions of pulmonary function and severe consequences for psychomotor development and quality of life. Besides BPD, the immature brain, eye, and gut are also exposed to inflammatory injuries provoked by infection, mechanical ventilation, and oxygen toxicity. Despite the tremendous progress in the understanding of disease pathologies, therapeutic interventions with proven efficiency remain restricted to a few drug therapies with restricted therapeutic benefit, partially considerable side effects, and missing option of applicability to the inflamed brain. The therapeutic potential of mesenchymal stromal cells (MSCs)—also known as mesenchymal stem cells—has attracted much attention during the recent years due to their anti-inflammatory activities and their secretion of growth and development-promoting factors. Based on a molecular understanding, this review summarizes the positive actions of exogenous umbilical cord-derived MSCs on the immature lung and brain and the therapeutic potential of reprogramming resident MSCs. The pathomechanistic understanding of MSC actions from the animal model is complemented by the promising results from the first phase I clinical trials testing allogenic MSC transplantation from umbilical cord blood. Despite all the enthusiasm towards this new therapeutic option, the caveats and outstanding issues have to be critically evaluated before a broad introduction of MSC-based therapies.

1. Introduction

Inflammatory diseases represent the biggest threat to the preterm infant. They affect all organs including the immature lung, brain, eye, and gastrointestinal tract with extensive and lifelong consequences for the patient's health. So far, efficient therapeutic interventions are restricted to a limited number of drugs and most pathomechanistic insights are available for the inflammatory damage to the immature lung. Therefore, this review is focused on the disease pathology of lung injury and on the therapeutic concepts to protect the immature lung from inflammatory damage. Exogenous mesenchymal stromal cells (MSCs) exert many positive effects on organ development and regeneration [1] and attenuation of all forms of inflammatory processes [2]. Resident MSCs can play an important role in fibrotic diseases including the

lung [3]. Therefore, MSC-based therapies have come into the focus of neonatologists. Here, we summarize the current evidence on resident MSCs and the therapeutic potential of exogenous MSCs to reduce the inflammatory damage to the preterm infant.

1.1. Epidemiology of Bronchopulmonary Dysplasia. Bronchopulmonary dysplasia is the chronic lung disease of the preterm infant which affects more than 60% with a gestational age < 28 weeks in the US and more than 30% of infants < 30 weeks in Europe [4, 5]. BPD is defined by the clinical criteria of dependency on oxygen or ventilator support at a corrected age of 36 weeks of gestation [6] with grading into "mild," "moderate," or "severe" forms [7]. But even infants not fulfilling these criteria display persisting limitations in lung function later in life. Cohort follow-up data substantiate the inability of lung catch-up growth and the persistence of alterations of pulmonary metabolism into adulthood [8]. The long lasting limitations probably lead to recurrent pulmonary sequelae in older age which resemble a COPD-like phenotype in the animal model [9]. Beyond the consequences for exercise capacity and life expectancy, pulmonary sequelae pose an important threat to the overall quality of life in former preterm infants with a close association between limitations in lung function and disorders of somatic growth and psychomotor development [10].

1.2. Distortion of Lung Development by Inflammation in the Preterm Infant. The pathogenesis of BPD is caused by the distortion of physiologic lung development in the critical period of the saccular stage. BPD constitutes a multifactorial disease which is caused by the interaction of a plenty of pre-, peri-, and postnatal factors. Being small for gestational age with intrauterine lung growth restrictions caused by placental insufficiency, smoke-induced injury, or diseases emerging during pregnancy, genetic predisposition and the immaturity of the lung with its insufficiency of anti-inflammatory surfactant production represent important prenatal conditions. Pre- and postnatal infections, mechanical ventilation, and oxygen supply are the central triggers of disease pathology with an overwhelming inflammatory reaction in the immature lung which induces or further aggravates lung injury [11, 12].

Characteristic features of lung damage initiation are the overweight of classical proinflammatory cytokines including IL-1β, IL-6, IL-8, and TNF-α and the absence or downregulation of anti-inflammatory cytokines including IL-4, IL-10, and IL-13 and of lung growth factors including growth factors such as FGFs, VEGFA, and PDGF-A which are required for further physiologic development of the epithelial, mesenchymal, and endothelial compartments of the lung [13]. The resulting leaks in the epithelial barrier promote the influx of inflammatory macrophages and neutrophils which further aggravate lung damage by the release of proinflammatory cytokines and monocyte chemoattractant proteins MCP-1, MCP-2, and MCP-3 and macrophage inflammatory proteins MIP-1α, MIP-1β, and TGF-β1 that augment and sustain the inflammatory reaction [14]. The inflammatory overweight turns the signaling pathway equilibrium into a self-perpetuating disbalance and exaggerated NFκB signaling which boosts inflammation and inflammatory cell survival and aggravates the distortion of the central pathways of lung development. PDGF receptor α, bone morphogenetic protein, fibroblast growth factors, PDGFR-α, Wnt/β-catenin, retinoic acid, VEGFA, and HIF signaling constitute critical pathways affected [15, 16]. Early alterations observed include the inactivation of surfactant proteins and the destruction of organ physiology by cell metaplasia and cell death induction [17]. The characteristic features described are not restricted to the epithelial barrier and alveolar development. Their impact on the mesenchymal and vascular compartment drives the remodeling of the extracellular matrix and the disturbance of vascular development [18]. Further vascular development is disturbed by the inflammatory overweight which suppresses HIF-1α, HIF-2α, and VEGFA activity [15]. In the mesenchyme, the inflammatory response dysregulates the central pathways of EGF receptor activity, TGF-β1-mediated TTG2, PLOD2, and FGF signaling, PDGFR-α-dependent VEGFA action, and myofibroblast and mesenchymal progenitor cell function [12, 16, 19–21]. Not surprisingly, dysregulation of myofibroblasts which originate from mesenchymal progenitors and are central drivers of lung development in the saccular stage represents a long-known hallmark of bronchopulmonary dysplasia [22]. Histopathology reveals a diffuse scattering of ACTA-2-positive myofibroblasts in the mesenchyme, and pathologic function is reflected by disturbed growth factor and extracellular matrix component secretion and fibroproliferation [23–26]. Of importance, derangement of any of the three compartments of the epithelium, mesenchyme, and endothelium leads to disruption of lung development [27] and impacts on the others. The reciprocal interplay between the different pathways and the negative consequences of both inhibition or overstimulation of the identical pathway as observed recently for TGF-β1 [28, 29], PDGFR-α, and NFκB add further levels of complexity but can explain why most promising preclinical therapies failed to prove superiority in the clinical setting [12, 28–33] (Figure 1).

Taken together, the critical hallmark of BPD is the dysregulation of the complex signaling network regulating lung development. Either the activation or inhibition of any central pathway involved in inflammation and/or lung growth results in severe derangement of pulmonary development. Similar pathomechanistic concepts are available for the inflammatory diseases of the cardiovascular system, immature brain, eye, and gut with overlapping and divergent central signaling pathways which have been reviewed in detail [34–39].

1.3. Limited Therapeutic Options to Prevent Lung Damage. Despite the progress in clinical care during the last decades, the overall incidence of morbidities of the preterm infant was not reduced due to the steadily increasing survival rates and the successful therapy of more and more immature and small infants [40–42]. Based on the pathomechanistic understanding, far more than 50 different approaches to prevent inflammatory injury or to promote organ development have been tested in clinical trials [43, 44]. In addition,

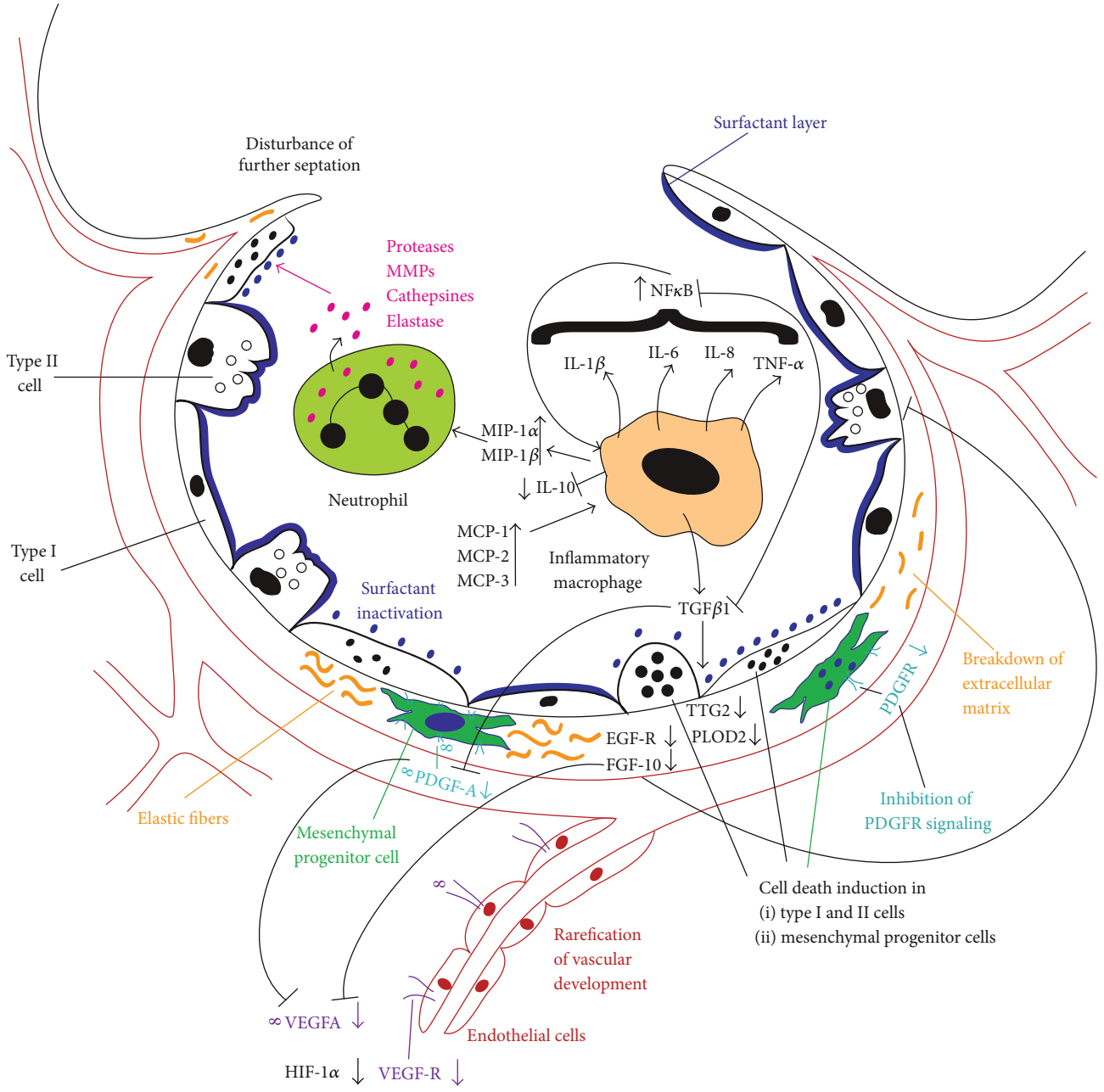


Figure 1: Pathogenesis of BPD: BPD is the consequence of distortion of alveolar, mesenchymal, and vascular development mainly in the saccular stage. Central to the disease is the inflammatory response which is characterized by a disbalance of cytokines and growth factors, the influx of inflammatory cells, cell death induction, and surfactant inactivation. Distortion of mesenchymal progenitor cell function represents a key event in disease pathogenesis.

no catch-up growth can be attained later in life [45]. For BPD, special focus has been drawn to the modulation of the pulmonary inflammatory response. Currently, the therapeutic options are restricted to the anti-inflammatory action of corticosteroids and surfactant and the promotion of retinoic acid signaling which are centered during the initial phase of clinical therapy. The efficacy of corticosteroids can be explained by the high potency and the impact on several central pathways of inflammation. Due to the severe side effects on psychomotor development, somatic growth, and endocrinological homeostasis, this therapeutic option is actually restricted to rescue therapy in case of pulmonary failure [46].

Therefore, new therapeutic options based on a molecular understanding are urgently needed. During the recent years, MSCs have gained great interest due to their broad anti-inflammatory and development and repair-promoting properties [47–49]. Therefore, we will first summarize physiologic progenitor cell functions in the preterm infant with special focus on the lung.

2. Progenitor Cells in Development and Disease

Progenitor cells play a central role in development and disease. The lung originates from the endoderm and the mesoderm. While the endoderm gives rise to the epithelium, the mesoderm develops into the mesenchyme and endothelium. Lung development is separated into four stages. The pseudoglandular stage with the formation of the first epithelial buds in week 5–16 is followed by the canalicular stage where narrowing of the interstitium takes place. At the border of viability between 22 and 24 weeks of gestation, saccular stage of lung development starts which is a very critical and vulnerable period of lung development. Proper lung development and septation in the saccular stage highly depend on the interaction between the epithelium, the mesenchyme, and the endothelium and the proper localization of mesenchymal progenitors [50]. Currently, we can just speculate on the impact of further cells like alveolar macrophages and the nervous system. Any noxious insult leads to disturbance of this highly orchestrated process. Epithelial and endothelial progenitor cells and MSCs play key roles in this critical period, and any disturbance of their physiologic function leads to disruption of lung development which was repeatedly demonstrated for MSCs and endothelial progenitor cells in vitro and in vivo [51–63].

MSCs are the source for several precursor cells in the immature lung including fibroblasts, lipofibroblasts, and myofibroblasts. They constitute central regulators of lung development in the saccular stage, and their multiple actions during lung development have been reviewed in detail [48, 64, 65]. A special role is ascribed to the production of the main components of the extracellular matrix including elastin and the secretion of the central drivers of lung development including FGF10 [66], Wnt, and PDGF-A [24]. The MSC population is not uniform but constitutes different subsets with cell-specific function in lung development [67–69]. Paracrine secretion by MSCs constitutes a central mechanism of action with impact on alveolar development and angiogenesis. Their antifibrotic and antiapoptotic properties are well accepted for a broad range of diseases [70, 71].

In contrast to terminally differentiated cells, progenitor cells from preterm infants are sensitive to noxious stimuli and more vulnerable than cells from term infants [59, 72] Oxidative stress and hyperoxic exposure of endothelial progenitor cells are associated with reduced numbers circulating in the blood probably due to increased susceptibility towards cell death and accelerated cellular senescence which both can serve as surrogate markers of endothelial dysfunction and distorted vascular development. Furthermore, hyperoxic exposure negatively impacts on paracrine cytokine secretion including VEGFA and NO synthesis [57–61, 63, 72–74]. Epigenetic gene silencing of SIRT-1 was identified to be the critical feature in endothelial progenitors leading to premature p16 stress-induced senescence. Vice versa, biochemical and molecular SIRT-1 rescue experiments provided strong evidence for its central role in prevention of premature senescence of endothelial progenitor cells and the promotion of vessel formation in vitro and in vivo [73]. Comparable insights are described for MSCs and epithelial progenitor cells [75–77]. Taking this into account, the consequences of reduction of progenitor cells following lung injury are well explained. They go along with an overweight of proinflammatory cytokines while protective growth factors and cytokines are deprived [74]. Genetic variants and antenatal factors further predispose to aggravated lung damage [11].

Similar observations are described for the immature brain where neuronal abnormalities and distorted white matter development resulting in hypomyelination are observed together with rarefication of progenitor cell populations. These cells, which are called preoligodendrocytes dominating the preterm brain, are responsible for myelin sheath formation but are extremely sensitive towards inflammatory and hyperoxic injury [78].

Therefore, therapeutic targeting of endogenous progenitor cells and application of exogenous pluripotent cells represent two intriguing strategies to prevent or repair inflammatory damage and to promote organ regrowth.

3. Distortion of Resident MSCs in the Pathogenesis of BPD

A decade ago, MSCs were first identified in the tracheal aspirate of preterm infants and were attributed an important role in the development of BPD [79]. MSC-specific characteristic were confirmed by the adherence to uncoated plastic, the expression of mesenchymal cell markers including CD73, CD90, and CD105, the absence of cell surface expression of CD34, CD45, CD11b, CD14, CD19, CD79α, and HLA-DR, and the potential of differentiation into adipocytes, osteoblasts, chondrocytes, and myofibroblasts [70, 80]. Overall, studies confirmed the pulmonary origin by cell surface marker expression and RNA profiling and demonstrated phenotype alterations not observed in MSCs derived from other organs and in MSCs from control infants [23]. Recent data proved the presence of MSCs in the tracheal aspirate of every preterm infant [81]. Despite the determination of the pulmonary origin by a lung-specific pattern, the precise origin of these cells from the proximal or distal sections in the lung is not verified [23]. One possible explanation for the appearance of these cells in the alveolar lumen is the promotion of migration by the proinflammatory cytokine overweight induced during lung injury [82]. Three specific changes in the pathway pattern were observed in MSCs from preterm infants later developing BPD. Expression of PDGF receptor α was reduced which is a characteristic feature of hypoalveolarization; TGF-β1 secretion and β-catenin signal transduction were increased which account for myofibroblast differentiation, lung fibrosis, and lung cell death induction. The results from primary MSCs were recapitulated under cell culture conditions when MSCs were exposed to hyperoxia and displayed reduced elastin production and differentiation into myofibroblasts [19, 83, 84]. MSCs obtained from the tracheal aspirates of preterm infants display a proinflammatory phenotype with higher secretion of proinflammatory cytokines including CXCL-1, IL-6, and IL-8 [23]. In a subsequent study, specific changes in MSC phenotype were ascribed a predictive value for the severity of BPD. The MSCs obtained

from preterm infants later developing severe BPD displayed more pronounced changes comprising a combined score of cell phenotype, protein expression, and signal transduction. The simultaneous increase in proliferation which was induced by increased NFκB activation was accompanied by the decrease of cellular α-SMA levels. The in vitro recapitulation of the inflammatory milieu confirmed the dependency of phenotype alteration on NFκB. The classical proinflammatory cytokines including IL-1β, IL-6, IL-8, or TNF-α induced the identical changes as observed in MSCs from preterm infants with severe BPD, and NFκB targeting reverted the proinflammatory phenotype [81]. These proof-of-principle data of phenotype reversibility argue for a special focus on pulmonary resident MSC characteristics during the evaluation of any new therapeutic intervention. Vice versa, augmentation of phenotype alteration or increased cell death induction will aggravate lung injury. In a recent animal study, the abrogation of TNF-α-mediated NFκB signaling was associated with increased apoptosis induction in PDGFR-α-positive mesenchymal cells mediated by predominant TGFβ1 signaling which explained the aggravated lung damage during mechanical ventilation with oxygen-rich gas [25]. These results are a further example for the complex interplay between the different signaling pathways and the need for a comprehensive evaluation [12, 31].

Taken together, data obtained from human observational studies and in vitro investigations clearly demonstrate that phenotype alterations of resident lung MSCs are associated with the development of BPD. We speculate that reprogramming of resident pulmonary MSCs represents a promising new therapeutic option. Next, we will focus on the therapeutic potential of exogenous MSCs.

4. Positive Effects of Exogenous MSCs on the Diseased Lung

During the recent years, a tremendous effort has been undertaken to investigate and optimize stem cell-based therapies in different acute and chronic diseases. Preclinical results mainly from the rodent model [74, 85–91] but also first clinical studies hint to a tremendous potential of stem cell-based therapies to alleviate or cure diseases of the preterm infant [92–94]. MSCs represent the most extensively studied cell population for therapeutic use, and MSCs from different tissues have been tested to prevent the damage to the immature lung or to alleviate the severity of lung damage. Despite the progress in animal studies, the precise molecular mechanisms how these therapies alleviate lung damage remain to be determined. The following chapter summarizes the preclinical evidence available to prevent or treat damage to the preterm lung by exogenous MSC application [71]. The main focus is drawn to give a comprehensive overview of the pathomechanistic understanding based on the studies in the different rodent models. Due to the limitations in space, no critical evaluation of differences in study quality and no separated consideration of MSCs from the umbilical cord blood or bone marrow are presented. We acknowledge the preclinical promising evidence of other cell-based therapies with cell products from the amniotic fluid, Wharton's Jelly, placenta and fetal membranes, and the work with endothelial progenitors and refer to actual comprehensive reviews [95, 96].

MSCs from the umbilical cord or bone marrow have attracted the greatest interest due to the easy accessibility, their multipotency, immune privilege, and low immunogenic potential, and their positive impact on the epithelial, mesenchymal, and vascular compartment of the lung. The first pioneering studies performed in rodents demonstrated beneficial effects of the application of bone marrow-derived exogenous MSCs on survival, lung morphology including alveolar septation, vessel density, and the functional parameters of pulmonary arterial pressure and lung function testing [74, 89, 91, 97, 98]. Beneficial effects were uniformly confirmed in all subsequent studies using the identical approach in the rodent model [85, 87, 88, 99–103]. Identified functions cover all property characteristics for MSC-based therapies and comprise plenty of lung protective mechanisms including lung development promoting anti-inflammatory, antioxidative, and antiapoptotic actions. These results increased survival, the promotion of alveolar and vascular growth, the proper composition of the extracellular matrix, and the inhibition of lung fibrosis [85, 90, 93, 99, 104, 105]. The identical positive effects were demonstrated for the important and frequent clinical situations of perinatal inflammatory exposure or intrauterine growth restriction followed by hyperoxic injury. Both settings revealed identical beneficial effects of MSC transplantation for all areas of interest [106, 107]. The positive effects of exogenous MSCs on lung architecture are not of transient nature, but follow-up examinations confirmed the long-term beneficial effects on inflammatory properties in the lung and lung morphometry. Thereby, the persistent long-term positive effects of both exogenous MSCs despite low engraftment and rapid fade away of the donor cells and of their conditioned media (100, 105) suggest that the paracrine protective effects induced by the transplanted stem cells might initially play a pivotal role in tissue repair (93, 100, 105). As the donor cells fade away, the intact host tissue that is protected by stem cell transplantation might be responsible for the sustained upregulation of various paracrine factors, thus the persistent beneficial effects.

On a molecular level, MSC therapy leads to a reduction of pulmonary inflammatory response to hyperoxic injury. MSCs have a positive impact on all characteristic proinflammatory features. The classical proinflammatory cytokine levels are reduced including IL-1β, IL-6, MIP-1α, and TNF-α, and the central growth factors and anti-inflammatory cytokines including VEGFA, HGF, and IL-10 are upregulated while TGF-β1 levels are decreased. This restoration of cytokine balance leads to an attenuated influx of inflammatory macrophages and neutrophils into the lung. Furthermore, the reduced pulmonary inflammatory response was accompanied by an increase in VEGFA and CTGF signaling and improved lung morphology [106]. MSC transplantation leads to the attenuation of indicators of oxidative stress, reduction of inhibitors of metalloproteinases, adhesion molecules like RANTES, L-selectin, and soluble intercellular

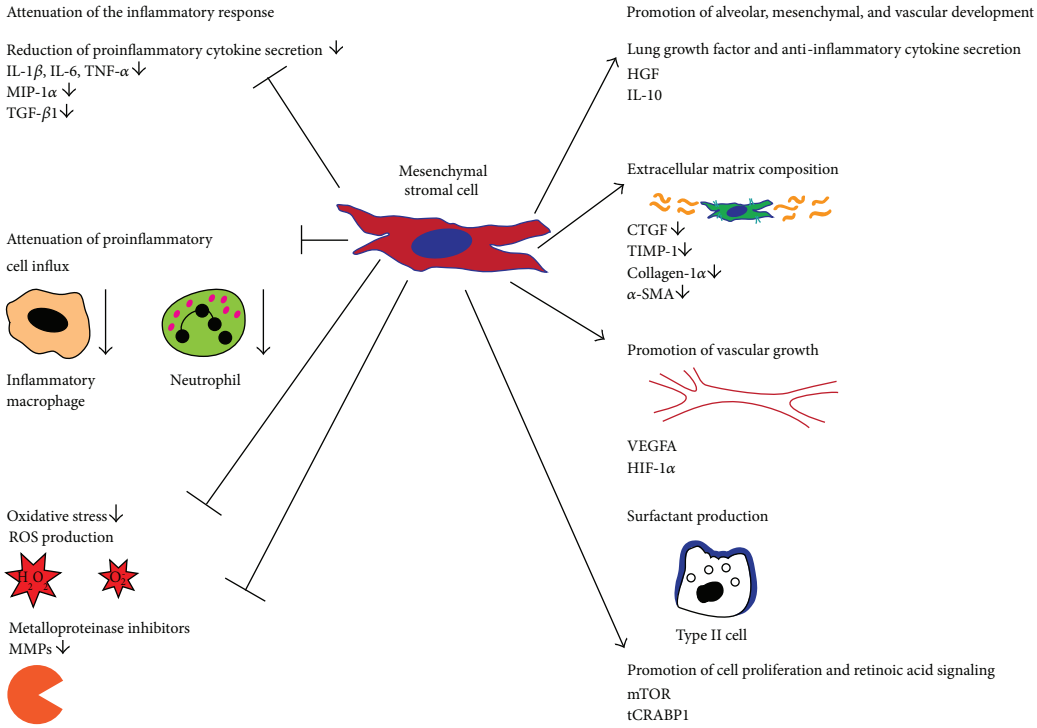


Figure 2: The therapeutic potential of allogenic mesenchymal stromal cells: allogenic MSC transplantation for prevention or therapy of lung injury has been studied in detail in the rodent model. The scheme summarizes all beneficial effects following allogenic MSC therapy with separation of the positive effects on inflammation and organ development.

adhesion molecule-1 [104], and the preservation of mTOR and tCRABP1 signaling which are key regulators of cell proliferation and retinoic acid pathway, respectively [107]. The last but not the least, application of exogenous MSCs preserves the production of surfactant proteins after hyperoxic injury [91]. The beneficial impact on lung growth with improvements in alveolar and vascular development is accompanied by reduced cell death induction. The reduced expression of genes involved in cell death including the gatekeeper of apoptosis p53 is accompanied by a diminished activation of effector caspases, fewer number of apoptotic cells, and attenuated interstitial lung fibrosis with a decrease in profibrotic genes like CTGF, TIMP-1, collagen-1α, α-SMA, and TGF-β1 [85, 86, 89, 102, 108] (Figure 2).

Besides the direct anti-inflammatory action, data from the bone marrow-derived MSC transplantation argue for a lung self-renewal effect as their application into the lung leads to an increase of bronchoalveolar stem cells. [101]. Experiments in the rodent provide evidence that MSC application contributed to the uprise of decreased growth factors like VEGFA and HGF by paracrine secretion [104, 109]. On a molecular level, RNA interference against VEGFA in exogenous MSCs before application to the injured lung prevented the described increase in VEGFA and concomitantly had a negative impact on all features of hyperoxic lung injury including influx of macrophages, proinflammatory cytokine release, cell death induction, and distortion of alveolar and vascular growth [109] (Figure 2).

The beneficial effects of MSCs are dose dependent, and the transplantation of low numbers of MSCs restricts the positive effects to some of the described MSC features. Different routes of administration have been compared, and intratracheal and intravenous application showed therapeutic efficacy which is in line with the data that stem cells from nonlung tissue can contribute to the attenuation of lung injury [90]. Evidence suggests that the probable dominant mode of action of MSCs is by paracrine effects. The results from animal trials on the other hand suggest also a direct cellular activity which cannot be substituted by cell-free supernatants or exosome preparations and includes mitochondrial transfer. The latter might be of high importance as pathogenesis of BPD is also ascribed to mitochondrial dysfunction [74, 100, 110, 111]. So far, detailed data on the differences between MSC cell-based therapies and application of cell-free extracts, isolated microparticles, or exosomes are missing despite the high enthusiasm about these advanced technologies [105]. Concerns have to be raised with respect to therapeutic equivalence as in the rat hyperoxic model, intratracheal application was superior to the intravenous route. Whether this discrepancy can be ascribed to the dose-response relationship remains unclear. The studies available suggest that the therapeutic benefit of MSC cell therapy is higher for alveolar morphology and lung fibrosis scores which was reproduced by two independent groups [100, 103, 108]. Ex vivo preconditioning might represent a possibility to improve therapeutic efficiency of cell-free extracts [88].

5. Further Areas of Research with Exogenous MSC Application

Besides the therapeutic potential of MSCs to prevent or reduce the severity of pulmonary sequelae, all other disease entities of the preterm infant with inflammatory properties and loss of cell function are potential targets for cell-based therapies. The current status of MSC-based therapies is summarized in Figure 3.

The central nervous system is the second organ most affected by injury to the preterm infant and therefore has attracted special attention during recent years. Data obtained in preclinical and clinical studies to date suggest a high therapeutic potential of MS-based therapies for cardiovascular diseases, gastrointestinal complications, and retinopathy of prematurity [112–114] which represent further disease entities with dominating inflammatory properties (reviewed in [95]). So far, most preclinical studies in the rodent model were performed for brain pathologies of the term newborn infant including hypoxic brain injury following perinatal asphyxia and neonatal stroke. Actual studies also evaluated the therapeutic potential of MSC therapy in periventricular leukomalacia and intraventricular hemorrhage [115–128]. For intraventricular hemorrhage, the contributions of inflammatory reaction to disease pathologies are well established and include brain damage by cell death induction of neurons and distortion of further myelination and gliosis [34, 129]. In analogy to the inflammatory damage to the immature lung, the selective therapeutic blockade of one inflammatory pathway leads only to a modest or no improvement in brain morphology. In addition, central regulatory pathways of organ damage differ partly from that involved in lung injury as observed for TGF-β1 signaling but can be common regulators as for macrophage attraction and proinflammatory cytokine production [129–135]. Therefore, the broader approach of targeting several inflammatory actions as achieved with MSC treatment is reasonable and proven efficient in the rodent model with respect to all relevant brain pathologies including astrogliosis, myelination, cell death induction, and prevention of posthemorrhagic hydrocephalus. Morphologic benefits were accompanied by a strong reduction in classical proinflammatory cytokines like IL-1α, IL-1β, IL-6, and TNF-α and improved performance in behavioral function tests [34, 122]. Brain-derived neurotrophic factor (BDNF) seems to play a pivotal role in the prevention of brain injury, as siRNA knockdown before MSC transplantation abrogated the beneficial effects of MSC transplantation on all morphologic and functional features in the rat model of intraventricular hemorrhage including inflammatory reaction, cell death induction, myelination, astrogliosis, attenuation of posthemorrhagic hydrocephalus, and behavioral test performance [34, 122, 136]. Comparable results were obtained for MSC therapy with cells overexpressing BDNF after hypoxic-ischemic insult and underline the dominant function of BDNF in the brain [137].

The intraventricular route tested in some animal models [120, 122] is restricted by the potential deleterious effects of direct intracerebral injection. Intravenous [138], intracardial [139], and intranasal [119, 125, 127] administration of MSCs proved therapeutic efficiency, and intravenous application revealed similar efficacy with respect to brain morphology parameters and functional read-outs compared to the intraventricular route despite a reduced MSC deposition in the brain tissue [123]. Adjustment of MSC numbers transplanted was a prerogative for comparable efficacy, and scientific data suggest a minimal effective dose for MSC transplantation. In contrast to the lung, repeated application proved superiority with positive effects on the inflammatory response and neuronal apoptosis, brain morphometry, and reactive astrogliosis [118, 119, 128, 140]. Optimal timing is much more critical as the inflammatory reaction following IVH is restricted to a short peak which restricts the therapeutic window [127, 136]. The limited time period can be explained by the cessation of MSC attraction to the site of injury after the short peak of cytokine secretion. In the model of periventricular white matter injury, bone marrow MSCs were attracted from the site of injection to the site of inflammatory injury. The lacking differentiation of these cells into neuronal cells suggests a paracrine neuroprotective effect [120, 124, 127, 141]. As the central nervous system represents a special compartment with reduced immunologic properties, special concerns have to be drawn to the persistent prevalence of MSCs in the brain which has not been studied for the IVH and periventricular white matter injury models so far. In the studies on hypoxic injury, emergence of neoplasia was not detected. [125].

Of importance, the results from the hypoxic ischemic brain injury model suggest that combination of MSC application with the currently practiced evidence-based therapy of therapeutic hypothermia proved superiority compared to each single strategy. The combined approach was the most efficient with respect to the reduction of proinflammatory cytokines, apoptotic cells in the brain, microgliosis, astrocytosis, and functional limitations [121]. These results encourage the pursuit of combinatorial treatment regimens not only in the brain but also in the lung together with therapies with proven efficacy to prevent BPD-like surfactant application.

6. MSC Application in Phase I Clinical Trials

A pioneering phase I study to reduce the severity of BPD performed in South Korea was published 3 years ago which demonstrated superiority of exogenous MSC application derived from the umbilical cord blood of healthy newborn infants [94]. MSCs were applied once between days 5 and 14 of life to $n = 9$ infants at high risk for BPD of 24 to 26 weeks of gestation requiring mechanical ventilation and showing deteriorating respiratory function. A significant reduction in BPD severity was observed compared to a control cohort of untreated infants. MSCs were applied in two slightly different dosages which do not allow any conclusion about the optimal number of cells. As expected from the animal experiments, the extent of proinflammatory cytokine response was reduced in the tracheal aspirates following MSC administration and no acute toxicities or side effects were observed until the age of approximately 3 months. The long-term follow-up of safety and efficiency is still pending. Recently, the follow-up at the age of 2 years was

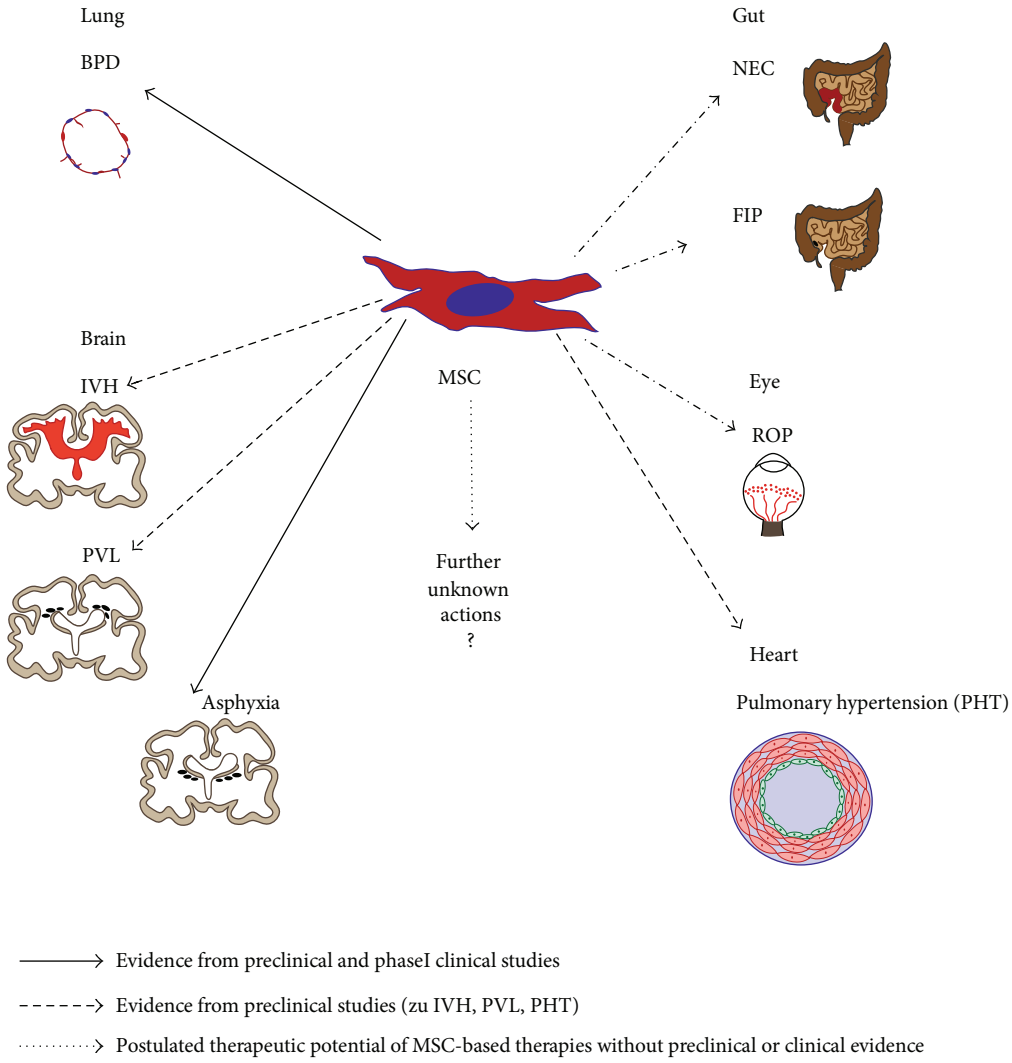


Figure 3: Therapeutic potential of MSC therapy in the preterm and newborn infant: beyond prevention or therapy of lung injury following preterm birth, MSC-based therapies have a therapeutic potential for all inflammatory diseases of the preterm and newborn infant. The scheme summarizes the current status of MSC-based therapies for the lung, brain, gut, eye, and cardiopulmonary system. The scientific knowledge is separated into evidence from phase I clinical trials and preclinical studies mainly in rodents and a presumed therapeutic potential.

published. Despite all the limitations due to the study size, promising data were added for home oxygen, rehospitalization due to pulmonary sequelae, somatic growth, and motor development [92].

In addition to the trials to prevent BPD, two studies on hypoxic ischemic brain encephalopathy and severe intraventricular hemorrhage were conducted during the recent years. The open-labeled study on hypoxic brain injury used autologous umbilical cord blood cells plus therapeutic hypothermia [142]. The evaluation of acute toxicities revealed no acute adverse reactions including cardiovascular compromise or nosocomial infection. The standardized follow-up at the age of 12 months revealed superiority of cell therapy with a combined reduction of neurodevelopmental compromise or death from 59% to 28%. The definite interpretation of the data is hampered by the incomplete follow-up. The enrollment within the IVH study is completed, and no acute toxicities were preliminarily described, but psychomotor follow-up is still awaiting publication [143].

The death of one infant in the MSC group due to sepsis at the age of 4 months in the BPD trial and two additional deaths due to viral infections in the intervention group in the MSC trial for hypoxic ischemic brain injury must be mentioned which occurred in severely handicapped infants [92, 142]. Due to the time period of several months between cell-based therapy and the onset of infection, a direct causal relationship is not probable, but infectious complications should be carefully monitored in the ongoing (follow-up) studies [144].

Taken together, the first phase I MSC trials render promising results and advocate the further evaluation of MSC therapy to reduce the deleterious effects of inflammatory injury to the preterm infant. As these lesions are not restricted to the lung and brain, actual and future studies

should carefully consider all other organs prone to inflammatory injury including the cardiovascular system, the gut, and the eye (Figure 3).

7. Current Therapeutic Obstacles to MSC Therapy

Despite the tremendous progress in the pathomechanistic understanding of the therapeutic potential of MSC therapy from the rodent models to overcome the deleterious effects of inflammatory diseases in the preterm infant, the broad application of exogenous MSCs to the preterm infant is still hampered by a huge gap of knowledge. Central obstacles include the following: (1) the selection of the most effective cell preparation fulfilling quality criteria which yet have to be defined, (2) the optimal number, time point, and route of MSC cell administration, and (3) the need for validation of clinical criteria to predict the infant at risk benefiting from such a therapy. At present, MSCs represent the most promising candidates for prevention or treatment of BPD and other inflammatory diseases of the preterm infant as MSCs proved therapeutic efficacy in several animal models mimicking BPD, intraventricular hemorrhage, or periventricular white matter injury. In contrast, data on alveolar and endothelial progenitor cells and on epithelial cells from the amniotic fluid [145] display considerable gaps in knowledge with respect to therapeutic efficiency and cell product preparation. Furthermore, experience with the therapeutic potential of endothelial progenitors is restricted to rodent models and might be limited to paracrine effects which can solely rescue vascular outgrowth while epithelial progenitors from the amniotic cavity mainly limit inflammatory processes. MSCs from umbilical cord blood from donor newborn infants can be prepared in sufficient quantity but harbor the risks of allogenic transplantation although the first phase I clinical trials did not show any short-term side effects [94, 142, 146]. Isolation and expansion techniques are still not standardized harboring the risk of variable quality of MSC cell preparations. Currently, MSC differentiation during expansion and related loss of function cannot be monitored by quality control assays, but expansion in cell culture using xeno-free media represents a promising research direction [147]. Furthermore, expansion of autologous MSCs should be taken into the conceptual consideration to overcome the risks and ethical concerns of allogenic transplantation. The application of cell-free supernatants represents an alternative approach to bypass the concerns of allogenic transplantation, but results from animal experiments suggest that the therapeutic efficiency of MSC therapy is mediated by paracrine effects and direct cell interaction at least for BPD [93, 105, 148]. Therefore, MSCs from the umbilical cord blood constitute currently the therapeutic option closest to clinical use. The beneficial effects of comparatively high dosages of MSCs in the rodent model were reproduced in the single phase I clinical trial in infants, but the optimal time point for administration and MSC number remains to be determined. Data from the rodent model suggest that the early preventive application is superior to the late administration to the injured lung and brain. For BPD, actual data do not allow the conclusion that repeated applications are superior to a single dose while repetitive treatments showed superiority for prevention of the deleterious effects of intraventricular hemorrhage [74, 104, 108, 118, 119, 123, 125, 127, 128, 140]. As the majority of preterm infants can be stabilized on noninvasive ventilator support with or without the additional application of surfactant during spontaneous breathing and are generally ventilated much shorter than during the past decades, the intratracheal route of application is restricted to the most severely affected preterms. Uniform distribution of MSCs as ensured for surfactant must be established for any noninvasive application procedure. The intravenous application constitutes an appealing alternative in the preterm infant but has not been studied at all and requires thorough preclinical evaluation. Whether the superiority of intratracheal application of MSCs in the rat model can be attributed to discrepancies in cell numbers delivered to the site of injury remains to be determined. The observed differences in therapeutic efficacy raise concerns about the therapeutic efficiency of the intravenous route [108]. Finally, the inflammatory injuries to the preterm infant result from complex pathophysiologies and resulting disorders currently cannot be predicted based on clinical parameters at birth. While risk calculators can narrow the population at risk, reliable biomarkers are urgently needed for reliable patient selection and monitoring of therapeutic efficiency. The trustworthy selection of infants at high risk is a prerogative for any further therapeutic evaluation of allogenic MSCs in larger scale controlled studies of MSC-based therapies at this stage.

Experimental data on exogenous and resident pulmonary MSCs suggest that both the application of exogenous physiologic cell preparation and the reprogramming of resident pulmonary MSCs represent highly promising therapeutic interventions. While the therapeutic potential of reprogramming resident pulmonary MSCs is still at the very beginning and limited to in vitro investigations, the application of exogenous MSCs requires thorough studies addressing the concerns raised above. One often ignored argument against exogenous MSC preparation is that these cells do not display all properties inherent to resident pulmonary MSCs and that safety issues must be taken into account concerning potential long-term side effects which require decades of research before definitive answers can be given for the preterm situation. It is clearly established that exogenous MSCs do not possess the capability to produce lung growth factors and cytokines and lung-specific proteins including that of the extracellular matrix and do not have the characteristic profile of upregulated transcriptions factors specific for the lung [23, 149, 150]. Special consideration has to be given to the inherent immune system as MSCs can impact on immune cell proliferation and function including dendritic cells, NK cells, and T and B lymphocytes [70, 71, 151]. Therefore, the reports on unexplained deaths due to severe infections as well as the potential risk of malignant transformation in the immune compromised preterm infant have to be evaluated carefully and with respect to the long-term outcome although current data suggest that MSCs in contrast to embryonic stem cells do not display, that is, teratogenic potential even during compromise of the immune system

[92, 142, 144, 152]. Finally, it is currently impossible to predict whether genomic instability during MSC expansion is responsible for the altered behavior in vitro during passaging and whether cell propagation gives rise to subpopulations with heterogeneous properties. To overcome these obstacles, MSC-derived supernatants or exosomes represent intriguing alternatives with promising results in first animal trials [153] but as long as it cannot be excluded that cellular MSC components like mitochondria contribute to the protection from inflammatory injury—especially since MSC-derived exosomes showed mitochondria-like aerobic metabolism in one study [154]—a lot of preclinical research activity is needed before safe introduction into clinical trials.

Taken together, MSC-based therapies represent a highly promising research field to ameliorate or overcome the deleterious effects of overwhelming inflammation in the preterm infant. Despite the tremendous progress in pathomechanistic understanding obtained from animal trials during the recent years and the first promising phase I clinical trials, a lot of research effort is needed to bring the optimized MSC product into the upcoming clinical trials. As we have learned from the multicenter studies from the past, the look at the long-term aspects is of central importance for the overall and sustained success of stem cell-based therapies in the preterm infant.

Disclosure

Judith Gronbach and Tayyab Shahzad share first authorship.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Judith Gronbach, Tayyab Shahzad, and Harald Ehrhardt drafted the manuscript and designed the figures. Tobias Reicherzer provided valuable intellectual input. Sarah Radajewski, Cho-Ming Chao, Saverio Bellusci, Rory E. Morty, and Tobias Reicherzer edited and revised the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgments

The authors highly appreciate the realization of figure layout by CE Marketing & PR Services, Germany. This work was supported by research grants from the University Medical Center Gießen and Marburg (UKGM 7-2013GI) and Von-Behring-Röntgen-Stiftung (63-0009 and COST BM1201).

References

[1] L. da Silva Meirelles, P. C. Chagastelles, and N. B. Nardi, "Mesenchymal stem cells reside in virtually all post-natal organs and tissues," *Journal of Cell Science*, vol. 119, no. 11, pp. 2204–2213, 2006.

[2] S. Aggarwal and M. F. Pittenger, "Human mesenchymal stem cells modulate allogeneic immune cell responses," *Blood*, vol. 105, no. 4, pp. 1815–1822, 2005.

[3] E. El Agha, R. Kramann, R. K. Schneider et al., "Mesenchymal stem cells in fibrotic disease," *Cell Stem Cell*, vol. 21, no. 2, pp. 166–177, 2017.

[4] E. Bancalari and A. Gonzalez, "Clinical course and lung function abnormalities during development of neonatal chronic lung disease," in *Chronic Lung Disease in Early Infancy*, R. D. Bland and J. J. Coalson, Eds., pp. 41–64, Marcel Dekker, New York City, NY, USA, 2000.

[5] L. Gortner, B. Misselwitz, D. Milligan et al., "Rates of bronchopulmonary dysplasia in very preterm neonates in Europe: results from the MOSAIC cohort," *Neonatology*, vol. 99, no. 2, pp. 112–117, 2011.

[6] A. H. Jobe and E. Bancalari, "Bronchopulmonary dysplasia," *American Journal of Respiratory and Critical Care Medicine*, vol. 163, no. 7, pp. 1723–1729, 2001.

[7] R. A. Ehrenkranz, M. C. Walsh, B. R. Vohr et al., "Validation of the National Institutes of Health consensus definition of bronchopulmonary dysplasia," *Pediatrics*, vol. 116, no. 6, pp. 1353–1360, 2005.

[8] L. W. Doyle, B. Faber, C. Callanan, N. Freezer, G. W. Ford, and N. M. Davis, "Bronchopulmonary dysplasia in very low birth weight subjects and lung function in late adolescence," *Pediatrics*, vol. 118, no. 1, pp. 108–113, 2006.

[9] L. Didon, A. B. Roos, G. P. Elmberger, F. J. Gonzalez, and M. Nord, "Lung-specific inactivation of CCAAT/enhancer binding protein α causes a pathological pattern characteristic of COPD," *European Respiratory Journal*, vol. 35, no. 1, pp. 186–197, 2010.

[10] N. Wood, K. Costeloe, A. T. Gibson et al., "The EPICure study: associations and antecedents of neurological and developmental disability at 30 months of age following extremely preterm birth," *Archives of Disease in Childhood-Fetal and Neonatal Edition*, vol. 90, no. 2, pp. F134–F140, 2005.

[11] P. M. Lavoie, C. Pham, and K. L. Jang, "Heritability of bronchopulmonary dysplasia, defined according to the consensus statement of the National Institutes of Health," *Pediatrics*, vol. 122, no. 3, pp. 479–485, 2008.

[12] T. Shahzad, S. Radajewski, C.-M. Chao, S. Bellusci, and H. Ehrhardt, "Pathogenesis of bronchopulmonary dysplasia: when inflammation meets organ development," *Molecular and Cellular Pediatrics*, vol. 3, no. 1, p. 23, 2016.

[13] V. Compernolle, K. Brusselmans, T. Acker et al., "Loss of HIF-2α and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice," *Nature Medicine*, vol. 8, no. 7, pp. 702–710, 2002.

[14] U. Weichelt, R. Cay, T. Schmitz et al., "Prevention of hyperoxia-mediated pulmonary inflammation in neonatal rats by caffeine," *European Respiratory Journal*, vol. 41, no. 4, pp. 966–973, 2013.

[15] A. Hilgendorff, I. Reiss, H. Ehrhardt, O. Eickelberg, and C. M. Alvira, "Chronic lung disease in the preterm infant. Lessons learned from animal models," *American Journal of Respiratory Cell and Molecular Biology*, vol. 50, no. 2, 2014.

[16] P. Oak, T. Pritzke, I. Thiel et al., "Attenuated PDGF signaling drives alveolar and microvascular defects in neonatal chronic lung disease," *EMBO Molecular Medicine*, vol. 9, no. 11, pp. 1504–1520, 2017.

[17] W. H. Northway Jr, R. C. Rosan, and D. Y. Porter, "Pulmonary disease following respirator therapy of hyaline-membrane disease — bronchopulmonary dysplasia," *The New England Journal of Medicine*, vol. 276, no. 7, pp. 357–368, 1967.

[18] K. R. Stenmark and S. H. Abman, "Lung vascular development: implications for the pathogenesis of bronchopulmonary dysplasia," *Annual Review of Physiology*, vol. 67, no. 1, pp. 623–661, 2005.

[19] A. P. Popova, J. Kelley Bentley, A. C. Anyanwu et al., "Glycogen synthase kinase-3β/β-catenin signaling regulates neonatal lung mesenchymal stromal cell myofibroblastic differentiation," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 303, no. 5, pp. L439–L448, 2012.

[20] T. J. Witsch, G. Niess, E. Sakkas et al., "Transglutaminase 2: a new player in bronchopulmonary dysplasia?," *European Respiratory Journal*, vol. 44, no. 1, pp. 109–121, 2014.

[21] T. J. Witsch, P. Turowski, E. Sakkas et al., "Deregulation of the lysyl hydroxylase matrix cross-linking system in experimental and clinical bronchopulmonary dysplasia," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 306, no. 3, pp. L246–L259, 2014.

[22] K. H. Albertine, G. P. Jones, B. C. STarcher et al., "Chronic lung injury in preterm lambs: disordered respiratory tract development," *American Journal of Respiratory and Critical Care Medicine*, vol. 159, no. 3, pp. 945–958, 1999.

[23] P. D. Bozyk, A. P. Popova, J. K. Bentley et al., "Mesenchymal stromal cells from neonatal tracheal aspirates demonstrate a pattern of lung-specific gene expression," *Stem Cells and Development*, vol. 20, no. 11, pp. 1995–2007, 2011.

[24] H. Boström, K. Willetts, M. Pekny et al., "PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis," *Cell*, vol. 85, no. 6, pp. 863–873, 1996.

[25] H. Ehrhardt, T. Pritzke, P. Oak et al., "Absence of TNF-α enhances inflammatory response in the newborn lung undergoing mechanical ventilation," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 310, no. 10, pp. L909–L918, 2016.

[26] S. K. Ahlfeld and S. J. Conway, "Aberrant signaling pathways of the lung mesenchyme and their contributions to the pathogenesis of bronchopulmonary dysplasia," *Birth Defects Research Part A: Clinical and Molecular Teratology*, vol. 94, no. 1, pp. 3–15, 2012.

[27] C.-M. Chao, E. El Agha, C. Tiozzo, P. Minoo, and S. Bellusci, "A breath of fresh air on the mesenchyme: impact of impaired mesenchymal development on the pathogenesis of bronchopulmonary dysplasia," *Frontiers in Medicine*, vol. 2, 2015.

[28] J. Gauldie, T. Galt, P. Bonniaud, C. Robbins, M. Kelly, and D. Warburton, "Transfer of the active form of transforming growth factor-β1 gene to newborn rat lung induces changes consistent with bronchopulmonary dysplasia," *The American Journal of Pathology*, vol. 163, no. 6, pp. 2575–2584, 2003.

[29] P. Bonniaud, M. Kolb, T. Galt et al., "Smad3 null mice develop airspace enlargement and are resistant to TGF-β-mediated pulmonary fibrosis," *The Journal of Immunology*, vol. 173, no. 3, pp. 2099–2108, 2004.

[30] T. S. Blackwell, A. N. Hipps, Y. Yamamoto et al., "NF-κB signaling in fetal lung macrophages disrupts airway morphogenesis," *The Journal of Immunology*, vol. 187, no. 5, pp. 2740–2747, 2011.

[31] C. Iosef, T.-P. Alastalo, Y. Hou et al., "Inhibiting NF-κB in the developing lung disrupts angiogenesis and alveolarization," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 302, no. 10, pp. L1023–L1036, 2012.

[32] S. McKenna, K. A. Michaelis, F. Agboke et al., "Sustained hyperoxia-induced NF-κB activation improves survival and preserves lung development in neonatal mice," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 306, no. 12, pp. L1078–L1089, 2014.

[33] Y. Hou, M. Liu, C. Husted et al., "Activation of the nuclear factor-κB pathway during postnatal lung inflammation preserves alveolarization by suppressing macrophage inflammatory protein-2," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 309, no. 6, pp. L593–L604, 2015.

[34] S. Y. Ahn, Y. S. Chang, and W. S. Park, "Mesenchymal stem cells transplantation for neuroprotection in preterm infants with severe intraventricular hemorrhage," *Korean journal of pediatrics*, vol. 57, no. 6, pp. 251–256, 2014.

[35] M. E. Hartnett and J. S. Penn, "Mechanisms and management of retinopathy of prematurity," *The New England Journal of Medicine*, vol. 367, no. 26, pp. 2515–2526, 2012.

[36] P. C. Ng, "Biomarkers of necrotising enterocolitis," *Seminars in Fetal & Neonatal Medicine*, vol. 19, no. 1, pp. 33–38, 2014.

[37] Z. Hodzic, A. M. Bolock, and M. Good, "The role of mucosal immunity in the pathogenesis of necrotizing enterocolitis," *Frontiers in Pediatrics*, vol. 5, p. 40, 2017.

[38] A. M. Nuyt, J.-C. Lavoie, I. Mohamed, K. Paquette, and T. M. Luu, "Adult consequences of extremely preterm birth: cardiovascular and metabolic diseases risk factors, mechanisms, and prevention avenues," *Clinics in Perinatology*, vol. 44, no. 2, pp. 315–332, 2017.

[39] M. Tare, J. G. Bensley, T. J. M. Moss et al., "Exposure to intrauterine inflammation leads to impaired function and altered structure in the preterm heart of fetal sheep," *Clinical Science*, vol. 127, no. 9, pp. 559–569, 2014.

[40] B. J. Stoll, N. I. Hansen, E. F. Bell et al., "Neonatal outcomes of extremely preterm infants from the NICHD neonatal research network," *Pediatrics*, vol. 126, no. 3, pp. 443–456, 2010.

[41] G. R. Alexander and M. Slay, "Prematurity at birth: trends, racial disparities, and epidemiology," *Developmental Disabilities Research Reviews*, vol. 8, no. 4, pp. 215–220, 2002.

[42] B. J. Stoll, N. I. Hansen, E. F. Bell et al., "Trends in care practices, morbidity, and mortality of extremely preterm neonates, 1993-2012," *JAMA*, vol. 314, no. 10, pp. 1039–1051, 2015.

[43] C. T. McEvoy, L. Jain, B. Schmidt, S. Abman, E. Bancalari, and J. L. Aschner, "Bronchopulmonary dysplasia: NHLBI workshop on the primary prevention of chronic lung diseases," *Annals of the American Thoracic Society*, vol. 11, Supplement 3, pp. S146–S153, 2014.

[44] K. S. Beam, S. Aliaga, S. K. Ahlfeld, M. Cohen-Wolkowiez, P. B. Smith, and M. M. Laughon, "A systematic review of randomized controlled trials for the prevention of bronchopulmonary dysplasia in infants," *Journal of perinatology*, vol. 34, no. 9, pp. 705–710, 2014.

[45] M. Sanchez-Solis, V. Perez-Fernandez, V. Bosch-Gimenez, J. J. Quesada, and L. Garcia-Marcos, "Lung function gain in preterm infants with and without bronchopulmonary dysplasia," *Pediatric Pulmonology*, vol. 51, no. 9, pp. 936–942, 2016.

[46] K. J. Barrington, "The adverse neuro-developmental effects of postnatal steroids in the preterm infant: a systematic review of RCTs," *BMC Pediatrics*, vol. 1, no. 1, p. 1, 2001.

[47] F. Gao, S. M. Chiu, D. A. L. Motan et al., "Mesenchymal stem cells and immunomodulation: current status and future prospects," *Cell Death & Disease*, vol. 7, no. 1, article e2062, 2016.

[48] J. J. Collins and B. Thébaud, "Lung mesenchymal stromal cells in development and disease: to serve and protect?," *Antioxidants & redox signaling*, vol. 21, no. 13, pp. 1849–1862, 2014.

[49] R. Rohban and T. R. Pieber, "Mesenchymal stem and progenitor cells in regeneration: tissue specificity and regenerative potential," *Stem Cells International*, vol. 2017, Article ID 5173732, 16 pages, 2017.

[50] M. E. Kumar, P. E. Bogard, F. Hernán Espinoza, D. B. Menke, D. M. Kingsley, and M. A. Krasnow, "Defining a mesenchymal progenitor niche at single-cell resolution," *Science*, vol. 346, no. 6211, article 1258810, 2014.

[51] K. U. Hong, S. D. Reynolds, S. Watkins, E. Fuchs, and B. R. Stripp, "In vivo differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 286, no. 4, pp. L643–L649, 2004.

[52] K. U. Hong, S. D. Reynolds, S. Watkins, E. Fuchs, and B. R. Stripp, "Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium," *The American Journal of Pathology*, vol. 164, no. 2, pp. 577–588, 2004.

[53] A. Giangreco, H. Shen, S. D. Reynolds, and B. R. Stripp, "Molecular phenotype of airway side population cells," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 286, no. 4, pp. L624–L630, 2004.

[54] R. Reddy, S. Buckley, M. Doerken et al., "Isolation of a putative progenitor subpopulation of alveolar epithelial type 2 cells," *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 286, no. 4, pp. L658–L667, 2004.

[55] J. Kajstura, M. Rota, S. R. Hall et al., "Evidence for human lung stem cells," *The New England Journal of Medicine*, vol. 364, no. 19, pp. 1795–1806, 2011.

[56] D. Irwin, K. Helm, N. Campbell et al., "Neonatal lung side population cells demonstrate endothelial potential and are altered in response to hyperoxia-induced lung simplification," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 293, no. 4, pp. L941–L951, 2007.

[57] V. Balasubramaniam, C. F. Mervis, A. M. Maxey, N. E. Markham, and S. H. Abman, "Hyperoxia reduces bone marrow, circulating, and lung endothelial progenitor cells in the developing lung: implications for the pathogenesis of bronchopulmonary dysplasia," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 292, no. 5, pp. L1073–L1084, 2007.

[58] C. D. Baker, V. Balasubramaniam, P. M. Mourani et al., "Cord blood angiogenic progenitor cells are decreased in bronchopulmonary dysplasia," *European Respiratory Journal*, vol. 40, no. 6, pp. 1516–1522, 2012.

[59] C. D. Baker, S. L. Ryan, D. A. Ingram, G. J. Seedorf, S. H. Abman, and V. Balasubramaniam, "Endothelial colony-forming cells from preterm infants are increased and more susceptible to hyperoxia," *American Journal of Respiratory and Critical Care Medicine*, vol. 180, no. 5, pp. 454–461, 2009.

[60] A. Borghesi, M. Massa, R. Campanelli et al., "Circulating endothelial progenitor cells in preterm infants with bronchopulmonary dysplasia," *American Journal of Respiratory and Critical Care Medicine*, vol. 180, no. 6, pp. 540–546, 2009.

[61] C. D. Baker, G. J. Seedorf, B. L. Wisniewski et al., "Endothelial colony-forming cell conditioned media promote angiogenesis in vitro and prevent pulmonary hypertension in experimental bronchopulmonary dysplasia," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 305, no. 1, pp. L73–L81, 2013.

[62] V. Balasubramaniam, S. L. Ryan, G. J. Seedorf et al., "Bone marrow-derived angiogenic cells restore lung alveolar and vascular structure after neonatal hyperoxia in infant mice," *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 298, no. 3, pp. L315–L323, 2010.

[63] R. S. Alphonse, A. Vadivel, M. Fung et al., "Existence, functional impairment, and lung repair potential of endothelial colony-forming cells in oxygen-induced arrested alveolar growth," *Circulation*, vol. 129, no. 21, pp. 2144–2157, 2014.

[64] M. A. Möbius and M. Rüdiger, "Mesenchymal stromal cells in the development and therapy of bronchopulmonary dysplasia," *Molecular and Cellular Pediatrics*, vol. 3, no. 1, p. 18, 2016.

[65] P. R. Amable, M. V. Teixeira, R. B. Carias, J. Granjeiro, and R. Borojevic, "Protein synthesis and secretion in human mesenchymal cells derived from bone marrow, adipose tissue and Wharton's jelly," *Stem Cell Research & Therapy*, vol. 5, no. 2, p. 53, 2014.

[66] C. M. Chao, F. Yahya, A. Moiseenko et al., "*Fgf10* deficiency is causative for lethality in a mouse model of bronchopulmonary dysplasia," *The Journal of Pathology*, vol. 241, no. 1, pp. 91–103, 2017.

[67] A. Moiseenko, V. Kheirollahi, C. M. Chao et al., "Origin and characterization of alpha smooth muscle actin-positive cells during murine lung development," *Stem Cells*, vol. 35, no. 6, pp. 1566–1578, 2017.

[68] D. M. Silva, C. Nardiello, A. Pozarska, and R. E. Morty, "Recent advances in the mechanisms of lung alveolarization and the pathogenesis of bronchopulmonary dysplasia," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 309, no. 11, pp. L1239–L1272, 2015.

[69] J. Ruiz-Camp and R. E. Morty, "Divergent fibroblast growth factor signaling pathways in lung fibroblast subsets: where do we go from here?," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 309, no. 8, pp. L751–L755, 2015.

[70] K. Le Blanc, "Mesenchymal stromal cells: tissue repair and immune modulation," *Cytotherapy*, vol. 8, no. 6, pp. 559–561, 2006.

[71] N. G. Singer and A. I. Caplan, "Mesenchymal stem cells: mechanisms of inflammation," *Annual Review of Pathology: Mechanisms of Disease*, vol. 6, no. 1, pp. 457–478, 2011.

[72] H. Fujinaga, C. D. Baker, S. L. Ryan et al., "Hyperoxia disrupts vascular endothelial growth factor-nitric oxide signaling and decreases growth of endothelial colony-forming cells from preterm infants," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 297, no. 6, pp. L1160–L1169, 2009.

[73] P. F. Vassallo, S. Simoncini, I. Ligi et al., "Accelerated senescence of cord blood endothelial progenitor cells in premature neonates is driven by SIRT1 decreased expression," *Blood*, vol. 123, no. 13, pp. 2116–2126, 2014.

[74] T. van Haaften, R. Byrne, S. Bonnet et al., "Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats," *American Journal of Respiratory and Critical Care Medicine*, vol. 180, no. 11, pp. 1131–1142, 2009.

[75] M. Shakibaei, P. Shayan, F. Busch et al., "Resveratrol mediated modulation of Sirt-1/Runx2 promotes osteogenic differentiation of mesenchymal stem cells: *potential role of Runx2 deacetylation*," *PLoS One*, vol. 7, no. 4, article e35712, 2012.

[76] H.-F. Yuan, C. Zhai, X. L. Yan et al., "SIRT1 is required for long-term growth of human mesenchymal stem cells," *Journal of Molecular Medicine*, vol. 90, no. 4, pp. 389–400, 2012.

[77] S. Minagawa, J. Araya, T. Numata et al., "Accelerated epithelial cell senescence in IPF and the inhibitory role of SIRT6 in TGF-β-induced senescence of human bronchial epithelial cells," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 300, no. 3, pp. L391–L401, 2011.

[78] O. Khwaja and J. Volpe, "Pathogenesis of cerebral white matter injury of prematurity," *Archives of Disease in Childhood-Fetal and Neonatal Edition*, vol. 93, no. 2, pp. F153–F161, 2008.

[79] K. T. Hennrick, A. G. Keeton, S. Nanua et al., "Lung cells from neonates show a mesenchymal stem cell phenotype," *American Journal of Respiratory and Critical Care Medicine*, vol. 175, no. 11, pp. 1158–1164, 2007.

[80] M. Dominici, K. le Blanc, I. Mueller et al., "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement," *Cytotherapy*, vol. 8, no. 4, pp. 315–317, 2006.

[81] T. Reicherzer, S. Häffner, T. Shahzad et al., "Pro-inflammatory NFκB activation alters the phenotype of tracheal aspirate MSC in preterm infants developing severe BPD," *AJP LUNG*, 2018.

[82] A. L. Ponte, E. Marais, N. Gallay et al., "The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities," *Stem Cells*, vol. 25, no. 7, pp. 1737–1745, 2007.

[83] A. P. Popova, P. D. Bozyk, A. M. Goldsmith et al., "Autocrine production of TGF-β1 promotes myofibroblastic differentiation of neonatal lung mesenchymal stem cells," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 298, no. 6, pp. L735–L743, 2010.

[84] A. P. Popova, J. K. Bentley, T. X. Cui et al., "Reduced platelet-derived growth factor receptor expression is a primary feature of human bronchopulmonary dysplasia," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 307, no. 3, pp. L231–L239, 2014.

[85] H. Zhang, J. Fang, H. Su et al., "Bone marrow mesenchymal stem cells attenuate lung inflammation of hyperoxic newborn rats," *Pediatric Transplantation*, vol. 16, no. 6, pp. 589–598, 2012.

[86] Y. Luan, W. Ding, Z.-Y. Ju, Z.-H. Zhang, X. Zhang, and F. Kong, "Bone marrow-derived mesenchymal stem cells protect against lung injury in a mouse model of bronchopulmonary dysplasia," *Molecular Medicine Reports*, vol. 11, no. 3, pp. 1945–1950, 2015.

[87] H. Zhang, J. Fang, Y. Wu, Y. Mai, W. Lai, and H. Su, "Mesenchymal stem cells protect against neonatal rat hyperoxic lung injury," *Expert Opinion on Biological Therapy*, vol. 13, no. 6, pp. 817–829, 2013.

[88] P. Waszak, R. Alphonse, A. Vadivel, L. Ionescu, F. Eaton, and B. Thébaud, "Preconditioning enhances the paracrine effect of mesenchymal stem cells in preventing oxygen-induced neonatal lung injury in rats," *Stem Cells and Development*, vol. 21, no. 15, pp. 2789–2797, 2012.

[89] M. Aslam, R. Baveja, O. D. Liang et al., "Bone marrow stromal cells attenuate lung injury in a murine model of neonatal chronic lung disease," *American Journal of Respiratory and Critical Care Medicine*, vol. 180, no. 11, pp. 1122–1130, 2009.

[90] Y. S. Chang, S. J. Choi, D. K. Sung et al., "Intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells dose-dependently attenuates hyperoxia-induced lung injury in neonatal rats," *Cell Transplantation*, vol. 20, no. 11-12, pp. 1843–1854, 2011.

[91] Y. S. Chang, W. Oh, S. J. Choi et al., "Human umbilical cord blood-derived mesenchymal stem cells attenuate hyperoxia-induced lung injury in neonatal rats," *Cell Transplantation*, vol. 18, no. 8, pp. 869–886, 2009.

[92] S. Y. Ahn, Y. S. Chang, J. H. Kim, S. I. Sung, and W. S. Park, "Two-year follow-up outcomes of premature infants enrolled in the phase I trial of mesenchymal stem cells transplantation for bronchopulmonary dysplasia," *The Journal of Pediatrics*, vol. 185, pp. 49–54.e2, 2017.

[93] S. Y. Ahn, Y. S. Chang, S. Y. Kim et al., "Long-term (postnatal day 70) outcome and safety of intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells in neonatal hyperoxic lung injury," *Yonsei Medical Journal*, vol. 54, no. 2, pp. 416–424, 2013.

[94] Y. S. Chang, S. Y. Ahn, H. S. Yoo et al., "Mesenchymal stem cells for bronchopulmonary dysplasia: phase 1 dose-escalation clinical trial," *The Journal of pediatrics*, vol. 164, no. 5, pp. 966–972.e6, 2014.

[95] D. Zhu, E. M. Wallace, and R. Lim, "Cell-based therapies for the preterm infant," *Cytotherapy*, vol. 16, no. 12, pp. 1614–1628, 2014.

[96] M. E. Fung and B. Thébaud, "Stem cell–based therapy for neonatal lung disease: it is in the juice," *Pediatric Research*, vol. 75, no. 1-1, pp. 2–7, 2014.

[97] Z. Tian, J. du, B. Wang, X. Y. Hong, and Z. C. Feng, "Intravenous infusion of rat bone marrow-derived mesenchymal stem cells ameliorates hyperoxia-induced lung injury in neonatal rats," *Nan fang yi ke da xue xue bao=Journal of Southern Medical University*, vol. 27, no. 11, pp. 1692–1695, 2007.

[98] Z. Tian, J. Du, X. M. Fu, B. Wang, X. Y. Hong, and Z. C. Feng, "Influence of human bone marrow-derived mesenchymal stem cells on the lung of newborn rats damaged by hyperoxia," *Zhonghua er ke za zhi= Chinese journal of pediatrics*, vol. 46, no. 1, pp. 4–8, 2008.

[99] G. Hansmann, A. Fernandez-Gonzalez, M. Aslam et al., "Mesenchymal stem cell-mediated reversal of bronchopulmonary dysplasia and associated pulmonary hypertension," *Pulmonary circulation*, vol. 2, no. 2, pp. 170–181, 2012.

[100] R. P. Sutsko, K. C. Young, A. Ribeiro et al., "Long-term reparative effects of mesenchymal stem cell therapy following neonatal hyperoxia-induced lung injury," *Pediatric Research*, vol. 73, no. 1, pp. 46–53, 2012.

[101] K. A. Tropea, E. Leder, M. Aslam et al., "Bronchioalveolar stem cells increase after mesenchymal stromal cell treatment in a mouse model of bronchopulmonary dysplasia," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 302, no. 9, pp. L829–L837, 2012.

[102] X. Zhang, H. Wang, Y. Shi et al., "Role of bone marrow-derived mesenchymal stem cells in the prevention of hyperoxia-induced lung injury in newborn mice," *Cell Biology International*, vol. 36, no. 6, pp. 589–594, 2012.

[103] S. Gülaşı, A. Atıcı, Ş. N. Yılmaz et al., "Mesenchymal stem cell treatment in hyperoxia-induced lung injury in newborn rats," *Pediatrics International*, vol. 58, no. 3, pp. 206–213, 2015.

[104] Y. S. Chang, S. J. Choi, S. Y. Ahn et al., "Timing of umbilical cord blood derived mesenchymal stem cells transplantation determines therapeutic efficacy in the neonatal hyperoxic lung injury," *PLoS One*, vol. 8, no. 1, article e52419, 2013.

[105] M. Pierro, L. Ionescu, T. Montemurro et al., "Short-term, long-term and paracrine effect of human umbilical cord-derived stem cells in lung injury prevention and repair in experimental bronchopulmonary dysplasia," *Thorax*, vol. 68, no. 5, pp. 475–484, 2013.

[106] H.-C. Chou, Y.-T. Li, and C.-M. Chen, "Human mesenchymal stem cells attenuate experimental bronchopulmonary dysplasia induced by perinatal inflammation and hyperoxia," *American Journal of Translational Research*, vol. 8, no. 2, pp. 342–353, 2016.

[107] D. Monz, E. Tutdibi, C. Mildau et al., "Human umbilical cord blood mononuclear cells in a double-hit model of bronchopulmonary dysplasia in neonatal mice," *PLoS One*, vol. 8, no. 9, article e74740, 2013.

[108] D. K. Sung, Y. S. Chang, S. Y. Ahn et al., "Optimal route for human umbilical cord blood-derived mesenchymal stem cell transplantation to protect against neonatal hyperoxic lung injury: gene expression profiles and histopathology," *PLoS One*, vol. 10, no. 8, article e0135574, 2015.

[109] Y. S. Chang, S. Y. Ahn, H. B. Jeon et al., "Critical role of vascular endothelial growth factor secreted by mesenchymal stem cells in hyperoxic lung injury," *American Journal of Respiratory Cell and Molecular Biology*, vol. 51, no. 3, pp. 391–399, 2014.

[110] R. L. Morton, D. Iklé, and C. W. White, "Loss of lung mitochondrial aconitase activity due to hyperoxia in bronchopulmonary dysplasia in primates," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 274, no. 1, pp. L127–L133, 1998.

[111] V. Ratner, A. Starkov, D. Matsiukevich, R. A. Polin, and V. S. Ten, "Mitochondrial dysfunction contributes to alveolar developmental arrest in hyperoxia-exposed mice," *American Journal of Respiratory Cell and Molecular Biology*, vol. 40, no. 5, pp. 511–518, 2009.

[112] C. Y. Lee, R. Kim, O. Ham et al., "Therapeutic potential of stem cells strategy for cardiovascular diseases," *Stem Cells International*, vol. 2016, 10 pages, 2016.

[113] C. Roubeix, A. Denoyer, F. Brignole-Baudouin, and C. Baudouin, "Mesenchymal stem cell therapy, a new hope for eye disease," *Journal francais d'ophtalmologie*, vol. 38, no. 8, pp. 764–775, 2015.

[114] G. Dothel, E. Raschi, R. Rimondini, and F. de Ponti, "Mesenchymal stromal cell-based therapy: regulatory and translational aspects in gastroenterology," *World Journal of Gastroenterology*, vol. 22, no. 41, pp. 9057–9068, 2016.

[115] C. T. van Velthoven, M. Dzietko, M. F. Wendland et al., "Mesenchymal stem cells attenuate MRI-identifiable injury, protect white matter, and improve long-term functional outcomes after neonatal focal stroke in rats," *Journal of Neuroscience Research*, vol. 95, no. 5, pp. 1225–1236, 2017.

[116] C. T. van Velthoven, R. A. Sheldon, A. Kavelaars et al., "Mesenchymal stem cell transplantation attenuates brain injury after neonatal stroke," *Stroke*, vol. 44, no. 5, pp. 1426–1432, 2013.

[117] C. T. van Velthoven et al., "Mesenchymal stem cells restore cortical rewiring after neonatal ischemia in mice," *Annals of Neurology*, vol. 71, no. 6, pp. 785–796, 2012.

[118] C. T. van Velthoven, A. Kavelaars, F. van Bel, and C. J. Heijnen, "Mesenchymal stem cell transplantation changes the gene expression profile of the neonatal ischemic brain," *Brain, Behavior, and Immunity*, vol. 25, no. 7, pp. 1342–1348, 2011.

[119] C. T. Van Velthoven, A. Kavelaars, F. van Bel, and C. J. Heijnen, "Nasal administration of stem cells: a promising novel route to treat neonatal ischemic brain damage," *Pediatric Research*, vol. 68, pp. 1–422, 2010.

[120] E. S. Kim, S. Y. Ahn, G. H. Im et al., "Human umbilical cord blood–derived mesenchymal stem cell transplantation attenuates severe brain injury by permanent middle cerebral artery occlusion in newborn rats," *Pediatric Research*, vol. 72, no. 3, pp. 277–284, 2012.

[121] W. S. Park, S. In Sung, S. Y. Ahn et al., "Hypothermia augments neuroprotective activity of mesenchymal stem cells for neonatal hypoxic-ischemic encephalopathy," *PLoS One*, vol. 10, no. 3, article e0120893, 2015.

[122] S. Y. Ahn, Y. S. Chang, D. K. Sung et al., "Mesenchymal stem cells prevent hydrocephalus after severe intraventricular hemorrhage," *Stroke*, vol. 44, no. 2, pp. 497–504, 2013.

[123] S. Y. Ahn, Y. S. Chang, D. K. Sung et al., "Optimal route for mesenchymal stem cells transplantation after severe intraventricular hemorrhage in newborn rats," *PLoS One*, vol. 10, no. 7, article e0132919, 2015.

[124] S. Y. Ahn, Y. S. Chang, D. K. Sung, S. I. Sung, J. Y. Ahn, and W. S. Park, "Pivotal role of brain derived neurotrophic factor secreted by mesenchymal stem cells in severe intraventricular hemorrhage in the newborn rats," *Cell Transplantation*, vol. 26, no. 1, pp. 145–156, 2016.

[125] V. Donega, C. H. Nijboer, C. T. J. van Velthoven et al., "Assessment of long-term safety and efficacy of intranasal mesenchymal stem cell treatment for neonatal brain injury in the mouse," *Pediatric Research*, vol. 78, no. 5, pp. 520–526, 2015.

[126] A. M. Comi, E. Cho, J. D. Mulholland et al., "Neural stem cells reduce brain injury after unilateral carotid ligation," *Pediatric Neurology*, vol. 38, no. 2, pp. 86–92, 2008.

[127] V. Donega, C. T. J. van Velthoven, C. H. Nijboer et al., "Intranasal mesenchymal stem cell treatment for neonatal brain damage: long-term cognitive and sensorimotor improvement," *PLoS One*, vol. 8, no. 1, article e51253, 2013.

[128] R. K. Jellema, T. G. A. M. Wolfs, V. Lima Passos et al., "Mesenchymal stem cells induce T-cell tolerance and protect the preterm brain after global hypoxia-ischemia," *PLoS One*, vol. 8, no. 8, article e73031, 2013.

[129] N. Hoque, M. Thoresen, K. Aquilina, S. Hogan, and A. Whitelaw, "Decorin and colchicine as potential treatments for post-haemorrhagic ventricular dilatation in a neonatal rat model," *Neonatology*, vol. 100, no. 3, pp. 271–276, 2011.

[130] C. T. van Velthoven, C. J. Heijnen, F. van Bel, and A. Kavelaars, "Osteopontin enhances endogenous repair after neonatal hypoxic–ischemic brain injury," *Stroke*, vol. 42, no. 8, pp. 2294–2301, 2011.

[131] K. Aquilina, C. Hobbs, A. Tucker, A. Whitelaw, and M. Thoresen, "Do drugs that block transforming growth factor beta reduce posthaemorrhagic ventricular dilatation in a neonatal rat model?," *Acta Paediatrica*, vol. 97, no. 9, pp. 1181–1186, 2008.

[132] J. Aronowski and C. E. Hall, "New horizons for primary intracerebral hemorrhage treatment: experience from preclinical studies," *Neurological Research*, vol. 27, no. 3, pp. 268–279, 2005.

[133] G. Vinukonda, A. Csiszar, F. Hu et al., "Neuroprotection in a rabbit model of intraventricular haemorrhage by cyclooxygenase-2, prostanoid receptor-1 or tumour necrosis factor-alpha inhibition," *Brain*, vol. 133, no. 8, pp. 2264–2280, 2010.

[134] K. Dummula, G. Vinukonda, P. Chu et al., "Bone morphogenetic protein inhibition promotes neurological recovery after intraventricular hemorrhage," *The Journal of Neuroscience*, vol. 31, no. 34, pp. 12068–12082, 2011.

[135] J. Wang and S. E. Tsirka, "Neuroprotection by inhibition of matrix metalloproteinases in a mouse model of intracerebral haemorrhage," *Brain*, vol. 128, no. 7, pp. 1622–1633, 2005.

[136] W. S. Park, S. I. Sung, S. Y. Ahn et al., "Optimal timing of mesenchymal stem cell therapy for neonatal intraventricular hemorrhage," *Cell Transplantation*, vol. 25, no. 6, pp. 1131–1144, 2016.

[137] C. T. Van Velthoven, L. Braccioli, H. L. D. M. Willemen, A. Kavelaars, and C. J. Heijnen, "Therapeutic potential of genetically modified mesenchymal stem cells after neonatal hypoxic-ischemic brain damage," *Molecular Therapy*, vol. 22, no. 3, pp. 645–654, 2014.

[138] C. T. van Velthoven, A. Kavelaars, F. van Bel, and C. J. Heijnen, "Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration," *Brain, Behavior, and Immunity*, vol. 24, no. 3, pp. 387–393, 2010.

[139] J. A. Lee, B. I. Kim, C. H. Jo et al., "Mesenchymal stem-cell transplantation for hypoxic-ischemic brain injury in neonatal rat model," *Pediatric Research*, vol. 67, no. 1, pp. 42–46, 2010.

[140] C. T. van Velthoven, A. Kavelaars, F. van Bel, and C. J. Heijnen, "Repeated mesenchymal stem cell treatment after neonatal hypoxia–ischemia has distinct effects on formation and maturation of new neurons and oligodendrocytes leading to restoration of damage, corticospinal motor tract activity, and sensorimotor function," *The Journal of Neuroscience*, vol. 30, no. 28, pp. 9603–9611, 2010.

[141] S. Grade, Y. C. Weng, M. Snapyan, J. Kriz, J. O. Malva, and A. Saghatelyan, "Brain-derived neurotrophic factor promotes vasculature-associated migration of neuronal precursors toward the ischemic striatum," *PLoS One*, vol. 8, no. 1, article e55039, 2013.

[142] C. M. Cotten, A. P. Murtha, R. N. Goldberg et al., "Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy," *The Journal of pediatrics*, vol. 164, no. 5, pp. 973–979.e1, 2014.

[143] Y. S. Chang, S. Y. Ahn, S. Sung, and W. S. Park, "Stem cell therapy for neonatal disorders: prospects and challenges," *Yonsei Medical Journal*, vol. 58, no. 2, pp. 266–271, 2017.

[144] M. M. Lalu, L. McIntyre, C. Pugliese et al., "Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials," *PLoS One*, vol. 7, no. 10, article e47559, 2012.

[145] D. Grisafi, M. Pozzobon, A. Dedja et al., "Human amniotic fluid stem cells protect rat lungs exposed to moderate hyperoxia," *Pediatric Pulmonology*, vol. 48, no. 11, pp. 1070–1080, 2013.

[146] I. Arutyunyan, A. Elchaninov, A. Makarov, and T. Fatkhudinov, "Umbilical cord as prospective source for mesenchymal stem cell-based therapy," *Stem Cells International*, vol. 2016, Article ID 6901286, 17 pages, 2016.

[147] A. Blázquez-Prunera, C. Almeida, and M. Barbosa, "Human bone marrow mesenchymal stem/stromal cells preserve their immunomodulatory and chemotactic properties when expanded in a human plasma derived xeno-free medium," *Stem Cells International*, vol. 2017, Article ID 2185351, 12 pages, 2017.

[148] M. Krampera, J. Galipeau, Y. Shi, K. Tarte, L. Sensebe, and MSC Committee of the International Society for Cellular Therapy (ISCT), "Immunological characterization of multipotent mesenchymal stromal cells—the International Society for Cellular Therapy (ISCT) working proposal," *Cytotherapy*, vol. 15, no. 9, pp. 1054–1061, 2013.

[149] N. Walker, L. Badri, S. Wettlaufer et al., "Resident tissue-specific mesenchymal progenitor cells contribute to fibrogenesis in human lung allografts," *The American Journal of Pathology*, vol. 178, no. 6, pp. 2461–2469, 2011.

[150] A. M. Hoffman, J. A. Paxson, M. R. Mazan et al., "Lung-derived mesenchymal stromal cell post-transplantation survival, persistence, paracrine expression, and repair of elastase-injured lung," *Stem Cells and Development*, vol. 20, no. 10, pp. 1779–1792, 2011.

[151] M. Franquesa, M. J. Hoogduijn, O. Bestard, and J. M. Grinyó, "Immunomodulatory effect of mesenchymal stem cells on B cells," *Frontiers in Immunology*, vol. 3, p. 212, 2012.

[152] L. Barkholt, E. Flory, V. Jekerle et al., "Risk of tumorigenicity in mesenchymal stromal cell–based therapies—bridging scientific observations and regulatory viewpoints," *Cytotherapy*, vol. 15, no. 7, pp. 753–759, 2013.

[153] C. Lee, S. A. Mitsialis, M. Aslam et al., "Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension," *Circulation*, vol. 126, no. 22, pp. 2601–2611, 2012.

[154] I. Panfoli, S. Ravera, M. Podestà et al., "Exosomes from human mesenchymal stem cells conduct aerobic metabolism in term and preterm newborn infants," *The FASEB Journal*, vol. 30, no. 4, pp. 1416–1424, 2016.

Hindawi
Stem Cells International
Volume 2018, Article ID 5832460, 15 pages
https://doi.org/10.1155/2018/5832460

Research Article

Mesenchymal Stromal Cells Cultured in Serum from Heart Failure Patients Are More Resistant to Simulated Chronic and Acute Stress

Timo Z. Nazari-Shafti,[1,2,3] **Zhiyi Xu**,[4] **Andreas Matthäus Bader**,[1] **Georg Henke**,[4] **Kristin Klose**,[4] **Volkmar Falk**,[1,2,4] **and Christof Stamm**[1,2,4]

[1]*Deutsches Herzzentrum Berlin (DHZB), Berlin, Germany*
[2]*Deutsches Zentrum für Herz-Kreislaufforschung (DZHK), Partner Site Berlin, Berlin, Germany*
[3]*Berlin Institute of Health (BIH), 10178 Berlin, Germany*
[4]*Berlin Center for Regenerative Therapies (BCRT), Berlin, Germany*

Correspondence should be addressed to Timo Z. Nazari-Shafti; nazari@dhzb.de

Received 11 August 2017; Revised 14 November 2017; Accepted 30 November 2017; Published 1 April 2018

Academic Editor: Fatemeh Sharifpanah

Despite regulatory issues surrounding the use of animal-derived cell culture supplements, most clinical cardiac cell therapy trials using mesenchymal stromal cells (MSCs) still rely on fetal bovine serum (FBS) for cell expansion before transplantation. We sought to investigate the effect of human serum from heart failure patients (HFS) on cord blood MSCs (CB-MSCs) during short-term culture under regular conditions and during simulated acute and chronic stress. Cell survival, proliferation, metabolic activity, and apoptosis were quantified, and gene expression profiles of selected apoptosis and cell cycle regulators were determined. Compared to FBS, HFS and serum from healthy donors (CS) showed similar effects by substantially increasing cell survival during chronic and acute stress and by increasing cell yields 5 days after acute stress. Shortly after the termination of acute stress, both HFS and CS resulted in a marked decrease in apoptotic cells. Transcriptome analysis suggested a decrease in TNF-mediated induction of caspases and decreased activation of mitochondrial apoptosis. Our data confirm that human serum from both healthy donors and heart failure patients results in increased cell yields and increased resistance to cellular stress signals. Therefore, we consider autologous serum a valid alternative to FBS in cell-based therapies addressing severe heart disease.

1. Introduction

To date, there have been several clinical studies investigating the use of mesenchymal stromal cells (MSCs) for treatment of cardiovascular disease. Based on the findings of Kawada et al. [1] and Chen et al. [2] demonstrating the regenerative potential of bone marrow MSCs (BM-MSCs), various BM-MSC preparations were transplanted into infarcted myocardium. It is currently assumed that, independent of their source, MSCs can prevent myocardial remodeling following acute myocardial infarction (AMI) via paracrine secretion of gene expression modulating and trophic factors [3, 4]. In addition, a number of trials tested the myocardial injection of MSCs in the setting of chronic ischemic cardiomyopathy, hoping to induce reverse myocardial remodeling [5–7]. Multiple factors influence the outcome of cellular therapy, and there have been attempts to address those recently (Figure 1), including the timing (TIME-Study [8]) and frequency of MSC administration, the route of administration, and the source of stromal cells. There have also been attempts to modify MSCs prior to transplantation to improve their engraftment efficacy and clinical effects (i.e., C-CURE [9], CHART-1 [10], and IMPACT-DCM [11]). Cells transplanted into damaged myocardium are exposed to acute

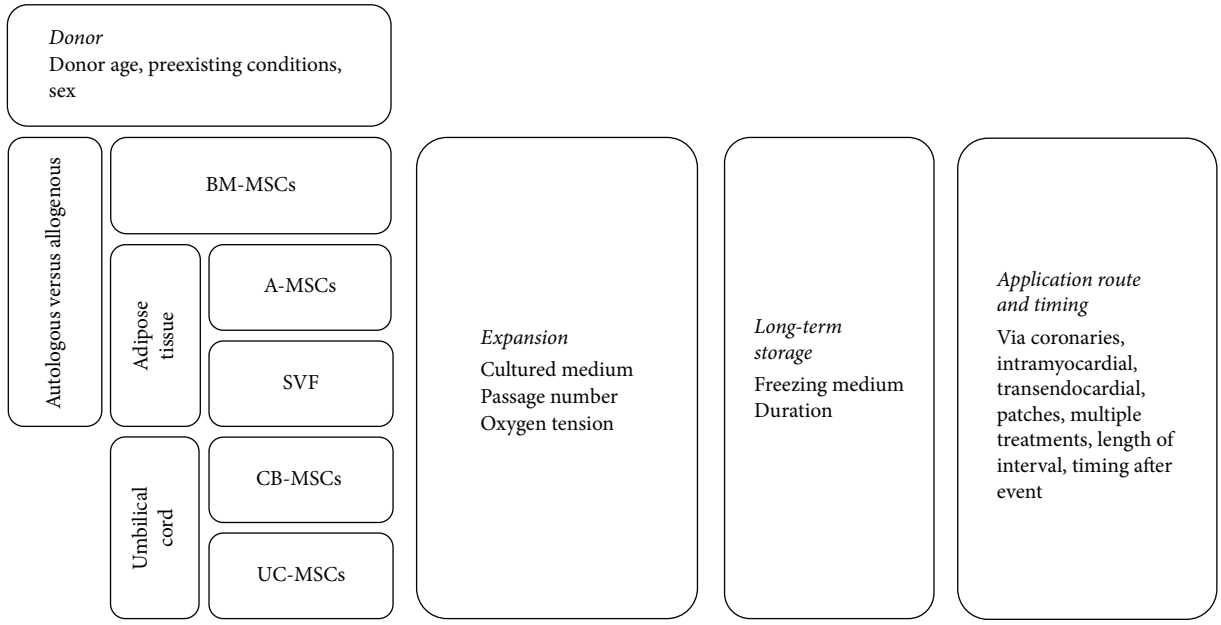


Figure 1: Examples of different variables that may influence the efficacy and efficiency of mesenchymal stromal cell transplantation in the setting of cardiovascular regeneration. BM-MSCs: bone marrow MSCs; A-MSCs: adipose tissue-derived MSCs; SVF: stromal vascular fraction; CB-MSCs: cord blood MSCs; UC-MSCs: umbilical cord matrix MSCs.

and chronic stress within a hypoxic and malnutritioned microenvironment. Additionally, MSC culture conditions greatly influence their stress responses after transplantation. It has been well established that cell culture introduces DNA damage and functional changes in MSCs [12]. The most common cell culture media used today contain heat-inactivated fetal bovine serum (FBS). However, in addition to its regulatory difficulties, FBS also introduces such variables in MSC products that are difficult to control, causing dysregulation of cell cycle and metabolism [13]. Consequently, many groups have been working on formulating a serum-free culture media with recombinant growth factors [14, 15]. However, some controversy around the effect of serum-free culture medium on MSC function remains [16–18]. An alternative approach to eliminate the issues surrounding FBS is the use of autologous recipient serum for MSC *in vitro* culture [19, 20]. Other groups, including our team, have previously shown that serum from patients with heart failure can impair MSC function [21]. In fact, a retrospective analysis of patients with chronic heart failure (CHF) treated with BM-MSCs that were cultured in either FBS or autologous serum demonstrated less variance in population doublings in the FBS group [22].

In the current study, we sought to investigate whether the short-term incubation of a "virginal" model cell product with human serum of CHF has an immediate effect on cell proliferation and metabolism. We chose cord blood-derived MSCs (CB-MSCs) as a model cell type, since CB-MSCs are more proliferative, not senescent, and have not been subjected to exogenous noxae [23]. CB-MSCs show no sign of DNA damage and telomere dysfunction at the time of isolation [24–26]. Additionally, they do not express HLA on their surface, facilitating potential allogenic applications [27]. We assume that, by using model cells free from intrinsic pathology, changes in biologic behavior should solely reflect the impact of the media composition.

2. Methods

2.1. Clinical Trial Analysis. A literature search on MEDLINE and clinicaltrials.gov was conducted to identify clinical trials testing MSCs for cardiovascular regeneration in the past five years. The search was limited to study protocols or results published between May 1, 2012, and May 31, 2017. A combination of terms for mesenchymal stem cells ("MSC," "mesenchymal cells," "bone marrow cells," "adipose-derived stem cells," "umbilical cord/blood MSC," and "stromal vascular fraction") and disease-related keywords ("cardiovascular disease," "acute/myocardial infarction," or "congestive/heart failure," and "ischemic/dilated cardiomyopathy") was used to identify relevant trials. Vocabulary and syntax were adjusted across databases. Each study protocol was screened for cell type used and, if applicable, for cell culture medium formulation used for ex vivo expansion of cells (Table 1).

2.2. Study Population. In accordance with the Declaration of Helsinki, this study was approved by the Ethics Committee of Charité-Universitätsmedizin Berlin, Berlin, with informed consents signed by all patients ($n = 12$) and volunteers ($n = 12$) (Table 2). A thorough medical history of all patients was obtained, and all current medications were documented. We included patients aged 64 ± 3 years with ischemic cardiomyopathy (ICMP), who had an average left ventricular ejection fraction of $22 \pm 2\%$ at the time of blood collection. Two-thirds of the patients had a history of past myocardial infarction, and all patients suffered

Table 1: Clinical trials investigating the therapeutic potential of MSCs in cardiovascular disease within the past five years. BM-MCSs: bone marrow mesenchymal stem cells; BM-MNCs: bone marrow mononuclear cells; FBS: fetal bovine serum; UC-MCSs: umbilical cord mesenchymal stem cells; SVF: stromal vascular fraction; A-MSCs: adipose tissue-derived mesenchymal stem cells; CHF: congestive heart failure; AMI: acute myocardial infarction; CAD: coronary artery disease without clinical signs of AMI.

Registry identifier	PMID of publications	Trial name/first author	Cell types	Medium supplementation	CV disease treated
NCT01768702	26662998	CHART-1	Autologous BM-MSCs, exposed to "cardiogenic cocktail"	Pooled human platelet lysate	CHF
NCT00810238	23583246	C-CURE	Autologous BM-MSCs, exposed to "cardiogenic cocktail"	Pooled human platelet lysate	CHF
NCT02323477	26123356	HUC-HEART	Umbilical cord MSCs	Pooled human AB serum	AMI
NCT00721045	26148930	Perin et al.	Autologous STRO-1 immunoselected BM-MSCs	FBS	CHF
NCT00644410	25926562	MSC-HF	Autologous BM-MSCs	FBS	CHF
NCT00883727	25484310	Chullikana et al.	Allogeneic pooled BM-MSCs	FBS	AMI
NCT01392105	24431901	Lee et al.	Autologous BM-MSCs	FBS	AMI
NCT01291329	26162993	Gao et al.	Allogeneic UC-MSCs	FBS	AMI
—	24975729	Wang et al.	Autologous BM-MSCs	FBS	AMI
NCT00768066	24247587	TAC-HFT	Autologous BM-MSCs	FBS	CHF
NTR1553 (Dutch trial registry)	23982478	Rodrigo et al.	Autologous BM-MSCs	FBS	AMI
ChiCTR-TRC-08000080 (Chinese clinical trial registry)	23651816	Gao et al.	Autologous BM-MSCs	FBS	AMI
NCT00587990	24565698	PROMETHEUS	Autologous BM-MSCs	FBS	CAD
NCT01087996	23117550	POSEIDON	Autologous and allogeneic BM-MSCs	FBS	CHF
NCT01392625	27856208	POSEIDON-DCM	Autologous and allogeneic BM-MSCs	FBS	CHF
NCT02467387	27856497	Butler et al.	"Ischemia tolerated" BM-MSCs	FBS	CHF
NCT00260338	24211066	Mathiasen et al.	Autologous BM-MSCs	FBS	CAD
NCT00418418	25797522	Lethinen et al.	BM-MNCs	No *in vitro* expansion	CHF
NCT01299324	26217065	REVIVE	Autologous BM-MNCs	No *in vitro* expansion	CHF
NCT01033617	23265095	IMPACT-CABG	Autologous $CD133^+$ BM-MNCs	No *in vitro* expansion	CAD
NCT00824005	22447880	FOCUS-CC TRN	BM-MNCs	No *in vitro* expansion	CHF
NCT00684021	23129008	TIME	BM-MNCs	No *in vitro* expansion	AMI
NCT00395811	25418212	Qi et al.	Autologous BM-MNCs	No *in vitro* expansion	CHF
NCT01502514	27255774	Parcero et al.	SVF	No *in vitro* expansion	CHF
NCT02052427	27148802	ATHENA I/II	SVF (A-MSCs)	No *in vitro* expansion	CHF
NCT00426868	24952864	PRECISE	SVF (A-MSCs)	No *in vitro* expansion	CHF
NCT01670981	27059887	ixCELL-DCM	"Ixmyelocel-T" from BM-MNCs	Proprietary culture system; supplement not disclosed	CHF
NCT01076920	26901787	MESAMI 1	Autologous BM-MSCs	Supplement not described	CHF

Table 2: Clinical characteristics of serum donors. Where applicable, data are shown as mean ± SEM. MI: myocardial infarction; CAD: coronary artery disease; NYHA: New York Heart Association functional classification; LVEDD: left ventricular end-diastolic diameter; LVEF: left ventricular ejection fraction. $^*p < 0.05$ compared to healthy volunteers.

	Healthy volunteers ($n = 12$)	Heart failure patients ($n = 12$)
History		
Age (years)	54 ± 1.60	64 ± 3.26*
Gender (male/female)	7/5	9/3
Smoker	2	2
Ex-smoker	3	10
Previous MI	—	8
Time since most recent MI (years)	—	8 ± 3.39
Extent of CAD		
Two vessels	—	2
Three vessels	—	10
NYHA functional class		
III	—	5
IV	—	7
Echocardiogram		
LVEDD (mm)	—	65 ± 2.73
LVEF (%)	—	22 ± 2.06
Pulmonary hypertension	—	3

from heart failure symptoms with New York Heart Association functional class III or IV. Patients with acute or recent myocardial infarction were not included in this study; the average time elapsed since the infarct was 8 ± 3.4 years. Control serum was collected from healthy volunteers aged 54 ± 1.6 years ($p < 0.05$ versus CHF patients) without a history of cardiovascular disease.

2.3. Serum Extraction. Venous blood was collected in S-Monovettes® (Sarstedt, Nümbrecht, Germany) by using the BD Vacutainer Safety-Lok blood collection set (BD Medical, Heidelberg, Germany). Whole blood samples were left undisturbed at room temperature for 30 minutes and then centrifuged at 3500g for 15 minutes at 4°C to remove the clot and all remaining cellular particles. Serum supernatants were then sterile filtered, aliquoted, flash frozen in liquid nitrogen, and then stored at −80°C for later use. Serum from each donor ($n = 12$) was tested individually on CB-MSCs to account for possible patient-specific confounding factors. CHF is associated with interstitial and intravascular volume retention, and patients usually show relative hyperproteinemia which could influence the quantitative bioactivity of heart failure serum (HFS) as compared to control serum (CS). Accordingly, the total protein concentration in CS was significantly higher than that in HFS (5.9 g/dL in CS, 5.5 g/dL in HFS, $p = 0.04$). We noted that the lowest serum protein concentration in one of our heart failure patients (4.6 g/dL) was within the range of protein concentration found in our FBS sample (4.5 g/dL). Since we hypothesized that differences in serum impact on cells arise from its bioactive contents, that is, cytokines, exosomes, short nucleotides, or other trophic factors, we decided to eliminate the variance in total protein concentration. In order to minimize this systematic error, serum concentrations were normalized concentration within our samples to 4.5 g/dL, by dilution with Dulbecco's phosphate-buffered saline (DPBS) containing Ca^{2+} and Mg^{2+} (Life Technologies, Darmstadt, Germany). Total protein concentration of each serum sample was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher, Waltham, MA, USA).

2.4. In Vitro Cultivation of Human CB-MSCs. Human CB-MSCs were provided by courtesy of Dr. K. Bieback, who isolated them from umbilical cord blood with the mothers' consent and approval of the local ethics committee and expanded the cells based on a previously published protocol [28]. Prior to experimentation, the CB-MSCs were thawed, washed, and expanded in 1 g/L glucose DMEM with 10% FBS, under antibiotic protection with 1% streptomycin/penicillin (all from Life Technologies, Carlsbad, CA, USA). We initially tested the effects of FBS from three different lots on proliferation, immunophenotype, and trilineage differentiation capacity of CB-MSCs. No difference was detected, and thus, FBS from one lot was used in all experiments (Life Technologies, Lot 41A1513K). Cells were seeded at 800–1000 cells/cm^2 in T175 flasks and cultured under 21% O_2 and 5% CO_2 at 37°C. Partial media changes were performed every 3 days. All cultured cells were screened for the presence of mycoplasma (MycoAlert™ assay, Lonza, Walkersville, MD, USA). All experiments were performed on cells between passages 4 and 6. CB-MSC phenotype and their ability to differentiate into nonhematopoietic cell types have been repeatedly confirmed in previous experiments by our group [21].

2.5. In Vitro Models for Acute and Chronic Stress on CB-MSCs

2.5.1. Chronic Stress (ChS). Glucose deprivation in a setting of low oxygen tension triggers oxidative stress in MSCs [29, 30]. To test the response of CB-MSCs to continued stress in culture with either human serum or FBS, we designed an experimental setup where CB-MSCs were cultured under low oxygen tension and glucose deprivation for 5 days (Figure 2(a)). CB-MSCs were seeded at a density of 4–6×10^3 cells/cm^2 into 96-well plates (Greiner Bio-One, Frickenhausen, Germany). After 24 hours of incubation under standard cell culture conditions to allow for attachment (day 0), cells were deprived of glucose and provided with either 10% human serum or FBS in their culture media and cultivated for 5 days at 1% O_2. In parallel, as a control condition, cells were kept under regular culture conditions with the exception of supplementing the media with FBS, CS, or HFS. Culture media was changed every other day.

2.5.2. Acute Stress (AcS). To test how CB-MSCs cultured with different sera behave in response to an acute stress trigger (AcS), we chose an *in vitro* model of "simulated ischemia reperfusion injury" where the cells are deprived of glucose, serum, and oxygen (0.2% O_2) for 4 hours and then transferred back into regular culture conditions (Figure 2(b)). This

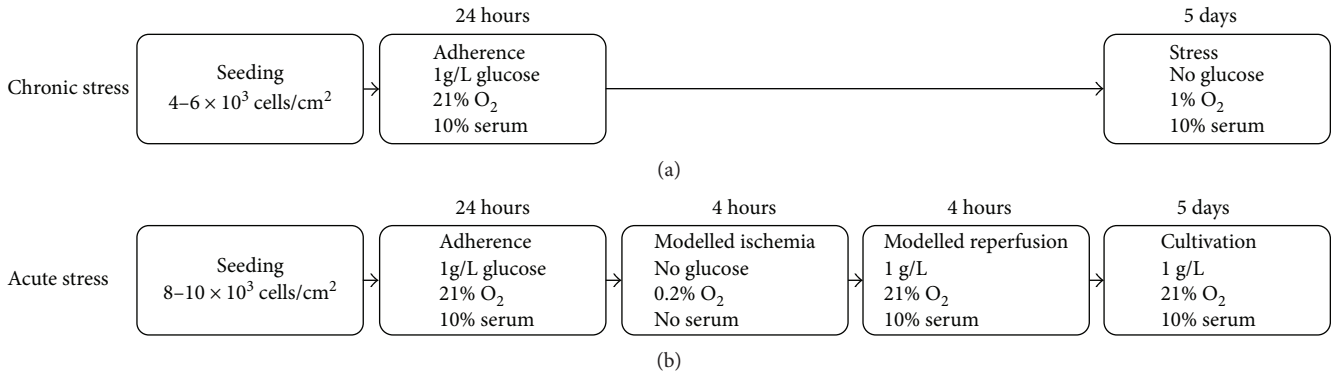


Figure 2: Flow chart depicting the experimental setup for the *in vitro* models of simulated chronic (a) and acute (b) stress. FBS, HFS, or CS was used as a serum supplement where indicated. In selected experiments, IL-6 was added to regular cell culture media supplemented with FBS.

setup was designed to test the immediate survival of cells after simulated ischemia, with the use of either CS, HFS, or FBS during their oxygenation phase. All oxygen-deprivation studies were performed in a hypoxic incubator (Binder, Tuttlingen, Germany). Cells were seeded at $8–10 \times 10^3$ cells/cm^2 and cultivated with FBS and 1 g/L glucose for 24 hours at normal cultivation conditions. Then, cells were exposed to hypoxia (0.2% O_2) as well as serum and glucose deprivation for 4 hours, followed by 4 hours of simulated reperfusion in normal culture settings. The proliferative capacity of CB-MSCs (or recovery) during the first 5 days after the acute stress trigger was tested in culture with the different sera (10% CS, HFS, or FBS).

2.6. Metabolic Activity, Cell Counts, and Proliferation Assays. One part of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) solution was added to 5 parts of culture media, and the cells were incubated for 4 hours at 37°C. Absorbance (OD) was measured at 490 nm and 650 nm as a reference wavelength. Subsequently, nuclei were stained with Hoechst 33342 (Life Technologies) in the dark for 20 minutes at room temperature and washed with DPBS. Cell numbers were counted in the Operetta High-Content Imaging System (PerkinElmer, Rodgau, Germany) at 380 nm excitation and 445 nm emission. Cell survival rate after AcS is depicted as the percentage of initially plated cells. Cell survival rate is depicted as the percentage of cells counted after AcS, as compared to the percentage of cells plated. BrdU incorporation was used (Roche, Mannheim, Germany) to quantify cell proliferation. Cells cultured in 96-well plates were incubated with BrdU labeling solution at 37°C for 4 hours, and the following steps were performed without interruptions according to the manufacturer's instructions. Absorbance was measured in the ELISA reader (Molecular Devices GmbH, Biberach an der Riss, Germany) at 370 nm with the reference wavelength set to 492 nm.

2.7. Apoptosis Detection Assays. Two different assays were performed to quantify apoptosis. Fluorochrome-labeled inhibitors of caspases (FLICA) assays were used to detect caspase activity in CB-MSCs. The polycaspase probe (SR-VAD-FMK), provided by ImmunoChemistry Technologies (LLC, Bloomington, MN, USA), recognizes all the different types of activated polycaspases. Cells were incubated with the polycaspase probe for 45 minutes at 37°C, under gentle agitation every 10 minutes. Then, nuclear counterstaining with Hoechst 33342 was performed. Cells were then scanned with the Operetta High-Content Imaging System at 570 nm and 380 nm excitation and 615 nm and 445 nm emission wavelengths. The data were analyzed using the Columbus software (PerkinElmer). Additionally, the differential detection of late and early stages of apoptosis was performed by FITC-Annexin V and ethidium homodimer III (EthD-III) staining, using a kit provided by Promokine (PromoCell GmbH, Heidelberg, Germany). CB-MSCs were harvested after the AcS and stained according to the manufacturer's protocol. Cells in normal culture were used as negative control. CB-MSCs treated for 24 hours with 200 μM H_2O_2 were used as Annexin V-positive control, while cells incubated on ice after 10 minutes of 65°C warm water bath were used as ethidium homodimer III-positive control. FITC- and/or EthD-III-positive cells were then quantified using the MACSQuant VYB (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and analyzed with FlowJo 10 (FlowJo, LLC, Ashland, OR, USA).

2.8. Semiquantitative Real-Time PCR. Total RNA was isolated using the NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany). The purity and integrity of isolated RNA were determined by spectrophotometry and gel electrophoresis. The RNA samples from biological replicates were pooled before cDNA synthesis to account for biological variation [26]. cDNA was synthesized by reverse transcription using the qScript SuperMix (QuantaBio, Beverly, Massachusetts, USA). The expression of 96 genes associated with apoptosis and cell cycle pathways was tested using RT-PCR array kits from http://realtimeprimers.com (Elkins Park, Philadelphia, USA), using their recommended amplification protocol. All the CT values were normalized to mRNA expression of HPRT1. We collected total RNA after 1 day of cultivation with FBS, CS, and HFS and then 4 hours after AcS, as well as 1, 3, and 5 days after

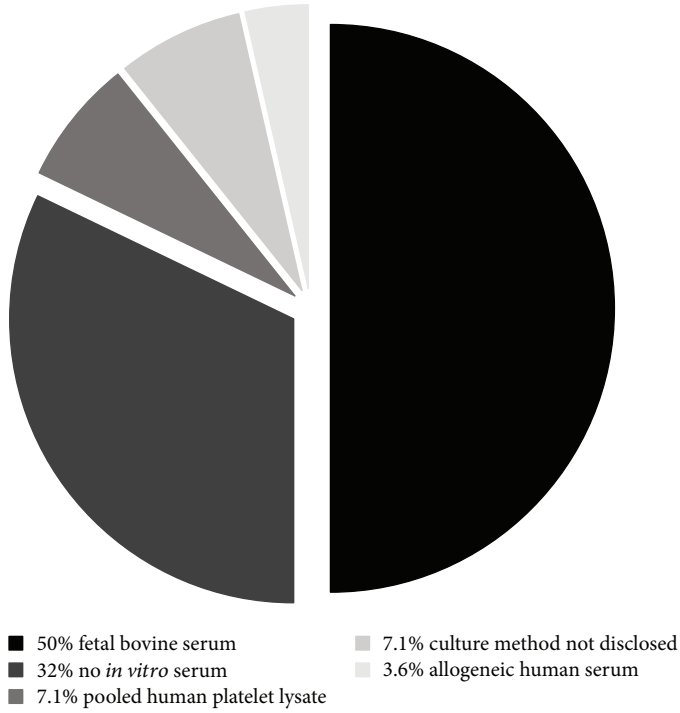


Figure 3: Analysis of culture media supplements used in clinical trials in the past five years (cutoff 07/2012) utilizing MSCs in cardiovascular regeneration. 28 trials were identified, of which only three used human blood products for media supplementation (1 with allergenic serum and 2 with platelet lysate). Trials without *in vitro* expansion ($n = 9$) were also included. Overall, 50% of the clinical trials relied on FBS as a supplement ($n = 14$). In two reports, the exact formulation of the culture media could not be identified based on the information provided.

AcS under continued cultivation with either FBS, CS, and HFS. The fold change of the mRNA expression before AcS of 119 genes was included in a heatmap, using average-linkage hierarchical clustering. The grouping of the genes in the heatmap was determined by calculating the distance among the groups by the Spearman rank correlation method.

2.9. Statistics. All results are shown as mean ± SEM. Unless stated otherwise, experiments were performed with group sizes of at least four control samples (FBS) and 12 human serum samples. Normal distribution and homogeneity of variances (Levene's test) were tested. With a normal distribution provided, ANOVA with Tukey's post hoc analysis was done; otherwise, the Kruskal-Wallis test or Mann–Whitney U test was applied to test for differences between groups. In cases where Levene's test showed heterogeneity, Welch ANOVA and Games-Howell post hoc tests were performed. The differences were considered statistically significant at $p < 0.05$. Repeated measures ANOVA was performed along with the experiments that had three or more time points. Statistical analyses were performed using the IBM SPSS Statistics software Version 22 (IBM Corporation, Somers, NY, USA).

3. Results and Discussion

3.1. Sera Used in Clinical MSC Trials. As shown in Figure 3 and Table 1, the results from 28 trials using MSCs for cardiovascular cell therapy were published within the past five years. The therapeutic effect of MSCs was tested in patients with congestive heart failure (61%), acute myocardial infarction (29%), or coronary artery disease without clinical signs of myocardial infarction (11%) (Table 1). In 50% of the studies, MSCs were expanded in FBS-containing cell culture medium prior to transplantation into patients. Only three clinical trials utilized either allogeneic or autologous human blood products. In two trials, allogeneic pooled human platelet lysate was used (C-CURE trial, NCT00810238; CHART-1, NCT01768702) to expand autologous BM-MSCs prior to implantation. In one trial (HUC-HEART trial, NCT02323477), human serum from healthy donors with the blood group AB was used to expand human umbilical cord MSCs. In one-third of the published trials, cell products that did not require any cell culture after isolation and prior to transplantation were used. This includes trials utilizing adipose-derived stromal vascular cells and bone marrow-derived mononuclear cell preparations.

3.2. Human Serum Improves Proliferative Capacity of CB-MSCs. For clinical use of CB-MSCs, the use of an autologous serum may reduce the regulatory burden and risks of foreign pathogens in cultured cells. However, heart failure serum factors may negatively influence cells [21]. Therefore, we tested whether the presence of heart failure in serum donors affects the proliferation profile of CB-MSCs during short-term cultivation. We previously showed that the cultivation of CB-MSCs with human serum neither from healthy individuals

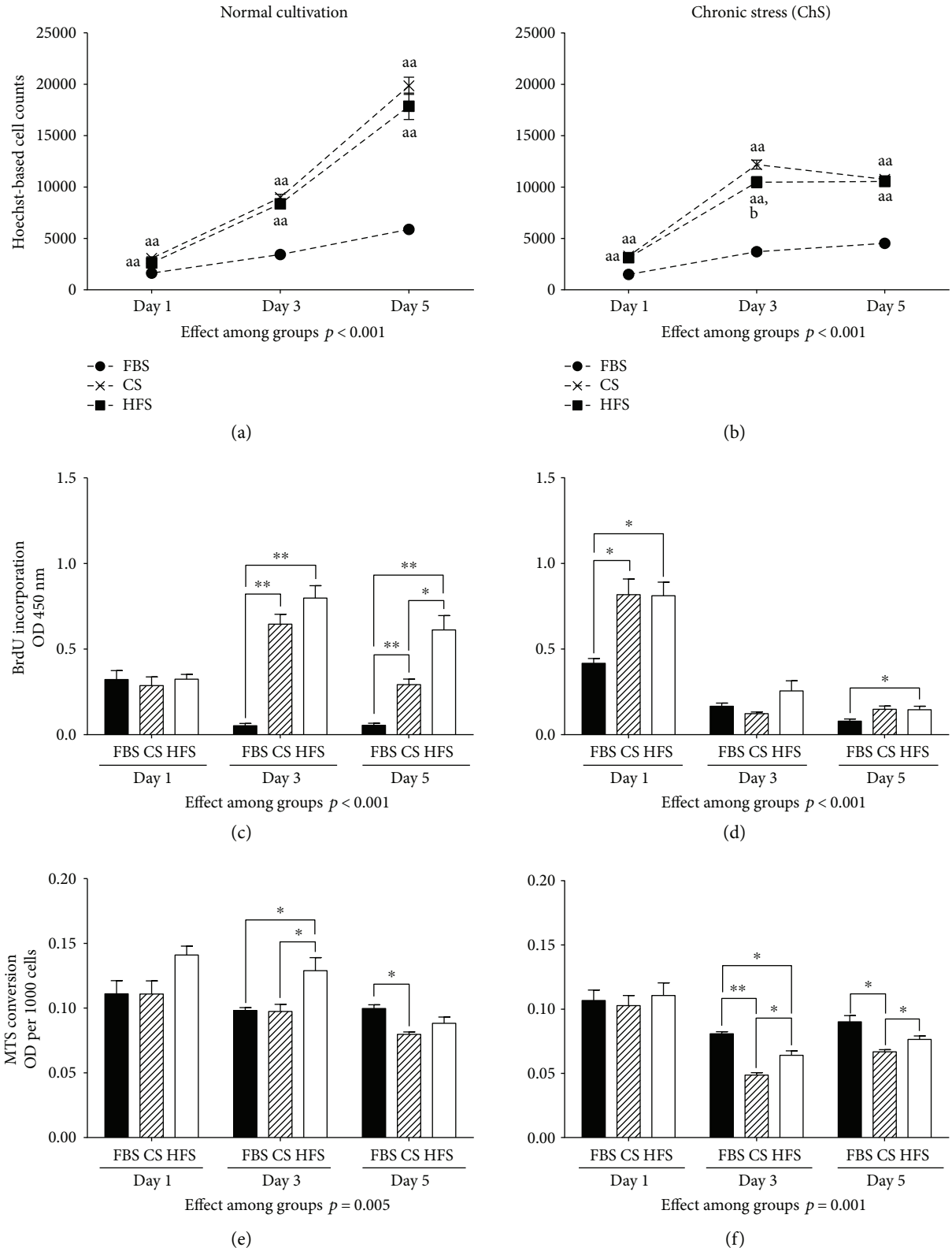


Figure 4: CB-MSC behavior during 5 days of incubation under normoxia and regular glucose supplementation (a, c, e) and under "chronic stress" (1% O_2 and glucose deprivation) (b, d, f). Shown are cell numbers (a, b), proliferation via BrdU incorporation (c, d), and metabolic activity via MTS conversion (e, f). There were significant proproliferative effects with human sera (CS and HFS). Compared to CS, HFS elevated cellular metabolic activities in both conditions. $^{*}p < 0.05$ and $^{**}p < 0.01$. $^{aa}p < 0.01$ compared to corresponding FBS; $^{b}p < 0.05$ HFS groups compared to corresponding CS groups.

nor from heart failure patients affects the overall morphology or immunophenotype of the cells [21]. In the current series of experiments, individual human serum (CS and HFS) supplementation resulted in a greater variability of the CB-MSC growth rate than FBS, which is a standardized, pooled tissue culture supplement (Figure 4(a)). Pooled human serum may have produced less variation but since we aimed to test whether the use of autologous HF serum is feasible for MSC cultivation, pooled serum would not reflect the potential translational scenario. Cell culture with CS and HFS resulted in significantly higher cell yields compared to FBS. (Figure 4(a)). The significant increase in DNA synthesis in HFS and CS compared to FBS treatment indicates that the higher cell yields are a result of increased cell proliferation (Figure 4(c)). Interestingly, CB-MSCs subjected to HFS maintained a high BrdU incorporation rate throughout day 5, whereas the proliferation rate declined in CS-treated cells on day 5, when they reached confluency, which in itself is an inhibitor of cell proliferation of MSCs. Similarly, MTS conversion rate on day 3 was higher with HFS than with CS (0.13 ± 0.01, as compared to 0.1 ± 0.02 with CS, $p = 0.03$) (Figure 4(e)), indicating increased metabolic activity and/or higher cell numbers. However, this effect was no longer detectable by day 5. Overall, our data confirm that the proliferation profiles of CB-MSCs are significantly better under the cultivation with human serum, regardless of whether the donor suffered from CHF or not. Kubo et al. recently reported that autologous serum from older donors with CHF prolonged the population doubling times of BM-MSCs, as compared to FBS and serum from donors without CHF [22]. "Aged" serum was also found to negatively affect mesenchymal stem cells in mice [31], and aging satellite cells could be rejuvenated when exposed to a young serum [32, 33]. Although we cannot eliminate the possibility that donor age affects the proliferation profile of CB-MSCs, we did not observe a clearly negative serum effect that could be attributed to age. Compared to the "age" of the CB-MSCs (neonatal), the difference in CS and HFS donor age (54 versus 64 years) may be too small to elicit a relevant functional response.

3.3. In Vitro "Chronic Ischemia" Model. As mentioned above, cells transplanted into ischemic or scarred myocardium are exposed to a poorly vascularized microenvironment, with low tissue oxygenation and impaired nutrition supply. MSCs heavily depend on a glycolytic metabolism under physiological conditions [30], and dependence on glycolytic pathway further increases in lower oxygen tension [29]. We therefore exposed the cells to a model of chronic "ischemic" stress by removing glucose from the cell culture media and reducing the oxygen supply during culture to 1% O_2. Combined with glucose starvation, this model significantly inhibited CB-MSC proliferation regardless of the source of the supplemented serum (Figure 4(b)). However, CB-MSCs cultured with human serum (both CS and HFS) initially maintained significantly higher BrdU incorporation than cells in FBS (Figure 4(d)). In all groups, on days 3 and 5, CB-MSCs cultured under glucose deprivation went into cell cycle arrest with almost no detectable BrdU incorporation. Interestingly, cells cultivated with FBS maintained higher levels of metabolic activity, when compared to those cultured with human CS or HFS (Figure 4(f)). This may indicate that the suppression of metabolic activity, a presumably protective measurement during stress, was more effective in CS and in HFS-treated CB-MSCs [34, 35].

3.4. In Vitro "Acute Ischemia" Model. Because cells are exposed to acute stress during the process of transplantation, we designed an *in vitro* model mimicking ischemia/reperfusion injury. In our preliminary experiments, we established that the combination of 4 hours of hypoxia at 0.2% O_2, combined with glucose and serum deprivation and followed by reoxygenation in normoxic culture conditions with serum and glucose, is sufficient for reproducibly inducing significant cell loss. Immediately following simulated acute ischemia, we observed that the rescue of cells cultured with HFS ($66.8\% \pm 5\%$) and CS ($72.9\% \pm 5.6\%$) was significantly higher than the cells treated with FBS ($48.7\% \pm 2.1\%$) ($p = 0.013$ versus $p = 0.004$, resp.) (Figure 5(a)). Moreover, when we performed Annexin V and EthD-III stainings after AcS, we found that the number of apoptotic cells was significantly lower in HFS than in cells treated with FBS (HFS, $14.4\% \pm 1.1\%$ versus FBS, $18.8\% \pm 1.4\%$, $p = 0.04$) (Figure 5(b)). During the reoxygenation period, cells incubated with CS or HFS recovered much faster and showed significantly higher proliferation rates (Figure 5(c)). As expected, AcS led to a significant decrease in metabolic activity in all treatment groups (Figure 5(d)). After the reoxygenation/recovery period, MTS conversion rates were markedly increased compared to baseline, independently from which serum was used. In CB-MSCs cultured with HFS or CS, MTS conversion rates returned to pre-AcS levels at day one. In FBS-cultured cells, metabolic activity did not return to baseline until day three. The increase in metabolic activity during the recovery period could be explained by the sudden increase in reactive oxygen species (ROS) during simulated reperfusion and the increased ATP demand to overcome the imbalance in intracellular calcium homeostasis induced by simulated ischemia [36]. The faster recovery to baseline metabolic rates further supports the hypothesis that HFS and CS protect CB-MSCs during acute cellular stress (Figure 5(d)).

3.5. The Role of Interleukin-6 as a Representative Heart Failure Serum Factor. In our previous work, we showed that IL-6 titers are significantly higher in sera from patients with CHF [21]. IL-6 facilitates proinflammatory signaling in the setting of acute and chronic injury, can also initiate apoptosis in tissues exposed to inflammation [37], and has been associated with cardiomyocyte hypertrophy and myocardial dysfunction. Therefore, we studied whether supplementation of FBS media with recombinant human IL-6 has an effect on growth rate, metabolic activity, and/or apoptosis of CB-MSCs in the acute injury model. IL-6 is vital for the innate and adaptive immune system [38] and has been shown to be present in increased concentrations in sera from heart failure patients [21]. IL-6 was shown to increase HIF-1a through the IL-6-STAT3-HIF1

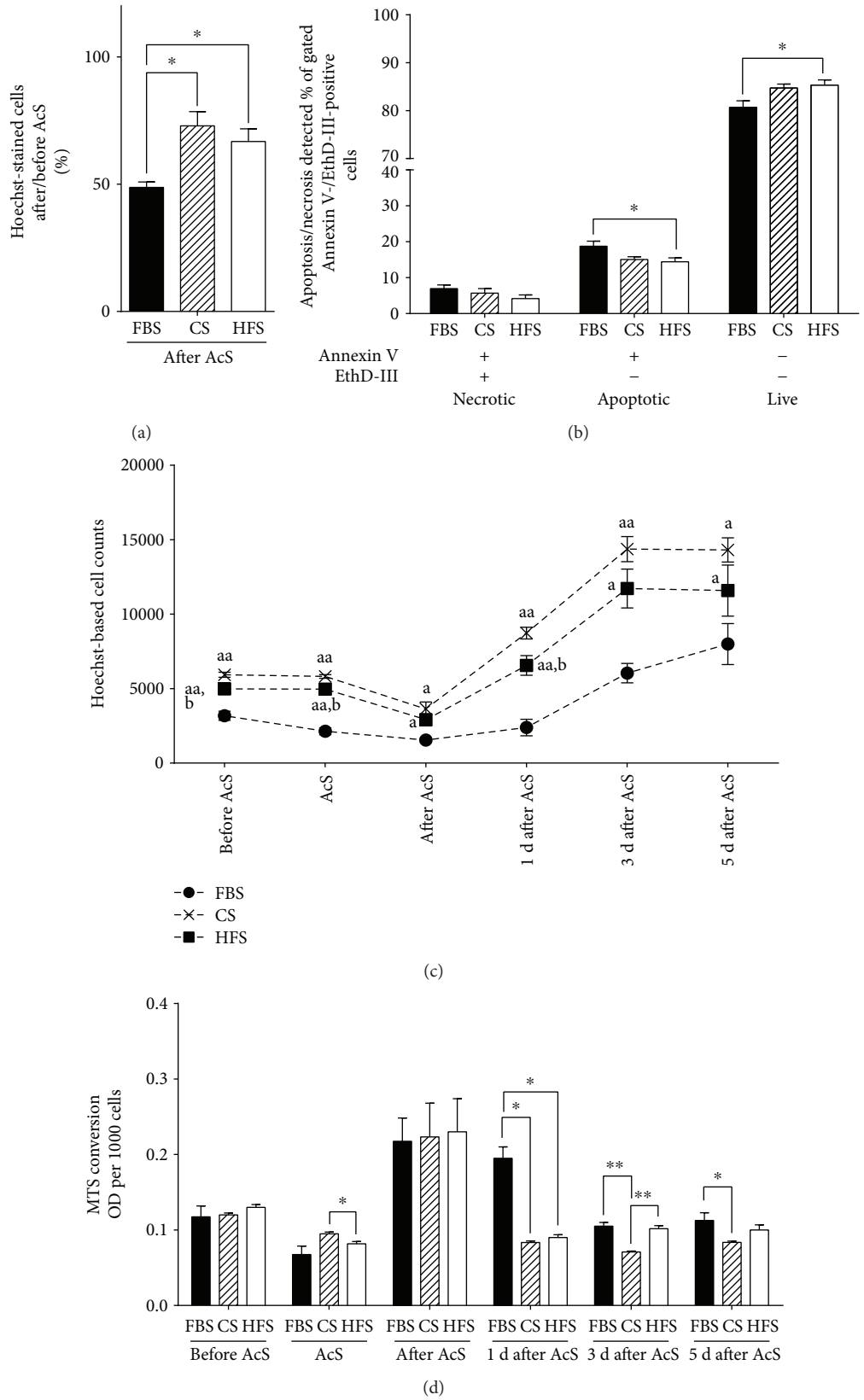


Figure 5: Overview of survival and recovery of CB-MSCs after HFS and CS pretreatment and AcS. Survival rates (a) were calculated by normalizing to the cell numbers before AcS. Percentages of apoptotic, necrotic, and live cells (b) were quantified by flow cytometry. CB-MSC recovery was assessed by cell counting for five days (c). Metabolic activity was measured throughout days 0, 1, 3, and 5 after AcS (d). $^{*}p < 0.05$ and $^{**}p < 0.01$; $^{a}p < 0.05$ and $^{aa}p < 0.05$ compared to the corresponding FBS groups and $^{b}p < 0.05$ HFS groups compared to the corresponding CS groups.

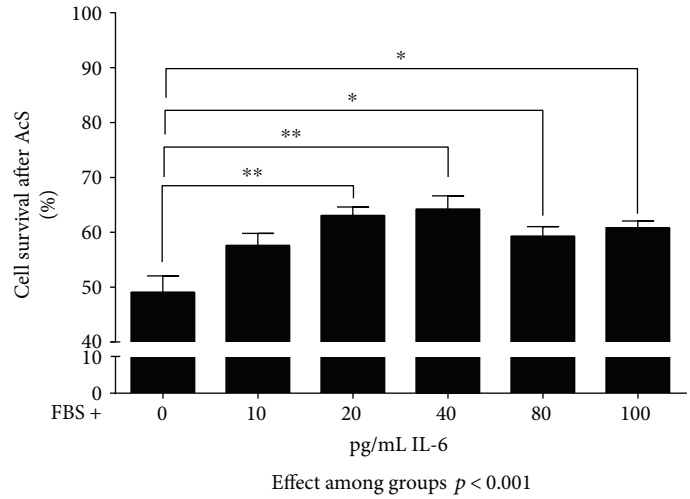


Figure 6: Preliminary experiments to establish the appropriate IL-6 concentration for *in vitro* testing. Cells were seeded at a density of $3 \times 10^3/\text{cm}^2$. After 1 day in cell culture, cells were subjected to 4-hour simulated acute "ischemia" and 4-hour reoxygenation (AcS). Cell numbers were quantified by Hoechst-based nucleated cell counts. Cell survival was calculated by dividing cell numbers from experimental groups by control group. $^{**}p < 0.001$ and $^{*}p < 0.05$.

signaling pathway [39], and HIF-1a exhibits a protective effect on cells under hypoxic stress [40]. Also, IL-6 was shown to increase cell survival under hypoxia and other different conditions [41, 42]. Thus, we hypothesized that pretreatment of IL-6 increases the resistance of CB-MSCs to "ischemic" stress. We first performed preliminary experiments for treating CB-MSCs with increasing concentrations of recombinant human IL-6, followed by exposure to "acute stress." As shown in Figure 6, a concentration of 40 pg/mL IL-6 indeed improved the survival of CB-MSCs when compared to the FBS control. However, the protective effects proved not to be statistically significant in the subsequent serial experiments over time (Figure 7). There was no significant difference in survival rates between FBS plus IL-6 and FBS immediately at the end of "simulated ischemia" (Figure 7(a)). Necrotic and apoptotic cells did not decrease, and live cells did not increase based on Annexin V/EthD-III stainings (Figure 7(b)). Similarly, there was no evidence of an improved MTS conversion rate in response to IL-6 (Figure 7(d)). In fact, total cell counts appeared to be lower in the presence of IL-6 on days 3 and 5 after AcS (Figure 7(c)).

3.6. Transcriptional Profiling. In order to better understand the pathways regulating apoptosis and cell cycle that are affected during acute stress under the influence of the different sera, we performed an RT-PCR array with panels of mRNAs coding for the proteins involved in apoptosis and cell cycle pathways (Figure 8). Our analysis suggests a differential expression of mRNAs related to the DNA damage response and induction of cell cycle arrest during the time course after acute injury. While the expression patterns remained similar in all the groups even 4 hours after AcS, proapoptotic genes like BNIP3 (BCL2-interacting protein 3) and antiproliferative genes like p15 (CDKN2B, cyclin-dependent kinase inhibitor 2B) were downregulated during the 5 days following AcS. Downregulation of BNIP3 in CB-MSCs during hypoxic preconditioning has been associated with an increased survival in response to stress via activation of BCL2 (B-cell lymphoma 2), which also showed an increased expression in HFS- and CS-treated cells on days 1, 3, and 5 [43]. Interestingly, CB-MSCs cultured in HFS, CS, and FBS alike demonstrated an increased response in growth arrest and DNA damage-inducible protein upregulation (GADD45A, growth arrest, and DNA damage-inducible 45 alpha) 4 hours after AcS. Consequently, the expression levels decreased in the CS and HFS cells, while the expression levels remained at a higher level than before AcS in the FBS-treated cells. The GADD45 family is a key player in stress-induced cell arrest, for example, through oxidative stress or DNA damage [44]. The lower levels of cell proliferation, observed in CB-MSCs treated with FBS, may in part be explained by the continued GADD45A-induced cell arrest after AcS.

As mentioned in the previous section, the increased yield of CB-MSCs cultured with human serum could only in part be explained by the increased proliferation. After AcS, we observed significantly lower cell loss and decrease in apoptosis in the cells treated with human serum. The array analysis confirmed the activation of expression of multiple antiapoptotic mRNAs in HFS- and CS-treated cells, especially at the early time points after AcS. The most prominent was an increase in the expression of TRAF1 (TNF receptor-associated factor 1) and TRAF2 as well as mRNA expression of the BIRC-family. By binding the TRAF1/2 complex to the intracellular domain of the TNF receptor, TRADD (tumor necrosis factor receptor type 1-associated DEATH domain protein) inhibits the activation of caspases [45]. BIRC2 (baculoviral IAP repeat containing) and BIRC3 are also known mediators of apoptosis suppression which also interact with the TNF-mediated activation of caspases [46]. These data suggest that the decrease in cell death, observed in CB-MSCs when cultured in human serum, is due to a

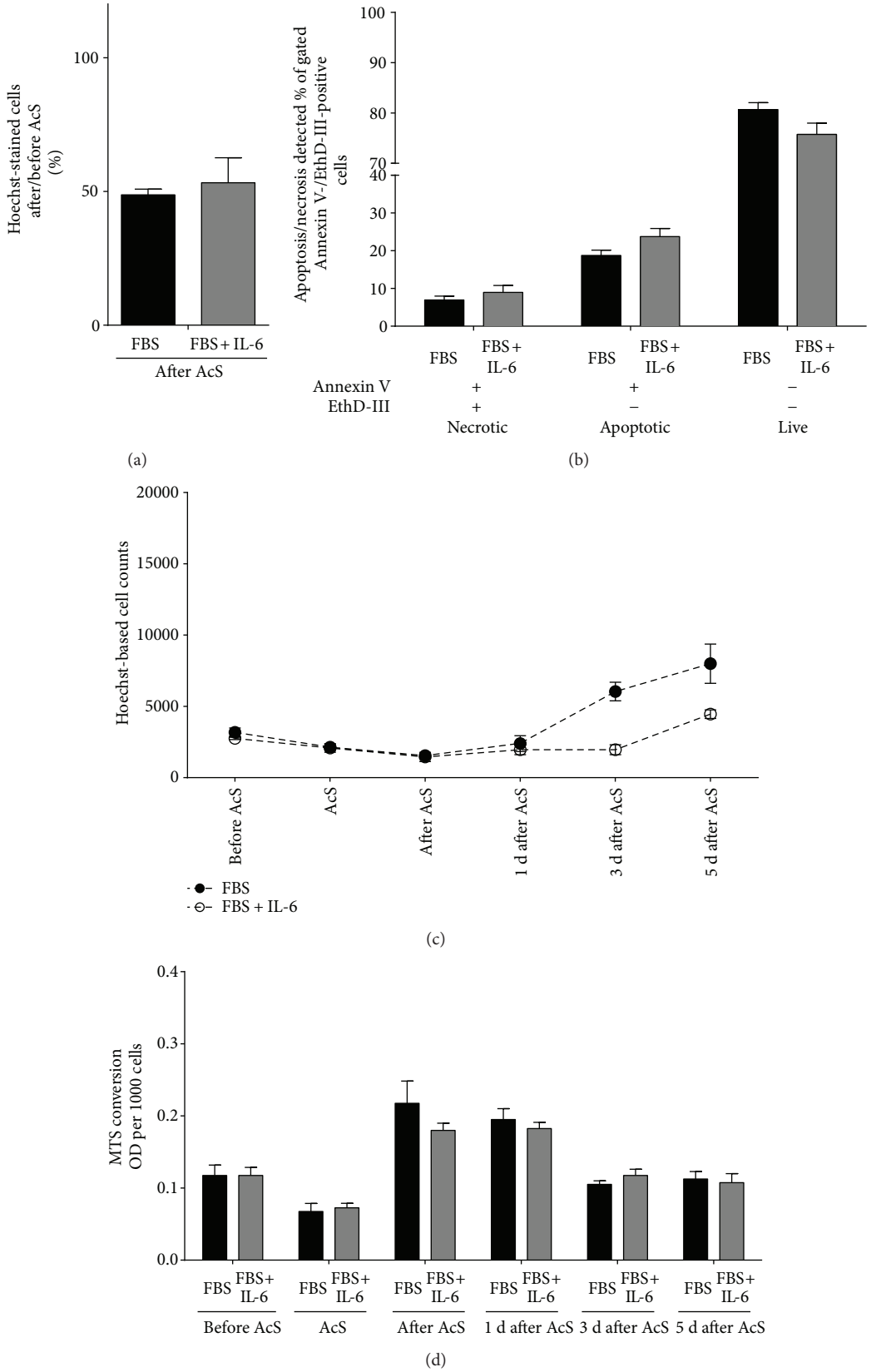


Figure 7: Survival and recovery of CB-MSCs after treatment with 40 pg/mL of human recombinant IL-6 protein and AcS. Survival rates (a) were calculated by normalizing to the cell numbers before AcS. Ratios of apoptotic, necrotic, and live cells (b) were quantified by flow cytometry. Cell numbers (c) and metabolic activity (d) were measured one day before AcS. Measurements were repeated 4 hours after AcS and at days 1, 3, and 5 after AcS.

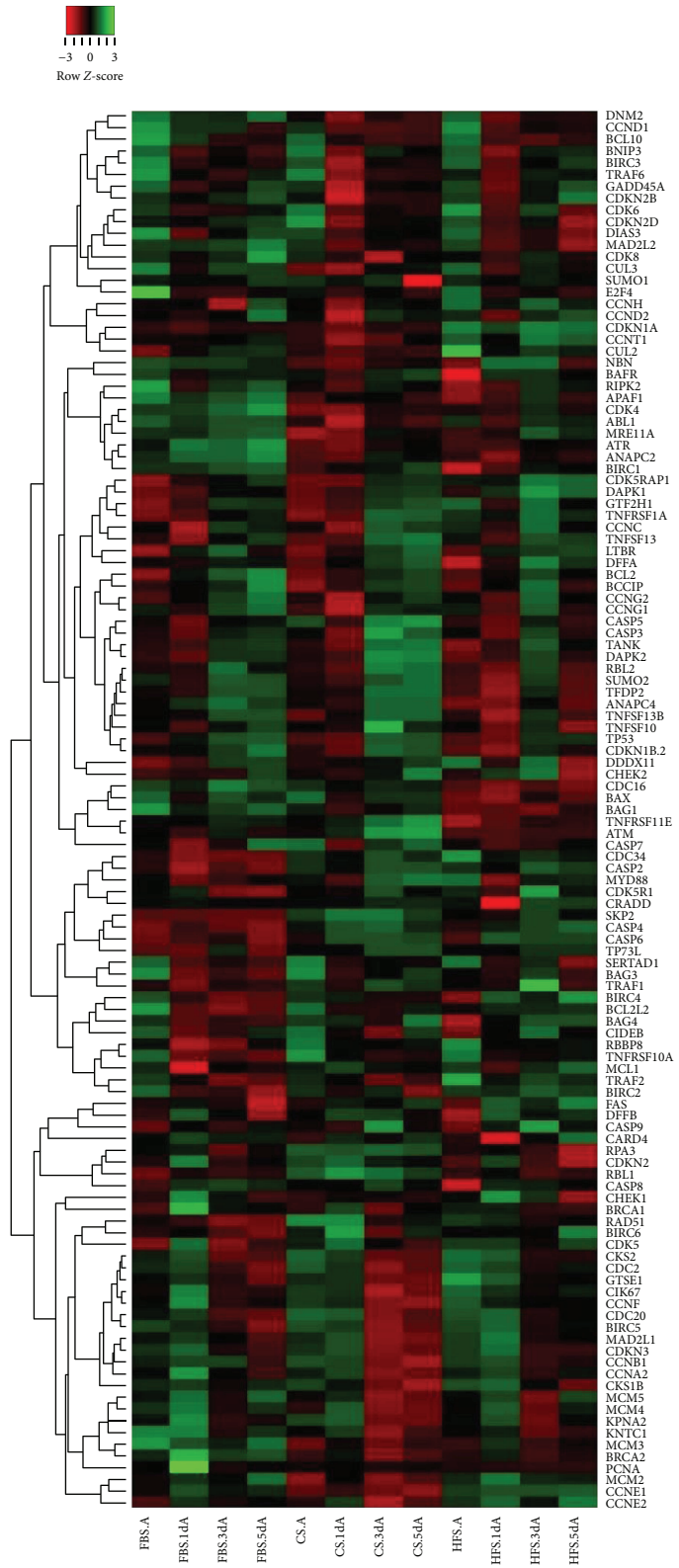


Figure 8: Gene regulation of CB-MSCs under FBS, HFS, and CS cultivation through 5 days of recovery after AcS. The fold changes of mRNA expression relative to unexposed cells of 119 genes were included in this heatmap, which was constructed by using average linkage hierarchical clustering. Gene list was sorted, and distances among groups were calculated by Spearman rank correlation method. Relative regulation levels were represented by different colors, which reflected the row z-score shown in the red-green key.

suppression of TNF-mediated activation of apoptosis. Accordingly, the PCR data also indicated that further downstream in the mitochondrial apoptosis pathway, one of the important mediators, APAF1 (apoptotic protease activating factor 1), is differentially expressed between FBS and human serum, with higher expressions post-AcS in FBS-treated CB-MSCs [47].

4. Limitations

Clearly, our *in vitro* hypoxia/reoxygenation model mirrors the situation in myocardial ischemia incompletely, but cell behavior cannot be directly observed in respective *in vivo* experimental models. The culture period of 5 days is short; in cell products requiring longer MSC expansion, the impact of the different sera may differ. We chose this period because we were interested in primary CB-MSC proliferative behavior and stress response. Because cells usually reached confluence at day 5, passaging/subcultivation would be required for longer periods, impeding direct readouts regarding cell number and so on. The average serum donor age differed by roughly 10 years between the CS and HFS groups. This may have impacted the biologic activity of the sera. However, given that both groups were more or less middle-aged while the cells were neonatal, this confounding factor should be of little relevance. Finally, it may be argued that FBS is a pooled product, while the human sera were used individually. We chose not to pool the human sera because we were interested in the heterogeneity of serum activity and because in the clinical setting, autologous human serum would obviously not be pooled. Taken together, we feel that our data support the concept of using autologous serum for cardiac cell therapy, but other experimental designs would be required to understand biology and therapeutic relevance in greater detail.

5. Conclusions

At odds with current clinical practice, human serum for MSC expansion in 2D culture is not only equivalent but also may even be superior to FBS in terms of proliferative capacity and resilience to acute and chronic "ischemic" stress. This is also the case when potentially autologous serum from patients with advanced heart failure is used. Using autologous serum simplifies GMP grade translational cell expansion, ought to reduce costs, avoids potential problems with xenogenic biomolecules, and may even have positive preconditioning effects on therapeutic cells.

Conflicts of Interest

The authors of this manuscript have no conflict of interest to declare.

Authors' Contributions

Timo Z. Nazari-Shafti and Zhiyi Xu contributed equally to this work.

Acknowledgments

The authors would like to thank Dr. Harald Stachelscheid for making the Operetta High-Content Imaging System available to them and his valuable support throughout this project. This work was supported by the German Federal Ministry of Education and Research and the federal states of Berlin and Brandenburg (FKZ 1315848A, FKZ 13GW0099), as well as by the China Scholar Council.

References

[1] H. Kawada, J. Fujita, K. Kinjo et al., "Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction," *Blood*, vol. 104, no. 12, pp. 3581–3587, 2004.

[2] S.-L. Chen, W.-W. Fang, F. Ye et al., "Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction," *The American Journal of Cardiology*, vol. 94, no. 1, pp. 92–95, 2004.

[3] Q. Mao, C.-X. Lin, X.-L. Liang, J.-S. Gao, and B. Xu, "Mesenchymal stem cells overexpressing integrin-linked kinase attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions," *Molecular Medicine Reports*, vol. 7, no. 5, pp. 1617–1623, 2013.

[4] D. Santos Nascimento, D. Mosqueira, L. M. Sousa et al., "Human umbilical cord tissue-derived mesenchymal stromal cells attenuate remodeling after myocardial infarction by proangiogenic, antiapoptotic, and endogenous cell-activation mechanisms," *Stem Cell Research & Therapy*, vol. 5, no. 1, p. 5, 2014.

[5] V. Dayan, G. Yannarelli, P. Filomeno, and A. Keating, "Human mesenchymal stromal cells improve scar thickness without enhancing cardiac function in a chronic ischaemic heart failure model," *Interactive Cardiovascular and Thoracic Surgery*, vol. 14, no. 5, pp. 516–520, 2012.

[6] S. Montanari, V. Dayan, G. Yannarelli et al., "Mesenchymal stromal cells improve cardiac function and left ventricular remodeling in a heart transplantation model," *The Journal of Heart and Lung Transplantation*, vol. 34, no. 11, pp. 1481–1488, 2015.

[7] C.-B. Liu, H. Huang, P. Sun et al., "Human umbilical cord-derived mesenchymal stromal cells improve left ventricular function, perfusion, and remodeling in a porcine model of chronic myocardial ischemia," *Stem Cells Translational Medicine*, vol. 5, no. 8, pp. 1004–1013, 2016.

[8] J. H. Traverse, T. D. Henry, D. E. Vaughn et al., "Rationale and design for TIME: a phase II, randomized, double-blind, placebo-controlled pilot trial evaluating the safety and effect of timing of administration of bone marrow mononuclear cells after acute myocardial infarction," *American Heart Journal*, vol. 158, no. 3, pp. 356–363, 2009.

[9] M. Mielewczik, G. D. Cole, A. N. Nowbar et al., "The C-CURE randomized clinical trial (cardiopoietic stem cell therapy in heart failURE)," *Journal of the American College of Cardiology*, vol. 62, no. 25, p. 2453, 2013.

[10] J. Bartunek, A. Terzic, B. A. Davison et al., "Cardiopoietic cell therapy for advanced ischaemic heart failure: results at 39 weeks of the prospective, randomized, double blind, sham-controlled CHART-1 clinical trial," *European Heart Journal*, vol. 38, no. 9, pp. ehw543–ehw560, 2017.

[11] T. D. Henry, J. H. Traverse, B. L. Hammon et al., "Safety and efficacy of ixmyelocel-T: an expanded, autologous multicellular therapy, in dilated cardiomyopathy," *Circulation Research*, vol. 115, no. 8, pp. 730–737, 2014.

[12] I. Hare, M. Gencheva, R. Evans et al., "*In vitro* expansion of bone marrow derived mesenchymal stem cells alters DNA double strand break repair of etoposide induced DNA damage," *Stem Cells International*, vol. 2016, Article ID 8270464, 11 pages, 2016.

[13] A. Shahdadfar, K. Frønsdal, T. Haug, F. P. Reinholt, and J. E. Brinchmann, "In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability," *Stem Cells*, vol. 23, no. 9, pp. 1357–1366, 2005.

[14] S. Mimura, N. Kimura, M. Hirata et al., "Growth factor-defined culture medium for human mesenchymal stem cells," *The International Journal of Developmental Biology*, vol. 55, no. 2, pp. 181–187, 2011.

[15] H. Mao, S. M. Kim, M. Ueki, and Y. Ito, "Serum-free culturing of human mesenchymal stem cells with immobilized growth factors," *Journal of Materials Chemistry. B, Materials for Biology and Medicine*, vol. 5, no. 5, pp. 928–934, 2017.

[16] R. Sawada, T. Yamada, T. Tsuchiya, and A. Matsuoka, "Microarray analysis of the effects of serum-free medium on gene expression changes in human mesenchymal stem cells during the in vitro culture," *Yakugaku Zasshi*, vol. 130, no. 10, pp. 1387–1393, 2010.

[17] P. Swamynathan, P. Venugopal, S. Kannan et al., "Are serum-free and xeno-free culture conditions ideal for large scale clinical grade expansion of Wharton's jelly derived mesenchymal stem cells? A comparative study," *Stem Cell Research & Therapy*, vol. 5, no. 4, p. 88, 2014.

[18] H. Agata, N. Watanabe, Y. Ishii et al., "Feasibility and efficacy of bone tissue engineering using human bone marrow stromal cells cultivated in serum-free conditions," *Biochemical and Biophysical Research Communications*, vol. 382, no. 2, pp. 353–358, 2009.

[19] K. Tateishi, W. Ando, C. Higuchi et al., "Comparison of human serum with fetal bovine serum for expansion and differentiation of human synovial MSC: potential feasibility for clinical applications," *Cell Transplantation*, vol. 17, no. 5, pp. 549–557, 2008.

[20] A. Nimura, T. Muneta, H. Koga et al., "Increased proliferation of human synovial mesenchymal stem cells with autologous human serum: comparisons with bone marrow mesenchymal stem cells and with fetal bovine serum," *Arthritis & Rheumatism*, vol. 58, no. 2, pp. 501–510, 2008.

[21] K. Klose, R. Roy, A. Brodarac et al., "Impact of heart failure on the behavior of human neonatal stem cells in vitro," *Journal of Translational Medicine*, vol. 11, no. 1, p. 236, 2013.

[22] H. Kubo, K. Takamura, N. Nagaya, and H. Ohgushi, "The effect of serum on the proliferation of bone marrow-derived mesenchymal stem cells from aged donors and donors with or without chronic heart failure," *Journal of Tissue Engineering and Regenerative Medicine*, 2017.

[23] T. Nagamura-Inoue and H. He, "Umbilical cord-derived mesenchymal stem cells: their advantages and potential clinical utility," *World Journal Stem Cells*, vol. 6, no. 2, pp. 195–202, 2014.

[24] Z. Wang, L. Wang, X. Su, J. Pu, M. Jiang, and B. He, "Rational transplant timing and dose of mesenchymal stromal cells in patients with acute myocardial infarction: a meta-analysis of randomized controlled trials," *Stem Cell Research & Therapy*, vol. 8, no. 1, p. 21, 2017.

[25] B. Liu, C.-Y. Duan, C.-F. Luo et al., "Effectiveness and safety of selected bone marrow stem cells on left ventricular function in patients with acute myocardial infarction: a meta-analysis of randomized controlled trials," *International Journal of Cardiology*, vol. 177, no. 3, pp. 764–770, 2014.

[26] A. Abdel-Latif, R. Bolli, I. M. Tleyjeh et al., "Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis," *Archives of Internal Medicine*, vol. 167, no. 10, pp. 989–997, 2007.

[27] K. B. Choo, L. Tai, K. S. Hymavathee et al., "Oxidative stress-induced premature senescence in Wharton's jelly-derived mesenchymal stem cells," *International Journal of Medical Sciences*, vol. 11, no. 11, pp. 1201–1207, 2014.

[28] K. Bieback, S. Kern, H. Klüter, and H. Eichler, "Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood," *Stem Cells*, vol. 22, no. 4, pp. 625–634, 2004.

[29] A. Laitinen, M. Lampinen, S. Liedtke et al., "The effects of culture conditions on the functionality of efficiently obtained mesenchymal stromal cells from human cord blood," *Cytotherapy*, vol. 18, no. 3, pp. 423–437, 2016.

[30] M. Pietilä, S. Palomäki, S. Lehtonen et al., "Mitochondrial function and energy metabolism in umbilical cord blood- and bone marrow-derived mesenchymal stem cells," *Stem Cells and Development*, vol. 21, no. 4, pp. 575–588, 2012.

[31] D. Zhang, X. Xiang, L. Chen, J. Wang, Z. Yubin, and J. Wang, "The study of effects of different aged serum on mesenchymal stem cells aging," *Chinese Journal of Cell Biology*, vol. 33, pp. 1109–1115, 2011.

[32] I. M. Conboy, M. J. Conboy, A. J. Wagers, E. R. Girma, I. L. Weissman, and T. A. Rando, "Rejuvenation of aged progenitor cells by exposure to a young systemic environment," *Nature*, vol. 433, no. 7027, pp. 760–764, 2005.

[33] H.-W. Snoeck, "Serum of youth?," *Nature Biotechnology*, vol. 23, no. 4, pp. 434-435, 2005.

[34] P. W. Hochachka, "Metabolic arrest," *Intensive Care Medicine*, vol. 12, no. 3, pp. 127–133, 1986.

[35] R. G. Boutilier, "Mechanisms of cell survival in hypoxia and hypothermia," *Journal of Experimental Biology*, vol. 204, Part 18, pp. 3171–3181, 2001.

[36] T. Kalogeris, C. P. Baines, M. Krenz, and R. J. Korthuis, "Cell biology of ischemia/reperfusion injury," *International Review of Cell and Molecular Biology*, vol. 298, pp. 229–317, 2012.

[37] M. T. Lin, C. Y. Juan, K. J. Chang, W. J. Chen, and M. L. Kuo, "IL-6 inhibits apoptosis and retains oxidative DNA lesions in human gastric cancer AGS cells through up-regulation of anti-apoptotic gene mcl-1," *Carcinogenesis*, vol. 22, no. 12, pp. 1947–1953, 2001.

[38] L. Tamariz and J. M. Hare, "Inflammatory cytokines in heart failure: roles in aetiology and utility as biomarkers," *European Heart Journal*, vol. 31, no. 7, pp. 768–770, 2010.

[39] M. S. Anglesio, J. George, H. Kulbe et al., "IL6-STAT3-HIF signaling and therapeutic response to the angiogenesis inhibitor sunitinib in ovarian clear cell cancer," *Clinical Cancer Research*, vol. 17, no. 8, pp. 2538–2548, 2011.

[40] G. Nanayakkara, A. Alasmari, S. Mouli et al., "Cardioprotective HIF-1α-frataxin signaling against ischemia-reperfusion injury," *American Journal of Physiology Heart and Circulatory Physiology*, vol. 309, no. 5, pp. H867–H879, 2015.

[41] J. A. Westberg, M. Serlachius, P. Lankila, M. Penkowa, J. Hidalgo, and L. C. Andersson, "Hypoxic preconditioning induces neuroprotective stanniocalcin-1 in brain via IL-6 signaling," *Stroke*, vol. 38, no. 3, pp. 1025–1030, 2007.

[42] S. Grivennikov, E. Karin, J. Terzic et al., "IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer," *Cancer Cell*, vol. 15, no. 2, pp. 103–113, 2009.

[43] E. Martin-Rendon, S. J. M. Hale, D. Ryan et al., "Transcriptional profiling of human cord blood $CD133^{+}$ and cultured bone marrow mesenchymal stem cells in response to hypoxia," *Stem Cells*, vol. 25, no. 4, pp. 1003–1012, 2007.

[44] A. J. Fornace, J. Jackman, M. C. Hollander, B. Hoffman-Liebermann, and D. A. Liebermann, "Genotoxic-stress-response genes and growth-arrest genes: gadd, MyD, and other genes induced by treatments eliciting growth arrest," *Annals of the New York Academy of Sciences*, vol. 663, no. 1, pp. 139–153, 1992.

[45] X. Li, Y. Yang, and J. D. Ashwell, "TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2," *Nature*, vol. 416, no. 6878, pp. 345–347, 2002.

[46] P. Liston, N. Roy, K. Tamai et al., "Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes," *Nature*, vol. 379, no. 6563, pp. 349–353, 1996.

[47] D. Dakhlallah, J. Zhang, L. Yu, C. B. Marsh, M. G. Angelos, and M. Khan, "MicroRNA-133a engineered mesenchymal stem cells augment cardiac function and cell survival in the infarct heart," *Journal of Cardiovascular Pharmacology*, vol. 65, no. 3, pp. 241–251, 2015.

Hindawi
Stem Cells International
Volume 2018, Article ID 3814747, 14 pages
https://doi.org/10.1155/2018/3814747

Research Article

A Loss of Function Screen of Epigenetic Modifiers and Splicing Factors during Early Stage of Cardiac Reprogramming

Yang Zhou[1,2], **Sahar Alimohamadi**[1,2], **Li Wang**[1,2], **Ziqing Liu**[1,2], **Joseph B. Wall**[1,2], **Chaoying Yin**[1,2], **Jiandong Liu**[1,2] and **Li Qian**[1,2]

[1]*Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC 27599, USA*
[2]*McAllister Heart Institute, University of North Carolina, Chapel Hill, NC 27599, USA*

Correspondence should be addressed to Li Qian; li_qian@med.unc.edu

Received 22 July 2017; Accepted 4 December 2017; Published 18 March 2018

Academic Editor: Hossein A. Ghofrani

Direct reprogramming of cardiac fibroblasts (CFs) to induced cardiomyocytes (iCMs) is a newly emerged promising approach for cardiac regeneration, disease modeling, and drug discovery. However, its potential has been drastically limited due to the low reprogramming efficiency and largely unknown underlying molecular mechanisms. We have previously screened and identified epigenetic factors related to histone modification during iCM reprogramming. Here, we used shRNAs targeting an additional battery of epigenetic factors involved in chromatin remodeling and RNA splicing factors to further identify inhibitors and facilitators of direct cardiac reprogramming. Knockdown of RNA splicing factors Sf3a1 or Sf3b1 significantly reduced the percentage and total number of cardiac marker positive iCMs accompanied with generally repressed gene expression. Removal of another RNA splicing factor Zrsr2 promoted the acquisition of CM molecular features in CFs and mouse embryonic fibroblasts (MEFs) at both protein and mRNA levels. Moreover, a consistent increase of reprogramming efficiency was observed in CFs and MEFs treated with shRNAs targeting Bcor (component of BCOR complex superfamily) or Stag2 (component of cohesin complex). Our work thus reveals several additional epigenetic and splicing factors that are either inhibitory to or required for iCM reprogramming and highlights the importance of epigenetic regulation and RNA splicing process during cell fate conversion.

1. Introduction

Mammalian hearts have limited ability to regenerate, thus deleterious insult such as myocardial infarction (MI) can result in a permanent loss of cardiomyocytes (CMs) and a progressive decline in heart function [1]. So far, there is limited treatment to fully restore heart function after cardiac injury, ultimately leading to heart failure that becomes the leading cause of death worldwide. Recently, several promising strategies emerged to replenish the lost endogenous CMs or replace the malfunctioning CMs, including the ones using autologous sources of CMs derived from cardiac progenitor/stem cells, pluripotent stem cell, or directly induced cardiomyocytes (iCMs) [2]. Among them, direct reprogramming of fibroblasts into iCMs has been vigorously pursued in recent years, because of its feasibility both in vitro and in vivo and its unique process without passing through a pluripotent or progenitor stage, which can potentially avoid the risk of tumorigenesis. It was first reported that three master transcription factors, Gata4, Mef2c, and Tbx5, are capable of directly converting mouse cardiac fibroblasts (CFs) into iCMs in vitro [3]. Subsequently, generation of iCMs in vivo became possible in a murine MI model, resulting in functional improvement and scar size reduction [4, 5]. Thereafter, a growing number of studies have been performed focusing on alternative cocktails that could improve efficiency and/or purity of iCMs [4, 6–16] and began to reveal the underlying molecular mechanisms during iCM reprogramming [17–22]. Despite these advances, the potential of iCM approach to be used on patients is still limited because of

the relatively low efficiency and largely unknown molecular mechanisms, which have to be fully elucidated before future clinical implementation.

Epigenetics is defined as stable and heritable changes in gene expression or cellular phenotype that does not involve changes in DNA sequence [23, 24]. Although the cell fate conversion requires instructive cues via ectopic expression of master transcription factors, the successful reprogramming relies on and can be greatly enhanced by epigenetic modification that is necessary for establishing and maintaining altered gene expression patterns over rounds of cell division. As such, epigenetic regulation is critical for cellular reprogramming as elaborated in other direct reprogramming processes [25]. We and others have shown that repatterning of H3K27me3, H3K4me3, and DNA methylation is accompanied with alternation in gene transcription during early stage of cardiac reprogramming from fibroblasts [3, 17, 19, 26], and removal of epigenetic barriers associated with histone modifications, such as Bmi1 and Mll1, significantly improved quantity and quality of iCMs [18, 21]. However, besides histone modifications and DNA methylation, the epigenetic processes that stably sustain gene expression also include chromatin remodeling and various RNA-mediated processes, and the role of the related epigenetic regulators remains largely unknown in direct cardiac reprogramming. Recent studies on heart development and cellular reprogramming demonstrated that the coordination of transcription factors and chromatin remodeling is critical for cell fate determination and conversion [25, 27, 28]. Therefore, despite what has been studied, it is important to identify key chromatin remodeling-related epigenetic regulators that orchestrate iCM induction. Characterization of each epigenetic modulator will help understand how cells with identical DNA reprogrammed into different lineages and delineate the role of epigenetic barriers and facilitators involved in not only iCM reprogramming but also maybe other cellular reprogramming processes.

RNA splicing is increasingly being recognized as an important layer of posttranslational gene regulation in the heart [29]. For instance, splicing factor Sf3b1, a component of U2 snRNPS involved in both constitutive and alternative splicing, is dysregulated in human and mouse models of pathological cardiac hypertrophy [30]. Moreover, reversion of global splicing pattern has been demonstrated to occur during somatic cell reprogramming [31]. Removal of splicing factors U2af1 and Srsf3 decreased reprogramming efficiency of induced pluripotent stem cells (iPSCs) [31]. Of note, we recently found that removal of alternative splicing factor Ptbp1 significantly promoted cardiac fate conversion from fibroblasts [22]. These studies highlighted the regulation of RNA splicing as part of the mechanisms underlying cellular reprogramming and pathogenesis of heart disease and implied the potential key role of RNA splicing factors for iCM reprogramming. Thus, identifying functional splicing factors during direct cardiac reprogramming will provide further insight into our understanding of the molecular mechanisms underlying iCM induction.

Here, we screened epigenetic modulators associated with different complexes and core splicing factors by shRNA-mediated loss of function assays and identified splicing factors Sf3a1 and Sf3b1 that are required for direct cardiac reprogramming. Meanwhile, the removal of another splicing factor Zrsr2 enhanced generation of iCMs from CFs and MEFs. Additional two epigenetic regulators, Bcor and Stag2, were implicated as independent epigenetic inhibitors to iCM reprogramming. These findings provide additional insights into the critical roles of epigenetic modulators and splicing factors on direct cardiac lineage conversion and the basis for future further investigation of epigenetic and RNA splicing-related mechanisms underlying reprogramming.

2. Materials and Methods

2.1. Mouse Lines. The transgenic mice carrying αMHC-GFP reporter were used for isolation of cardiac fibroblasts (CFs) and mouse embryonic fibroblasts (MEFs) [3, 5]. All mouse protocols were approved by the Institutional Animal Care and Use Committee (IACUC), University of North Carolina, Chapel Hill. Animal care was performed in accordance with the guidelines established by the University of North Carolina, Chapel Hill.

2.2. Plasmids. The polycistronic construct pMXs-puro-MGT was constructed as previously described [14]. The plasmid map of pMXs-puro-MGT is provided in Figure S1. shRNA lentiviral vectors with pLKO.1 backbone were obtained from Sigma-Aldrich. Packaging and envelop vectors for lentivirus were psPAX2 and pMD2.G (Addgene).

2.3. Virus Packaging and Transduction. PlatE cells were cultured in 293T media (10% fetal bovine serum (FBS)/1x penicillin/streptomycin (P/S)/0.1 mM nonessential amino acids (NEAA)/DMEM) (Life Technologies). Four to five million platE cells seeded onto a 10 cm dish were used for transfection. The next day, pMXs-puro-MGT were introduced into platE cells using Nanofect (Alstem) according to manufacturer's instructions. Briefly, 20 μg of pMXs-puro-MGT and 45 μl of Nanofect were mixed with 500 μl of DMEM in separate tubes, and the mixture was combined and vortexed for a few seconds. After 15 minutes of incubation at room temperature, 1 ml of total mixture was added dropwise to platE cells. Fresh 293T medium without P/S was replaced before transfection. 16 hours posttransfection, media were changed with regular 293T media. Supernatant containing retroviruses was collected 48 and 72 hours after transfection, filtered through a 0.45 μm filter (Thermo Scientific), and incubated with 8% of PEG6000 (Sigma) at 4°C overnight. Viral particles were pelleted with centrifuge at 3900 rpm for 30 minutes at 4°C and resuspended with 100 μl of DMEM. For CFs or MEFs in one well of 24 well plate, 10 μl of pMX-puro-MGT supplemented with 4 μg/ml polybrene (Life Technologies) was added for cardiac reprogramming.

Five million 293T cells seeded and cultured overnight in one 10 cm dish with 293T media were used for lentiviral packaging. 10 μg of pLKO.1 mixture with shRNAs (equal amount) targeting one gene, 7 μg of psPAX2, and 3 μg of pMD2.G were mixed in 500 μl of DMEM. The DNA mixture was combined with transfection reagent mixture containing

45 μl of Nanofect and 500 μl of DMEM, vortexed, and incubated at room temperature for 20 minutes. After media exchange 16 hours posttransfection, virus-containing media were collected at 48 and 72 hours posttransfection. Filtered media were incubated with 8% PEG6000 overnight at 4°C and centrifuged at 3900 rpm, 30 minutes at 4°C to obtain viral particles. 100 μl of DMEM was used to resuspend viral particles. 10 μl of lentiviruses was used for cells seeded on each well of 24-well plate coinfected with MGT retroviruses.

2.4. Isolation of CFs. Explant and fresh isolation of CFs were performed according to the protocols described as previously [14, 32]. Briefly, hearts were dissected from postnatal 1.5 (p1.5) mice, rinsed with cold PBS, and cut into small pieces with a sterile blade. For explant CFs, small tissues were plated onto gelatin-coated dishes and cultured in fibroblast (FB) media (IMDM/20% FBS/1xPen/Strep) for 7 days. Before magnetic-activated cell sorting (MACS), explanted heart cells were trypsinized and filtered through 40 μm cell strainer (BD). To isolate fresh CFs, heart tissues were digested with 0.05% trypsin at 37°C for 10 minutes and 0.2% collagenase type II/HBSS (Life Technologies) at 37°C for 5 minutes followed by 1 minute of vortexing for 5 times. Each time, supernatant containing single cells was filtered through 40 μm cell strainer (BD) and neutralized in equal volume of FB media. Red cells were removed using red cell lysis buffer (150 ml NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA) for 1 minute on ice. Then, MACS was performed to enrich Thy1.2-positive fibroblasts. Cells were incubated with 10 μl of biotin anti-Thy1.2 antibody (Biolegend) in FACS buffer (DPBS/2% FBS/2 mM EDTA) for 30 minutes at 4°C and then with 10 μl of Anti-Biotin Microbeads (Miltenyi Biotec.) in MACS buffer (DPBS/0.5% BSA/2 mM EDTA) at 4°C for 30 minutes. After that, cells were washed and resuspended in MACS buffer and applied to calibrated LS column (Miltenyi Biotec.). Thy1.2-positive cells were flushed out and seeded for reprogramming.

2.5. Preparation of MEFs. MEFs were isolated from E13.5 αMHC-GFP pups from CD1 strain as previously described [33]. Briefly, embryos isolated from pregnant mice at E13.5 were dissected out heads and red organs and then mined with a sterile blade. Small tissues were dissociated in 1 ml of 0.05% trypsin/EDTA supplemented with 100 units of DNase I for 15 minutes at 37°C and then neutralized in MEF media (10%FBS/1XP/S/DMEM). After centrifuge, the cells were resuspended into MEF media and plated into gelatin-coated dishes. Then, MEFs with a low passage number ($n = 2$–6) were used for iCM reprogramming assays.

2.6. Direct Cardiac Reprogramming. ExCFs, fCFs, and MEFs were seeded onto gelatin-coated wells of 24-well plates at a cell density of 2×10^4 one day before infection. iCM media (10% FBS/20% M199/DMEM) with 10 μl of retroviral puro-MGT, 10 μl of lentiviral shRNAs, and 4 μg/ml polybrene were replaced for FB media at reprogramming day 0. iCM media with 1 μg/ml puromycin were used at day 3 and replaced by regular iCM media at day 6. At day 10, reprogramming cells were collected in TRIzol for RNA extraction or fixed with 4% paraformaldehyde (PFA) for immunostaining (Figure 1(a)).

2.7. Flow Cytometry and Immunocytochemistry (ICC). For flow cytometry, reprogrammed cells were trypsinized with 0.05% trypsin/EDTA (Life Technologies), fixed with Fixation/Permeabilization Solution (BD Biosciences) for 30 minutes at 4°C. Perm/Wash Solution (BD Bioscience) was used for wash between each step. Cells were incubated with primary antibodies (GFP, 1 : 500, Invitrogen; cTnT, 1 : 400, Thermo Scientific) diluted in BD Perm/Wash Solution for 30 minutes at 4°C and Alex Fluor 488-conjugated or Alex Fluor 647-conjugated secondary antibodies (1 : 500, Jackson ImmunoResearch Inc.) for 30 minutes at 4°C. Cells were run on Beckman Coulter CyAn ADP flow cytometer. Data analyses were performed by FlowJo software (Tree Star). For ICC, 4% PFA-fixed cells were permeablized with 0.1% Triton-X100 for 20 minutes, blocked by 5% BSA for 30 minutes at room temperature, and then incubated with primary antibody (GFP, 1 : 500, Invitrogen; αActinin, 1 : 500, Sigma-Aldrich; cTnT, 1 : 400, Thermo Scientific) at 4°C overnight and Alex Fluor 488-conjugated or Alex Fluor 647-conjugated secondary antibodies (1 : 500, Jackson ImmunoResearch Inc.) for 1 hour at room temperature. Finally, Hoechst 33342 (Life Technologies) was used to label nuclei. PBS was used for wash between each step. Images were captured using EVOS® FL Auto Cell Imaging System (Life Technologies). For the quantification of ICC, 10 images were randomly acquired under 20x magnification at the same exposure setting. Then the indicated cells were counted manually in a blind fashion.

2.8. RNA Extraction and Reverse Transcription Following Quantitative PCR (RT-qPCR). According to the manufacturer's instruction, cell lysates in TRIzol reagent (Invitrogen) were separated with chloroform. RNA in the aqueous phase was precipitated with isopropanol, pelleted with centrifuge, washed with ethanol, and eluted in DNase-free and RNase-free water. Purified RNA was quantified by Nanodrop (Thermo Scientific) and reverse-transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen). qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) on the ABI ViiA 7 Real-Time PCR system (Applied Biosystems). Additional primer sequences for RT-qPCR are provided in Supplemental Table S2.

2.9. Statistical Analyses. For each experiment, 3-4 biological replicates were examined with technical duplicates. Negative controls (i.e., mock-treated and/or nontargeting shRNA control transduced cells) and positive controls (i.e., shBmi1-transfected cells) were used in every single experiment. Average number from technical duplicates was used for statistics. For ICC, quantification was performed from 10 images randomly taken under 20x magnification at the same exposure setting in a blind fashion, and averaged numbers were used for final statistics. Where appropriate, values are presented as the mean ± SEM of replicate experiments. Statistical analyses were performed with two-way unpaired Student t-test or one-way ANOVA. * indicates significant

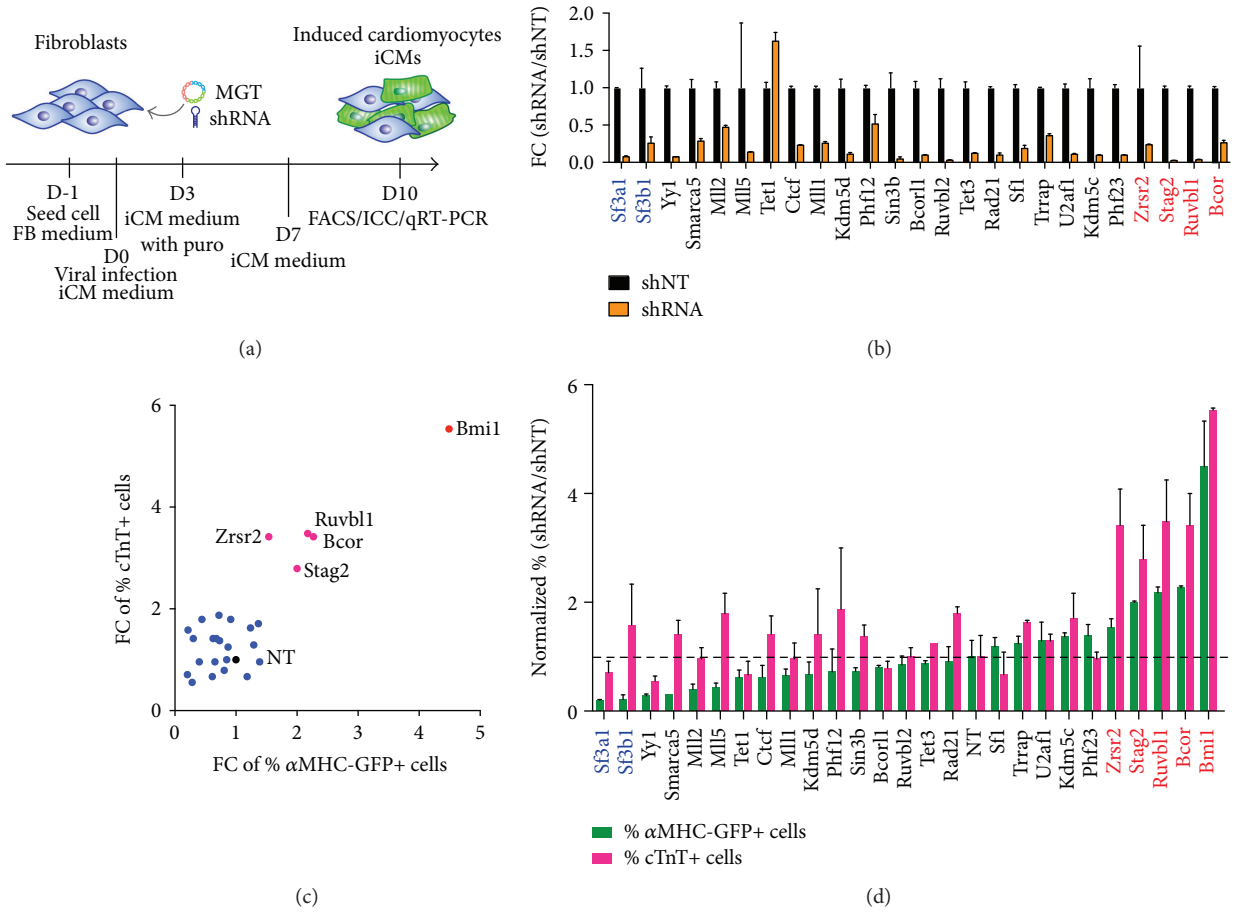


Figure 1: Knockdown of various epigenetic regulators influenced direct cardiac reprogramming. (a) Schematic of experimental design to determine the effect of candidate factors on iCM reprogramming via shRNA-mediated RNAi. FB stands for fibroblasts. (b) Knockdown efficiency of indicated shRNAs measured by RT-qPCR. Expression values were normalized to those measured in shNT-infected cells at reprogramming day 10. FC stands for fold change. (c) 25 selected chromatin modulators and splicing factors were knocked down in CFs coinfected with MGT for cardiac reprogramming. αMHC-GFP+ and cTnT+ cells were measured by flow cytometry at day 10 posttransduction. The percentage of marker positive cells was normalized to shNT-infected control cells shown in black. Bmi1 highlighted in red was used as a positive control for screening. (d) Histogram of normalized percentage of αMHC-GFP+ and cTnT+ cells after infection of MGT and shRNA lentiviruses targeting individual genes as indicated.

difference between two groups with a p value of $p < 0.05$, and $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ indicates highly significant difference.

3. Results

3.1. RNAi Screening for Epigenetic Regulators during Early Stage of Cardiac Reprogramming. We previously showed that removal of the key epigenetic barrier Bmi1 promotes the efficiency and quality of iCMs generated via transcription factor-mediated direct reprogramming [21]. In an effort to further examine the influence of additional epigenetic factors on iCM reprogramming, we used a similar loss-of-function screen to determine the role of 25 selected genes related to epigenetic modification and chromatin remodeling (Table 1). A pool of 4–6 short hairpins was used for knockdown of each gene (Table S1). We infected cardiac fibroblasts isolated using explant method (ExCFs, [3]) from αMHC-GFP transgenic pups at P1.5 with shRNA pools, subsequently transduced them with the polycistronic reprogramming vector expressing Mef2c, Gata4, and Tbx5 (MGT in short). We then determined the percentage of reprogrammed iCMs expressing αMHC-GFP and cardiac troponin T (cTnT) by flow cytometry (Figure 1(a)). Nontargeting shRNA and oligo-targeting Bmi1 were served as the negative and positive control, respectively [21]. After 10 days of infection, knockdown efficiency of shRNAs was first determined by real-time quantitative PCR (RT-qPCR) (Figure 1(b)). Then, generation of iCMs upon knockdown of various genes was scored by the fold changes relative to shNT control in the percentage of αMHC-GFP+ cells and cTnT+ cells. We found that knockdown of four genes showed a significant increase in reprogramming efficiency when compared to control shNT, but still lower than that when Bmi1 was knocked down (Figure 1(c)). Among the target genes are BCL6-interacting corepressor (*Bcor*) and RuvB-like protein 1(*Ruvbl1*), two chromatin modifiers studied in various chromatin complexes [34–38]. The remaining two shRNAs

Table 1: Categories and functional description of shRNA target genes.

shRNA target	Category	Description
Kdm5c/Jarid1c/Smcx	Epigenetic modulator	Histone H3 lysine 4 demethylase
Kdm5d/Jarid1d/Smcy	Epigenetic modulator	Histone H3 lysine 4 demethylase
Kmt2a/Mll1	Epigenetic modulator	Member of SET1/MLL complexes
Kmt2d/Mll2	Epigenetic modulator	Member of SET1/MLL complexes
Kmt2e/Mll5	Epigenetic modulator	Member of SET1/MLL complexes
Phf23	Epigenetic modulator	Redear of H3K4me3/2
Ctcf	Epigenetic modulator	Chromatin insulator
Bcorl1	Epigenetic modulator	Component of BCOR complex (subtype of PRC1, PRC1.1)
Bcor	Epigenetic modulator	Component of BCOR complex (subtype of PRC1, PRC1.1)
Rad21	Epigenetic modulator	Component of cohesin complex
Stag2/SA2	Epigenetic modulator	Component of cohesin complex
Phf12	Epigenetic modulator	Component of EMSY/KDM5A/SIN3B complex
Sin3b	Epigenetic modulator	Component of EMSY/KDM5A/SIN3B complex
Trrap	Epigenetic modulator	Component of TIP60 complex
Yy1	Epigenetic modulator	Component of INO80 complex
Ruvbl2/Tip48	Epigenetic modulator	Component of INO80 subfamily (INO80, TIP60, and SWR complexes)
Ruvbl1/Tip49	Epigenetic modulator	Component of INO80 subfamily (INO80, TIP60, and SWR complexes)
Smarca5	Epigenetic modulator	Component of ISWI complex (SWI/SNF)
Tet1	Epigenetic modulator	DNA hydroxymethyltransferase
Tet3	Epigenetic modulator	DNA hydroxymethyltransferase
Sf1	Splicing factor	Splicing factor for spliceosome assembly
Sf3a1/PRP21	Splicing factor	Splicing factor for spliceosome assembly (U2 snRNP)
Sf3b1	Splicing factor	Splicing factor for spliceosome assembly (U2 snRNP)
U2af1	Splicing factor	Splicing factor for spliceosome assembly
Zrsr2/URP	Splicing factor	Splicing factor for spliceosome assembly

targeted one of the components of cohesin complex, stromal antigen 2 (*Stag2*), and one of the splicing factors for spliceosome assembly, zinc finger (CCCH type) RNA binding motif, and serine/arginine rich 2 (*Zrsr2*). On the contrary, knockdown of several members of SET1/MLL protein family, lysine- (K-) specific methyltransferase 2A (*Mll1*/*Kmt2a*), lysine- (K-) specific methyltransferase 2D (*Mll2*/*Kmt2d*), and lysine- (K-) specific methyltransferase 2E (*Mll5*/*Kmt2e*), showed repressive effects on generation of αMHC-GFP+ iCMs, suggesting the essential roles of H3K4 methylation for cardiac reprogramming (Figure 1(d)). Interestingly, the top targets, knocking down of which caused 5-fold decreases in the percentage of αMHC-GFP+ iCMs, are splicing factor 3a, subunit 1 (*Sf3a1*), and splicing factor 3b, subunit 1 (*Sf3b1*). Both belong to splicing factors of spliceosome similarly as Zrsr2 but have opposite phenotypes upon depletion compared to deletion of Zrsr2. Taking advantage of shRNA-mediated RNAi screen, we identified various epigenetic modulators that are either required for or inhibitory to iCM reprogramming.

3.2. Impaired Cardiac Reprogramming after Knockdown of Sf3a1 or Sf3b1. Spliceosome is a complex and highly dynamic molecular machinery that recognizes the splice sites and removes introns from precursor messenger RNAs (pre-mRNAs) [39, 40]. Five small ribonuclear protein particles (snRNPs) and various accessory proteins are assembled to form the spliceosome [39, 40]. Sf3a1 and Sf3b1 are components of U2 snRNP, which stabilizes U2 snRNA binding to the branch point sequence in introns [41, 42]. The mutations of these two splicing factors are found to be associated with myelodysplastic syndrome [43]. However, it is still largely unknown about the cellular functions of Sf3a1 or Sf3b1. Through our shRNA screen, we are intrigued to find that knockdown of two splicing factors involved in spliceosome assembly led to inhibition of iCM generation. We then confirmed shRNA screen results by both flow cytometry and quantification of iCMs generated from ExCFs. At day 10 after infection of shSf3a1 or shSf3b1 lentiviruses on MGT-transduced ExCFs, the percentage of cells expressing cardiac marker, αMHC-GFP, or cTnT and positive cells dropped dramatically (Figure 2(a)). To further confirm the essential roles of Sf3a1 and Sf3b1 for iCM reprogramming, we performed knockdown experiments on freshly isolated CFs (fCFs), which seems most amenable for MGT-mediated cardiac reprogramming [21], and assessed reprogramming efficiency by flow and ICC analyses of iCM-expressing cardiac markers. Consistently, knockdown of *Sf3a1* or *Sf3b1* repressed the generation of αMHC-GFP and/or cTnT-positive iCMs derived from fCFs when compared to those from control shNT-infected CFs (Figure 2(b)). ICC images and quantifications showed similar decrease of reprogrammed cells

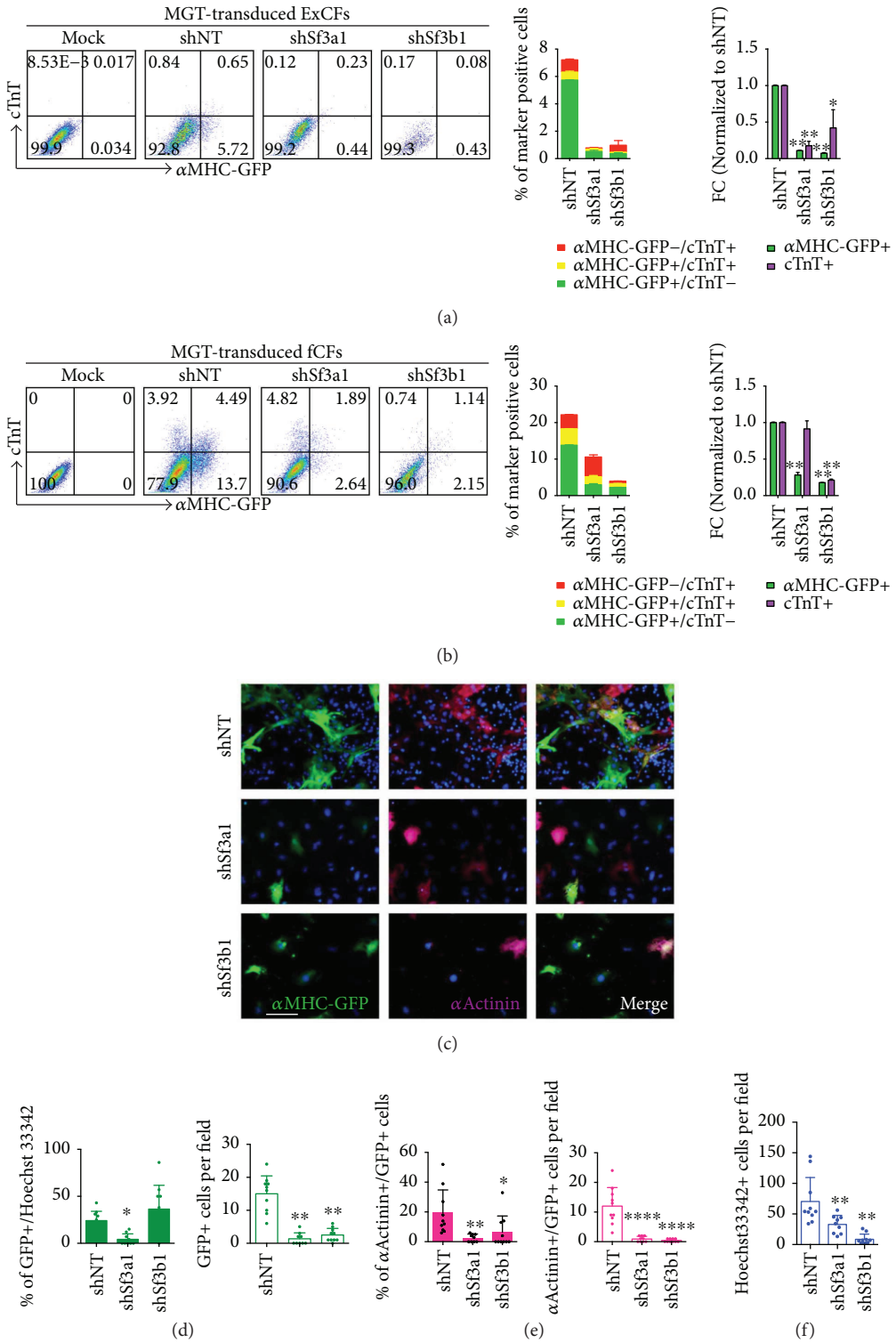


Figure 2: Knockdown of *Sf3a1* or *Sf3b1* inhibited the generation of iCMs. (a) Flow cytometry analysis (left) for αMHC-GFP+ and cTnT+ cells reprogrammed from ExCFs 10 days after infection of MGT with shNT, shSf3a1, or shSf3b1. The histograms (right) showed percentage and normalized fold change of αMHC-GFP+ and/or cTnT+ cells measured by flow cytometry. (b) Flow cytometry analysis (left) for αMHC-GFP+ and cTnT+ cells reprogrammed from fCFs 10 days after infection of MGT with shNT, shSf3a1, or shSf3b1. The histogram (right) showed percentage and normalized fold change of αMHC-GFP+ and/or cTnT+ cells measured by flow cytometry. (c) Representative ICC images for αMHC-GFP+ and αActinin+ cells on MGT-transduced ExCFs coinfected with shNT, shSf3a1, or shSf3b1. Scale bar, 100 μm. (d) ICC quantification of percentage and cell number of αMHC-GFP+ cells indicated in (c). (e) ICC quantification of percentage and cell number of cells expressing both αMHC-GFP+ and αActinin+ in (c) samples. (f) Total cell number of cells in (c) labeled by Hoechst 33342. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$.

expressing αMHC-GFP reporter and cardiac Z-disc protein, αActinin upon knockdown of *Sf3a1* or *Sf3b1* (Figures 2(c)–2(e)). Meanwhile, we found that knockdown of these two splicing factors during reprogramming resulted in a significantly reduced total cell number indicated by Hoechst 33342 staining (Figure 2(f)). These data suggest that depletion of splicing factors not only decreased iCM reprogramming efficiency but also influenced cell survival under such context.

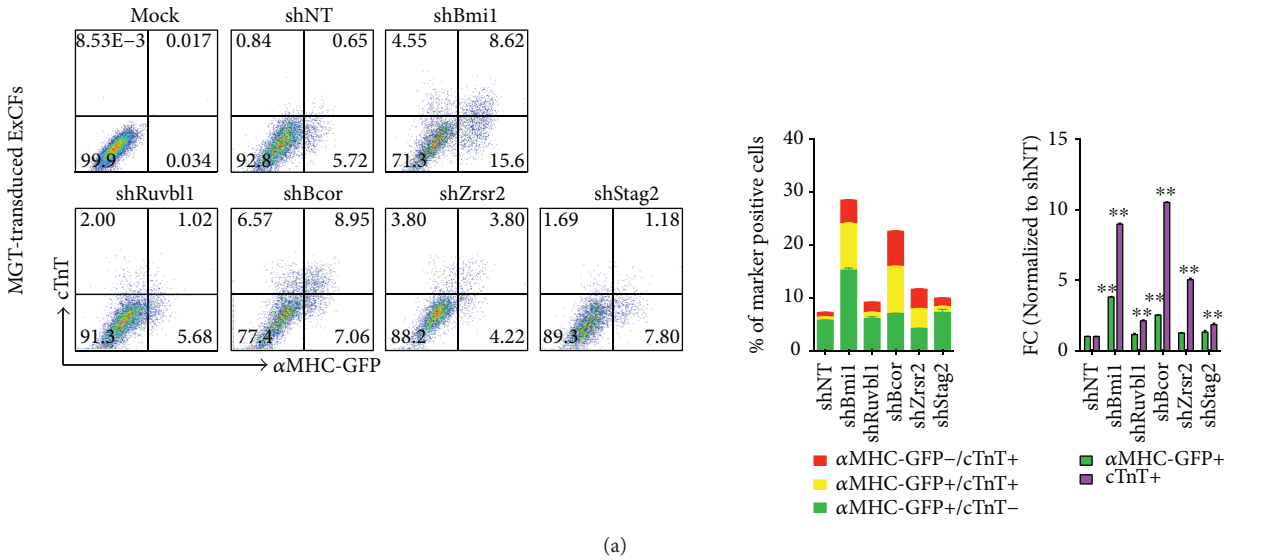
3.3. Enhanced Conversion of iCMs from CFs upon Knockdown of Ruvbl1, Bcor, Zrsr2, or Stag2. On the other hand, we further confirmed the phenotypes resulted from knockdown of the top four hits, *Ruvbl1*, *Bcor*, *Zrsr2*, and *Stag2* that seem to be inhibitors of direct cardiac reprogramming. Ruvbl1 (or Tip48, potin) belongs to the AAA+ ATPase (ATPase associated with multiple activities) family [36]. A number of chromatin-remodeling complexes contain Ruvbl1, like INO80 complex, TIP60 complex, and SWR1 complex, to facilitate the assembling and maintenance of the catalytic activity of ATPase [36, 38, 44]. It has been reported that INO80 complex is required for embryonic stem cell self-renewal and pluripotency via exchange and deposition of histone variant H2A.Z [45]. Here, to further confirm the role of Ruvbl1 as the potential inhibitor for cardiac reprogramming, we assessed iCM generation from ExCFs and fCFs by flow cytometry and ICC staining. shNT was used as a negative control, while shBmi1 as a positive control. Flow cytometry results showed a 2-fold increase in the percentage of cTnT+ iCMs from MGT-transduced ExCFs and fCFs after infection of shRuvbl1 lentiviruses (Figures 3(a) and 3(b)). In addition, loss of *Ruvbl1* led to a significant increase in the percentage of αMHC-GFP+ cells derived from fCFs (Figures 3(c) and 3(d)). Interestingly, knockdown of the other RVB gene Ruvbl2 did not appear to affect the reprogramming efficiency (Figure 1(d)), indicating distinct roles of different RVB genes during conversion from fibroblasts to iCMs.

Bcor has been identified as a transcriptional corepressor and known to regulate gene expression in association with epigenetic-modifying complexes including Polycomb group (PcG) proteins, Skp-Cullin-F-box (SCF) ubiquitin ligase, and histone demethylase [34, 35]. *Bcor* is a ubiquitously expressed gene and related to X-linked oculofaciocardiodental (OFCD) syndrome exemplified by multiple defects in human, such as cardiac atrial septal defect [34, 46]. Study of Bcor loss of function mutant mice showed a strong parent-of-origin effect, indicating a possible regulatory role of Bcor in extraembryonic tissues during early development [46]. Moreover, Bcor is required for proper differentiation of embryonic stem cells into ectoderm, mesoderm, and downstream hematopoietic lineages [46]. This is the first time that *Bcor* was investigated in iCM reprogramming. We found that knockdown of *Bcor* resulted in 10-fold and 3-fold increases in percentage of cTnT+ iCMs derived from ExCFs and fCFs, respectively, when compared to treatment of shNT (Figures 3(a) and 3(b)). Increased percentages of additional cardiac markers αMHC-GFP and αActinin-positive iCMs upon *Bcor* knockdown were confirmed by ICC staining (Figures 3(c) and 3(d)). Notably, based on cardiac marker expression measured by flow and ICC, knockdown of *Bcor* resulted in the highest increase in reprogramming efficiency among the top four candidates.

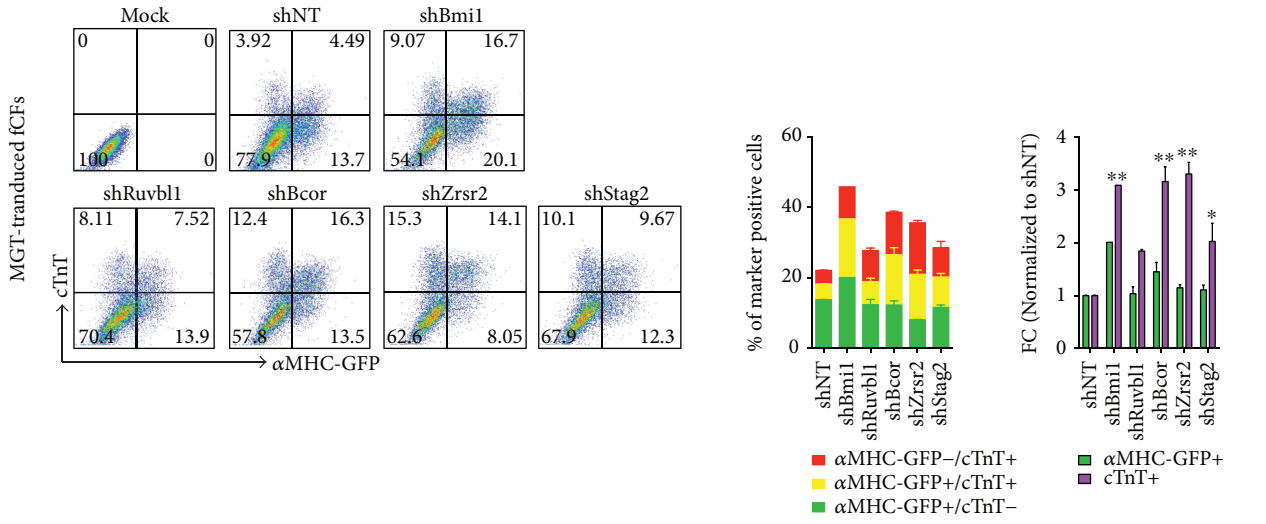
Zrsr2 (a homolog of U2AF35) is another essential component of the spliceosome [47]. Zrsr2 (or Urp) physically interacts with U2AF65 and serine/arginine-rich (SR) proteins to facilitate the recognition of exon/intron boundary and spliceosome assembly [47, 48]. After 10 days of MGT transduction, a significant increase in the percentage of cTnT+ cells was detected under treatment of shZrsr2 in both ExCFs and fCFs when compared to that obtained with control shNT treatment (Figures 3(a) and 3(b)). Moreover, the percentage as well as the absolute number of αMHC-GFP+ or αActinin+ cells significantly increased upon the removal of *Zrsr2* (Figures 3(c) and 3(d)). Taken together, our data indicated that the loss of function of *Zrsr2* enhanced conversion from cardiac fibroblasts to iCMs.

Stag2 (or SA2) encodes one of the core subunits of cohesin complex, which holds sister chromatids in dividing cells and is essential for chromatin segregation [49, 50]. In addition, cohesin has been recently implicated in chromatin looping and insulation via its direct interaction with CTCF to control chromatin structure and gene regulation [51–53]. Similarly, independent of different isolation methods to prepare the starting CFs, loss of *Stag2* always led to significant increases in both percentage and number of iCMs as shown by flow cytometry for αMHC-GFP+/cTnT+ cells and ICC analysis of αMHC-GFP+ or αActinin+ cells (Figures 3(a)–3(d)). However, knockdown of another component of cohesion complex *Rad21* did not affect iCM reprogramming (Figure 1(d)), suggesting the complexity of underlying mechanism by which Stag2 and associated cohesion complexes function during direct cardiac reprogramming.

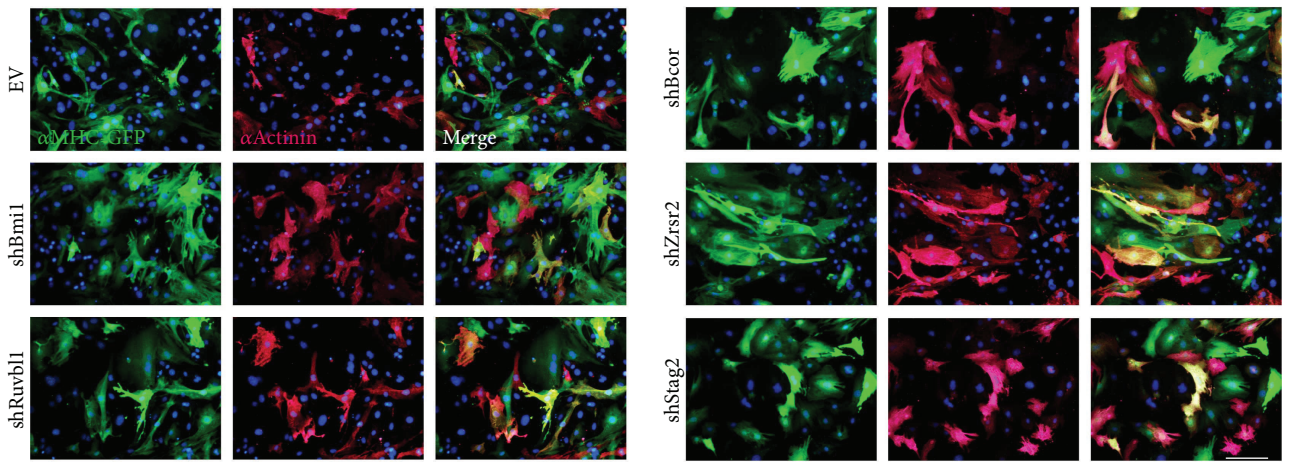
3.4. Enhanced iCM Generation from MEFs Depleted with Bcor, Zrsr2, or Stag2. To rule out the cell type-specific roles of candidate epigenetic factors on cardiac reprogramming, we utilized mouse embryonic fibroblasts (MEFs) isolated from E13.5 pups from αMHC-GFP transgenic mice to test the effects of knockdown of *Ruvbl1*, *Bcor*, *Zrsr2*, and *Stag2*. After 10 days of MGT induction, αMHC-GFP+ and/or cTnT+ iCMs were assessed by flow cytometry on MEFs infected with shRNAs targeting *Ruvbl1*, *Bcor*, *Zrsr2*, *Stag2*, or control sequences (Figure 4(a)). Noticeably, knockdown of *Bcor*, *Zrsr2*, or *Stag2* resulted in about 5-fold increase in the percentage of αMHC-GFP+ iCMs when compared to shNT-treated cells (Figure 4(a)). However, knockdown of *Ruvbl1* led to merely 2-fold increase in the percentage of αMHC-GFP+ cells (Figure 4(a)). Generally, the fold change of the percentage of cTnT+ cells increased to various degree upon depletion of candidate four genes, but the percentage of cTnT+ cells derived from MEFs was always lower than that obtained from CFs (Figures 3(a) and 4(a)), suggesting the varied plasticity of fibroblasts from different origin with cardiac fibroblasts being most amenable. Interestingly, the bright field cell images showed that MGT-transduced cells became flat after knockdown of *Bcor* or *Zrsr2* and extensive cell death was observed from shStag2-infected



(a)



(b)



(c)

Figure 3: Continued.

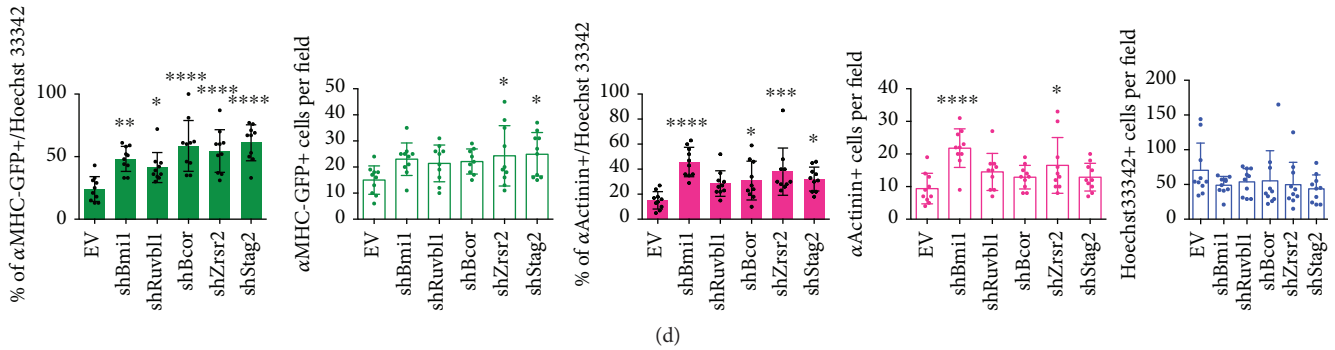


Figure 3: Knockdown of *Ruvbl1*/*Bcor*/*Zrsr2*/*Stag2* enhanced the efficiency of cardiac reprogramming from CFs. (a) Representative flow plots (left) with quantification (right) for αMHC-GFP+ and cTnT+ cells derived from ExCFs 10 days postinfection of MGT and shRNA lentiviruses as indicated. (b) Representative flow plots (left) with quantification (right) for αMHC-GFP+ and cTnT+ cells derived from fCFs 10 days postinfection of MGT and shRNA lentiviruses as indicated. (c) Representative images of ICC for αMHC-GFP+ and αActinin+ cells derived from fCFs after infection of MGT and indicated shRNAs at reprogramming day 10. (d) ICC quantification for αMHC-GFP+ and αActinin+ cells and total cells indicated by Hoechst 33342 in (c). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

MEFs (Figure 4(b)), confirmed by Hoechst 33342 staining (Figure 4(d), third graph). However, the total cell number when reprogramming fCFs was unaltered (Figure 3(d), fifth graph), suggesting that starting fibroblasts with diverse origins responded differently upon loss of epigenetic factors involved in a wide spectrum of chromatin regulatory complexes. Moreover, we performed ICC staining of cardiac reporter αMHC-GFP and cTnT on MGT-transduced MEFs treated with shRNAs targeting candidate genes. Noticeably, the percentage of αMHC-GFP+ cells was only significantly increased in shStag2-treated (2-fold increase) MEFs when compared to that in shNT-treated cells (Figures 4(c) and 4(d)). However, the absolute number of not only αMHC-GFP+ iCMs but also total cells was reduced after knockdown of *Bcor*/*Zrsr2*/*Stag2* (Figure 4(d)), suggesting the influence of epigenetic disruption on basic cell survival and growth of MEFs. Meanwhile, we found that loss of *Ruvbl1* did not affect iCM reprogramming efficiency from MEFs at day 10 upon transduction of MGT (Figures 4(a), 4(c), and 4(d)), indicating that the repressive function of Ruvbl1 might be CF specific and suggesting the potential variability of epigenetic status among multiple cell types. Taken these data together, we suggest Bcor/Zrsr2/Stag2 as inhibitory epigenetic regulatory factors during cardiac reprogramming from several fibroblast types.

3.5. Gene Expression Analyses of Reprogramming Cells after Knockdown of Epigenetic and Splicing Factors. To further explore how knockdown of epigenetic and splicing regulators influenced the expression profile of iCMs, we performed RT-qPCR with a set of CM marker genes related to sarcomere structure formation, ion channel, and fibroblast marker genes in reprogramming ExCFs coinfected with shRNA lentiviruses. Interestingly, loss of *Zrsr2* resulted in the highest increase in cardiac marker expression yet with no change in fibroblast marker expression (Figure 5(a)). Meanwhile, knockdown of *Stag2* significantly repressed fibroblast gene expression, as well as the expression of cardiac genes (Figure 5(a)), suggesting the essential role of Stag2 on global gene expression. On the other hand, depletion of core spliceosome factor *Sf3a1* and *Sf3b1* seems generally interfered with expression of all marker genes regardless of cell lineage. A dramatic decrease of these maker genes was found under treatment of shSf3a1 or shSf3b1 (Figure 5(b)), suggesting the essential role of U2-dependent spliceosome in maintaining gene expression in fibroblasts. Moreover, similar phenotypes after knockdown of *Bcor*/*Zrsr2*/*Stag2* were obtained by RT-qPCR of the same marker genes in MGT-transduced MEFs (Figure 5(c)). Therefore, we discovered gene expression patterns of reprogramming cells resulted from knockdown of distinct epigenetic factors, indicating the varied molecular response and potential underlying mechanisms upon manipulation of different epigenetic complexes or splicing factors that potentially orchestrate the expression of cardiac and fibroblast-related genes.

4. Discussion

In this study, we performed a shRNA-mediated loss of function screen for epigenetic modulators involved in chromatin remodeling and RNA splicing factors during direct cardiac reprogramming. We demonstrated that splicing factors Sf3a1 and Sf3b1 are required for cardiac reprogramming, while Zrsr2 is inhibitory to iCM induction. Moreover, we found that removal of *Bcor* and *Stag2* increased reprogramming efficiency regardless of the origins of starting fibroblasts, indicating that Bcor-related BCOR complex and Stag2-involved cohesin complex may play suppressive roles during conversion of iCMs. Although the detailed mechanisms by which these factors orchestrate iCM reprogramming remain to be elucidated, our results reveal additional regulators participating in the molecular networks underlying direct conversion from fibroblasts to iCMs.

Taken together with our previous finding, splicing factors have been demonstrated to play critical roles during direct cardiac reprogramming [22]. Knockdown of *Sf3a1* and *Sf3b1*, which are core components of U2 snRNP assembling U2-dependent major spliceosome, drastically reduced total reprogramming cell number and suppressed the mRNA level of both cardiac and fibroblast genes. However, knockdown of

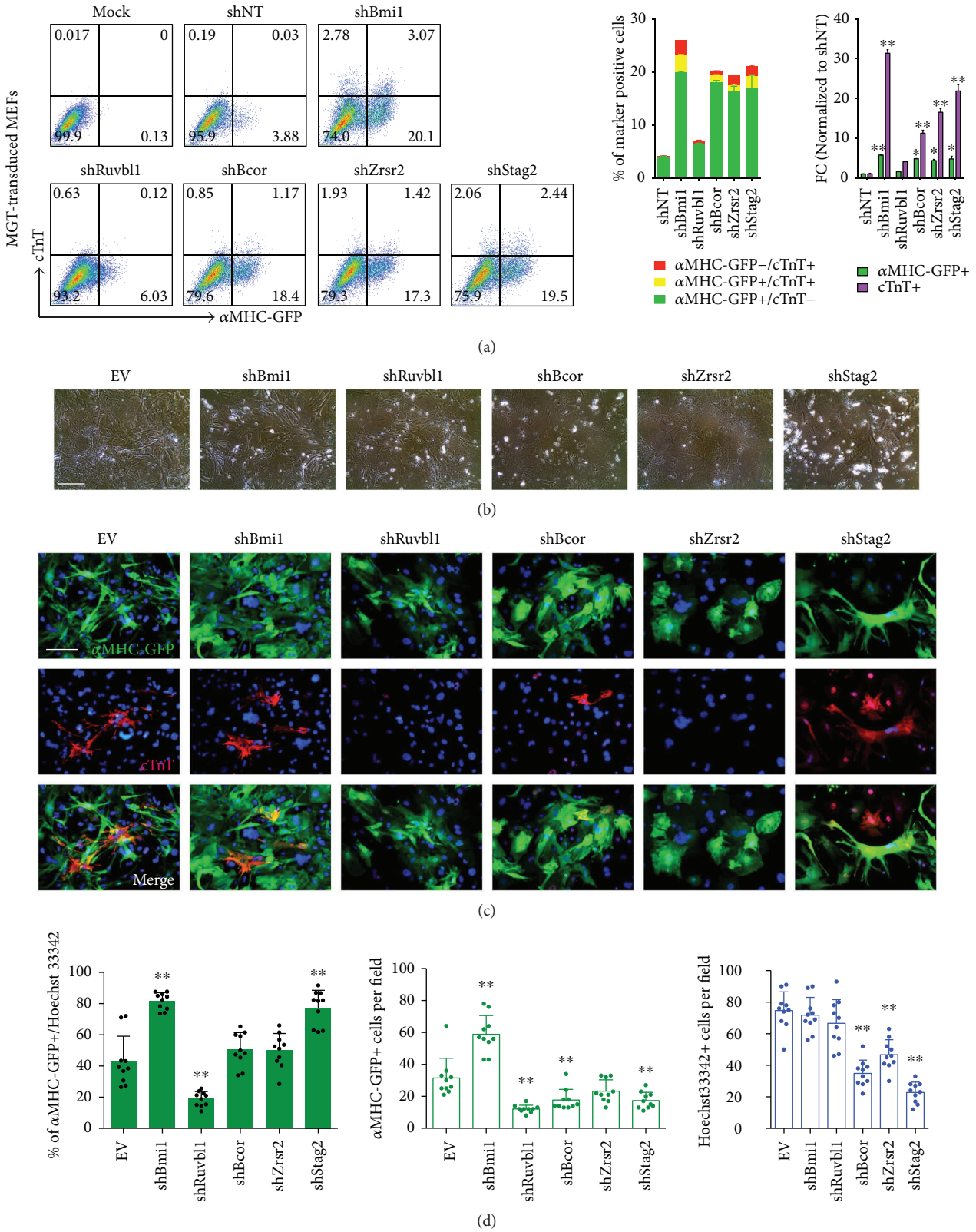


FIGURE 4: Knockdown of *Bcor*/Zrsr2/Stag2 promoted iCM conversion from MEFs. (a) Representative flow plots (left) and quantification (right) for αMHC-GFP+ and/or cTnT+ cells on MEFs after infection of MGT with indicated shRNA lentiviruses for 10 days. (b) Representative phase-contrast images of MGT-infected MEFs at day 10 after transduction of shRNAs as indicated. EV, empty vector, was used as a negative control. (c) Representative ICC images for αMHC-GFP or cTnT expressed iCMs derived from MEFs transduced with MGT and indicated shRNAs at reprogramming day 10. EV, empty vector, was used as a negative control. (d) Quantification for αMHC-GFP+ cells and total cells indicated by Hoechst 33342 in (c). $^{*}p < 0.05$, $^{**}p < 0.01$.

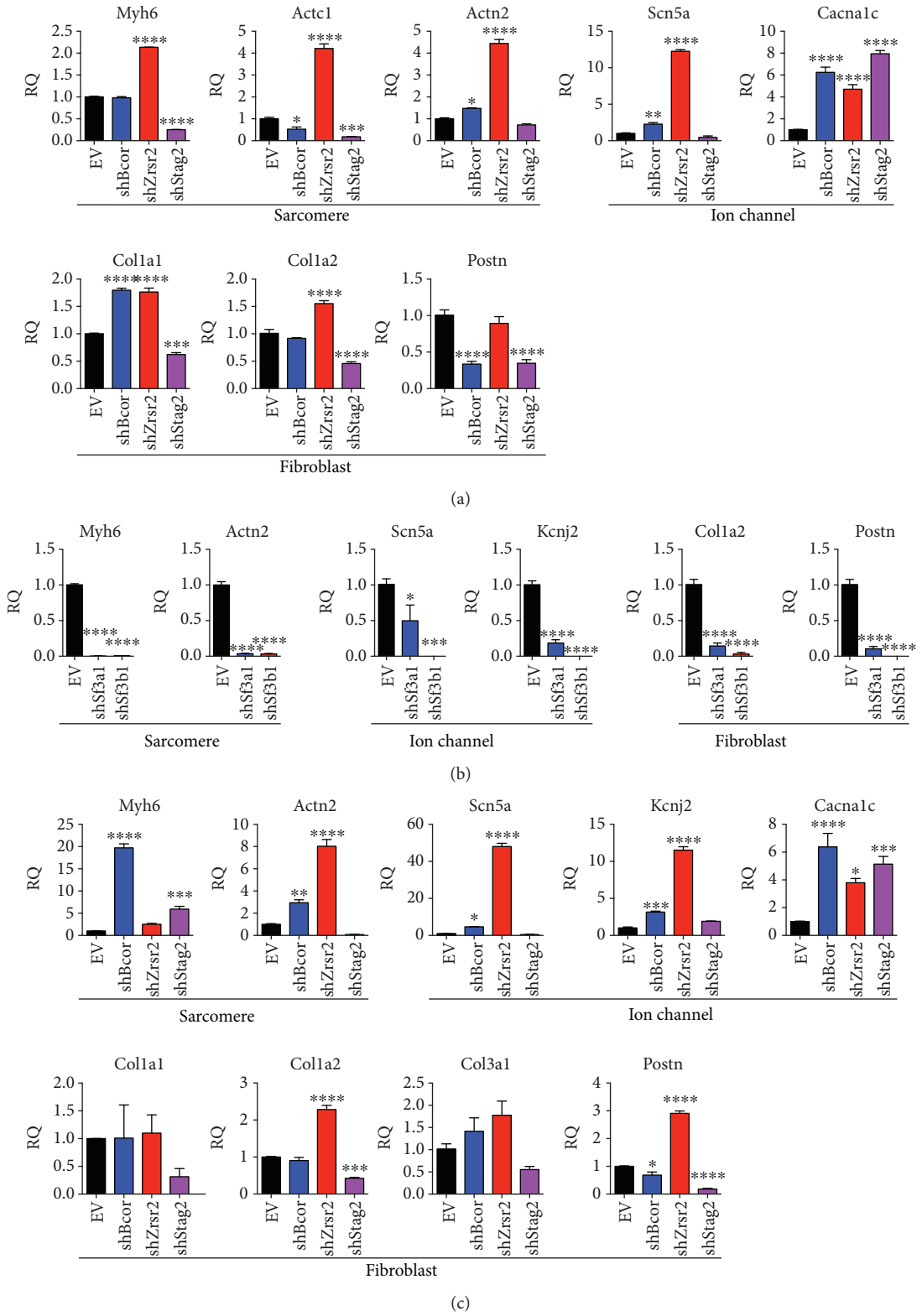


Figure 5: Knockdown of epigenetic factors changed the molecular features of reprogrammed cells. (a) Relative expression of cardiomyocyte-related sarcomere and ion channel genes or fibroblast marker genes in ExCFs infected with MGT and EV, shBcor, shZrsr2, or shStag2 at 10 days after infections. EV, empty vector, was used as a negative control. (b) Relative expression of sarcomere, ion channel, and fibroblast marker genes in MGT-infected ExCF at day 10 postinfection of EV, shSf3a1, or shSf3b1. EV, empty vector, was used as a negative control. $^{*}p < 0.05$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. (c) Relative expression of cardiomyocyte-related sarcomere and ion channel genes or fibroblast marker genes in MEFs infected with MGT and indicated shRNAs at 10 days after infections. EV, empty vector, was used as a negative control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Zrsr2, which not only participates in the U2-dependent major splicing but is also required for U12-dependent minor splicing [48], enhanced cardiac reprogramming with increased iCM percentage and gene expression of cardiac markers. The opposite phenotypes might be associated with the complexity and dynamics of spliceosome and cell type-specific function of splicing factors. Likewise, we observed different effects of U2af1 knockdown on iCM generation from that on iPSC reprogramming [31], suggesting distinct RNA splicing regulation between iCM and iPSC reprogramming processes.

Although cohesin has been widely considered to be required for ESC self-renewal and identified as facilitators of iPSC reprogramming [53–55], it is intriguing to find that knockdown of the core component of cohesin *Stag2* facilitates iCM generation accompanied with decreased expression of fibroblast markers, suggesting cohesin as a potential barrier to direct cardiac reprogramming. In addition, cohesin-depleted ESCs and iPSCs are difficult to maintain or establish pluripotency gene expression, which could be explained by a loss of long-range interactions [54, 55] or by DNA damage responses resulted from defects in proliferation [56]. However, studies that excluded the influence of cell proliferation demonstrated that cohesin depletion enhanced the ability of ES cells to initiate somatic cell reprogramming [57]. Likely, in our study, the nondividing features of fully reprogrammed iCMs might partially explain the opposite roles of cohesin in iCM versus iPSC reprogramming. Additionally, cohesin also contributes to the establishment and maintenance of tissue-specific gene expression [52, 58]. Therefore, it will be particularly interesting to investigate cell type-specific role of cohesin in gene regulation during different cell fate conversion processes.

5. Conclusions

In this study, we employed shRNA-mediated RNAi screen and identified splicing factors Sf3a1 and Sf3b1 as essential regulators while splicing factor Zrsr2 and epigenetic modulators Bcor and Stag2 as inhibitory barriers for direct cardiac reprogramming. Our finding provides not only insights into understanding of molecular mechanisms of iCM reprogramming but also potential RNAi-based approach to improve reprogramming efficiency.

Conflicts of Interest

The authors indicate no potential competing interests.

Acknowledgments

The authors are grateful for the expert technical assistance from the UNC Flow Cytometry Core. They thank members of the Qian lab and the Liu lab for the helpful discussions and critical reviews of the manuscript. They thank Dr. Greg G. Wang for kindly providing them the critical reagents for their experiments. This study was supported by NIH/NHLBI R00HL109079 grant and American Heart Association (AHA) 15GRNT25530005 grant to Jiandong Liu and AHA 13SDG17060010, the Ellison Medical Foundation (EMF) Grant AG-NS-1064-13, and NIH/NHLBI R01HL128331 to Li Qian.

Supplementary Materials

Figure S1: plasmid map of pMXs-puro-MGT. Diagram of the polycistronic retroviral vector-expressing Mef2c, Gata4, and Tbx5, separated by P2A and T2A. Table S1: sequences of shRNA oligoes used for RNAi screen. Table S2: list of primers and Taqman probes. *(Supplementary Materials)*

References

[1] M. A. Laflamme and C. E. Murry, "Heart regeneration," *Nature*, vol. 473, no. 7347, pp. 326–335, 2011.

[2] S. A. Doppler, M. A. Deutsch, V. Serpooshan et al., "Mammalian heart regeneration," *Circulation Research*, vol. 120, no. 4, pp. 630–632, 2017.

[3] M. Ieda, J. D. Fu, P. Delgado-Olguin et al., "Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors," *Cell*, vol. 142, no. 3, pp. 375–386, 2010.

[4] K. Song, Y.-J. Nam, X. Luo et al., "Heart repair by reprogramming non-myocytes with cardiac transcription factors," *Nature*, vol. 485, no. 7400, pp. 599–604, 2012.

[5] L. Qian, Y. Huang, C. I. Spencer et al., "In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes," *Nature*, vol. 485, no. 7400, pp. 593–598, 2012.

[6] R. C. Addis, J. L. Ifkovits, F. Pinto et al., "Optimization of direct fibroblast reprogramming to cardiomyocytes using calcium activity as a functional measure of success," *Journal of Molecular and Cellular Cardiology*, vol. 60, pp. 97–106, 2013.

[7] H. Hirai, N. Katoku-Kikyo, S. A. Keirstead, and N. Kikyo, "Accelerated direct reprogramming of fibroblasts into cardiomyocyte-like cells with the MyoD transactivation domain," *Cardiovascular Research*, vol. 100, no. 1, pp. 105–113, 2013.

[8] T. M. Jayawardena, B. Egemnazarov, E. A. Finch et al., "MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes," *Circulation Research*, vol. 110, no. 11, pp. 1465–1473, 2012.

[9] S. Protze, S. Khattak, C. Poulet, D. Lindemann, E. M. Tanaka, and U. Ravens, "A new approach to transcription factor screening for reprogramming of fibroblasts to cardiomyocyte-like cells," *Journal of Molecular and Cellular Cardiology*, vol. 53, no. 3, pp. 323–332, 2012.

[10] H. Zhou, M. E. Dickson, M. S. Kim, R. Bassel-Duby, and E. N. Olson, "Akt1/protein kinase B enhances transcriptional reprogramming of fibroblasts to functional cardiomyocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 38, pp. 11864–11869, 2015.

[11] M. Abad, H. Hashimoto, H. Zhou et al., "Notch inhibition enhances cardiac reprogramming by increasing MEF2C transcriptional activity," *Stem Cell Reports*, vol. 8, no. 3, pp. 548–560, 2017.

[12] T. M. A. Mohamed, N. R. Stone, E. C. Berry et al., "Chemical enhancement of in vitro and in vivo direct cardiac reprogramming," *Circulation*, vol. 135, no. 10, pp. 978–995, 2017.

[13] Y.-J. Nam, C. Lubczyk, M. Bhakta et al., "Induction of diverse cardiac cell types by reprogramming fibroblasts with cardiac

transcription factors," *Development*, vol. 141, no. 22, pp. 4267–4278, 2014.

[14] L. Wang, Z. Liu, C. Yin et al., "Stoichiometry of Gata4, Mef2c, and Tbx5 influences the efficiency and quality of induced cardiac myocyte reprogramming," *Circulation Research*, vol. 116, no. 2, pp. 237–244, 2015.

[15] H. Yamakawa, N. Muraoka, K. Miyamoto et al., "Fibroblast growth factors and vascular endothelial growth factor promote cardiac reprogramming under defined conditions," *Stem Cell Reports*, vol. 5, no. 6, pp. 1128–1142, 2015.

[16] Y. Zhao, P. Londono, Y. Cao et al., "High-efficiency reprogramming of fibroblasts into cardiomyocytes requires suppression of pro-fibrotic signalling," *Nature Communications*, vol. 6, no. 1, p. 8243, 2015.

[17] S. Dal-Pra, C. P. Hodgkinson, M. Mirotsou, I. Kirste, and V. J. Dzau, "Demethylation of H3K27 is essential for the induction of direct cardiac reprogramming by miR combo," *Circulation Research*, vol. 120, no. 9, pp. 1403–1413, 2017.

[18] L. Liu, I. Lei, H. Karatas et al., "Targeting Mll1 H3K4 methyltransferase activity to guide cardiac lineage specific reprogramming of fibroblasts," *Cell Discovery*, vol. 2, no. 1, 2016.

[19] Z. Liu, O. Chen, M. Zheng et al., "Re-patterning of H3K27me3, H3K4me3 and DNA methylation during fibroblast conversion into induced cardiomyocytes," *Stem Cell Research*, vol. 16, no. 2, pp. 507–518, 2016.

[20] N. Muraoka, H. Yamakawa, K. Miyamoto et al., "MiR-133 promotes cardiac reprogramming by directly repressing Snai1 and silencing fibroblast signatures," *The EMBO Journal*, vol. 33, no. 14, pp. 1565–1581, 2014.

[21] Y. Zhou, L. Wang, H. R. Vaseghi et al., "Bmi1 is a key epigenetic barrier to direct cardiac reprogramming," *Cell Stem Cell*, vol. 18, no. 3, pp. 382–395, 2016.

[22] Z. Liu, L. Wang, J. D. Welch et al., "Single-cell transcriptomics reconstructs fate conversion from fibroblast to cardiomyocyte," *Nature*, vol. 551, no. 7678, pp. 100–104, 2017.

[23] A. Bird, "Perceptions of epigenetics," *Nature*, vol. 447, no. 7143, pp. 396–398, 2007.

[24] A. D. Goldberg, C. D. Allis, and E. Bernstein, "Epigenetics: a landscape takes shape," *Cell*, vol. 128, no. 4, pp. 635–638, 2007.

[25] L. Luna-Zurita and B. G. Bruneau, "Chromatin modulators as facilitating factors in cellular reprogramming," *Current Opinion in Genetics & Development*, vol. 23, no. 5, pp. 556–561, 2013.

[26] J. D. Fu, N. R. Stone, L. Liu et al., "Direct reprogramming of human fibroblasts toward a cardiomyocyte-like state," *Stem Cell Reports*, vol. 1, no. 3, pp. 235–247, 2013.

[27] B. G. Bruneau, "Chromatin remodeling in heart development," *Current Opinion in Genetics & Development*, vol. 20, no. 5, pp. 505–511, 2010.

[28] H. Lickert, J. K. Takeuchi, I. von Both et al., "Baf60c is essential for function of BAF chromatin remodelling complexes in heart development," *Nature*, vol. 432, no. 7013, pp. 107–112, 2004.

[29] M. M. G. van den Hoogenhof, Y. M. Pinto, and E. E. Creemers, "RNA splicing," *Circulation Research*, vol. 118, no. 3, pp. 454–468, 2016.

[30] P. Mirtschink, J. Krishnan, F. Grimm et al., "HIF-driven *SF3B1* induces *KHK-C* to enforce fructolysis and heart disease," *Nature*, vol. 522, no. 7557, pp. 444–449, 2015.

[31] S. Ohta, E. Nishida, S. Yamanaka, and T. Yamamoto, "Global splicing pattern reversion during somatic cell reprogramming," *Cell Reports*, vol. 5, no. 2, pp. 357–366, 2013.

[32] L. Wang, Z. Liu, C. Yin, Y. Zhou, J. Liu, and L. Qian, "Improved generation of induced cardiomyocytes using a polycistronic construct expressing optimal ratio of Gata4, Mef2c and Tbx5," *Journal of Visualized Experiments*, no. 105, article e53426, 2015.

[33] J. Jozefczuk, K. Drews, and J. Adjaye, "Preparation of mouse embryonic fibroblast cells suitable for culturing human embryonic and induced pluripotent stem cells," *Journal of Visualized Experiments*, no. 64, article e3854, 2012.

[34] K. D. Huynh, W. Fischle, E. Verdin, and V. J. Bardwell, "BCoR, a novel corepressor involved in BCL-6 repression," *Genes & Development*, vol. 14, no. 14, pp. 1810–1823, 2000.

[35] M. D. Gearhart, C. M. Corcoran, J. A. Wamstad, and V. J. Bardwell, "Polycomb group and SCF ubiquitin ligases are found in a novel BCOR complex that is recruited to BCL6 targets," *Molecular and Cellular Biology*, vol. 26, no. 18, pp. 6880–6889, 2006.

[36] S. Jha, A. Gupta, A. Dar, and A. Dutta, "RVBs are required for assembling a functional TIP60 complex," *Molecular and Cellular Biology*, vol. 33, no. 6, pp. 1164–1174, 2013.

[37] T. Ikura, V. V. Ogryzko, M. Grigoriev et al., "Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis," *Cell*, vol. 102, no. 4, pp. 463–473, 2000.

[38] X. Shen, G. Mizuguchi, A. Hamiche, and C. Wu, "A chromatin remodelling complex involved in transcription and DNA processing," *Nature*, vol. 406, no. 6795, pp. 541–544, 2000.

[39] C. L. Will and R. Lührmann, "Spliceosome structure and function," *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 7, 2011.

[40] M. C. Wahl, C. L. Will, and R. Lührmann, "The spliceosome: design principles of a dynamic RNP machine," *Cell*, vol. 136, no. 4, pp. 701–718, 2009.

[41] O. Gozani, R. Feld, and R. Reed, "Evidence that sequence-independent binding of highly conserved U2 snRNP proteins upstream of the branch site is required for assembly of spliceosomal complex A," *Genes & Development*, vol. 10, no. 2, pp. 233–243, 1996.

[42] P. D. Zamore and M. R. Green, "Identification, purification, and biochemical characterization of U2 small nuclear ribonucleoprotein auxiliary factor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 23, pp. 9243–9247, 1989.

[43] K. Yoshida, M. Sanada, Y. Shiraishi et al., "Frequent pathway mutations of splicing machinery in myelodysplasia," *Nature*, vol. 478, no. 7367, pp. 64–69, 2011.

[44] Z. O. Jónsson, S. Jha, J. A. Wohlschlegel, and A. Dutta, "Rvb1p/Rvb2p recruit Arp5p and assemble a functional Ino80 chromatin remodeling complex," *Molecular Cell*, vol. 16, no. 3, pp. 465–477, 2004.

[45] L. Wang, Y. Du, J. M. Ward et al., "INO80 facilitates pluripotency gene activation in embryonic stem cell self-renewal, reprogramming, and blastocyst development," *Cell Stem Cell*, vol. 14, no. 5, pp. 575–591, 2014.

[46] J. A. Wamstad, C. M. Corcoran, A. M. Keating, and V. J. Bardwell, "Role of the transcriptional corepressor *Bcor* in embryonic stem cell differentiation and early embryonic development," *PLoS One*, vol. 3, no. 7, article e2814, 2008.

[47] H. Tronchre, J. Wang, and X.-D. Fu, "A protein related to splicing factor $U2AF^{35}$ that interacts with $U2AF^{65}$ and SR proteins in splicing of pre-mRNA," *Nature*, vol. 388, no. 6640, pp. 397–400, 1997.

[48] H. Shen, X. Zheng, S. Luecke, and M. R. Green, "The U2AF35-related protein Urp contacts the 3′ splice site to promote U12-type intron splicing and the second step of U2-type intron splicing," *Genes & Development*, vol. 24, no. 21, pp. 2389–2394, 2010.

[49] A. Losada, T. Yokochi, R. Kobayashi, and T. Hirano, "Identification and characterization of SA/Scc3p subunits in the *Xenopus* and human cohesin complexes," *The Journal of Cell Biology*, vol. 150, no. 3, pp. 405–416, 2000.

[50] C. Michaelis, R. Ciosk, and K. Nasmyth, "Cohesins: chromosomal proteins that prevent premature separation of sister chromatids," *Cell*, vol. 91, no. 1, pp. 35–45, 1997.

[51] J. Zuin, J. R. Dixon, M. I. J. A. van der Reijden et al., "Cohesin and CTCF differentially affect chromatin architecture and gene expression in human cells," *Proceedings of the National Academy of Sciences*, vol. 111, no. 3, pp. 996–1001, 2014.

[52] D. Schmidt, P. C. Schwalie, C. S. Ross-Innes et al., "A CTCF-independent role for cohesin in tissue-specific transcription," *Genome Research*, vol. 20, no. 5, pp. 578–588, 2010.

[53] M. H. Kagey, J. J. Newman, S. Bilodeau et al., "Mediator and cohesin connect gene expression and chromatin architecture," *Nature*, vol. 467, no. 7314, pp. 430–435, 2010.

[54] H. Zhang, W. Jiao, L. Sun et al., "Intrachromosomal looping is required for activation of endogenous pluripotency genes during reprogramming," *Cell Stem Cell*, vol. 13, no. 1, pp. 30–35, 2013.

[55] Z. Zhao, G. Tavoosidana, M. Sjölinder et al., "Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions," *Nature Genetics*, vol. 38, no. 11, pp. 1341–1347, 2006.

[56] P. Gupta, T. Lavagnolli, H. Mira-Bontenbal, A. G. Fisher, and M. Merkenschlager, "Cohesin's role in pluripotency and reprogramming," *Cell Cycle*, vol. 15, no. 3, pp. 324–330, 2016.

[57] T. Lavagnolli, P. Gupta, E. Hörmanseder et al., "Initiation and maintenance of pluripotency gene expression in the absence of cohesin," *Genes & Development*, vol. 29, no. 1, pp. 23–38, 2015.

[58] M. Merkenschlager and D. T. Odom, "CTCF and cohesin: linking gene regulatory elements with their targets," *Cell*, vol. 152, no. 6, pp. 1285–1297, 2013.

Hindawi
Stem Cells International
Volume 2018, Article ID 1435746, 10 pages
https://doi.org/10.1155/2018/1435746

Review Article

Direct Cardiac Reprogramming: Progress and Promise

James L. Engel[1,2,3] **and Reza Ardehali**[1,2,3]

[1]*Molecular, Cellular and Integrative Physiology Graduate Program, University of California, Los Angeles, CA 90095, USA*
[2]*Division of Cardiology, Department of Internal Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA*
[3]*Eli and Edythe Broad Stem Cell Research Center, University of California, Los Angeles, CA 90095, USA*

Correspondence should be addressed to Reza Ardehali; RArdehali@mednet.ucla.edu

Received 1 September 2017; Revised 15 December 2017; Accepted 9 January 2018; Published 13 March 2018

Academic Editor: Fatemeh Sharifpanah

The human adult heart lacks a robust endogenous repair mechanism to fully restore cardiac function after insult; thus, the ability to regenerate and repair the injured myocardium remains a top priority in treating heart failure. The ability to efficiently generate a large number of functioning cardiomyocytes capable of functional integration within the injured heart has been difficult. However, the ability to directly convert fibroblasts into cardiomyocyte-like cells both *in vitro* and *in vivo* offers great promise in overcoming this problem. In this review, we describe the insights and progress that have been gained from the investigation of direct cardiac reprogramming. We focus on the use of key transcription factors and cardiogenic genes as well as on the use of other biological molecules such as small molecules, cytokines, noncoding RNAs, and epigenetic modifiers to improve the efficiency of cardiac reprogramming. Finally, we discuss the development of safer reprogramming approaches for future clinical application.

1. Introduction

Heart failure (HF), the leading cause of death and hospitalizations worldwide, results from a myriad of cardiovascular diseases that lead to the death or dysfunction of cardiomyocytes. With a prevalence of 38 million people worldwide, it places a significant financial burden on health care systems, with an estimated $30 billion of annual spending in just the United States alone [1, 2]. Despite recent advances in the care and management of HF patients, the prognosis of advanced HF remains dismal at 50% survival at 5 years, a rate often lower than that of many cancers [3, 4]. Considering that the pathophysiology of HF involves death or dysfunction of the cardiac myocyte, new therapeutic strategies for heart regeneration may offer hope to this intractable disease.

The human adult heart lacks endogenous repair mechanisms to fully restore cardiac function after an insult; thus, the ability to regenerate and repair the injured myocardium remains a top priority in treating HF. However, the ability to efficiently generate a large number of functioning cardiomyocytes capable of functional integration within the injured heart has remained an obstacle. Current cell therapies are focused on three main approaches: (1) induction of endogenous cardiomyocytes to undergo proliferation and repopulate the damaged myocardium, (2) transplantation of cardiovascular progenitor cells (CPCs) or cardiomyocytes generated through the differentiation of pluripotent stem cells, and (3) direct reprogramming of somatic cells to cardiomyocytes or expandable CPCs without transitioning through a pluripotent intermediate. This review is focused on the last approach. Direct reprogramming was first reported in 1987 when a single cDNA encoding MyoD was transfected into fibroblasts converting them into muscle myoblasts [5]. A few years later, MyoD was identified as the master regulator gene for skeletal muscle development [6]. The ability to directly reprogram adult cells to a desirable fate demonstrates an immense potential of this powerful

tool for tissue regeneration and replacement. Since the identification of MyoD, there has been extensive focus on the identification of master regulator(s) for other cell lineages and this search has led to the successful conversion of mature cells into other cells types including myoblasts, neurons, hepatocytes, intestinal cells, blood progenitor cells, and cardiomyocytes [5, 7–11].

In this review, we describe the insights and progress that have been gained from the investigation of direct cardiac reprogramming, with a focus on the use of key transcription factors and other cardiogenic genes. Furthermore, we discuss the use of other biologics and small molecules to improve the efficiency of cardiac reprogramming and the development of safe reprogramming approaches for clinical application.

2. Reprogramming of Somatic Cells to Cardiomyocyte-Like Cells by Overexpression of Key Cardiac Transcription Factors

Direct reprogramming of fibroblasts into cardiomyocyte-like cells was first reported in 2010 using viral overexpression of three important cardiac developmental transcription factors (TFs), *Gata4*, *Mef2c*, and *Tbx5* (GMT) in mouse cardiac and tail-tip fibroblasts [11]. Ieda et al. used an iterative screening approach in which 14 factors were removed one by one to identify those that were dispensable for direct reprogramming. This process ultimately identified GMT as the factors sufficient to induce conversion of fibroblasts to cardiomyocyte-like cells without transitioning through a progenitor state. TBX5 is an important T-box TF involved in early cardiac development that directs formation of the primary heart field through a coordinated but yet complex interaction with other TFs [12]. One such interaction is with GATA4, a member of the GATA family zinc-finger TFs, which modifies the chromatin structure allowing other TFs such as NKX2–5 to bind to their targets and fully activate the cardiac transcriptional program [13]. MEF2c, a MADS box transcription enhancer factor, is important for the formation of the secondary heart field through its interaction with other cardiac TFs [14]. After the establishment of GMT as the core TFs for direct cardiac reprograming, much of the focus transitioned to improving the reprogramming efficiency and/or the function of the induced cardiomyocyte-like cells (iCMs) through addition of other important cardiac TFs to GMT. This was mainly due to the poor efficiency of reprogramming, reported to be 4.8% cardiac troponin T + (cTnT+) cells in the original paper. Additionally, it was soon noted that GMT alone was insufficient to convert human fibroblasts to iCMs.

One of the first TFs added to GMT was the bHLH TF HAND2 (referred to as GMHT). In cardiac development, HAND2 plays an important role in the formation of the ventricular chambers through interaction with GATA4 and NKX2–5 [15]. GMHT treatment of mouse embryonic fibroblasts (MEFs) resulted in iCMs expressing low levels of sarcomeric proteins and displayed immature characteristics of the main cardiac cell types (atrial, ventricular, and pacemaker) [16]. In an effort to increase transcriptional activity of GMHT, the transactivation domain of MyoD was fused to each G, M, H, or T and overexpressed in mouse fibroblasts. When *MyoD* was fused to *Mef2c*, a 15-fold increase in reprogramming efficiency was observed [17]. Other TFs that are essential during cardiovascular development have also been studied for direct reprogramming. NKX2–5, a homeobox TF critical for normal heart morphogenesis, was overexpressed in mouse fibroblasts in addition to GMHT. This combination resulted in a more than 50-fold increase in the efficiency of cardiac reprogramming compared to GMT alone and produced iCMs with mature cardiomyocyte marker expression, robust calcium oscillation, and spontaneous beating [18].

Additionally, an alternative screening approach that surveyed triplet combinations of 10 important cardiac TFs revealed that *Tbx5*, *Mef2c*, and *Myocd*, a developmental regulator of cardiomyocytes and smooth muscle cells, were able to induce a more complete cardiac phenotype than GMT in mouse fibroblasts [19]. Likewise, a combinatorial screen of 10 transcription factors added to GMT in MEFs identified a combination of cocktails that resulted in successful reprogramming. GMT plus *Myocd* and *Srf*, a TF important in mesoderm formation, or GMT plus *Myocd*, *Srf*, *Mesp1*, another mesoderm-inducing TF, and *Smarcd3*, a chromatin structure-altering protein, enhanced reprogramming and the expression of cardiac sarcomeric proteins over GMT alone [20].

Despite the successes of TF overexpression to reprogram murine cells, similar approaches to reprogram human somatic cells have been more difficult to achieve. Only a few studies have reported successful reprogramming of human cells to iCMs using TFs alone. The first of these studies reported a combination of the E26 transformation-specific (ETS) TF family member *ETS2* and *MESP1* proteins to induce reprogramming of human dermal fibroblasts to cardiac progenitors [21]. Another study using GMT with *MESP1* and *MYOCD* in human cardiac and dermal fibroblasts was sufficient to induce expression of multiple cardiac-specific proteins, increase a broad range of cardiac genes, and exhibit spontaneous calcium transients [22]. The third report showed that expressing GMT along with *ESSRG* (a transcriptional activator), *MESP1*, *MYOCD*, and *ZFPM2* (a modulator of GATA proteins) in human fetal cardiac fibroblasts and neonatal skin fibroblasts enhanced cardiac reprogramming, sarcomere formation, calcium transients, and action potentials [23]. Results of TF-based reprogramming are summarized in Table 1.

3. Improving the Efficiency of Direct Reprogramming with Biological Molecules

Despite the successes of direct reprogramming using forced expression of cardiac TFs, the efficiency remains low. The initial report on direct conversion of fibroblasts to cardiomyocyte-like cells noted an efficiency of 4.8%. In an effort to improve reprogramming efficiency, many

Table 1: Summary of transcription factor direct cardiac reprogramming results.

Reprogramming factors	Mouse/human	*In vitro*/*in vivo*	Reported efficiency	Analysis method	Reference
GMT	Mouse	Both	4.8% cTnT+ *(in vitro)* 17% αMHC+ *(in vitro)*	FC	[11, 54]
GMHT	Mouse	Both	27.6% cTnT+ *(in vitro)*	FC	[16, 53]
GMHT, *MyoD* transactivation domain	Mouse	*In vitro*	19% cTnT+	IF	[17]
GMHT, *Nkx2–5*	Mouse	*In vitro*	1.6% GCaMP+	IF	[18]
MT, *Myocd*	Mouse	*In vitro*	12% cTnT+	FC	[19]
GMT, *Myocd*, *Srf*, *Mesp1*, *Smarcd3*	Mouse	*In vitro*	2.4% αMHC+	FC	[20]
ETS2, *MESP1*	Human	*In vitro*	13.7% αMHC+	FC	[21]
GMT, *MESP1*, *MYOCD*	Human	*In vitro*	5.9% cTnT+	FC	[22]
GMT, *ESSRG*, *MESP1*, *MYOCD*, *ZFPM2*	Human	*In vitro*	18.1% αMHC+	FC	[23]

FC: flow cytometry; IF: immunofluorescence.

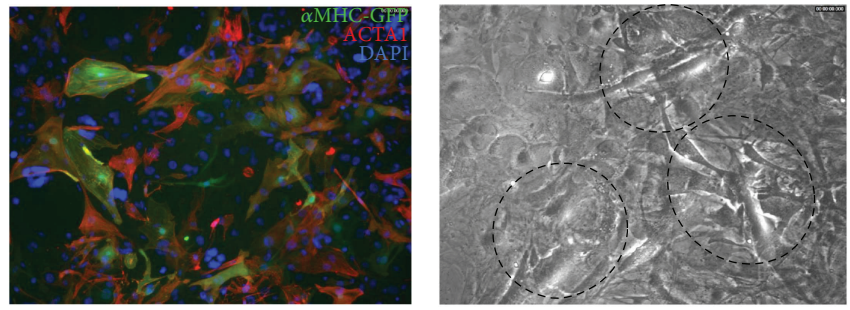
Table 2: Summary of direct cardiac reprogramming results.

Reprogramming factors	Mouse/human	*In vitro*/*in vivo*	Reported efficiency	Analysis method	Reference
GMHT, SB431542	Mouse	*In vitro*	9.3% GCaMP+	IF	[26]
GMHT, DAPT	Mouse	*In vitro*	38% cTnT+	IF	[28]
GM(H)T, FGF2, FGF10, VEGF	Mouse	*In vitro*	2.9% cTnT+	FC	[32]
miRNA-1, miRNA-133, miRNA-208, miRNA-499	Mouse	*In vivo*	12% tdTomato+cTnT+	IHC	[36]
miRNA-1, miRNA-133, miRNA-208, miRNA-499, JAK inhibitor I	Mouse	Both	28% αMHC+ (*in vitro*)	FC	[30]
GMT, *Mesp1*, *Myocd*, miRNA-133	Mouse Human	*In vitro*	12.9% cTnT+ 27.8% cTnT+	FC	[35]
GHT, *MYOCD*, miRNA-1, miRNA-133	Human	*In vitro*	34.1% cTnT+	FC	[37]
GMHT, miRNA-1, miRNA-133, miRNA-208, miRNA-499, Y-27632, A83-01	Mouse	*In vitro*	60% cTnT+	IF	[29]
GMT, miRNA-590	Human Porcine	*In vitro*	4.6% cTnT+	FC	[39]
Ascorbic acid, RepSox, forskolin, valproic acid, CHIR99021	Mouse	*In vitro*	9% αMHC+	FC	[60]
CHIR99021, BIX01294, A83-01, AS8351, SC1, OAC2, Y27632, SU16F, JNJ10198409	Human	*In vitro*	6.6% cTnT+	FC	[61]

FC: flow cytometry; IF: immunofluorescence; IHC: immunohistochemistry.

methods have been developed using additional molecules. These additives can be classified into three major categories: inhibitors/cytokines, noncoding RNAs, and epigenetic modifiers. A summary of these reprogramming experiments is presented in Table 2.

3.1. Inhibitors/Cytokines. A potential approach to improving reprogramming is to inhibit the endogenous signaling pathways and gene programs that maintain the distinct properties of fibroblasts. One of the major signaling pathways active in fibroblasts is the transforming growth factor-(TGF-) β pathway. TGF-β has diverse and pleotropic effects through its activation and signaling. The downstream effect of the TGF-β signaling pathway involves phosphorylation of receptor-regulated SMADs that ultimately activate TFs that participate in the regulation of target gene expressions, many of which are critical in fibroblast activation and proliferation. Since inhibition of TGF-β has been shown to increase mouse embryonic stem cell differentiation to cardiomyocytes [24, 25], it was hypothesized that TGF-β inhibition could improve reprogramming. The TGF-β inhibitors SB431542 and A83-01 have been added to various reprogramming combinations and have shown an increase in reprogramming efficiency. SB431542 is a selective and potent inhibitor of the TGF-β pathway through suppression of the activin A receptors ALK5, ALK4, and ALK7. A83-01 is also a selective inhibitor of ALK5, ALK4, and ALK7 but is more potent than SB431542 in its inhibition and effectively blocks phosphorylation of Smad2. When SB431542 was combined with GMHT, a 5-fold increase in reprogramming efficiency was observed in both MEFs and mouse adult cardiac fibroblasts [26]. Likewise, we observed an increase in



(a) (b)

Figure 1: GMT + A83-01 reprogramming in MEFs. (a) Immunocytochemistry for the cardiac markers αMHC-GFP and α-actinin (ACTA1). (b) Video snapshot showing beating areas of reprogrammed cells (outlined areas).

reprogramming efficiency, with frequent areas of spontaneous contraction and enhanced expression of cardiac contractile proteins when we reprogrammed MEFs with GMT and A83-01 cells (Figure 1). Furthermore, when GMT and SB431542 were combined with WNT inhibition by XAV939, reprogramming efficiency was increased 8-fold in cardiac fibroblasts with respect to GMT alone [27]. In addition to the TGF-β pathway, other profibrotic and intracellular signaling pathways such as the Rho-associated kinase, JAK/STAT, Notch and Akt pathways have been targeted to improve reprogramming [28–31].

The utility of other molecules to enhance cardiac reprogramming has been inspired by using cytokines and/or modulators that are critical in mammalian cardiac development, many of which are commonly used in the differentiation of cardiomyocytes from pluripotent stem cells. Fibroblast growth factor-2 (FGF2), FGF10, and vascular endothelial growth factor (VEGF) in combination with GMT or GMHT in MEFs and mouse tail-tip fibroblasts showed an increase in the number of iCMs that spontaneously contract [32]. These approaches also accelerated maturation of iCMs *in vitro*, and thus, the activation of the important developmental pathway during reprogramming warrants further research.

3.2. Noncoding RNAs. MicroRNAs (miRNAs) are small noncoding RNAs that induce degradation or inhibit translation of target mRNAs. miRNAs are an attractive additive to reprogramming since they play important roles in the posttranscriptional regulation of cardiac gene expression and have critical function in almost every stage of heart development. miRNA-1, miRNA-133, miRNA-208, and miRNA-499 have been shown to be cardiac- and muscle-specific and play important roles in cardiac development and function. miRNA-1, contributing to ~40% of total miRNAs in the heart, has been shown to promote cardiomyocyte proliferation and suppress apoptosis during development; however, its function in cardiac reprogramming remains unknown [33]. miRNA-133 is important in orchestrating cardiac development, gene expression, growth, and function [34]. It also promotes cardiomyocyte proliferation by repressing the transcriptional regulator SNAI1 and silences fibroblast gene signatures during reprogramming [35]. These two miRNAs have been used in combination with other factors to successfully enhance cardiac reprogramming. A novel approach was reported recently where a combination of miRNAs promoted direct conversion of cardiac fibroblasts into cardiomyocyte-like cells without the need for forced expression of exogenous TFs. A combination of miRNA-1, miRNA-133, miRNA-208, and miRNA-499 was reported to be sufficient to convert mouse cardiac fibroblasts into iCMs without the addition of other factors *in vivo* [36]. The potential mechanism for this effect is thought to be due to altered H3K27 methyltransferase and demethylase expression, which leads to changes in the epigenetic landscape of fibroblasts to induce their conversion into cardiomyocyte-like cells. A 10-fold increase in miRNA-mediated murine cardiac fibroblast reprogramming was observed when miRNA-1, miRNA-133, miRNA-208, and miRNA-499 were combined with JAK inhibitor I [30].

Similarly, when miRNA-133 was used in conjunction with GMT, *Mesp1*, and *Myocd* or GHT, *Myocd*, and miRNA-1, the reprogramming efficiency was increased in both human and mouse fibroblasts by repressing Snai1 and silencing fibroblast gene signatures [35, 37]. Zhao et al. used a combination of GMHT, miRNA-1, miRNA-133, miRNA-208, miRNA-499, Y-27632, and A83-01 in MEFs and mouse adult fibroblasts to achieve ~60% cardiac troponin T+ and 60% α-actinin+ iCMs [29]. miRNA-590, a miRNA that can induce adult cardiomyocyte proliferation, was recently shown to be able to replace *HAND2* and *MYOCD* in GMT direct reprogramming experiments using human and porcine fibroblasts [38, 39]. While GMT was initially shown to be sufficient for cardiac reprogramming, further studies have indicated that a multiprong approach may be necessary to enhance reprogramming and could hold great promise for future *in vivo* clinical application.

Long noncoding RNAs (lncRNAs) are a heterogeneous group of transcripts longer than 200 nucleotides that exert major regulatory roles in gene expression during development and disease through many different mechanisms. Recent advances in sequencing and analysis technologies have allowed many lncRNAs to be identified, but due to their complex and multiple mechanisms of action as well as to the low interspecies conservation, it

has been difficult to decipher biological functions of many lncRNAs [40]. A list of cardiac lncRNAs that are involved in cardiac differentiation, development, and contractile function has been reviewed [41]. Braveheart (*Bvht*) and *Fendrr* play a critical role in cardiac lineage commitment by regulating the transition from mesoderm to CPCs through activation of key cardiac development genes and TFs including some of those studied above [42–44]. *Hotair*, *Chaer*, and other lncRNAs have also been shown to regulate the epigenetic landscape in cardiac development by regulating proteins involved in histone modification at targeted promoters [45, 46]. lncRNAs play an extensive role in the regulation of cardiac development and gene expression; therefore, it may be advantageous to explore the use of lncRNAs in direct reprogramming studies; however, no direct reprogramming studies have been published using lncRNAs.

3.3. Epigenetic Modifiers. Reprogramming of one somatic cell type to another requires the activation and repression of multiple sets of genes, leading to vast genomic changes. The epigenetic landscape plays an important role in determining the reprogramming efficiency as accessibility of TFs to their DNA targets is critical. During reprogramming, epigenetic marks such as histone methylation, acetylation, and ubiquitination must be added and removed from fibroblast- and cardiac-specific genes. These modifications will suppress expression of fibroblast genes while activating cardiac genes by remodeling chromatin structure to allow or restrict the access of TFs to their target genes. It has been shown that during cardiac direct reprogramming, the trimethylated histone H3 of lysine 27 (H3K27me3), which marks inactive chromatin, increases at fibroblast promoters and decreases at cardiac promoters while the activated chromatin mark H3K4me3 shows the opposite pattern at important loci [11, 47]. Moreover, the activating H3K4me2 histone mark has been shown to be increased at the regulatory regions of miRNA-1 and miRNA-133 [29]. To this end, attempts to improve direct cardiac reprogramming have been carried out using modulators of epigenetic marks. Bmi1 was identified as a barrier to reprogramming by modifying histone marks at key cardiogenic loci, thus inhibiting iCM induction. When *Bmi1* activity was knocked down, the active histone mark, H3K4me3, was increased while the repressive H2AK119ub mark was reduced, leading to increased cardiac gene expression at important loci [48]. In nonintegrative and *in vivo* reprogramming experiments discussed later in this review, other epigenetic modifiers that inhibit histone methyl transferases and histone demethylase have been used. The importance of epigenetic landscape and changes that happen during reprogramming have recently begun to be unraveled using a single-cell transcriptomic approach by Liu et al. [49]. These results highlight the complexity of the reprogramming process and the importance of the influence of a variety of factors, warranting additional research into the sequential addition of TFs, noncoding RNAs, cytokines, inhibitors, and epigenetic modifiers to further improve the reprogramming efficiency.

4. Direct Reprogramming to Expandable Cardiac Progenitor Cells

Another recent approach of clinical promise is the generation of expandable CPCs by direct reprogramming. The goal of this approach is to safely create CPCs *in vitro* that can then be expanded in culture before transplantation into the injured heart. Upon transplantation, the CPCs will differentiate into three major cells of the heart; cardiomyocytes, endothelial cells, and smooth muscle cells. Two groups were able to successfully generate expandable CPCs using unique reprogramming cocktails containing a variety of biomolecules described above. Lalit et al. generated CPCs from fibroblasts using Mesp1, Tbx5, Gata4, Nkx2–5, and Baf60c, a chromatin remodeling protein. *In vitro* expansion and maintenance of a CPC state were achieved using a Wnt activator, BIO, and a JAK/STAT activator, LIF [50, 51]. On the other hand, Zhang et al. used a chemical approach to reprogramming fibroblasts to CPCs. Generation, expansion, and maintenance of CPCs were achieved by the addition of BMP4, activin A, CHIR99021, and SU504 (a FGF, VEGF, and PDGF signaling inhibitor) [52]. Both groups were able to show that their reprogrammed CPCs maintained their characteristics for many passages in culture and could generate cardiomyocytes, endothelial cells, and smooth muscle cells both *in vitro* and *in vivo* when transplanted. Direct reprogramming of fibroblasts to CPCs represents a scalable method for the generation of multiple cardiac cell types for clinical applications; however, this approach has not yet been applied to human cells.

5. Progress of *In Vivo* Direct Reprogramming

The ultimate goal of direct reprogramming is to be able to repair the damaged myocardium after injury. Direct reprogramming offers two potential approaches for heart regeneration: (1) transplantation of reprogrammed fibroblasts into the infarcted heart and (2) reprogramming resident cardiac fibroblasts directly to cardiomyocytes. The first attempt at cardiac regeneration using direct reprogramming was carried out using cardiac fibroblasts that were transduced with GMT for 1 day and then transplanted into mouse hearts [11]. Analysis of these cells posttransplantation revealed that they successfully generated cardiomyocyte-like cells *in vivo*. Other studies have used *in vivo* transplantation of reprogrammed cells to test their regenerative potential. However, cell transplantation is complicated by many factors such as cell retention, viability, structural and functional integration, and immune rejection. Therefore, in situ repair of the heart is best studied by targeting endogenous cardiac fibroblasts through viral transfection of the infarct zone. This approach was attempted in 2012 by Song et al. and Qian et al., in which local delivery of GM(H)T viruses induced reprogramming of nonmyocytes into iCMs by 4 weeks postsurgery [53, 54]. Additionally, Qian et al. reported that codelivery of thymosin β4 and GMT viruses further improved ejection fraction and reduced scar

formation. Interestingly, it has been reported that the *in vivo* cardiac niche may improve the efficiency of reprogramming; however, the mechanisms underlying this observation remain elusive [27, 36, 53, 54]. Several studies have improved the *in vivo* reprogramming efficiency even further by optimizing polycistronic expression vectors to control the stoichiometry of TF expression or by the addition of small molecules delivered with TFs [27, 55–57].

6. Are All Fibroblasts Created Equal?

Cardiac fibroblasts are ideal targets for direct reprogramming as they are the most prominent cell type within the heart and play key roles in regulating normal myocardial function as well as adverse remodeling following injury. Various mouse and/or human fibroblast sources have been tested, including mouse embryonic fibroblasts, tail-tip fibroblasts, and dermal fibroblasts, with varying results, suggesting the importance of the starting cell type for direct reprogramming. It is also interesting to note that *in vivo* reprogramming has been reported to be more efficient than *in vitro* reprogramming, despite the fact that upon injury, cardiac fibroblast expresses TGF-β, which has been shown to be inhibitory to reprogramming *in vitro*. Furthermore, our lab has shown that cardiac fibroblasts are a heterogeneous population from different embryonic origins [58]. It is possible to postulate that perhaps a subpopulation of cardiac fibroblasts may be more susceptible to reprogramming depending on their developmental origin. Further understanding of the epigenetic landscape of fibroblasts and their susceptibility to direct reprogramming would be of great use to the field. This would also open up the possibility for repairing the heart by targeting specific fibroblast populations.

7. Nonintegrative Methods of Direct Reprogramming for Future *In vivo* Applications

The reprogramming results shown thus far suggest that direct reprogramming of fibroblasts can be a feasible therapeutic approach to repairing the injured myocardium. However, relatively safe methods for the delivery of various reprogramming factors need to be explored for *in vivo* applications. Adeno-associated virus (AAV) vectors are attractive tools for TF delivery, but the limited capacity of about 4.5 kb complicates the expression of multiple TFs in a single vector and still involves the use of an integrative viral system. Sendai virus reprogramming is an appealing alternative to AAV since it does not integrate into the host genome and has been successfully used to reprogram many different cell types to pluripotency; however, its use in direct reprogramming has not yet been explored. A recent study showed that acute expression of GMT in nonintegrating adenoviral vectors was as efficient as lentiviral vectors at reprogramming in a rat infarct model, which has increased the clinical applicability of *in vivo* reprogramming. As described in two recent reports, the temporal control and stoichiometric control of TFs are also important in determining reprogramming efficiency [57, 59]. Unfortunately, current *in vivo* viral reprogramming tools are unable to control dosage and temporal expression of TFs but warrant further investigation to improve reprogramming efficiency.

Compared to TFs and miRNAs, small molecules have many advantages such as more effective cell delivery and being nonimmunogenic and less expensive and are generally safer. Moreover, it is more convenient to control the process of reprogramming through varying small molecule concentrations and combinations *in vitro*. A combination of ascorbic acid, RepSox (a TGF-β inhibitor), forskolin, valproic acid, and CHIR99021 (a WNT pathway activator through the inhibition of glycogen synthase kinase 3) was shown to reprogram MEFs and mouse tail-tip fibroblasts to iCMs *in vitro* [60]. Cao et al. were able to use a cocktail of 9 small molecules (CHIR99021, A83-01, BIX01294, AS8351, SC1, OAC2, Y27632, SU16F, and JNJ10198409) to direct cardiac reprogramming of human foreskin fibroblasts *in vitro* before transplantation in injured murine hearts [61]. Among these small molecules were the epigenetic modifiers BIX01294 (a methyltransferase inhibitor) and AS8351 (a histone demethylase inhibitor), SC1 (an ERK2 and Ras-GAP inhibitor), OAC2 (an Oct4 activator), SU16F (a PDGFRβ inhibitor), and JNJ10198409 (a PDGF receptor tyrosine kinase inhibitor). However, the use of small molecules for *in vivo* reprogramming poses some unanswered questions. Small molecules can enter the blood stream and spread to other organs with unknown consequences. Additionally, the ability of timely uptake into specific target cell type remains a challenge. Development of novel biomaterials for local delivery, controlled release, and retention of small molecules is still needed.

Another promising nonviral method of direct cardiac reprogramming is the use of modified mRNAs (modRNAs) [62]. ModRNAs are noncytopathic and do not integrate into the host genome, thus offering a safer approach to reprogramming. ModRNAs have been used successfully to generate induced pluripotent stem cells from somatic cells through transient expression of mRNAs that direct cell fate. ModRNAs are produced using an *in vitro* transcription system to generate mRNAs that contain a synthetic 5′ guanine cap and poly-A tail, which improves half-life and stability, as well as modified nucleotide bases that reduce the innate immune response of the host cell. This technology is endowed with a number of attractive properties that would make it a potentially powerful platform for direct cardiac reprogramming. ModRNAs can mediate robust and dose-titratable expression of key TFs over a specified time and in a particular sequence. Previous studies outlined in this review have highlighted the fact that direct cardiac reprogramming is a complex process that may require sequential treatments to better overcome the reprogramming barrier. ModRNAs may be ideal for direct reprogramming as they have a relatively short half-life; therefore, distinct factors can be expressed for a short period of time and then removed from the reprogramming cocktail or added again to continue expression. ModRNAs may open the

door to following a more developmentally relevant sequence of TFs to improve transcription. It is also foreseeable that modRNAs could be combined with other small molecules, cytokines, and noncoding RNAs discussed in this review.

8. Roadblocks and Challenges

There has been significant progress in recent years with direct cardiac reprogramming through important discoveries in understanding the mechanism of reprogramming and the biology of cardiac development. However, several challenges must be addressed prior to clinical translation of this technology. The reprogramming efficiency must be increased in order to generate enough cells *in vitro* for transplantation. One avenue that has the potential to generate the number of cells needed for transplantation is reprogramming to CPCs, which can be expanded *in vitro* before transplantation. The retention, integration, and maturation of iCMs or CPCs after transplantation remain a concern. Multiplex immunostaining and patch clamp analysis have also revealed the presence of all three cardiomyocyte types (atrial, ventricular, and pacemaker) in iCMs, therefore increasing the risk of arrhythmias [16]. There is a need to develop techniques to generate specific subtypes of cardiomyocytes for both *in vitro* and *in vivo* direct reprogramming. A safe and effective approach to delivering and targeting reprogramming factors *in vivo* will be needed to circumvent *in vitro* reprogramming completely.

Transcription factors, inhibitors, cytokines, noncoding RNAs, and epigenetic modulators have been shown to be important for direct cardiac reprogramming. However, studies have uncovered variable reproducibility between different labs, leading to wide differences in reprogramming efficiency, maturity, and characteristics of the iCMs. These inconsistencies can be attributed to many factors other than the reprogramming factors themselves. First, the components of culture media used during reprogramming widely vary from group to group along with the duration of reprogramming before analysis. Additionally, the induction time, the type of fibroblasts, and the amount and sequence of factors used along with the time exposed to reprogramming factors are different between protocols. Moreover, the criteria used to measure the outcome and success are inconsistent and not standardized in the field. Reprogramming success is measured by some as the presence of cardiac-related structural proteins on immunostaining, while others employ a much more detailed approach including appearance of spontaneous beating along with gene and protein expression data. Even differences in the cardiac markers used to analyze the reprogramming efficiency, cardiac troponin T (cTnT) versus alpha myosin heavy chain (αMHC) versus GCaMP activity, and the method of measurement, flow cytometry versus immunofluorescence (IF), make comparisons among studies difficult. A myriad of criteria and stringency that have been used to evaluate reprogramming efficiency have been summarized by Addis and Epstein and are presented in Table 3 [63].

Table 3: Criteria to evaluate reprogramming efficiency. Adapted from Addis and Epstein [63].

Characteristic	Stringency	Assay technique(s)
Gene expression	Low	RT-qPCR Reporter transgene
Protein expression	Low	Immunostaining Flow cytometry Western blot
Transcriptome and epigenetic analysis	High	Microarray RNA-seq ChIP-seq ATAC-seq
Contraction and force generation	High	Spontaneous Chemical stimulation Electrical stimulation Three-dimensional bioengineered platforms
Electrophysiological	High	Patch clamp Microelectrode arrays Optical mapping
Calcium transients and electrical coupling	High	Calcium-sensitive dyes Genetically encoded indicators (GCaMP) Optical mapping
Functional improvement	High	Echocardiography

Optimization of the minimal yet sufficient combination of factors to improve reprogramming requires further research. Studies presented here have revealed that simply expressing a few core transcription factors is not sufficient for efficient cardiac reprogramming [64]. There may also be a dosage and temporal requirement for reprogramming factors [57, 59]. Other factors such as activated cellular signaling processes and epigenetic landscape should be considered to improve efficiency and quality. For example, when the TGF-β signaling pathway was disrupted by small molecules or when important cardiac regulatory miRNAs were added, an increase in reprogramming was observed, supporting the hypothesis that a multifaceted approach is likely necessary to achieve high reprogramming efficiency. Furthermore, these studies highlight the significant differences between mouse and human reprogramming as well as the effect of the starting fibroblast type (MEFs, tail-tip fibroblasts, or cardiac fibroblasts). Differences in fibroblast populations may be attributed to differences in epigenetic landscape, which can be influenced by many factors such as the cell environment or developmental origin. Further research on the heterogeneity of fibroblast epigenetic landscapes is warranted and will be of great benefit to direct reprogramming.

9. Conclusion

In this review, we discussed the reprogramming of fibroblasts into cardiomyocyte-like cells and expandable CPCs using

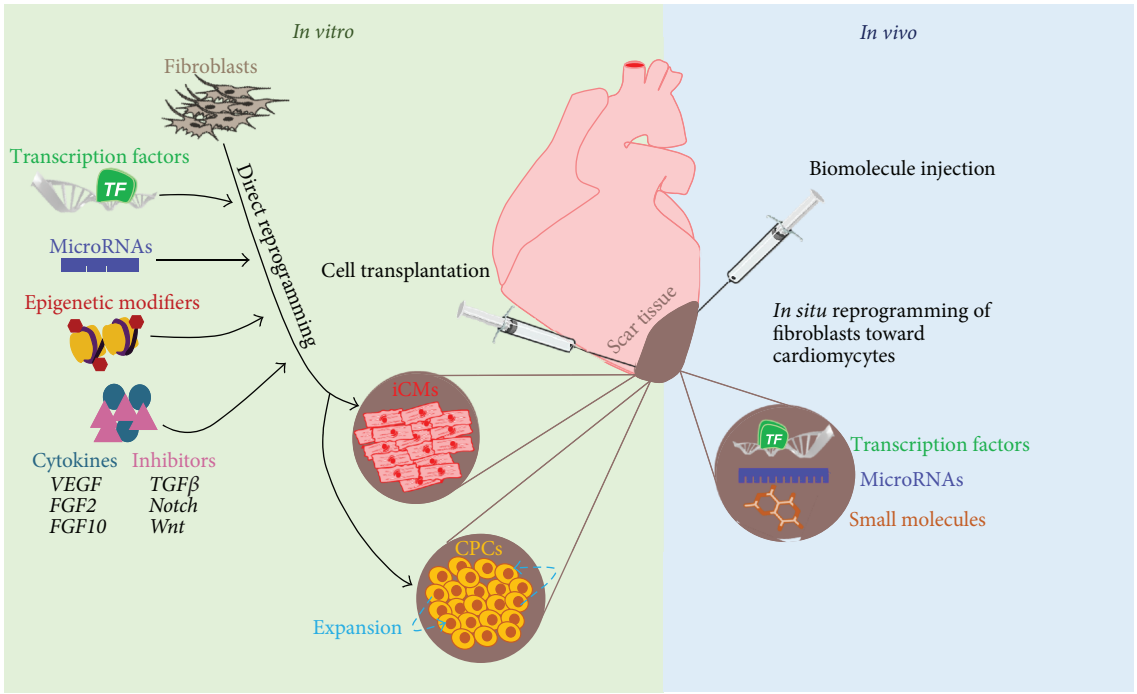


Figure 2: Schematic showing the current and future applications of direct cardiac reprogramming.

transcription factors, small molecules, noncoding RNAs, and other biologics for the treatment of heart failure (Figure 2). Despite the current limitations that exist with direct cardiac reprogramming, this technology offers great promise for cardiac regeneration therapy. It is clear that the reprogramming process is very complex and that many factors have profound influence over this process. Continued research of key transcription factors, noncoding RNAs, small molecules, reprogramming mechanisms, delivery and targeting methods, and biomaterials will help advance direct cardiac reprogramming to large animal models and ultimately for the treatment of heart failure.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors would like to thank Sara Ranjbarvaziri for her help in figure preparation and Ngoc B. Nguyen for critically reading this review. This work was supported in part by grants from the National Institutes of Health (NIH) (DP2 HL127728) and the California Institute for Regenerative Medicine (CIRM) (RN3-06378).

References

[1] E. Braunwald, "The war against heart failure: the *Lancet* lecture," *The Lancet*, vol. 385, no. 9970, pp. 812–824, 2015.

[2] P. A. Heidenreich, N. M. Albert, L. A. Allen et al., "Forecasting the impact of heart failure in the United States: a policy statement from the American Heart Association," *Circulation: Heart Failure*, vol. 6, no. 3, pp. 606–619, 2013.

[3] T. J. Cahill, R. P. Choudhury, and P. R. Riley, "Heart regeneration and repair after myocardial infarction: translational opportunities for novel therapeutics," *Nature Reviews Drug Discovery*, vol. 16, no. 10, pp. 699–717, 2017.

[4] C. J. Taylor, A. K. Roalfe, R. Iles, and F. D. Hobbs, "Ten-year prognosis of heart failure in the community: follow-up data from the Echocardiographic Heart of England Screening (ECHOES) study," *European Journal of Heart Failure*, vol. 14, no. 2, pp. 176–184, 2012.

[5] R. L. Davis, H. Weintraub, and A. B. Lassar, "Expression of a single transfected cDNA converts fibroblasts to myoblasts," *Cell*, vol. 51, no. 6, pp. 987–1000, 1987.

[6] J. Choi, M. L. Costa, C. S. Mermelstein, C. Chagas, S. Holtzer, and H. Holtzer, "MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 20, pp. 7988–7992, 1990.

[7] T. Vierbuchen, A. Ostermeier, Z. P. Pang, Y. Kokubu, T. C. Südhof, and M. Wernig, "Direct conversion of fibroblasts to functional neurons by defined factors," *Nature*, vol. 463, no. 7284, pp. 1035–1041, 2010.

[8] B. Yu, Z. Y. He, P. You et al., "Reprogramming fibroblasts into bipotential hepatic stem cells by defined factors," *Cell Stem Cell*, vol. 13, no. 3, pp. 328–340, 2013.

[9] P. Huang, Z. He, S. Ji et al., "Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors," *Nature*, vol. 475, no. 7356, pp. 386–389, 2011.

[10] H. Xie, M. Ye, R. Feng, and T. Graf, "Stepwise reprogramming of B cells into macrophages," *Cell*, vol. 117, no. 5, pp. 663–676, 2004.

[11] M. Ieda, J. D. Fu, P. Delgado-Olguin et al., "Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors," *Cell*, vol. 142, no. 3, pp. 375–386, 2010.

[12] Y. Hiroi, S. Kudoh, K. Monzen et al., "Tbx5 associates with Nkx2-5 and synergistically promotes cardiomyocyte differentiation," *Nature Genetics*, vol. 28, no. 3, pp. 276–280, 2001.

[13] C. Perrino and H. A. Rockman, "GATA4 and the two sides of gene expression reprogramming," *Circulation Research*, vol. 98, no. 6, pp. 715-716, 2006.

[14] E. Dodou, M. P. Verzi, J. P. Anderson, S. M. Xu, and B. L. Black, "*Mef2c* is a direct transcriptional target of ISL1 and GATA factors in the anterior heart field during mouse embryonic development," *Development*, vol. 131, no. 16, pp. 3931–3942, 2004.

[15] Y. S. Dai, P. Cserjesi, B. E. Markham, and J. D. Molkentin, "The transcription factors GATA4 and dHAND physically interact to synergistically activate cardiac gene expression through a p300-dependent mechanism," *Journal of Biological Chemistry*, vol. 277, no. 27, pp. 24390–24398, 2002.

[16] Y. J. Nam, C. Lubczyk, M. Bhakta et al., "Induction of diverse cardiac cell types by reprogramming fibroblasts with cardiac transcription factors," *Development*, vol. 141, no. 22, pp. 4267–4278, 2014.

[17] H. Hirai, N. Katoku-Kikyo, S. A. Keirstead, and N. Kikyo, "Accelerated direct reprogramming of fibroblasts into cardiomyocyte-like cells with the MyoD transactivation domain," *Cardiovascular Research*, vol. 100, no. 1, pp. 105–113, 2013.

[18] R. C. Addis, J. L. Ifkovits, F. Pinto et al., "Optimization of direct fibroblast reprogramming to cardiomyocytes using calcium activity as a functional measure of success," *Journal of Molecular and Cellular Cardiology*, vol. 60, pp. 97–106, 2013.

[19] S. Protze, S. Khattak, C. Poulet, D. Lindemann, E. M. Tanaka, and U. Ravens, "A new approach to transcription factor screening for reprogramming of fibroblasts to cardiomyocyte-like cells," *Journal of Molecular and Cellular Cardiology*, vol. 53, no. 3, pp. 323–332, 2012.

[20] N. Christoforou, M. Chellappan, A. F. Adler et al., "Transcription factors MYOCD, SRF, Mesp1 and SMARCD3 enhance the cardio-inducing effect of GATA4, TBX5, and MEF2C during direct cellular reprogramming," *PLoS One*, vol. 8, no. 5, article e63577, 2013.

[21] J. F. Islas, Y. Liu, K. C. Weng et al., "Transcription factors ETS2 and MESP1 transdifferentiate human dermal fibroblasts into cardiac progenitors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 32, pp. 13016–13021, 2012.

[22] R. Wada, N. Muraoka, K. Inagawa et al., "Induction of human cardiomyocyte-like cells from fibroblasts by defined factors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 31, pp. 12667–12672, 2013.

[23] J. D. Fu, N. R. Stone, L. Liu et al., "Direct reprogramming of human fibroblasts toward a cardiomyocyte-like state," *Stem Cell Reports*, vol. 1, no. 3, pp. 235–247, 2013.

[24] A. Ao, J. Hao, C. R. Hopkins, and C. C. Hong, "DMH1, a novel BMP small molecule inhibitor, increases cardiomyocyte progenitors and promotes cardiac differentiation in mouse embryonic stem cells," *PLoS One*, vol. 7, no. 7, article e41627, 2012.

[25] W. Cai, R. M. Guzzo, K. Wei, E. Willems, H. Davidovics, and M. Mercola, "A nodal-to-TGFβ cascade exerts biphasic control over cardiopoiesis," *Circulation Research*, vol. 111, no. 7, pp. 876–881, 2012.

[26] J. L. Ifkovits, R. C. Addis, J. A. Epstein, and J. D. Gearhart, "Inhibition of TGFβ signaling increases direct conversion of fibroblasts to induced cardiomyocytes," *PLoS One*, vol. 9, no. 2, article e89678, 2014.

[27] T. M. Mohamed, N. R. Stone, E. C. Berry et al., "Chemical enhancement of in vitro and in vivo direct cardiac reprogramming," *Circulation*, vol. 135, no. 10, pp. 978–995, 2017.

[28] M. Abad, H. Hashimoto, H. Zhou et al., "Notch inhibition enhances cardiac reprogramming by increasing MEF2C transcriptional activity," *Stem Cell Reports*, vol. 8, no. 3, pp. 548–560, 2017.

[29] Y. Zhao, P. Londono, Y. Cao et al., "High-efficiency reprogramming of fibroblasts into cardiomyocytes requires suppression of pro-fibrotic signalling," *Nature Communications*, vol. 6, p. 8243, 2015.

[30] T. M. Jayawardena, B. Egemnazarov, E. A. Finch et al., "MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes," *Circulation Research*, vol. 110, no. 11, pp. 1465–1473, 2012.

[31] H. Zhou, M. E. Dickson, M. S. Kim, R. Bassel-Duby, and E. N. Olson, "Akt1/protein kinase B enhances transcriptional reprogramming of fibroblasts to functional cardiomyocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 38, pp. 11864–11869, 2015.

[32] H. Yamakawa, N. Muraoka, K. Miyamoto et al., "Fibroblast growth factors and vascular endothelial growth factor promote cardiac reprogramming under defined conditions," *Stem Cell Reports*, vol. 5, no. 6, pp. 1128–1142, 2015.

[33] P. K. Rao, Y. Toyama, H. R. Chiang et al., "Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure," *Circulation Research*, vol. 105, no. 6, pp. 585–594, 2009.

[34] N. Liu, S. Bezprozvannaya, A. H. Williams et al., "MicroRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart," *Genes & Development*, vol. 22, no. 23, pp. 3242–3254, 2008.

[35] N. Muraoka, H. Yamakawa, K. Miyamoto et al., "MiR-133 promotes cardiac reprogramming by directly repressing Snai1 and silencing fibroblast signatures," *The EMBO Journal*, vol. 33, no. 14, pp. 1565–1581, 2014.

[36] T. M. Jayawardena, E. A. Finch, L. Zhang et al., "MicroRNA induced cardiac reprogramming in vivo: evidence for mature cardiac myocytes and improved cardiac function," *Circulation Research*, vol. 116, no. 3, pp. 418–424, 2015.

[37] Y. J. Nam, K. Song, X. Luo et al., "Reprogramming of human fibroblasts toward a cardiac fate," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 14, pp. 5588–5593, 2013.

[38] A. Eulalio, M. Mano, M. D. Ferro et al., "Functional screening identifies miRNAs inducing cardiac regeneration," *Nature*, vol. 492, no. 7429, pp. 376–381, 2012.

[39] V. P. Singh, M. Mathison, V. Patel et al., "MiR-590 promotes transdifferentiation of porcine and human fibroblasts toward a cardiomyocyte-like fate by directly repressing specificity protein 1," *Journal of the American Heart Association*, vol. 5, no. 11, article e003922, 2016.

[40] P. Johnsson, L. Lipovich, D. Grandér, and K. V. Morris, "Evolutionary conservation of long non-coding RNAs; sequence, structure, function," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1840, no. 3, pp. 1063–1071, 2014.

[41] C. P. C. Gomes, H. Spencer, K. L. Ford et al., "The function and therapeutic potential of long non-coding RNAs in cardiovascular development and disease," *Molecular Theraphy Nucleic Acids*, vol. 8, pp. 494–507, 2017.

[42] C. A. Klattenhoff, J. C. Scheuermann, L. E. Surface et al., "*Braveheart*, a long noncoding RNA required for cardiovascular lineage commitment," *Cell*, vol. 152, no. 3, pp. 570–583, 2013.

[43] Z. Xue, S. Hennelly, B. Doyle et al., "A G-rich motif in the lncRNA *Braveheart* interacts with a zinc-finger transcription factor to specify the cardiovascular lineage," *Molecular Cell*, vol. 64, no. 1, pp. 37–50, 2016.

[44] P. Grote, L. Wittler, D. Hendrix et al., "The tissue-specific lncRNA *Fendrr* is an essential regulator of heart and body wall development in the mouse," *Developmental Cell*, vol. 24, no. 2, pp. 206–214, 2013.

[45] Z. Wang, X. J. Zhang, Y. X. Ji et al., "The long noncoding RNA *Chaer* defines an epigenetic checkpoint in cardiac hypertrophy," *Nature Medicine*, vol. 22, no. 10, pp. 1131–1139, 2016.

[46] Z. Wang and Y. Wang, "Dawn of the epi-LncRNAs: new path from Myheart," *Circulation Research*, vol. 116, no. 2, pp. 235-236, 2015.

[47] Z. Liu, O. Chen, M. Zheng et al., "Re-patterning of H3K27me3, H3K4me3 and DNA methylation during fibroblast conversion into induced cardiomyocytes," *Stem Cell Research*, vol. 16, no. 2, pp. 507–518, 2016.

[48] Y. Zhou, L. Wang, H. R. Vaseghi et al., "Bmi1 is a key epigenetic barrier to direct cardiac reprogramming," *Cell Stem Cell*, vol. 18, no. 3, pp. 382–395, 2016.

[49] Z. Liu, L. Wang, J. D. Welch et al., "Single-cell transcriptomics reconstructs fate conversion from fibroblast to cardiomyocyte," *Nature*, vol. 551, no. 7678, pp. 100–104, 2017.

[50] P. A. Lalit, M. R. Salick, D. O. Nelson et al., "Lineage reprogramming of fibroblasts into proliferative induced cardiac progenitor cells by defined factors," *Cell Stem Cell*, vol. 18, no. 3, pp. 354–367, 2016.

[51] P. A. Lalit, A. M. Rodriguez, K. M. Downs, and T. J. Kamp, "Generation of multipotent induced cardiac progenitor cells from mouse fibroblasts and potency testing in ex vivo mouse embryos," *Nature Protocols*, vol. 12, no. 5, pp. 1029–1054, 2017.

[52] Y. Zhang, N. Cao, Y. Huang et al., "Expandable cardiovascular progenitor cells reprogrammed from fibroblasts," *Cell Stem Cell*, vol. 18, no. 3, pp. 368–381, 2016.

[53] K. Song, Y. J. Nam, X. Luo et al., "Heart repair by reprogramming non-myocytes with cardiac transcription factors," *Nature*, vol. 485, no. 7400, pp. 599–604, 2012.

[54] L. Qian, Y. Huang, C. I. Spencer et al., "*In vivo* reprogramming of murine cardiac fibroblasts into induced cardiomyocytes," *Nature*, vol. 485, no. 7400, pp. 593–598, 2012.

[55] H. Ma, L. Wang, C. Yin, J. Liu, and L. Qian, "*In vivo* cardiac reprogramming using an optimal single polycistronic construct," *Cardiovascular Research*, vol. 108, no. 2, pp. 217–219, 2015.

[56] M. Mathison, V. P. Singh, R. P. Gersch et al., ""Triplet" polycistronic vectors encoding Gata4, Mef2c, and Tbx5 enhances postinfarct ventricular functional improvement compared with singlet vectors," *The Journal of Thoracic and Cardiovascular Surgery*, vol. 148, no. 4, pp. 1656–1664.e2, 2014.

[57] L. Wang, Z. Liu, C. Yin et al., "Stoichiometry of Gata4, Mef2c, and Tbx5 influences the efficiency and quality of induced cardiac myocyte reprogramming," *Circulation Research*, vol. 116, no. 2, pp. 237–244, 2015.

[58] S. R. Ali, S. Ranjbarvaziri, M. Talkhabi et al., "Developmental heterogeneity of cardiac fibroblasts does not predict pathological proliferation and activation," *Circulation Research*, vol. 115, no. 7, pp. 625–635, 2014.

[59] T. C. Umei, H. Yamakawa, N. Muraoka et al., "Single-construct polycistronic doxycycline-inducible vectors improve direct cardiac reprogramming and can be used to identify the critical timing of transgene expression," *International Journal of Molecular Sciences*, vol. 18, no. 8, 2017.

[60] Y. Fu, C. Huang, X. Xu et al., "Direct reprogramming of mouse fibroblasts into cardiomyocytes with chemical cocktails," *Cell Research*, vol. 25, no. 9, pp. 1013–1024, 2015.

[61] N. Cao, Y. Huang, J. Zheng et al., "Conversion of human fibroblasts into functional cardiomyocytes by small molecules," *Science*, vol. 352, no. 6290, pp. 1216–1220, 2016.

[62] L. Warren, P. D. Manos, T. Ahfeldt et al., "Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA," *Cell Stem Cell*, vol. 7, no. 5, pp. 618–630, 2010.

[63] R. C. Addis and J. A. Epstein, "Induced regeneration–the progress and promise of direct reprogramming for heart repair," *Nature Medicine*, vol. 19, no. 7, pp. 829–836, 2013.

[64] J. X. Chen, M. Krane, M. A. Deutsch et al., "Inefficient reprogramming of fibroblasts into cardiomyocytes using Gata4, Mef2c, and Tbx5," *Circulation Research*, vol. 111, no. 1, pp. 50–55, 2012.

Hindawi
Stem Cells International
Volume 2018, Article ID 8283648, 9 pages
https://doi.org/10.1155/2018/8283648

Review Article

Cardiac Progenitor Cells in Basic Biology and Regenerative Medicine

Nevin Witman[1,2] **and Makoto Sahara**[1,2]

[1]*Department of Cell and Molecular Biology, Karolinska Institutet, 171 77 Stockholm, Sweden*
[2]*Department of Medicine-Cardiology, Karolinska Institutet, 171 77 Stockholm, Sweden*

Correspondence should be addressed to Makoto Sahara; makoto.sahara@ki.se

Received 9 August 2017; Revised 29 November 2017; Accepted 25 December 2017; Published 5 February 2018

Academic Editor: Fatemeh Sharifpanah

Major cardiovascular events including myocardial infarction (MI) continue to dominate morbidity rates in the developed world. Although multiple device therapies and various pharmacological agents have been shown to improve patient care and reduce mortality rates, clinicians and researchers alike still lack a true panacea to regenerate damaged cardiac tissue. Over the previous two to three decades, cardiovascular stem cell therapies have held great promise. Several stem cell-based approaches have now been shown to improve ventricular function and are documented in preclinical animal models as well as phase I and phase II clinical trials. More recently, the cardiac progenitor cell has begun to gain momentum as an ideal candidate for stem cell therapy in heart disease. Here, we will highlight the most recent advances in cardiac stem/progenitor cell biology in regard to both the basics and applied settings.

1. Introduction

Due to marginal improvements in heart failure treatments, a greater number of elderly patients are living longer with chronic heart failure. However, no treatment regime is capable of fully reversing pathological remodeling or completely restoring ventricular function after a major cardiovascular event, such as MI. In fact, many patients progress steadily towards New York Heart Association (NYHA) class III-IV heart failure where the only curative therapy is heart transplantation. Due to the unbalanced need of donor hearts, alternative regenerative therapeutic approaches aim to build up lost functional ventricular muscle.

Cell-based therapies have been conceptualized to alleviate some of the barriers limiting cardiac regeneration. The golden objective in cell-based therapies is to repopulate parts of damaged myocardium with engrafted, functional cells that restore lost cardiac function, enabling sufficient oxygen and nutrient circulation to all the vital organs of the body. Several technological, financial, and ethical hurdles impede such a medicinal feat, yet the field continues to move forward with the collaborative efforts between stem cell biologists, who are investigating novel mechanisms of cardiac regeneration, and medical teams in cardiology.

Much effort has been made in replacing damaged myocardium with adult/mature cardiomyocytes (CMs), those of which are derived from pluripotent stem cells or reprogramming strategies [1, 2]. However, several major technical limitations are compromising the success of an implantable, mature, cardiac muscle patch, including low numbers of surviving implanted CMs and the lack of electromechanical and structural integration between the host and donor CMs [3, 4]. More recently, emerging scientific evidence has begun to emphasize the use of cardiac progenitor cells (CPCs), rather than differentiated CMs, as a novel treatment strategy for cardiac regeneration. This is due to the notion that CPCs, which imply both embryonic/developmental and adult CPCs, are more capable of engrafting to host myocardium, in part by their strong proliferative potential and also their ability to generate multiple cardiac derivatives (Figure 1). Unlocking the use of such CPC technologies could potentially eliminate the limitations seen with mature CMs and

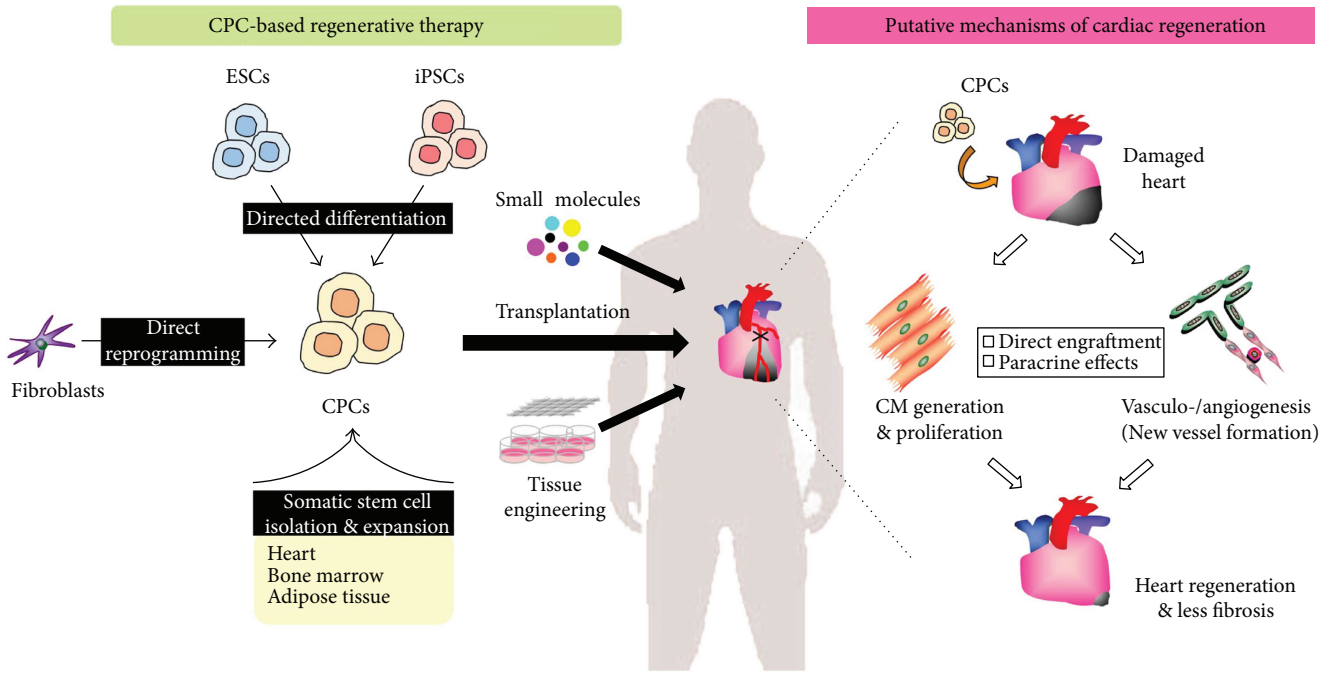


Figure 1: CPC-based regenerative therapy for heart disease. Cardiac progenitor cells (CPCs) can be obtained through several approaches (left). Directed differentiation of pluripotent stem cells such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) can generate "developmental (embryonic)" CPCs, while isolation and expansion of tissue- (i.e., heart) resident stem/progenitor cells can generate "adult" CPCs. Recently, an alternative approach by employing direct reprogramming can also generate "inducible" CPCs. These purified and expanded CPCs combined with small molecules and/or tissue engineering can be therapeutically transplanted into the damaged hearts of patients, such as those suffering from ischemic cardiomyopathy. Putative cellular mechanisms of cardiac regeneration by CPC-based therapy (right). Transplanted CPCs can be engrafted directly into the damaged host cardiac tissue and differentiated into mature cardiomyocytes as well as vascular cells (smooth muscle cells and endothelial cells). Simultaneously, the CPCs can potentially promote proliferation of preexisting cardiomyocytes in the damaged heart and also induce vasculo-/angiogenesis in the ischemic regions through secretion of the paracrine factors. Theoretically, increased working cardiomyocytes and newly formed vessels could lead to effective heart regeneration and a reduction in cardiac fibrosis in a coordinated fashion. Further details for cell-free approaches (e.g., small molecules and tissue engineering), somatic stem cell-expansion derived from bone marrow and adipose tissue, and CPC therapy-related mechanisms for cardiac regeneration have been reviewed elsewhere [6, 7].

provide long-term therapeutic effects, although the CPC therapy may bring the new challenges of obtaining efficient and committed differentiation of CPCs into CMs *in vivo* under pathological conditions, such as the ischemic and/or injured microenvironment [3, 5].

In this minireview, we discuss briefly the recent advances and knowledge of CPCs in basic biology and also clinical settings. For a more in-depth review of cell-free and cell-based approaches to cardiac regeneration, we refer the reader to the following reviews [6, 7].

2. Embryonic and Adult Cardiac Progenitor Cells

Conceptually, there are two distinct types of CPCs: embryonic/developmental CPCs and adult CPCs [8, 9]. Embryonic CPCs exist in the developmental mammalian heart, where they derive from a common mesodermal lineage. During cardiac development, two heart fields emerge termed the First Heart Field (FHF) and Second Heart Field (SHF). The FHF forms the cardiac crescent at embryonic day (E) 7.5 in mice and during embryonic days 16 to 18 in human and is marked by the transcription factor *NKX2-5* [9, 10] and the cyclic nucleotide-gated ion channel *HCN4* [11, 12]. The FHF then fuses at the midline and eventually forms the primitive heart tube that will begin to pump blood. The SHF is instead specifically marked by Islet-1 (*ISL1*) expression and lies medially and posteriorly to the crescent/FHF [13]. The SHF progenitors migrate behind the heart tube and extend anteriorly and posteriorly into the pharyngeal mesoderm to lengthen the outflow tract and form the looping heart tube at E8.5–9.0 in mice and during embryonic days 23 to 28 in human, in concert with the FHF progenitors [8, 14, 15]. FHF derivatives give rise to left ventricular myocardium with partial contribution to the atria, whereas SHF derivatives contribute to myocardium of the right ventricle, parts of the atria, and the outflow tract. The CPCs derived from the FHF and SHF will go on to give rise to many of the intermediates that are responsible for generating all the major cell types in the heart, including CMs, vascular smooth muscle cells (SMCs), arterial and venous endothelial cells (ECs), fibroblasts, and conductive cells of the cardiac conduction system. Much work is currently ongoing to understand the molecular underpinnings that regulate the spatiotemporal aspects of multipotent CPCs, as well as the signals that promote their differentiation into the diverse cell types that create the beating heart [16].

In addition to embryonic FHF and SHF CPCs, other progenitor cell populations, including epicardium-derived cells (EPDCs) and cardiac neural crest cells (cNCCs), also contribute to the formation of the developmental heart. Embryonic EPDCs are likely to contribute the SMCs, ECs, fibroblasts, and a small population of CMs in the heart through epithelial-to-mesenchymal transition, although EPDCs are heterogeneous and their contribution to CMs is still under debate [17–19]. cNCCs, which originate from the dorsal neural tube and migrate through the posterior pharyngeal arches to the arterial pole of the heart tube, give rise to SMCs of the outflow tract and contribute to outflow tract septation and valve formation [20, 21].

Embryonic-like CPCs, which are referred to as "developmental" CPCs, can be generated *in vitro* from pluripotent stem cells such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) [3, 5, 6]. CPCs in general are defined by having self-renewing and clonogenic properties, as well as multipotent differentiation capabilities to give rise to different cardiac lineages such as CMs, SMCs, and ECs, both *in vitro* and *in vivo* [22].

In contrast to the embryonic/developmental CPCs, to date, several kinds of endogenous CPCs, referred to as "adult" CPCs, have been isolated from adult rodent and human hearts, although their role in homeostasis or potential reparative function remains controversial [23]. The cell-surface marker tyrosine kinase receptor *c-kit* has been routinely used to identify the adult CPCs [22, 24]. Cardiac c-kit$^+$ cells isolated from adult human heart and injected into the infarcted rodent myocardium have been shown to increase cardiac function and improve cardiac structure [24, 25]. However, more recently, it was reported that very few cardiomyocytes are generated from c-kit$^+$ cells based on genetic lineage tracing technology [26], although in the mouse model used in [26], all the c-kit$^+$ cells were constitutively tagged, and thereby, the cardiac-derived c-kit$^+$ cells localized in the ageing or injured heart could not be distinguished from the bone marrow-derived c-kit$^+$ cells identified in the heart. There continues to be abundant controversy around the origin of c-kit$^+$ cells as they are broadly expressed in cells of the hematopoietic lineage [27], and a large number of c-kit$^+$ cells in the heart after MI appear to be bone marrow-derived [28]. Interestingly, the latest report has revealed that majority (≈90%) of the resident c-kit$^+$ cells in the rodent heart are blood/endothelial lineage-committed cells, while cardiac c-kit$^+$ (blood/endothelial lineage-negative) cells represent ≤ 10% of the total c-kit$^+$ cells in the heart [29]. It is speculated that the positive effects seen from the delivered c-kit$^+$ cells in the post-MI setting could be due to the release of signaling molecules, rather than the engrafted cells themselves [30, 31].

Previously, seminal works identified vascular endothelial growth factor type 2 receptor Flk-1, also known as kinase insert domain protein receptor (KDR) in human, and the platelet-derived growth factor receptor alpha (PDGFR-α) as some of the earliest cardiovascular progenitor cell markers involved in early stages of human cardiac development [32–35]. To date, a KDR$^+$/PDGFRα^+ population has become widely accepted as a classical CPC marker profile. Researchers are using this population and others as a means to enrich cardiac progenitors to possibly enhance applications of downstream cell-based therapies and disease modeling.

Apart from c-kit$^+$ or KDR$^+$/PDGFRα^+ cells, additional progenitor-like cell populations have been identified as adult CPC-like cells, including Sca1$^+$ cardiac cells [36, 37], cardiosphere-derived cells [38], and cardiac side population cells [39]. These cell types are heterogeneous in nature, and populations identified with different markers or approaches may have both unique and overlapping subsets in regard to molecular and physiological characteristics.

3. Recent Findings of Embryonic CPCs

A multipotent progenitor cell type that can intrinsically expand within the cardiac lineage has great potential as a regenerative therapy. In order to employ the correct cell type for regenerative purposes against heart disease, it is imperative to understand the role of the CPCs in development. Embryonic/developmental CPCs can be found in early embryonic stages of cardiac development, as mentioned above; however, they can also be generated *in vitro* from pluripotent stem cell technologies. The assessment of such CPCs both *in vivo* and *in vitro* provide a means for answering unresolved questions about the diversity and commitment of their nature. Furthermore, advanced technologies involving elegant lineage tracing strategies, deep RNA-sequencing tools, and CRISPR-CAS genome editing have allowed researchers to better identify new and novel markers of the embryonic CPCs [40]. Below, we will highlight several recent papers that have elucidated novel markers and molecular mechanisms of embryonic CPCs through a combination of these technologies.

A report by Jain et al. identified a transcription factor Hopx$^+$ cell population that is committed to cardiomyocyte fate [41]. By employing a knock-in approach, the authors showed that *Hopx* expression initiates shortly after the expression of FHF marker *Nkx2-5*. The use of fate-mapping experiments illustrated that Hopx$^+$ cells were distributed in all four chambers of the developing heart, and the Hopx$^+$ derivatives were comprised entirely of cardiac myocytes. The mechanism by which Hopx promotes myogenesis through the repression of Wnt signaling was clearly elucidated by employment of a previously published *in vitro* ESC differentiation protocol [34]. Finally, the authors showed that Hopx deficiency gave rise to a thinning myocardium and cardiac rupture in developing mouse embryos. Whether genetic alterations in the *Hopx* gene could give rise to similar congenital impairments during human cardiac development remains elusive, yet the discovery of a specific CPC subtype that gives rise solely to cardiac muscle could provide profound insights for rebuilding damaged and/or atrophic myocardium.

More recently, another report highlighted a member of the forkhead class of DNA-binding proteins, Foxa2, as a marker of a novel progenitor population, which unlike that of the 4-chamber cardiac identifier Hopx gave rise primarily to CMs exclusively in the ventricles [42]. The use of a Foxa2

lineage tracing model system clearly revealed the expression profile of Foxa2, which was found predominantly in the node, midline, and visceral endoderm as well as regions of migrating mesoderm cells during late stages of gastrulation at E7.5 in mice. As the heart continued to develop into four distinct chambers (E9.5–E17), Foxa2$^+$ derivatives became localized to the ventricular chambers, with very few being expressed in the atria. Next, Bardot et al. [42] employed a murine ESC cardiac differentiation protocol in order to see if embryonic Foxa2$^+$ CPCs could be generated *in vitro*. The group showed that a large portion of a KDR$^+$/PDGFRα^+ CPC population also coexpressed Foxa2. By employing cardiovascular lineage analysis together with immunohistochemistry and flow cytometry, Foxa2 expression was revealed predominantly in the ventricular CMs and in equal proportions between the left and right ventricles [42].

Work produced by Ishida et al. showcased that Gfra2 (GPI-anchored neurotrophic factor receptor) expression labels a specific population of embryonic CPCs in mouse cardiac development, which is required for cardiac compaction [43]. According to single-cell profiling studies during murine heart development, the authors showed that Gfra2 was coexpressed with Mesp1, a well-known early cardiac mesodermal marker. Using whole mount in situ hybridization studies and immunohistochemistry procedures, Ishida et al. showed the localization of Gfra2 and concluded a Gfra2 expression pattern that labels some subsets of embryonic CPCs in both the FHF and SHF. The authors also demonstrated that Gfra2 expression marks a human developmental CPC population during ESC/iPSC differentiation. The expression profile of the *Gfra2* gene appears to peak just before embryoid bodies begin to beat in culture. The proportion of KDR$^+$/PDGFRα^+ cells expressing Gfra2 is quite low but give rise to mature CMs. However, in their differentiation protocol, a Gfra2-negative KDR$^+$/PDGFRα^+ population failed to give rise to differentiated CMs, supporting the notion of a strong specificity of Gfra2 to give rise to a distinct CPC population [43]. Furthermore, the emergence of a surface receptor to label and isolate embryonic (or adult) CPCs is enticing for future cell-based therapies, as many well-known markers of embryonic CPCs are transcription factors that require fixation of the cells for successful labeling and as such cannot be used for downstream *in vivo* applications.

4. Expansion, Maintenance, and Preclinical Use of Embryonic or Inducible CPCs

The creation of a technology platform capable of expanding a multipotent and clonogenic CPC population that produces mature cardiomyocytes and vascular cells has been challenging. The exploited accomplishment of which has direct implications in understanding developmental cardiogenesis, cardiac disease modeling, and regeneration research, as well as cardiotoxicity studies for novel pharmacological agents. Several recent reports have paved great progress in the field, and below, we will highlight a few selected works, showing novel findings for effectively expanding embryonic (developmental) or inducible CPCs and improving renewable cardiac precursor technologies.

A finding produced from the Mummery lab attractively illustrated a technique by which developmental CPCs could be restrained from further differentiation through the control of oncogene *Myc* expression and simultaneously expanded using IGF-1 and a hedgehog pathway agonist [44]. Using a human ESC line and a Tet-On system, the group could regulate expression of Myc in a fine-tuned manner with doxycycline administration during differentiation, thereby halting CM differentiation, whereas in the absence of doxycycline, the cells formed beating CMs. Birket et al. [44] also demonstrated long-term expansion of the developmental CPCs, undergoing over 40 population doublings, which did not alter the multipotent capacity of the CPCs; as even the highly expanded CPCs could generate large numbers of successfully differentiated CMs and ECs.

Yet more recently, two independent research groups reported two different strategies for the expansion of "inducible" CPCs from reprogrammed adult mouse fibroblasts [45–47]. Using a combination of transcription factors, which were 5 cardiac genes for direct reprogramming of fibroblasts into CPCs [45] or 4 Yamanaka factors for generating iPSC-like cells first, followed by committed differentiation into CPCs [47], and a defined media containing growth factors and small molecules, both groups were able to produce and maintain a cell population that was highly expandable and could give rise to CMs, ECs, and SMCs. The CPCs produced by both groups, referred to as "inducible" CPCs, could be expanded $> 10^{10}$-fold under chemically defined conditions with BIO and LIF to activate the Wnt and JAK/STAT pathways, respectively [45], or with a JAK inhibitor and BACS (BMP4, Activin A, CHIR99021 (a GSK inhibitor), and SU5402 (an inhibitor of FGF, VEGF, and PDGF)) [47], allowing for the propagation and expansion of desirable cell numbers for *in vivo* experiments. Both Lalit et al. [45] and Zhang et al. [47] went on to demonstrate that morphologically, the delivery of the inducible CPCs can reduce major architectural remodeling and improve cardiac function when delivered to the murine heart at the onset of MI, which was depicted by decreased scar sizes several months following the injury and implantation. In the results, the inducible CPC-derived exogenous CMs were found engrafted deeply within the heart scar tissue where they exhibited expression of marker genes indicative to differentiated and mature CMs, and thereby, both groups concluded that the beneficial effects seen in these studies appear to be based on direct engraftment of the injected inducible CPCs *in vivo* [45, 47]. Further studies are needed to more clearly decipher the ideal transplantable number of the inducible CPCs, which can promote cardiac repair and enhance long-term engraftment *in vivo*.

A study from the Murry lab sought to directly compare the regenerative capabilities of implanted human cardiac cell types; cardiomyocytes derived from human ESCs (hESC-CM), cardiovascular progenitors derived from human ESCs and expressing KDR$^+$/PDGFR-α^+ (hESC-CVP), and human bone marrow mononuclear cells (BMMC). The group administered these cell populations at the onset of a reperfusion MI injury in the nude rat heart [48]. The study concluded that the administration of both hESC-CMs and

Table 1: Selected clinical trials employing CPC therapy for cardiac regeneration against ischemic cardiomyopathy.

Trial name/reference	Classification	Cell type	Delivery route	Patient number	Follow-up time	Outcome	Side effects
SCIPIO (Chugh et al., 2012)	Phase I	c-kit$^+$ CPCs	Intracoronary	33	4 & 12 mo	LVEF: 8% ↑ at 12 mo versus baseline Scar size: 30% ↓ at 12 mo versus baseline	None
CONCERT-HF (NCT02501811)	Phase II	c-kit$^+$ CPCs & MSCs	Transendocardial	Est 144	6 & 12 mo	Currently ongoing	N/A
CADUCEUS (Malilarus et al., 2014)	Phase I	CDCs	Intracoronary	25	6 & 12 mo	LVEF: unchanged at 12 mo versus baseline Scar size: 12.3% ↓ at 12 mo versus baseline	1 patient death
ALCADIA (NCT00981006)	Phase I	CDCs	Intramyocardial with CABG	6	12 mo	LVEF: 12% ↑ at 6 mo versus baseline Scar size: 3.3% ↓ at 6 mo versus baseline	None
ALLSTAR (NCT01458405)	Phase I/II	CDCs	Intracoronary	Est 132	12 mo	Currently ongoing	N/A
HOPE (NCT02485938)	Phase I/II	CDCs	Intracoronary	Est 34	12 mo	Currently ongoing	N/A
DYNAMIC (NCT02293603)	Phase I	CDCs	Intracoronary	Est 42	12 mo	Currently ongoing	N/A
CAREMI (NCT02439398)	Phase I/II	CDCs	Intracoronary	Est 55	1, 6 & 12 mo	Currently ongoing	N/A
ESCORT (NCT02057900)	Phase I	ESC-derived ISL1$^+$/ CD15$^+$	Epicardial patch	N/A	N/A	Currently recruiting	N/A

For ongoing and currently recruiting trials with no published results, the NCT (national clinical trial) identifier has been indicated as referenced by http://ClinicalTrials.gov; CPCs: cardiac progenitor cells, MSCs: mesenchymal stem cells, CDCs: cardiosphere-derived cells, CABG: coronary artery bypass graft, LVEF: left ventricular ejection fraction, mo: month, Est: estimated, N/A: not applicable, ↑: increase, ↓: decrease.

hESC-CVPs were capable of improving cardiac function one month following the ischemic reperfusion injury, more efficiently than the human BMMCs. Interestingly, the hESC-CVPs did not appear to yield a larger graft or give rise to a more significant number of human vessels in the grafted region, compared with hESC-CMs. However, there may exist several issues regarding an ideal number of the transplanted cells as well as a special time window in which the developmental CPCs must be administered as to not lose their proliferative and regenerative properties, which the authors did not address. Further experiments with variations in cell numbers, different cell populations, and timings of administration are needed to reach a more valid conclusion.

5. Adult and Developmental CPCs in Clinical Trials

There has now been a multitude of clinical trials that have employed stem cell technologies for patients with ischemic cardiomyopathy, the findings of which support the use of stem cell therapies in the heart to be safe [49]. Infusions of bone marrow-derived cells (BMCs) represent the largest number of clinical studies for MI. There are many cell populations that fall under the BMC umbrella including hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). To report the findings of BMCs in clinical cardiac studies would outweigh the scope of this review; however, for a comprehensive overview of such clinical studies, we direct the reader to the following review [50]. Here, we will focus on the clinical trials using purified adult or developmental CPCs as a regenerative therapy for ischemic heart disease (Table 1).

The SCIPIO study was the first CPC clinical trial to investigate the therapeutic effects of autologous CPCs (cardiac c-kit$^+$ cells) in patients with ischemic cardiomyopathy [51]. The cells were isolated from cardiac tissue of patients during surgery and expanded ex vivo, and later delivered via intracoronary infusion. Results from the SCIPIO trial showed an increase in several functional parameters and no evidence of tumor formation at 1 yr follow-up, although it must be noted that concerns regarding patient randomization and the integrity of certain data generated in the SCIPIO trial have been raised [52]. Following the SCIPIO trial, a new trial CONCERT-HF (NCT02501811) will aim to deliver a combination therapy utilizing both MSCs and cardiac c-kit$^+$ cells for the treatment of ischemic cardiomyopathy, as MSCs have been shown to increase several parameters of cardiac function when administered to the heart after MI, effects of which are thought to be paracrine-mediated [53, 54].

Adult CPC-like cells can also be obtained through human myocardial biopsies, where cultured pieces of myocardial tissue give rise to spherical clusters of stem cell-like cells coined

cardiospheres [38, 55]. Several phase I clinical trials including CADUCEUS and ALCADIA (NCT00981006) tested the efficacy and safety of intracoronary delivery of cardiosphere-derived cells (CDCs) in patients with ischemic cardiomyopathy and reported small improvements in regional but not global function, as well as decreased scar sizes [56, 57]. Although some concerns exist in regard to capillary plugging due to the size of the cardiospheres, several ongoing clinical trials including ALLSTAR (NCT01458405), HOPE (NCT02485938), and DYNAMIC (NCT02293603) are aiming to address the real regenerative potential of CDCs for ischemic cardiomyopathy and also to evaluate safe dosage limits as well as differences between an allogeneic and autologous cell source of CDCs.

An additional clinical trial CAREMI (NCT02439398) is currently ongoing to test the feasibility and safety of delivering an allogeneic adult CPC population in human, isolated from right atrial appendages and expanded *in vitro*.

Overcoming the technological hurdle of deriving functional CMs and their progenitors from ESCs/iPSCs is beginning to pave great insight for their potential uses in the clinic [58, 59]. Although most of human ESC and iPSC-derived CM protocols can give rise to efficient numbers of beating cells, much optimization is required to generate highly enriched populations of CMs devoid of alternate cell types or undifferentiated stem cells, at low cost and in a timely manner. It should be also noted that the difficulties of obtaining fully differentiated CMs from ESCs/iPSCs are frequently observed, as the previous report indicated immaturity of ESC/iPSC-derived CMs compared with native ventricular tissue-derived CMs [60]. Even with such drawbacks, the clinical trial ESCORT (NCT02057900) is recruiting patients with severe ischemic heart failure (LVEF $\leq$ 35%) in order to evaluate the regenerative effects of a human ESC-derived developmental CPC denoted by $CD15^+/ISL1^+$ coexpression. Patients will receive a fibrin gel embedded with the human ESC-derived $CD15^+/ISL1^+$ CPCs at the onset of coronary artery bypass grafting. The generation and survival of the patch, as well as the efficacy on patient cardiac function, will assess the overall feasibility of the study (Table 1).

6. Unresolved Issues and Future Perspectives

Overall, the use of CPCs as a regenerative therapy in the clinic to date has shown varying degrees of benefits; the outcomes of which we hope may one day provide alternate options when conventional medical treatments fail. Several engaging and ongoing clinical trials are still deciphering optimal cell types and doses, and we anxiously await the feasibility and safety of such approaches. However, before directly applying CPC therapy in the clinic, many critical issues, including the challenges of electrical coupling, undetermined mechanistic aspects, long-term engraftment, and the direct reprogramming of the (inducible) CPCs as an alternative approach, should all be addressed [6, 7].

One major caveat associated with the CPC/CM-based therapy is the risk of arrhythmias due to incomplete electrical coupling of the transplanted cells with the host cardiac tissue. Indeed, few studies have thoroughly evaluated the electrical integrity of the cardiac system following the administration of human ESC-derived CMs in ischemic models of nonhuman primates or guinea pigs, but those studies have obtained varying results [61–63]. Ideally, transplanted cells have to align, engraft, and couple with host cardiomyocytes in an ordered fashion. Further studies are required to determine how this process is precisely orchestrated [4].

The mechanisms of action by which CPCs contribute to the generation of new CMs, promotion of preexisting CM proliferation, and/or development of vasculo-/angiogenesis remain to be fully elucidated (Figure 1). It is commonly speculated that direct engraftment of the injected adult CPCs is a relatively rare event and that the functional benefits associated with the administration of the CPCs are derived predominantly from their paracrine effects [64, 65]. However, the latest studies have revealed that the transplanted inducible CPCs exert beneficial effects based on direct engraftment *in vivo*, as described above [45, 47]. To improve the long-term cell engraftment in the ischemic environment, cardiac tissue engineering with natural or synthetic biomaterials is most likely to serve as an excellent tool [66, 67]. Yet the potential paracrine effects, such as cytokines and growth factors released by the transplanted adult CPCs or human ESC-derived CMs, are still considered to be indispensable on the CPC/CM therapy-mediated cardiac protection and repair after injury [64, 65, 68].

Alternative approaches to cell therapies for cardiac repair also include reprogramming strategies using fibroblasts [2, 6]. Cardiac reprogramming of fibroblasts can be achieved through direct conversion by employing a unique combination of cardiac-specific transcription factors, miRNAs, and/or chemical molecules *in vitro* and *in vivo* [2, 6, 69, 70]. To date, these *in vivo* studies have shown only direct reprogramming of cardiac fibroblasts into an "induced CM-like cell" but not adequate CPCs, although several *in vitro* studies have shown direct reprogramming of fibroblasts into an "inducible CPC" (Figure 1) [45, 47]. Several reprogramming strategies to generate cardiac cell lineages from fibroblasts, including inducible CPCs to differentiated CMs *in vitro* and *in vivo*, continue to be investigated [71].

Regardless of the several critical issues as described above, the concept of enhancing stem cell properties through a combination of strategies could go some way in obtaining better outcomes for patients. An innovative focus that aims to synergize cell-based and cell-free therapies such as combining "ideal" CPC types with gene therapy, small molecules, and/or tissue engineering strategies should be conceptualized as a plausible clinical treatment for the enhancement of regenerative therapies in cardiovascular disease (Figure 1). Continuous and collective efforts by stem cell biologists and medical teams in cardiology must open the door and generate novel paths toward a goal of successfully establishing cardiac regenerative therapeutics in the near future.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

Makoto Sahara is supported by research grants provided by Swedish Heart and Lung Foundation and Karolinska Institutet.

References

[1] O. Caspi, I. Huber, I. Kehat et al., "Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts," *Journal of the American College of Cardiology*, vol. 50, no. 19, pp. 1884–1893, 2007.

[2] L. Qian, Y. Huang, C. I. Spencer et al., "*In vivo* reprogramming of murine cardiac fibroblasts into induced cardiomyocytes," *Nature*, vol. 485, no. 7400, pp. 593–598, 2012.

[3] S. K. Sanganalmath and R. Bolli, "Cell therapy for heart failure: a comprehensive overview of experimental and clinical studies, current challenges, and future directions," *Circulation Research*, vol. 113, no. 6, pp. 810–834, 2013.

[4] L. M. Monteiro, F. Vasques-Nóvoa, L. Ferreira, P. Pinto-do-Ó, and D. S. Nascimento, "Restoring heart function and electrical integrity: closing the circuit," *npj Regenerative Medicine*, vol. 2, no. 1, p. 9, 2017.

[5] A. Mauretti, S. Spaans, N. A. M. Bax, C. Sahlgren, and C. V. C. Bouten, "Cardiac progenitor cells and the interplay with their microenvironment," *Stem Cells International*, vol. 2017, Article ID 7471582, 20 pages, 2017.

[6] M. Sahara, F. Santoro, and K. R. Chien, "Programming and reprogramming a human heart cell," *The EMBO Journal*, vol. 34, no. 6, pp. 710–738, 2015.

[7] E. Tzahor and K. D. Poss, "Cardiac regeneration strategies: staying young at heart," *Science*, vol. 356, no. 6342, pp. 1035–1039, 2017.

[8] A. Moretti, L. Caron, A. Nakano et al., "Multipotent embryonic *isl1*$^+$ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification," *Cell*, vol. 127, no. 6, pp. 1151–1165, 2006.

[9] S. M. Wu, Y. Fujiwara, S. M. Cibulsky et al., "Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart," *Cell*, vol. 127, no. 6, pp. 1137–1150, 2006.

[10] T. Brade, L. S. Pane, A. Moretti, K. R. Chien, and K. L. Laugwitz, "Embryonic heart progenitors and cardiogenesis," *Cold Spring Harbor Perspectives in Medicine*, vol. 3, no. 10, p. a013847, 2013.

[11] X. Liang, G. Wang, L. Lin et al., "HCN4 dynamically marks the first heart field and conduction system precursors," *Circulation Research*, vol. 113, no. 4, pp. 399–407, 2013.

[12] D. Später, M. K. Abramczuk, K. Buac et al., "A HCN4+ cardiomyogenic progenitor derived from the first heart field and human pluripotent stem cells," *Nature Cell Biology*, vol. 15, no. 9, pp. 1098–1106, 2013.

[13] C. L. Cai, X. Liang, Y. Shi et al., "Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart," *Developmental Cell*, vol. 5, no. 6, pp. 877–889, 2003.

[14] L. Bu, X. Jiang, S. Martin-Puig et al., "Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages," *Nature*, vol. 460, no. 7251, pp. 113–117, 2009.

[15] K. L. Laugwitz, A. Moretti, J. Lam et al., "Postnatal isl1$^+$ cardioblasts enter fully differentiated cardiomyocyte lineages," *Nature*, vol. 433, no. 7026, pp. 647–653, 2005.

[16] M. Noseda, T. Peterkin, F. C. Simoes, R. Patient, and M. D. Schneider, "Cardiopoietic factors: extracellular signals for cardiac lineage commitment," *Circulation Research*, vol. 108, no. 1, pp. 129–152, 2011.

[17] C. L. Cai, J. C. Martin, Y. Sun et al., "A myocardial lineage derives from *Tbx18* epicardial cells," *Nature*, vol. 454, no. 7200, pp. 104–108, 2008.

[18] B. Zhou, Q. Ma, S. Rajagopal et al., "Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart," *Nature*, vol. 454, no. 7200, pp. 109–113, 2008.

[19] T. C. Katz, M. K. Singh, K. Degenhardt et al., "Distinct compartments of the proepicardial organ give rise to coronary vascular endothelial cells," *Developmental Cell*, vol. 22, no. 3, pp. 639–650, 2012.

[20] M. R. Hutson and M. L. Kirby, "Model systems for the study of heart development and disease: cardiac neural crest and conotruncal malformations," *Seminars in Cell & Developmental Biology*, vol. 18, no. 1, pp. 101–110, 2007.

[21] V. Hildreth, S. Webb, L. Bradshaw, N. A. Brown, R. H. Anderson, and D. J. Henderson, "Cells migrating from the neural crest contribute to the innervation of the venous pole of the heart," *Journal of Anatomy*, vol. 212, no. 1, pp. 1–11, 2008.

[22] A. P. Beltrami, L. Barlucchi, D. Torella et al., "Adult cardiac stem cells are multipotent and support myocardial regeneration," *Cell*, vol. 114, no. 6, pp. 763–776, 2003.

[23] J. J. H. Chong, E. Forte, and R. P. Harvey, "Developmental origins and lineage descendants of endogenous adult cardiac progenitor cells," *Stem Cell Research*, vol. 13, no. 3, pp. 592–614, 2014.

[24] C. Bearzi, M. Rota, T. Hosoda et al., "Human cardiac stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 35, pp. 14068–14073, 2007.

[25] G. M. Ellison, C. Vicinanza, A. J. Smith et al., "Adult c-kit^{pos} cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair," *Cell*, vol. 154, no. 4, pp. 827–842, 2013.

[26] J. H. van Berlo, O. Kanisicak, M. Maillet et al., "c-kit$^+$ cells minimally contribute cardiomyocytes to the heart," *Nature*, vol. 509, no. 7500, pp. 337–341, 2014.

[27] A. J. Smith, F. C. Lewis, I. Aquila et al., "Isolation and characterization of resident endogenous c-kit$^+$ cardiac stem cells from the adult mouse and rat heart," *Nature Protocols*, vol. 9, no. 7, pp. 1662–1681, 2014.

[28] S. Fazel, M. Cimini, L. Chen et al., "Cardioprotective c-kit$^+$ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines," *The Journal of Clinical Investigation*, vol. 116, no. 7, pp. 1865–1877, 2006.

[29] C. Vicinanza, I. Aquila, M. Scalise et al., "Adult cardiac stem cells are multipotent and robustly myogenic: c-kit expression is necessary but not sufficient for their identification," *Cell Death & Differentiation*, vol. 24, no. 12, pp. 2101–2116, 2017.

[30] K. U. Hong, Y. Guo, Q. H. Li et al., "c-kit+ cardiac stem cells alleviate post-myocardial infarction left ventricular dysfunction despite poor engraftment and negligible retention in the recipient heart," *PLoS One*, vol. 9, no. 5, article e96725, 2014.

[31] J. L. Torán, S. Aguilar, J. A. López et al., "CXCL6 is an important paracrine factor in the pro-angiogenic human cardiac

progenitor-like cell secretome," *Scientific Reports*, vol. 7, no. 1, article 12490, 2017.

[32] J. J. H. Chong, H. Reinecke, M. Iwata, B. Torok-Storb, A. Stempien-Otero, and C. E. Murry, "Progenitor cells identified by PDGFR-alpha expression in the developing and diseased human heart," *Stem Cells and Development*, vol. 22, no. 13, pp. 1932–1943, 2013.

[33] S. J. Kattman, T. L. Huber, and G. M. Keller, "Multipotent flk-1^+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages," *Developmental Cell*, vol. 11, no. 5, pp. 723–732, 2006.

[34] S. J. Kattman, A. D. Witty, M. Gagliardi 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*, vol. 8, no. 2, pp. 228–240, 2011.

[35] L. Yang, M. H. Soonpaa, E. D. Adler et al., "Human cardiovascular progenitor cells develop from a KDR^+ embryonic-stem-cell-derived population," *Nature*, vol. 453, no. 7194, pp. 524–528, 2008.

[36] K. Matsuura, T. Nagai, N. Nishigaki et al., "Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes," *The Journal of Biological Chemistry*, vol. 279, no. 12, pp. 11384–11391, 2004.

[37] H. Oh, S. B. Bradfute, T. D. Gallardo et al., "Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 21, pp. 12313–12318, 2003.

[38] E. Messina, L. De Angelis, G. Frati et al., "Isolation and expansion of adult cardiac stem cells from human and murine heart," *Circulation Research*, vol. 95, no. 9, pp. 911–921, 2004.

[39] C. M. Martin, A. P. Meeson, S. M. Robertson et al., "Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart," *Developmental Biology*, vol. 265, no. 1, pp. 262–275, 2004.

[40] D. M. DeLaughter, A. G. Bick, H. Wakimoto et al., "Single-cell resolution of temporal gene expression during heart development," *Developmental Cell*, vol. 39, no. 4, pp. 480–490, 2016.

[41] R. Jain, D. Li, M. Gupta et al., "Integration of Bmp and Wnt signaling by Hopx specifies commitment of cardiomyoblasts," *Science*, vol. 348, no. 6242, article aaa6071, 2015.

[42] E. Bardot, D. Calderon, F. Santoriello et al., "Foxa2 identifies a cardiac progenitor population with ventricular differentiation potential," *Nature Communications*, vol. 8, article 14428, 2017.

[43] H. Ishida, R. Saba, I. Kokkinopoulos et al., "GFRA2 identifies cardiac progenitors and mediates cardiomyocyte differentiation in a RET-independent signaling pathway," *Cell Reports*, vol. 16, no. 4, pp. 1026–1038, 2016.

[44] M. J. Birket, M. C. Ribeiro, A. O. Verkerk et al., "Expansion and patterning of cardiovascular progenitors derived from human pluripotent stem cells," *Nature Biotechnology*, vol. 33, no. 9, pp. 970–979, 2015.

[45] P. A. Lalit, M. R. Salick, D. O. Nelson et al., "Lineage reprogramming of fibroblasts into proliferative induced cardiac progenitor cells by defined factors," *Cell Stem Cell*, vol. 18, no. 3, pp. 354–367, 2016.

[46] N. Witman and M. Sahara, "Expansion of cardiac progenitors from reprogrammed fibroblasts as potential novel cardiovascular therapy," *Stem Cell Investigation*, vol. 3, p. 34, 2016.

[47] Y. Zhang, N. Cao, Y. Huang et al., "Expandable cardiovascular progenitor cells reprogrammed from fibroblasts," *Cell Stem Cell*, vol. 18, no. 3, pp. 368–381, 2016.

[48] S. Fernandes, J. J. H. Chong, S. L. Paige et al., "Comparison of human embryonic stem cell-derived cardiomyocytes, cardiovascular progenitors, and bone marrow mononuclear cells for cardiac repair," *Stem Cell Reports*, vol. 5, no. 5, pp. 753–762, 2015.

[49] A. R. Khan, T. A. Farid, A. Pathan et al., "Impact of cell therapy on myocardial perfusion and cardiovascular outcomes in patients with angina refractory to medical therapy: a systematic review and meta-analysis," *Circulation Research*, vol. 118, no. 6, pp. 984–993, 2016.

[50] M. Hao, R. Wang, and W. Wang, "Cell therapies in cardiomyopathy: current status of clinical trials," *Analytical Cellular Pathology*, vol. 2017, Article ID 9404057, 20 pages, 2017.

[51] R. Bolli, A. R. Chugh, D. D'Amario et al., "Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial," *The Lancet*, vol. 378, no. 9806, pp. 1847–1857, 2011.

[52] A. N. Nowbar, M. Mielewczik, M. Karavassilis et al., "Discrepancies in autologous bone marrow stem cell trials and enhancement of ejection fraction (DAMASCENE): weighted regression and meta-analysis," *BMJ*, vol. 348, apr28 1, p. g2688, 2014.

[53] J. M. Hare, J. E. Fishman, G. Gerstenblith et al., "Comparison of allogeneic vs autologous bone marrow–derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial," *Journal of the American Medical Association*, vol. 308, no. 22, pp. 2369–2379, 2012.

[54] V. Karantalis, D. L. DiFede, G. Gerstenblith et al., "Autologous mesenchymal stem cells produce concordant improvements in regional function, tissue perfusion, and fibrotic burden when administered to patients undergoing coronary artery bypass grafting: the Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery (PROMETHEUS) trial," *Circulation Research*, vol. 114, no. 8, pp. 1302–1310, 2014.

[55] I. Chimenti, R. Gaetani, L. Barile et al., "Isolation and expansion of adult cardiac stem/progenitor cells in the form of cardiospheres from human cardiac biopsies and murine hearts," *Methods in Molecular Biology*, vol. 879, pp. 327–338, 2012.

[56] K. Malliaras, R. R. Makkar, R. R. Smith et al., "Intracoronary cardiosphere-derived cells after myocardial infarction: evidence of therapeutic regeneration in the final 1-year results of the CADUCEUS trial (CArdiosphere-Derived aUtologous stem CElls to reverse ventricULar dySfunction)," *Journal of the American College of Cardiology*, vol. 63, no. 2, pp. 110–122, 2014.

[57] M. H. Yacoub and J. Terrovitis, "CADUCEUS, SCIPIO, ALCADIA: cell therapy trials using cardiac-derived cells for patients with post myocardial infarction LV dysfunction, still evolving," *Global Cardiology Science & Practice*, vol. 2013, no. 1, pp. 3–8, 2013.

[58] P. W. Burridge, E. Matsa, P. Shukla et al., "Chemically defined generation of human cardiomyocytes," *Nature Methods*, vol. 11, no. 8, pp. 855–860, 2014.

[59] C. E. Murry and G. Keller, "Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development," *Cell*, vol. 132, no. 4, pp. 661–680, 2008.

[60] J. Xi, M. Khalil, N. Shishechian et al., "Comparison of contractile behavior of native murine ventricular tissue and cardiomyocytes derived from embryonic or induced pluripotent stem cells," *The FASEB Journal*, vol. 24, no. 8, pp. 2739–2751, 2010.

[61] J. J. H. Chong, X. Yang, C. W. Don et al., "Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts," *Nature*, vol. 510, no. 7504, pp. 273–277, 2014.

[62] Y. Shiba, S. Fernandes, W. Z. Zhu et al., "Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts," *Nature*, vol. 489, no. 7415, pp. 322–325, 2012.

[63] Y. Shiba, D. Filice, S. Fernandes et al., "Electrical integration of human embryonic stem cell-derived cardiomyocytes in a guinea pig chronic infarct model," *Journal of Cardiovascular Pharmacology and Therapeutics*, vol. 19, no. 4, pp. 368–381, 2014.

[64] X. L. Tang, G. Rokosh, S. K. Sanganalmath et al., "Intracoronary administration of cardiac progenitor cells alleviates left ventricular dysfunction in rats with a 30-day-old infarction," *Circulation*, vol. 121, no. 2, pp. 293–305, 2010.

[65] R. Bolli, X. L. Tang, S. K. Sanganalmath et al., "Intracoronary delivery of autologous cardiac stem cells improves cardiac function in a porcine model of chronic ischemic cardiomyopathy," *Circulation*, vol. 128, no. 2, pp. 122–131, 2013.

[66] E. Cimetta, A. Godier-Furnemont, and G. Vunjak-Novakovic, "Bioengineering heart tissue for *in vitro* testing," *Current Opinion in Biotechnology*, vol. 24, no. 5, pp. 926–932, 2013.

[67] M. N. Hirt, A. Hansen, and T. Eschenhagen, "Cardiac tissue engineering: state of the art," *Circulation Research*, vol. 114, no. 2, pp. 354–367, 2014.

[68] A. Tachibana, M. R. Santoso, M. Mahmoudi et al., "Paracrine effects of the pluripotent stem cell-derived cardiac myocytes salvage the injured myocardium," *Circulation Research*, vol. 121, no. 6, pp. e22–e36, 2017.

[69] M. Ieda, J. D. Fu, P. Delgado-Olguin et al., "Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors," *Cell*, vol. 142, no. 3, pp. 375–386, 2010.

[70] K. Song, Y. J. Nam, X. Luo et al., "Heart repair by reprogramming non-myocytes with cardiac transcription factors," *Nature*, vol. 485, no. 7400, pp. 599–604, 2012.

[71] H. Yamakawa and M. Ieda, "Strategies for heart regeneration: approaches ranging from induced pluripotent stem cells to direct cardiac reprogramming," *International Heart Journal*, vol. 56, no. 1, pp. 1–5, 2015.

Hindawi
Stem Cells International
Volume 2017, Article ID 7849851, 10 pages
https://doi.org/10.1155/2017/7849851

Review Article

Myocardial Regeneration via Progenitor Cell-Derived Exosomes

Janita A. Maring,[1] Christien M. Beez,[1] Volkmar Falk,[2,3] Martina Seifert,[1] and Christof Stamm[1,2,3]

[1]*Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Charité-Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany*
[2]*German Center for Cardiovascular Research (DZHK), Partner Site Berlin, Berlin, Germany*
[3]*Deutsches Herzzentrum Berlin (DHZB), Berlin, Germany*

Correspondence should be addressed to Janita A. Maring; Janita-aline.maring@charite.de

Received 11 August 2017; Accepted 11 October 2017; Published 23 November 2017

Academic Editor: Heinrich Sauer

In the past 20 years, a variety of cell products has been evaluated in terms of their capacity to treat patients with acute myocardial infarction and chronic heart failure. Despite initial enthusiasm, therapeutic efficacy has overall been disappointing, and clinical application is costly and complex. Recently, a subset of small extracellular vesicles (EVs), commonly referred to as "exosomes," was shown to confer cardioprotective and regenerative signals at a magnitude similar to that of their donor cells. The conceptual advantage is that they may be produced in industrial quantities and stored at the point-of-care for off-the-shelf application, ideally without eliciting a relevant recipient immune response or other adverse effects associated with viable cells. The body of evidence on beneficial exosome-mediated effects in animal models of heart diseases is rapidly growing. However, there is significant heterogeneity in terms of exosome source cells, isolation process, therapeutic dosage, and delivery mode. This review summarizes the current state of research on exosomes as experimental therapy of heart diseases and seeks to identify roadblocks that need to be overcome prior to clinical application.

1. Introduction

Regenerative therapy has the ultimate goal of repairing damaged tissue by limiting the extent of tissue damage following injury (cytoprotection), stimulating the endogenous regenerative capacity of a tissue (regeneration), and/or providing new cells or tissues as a replacement (cell therapy, tissue engineering). Although resident cardiac progenitor cells (CPC) have been described to persist within the adult mammalian myocardium [1–3], the myocardium has no clinical relevant intrinsic regenerative capacity due to a lack of postnatal cardiomyocyte mitosis [4]. Exogenous somatic cells transplanted into the diseased human heart failed to induce a meaningful improvement of heart function in clinical trials [5–8]. Cardiomyocyte cell products derived from embryonic or induced pluripotent stem cells (ES or iPS) have not been tested yet in controlled clinical trials, largely due to the complexity and costs of a clinical-grade production process. Concerns about genomic integrity of pluripotent-derived cells and unresolved issues such as cellular immaturity, coupling with host cells and possible arrhythmia, are difficult to rule out in suitable preclinical models.

Nevertheless, a variety of beneficial effects following experimental cell therapy in cardiac disease models has been observed even in the absence of transplanted cell persistence or stem/progenitor cell differentiation [9]. For instance, studies in which mesenchymal stromal cells (MSCs) were transplanted failed to show permanent engraftment of the transplanted cells but still yielded a sustained increase in cardiac function [10, 11]. Indeed, subsequent research has shown that conditioned medium collected from MSCs has cardioprotective effects and their secreted factors alone were already able to reduce infarct size and increase cardiac function in an animal myocardial infarction (MI) model [12, 13]. Investigations regarding the active factors secreted by the MSCs have shown that extracellular vesicles (EV) rather than single growth factors convey this beneficial effect [12].

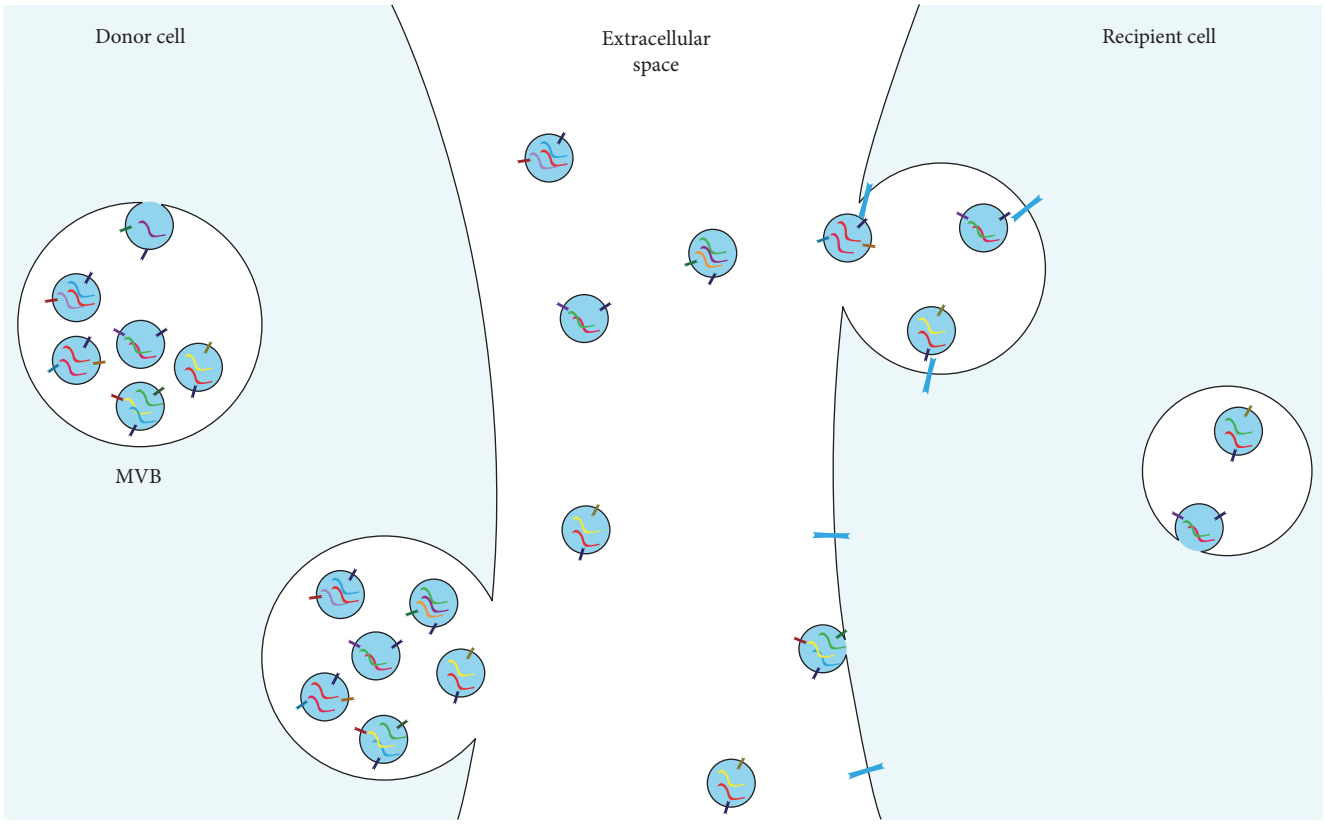


Figure 1: Exosomes are formed by invaginations of intercellular vesicles such as endosomes, which then form multivesicular bodies (MVBs). Exosomes are released into the extracellular space by fusion of the MVB with the cell membrane. Recipient cells take up the exosomes through direct fusion with the cell membrane, through internalisation or through receptor-ligand interaction on the recipient cell membrane.

Intercellular communication was long thought to be restricted to single agent messengers such as secreted growth factors and cytokines. Nowadays, the mode of paracrine signalling is extended by the incorporation of EVs as a major player in cell-to-cell as well as organ-to-organ communication. Vesicular structures were found in the extracellular space (and in body fluids) consisting of exosomes (30–100 nm), microvesicles (100–1000 nm), and apoptotic bodies (up to 5000 nm) [14]. Extracellular vesicle discovery is commonly attributed to the context of platelet maturation and the first use of the term dates back to the 1970s [15]. Extracellular vesicles are surrounded by a phospholipid membrane, and they are believed to contain up to 20,000 different protein molecules with preserved catalytic and ligand-binding activities [16–18]. More recently, vesicles were shown to also contain mRNAs and miRNAs, which seem to play an important role in cell-to-cell information transfer [19]. Extracellular vesicles are secreted in a diverse matter such as secretion via multivesicular bodies or pinching from the cell membrane. Exosomes are a distinct microvesicle subpopulation due to their particular biogenesis and the lack of proteins from certain organelles such as the Golgi apparatus and the endoplasmic reticulum, which can be found in other vesicles, for example, apoptotic bodies [20, 21].

Exosomes are formed through invaginations of endosomes, forming multivesicular bodies (MVBs) in the cytoplasm. Their content of RNAs, lipids, and proteins is partially a reflection of the composition of the cell. Therefore, part of the constituents found in exosomes is highly dependent on the state of the cell and changes upon, for example, hypoxia, mitochondrial stress, and differentiation [22]. Some components can be selectively sorted into the vesicles, for example, sorting of proteins into the exosomes is depended on the ESCRT (endosomal sorting complexes required for transport) machinery [23] or sphingolipids [24]. Interestingly, several studies have shown that the RNA content is not necessarily identical between donor cell and their secreted exosomes, since some microRNAs (miRNAs) are more abundantly present in vesicles compared to their originating cells, while others were completely absent [25–27].

Exosomes are secreted through transport and subsequent fusion of the MVBs to the cell membrane. This process is mediated by proteins of the Rab family, with Rab27A and Rab27B being the most prominent. Knockdown of either of these two proteins results in a significantly reduced exosome secretion [28, 29]. The process of exosome formation, secretion, and uptake known to date is shown in Figure 1. The bilipid membrane of the exosomes protects the content from degradation and thus enables signalling over long distances [19]. Investigations into the signalling range have revealed that the application of cells engineered to express Cre was able to rearrange the genome of susceptible cells containing a LoxP site on the contralateral site of the mice via their released EVs [30].

2. Suitable Cell Sources for Exosomal Therapy

Several cell sources could be used for a clinical approach with exosomes as a therapeutic agent. Multiple studies in several research areas have shown the effects of exosomes on, for instance, cell survival, angiogenesis, and migration [31, 32]. With the experience of the cells previously used in cell therapy, which were shown to be safe and effective, these donor cells are widely considered to be the most efficient sources for regenerative exosome generation (Figure 2).

The first cell type to be studied in preclinical and clinical settings for cardiac repair was bone marrow-derived cells, due to the fact that these cells are easily obtained in an autologous fashion. Even though it was hypothesized that bone marrow-derived cells were capable of transdifferentiating into cardiomyocytes [33], no conclusive evidence has been found that this is indeed the case [34, 35]. Although these cells were able to increase cardiac function when injected after myocardial infarction *in vivo* [33], they have varying results in clinical trials [5, 6].

More recently, MSCs have been investigated for clinical cardiac repair in autologous as well as allogeneic setting. In general, MSCs can be found in many tissues, such as bone marrow, adipose tissue, and cord blood. A hallmark is their capability to differentiate towards osteogenic, chondrogenic, and adipose lineages [36]. Differentiation into cardiomyocytes has so far only been seen in foetal MSCs, but with low efficiency *in vitro* [37]. The results on cardiac function in clinical trials have been very modest and not to the same extent as in the preclinical models [7, 8]. Moreover, MSCs have also been shown to not differentiate *in vivo* to cardiomyocytes and are not retained in the heart, suggesting that paracrine factors of these cells are the acting agents [10].

With the finding that the heart contains progenitor cells (CPC), actual cardiac regeneration seemed obtainable. Indeed, cardiac progenitors have the capability to differentiate into cardiomyocytes *in vitro* and *in vivo* [1–3, 38]. Moreover, they have been shown in preclinical models to be effective in increasing cardiac function after myocardial infarction [1–3, 38]. This concurred with the differentiation into cardiomyocytes and endothelial cells and, for example, an increase in angiogenesis in the borderzone of the infarction [9, 38]. Two clinical trials have been carried out so far to assess the safety of injecting cardiac progenitor cells. Both trials—CADUCEUS and SCIPIO—have indeed shown that injecting cardiac progenitor cells one month or more after myocardial infarction is safe, with some minor decreases in scar size and increases in local ejection fraction [39, 40].

Other cell sources are ES and iPS cell pluripotent stem cells, which have been under investigation for a wide range of regenerative processes since they still contain the ability to differentiate towards every cell type in the body. Therefore, pluripotent stem cells and cardiomyocytes derived from pluripotent stem cells have also been evaluated after myocardial infarction in preclinical models. In these studies, it was shown that these cells were indeed able to engraft into the heart and increase cardiac function significantly [41–44]. However, additional risks have emerged by using these cells, such as arrhythmias and teratoma formation [42–44].

3. Exosomes as Therapeutics in Cardiovascular Repair

Exosomes have been shown to be involved in a plethora of cellular processes, such as migration, differentiation, survival, and immune modulation [31, 32, 45]. Therefore, exosomes generated from the proper regenerative cell source could have profound beneficial effects in the regenerative processes after myocardial infarction, making them interesting new therapeutic agents. Due to the fact that the cells investigated in cell therapy after MI have been shown to convey their effects mainly through paracrine signalling, research has focused on evaluating the regenerative potential of the exosomes from these cell sources. Table 1 provides an overview of the experimental studies performed so far, their major findings, as well as involved pathways or proposed active molecules.

One of the first studies evaluating the paracrine mechanism in cardiac repair used exosomes from human MSCs. Here, they showed that injection of MSC-derived exosomes into the tail vein of mice 5 minutes before reperfusion of the cardiac tissue significantly reduced infarct size 24 hours postoperation. Additionally, animals treated with exosomes had increased cardiac function compared to control animals over a 28-day time course. Analysis of the hearts showed that in the first 24 hours, there was an increase in activation of pAkt and pGSK3 (glycogen synthase kinase 3), which are both involved in cell survival pathways, whereas immune cell infiltration was decreased [32]. In another study, the cardioprotective effect of human MSCs was compared with their exosomes, both after injection into the infarcted borderzone. Both cells and exosomes comparably decreased infarct size after 28 days and increased cardiac function. Additionally, vessel density was also increased after the treatment with exosomes [46]. This proangiogenic effect of MSC exosomes has indeed been verified *in vitro* by these and other studies, where a significant induction of proliferation, tubule formation, and migration of endothelial cells was observed [46, 47]. This effect might be explained by the presence of several proangiogenic proteins in the exosomes, such as vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP)-9 [47].

Exosomes from rat MSCs were shown to increase the proliferation, migration, and tubule formation of cardiac stem cells *in vitro*. Interestingly, it was demonstrated that several miRNAs were changed into these cells upon exosome treatment, such as upregulation of miR-147 and miR-503-3p and downregulation of miR-207, miR-326-5p, and miR-702-5p [48]. When cardiac stem cells were treated with exosomes from rat MSCs prior to injection into the heart, an increase in cardiac function and vessel density at the infarct site could be seen after 28 days [48].

Exosomes from iPS or ES cells were also able to increase proliferation and tubule formation in CPCs [49], while apoptosis was reduced *in vitro* in both CPCs and cardiomyocytes [49, 50]. Furthermore, the expression of several endothelial and cardiomyocyte genes was upregulated in CPCs after stimulation with ES-derived exosomes. When injected into the heart after MI, the exosomes were able to reduced

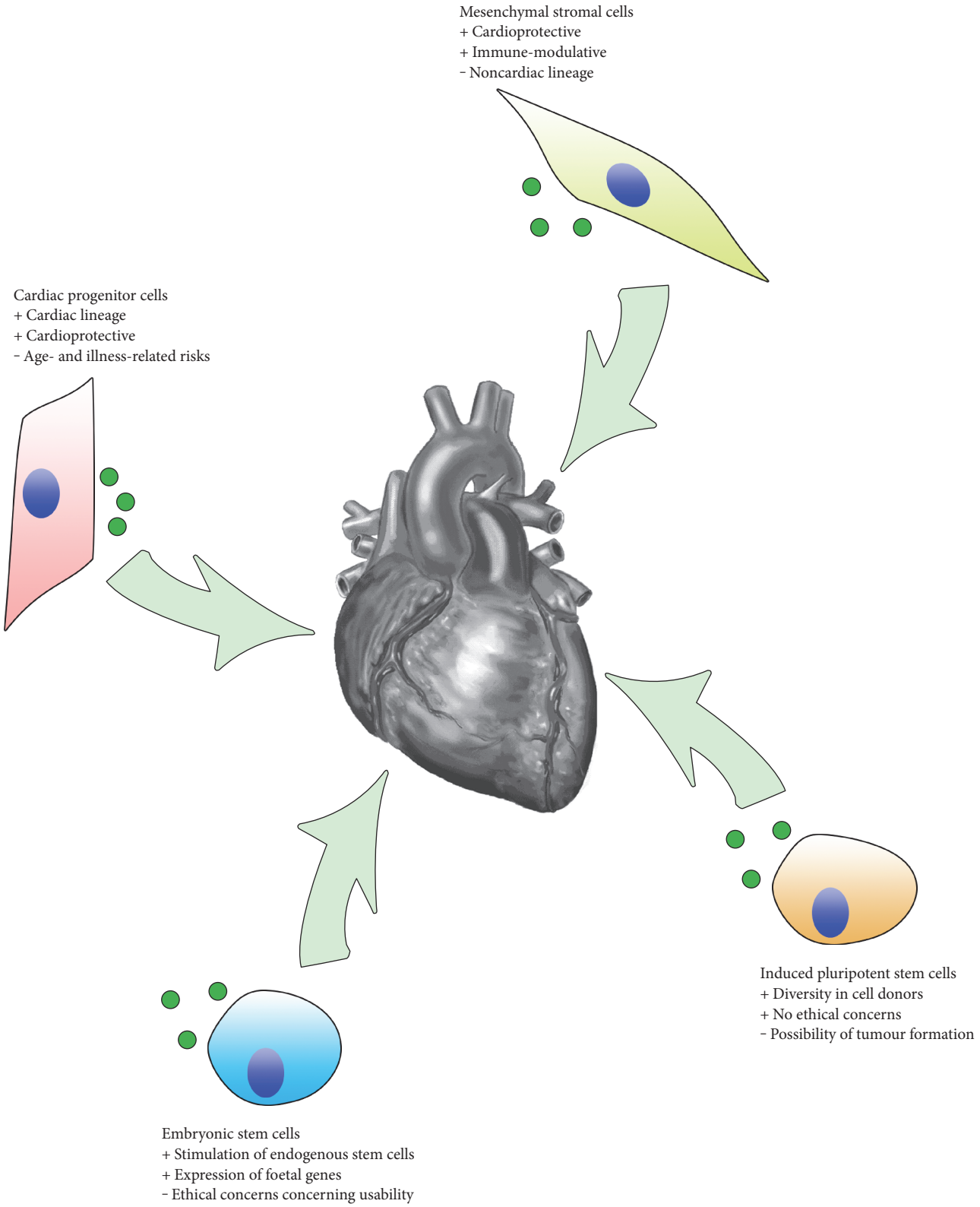


Figure 2: Several cell sources are being examined for use as an exosome therapy, most prominently cardiac progenitor cells, mesenchymal stem cells, and induced pluripotent stem cells as well as embryonic stem cells. Each of them has distinct advantages and disadvantages regarding cardiac regeneration.

Table 1: Overview of exosomes as therapeutics in preclinical MI studies.

Cell type	Species	Donor cell specifics	Cardiac effect					Other effects	Active pathways/ molecules	Ref
			Diminished infarct size	Increased cardiac function	Higher vessel density	Reduced fibrosis	Decreased apoptosis			
MSC	Human		✓	✓	✓					[46]
	Human		✓	✓						[32]
	Rat	CPCs prestimulated with exosomes		✓	✓	✓				[48]
	Mouse	Ischemic preconditioning				✓		Increase in pAkt and pGsk3, reduced immune cell infiltration	miR-22	[58]
CPC	Human	Cardiac explant cells from cardiovascular patients		Reduced				Decrease in exosome production	IL-6	[65]
	Human		✓	✓	✓	✓	✓		miR-132	[53]
	Human		✓	✓	✓	✓			miR-146a	[54]
	Rat			✓		✓			miR-292	[52]
	Mouse						✓		miR-451	[51]
ES	Mouse		✓	✓	✓		✓	Increase in CPC proliferation	miR-291, miR-294, miR-295/Akt	[49]
iPS	Mouse						✓		miR-21, miR-210	[50]
Other	Human	hCD34+ SHH+						Transfer of SHH to endothelial cells	SHH	[55]
	Rat/ human	Plasma from remote ischemic preconditioning	✓						Erk, Akt	[71]
	Rat	Perfused hearts with ischaemia (conditioning)	✓							[59]

apoptosis after 48 hours [50] and 4 weeks [49]. Furthermore, injection of ES-derived exosomes increased proliferation, vessel density, and cardiac function, while injection of CPCs prestimulated with ES-derived exosomes, increased cardiac function, and reduced infarct size. The cardioprotective effect could be due to the presence of several known protective miRNAs present in the exosomes, such as miR-21 and miR-210 [50]. The importance of miR-291, miR-294, and miR-295 was confirmed when the use of a miRNA mimic was able to reproduce the induction of CPC proliferation *in vitro* [49].

CPC-derived exosomes are currently being investigated regarding their regenerative potential. Mice CPC-derived exosomes have been shown to reduce the apoptosis of cardiomyocytes *in vitro* and *in vivo* after MI [51]. Exosomes from rat CPCs were able to increase tubule formation of endothelial cells. Furthermore, the mRNA levels of several fibrosis-related genes were significantly downregulated. *In vivo* analysis of these rat CPC-derived exosomes showed an increase in cardiac function, while fibrosis was reduced [52]. Also, exosomes from human CPCs have been evaluated whether they are able to reproduce the positive effects of the originating cell type when injected after MI. Those CPC-derived exosomes were capable to reduce the infarct size in between 7 and 30 days after MI, accompanied by an increase in cardiac function and vessel density. Furthermore, fibrosis was markedly reduced [53, 54]. It became apparent that miR-146a seems to be an important mediator in those exosomes, as a mimic of the miRNA was able to partly reproduce the antiapoptotic effect of the exosomes *in vitro*. Further investigations regarding the role of miR-146a *in vivo* by a knockout mice model showed that without miR-146a infarct size was not reduced and cardiac function did not improve, while injecting a mimic or miR-146a after MI did [54]. The overall proangiogenic effect of CPC-derived exosomes has been implicated in the abovementioned studies. Indeed, exosomes from CPCs are able to promote several parameters of the angiogenesis process *in vitro*. Endothelial cells stimulated

with human CPC-derived exosomes markedly increase migration, tubule formation, and spheroid sprouting [31, 47]. Moreover, in a mouse matrigel-based plug assay, overall cell migration into the plug and blood vessel formation was increased in the presence of exosomes. Several proangiogenic factors were present in these exosomes, such as VEGF, MMP-9, and EMMPRIN (extracellular matrix metalloproteinase inducer). Knockdown of EMMPRIN showed a significant decrease in angiogenesis *in vitro* and *in vivo*, implying that EMMPRIN is a major factor in CPC-derived exosome-induced angiogenesis [47].

4. Potential of Modified Exosomes

Since the protein and miRNA content of exosomes depended on the state and content of their donor cells, several approaches are possible to increase the efficiency of exosomes on cardiac repair. $CD34^+$ cells were genetically modified to overexpress sonic hedgehog (SHH), which resulted in an increased cardiac function and vessel density after MI. It was found that this was (partly) due to the fact that the exosomes from these cells contain SHH, which can be transferred to the recipient cells within the heart, and increase SHH signalling [55].

Overexpression of GATA binding protein 4 (GATA4) in rat bone marrow-derived MSCs leads to a reduction of hypoxia-induced apoptosis of cardiomyocytes when treated with the exosomes from these cells. Moreover, GATA4-overexpressing exosomes restored mitochondrial integrity and had an increase in miR-19 content. MiR-19 is an important effector in survival pathways. *In vivo* analysis indeed showed an increase in miR-19 when the GATA4-overexpressing exosomes were injected after MI. This also leads to an increase in cardiac function and a reduction in infarct size [56].

Besides genetically altering the donor cell to increase or decrease the expression of certain proteins, exosomes can also be altered by changing the conditions of the donor cell at the time of exosome generation. For example, hypoxia is well known to have profound effects on cells, and (remote) ischemic preconditioning has been shown to have positive effects in the clinic [57]. When exosomes were isolated from the hearts or MSCs that have undergone ischemic preconditioning, these exosomes reduced infarct size and fibrosis after MI [58, 59]. *In vitro*, they were able to reduce apoptosis in cardiomyocytes [58].

5. Translation to Preclinical Large Animal Models

Recently, the Marban group reported on a series of experiments in a clinical relevant pig model, where exosomes secreted by human cardiosphere-derived cells were delivered either intracoronary or intramyocardial following reperfused myocardial infarction [60]. In the acute infarct model, only intramyocardial exosome injection resulted in reserved LV function. Moreover, intramyocardial injection was also beneficial in chronic ischemia. The obvious conclusion is that exosomes need to be delivered directly into the myocardium, which may render clinical application even more complex. However, cohort size was small, and the average particle diameter was 197 nm rather than the typical diameter size of 30–100 nm. The authors attribute this to their use of nanoparticle tracking analysis, but it cannot be ruled out that this reflects a different vesicle population [60]. The notion that exosomes are poorly taken up after intracoronary delivery indeed needs serious consideration. Within tissue, cell-to-cell transfer of exosomes between neighbouring cells is feasible. For adhesion to and uptake by the vascular endothelium, however, a minimum density and number of specific adhesion molecules presented on the cell surface are required. On nanoscale exosomes formed in MVBs, a small number of surface proteins may be randomly incorporated, and exosomes should transit the capillary bed at a higher speed than the entire cell. It remains to be investigated whether the exosomes secreted from stimulated cells with distinct endothelial adhesion are predisposed to be taken up by the targeted cells. Interestingly, the Marban group also showed in acute ischemia-reperfusion experiments in rats and mice that CPC-derived exosomes confer cardioprotection by modulating macrophage polarization, induced by transfer of miR-181b leading to inhibition of protein kinase C (PKC)δ [61]. Interestingly, these effects were observed after intracoronary infusion of the exosomes in a rat model and after intramuscular delivery in pigs [60, 61]. In another study, the same group implicated that the Y RNA fragment (EV-YF1) is responsible for cardiomyocyte protection form hypoxia-reoxygenation/ischemia-reperfusion injury by being transferred to macrophages and inducing IL-10 secretion [62]. The means by which exosomes modulate the local tissue immune balance via small RNAs is clearly emerging. Individual pathways and mediators can be identified in specific experimental models, but there is consensus that the multitude of exosome nucleic acids (and proteins) acting in concert are responsible for their pronounced and sustained cardioprotective effects.

6. Future Perspectives

Exosomes from sources such as MSCs or CPCs have been investigated as interesting new therapeutical agents. So far, the first preclinical studies have shown that these exosomes have indeed large regenerative potential and are able to positively influence important processes after myocardial infarction. These effects were observed on a cellular as well as on the whole organ as an overall improvement in cardiac function.

6.1. Clinical Translation and Commercial Exploitation. A number of companies have a secured intellectual property of exosome technology, such as Esperite (immunology applications), Aegle Therapeutics (burns, chronic wounds, etc.), ReNeuron (neural stem cells), SystemsBiosciences (extracellular vesicle precipitation), Anosys (dendritic cell-derived ECV for malignancies), and Capricor. Capricor is a Cedars Sinai Medical Center Spin-off run by the Marban family that developed and evaluated cardiosphere-derived progenitor cells for treatment of postischemic myocardial dysfunction.

Their allogeneic CPC product, CAP-1002, apparently did not meet the efficacy primary outcome in a controlled clinical trial, and the company now focuses on clinical translational of their CPC-derived exosome product (CAP-2003). Their claim, that only exosomes derived from CPCs are effective in ischemic heart disease, is supported by data published by the Marban research group, [54, 60, 62]. However, other groups showed that exosomes from other cell sources are cardioprotective, too [32, 63].

6.2. Exosomes and Immunology. Exosomes derived from dendritic cells or B-cells were shown to present functional surface MHC class I and II antigens [17, 18]. This phenomenon is the basis for the concept of using exosomes/vesicles designed to contain specific tumour antigens as tumour vaccines. Similarly, vesicles carrying antigens of infectious pathogens may be used to elicit a specific immune response for vaccination or treatment. The current assumption is that exosomes from nonimmunological cells are immune-privileged, and indeed most animal experiments so far have been performed with exosomes derived from allogeneic or xenogeneic cells. Even if transplanted exosomes are ultimately eliminated by phagocytic cells, this does not seem to influence their therapeutic capacity. In contrast, in a recent study from Sicco et al., the immune-modulating property of exosomes from MSCs was investigated regarding their effects on macrophages. Here, they could show *in vitro* that a switch towards alternative activated macrophages can be induced by MSC-derived exosomes, which might explain to a certain extent their therapeutic potential [64]. Whether an unwanted sensitization can be induced by exosome-related alloantigens or allogeneic exosomes are less effective in allosensitized patients is not known, yet, but needs further attention.

6.3. Autologous Exosomes. In principle, exosomes can be obtained from autologous cells, provided that the primary cell source possesses robust viability and proliferation capacity in cell culture in order to yield sufficient high cell numbers and, if needed, responds to stimulation for collection of large quantities of conditioned media. MSCs, fibroblasts, endothelial cells, and also cells of cardiac origin (e.g., CPC) generally meet these criteria, but cultivation may be difficult or fail in patients of advanced age or severe disease, as well as a result in less effective exosomes [65]. However, the process of obtaining therapeutic doses of autologous exosomes could be time-consuming and costly, and their application in emergency situations such as acute myocardial infarction is not feasible.

6.4. Exosomes as a Therapeutic Product. While exosomes are not organisms, they do contain a nonuniform mix of proteins and are derived from cells that are either allogeneic or autologous but subjected to nonhomologous use. Therefore, they meet the key criteria for classification as biological medicinal products or, if derived from manipulated cells, as advanced therapeutic medicinal product (ATMP) or gene therapy products by most authorities. An algorithm for regulatory classification of extracellular vesicles has recently been proposed in a position paper of the International Society for Extracellular Vesicles (ISEV) [66]. As outlined by Brindley et al., the characterization of clinical-grade exosomes and their cGMP-compatible production process should encompass a defined size range including the use of a standardized detection method(s), identification by defined biochemical markers, exosome purity (freedom from cells, cell debris, and macromolecules), scalable isolation methods including serum-free source cell culture, and methods for exosome stabilization and storage [67]. It has been suggested that exosomes may be commercialized as "by-products" of cell manufacturing, but we consider it likely that dedicated exosome production processes will be required. Several groups concentrate on using iPS-derived cells as donor cells to generate exosomes for therapeutic use, such as iPS-derived MSCs. The idea is to use a standardized, "perfect" cell source free from any pathogens of genetic aberrations, possibly customized to enhance exosome function, to yield a uniform exosome product that is "streamlined" in terms of compliance with regulatory demands.

6.5. Optimizing Exosomes. In addition to their intrinsic therapeutic potential, exosomes may be used as vehicles for delivery of small molecule drugs, proteins, and nucleic acids (comprehensively reviewed by Ha et al.) [68]. This may be particularly useful when the active compound is unstable, such as siRNA. Moreover, exosomes may enable drugs to enter the central nervous system that are not able to cross the blood-brain barrier in the "naked" form. Specific delivery of cytostatic drugs to tumour tissue via integration into exosomes has also been described [69]. Compared to artificial liposomes for drug delivery, exosomes are believed to persist longer and to possess additional biologic effects [70].

To enhance therapeutic efficacy in the heart, it has been suggested to harvest exosomes from cells that were stimulated by stressors such as hypoxia, leading to an accumulation of stress-response proteins and possibly nucleic acids. While exosomes contain integrins, not all adhesion molecules present on exosomes have been identified yet. This might be the reason that not all exosomes are effective upon systemic or intracoronary delivery, as was shown by the Marban group in a large animal myocardial infarction model [60]. However, in numerous rodent models, intravascular exosome delivery led to improved cardiac function and it is entirely unclear whether this phenomenon has specific biologic reasons or merely reflects local underdosing [32, 71]. Guiding exosomes to specific tissues and improving their uptake by manipulating their surface protein profile are a field of research that will be important for therapeutic success.

7. Conclusion

Extracellular vesicles, most notably exosomes, have been known for almost 50 years, but their potential for therapeutic use in regenerative medicine has only recently been acknowledged. Numerous basic research and preclinical studies have shown beneficial effects in models of different heart diseases, including the acutely or chronically ischemic heart. Compared to viable cell products, development of a readily

available off-the-self therapy should be less complex. However, as was the case with "whole cell" therapy for heart disease in the past 20 years, exosomes from easily obtainable unmodified cells may have limited therapeutic efficacy, and future clinical studies will soon clarify this. Many facets of current translational and commercial activities surrounding the use of exosomes in cardiac regeneration are evocative of the recent history of somatic cell therapy. It remains to be seen whether exosome biology indeed offers fundamental advantages and is able to make a relevant contribution to the development of novel therapies for heart disease.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

[1] R. R. Smith, L. Barile, H. C. Cho et al., "Regenerative potential of cardiosphere-derived cells expanded," *Circulation*, vol. 115, no. 7, pp. 896–908, 2007.

[2] A. P. Beltrami, L. Barlucchi, D. Torella et al., "Adult cardiac stem cells are multipotent and support myocardial regeneration," *Cell*, vol. 114, no. 6, pp. 763–776, 2003.

[3] M.-J. Goumans, T. P. de Boer, A. M. Smits et al., "TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes *in vitro*," *Stem Cell Research*, vol. 1, no. 2, pp. 138–149, 2007.

[4] S. D. Prabhu and N. G. Frangogiannis, "The biological basis for cardiac repair after myocardial infarction," *Circulation Research*, vol. 119, no. 1, pp. 91–112, 2016.

[5] V. Jeevanantham, M. Butler, A. Saad, A. Abdel-Latif, E. K. Zuba-Surma, and B. Dawn, "Adult bone marrow cell therapy improves survival and induces long-term improvement in cardiac parameters: a systematic review and meta-analysis," *Circulation*, vol. 126, no. 5, pp. 551–568, 2012.

[6] P. Nigro, B. Bassetti, L. Cavallotti, V. Catto, C. Carbucicchio, and G. Pompilio, "Cell therapy for heart disease after 15 years: unmet expectations," *Pharmacological Research*, 2017.

[7] A. R. Williams and J. M. Hare, "Mesenchymal stem cells," *Circulation Research*, vol. 109, no. 8, pp. 923–940, 2011.

[8] S. F. Rodrigo, J. Van Ramshorst, G. E. Hoogslag et al., "Intramyocardial injection of autologous bone marrow-derived ex vivo expanded mesenchymal stem cells in acute myocardial infarction patients is feasible and safe up to 5 years of follow-up," *Journal of Cardiovascular Translational Research*, vol. 6, no. 5, pp. 816–825, 2013.

[9] M. C. den Haan, R. W. Grauss, A. M. Smits et al., "Cardiomyogenic differentiation-independent improvement of cardiac function by human cardiomyocyte progenitor cell injection in ischaemic mouse hearts," *Journal of Cellular and Molecular Medicine*, vol. 16, no. 7, pp. 1508–1521, 2012.

[10] M. Mazo, J. J. Gavira, G. Abizanda et al., "Transplantation of mesenchymal stem cells exerts a greater long-term effect than bone marrow mononuclear cells in a chronic myocardial infarction model in rat," *Cell Transplantation*, vol. 19, no. 3, pp. 313–328, 2010.

[11] B. Tao, M. Cui, C. Wang et al., "Percutaneous intramyocardial delivery of mesenchymal stem cells induces superior improvement in regional left ventricular function compared with bone marrow mononuclear cells in porcine myocardial infarcted heart," *Theranostics*, vol. 5, no. 2, pp. 196–205, 2015.

[12] L. Timmers, S. K. Lim, F. Arslan et al., "Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium," *Stem Cell Research*, vol. 1, no. 2, pp. 129–137, 2007.

[13] L. Timmers, S. K. Lim, I. E. Hoefer et al., "Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction," *Stem Cell Research*, vol. 6, no. 3, pp. 206–214, 2011.

[14] S. Ailawadi, X. Wang, H. Gu, and G. C. Fan, "Pathologic function and therapeutic potential of exosomes in cardiovascular disease," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1852, no. 1, pp. 1–11, 2015.

[15] N. Crawford, "The presence of contractile proteins in platelet microparticles isolated from human and animal platelet-free plasma," *British Journal of Haematology*, vol. 21, no. 1, pp. 53–69, 1971.

[16] H. Dvorak, S. Quay, N. Orenstein et al., "Tumor shedding and coagulation," *Science*, vol. 212, no. 4497, pp. 923-924, 1981.

[17] L. Zitvogel, A. Regnault, A. Lozier et al., "Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes," *Nature Medicine*, vol. 4, no. 5, pp. 594–600, 1998.

[18] G. Raposo, H. W. Nijman, W. Stoorvogel et al., "B lymphocytes secrete antigen-presenting vesicles," *Journal of Experimental Medicine*, vol. 183, no. 3, pp. 1161–1172, 1996.

[19] H. Valadi, K. Ekstrom, A. Bossios, M. Sjöstrand, J. J. Lee, and J. O. Lötvall, "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells," *Nature Cell Biology*, vol. 9, no. 6, pp. 654–659, 2007.

[20] C. Théry, S. Amigorena, G. Raposo, and A. Clayton, "Isolation and characterization of exosomes from cell culture supernatants," *Current Protocols in Cell Biology*, Chapter 3, pp. 1–29, 2006.

[21] D. K. Jeppesen, A. Nawrocki, S. G. Jensen et al., "Quantitative proteomics of fractionated membrane and lumen exosome proteins from isogenic metastatic and nonmetastatic bladder cancer cells reveal differential expression of EMT factors," *Proteomics*, vol. 14, no. 6, pp. 699–712, 2014.

[22] P. Kucharzewska, H. C. Christianson, J. E. Welch et al., "Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 18, pp. 7312–7317, 2013.

[23] J. Klumperman and G. Raposo, "The complex ultrastructure of the endolysosomal system," *Cold Spring Harbor Perspectives in Biology*, vol. 6, no. 10, article a016857, 2014.

[24] K. Trajkovic, C. Hsu, S. Chiantia et al., "Ceramide triggers budding of exosome vesicles into multivesicular endosomes," *Science*, vol. 319, no. 5867, pp. 1244–1247, 2008.

[25] M. Mittelbrunn, C. Gutiérrez-Vázquez, C. Villarroya-Beltri et al., "Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells," *Nature Communications*, vol. 2, p. 282, 2011.

[26] J. Liao, R. Liu, L. Yin, and Y. Pu, "Expression profiling of exosomal miRNAs derived from human esophageal cancer cells by Solexa high-throughput sequencing," *International Journal of Molecular Sciences*, vol. 15, no. 9, pp. 15530–15551, 2014.

[27] B. S. Hong, J.-H. Cho, H. Kim et al., "Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells," *BMC Genomics*, vol. 10, no. 1, p. 556, 2009.

[28] M. Ostrowski, N. B. Carmo, S. Krumeich et al., "Rab27a and Rab27b control different steps of the exosome secretion pathway," *Nature Cell Biology*, vol. 12, no. 1, pp. 19–30, 2010.

[29] J. Kowal, M. Tkach, and C. Théry, "Biogenesis and secretion of exosomes," *Current Opinion in Cell Biology*, vol. 29, pp. 116–125, 2014.

[30] A. Zomer, C. Maynard, F. J. Verweij et al., "In vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior," *Cell*, vol. 161, no. 5, pp. 1046–1057, 2015.

[31] K. R. Vrijsen, J. P. G. Sluijter, M. W. L. Schuchardt et al., "Cardiomyocyte progenitor cell-derived exosomes stimulate migration of endothelial cells," *Journal of Cellular and Molecular Medicine*, vol. 14, no. 5, pp. 1064–1070, 2010.

[32] F. Arslan, R. C. Lai, M. B. Smeets et al., "Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury," *Stem Cell Research*, vol. 10, no. 3, pp. 301–312, 2013.

[33] D. Orlic, J. Kajstura, S. Chimenti et al., "Bone marrow cells regenerate infarcted myocardium," *Nature*, vol. 410, no. 6829, pp. 701–705, 2001.

[34] C. E. Murry, M. H. Soonpaa, H. Reinecke et al., "Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts," *Nature*, vol. 428, no. 6983, pp. 664–668, 2004.

[35] L. B. Balsam, A. J. Wagers, J. L. Christensen, T. Kofidis, I. L. Weissman, and R. C. Robbins, "Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium," *Nature*, vol. 428, no. 6983, pp. 668–673, 2004.

[36] M. Dominici, K. Le Blanc, I. Mueller et al., "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement," *Cytotherapy*, vol. 8, no. 4, pp. 315–317, 2006.

[37] A. A. Ramkisoensing, D. A. Pijnappels, S. F. A. Askar et al., "Human embryonic and fetal mesenchymal stem cells differentiate toward three different cardiac lineages in contrast to their adult counterparts," *PLoS One*, vol. 6, no. 9, article e24164, 2011.

[38] A. M. Smits, L. W. van Laake, K. den Ouden et al., "Human cardiomyocyte progenitor cell transplantation preserves long-term function of the infarcted mouse myocardium," *Cardiovascular Research*, vol. 83, no. 3, pp. 527–535, 2009.

[39] K. Malliaras, R. R. Makkar, R. R. Smith et al., "Intracoronary cardiosphere-derived cells after myocardial infarction," *Journal of the American College of Cardiology*, vol. 63, no. 2, pp. 110–122, 2014.

[40] A. R. Chugh, G. M. Beache, J. H. Loughran et al., "Administration of cardiac stem cells in patients with ischemic cardiomyopathy: the SCIPIO trial," *Circulation*, vol. 126, no. 11, Supplement 1, pp. S54–S64, 2012.

[41] M. A. Laflamme, K. Y. Chen, A. V. Naumova et al., "Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts," *Nature Biotechnology*, vol. 25, no. 9, pp. 1015–1024, 2007.

[42] O. Caspi, I. Huber, I. Kehat et al., "Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts," *Journal of the American College of Cardiology*, vol. 50, no. 19, pp. 1884–1893, 2007.

[43] J. J. H. Chong, X. Yang, C. W. Don et al., "Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts," *Nature*, vol. 510, no. 7504, pp. 273–277, 2014.

[44] G. Blin, D. Nury, S. Stefanovic et al., "A purified population of multipotent cardiovascular progenitors derived from primate pluripotent stem cells engrafts in postmyocardial infarcted nonhuman primates," *The Journal of Clinical Investigation*, vol. 120, no. 4, pp. 1125–1139, 2010.

[45] S. Yu, H. Cao, B. Shen, and J. Feng, "Tumor-derived exosomes in cancer progression and treatment failure," *Oncotarget*, vol. 6, no. 35, pp. 37151–37168, 2015.

[46] S. Bian, L. Zhang, L. Duan, X. Wang, Y. Min, and H. Yu, "Extracellular vesicles derived from human bone marrow mesenchymal stem cells promote angiogenesis in a rat myocardial infarction model," *Journal of Molecular Medicine*, vol. 92, no. 4, pp. 387–397, 2014.

[47] K. R. Vrijsen, J. A. Maring, S. A. J. Chamuleau et al., "Exosomes from cardiomyocyte progenitor cells and mesenchymal stem cells stimulate angiogenesis via EMMPRIN," *Advanced Healthcare Materials*, vol. 5, no. 19, pp. 2555–2565, 2016.

[48] Z. Zhang, J. Yang, W. Yan, Y. Li, Z. Shen, and T. Asahara, "Pretreatment of cardiac stem cells with exosomes derived from mesenchymal stem cells enhances myocardial repair," *Journal of the American Heart Association*, vol. 5, no. 1, article e002856, 2016.

[49] M. Khan, E. Nickoloff, T. Abramova et al., "Embryonic stem cell-derived exosomes promote endogenous repair mechanisms and enhance cardiac function following myocardial infarction," *Circulation Research*, vol. 117, no. 1, pp. 52–64, 2015.

[50] Y. Wang, L. Zhang, Y. Li et al., "Exosomes/microvesicles from induced pluripotent stem cells deliver cardioprotective miRNAs and prevent cardiomyocyte apoptosis in the ischemic myocardium," *International Journal of Cardiology*, vol. 192, pp. 61–69, 2015.

[51] L. Chen, Y. Wang, Y. Pan et al., "Cardiac progenitor-derived exosomes protect ischemic myocardium from acute ischemia/reperfusion injury," *Biochemical and Biophysical Research Communications*, vol. 431, no. 3, pp. 566–571, 2013.

[52] W. D. Gray, K. M. French, S. Ghosh-Choudhary et al., "Identification of therapeutic covariant microRNA clusters in hypoxia-treated cardiac progenitor cell exosomes using systems biology," *Circulation Research*, vol. 116, no. 2, pp. 255–263, 2015.

[53] L. Barile, V. Lionetti, E. Cervio et al., "Extracellular vesicles from human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction," *Cardiovascular Research*, vol. 103, no. 4, pp. 530–541, 2014.

[54] A. G.-E. Ibrahim, K. Cheng, and E. Marbán, "Exosomes as critical agents of cardiac regeneration triggered by cell therapy," *Stem Cell Reports*, vol. 2, no. 5, pp. 606–619, 2014.

[55] A. R. Mackie, E. Klyachko, T. Thorne et al., "Sonic hedgehog-modified human CD34+ cells preserve cardiac function after acute myocardial infarction," *Circulation Research*, vol. 111, no. 3, pp. 312–321, 2012.

[56] B. Yu, H. W. Kim, M. Gong et al., "Exosomes secreted from GATA-4 overexpressing mesenchymal stem cells serve as a reservoir of anti-apoptotic microRNAs for cardioprotection," *International Journal of Cardiology*, vol. 182, pp. 349–360, 2015.

[57] Y. J. Cho, E.-H. Lee, K. Lee et al., "Long-term clinical outcomes of remote ischemic preconditioning and postconditioning outcome (RISPO) trial in patients undergoing cardiac surgery," *International Journal of Cardiology*, vol. 231, pp. 84–89, 2017.

[58] Y. Feng, W. Huang, M. Wani, X. Yu, and M. Ashraf, "Ischemic preconditioning potentiates the protective effect of stem cells through secretion of exosomes by targeting Mecp2 via miR-22," *PLoS One*, vol. 9, no. 12, article e88685, p. e114139, 2014.

[59] Z. Giricz, Z. V. Varga, T. Baranyai et al., "Cardioprotection by remote ischemic preconditioning of the rat heart is mediated by extracellular vesicles," *Journal of Molecular and Cellular Cardiology*, vol. 68, pp. 75–78, 2014.

[60] R. Gallet, J. Dawkins, J. Valle et al., "Exosomes secreted by cardiosphere-derived cells reduce scarring, attenuate adverse remodelling, and improve function in acute and chronic porcine myocardial infarction," *European Heart Journal*, vol. 38, no. 3, pp. 201–211, 2017.

[61] G. de Couto, R. Gallet, L. Cambier et al., "Exosomal microRNA transfer into macrophages mediates cellular postconditioning clinical perspective," *Circulation*, vol. 136, no. 2, pp. 200–214, 2017.

[62] L. Cambier, G. de Couto, A. Ibrahim et al., "Y RNA fragment in extracellular vesicles confers cardioprotection via modulation of IL-10 expression and secretion," *EMBO Molecular Medicine*, vol. 9, no. 3, pp. 337–352, 2017.

[63] R. C. Lai, F. Arslan, M. M. Lee et al., "Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury," *Stem Cell Research*, vol. 4, no. 3, pp. 214–222, 2010.

[64] C. Lo Sicco, D. Reverberi, C. Balbi et al., "Mesenchymal stem cell-derived extracellular vesicles as mediators of anti-inflammatory effects: endorsement of macrophage polarization," *Stem Cells Translational Medicine*, vol. 6, no. 3, pp. 1018–1028, 2017.

[65] A. E. Mayfield, M. E. Fitzpatrick, N. Latham et al., "The impact of patient co-morbidities on the regenerative capacity of cardiac explant-derived stem cells," *Stem Cell Research & Therapy*, vol. 7, no. 1, p. 60, 2016.

[66] T. Lener, M. Gimona, L. Aigner et al., "Applying extracellular vesicles based therapeutics in clinical trials – an ISEV position paper," *Journal of Extracellular Vesicles*, vol. 4, no. 1, article 30087, 2015.

[67] D. A. Brindley, K. Bure, A. Smith James et al., "Extracellular vesicles commercial potential as byproducts of cell manufacturing for research and therapeutic use," *Bioprocess International*, 2015, August 2017, http://www.bioprocessintl.com/manufacturing/cell-therapies/extracellular-vesicles-commercial-potential-as-byproducts-of-cell-manufacturing-for-research-and-therapeutic-use/.

[68] D. Ha, N. Yang, and V. Nadithe, "Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges," *Acta Pharmaceutica Sinica B*, vol. 6, no. 4, pp. 287–296, 2016.

[69] K. Tang, Y. Zhang, H. Zhang et al., "Delivery of chemotherapeutic drugs in tumour cell-derived microparticles," *Nature Communications*, vol. 3, p. 1282, 2012.

[70] K. B. Johnsen, J. M. Gudbergsson, M. N. Skov, L. Pilgaard, T. Moos, and M. Duroux, "A comprehensive overview of exosomes as drug delivery vehicles — endogenous nanocarriers for targeted cancer therapy," *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, vol. 1846, no. 1, pp. 75–87, 2014.

[71] J. M. Vicencio, D. M. Yellon, V. Sivaraman et al., "Plasma exosomes protect the myocardium from ischemia-reperfusion injury," *Journal of the American College of Cardiology*, vol. 65, no. 15, pp. 1525–1536, 2015.

Hindawi
Stem Cells International
Volume 2017, Article ID 2653142, 7 pages
https://doi.org/10.1155/2017/2653142

Review Article

Stem and Progenitor Cells in Human Cardiopulmonary Development and Regeneration

Silvana Bardelli[1] and Marco Moccetti[2]

[1]*Swiss Institute for Regenerative Medicine, Foundation for Cardiological Research and Education, Via ai Söi 24, 6807 Taverne, Switzerland*
[2]*Cardiology Department, Cardiocentro Ticino Foundation, Via Tesserete 48, 6900 Lugano, Switzerland*

Correspondence should be addressed to Silvana Bardelli; silvana.bardelli@cardiocentro.org

Received 28 April 2017; Accepted 15 August 2017; Published 17 September 2017

Academic Editor: Fatemeh Sharifpanah

Already during embryonic development, the heart and the lung are thoroughly connected organs. Their interdependence allows our survival in the terrestrial environment by coupling cardiac output and gas exchange. The knowledge on developmental processes involving stem and progenitor cells is crucial to understand the onset of human cardiopulmonary diseases. The precise identification of various adult endogenous progenitors is still incomplete. Thus, caution should be exercised on newly available stem cell-based treatments until specific mechanisms of action are disclosed. The objective is to provide in the nearest future feasible and safer cell therapeutics for the complex pathological condition of human cardiopulmonary diseases. In this paper, we highlight the significant knowledge advancement concerning stem and progenitor cells in the cardiopulmonary field: from embryonic development to adult progenitors until early preclinical models for cardiopulmonary regeneration.

1. Development of the Cardiopulmonary System: The Contribution of Stem and Progenitor Cells

Adaptation to terrestrial life happened recently in our evolutionary history. As a consequence of this event, the cardiac and the pulmonary systems developed in parallel to allow the coupling of cardiac function and gas exchange in the lung. In mammals, the cooperation of these two systems is already apparent during embryonic development: while the heart tube loops and asymmetrically divides into the mature cardiac chambers, the lung anterior endoderm protrudes into the cardiac embryonic mesoderm. This interdependence forms the cardiopulmonary circulation, a specialized compartment that connects the heart and the lungs: it receives the cardiac output to allow gas exchange and to provide oxygenated blood to the systemic circulation.

Cardiac morphogenesis occurs prior to lung development [1, 2]. The embryonic heart early provides pump function that is fundamental for fetal and postnatal life. Heart development is regulated by highly conserved tissue-specific transcription factors, signaling molecules, and noncoding RNAs. Central to this network are the transcription factors Wnt, NKX2-5, GATA4, and SRF, which, together with their target DNA elements, form an evolutionarily conserved subcircuit essential for development [3]. The process of looping morphogenesis brings the venous pole ventral to the foregut endoderm. This mesoderm-endoderm interaction is crucial to lung development. The embryonic lung evaginates from the anterior endoderm which will form also the trachea and larynx. The organ's epithelium derives from the endoderm, while lung mesenchyme is of mesodermal origin. From the distal region of the laryngotracheal groove [4, 5], embryonic epithelial progenitors divide rapidly and generate sequentially the primary and secondary bronchial airways and the alveolar structures. As a consequence, the adult respiratory tree is formed [6, 7].

The spatially and temporally coordinated development of the embryonic heart and lung raises the possibility of a common multipotent progenitor originating in both organs and their physiologic connection in terrestrial mammals. Recently, Peng et al. [8] reported a novel population of

multipotent cardiopulmonary mesoderm progenitors (CPPs) that arises from cardiac posterior pole prior to lung development. $Wnt2^+/Gli1^+/Isl1^+$ CPPs were identified by lineage tracing and clonal analysis experiments and proved to generate the mesoderm lineages of the cardiac inflow tract, pulmonary vascular and airway smooth muscle, lung proximal endothelium, pericyte-like cells, and also cardiomyocytes. The foregut endoderm that is required to connect the pulmonary vasculature to the heart regulates the development of CPPs through the Sonic Hedgehog (Shh) network. Shh activates its effector Gli1 that is coexpressed with Wnt2 and Isl1 in CPP cells. According to lineage-tracing experiments, the authors observed that Hedgehog signaling is required to direct the development of CPPs towards the lung smooth muscle lineage and initiates the cardiopulmonary connection. The authors reported that the earliest cardiac progenitors that are located in the second heart field are characterized by the expression of Isl1. The Isl1-positive ($Isl1^+$) population further subdivides into $Isl1^+/Nkx2.5^+$ cells in the ventral/medial domain and the $Isl1^+/Nkx2.5^-$ subpopulation in the lateral/dorsal domain. This latter subpopulation, characterized only by the expression of Isl1, generates all layers of the lung vasculature and the myocardial inflow tract at E8.5. Specifically, $Isl1^+$ progenitor cells generate the ventral lung mesenchyme that connects to the cardiac inflow tract, while Nkx2.5-positive progenitors give rise to the myocardium close to the pulmonary vein. Wnt2-positive progenitors, located exclusively in the posterior pole of the developing heart at E8.5, form cells within the cardiac inflow tract, not within the outflow tract. These cells are the ones that move to the lung bud in its early development. Later during embryonic development, $Wnt2^+$ cardiac progenitor cells generate all mesodermal lineages of the heart including cardiomyocytes and the endocardium. Additionally, they give rise to the pulmonary vasculature, lung pericytes, and airway smooth muscle cells in the developing lung. Therefore, the authors demonstrated that $Wnt2^+$ cells represent multipotent progenitors in the developing lung and inflow tract of the heart. The authors also reported that the subpopulation of cardiac Gli1-positive cells contributes to the cardiac mesodermal compartment as well as the early lung bud. Overall, the population of $Wnt2^+/Gli1^+/Isl1^+$ cells generates the majority of mesodermal cells in the cardiac inflow tract and in the lung. Therefore, pulmonary vascular and airway smooth muscle cells, proximal endothelium, and pericyte-like cells derive clonally from these progenitors. Importantly, alterations of this developmental pattern cause congenital defects such as tetralogy of Fallot syndrome in the newborn or persistent pulmonary hypertension. Ultimately, understanding the role of cardiac mesoderm and lung endoderm interaction during development would provide mechanistic insights into the congenital cardiopulmonary diseases where vascular patterning and differentiation are perturbed. Furthermore, deciphering the signaling pathways necessary for pulmonary vascular development could potentially shed light on mechanisms involved in vascular regeneration and remodeling in adult pulmonary diseases. Adult cardiac diseases such as myocardial infarction (MI) result in a massive loss of cardiomyocytes that leads to heart failure. Successful therapies for these diseases are lacking. There is an urgent need to clarify the mechanisms that regulate heart and lung development to design effective approaches for cardiopulmonary regeneration.

2. Adult Progenitor Cells in Human Heart and Lung Regeneration

Cardiac and pulmonary diseases are frequent. They impact significantly on healthcare costs. A recurring question in biology is whether regeneration occurs in these adult organs.

In the heart, the focus is whether adult cardiomyocytes (CMs) proliferate and to what extent. This long-debated question raises controversies in the field. In the last decades, human cardiomyocyte proliferation was documented, as well as its steady state. Bergmann et al. [9] presented a study on human CM stereology combined with quantification of genomic ^{14}C concentrations in cardiomyocyte nuclei (retrospective birth dating). They reported that, according to the analysis of CM volume and nuclear DNA synthesis, the CM number did not change substantially in postnatal life and remained constant throughout the whole human life span. Specifically, compared to both cardiac endothelial and mesenchymal cells, cardiomyocytes showed the highest extrapolated turnover rate restricted to the first decade of life; cardiomyocyte turnover decreased with age exponentially and was $\leq 1\%$ in adults. Mollova et al. [10] with the same technique, that is, stereology, found that most postnatally born CMs are generated in young humans: their number increased by 3.4-fold over the first 20 years of life, indicating that the highest cardiac cell proliferation rate occurred in young adults. No consensus exists on the magnitude of adult cardiomyocyte renewal, with estimates ranging from no turnover rate to complete cell exchange in a few year lifespan [11, 12].

Overall, the reported results suggest that the mammalian heart possesses a measurable capacity for renewal. Importantly, intense debate exists concerning the source of the newly generated cardiomyocytes: it is not yet clear whether cardiomyocytes are renewed through differentiation from a stem/progenitor population or through cell division by existing cardiomyocytes [13, 14]. Nevertheless, these two possibilities are not mutually exclusive, and both represent possible opportunities to increase cardiomyocyte generation for cardiac regenerative therapies. In the field of cardiac regeneration, there is a considerable interest in whether transdifferentiation events might generate new cardiomyocytes. Bone marrow-derived cells like hematopoietic stem cells and mesenchymal stem cells [15, 16] were thought to differentiate to cardiac muscle and contribute to functional recovery after MI. However, results from subsequent studies indicate that these cell types may contribute to heart repair by indirect paracrine mechanisms, as opposed to direct differentiation into myocardial cells [17, 18]. The mechanism of cardiomyocyte dedifferentiation might also occur. This process is characterized by a reduction of sarcomere structures and the expression of fetal gene markers. A significant advancement in the field will be to understand how dedifferentiation is initiated and identify the target molecules that induce these phenotypic changes [19, 20].

Based on the ongoing debate on the actual capacity of the adult human heart to renew cardiomyocytes, alternative therapeutic approaches to augment endogenous regeneration are explored such as the administration of stem or progenitor cells to the heart or the stimulation of endogenous cardiac progenitors.

To this extent, however, a clear definition of endogenous cardiac progenitors is necessary. The issue is still elusive and controversial as of today.

Numerous putative adult cardiac progenitors have been characterized by the positivity of different markers. Specifically, in 2003, Oh et al. documented a cardiac progenitor cell based on the expression of murine Sca1 antigen [21]. This population can be enriched for either high efflux of Hoescht dye through an ATP-binding cassette transporter (side population cells) or high expression of PDGFRa. The enriched population shows multilineage potential and differentiation towards cardiomyocytes in vivo. In the same year, Beltrami et al. documented an alternative cardiac progenitor characterized by the expression of the receptor c-kit (CD117, Stem Cell Factor receptor) [22]. In more recent years, Ellison et al. [23] concluded that c-kit-positive cells are necessary and sufficient to regenerate an acute adult myocardial injury based on a cardiotoxic isoproterenol treatment model. In 2004, Messina et al. reported the isolation of adult cardiac progenitors that grow in adherent spheres, named cardiospheres [24]. Cardiospheres are composed of a combination of progenitor cells, cardiac myocyte-like cells, and vascular cells. The authors suggested that these cell types are the progeny of a small subset of undifferentiated cells that express different stem cell markers such as c-kit and Sca-1. Cardiosphere-derived cells are isolated from adult murine and a human heart and can be expanded in vitro for therapeutic use. The identification of multiple progenitors and the concomitant-limited therapeutic regeneration observed in studies performed so far led some investigators to conclude that most progenitors are the same cell at different stages of differentiation [25–29].

The adult mammalian lung is organized into two major compartments: the airways that conduct gases and the alveoli where gas exchange occurs. Approximately 40 different cell types exist within the adult lung. The epithelial lineages are the best defined. Their characterization is based on murine lineage-tracing studies. These studies might reflect the organization of the adult human lung; however, human lung epithelium might possess unique properties.

The steady state lung is a low cellular turnover tissue that includes quiescent stem or progenitor cells. These cells participate in the repair of the damaged lung [30–33]. Basal cells are characterized by a small height compared to adjacent luminal cells, and they are located at the basement membrane [34]. Basal cells express the N-terminus-truncated isoform of TRP63 (p63), cytokeratin 5 (KRT5), nerve growth factor receptor (NGFR), and podoplanin (PDPN) [35]. These cells are self-renewing and multipotent: they generate other basal cells and also secretory and ciliated cells [36]. Recent studies by Pardo-Saganta et al. [37] demonstrated that, under steady state conditions, the basal cell population is heterogeneous: they express activated Notch2 intracellular domain (Notch2ICD) and c-myb (Myb) in secretory and ciliated cells, respectively. Basal cells are located in the murine trachea and bronchi while in humans, they are found more distally, in the small bronchioles.

Secretory or club cells (formerly known as Clara cells) are dome shaped and possess secretory granules in their cytoplasm. Murine secretory cells are self-renewing and differentiate into ciliated cells. These cells are present in the murine trachea, bronchi and bronchioles, and throughout the human airway epithelium. Recent studies by Tata et al. [38] indicated that they are highly heterogeneous.

Ciliated cells are also present throughout the large and small airways. They are characterized by multicilia on their apical surface and are positive for the nuclear transcription factor FoxJ1. Lineage-tracing studies document that they are terminally differentiated cells. Ciliated cells are produced directly from basal cells following injury. Neuroendocrine cells are single cells or organized clusters in close contact with nerve fibers. They are characterized by the expression calcitonin gene-related peptide (CALCA), chromogranin A, and achete-scute homolog 1 (ASCL1). They are present in murine large and small airways and are enriched at the branch points of airways. Pulmonary neuroendocrine cells perform multiple functions such as oxygen sensing and mechanotransduction.

Alveolar epithelial type 2 and type 1 cells are cuboidal surfactant-producing and gas-exchanging cells, respectively. Recent studies through lineage-tracing analysis demonstrated that type 2 cells maintain the homeostatic turnover of type 1 cells and clonally generate more type 2 cells in the adult lung [39]. The zone of transition from the bronchioles to the alveoli is referred to as the bronchioalveolar duct junction (BADJ). Within this region, bronchioalveolar stem cells (BASCs) are present. They were identified based on their proliferation after bleomycin injury [40]. In humans, BASCs have not been clearly characterized.

Interestingly, cellular plasticity is now an emerging concept in the biology of multiple adult organs. Multiple studies recently indicated that in various tissues, cellular plasticity is a common phenomenon in the process of repair after injury [41–43]. In the lung, evidence for plasticity derives from cell ablation experiments. Tata et al. [44] reported that in the tracheal epithelium, fully mature secretory cells dedifferentiated into basal stem cells following diphtheria toxin-induced stem cell ablation. Interestingly, secretory cells started to replicate when over 80% of the basal cells were ablated by the treatment. The signals that regulate cell plasticity are yet to be defined. Tata and Rajagopal reported that transdifferentiation can also occur [45]: fully differentiated neuroendocrine cells in the small airways generate secretory cells as well as ciliated cells following naphthalene-induced injury or after H1N1 influenza-induced injury. Lineage-tracing experiments demonstrate that epithelial stem and progenitor cells maintain a stable identity during steady state conditions but can display remarkable lineage plasticity following injury. In humans, our knowledge on cellular plasticity is preliminary. In vitro results demonstrate the plasticity of human lung epithelial cells. However, the results might not reflect the plasticity observed in living organisms.

Further advancement of the concept of cellular plasticity will certainly need confirmation in the next decades.

In the human heart, there is a lack of consensus on the composition of the nonmyocyte cell population. Very interestingly, a recent study by Pinto et al. revealed that fibroblasts represent a relatively minor cell population and that endothelial cells are the most abundant cell type in healthy adult human hearts [46]. The authors used newly available genetic trackers, flow cytometric analysis, and an unsupervised clustering algorithm (SPADE, Spanning-tree Progression Analysis of Density-normalized Events). The analysis showed that approximately 65% of cardiac cells are endothelial cells, 10% are leukocytes, and about 25% are cardiomyocytes. These unexpected results highlight the fact that the cardiac fibroblast population is much smaller than previously reported [47]. Furthermore, a comprehensive understanding of cardiac cellular composition will guide the development of new therapeutics to promote heart repair and regeneration. Overall, these findings redefine the cellular composition of the adult murine and human heart and indicate that the endothelial cell compartment might play a potentially important role in cardiac homeostasis, disease, and regeneration.

3. Current Stem Cell-Based Therapeutic Approaches for Cardiopulmonary Diseases

Pulmonary arterial hypertension (PAH) is associated with right ventricular hypertrophy or failure. This is the result of pressure overload in the right ventricle. Current therapeutic approaches are still experimental, and we need to be cautious in stating their efficacy. However, potentiality exists and current treatment options might expand in the next decades.

Overall, stem and progenitor cell therapy in cardiopulmonary diseases demonstrates to be effective in animal models of PAH. Mainly, these stem cell-based experimental models lay on the observation that stem and progenitor cells might regenerate pulmonary vasculature. Accordingly, endothelial progenitor cells (EPCs) are good candidates towards this goal: endothelial progenitors are circulating cells derived from the bone marrow. They are able to differentiate into mature endothelial cells to repair the vasculature. It is still not clear how endothelial stem or progenitor cells exert their effect when administered to the lung. Proper engraftment in the lung tissue is thought to happen rarely. A combination of concomitant biological mechanisms is more likely to occur, including stem cell-induced paracrine effect due to the release of microvesicles or exosomes. Noncoding microRNAs are more recent players in this field. Interestingly, Spees et al. investigated the effect of monocrotalin (MCT) on the engraftment and differentiation of GFP-positive bone marrow-derived cells in rodent models of PAH [48]. The authors observed the engraftment of the administered cells in the lungs and their differentiation into pulmonary epithelial cells (Clara cells), vascular endothelial cells, and smooth muscle cells. Furthermore, GFP-positive cells engrafted in both the right and the left ventricles of hyperthophic rat hearts. In the right ventricles, administered cells differentiated mainly into vascular cells and cardiomyocytes. No cell fusion events were observed between endogenous cardiac cells and administered bone marrow-derived cells. Combination therapy including the administration of stem or progenitor cells together with pharmacological agents is in general more effective. Sun et al. administered cilostazol, a phosphodiesterase III inhibitor, together with EPCs three days after MCT injection [49]. The authors observed reduced remodeling of pulmonary resistance arteries resulting from proliferation of endothelial cells and vascular smooth muscle cells. In general, combination therapy was more successful than EPCs or citostazol alone in preventing vascular remodeling due to MCF-induced PAH.

Takemiya et al. observed that intravenous administration of mesenchymal stem cells (MSCs) in rat lungs affected by MCT-induced PAH was not sufficient to lower pulmonary artery pressure. However, when MSCs were delivered in combination with prostacyclin synthase, the authors reported a significant decrease in pulmonary artery systolic pressure and right ventricular dilation. Notably, paracrine effect due to cell-mediated release of soluble factors rather than massive cell engraftment is thought to exert the effects observed.

In the clinical condition of emphysema, the alveolar epithelium is damaged and repair processes are unlikely to occur. The role of all transretinoic acid (ATRA) is currently under investigation in the therapeutic treatment of emphysema. Retinoic acid is the active metabolite of vitamin A (i.e., retinol) that is essential for multiple cellular functions such as cell homeostasis and differentiation. Retinoic acid is acquired from diet. However, the long-term use of oral retinoic acid causes side effects such as dry skin, headache, hyperlipidemia, muscle, and bone soreness. Specifically, Mao et al. performed a double-blind, placebo-controlled feasibility trial to test the long-term administration of ATRA. Patients affected by moderate to severe emphysema were subjected to the standard of care plus twice-daily oral administration of ATRA for 12 weeks [50]. The study did not show any therapeutic effect on emphysema, and side effects were observed. Brooks et al. tested the effect of aerosolized ATRA in rodent models of emphysema and demonstrated that it is feasible and represents a safer alternative to oral retinoic acid [51].

The processes involved in lung epithelial repair are currently unknown despite the significant advances in stem cell research over the past decades [52–54].

Personalized medicine approaches are essential for the treatment of cystic fibrosis. Over 1500 known mutations of the CFTR (cystic fibrosis transmembrane conductance regulator) gene exist. Each of them results in distinct functional pathologic variables. CFTR is expressed on the surface of plasma membranes, specifically in ciliated cells. It is a chloride channel that, when alterated, produces impaired chloride and bicarbonate secretion resulting in thicker mucus and recurrent infections. The generation of patient-specific in vitro models for this clinical condition is crucial. Culture of patient-derived primary human airway or nasal epithelial cells and their targeted differentiation may constitute a valuable objective of therapeutic investigation in this field. Alternatively, the differentiation of patient-specific-induced pluripotent stem cells (iPSCs) into adult epithelial cells might

be pursued. Patient-specific stem or progenitor cell treatments in preclinical models of cystic fibrosis will thus allow drug development in the future [55].

Notably, the current position of the COPD Foundation (https://www.copdfoundation.org) on stem cell therapy is cautious. The foundation warns on several clinics providing alleged stem cell-based treatments for incurable lung diseases, including chronic obstructive pulmonary disease (COPD). FDA did not approve such treatments. Therefore, the COPD Foundation does not recommend the use of autologous stem cell therapy for the treatment of COPD or other lung diseases until more convincing proof of effectiveness is provided. The COPD Foundation encourages patients to participate in the clinical trial that tests the development and potential benefit of this approach.

Overall, stem cell-based therapeutic approaches on human cardiopulmonary diseases are still at their preliminary stage. We acquired valuable information of endogenous cardiac and pulmonary stem or progenitor cells that are distributed in different compartments of these organs. Stem and progenitor cells may represent key protagonists of newly available treatments. The knowledge we acquired so far, although insufficient to guarantee an immediate therapeutic use, warrants further studies to impact on this massive clinical demand.

4. Reflections on Current Therapeutic Developments

As mentioned earlier, no consensus exists so far on the characterization of endogenous pulmonary and cardiac stem or progenitor cells. Many authors raise the possibility that the same cell at subsequent differentiation stages was characterized by different groups.

Once properly identified, specific progenitors might be successfully employed in lineage-tracing studies to understand their role in animal models of disease. Furthermore, specific sorting of surface markers through fluorescence-activated cell sorter (FACS) might be used to enrich candidate progenitor cells more homogeneously. This targeted approach will shed light on the specific role of the sorted cells when administered in vivo.

Administration of endothelial progenitor cells (EPCs) or mesenchymal stromal cells (MSCs) is the emerging strategy for the treatment of severe cardiopulmonary diseases such as pulmonary arterial hypertension. These studies are preliminary and rely mainly on preclinical animal models. MSCs are thought to exert their effect through immunomodulatory properties. Nevertheless, the precise mechanisms that allow stem or progenitor cells to act in cardiopulmonary remodeling are still unknown. Possibly, multiple concomitant biological, biochemical, and biomolecular cues are involved.

Induced pluripotent stem cells (iPSCs) represent an additional cell source. They are patient specific and might potentially serve as a renewable source. The immediate impact of iPSC technology does not lie in regenerative medicine applications but mainly in the study of the cellular mechanisms that generate cardiopulmonary diseases. This allows potential patient-specific drug screening and future gene therapy, a powerful approach within the field of personalized medicine.

On the other hand, increasing knowledge on the mechanisms that control embryonic cardiopulmonary development might highlight key molecular effectors. The same pathways are frequently impaired at the onset of cardiopulmonary diseases. Additionally, the recent identification of a common progenitor cell that directs development of the cardiopulmonary circulation further strengthens the connection of these two organs.

5. Conclusions

Accumulated knowledge in preclinical models and in preliminary clinical trials suggests that stem cell-based therapies may represent potential strategies for cardiopulmonary repair after injury. In parallel, further characterization of endogenous stem and progenitor cells in the lung and in the heart provides a sound scientific basis for therapeutic use in cardiopulmonary diseases. This approach lies on the precise identification of specific markers for each progenitor cell type.

Remarkable advances of basic research on human cardiac and pulmonary stem cells in the past decades have sustained the submission of numerous investigational new drug applications for clinical trials in humans. Although the current understanding is still limited to guarantee a safe human application for cardiopulmonary diseases, autologous stem and progenitor cells are emerging as key players for newly available therapies. The nearest future will hold better insights to develop safer and feasible therapeutic options. This further advancement will happen only if a scientifically sound approach leads the studies of human cardiopulmonary diseases that still constitute an area of unmet clinical need.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

[1] V. M. Christoffels and W. T. Pu, "Developing insights into cardiac regeneration," *Development*, vol. 140, no. 19, pp. 3933–3937, 2013.

[2] S. Zaffran and R. G. Kelly, "New developments in the second heart field," *Differentiation*, vol. 84, no. 1, pp. 17–24, 2012.

[3] E. H. Davidson and D. H. Erwin, "Gene regulatory networks and the evolution of animal body plans," *Science*, vol. 311, no. 5762, pp. 796–800, 2006.

[4] J. Que, M. Choi, J. W. Ziel, J. Klingensmith, and B. L. M. Hogan, "Morphogenesis of the trachea and esophagus: current players and new roles for noggin and Bmps," *Differentiation*, vol. 74, no. 7, pp. 422–437, 2006.

[5] J. Que, T. Okubo, J. R. Goldenring et al., "Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm," *Development*, vol. 134, no. 13, pp. 2521–2531, 2007.

[6] J. R. Rock and B. L. M. Hogan, "Epithelial progenitor cells in lung development, maintenance, repair, and disease,"

Annual Review of Cell and Developmental Biology, vol. 27, pp. 493–512, 2011.

[7] J. R. Rock, S. H. Randell, and B. L. M. Hogan, "Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling," *Disease Models & Mechanisms*, vol. 3, no. 9-10, pp. 545–556, 2010.

[8] T. Peng, Y. Tian, C. J. Boogerd et al., "Coordination of heart and lung co-development by a multipotent cardiopulmonary progenitor," *Nature*, vol. 500, no. 7464, pp. 589–592, 2013.

[9] O. Bergmann, S. Zdunek, A. Felker et al., "Dynamics of cell generation and turnover in the human heart," *Cell*, vol. 161, no. 7, pp. 1566–1575, 2015.

[10] M. Mollova, K. Bersell, S. Walsh et al., "Cardiomyocyte proliferation contributes to heart growth in young humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 4, pp. 1446–1451, 2013.

[11] O. Bergmann and S. Jovinge, "Cardiac regeneration in vivo: mending the heart from within?," *Stem Cell Research*, vol. 13, no. 3, Part B, pp. 523–531, 2014.

[12] J. Kajstura, N. Gurusamy, B. Ogórek et al., "Myocyte turnover in the aging human heart," *Circulation Research*, vol. 107, no. 11, pp. 1374–1386, 2010.

[13] K. Kikuchi and K. D. Poss, "Cardiac regenerative capacity and mechanisms," *Annual Review of Cell and Developmental Biology*, vol. 28, pp. 719–741, 2012.

[14] D. Später, E. M. Hansson, L. Zangi, and K. R. Chien, "How to make a cardiomyocyte," *Development*, vol. 141, no. 23, pp. 4418–4431, 2014.

[15] D. Orlic, J. Kajstura, S. Chimenti et al., "Bone marrow cells regenerate infarcted myocardium," *Nature*, vol. 410, no. 6829, pp. 701–705, 2001.

[16] C. Toma, M. F. Pittenger, K. S. Cahill, B. J. Byrne, and P. D. Kessler, "Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart," *Circulation*, vol. 105, no. 1, pp. 93–98, 2002.

[17] M. Mirotsou, Z. Zhang, A. Deb et al., "Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 5, pp. 1643–1648, 2007.

[18] L. Bao, Q. Meng, Y. Li et al.L. Zhang, H. Fan et al., "C-kit positive cardiac stem cells and bone marrow-derived mesenchymal stem cells synergistically enhance angiogenesis and improve cardiac function after myocardial infarction in a paracrine manner," *Journal of Cardiac Failure*, vol. 23, no. 5, pp. 403–415, 2017.

[19] T. Kubin, J. Poling, S. Kostin et al., "Oncostatin M is a major mediator of cardiomyocyte dedifferentiation and remodeling," *Cell Stem Cell*, vol. 9, no. 5, pp. 420–432, 2011.

[20] G. D'Uva, A. Aharonov, M. Lauriola et al., "ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation," *Nature Cell Biology*, vol. 17, no. 5, pp. 627–638, 2015.

[21] H. Oh, S. B. Bradfute, T. D. Gallardo et al., "Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 21, pp. 12313–12318, 2003.

[22] A. P. Beltrami, L. Barlucchi, D. Torella et al., "Adult cardiac stem cells are multipotent and support myocardial regeneration," *Cell*, vol. 114, no. 6, pp. 763–776, 2003.

[23] G. M. Ellison, C. Vicinanza, A. J. Smith et al., "Adult c-kit^{pos} cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair," *Cell*, vol. 154, no. 4, pp. 827–842, 2013.

[24] E. Messina, L. De Angelis, G. Frati et al., "Isolation and expansion of adult cardiac stem cells from human and murine heart," *Circulation Research*, vol. 95, no. 9, pp. 911–921, 2004.

[25] G. M. Ellison, V. Galuppo, C. Vicinanza et al., "Cardiac stem and progenitor cell identification: different markers for the same cell?," *Frontiers in Bioscience*, vol. 2, pp. 641–652, 2010.

[26] S. Koudstaal, S. J. Jansen of Lorkeers, R. Gaetani et al., "Concise review: heart regeneration and the role of cardiac stem cells," *Stem Cells Translational Medicine*, vol. 2, no. 6, pp. 434–443, 2013.

[27] A. Itzhaki-Alfia and J. Leor, "Resident cardiac progenitor cells," in *Adult and Pluripotent Stem Cells, Chapter 2*, J. Hescheler and E. Hofer, Eds., Springer Science+Business Media, Dordrecht, 2014.

[28] P. Dixit and R. Katare, "Challenges in identifying the best source of stem cells for cardiac regeneration therapy," *Stem Cell Research & Therapy*, vol. 6, p. 26, 2015.

[29] C. F. Leite, T. R. Almeida, C. S. Lopes, and V. J. Dias da Silva, "Multipotent stem cells of the heart—do they have therapeutic promise?," *Frontiers in Physiology*, vol. 6, p. 123, 2015.

[30] M. Herriges and E. E. Morrisey, "Lung development: orchestrating the generation and regeneration of a complex organ," *Development*, vol. 141, no. 3, pp. 502–513, 2014.

[31] E. E. Morrisey and B. L. M. Hogan, "Preparing for the first breath: genetic and cellular mechanisms in lung development," *Developmental Cell*, vol. 18, no. 1, pp. 8–23, 2010.

[32] B. L. M. Hogan, C. E. Barkauskas, H. A. Chapman et al., "Repair and regeneration of the respiratory system: complexity, plasticity, and mechanisms of lung stem cell function," *Cell Stem Cell*, vol. 15, no. 2, pp. 123–138, 2014.

[33] D. N. Kotton and E. E. Morrisey, "Lung regeneration: mechanisms, applications and emerging stem cell populations," *Nature Medicine*, vol. 20, no. 8, pp. 822–832, 2014.

[34] M. J. Evans and C. G. Plopper, "The role of basal cells in adhesion of columnar epithelium to airway basement membrane," *The American Review of Respiratory Disease*, vol. 138, no. 2, pp. 481–483, 1988.

[35] J. R. Rock, M. W. Onaitis, E. L. Rawlins et al., "Basal cells as stem cells of the mouse trachea and human airway epithelium," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 31, pp. 12771–12775, 2009.

[36] J. Y. Liu, P. Nettesheim, and S. H. Randell, "Growth and differentiation of tracheal epithelial progenitor cells," *The American Journal of Physiology*, vol. 266, no. 3, Part 1, pp. L296–L307, 1994.

[37] A. Pardo-Saganta, B. M. Law, P. R. Tata et al., "Injury induces direct lineage segregation of functionally distinct airway basal stem/progenitor cell subpopulations," *Cell Stem Cell*, vol. 16, no. 2, pp. 184–197, 2015.

[38] P. R. Tata and J. Rajagopal, "Plasticity in the lung: making and breaking cell identity," *Development*, vol. 144, no. 5, pp. 755–766, 2017.

[39] C. E. Barkauskas, M. J. Cronce, C. R. Rackley et al., "Type 2 alveolar cells are stem cells in adult lung," *The Journal of Clinical Investigation*, vol. 123, no. 7, pp. 3025–3036, 2013.

[40] C. F. B. Kim, E. L. Jackson, A. E. Woolfenden et al., "Identification of bronchioalveolar stem cells in normal lung and lung cancer," *Cell*, vol. 121, no. 6, pp. 823–835, 2005.

[41] Y. Atlasi and H. G. Stunnenberg, "The interplay of epigenetic marks during stem cell differentiation and development," *Nature Reviews Genetics*, 2017.

[42] P. W. Tetteh, H. F. Farin, and H. Clevers, "Plasticity within stem cell hierarchies in mammalian epithelia," *Trends in Cell Biology*, vol. 25, no. 2, pp. 100–108, 2015.

[43] P. W. Tetteh, O. Basak, H. F. Farin et al., "Replacement of lost Lgr5-positive stem cells through plasticity of their enterocyte-lineage daughters," *Cell Stem Cell*, vol. 18, no. 2, pp. 203–213, 2016.

[44] P. R. Tata, H. Mou, A. Pardo-Saganta et al., "Dedifferentiation of committed epithelial cells into stem cells in vivo," *Nature*, vol. 503, no. 7475, pp. 218–223, 2013.

[45] P. R. Tata and J. Rajagopal, "Cellular plasticity: 1712 to the present day," *Current Opinion in Cell Biology*, vol. 43, pp. 46–54, 2016.

[46] A. R. Pinto, A. Ilinykh, M. J. Ivey et al., "Revisiting cardiac cellular composition," *Circulation Research*, vol. 118, no. 3, pp. 400–409, 2016.

[47] M. B. Furtado, H. T. Nim, S. E. Boyd, and N. A. Rosenthal, "View from the heart: cardiac fibroblasts in development, scarring and regeneration," *Development*, vol. 143, no. 3, pp. 387–397, 2016.

[48] J. L. Spees, M. J. Whitney, D. E. Sullivan et al., "Bone marrow progenitor cells contribute to repair and remodeling of the lung and heart in a rat model of progressive pulmonary hypertension," *The FASEB Journal*, vol. 22, no. 4, pp. 1226–1236, 2008.

[49] C. K. Sun, F. Y. Lee, J. J. Sheu et al., "Early combined treatment with cilostazol and bone marrow-derived endothelial progenitor cells markedly attenuates pulmonary arterial hypertension in rats," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 330, no. 3, pp. 718–726, 2009.

[50] J. T. Mao, J. G. Goldin, J. Dermand et al., "A pilot study of all-trans-retinoic acid for the treatment of human emphysema," *American Journal of Respiratory and Critical Care Medicine*, vol. 165, no. 5, pp. 718–723, 2002.

[51] A. D. Brooks, W. Tong, F. Benedetti, Y. Kaneda, V. Miller, and R. P. Warrell Jr, "Inhaled aerosolization of all-trans-retinoic acid for targeted pulmonary delivery," *Cancer Chemotherapy and Pharmacology*, vol. 46, no. 4, pp. 313–318, 2000.

[52] J. Lenssen and J. Stolk, "Pulmonary stem cells and the induction of tissue regeneration in the treatment of emphysema," *International Journal of Chronic Obstructive Pulmonary Disease*, vol. 2, no. 2, pp. 131–139, 2007.

[53] A. van der Laarse, C. M. Cobbaert, and S. Umar, "Stem and progenitor cell therapy for pulmonary arterial hypertension: effects on the right ventricle (2013 Grover Conference Series)," *Pulmonary Circulation*, vol. 5, no. 1, pp. 73–80, 2015.

[54] D. J. Weiss, J. H. T. Bates, T. Gilbert et al., "Stem cells and cell therapies in lung biology and diseases: conference report," *Annals of the American Thoracic Society*, vol. 10, no. 5, pp. S25–S44, 2013.

[55] H. Mou, K. Brazauskas, and J. Rajagopal, "Personalized medicine for cystic fibrosis: establishing human model systems," *Pediatric Pulmonology*, vol. 50, Supplement 40, pp. S14–S23, 2015.

Hindawi
Stem Cells International
Volume 2017, Article ID 7304121, 10 pages
https://doi.org/10.1155/2017/7304121

Review Article

Mesenchymal Stem Cells in Sepsis and Associated Organ Dysfunction: A Promising Future or Blind Alley?

Jan Horák,[1,2] Lukáš Nalos,[2] Vendula Martínková,[2,3] Jan Beneš,[2] Milan Štengl,[2] and Martin Matějovič[1,2]

[1]*1st Medical Department, Faculty of Medicine in Pilsen, Charles University, Prague, Czech Republic*
[2]*Experimental Intensive Care Unit, Biomedical Centre, Faculty of Medicine in Plzen, Charles University, Alej Svobody 1655/76, Plzen, Czech Republic*
[3]*Third Department of Surgery, University Hospital Motol and First Medical School, Charles University, Prague, Czech Republic*

Correspondence should be addressed to Martin Matějovič; matejovic@fnplzen.cz

Received 21 June 2017; Accepted 6 August 2017; Published 14 September 2017

Academic Editor: Heinrich Sauer

Sepsis, newly defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection, is the most common cause of death in ICUs and one of the principal causes of death worldwide. Although substantial progress has been made in the understanding of fundamental mechanisms of sepsis, translation of these advances into clinically effective therapies has been disappointing. Given the extreme complexity of sepsis pathogenesis, the paradigm "one disease, one drug" is obviously flawed and combinations of multiple targets that involve early immunomodulation and cellular protection are needed. In this context, the immune-reprogramming properties of cell-based therapy using mesenchymal stem cells (MSC) represent an emerging therapeutic strategy in sepsis and associated organ dysfunction. This article provides an update of the current knowledge regarding MSC in preclinical models of sepsis and sepsis-induced acute kidney injury. Recommendations for further translational research in this field are discussed.

1. Introduction

Stem cells may be defined as cells capable of self-renewal and at the same time endowed with the ability to differentiate practically into all types of human cells. There are basically two main groups of stem cells—the first consists of embryonic stem cells, ESC, which are located in the inner cell mass of the emerging blastocyst and which may differentiate into cells of all the three primary germ layers. The second group then consists of adult stem cells, ASC, which are present in all tissues but have limited differentiation potential. Adult stem cells include haematopoietic stem cells (HSC) located in the bone marrow and representing haematopoiesis progenitors and so-called nonhaematopoietic stem cells (NHSC) of which the so-called *mesenchymal stem cells* (MSC) are a subgroup.

MSC were first described in the 1950s by the Russian haematologist A. Friedenstein. He thus followed in the footsteps of his senior colleague A. Maximow whose pioneering work is reflected in haematopoietic stem cell transplantation that today saves thousands of lives worldwide [1]. MSC are a heterogeneous group of multipotent cells, morphologically akin to fibroblasts, that form colonies and are capable of differentiation into mesenchymal tissue (osteocytes, chondrocytes, or adipocytes) [2–4]. It should be pointed out that although the term "mesenchymal stem cells" is commonly used in current literature, it does not reflect the essence of the definition of a stem cell, that is, the ability to differentiate into all cell types. The "alternative" term "mesenchymal stromal cells" is not appropriate either, as it has not been demonstrated thus far that these cells are involved in the formation of tissue stroma. However, for the purpose of this text, we will respect the general designation MSC (mesenchymal stem cells). In this article, we performed an update review on the potential therapeutic efficacy of MSC in preclinical models of sepsis and associated organ dysfunction. Literature was sourced by

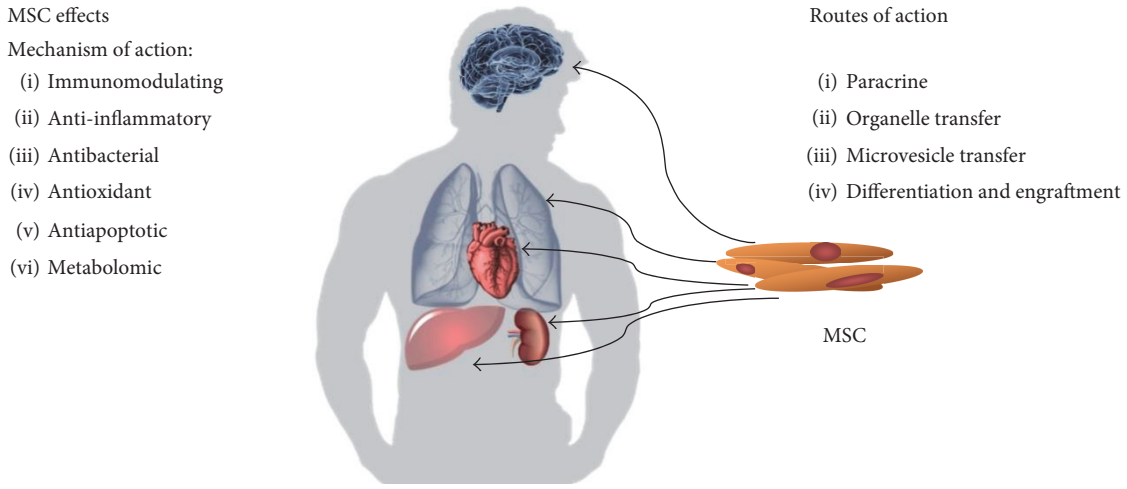


Figure 1: Mechanisms and means of MSC action.

conducting a search of PubMed database using phrases and synonyms for "mesenchymal stem cells," "sepsis," "endotoxemia," "acute kidney injury," "organ dysfunction," and "cardiovascular." The search was limited to articles published from 2012 to March 2017.

2. Properties and Mechanisms of Action

MSC are currently the focus of much attention thanks to a number of their unique properties. Over the previous years, it has been demonstrated using animal models that transplantation of allogeneic or autologous MSC ameliorates symptoms caused by inflammation, ischemia, or physical damage to living tissues [5, 6]. Absence of surface MHC complex II expression enables these cells to avoid mechanisms of immune allorecognition [7] and this property in combination with the ability to suppress autoimmunity and the "graft-versus-host" reaction [8] means that MSC are a suitable basic material for potential cell therapies. Last but not least, the ability of so-called transdifferentiation has been described *in vitro*. This is a process whereby the stem cells of one germ line differentiate into cells of another germ line [2]. The reparative mechanism applied by MSC when renewing damaged tissues has not as yet been satisfactorily clarified. The concept held until recently that this involved MSC migration, engraftment, and differentiation at the site of damage appears obsolete. It has been demonstrated that during tissue repair, MSC do not migrate in a sufficient amount and do not engraft sufficiently long enough to satisfactorily explain tissue reparation via this mechanism [9]. Spees et al. have summarised the possible mechanisms of action used by MSC during the process of tissue repair [9] and these are shown in Figure 1:

(1) Differentiation of MSC into the cells of the damaged tissue

(2) Reparation of damaged cells by their fusion with MSC

(3) Paracrine secretion of signalling molecules that stimulate tissue repair/prevent further damage has immunomodulatory functions

(4) Transport of organelles and/or molecules from the MSC into the damaged cell via tunnelling nanotubes (TNT)

(5) Molecule transfer via exosomes or microvesicles that separate from the MSC.

The means by which MSC modify immune system processes are being studied intensively, as it is the process of immunomodulation and the possibility of influencing the course of inflammatory reactions that has made MSC the focus of great attention in experimental intensive medicine. Figure 2 describes the pathways known to date through which MSC paracrine secretion affects immunocompetent cells.

One of the MSC molecular immunomodulatory mechanisms involves suppression of proliferation and activation of T-lymphocytes with concurrent activation of T-regulatory lymphocyte (Treg) proliferation on the basis of *IDO* (indoleamine 2,3-dioxygenase) and *prostaglandin E2* (PGE2) secretion following MSC stimulation by INF-γ [4, 10]. Macrophage stimulation via PGE2 and IDO leads to increased expression of anti-inflammatory IL-10 [10]; PGE2 and IDO also inhibit differentiation, maturation, and the process of antigen presentation by dendritic cells [10]. In costimulation with the MSC-produced *HLA-G5* (human leukocyte antigen G5) and IL-10, these PGE2 and IDO then inhibit activation and proliferation of NK cells and, on the contrary, potentiate the production of $CD73^+$ NK cells [10–12] that play an important role in antitumour immunity [11]. Secretion of HLA-G5 then affects in a similar manner to IDO and PGE2 the proliferation of T-lymphocytes [12]. Other cytokines produced by MSC include *TGF-β* (transforming growth factor-β) [10, 13]. This protein, produced by all cells of the myeloid haematopoietic lineage in three isoforms

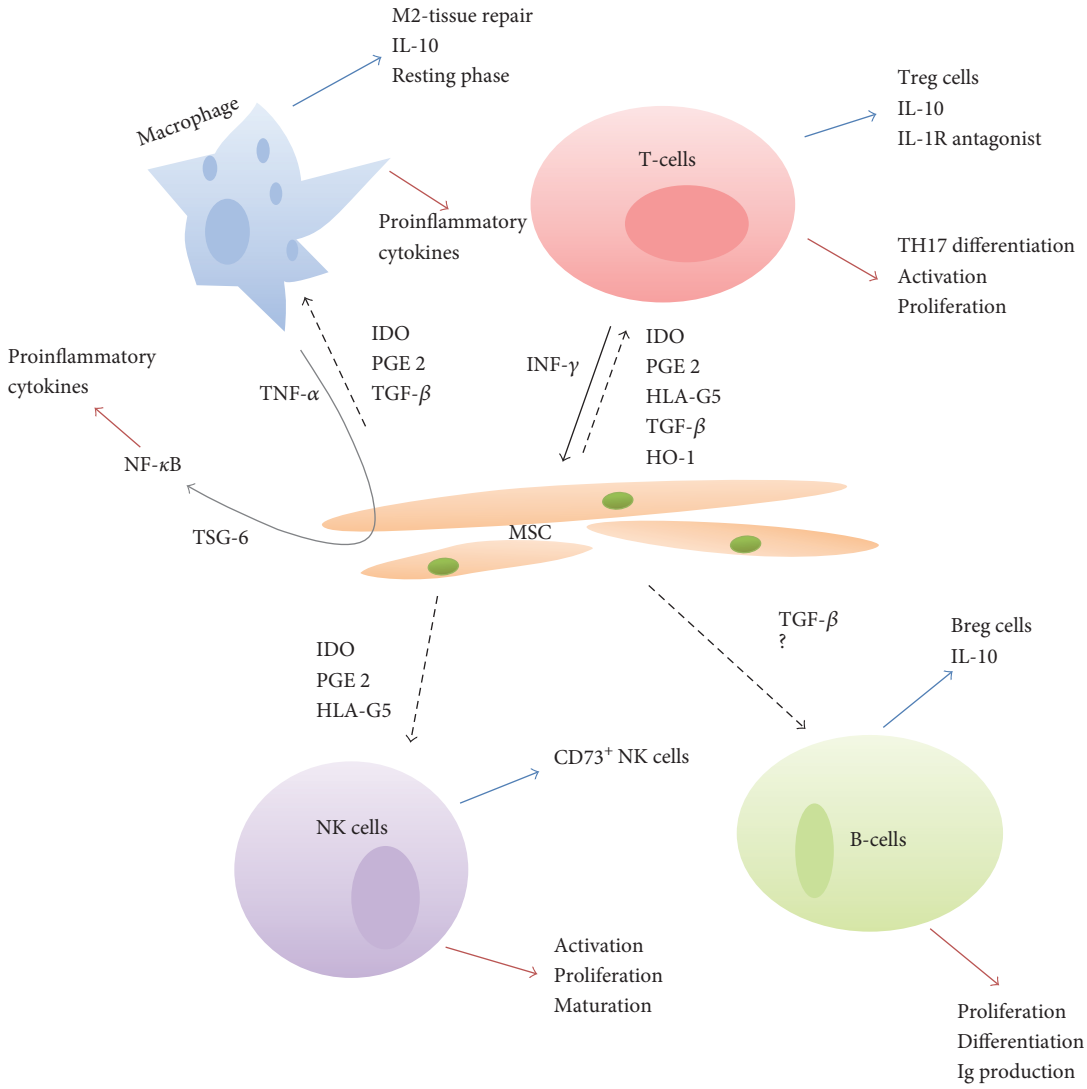


Figure 2: Known mechanisms of MSC immunomodulatory activity in sepsis.

(TGF-β1–3), ranks among polyfunctional cytokines. It stimulates Treg in the sense of IL-10 production, prevents differentiation of T-helper lymphocytes into the TH17 form producing proinflammatory cytokines, inhibits B-lymphocyte proliferation [14], and last but not least inhibits macrophage activity and by inhibiting NF-κB decreases the production of proinflammatory cytokines within macrophages [13, 15]. *HO-1* (heme-oxygenase-1), also produced by MSC [10], is another cytokine with an immunomodulatory function. Expression of HO-1 by MSC is induced by proinflammatory interleukins. The role of HO-1 in the process of immunomodulation involves stimulation of Treg and production of IL-10 [10, 16] and production of the IL1R (IL-1 receptor) antagonist as well as induction of mitochondrial biogenesis [16]. The *TSG6 protein* (TNFα-stimulated gene protein 6) secreted by MSC following their stimulation by TNFα also plays a role in the inhibition of the expression of proinflammatory cytokines (via NF-κB) through negative feedback [10, 17].

The protective effect of MSC on damaged cells and tissues or those exposed to stress may also be mediated by the mechanism of organelle or functional molecule transfer via so-called TNTs (tunnelling nanotubes) [9]. This mechanism was originally described in LPS-induced ARDS in mice [18] and later also in tissues outside the lung parenchyma [19] including tumour tissues [20]. These are protein channels of the gap junction type consisting of F-actin [21] and connexin 43 [18]. Active substances and some organelles, especially mitochondria, are transferred via these channels, which enable MSC to increase ATP production and thus partially or completely restore bioenergetic processes within a damaged cell.

Apart from immunomodulating properties, MSC are also endowed with the ability to directly affect the infectious agent [22]. These antimicrobial effects are mediated on the one hand by secretion of antibacterial peptides (LL-37 or lipocalin-2) and on the other by intensification of phagocytosis following MSC-induced transformation of type 1 macrophages into

Table 1: Preclinical models of sepsis and role of MSC.

Authors/year	Sepsis model	MSC type/ combination	Biological effect	Clinical effect	Source ref.
Asano et al. (2015)	TSS SEA + LPS mouse model	A-MSC (1×10^6)	↓ INF-γ, TNF-α, IL-6, IL-2 = Treg, IL-10	↓ 40 h mortality	[25]
Kim et al. (2014)	TSS SEB mouse model	hMSC, mMSC (2×10^5)	↓ TNF-α, IL-2, IL-6	= mortality No difference between hMSC and mMSC	[26]
Ou et al. (2016)	LPS mouse model	A-MSC, BM-MSC	↓ IL-8 (A-MSC) ↓ proinflammatory cytokines (both types)	↓ mortality	[27]
Pedrazza et al. (2014)	*E. coli*-induced peritonitis	A-MSC (1×10^6)	↓ IL-6, MCP-1 ↓ AST, ALT ↓ splenocyte apoptosis	↓ 26 h mortality	[28]
Chao et al. (2014)	CLP-polymicrobial mouse model	BM-MSC UC-MSC (5×10^6)	↓ IL-6 and TNF-α ↑ $CD3^+CD4^+CD25^+$ Treg	↓ 7- and 14-day mortality	[29]
Alcayaga-Miranda et al. (2015)	CLP-polymicrobial mouse model	Men-MSC, A-MSC, BM-MSC (2×10^6)/ enrofloxacin	*In vitro*: ↑ inhibition of bacterial growth (Men-MSC) No difference—Men-MSC versus A-MSC/BM-MSC in the dynamics of cytokines	Men-MSC + ATB ↓ 96 h mortality	[30]
Wang et al. (2015)	CLP-polymicrobial mouse model	D-MSC (2×10^6)	↓ IL-1, IL-6 ↑ IL-4, IL-5 IL-10 without significant changes *In vitro*: inhibition of macrophage apoptosis, increased migration intensity	↓ 10-day mortality	[31]
Liu et al. (2016)	CLP-polymicrobial mouse model	Unspecified (1×10^6)	↓ NK ↓ TNF-α, IL-6, INF-γ ↑ IL-10	↓ 72 h mortality	[35]
Sepúlveda et al. (2014)	LPS mouse model	BM-MSC (1×10^5)	*In vitro*: no difference between senescent versus immortalised *In vivo*: no reduction of proinflammatory cytokine levels in senescent cells	Immortalised MSC: ↓ 24, 48, 72, 96, 120, and 144 h mortality versus senescent type	[36]
Wu et al. (2016)	CLP-polymicrobial sepsis	UC-MSC	↓ TNF-α, MCP-1, IL1, 6 ↑ IL-10 ↓ mRNA MyD88 ↓ phosphorylation NF-κB	↓ 6 h mortality	[37]

TSS = toxic shock syndrome; SEA, B = staphylococcal enterotoxin A, B; LPS = lipopolysaccharide; CLP = cecal ligation and puncture; A-MSC = adipose tissue-derived MSC; hMSC = human MSC; mMSC = mouse MSC; Men-MSC = menstrual-derived MSC; BM-MSC = bone marrow-derived MSC; D-MSC = dermal MSC; UC-MSC = umbilical cord MSC.

type 2. Devaney et al. [23] demonstrated in a mouse model of *E. coli*-induced pneumonia a lesser intensity of lung damage, lower bacterial load, higher intensity of phagocytosis, and higher levels of LL-37 following the intratracheal administration of MSC.

The antioxidant and antiapoptotic effects of stem cells also play a protective role in the process of organ damage [9, 22]. The products secreted by stem cells help ameliorate oxidative tissue damage (especially of the lungs, liver, and kidneys) [22]. Some recent works using animal models of sepsis-induced organ damage also describe MSC-associated increased secretion of a whole range of growth factors [23, 24].

3. MSC in Sepsis

Table 1 summarises studies dealing with the utilisation of MSC in the treatment of sepsis using preclinical models. The study of the Japanese team [25], which used the intraperitoneal application of adipose tissue-derived mesenchymal stem cells (A-MSC) in the treatment of toxic shock syndrome (TSS; sepsis induced by staphylococcal enterotoxin A) potentiated by the application of a lipopolysaccharide in a mouse model demonstrated a lower 40-hour mortality (A-MSC versus placebo—73% versus 87.5%) and suppression of INF-γ, TNF-α, IL-6, and IL-2 expression measured 18 hours after induction of sepsis. On the other hand, Kim et al. [26] did

not demonstrate a significant positive effect of MSC on mortality in a mouse model of TSS induced by staphylococcal enterotoxin B. However, the authors describe a decrease in the level of proinflammatory cytokines IL-2, IL-6, and TNF-α in the group of mice who received MSC compared to the control group. Ou et al. [27] compared the effect of MSC and placebo on mortality, evolution of biochemical markers, and expression of pro- and anti-inflammatory cytokines during LPS-induced sepsis in a mouse model. The BMSCs and ADMSCs significantly reduced mortality rates and majority of proinflammatory cytokine levels. Their work then compared the effect of the individual conventional types of MSC (adipose-derived MSC (A-MSC) and bone marrow-derived MSC (BM-MSC)) on the course of sepsis with decreased concentration of IL-8 in the group treated with A-MSC compared to the BM-MSC group. Pedrazza et al. [28] studied the effects of A-MSC application in a mouse model of sepsis induced by the administration of *E. coli* into the peritoneal cavity. There was a significant decrease in 26-hour mortality in the group that received MSC compared to the control group. Following MSC application, these animals also presented with lower levels of proinflammatory cytokines (IL-6, MCP-1), significantly lower levels of alanine (ALT) and aspartate (AST) aminotransferase, and significantly lower apoptotic activity in spleen cells. Chao et al. [29] then compared the efficacy of MSC from other sources (BM-MSC and UC-MSC, i.e., umbilical cord-derived MSC). They demonstrated in an animal model of sepsis induced by cecal ligation and puncture (CLP) lower mortality of the animals that receive both types of MSC. The control, untreated group also showed higher levels of proinflammatory cytokines IL-6 and TNF-α as well as significantly lower levels of Treg lymphocytes ($CD3^+CD4^+CD25^+$) than did the animals that received MSC. This study is one of the first to define the pathways of the immunomodulatory cellular effects of MSC in inflammation. Alcayaga-Miranda et al. [30] used MSC obtained from menstrual blood (Men-MSC) separately or in combination with antibiotics in a mouse model of CLP-induced sepsis. Their results showed the superiority of Men-MSC over those derived from bone marrow or adipose tissue (BM-MSC or A-MSC) in the inhibition of *in vitro* bacterial growth. *In vivo*, the combination of ATB (enrofloxacin) and Men-MSC represented the most effective treatment modality (i.e., reduction of 96-hour mortality). Wang et al. used an attractive source of mesenchymal stem cells (dermal stem cells (D-MSC)) in their experiment [31]. Cells acquired from the skin of newborn mice were administered to a group of animals with CLP-induced sepsis. This group of animals demonstrated a milder course of the sepsis with lower ten-day mortality compared to the control group. At the same time, there was a decrease in the level of proinflammatory cytokines (IL-1, IL-6) and an increase in the level of interleukins 4 and 5. We have already mentioned the inhibitory effect of MSC products (especially IDO, HLA-G5, and PGE2) on NK cells. Their proliferative and increased secretory activity, in particular in the initial phases of the process, appears to be a factor negatively affecting the course and mortality of septic states according to studies published so far [32–34]. In a recent study of Liu et al., the authors demonstrated inhibitory effect on proliferation and maturation (identification of $CD3e^+$ forms) of septic NK cells (sNK) following their culture together with MSC *in vitro* [35]. In addition, the authors demonstrated a lower level of circulating sNK *in vivo* in CLP mice 72 hours following MSC application. The 72-hour survival interval in this experiment was longer in septic mice that received MSC (60% versus 25% in CLP mice without MSC administration, versus 90% in sham controls). *In vitro* and *in vivo* determination of cytokine levels concurred with the conclusions of other experiments, with significantly lower levels of TNF-α, IL-6, and INF-γ and higher levels of IL-10 in NK cultures with cocultivation of MSC and in the model that received MSC. An interesting paper was published by Sepúlveda et al. [36] dealing with the immunomodulatory effect of senescent MSC. In their experiment, the authors created 2 types of MSC: a senescent type cultured during concurrent γ radiation and a second type of MSC immortalised by the transduction of hTERT (recombinant telomerase-reverse transcriptase), whereby they attempted to simulate the activity of MSC in an ageing and young organism, respectively. *In vitro*, there was no difference in the inhibitory activity of senescent MSC on the lymphocyte population compared to the immortalised type of MSC. In the *in vivo* model of endotoxemia in mice, however, application of the senescent type of MSC did lead neither to a decrease in 24-, 48-, 72-, 96-, 120-, and 144-hour mortality nor to any effect on the level of proinflammatory cytokines compared to the immortalised and wild-type MSC. The authors considered that senescent MSC were noneffective because of the decline in their migratory potential in reaction to proinflammatory cytokines, whereby the secretory and immunomodulatory function of MSC was not significantly compromised. The possibility of influencing innate immune processes, specifically the signalling pathway of toll-like receptor 4 (TLR-4), was studied by Wu et al. [37]. Activation of TLR-4 (most often by binding to a lipopolysaccharide) leads to an increased expression of proinflammatory cytokines [38]. The results of this experiment replicate the aforementioned works in the sense of lower mortality, reduction of proinflammatory interleukin levels, and increase in anti-inflammatory interleukin levels. In the group of animals that received MSC, lower expression of mRNA coding the second messenger associated with TLR-4 (protein MyD88) was demonstrated in the liver tissue. In parallel, there was also a lower ratio of phosphorylated (activated) NF-κB genes.

In summary, application of MSC in rodent animal models of sepsis is associated with lower mortality, amelioration of the course of sepsis due to inhibition of proactive elements of the immune system, and a change in the pro- and anticytokine ratio both in *vitro* and *in vivo*. No study published in literature so far has demonstrated adverse effects associated with the application of MSC in animal models of sepsis.

4. MSC in Sepsis-Associated Acute Kidney Injury

Sepsis is the most frequent cause of acute kidney injury in intensive care units (sepsis-associated AKI (S-AKI)) [39].

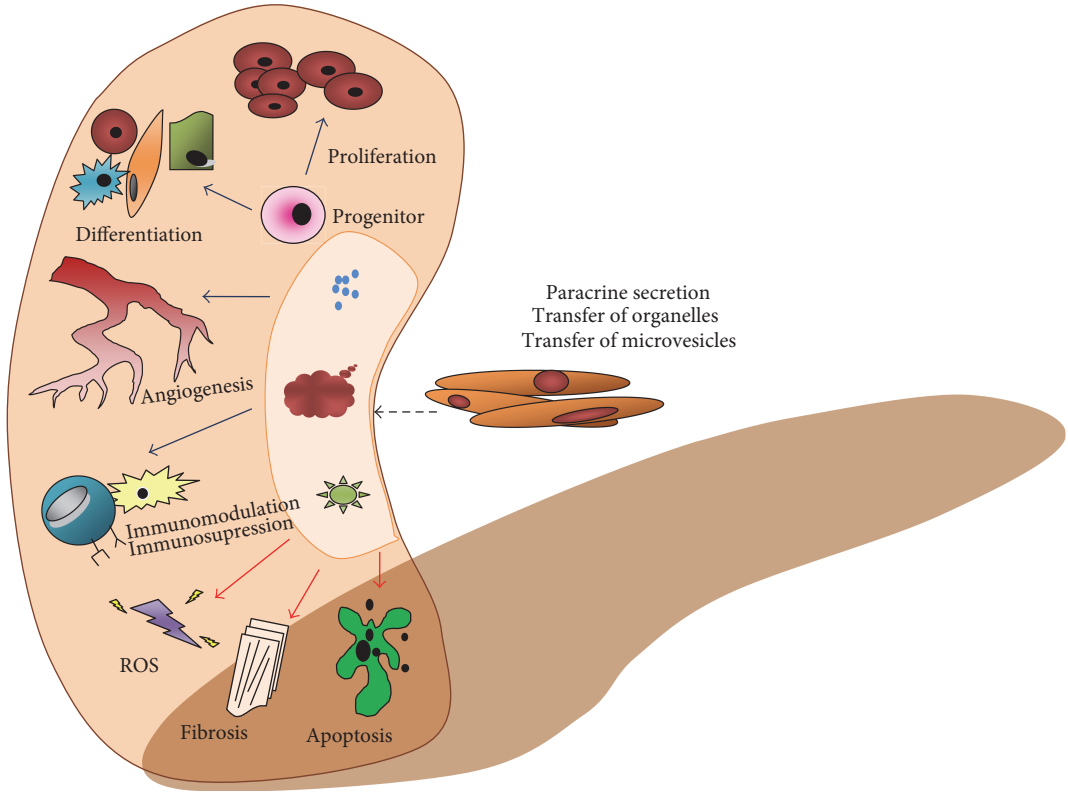


Figure 3: Mechanisms of the protective effect of MSC in the process of acute kidney injury (ROS = reactive oxygen species; blue arrow = process activation; red arrow = process inhibition) (adapted from [57]).

The pathophysiology of this process has not been elucidated satisfactorily. However, the prevailing view today is that an important role in this process is played by inflammation, together with abnormalities of renal microcirculation and changes in cell bioenergetics [40]. The mechanisms underlying the potential therapeutic effect of MSC in S-AKI are summarised in Figure 3.

Table 2 summarises studies dealing with the application of MSC in the treatment of S-AKI using preclinical models. Using a model of CLP-induced sepsis in mice that received MSC at a dose of 10^6 cells 3 hours after sepsis induction, Luo et al. demonstrated a lower incidence of S-AKI [41]. Histological examination of the kidneys showed a significantly lower score of acute tubular damage in animals that received MSC compared to the septic controls. Another issue that this experiment addressed involved the possibility of influencing IL-7 production by MSC paracrine secretion as a potential therapeutic target within the pathophysiology of S-AKI. The research team also demonstrated significantly lower levels of all proinflammatory cytokines including IL-17 and CXC as well as CCL chemokines *in vivo* in the group that received MSC 24 hours after induction. A secondary outcome of this study involved significantly lower 7-day mortality in mice that received MSC.

The effect of MSC derived from Wharton's jelly (WJ-MSC) on the development and course of S-AKI in an animal model was studied by Cóndor et al. [42]. They randomized rats into three groups: sham, CLP-polymicrobial sepsis, and CLP + WJ-MSC (administered 6 hours from sepsis induction). This work reported lower 5-day mortality in animals from the CLP + WJ-MSC group compared to the CLP group (12.5% versus 44.4%). Insulin clearance was lower in the CLP group after 6 and 24 hours from sepsis induction compared to the control group, while the 24-hour glomerular filtration rate did not differ significantly between the WJ-MSC and control groups. Histological examination of the kidneys demonstrated greater infiltration by macrophages and monocytes in the CLP group, as well as a greater intensity of apoptosis compared to the group treated with MSC. Expression of NF-κB and the levels of studied proinflammatory cytokines (IL-1, IL-6, and INF-γ) were also higher in the CLP group compared to the other two. However, the level of TNF-α and of the anti-inflammatory cytokines (IL-4 and IL-10) did not reach a level of significance in both septic groups. In their work, Sung et al. [43] focused on the effect of apoptotic MSC, that is, MSC cultured in a stress environment. In the experiment involving a CLP model of polymicrobial sepsis in mice, they demonstrated a higher efficacy of the cultured (apoptotic) MSC compared to standard cultures in terms of a decrease in TNF-α and serum creatinine levels after 72 hours from sepsis induction. A similar work undertaken by Tsoyi et al. [44], who used MSC preconditioned with carbon monoxide in the treatment of CLP-induced sepsis, showed not only a higher 7-day survival but also a lower incidence of AKI in a mice model. It also appears that combined therapy with ATB (ciprofloxacin) and A-MSC may be more

Table 2: Preclinical models of S-AKI and the effect of MSC.

Team/year	Animal model	Type of MSC/combination	Effect of MSC	Ref.
Luo et al. (2014)	CLP-polymicrobial mouse model	Unspecified MSC (1×10^6)	↓ urea, creatinine ↓ IL-17, CXC, CCL ↓ ATN score	[41]
Cóndor et al. (2016)	CLP-polymicrobial rat	WJ-MSC (1×10^6)	↑ glomerular filtration (inulin clearance) ↓ apoptosis intensity in the renal parenchyma ↓ kidney infiltration by immunocompetent cells	[42]
Sung et al. (2013)	CLP-polymicrobial mouse model	Apoptotic MSC (1.2×10^6)	↓ TNF-α ↓ serum creatinine	[43]
Tsoyi et al. (2016)	CLP-polymicrobial mouse (BALB/C)	MSC ($2.5–5 \times 10^5$) CO preconditioning	↓ incidence of AKI	[44]
Sung et al. (2016)	CLP-polymicrobial mouse model l	A-MSC (5×10^5)/ciprofloxacin (3 mg/kg/5 days)	↓ expression of proinflammatory cytokines in the kidney	[45]
Chen et al. (2014)	CLP-polymicrobial rat	A-MSC (1.2×10^6)/melatonin (20 mg/kg)	↓ levels of proinflammatory cytokines ↓ expression of NF-κB in the kidney	[46]

WJ-MSC = MSC derived from Wharton's jelly; AKI = acute kidney injury; CO = carbon monoxide.

effective than A-MSC administered alone in terms of decreasing mortality and incidence of S-AKI in a CLP model of polymicrobial sepsis [45]. In contrast, combined therapy using MSC and melatonin, which demonstrated its benefit as an antioxidant in experiments using animal models of reperfusion injury in a whole range of organs, did not prove more effective from the aspect of S-AKI incidence compared to the application of A-MSC alone in a CLP model of sepsis. However, this combination did have a positive effect on mortality, the inhibition of proinflammatory cytokines, and NF-κB expression in the kidneys compared to the group treated with A-MSC only [46].

5. MSC and Cardiovascular System in Sepsis

Hyperdynamic circulation and myocardial depression develop in most patients with septic shock [47]. However, only a few studies addressed potential beneficial effects of MSC on cardiovascular system in sepsis. In mice with endotoxemia, the cardiac function was impaired (reduced ejection fraction and fractional shortening) and application of bone marrow MSC prevented these functional changes [48]. Furthermore, MSC also reduced elevated levels of inflammatory mediators (IL-1β, IL-6, TNF-α, and IL-10) in both serum and myocardium. Expression of TLR-4, p65-nuclear factor-κB, and phosphorylated p38 in endotoxemic myocardium was also reduced by MSC treatment. The data suggest that anti-inflammatory actions of MSC were able to reverse the detrimental effects of endotoxemia in the heart.

Similarly, in rats with endotoxemia, the treatment with MSC (both intraperitoneal and intravenous application) ameliorated the myocardial depression and reduced both serum and myocardial levels of inflammatory mediators (TNF-α, IL-1β, and IL-6). On the other hand, in contrast to mice, the serum and myocardial levels of an anti-inflammatory cytokine IL-10, which were increased by endotoxemia, were increased by MSC treatment even further [49].

The functional state, type, and gender of MSC may have significant impact on the potential therapeutic outcome of MSC treatment. Apoptotic adipose-derived MSC were reported to be superior to healthy adipose-derived MSC in treating rat sepsis induced by cecal ligation and puncture in terms of both reducing mortality and preserving organ function [50]. When effects of female and male MSC on myocardial function in rat endotoxemia were compared, female MSC treatment resulted in greater preservation of myocardial function [51]. The superior preservation of myocardial function with female MSC treatment was probably not related to anti-inflammatory effects of MSC since both serum and myocardial levels of cytokines were comparable between rats given MSC from male or female donors and also myocardial levels of phosphorylated p38 MAPK were similarly reduced by both male and female MSC. Endotoxemia was associated, however, with a decreased ratio of antiapoptotic and proapoptotic proteins suggesting a shift to increased myocardial apoptosis. Since this ratio was found to be significantly more increased in female MSC treatment than in male MSC treatment, the superior antiapoptotic effects of female MSC were suggested to contribute to better preservation of cardiac function with female MSC [51].

The mechanisms of beneficial cardiovascular effects of MSC will obviously require further attention and clarification. It has been documented that MSC, when infused systemically in septic animal models, home mainly to the lung and the liver but not the heart [52]. Therefore, the beneficial cardiac effects of MSC in sepsis are probably due to their systemic effects rather than local actions. Beside general anti-inflammatory and antiapoptotic effects, an interesting mechanism with potential therapeutic implications was reported recently: exosomal transfer of miR-223 [53]. In this study, MSC were shown to secrete miR-223-enriched exosomes, which were taken up by macrophages and cardiomyocytes. Consequently, the miR-223 targets were downregulated, leading to the inhibition of inflammatory response in macrophages and attenuation of cardiomyocyte death.

It should be emphasized that all the beneficial cardiovascular effects of MSC in sepsis were only described in small animal (mice, rats) models with limited clinical relevance so far. A thorough investigation of MSC effects in clinically relevant large animal models will be necessary before translation to clinical level.

6. Discussion

The immunomodulatory, anti-inflammatory, antiapoptotic, metabolomic, and antimicrobial effects of MSC undoubtedly form a legitimate biological basis for the scientific verification of their benefits and impact when used as adjuvant treatment not only in sepsis but also in a number of other critical conditions. Although this article is not an exhaustive systemic analysis or meta-analysis, it illustrates the comparable positive effects of MSC used in a relatively wide range of preclinical models of sepsis. These predominantly involve a positive effect on the mortality of septic animals and on the amelioration of AKI severity, as one of the most frequent end-organ dysfunction in sepsis. Does this mean that there is sufficient scientific basis for translating this research evidence into clinical practice, that is, for launching clinical trials? Certainly not!

The excitement sparked off by the potential therapeutic applications of MSC in medicine is understandable. However, there are a number of important reasons supporting a more reserved position on this issue. If we are to abide by the principles of scientific evidence, we must first and foremost unequivocally demonstrate not only efficacy but also safety. The clinical application of MSC that we are currently witnessing in the field of orthopaedics or neurology and which is based on minimal evidence of benefit and safety represents a path that critical care medicine should avoid. Its history has repeatedly shown that, thus far, no new therapeutic approach that was successfully tested in preclinical models was found to be effective in clinical testing (or on the contrary was shown to actually have a negative effect) [54]. There are specific reasons why generally homogenous and encouraging results attained by current preclinical testing cannot be considered as sufficient arguments for launching clinical trials. Firstly, there is a high risk that the effect of MSC is overstated given that a number of studies with negative results have not been published. For example, it has been documented that in the field involving research of stroke, 1 out of 6 studies was not published [55]. Secondly, all studies published thus far have exclusively involved rodents, mainly mice. The marked difference in the immune-inflammatory response to insults between rodents and humans is well documented [56]. Moreover, all preclinical studies discussed above have been carried out on inbred, young, healthy animals with a uniform genetic makeup and thus expressing none of the comorbidities. These models, however, might not reliably mirror the typical septic patient. Both aging and comorbidities not only increase the susceptibility to sepsis and sepsis-driven multiorgan dysfunction but may also influence the immune-inflammatory phenotype and, thus, the efficacy of MSC. Ideally, preclinical studies should use animal population of advanced age and with various comorbidities, such as diabetes mellitus, hypertension, atherosclerosis, and chronic kidney disease. Thirdly, in a number of experiments, the model of sepsis does not correspond to current requirements for clinically relevant biomodels (e.g., induction by endotoxins, rapidly lethal models, and absence of standard supportive treatment of sepsis, i.e., fluid resuscitation, vasopressors, antibiotics, and artificial lung ventilation). Long-term (days) realistic models with true focus on infection allowing the animals to develop full spectrum of typical hemodynamic, metabolic, immune-inflammatory, and tissue morphological responses rather than short-term (hours), rapidly lethal models should be used in examining both safety and efficacy of MSC in sepsis and multiorgan dysfunction. Fourthly, the source, dose, and timing of MSC are highly heterogeneous and remain open for discussion. Again, in many experiments, MSC were administered either concurrently or shortly after sepsis induction, a fact that significantly limits the translational potential of these results. Fifthly, and the last, the long-term consequences of treatment involving MSC are not known (e.g., the risk of developing malignancies, autoimmune states). Taken together, all the abovementioned facts should be appreciated and precisely elucidated before the results of any experimental work obtained from a single species/model are applied to other animals or even humans.

7. Conclusion

In summary, we may conclude that the encouraging results of experiments with MSC in sepsis represent sufficient background for further scientific analysis in the form of properly randomised trials using clinically relevant animal models. Only such models may confirm both the internal (methodological quality, bias risk) and external (i.e., generalisation) validity of experiments conducted to date. The decision to move from experiments to clinical studies should always be preceded by robust preclinical evaluation extending from small animal models to highly complex large models, ideally in the form of multicentre projects in several world-renowned experimental laboratories. One such monocentric project is currently under way at the authors' institution (project AZV, 15-32801A) and results maybe expected next year. It is thus even more surprising that two clinical studies of MSC in sepsis have already been registered (NCT02883803, Effects of Administration of Mesenchymal Stem Cells on Organ Failure During the Septic Shock (CSM choc); NCT02421484 Cellular Immunotherapy for Septic Shock: A Phase I Trial).

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This work was supported by the program for the Development of Scientific Fields of Charles University (Progres Q39) and an AZV grant (Project 15-32801A).

References

[1] B. V. Afanasyev, E. E. Elstner, and A. R. Zander, "A. J. Friedenstein, founder of the mesenchymal stem cell concept," *Cellular Therapy and Transplantation*, vol. 3, pp. 35-36, 2009.

[2] A. Uccelli, L. Moretta, and V. Pistoia, "Mesenchymal stem cells in health and disease," *Nature Reviews Immunology*, vol. 8, no. 9, pp. 726–736, 2008.

[3] S. K. Cribbs, M. A. Matthay, and G. S. Martin, "Stem cells in sepsis and acute lung injury," *Critical Care Medicine*, vol. 38, no. 12, pp. 2379–2385, 2010.

[4] J. A. Zimmermann, M. H. Hettiaratchi, and T. C. McDevitt, "Enhanced immunosuppression of T cells by sustained presentation of bioactive interferon-gamma within three-dimensional mesenchymal stem cell constructs," *Stem Cells Translational Medicine*, vol. 6, no. 1, pp. 223–227, 2016.

[5] J. J. Minguell, P. Conget, and A. Erices, "Biology and clinical utilization of mesenchymal progenitor cells," *Brazilian Journal of Medical and Biological*, vol. 33, no. 8, pp. 881–887, 2009.

[6] K. N. Yarygin, A. Y. Lupatov, and G. T. Sukhikh, "Modulation of immune responses by mesenchymal stromal cells," *Bulletin of Experimental Biology and Medicine*, vol. 161, no. 4, pp. 561–565, 2016.

[7] K. L. Blanc, C. Tammik, K. Rosendahl, E. Zetterberg, and O. Ringden, "HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells," *Experimental Hematology*, vol. 31, no. 10, pp. 890–896, 2003.

[8] J. D. Glenn and K. A. Whartenby, "Mesenchymal stem cells: emerging mechanisms of immunomodulation and therapy," *World Journal of Stem Cells*, vol. 6, no. 5, pp. 526–539, 2014.

[9] J. L. Spees, R. H. Lee, and C. A. Gregory, "Mechanisms of mesenchymal stem/stromal cell function," *Stem Cell Research Therapy*, vol. 7, no. 1, p. 125, 2016.

[10] M. Najar, G. Raicevic, H. Fayyad-Kazan, D. Bron, M. Toungouz, and L. Lagneaux, "Mesenchymal stromal cells and immunomodulation: a gathering of regulatory immune cells," *Cytotherapy*, vol. 18, no. 2, pp. 160–171, 2016.

[11] P. A. Beavis, J. Stagg, P. K. Darcy, and M. J. Smyth, "CD73: a potent suppressor of antitumor immune responses," *Trends in Immunology*, vol. 33, no. 5, pp. 231–237, 2012.

[12] Z. Selmani, A. Naji, I. Zidi et al., "Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells," *Stem Cells*, vol. 26, no. 1, pp. 212–222, 2008.

[13] N. Morishima, I. Mizoguchi, K. Takeda, J. Mizuguchi, and T. Yoshimoto, "TGF-β is necessary for induction of IL-23R and Th17 differentiation by IL-6 and IL-23," *Biochemical and Biophysical Research Communications*, vol. 386, no. 1, pp. 105–110, 2009.

[14] M. Arsura, M. Wu, and G. E. Sonenshein, "TGF-β inhibits NF-κB/Rel activity inducing apoptosis of B cells: transcriptional activation," *Immunity*, vol. 5, no. 1, pp. 31–40, 2016.

[15] L. E. Smythies, M. Sellers, R. H. Clements et al., "Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity," *Journal of Clinical Investigation*, vol. 115, no. 1, pp. 66–75, 2005.

[16] C. A. Piantadosi, C. M. Withers, R. R. Bartz et al., "Heme oxygenase-1 couples activation of mitochondrial biogenesis to anti-inflammatory cytokine expression," *Journal of Biological Chemistry*, vol. 286, no. 18, pp. 16374–16385, 2011.

[17] S. Ma, N. Xie, W. Li, B. Yuan, Y. Shi, and Y. Wang, "Immunobiology of mesenchymal stem cells," *Cell Death Differentiation*, vol. 21, no. 2, pp. 216–225, 2014.

[18] M. N. Islam, S. R. Das, M. T. Emin et al., "Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury," *Nature Medicine*, vol. 18, no. 5, pp. 759–765, 2012.

[19] K. Liu, K. Ji, L. Guo et al., "Mesenchymal stem cells rescue injured endothelial cells in an in vitro ischemia–reperfusion model via tunneling nanotube like structure-mediated mitochondrial transfer," *Microvascular Research*, vol. 92, pp. 10–18, 2014.

[20] I. A. Barbosa, N. G. Machado, A. J. Skildum, P. M. Scott, and P. J. Oliveira, "Mitochondrial remodeling in cancer metabolism and survival: potential for new therapies," *Biochimica et Biophysica Acta*, vol. 1826, no. 1, pp. 238–254, 2011.

[21] A. Rustom, R. Saffrich, I. Markovic, P. Walther, and H.-H. Gerdes, "Nanotubular highways for intercellular organelle transport," *Science*, vol. 303, no. 5660, pp. 1007–1010, 2004.

[22] M. A. Matthay, S. Pati, and J.-W. Lee, "Concise review: mesenchymal stem, no. stromal cells: biology and preclinical evidence for therapeutic potential for organ dysfunction following trauma or sepsis," *Stem Cells*, vol. 35, no. 2, pp. 316–324, 2017.

[23] J. Devaney, S. Horie, C. Masterson et al., "Human mesenchymal stromal cells decrease the severity of acute lung injury induced by *E. coli* in the rat," *Thorax*, vol. 70, no. 7, pp. 625–635, 2015.

[24] B. Imberti, M. Morigi, S. Tomasoni et al., "Insulin-like growth factor-1 sustains stem cell mediated renal repair," *Journal of the American Society of Nephrology*, vol. 18, no. 11, pp. 2921–2928, 2007.

[25] K. Asano, S. Yoshimura, and A. Nakane, "Adipose tissue-derived mesenchymal stem cells attenuate staphylococcal enterotoxin A-induced toxic shock," *Infection and Immunity*, vol. 83, no. 9, pp. 3490–3496, 2015.

[26] H. Kim, I. Darwish, M.-F. Monroy, D. J. Prockop, W. C. Liles, and K. C. Kain, "Mesenchymal stromal, no. stem cells suppress pro-inflammatory cytokine production but fail to improve survival in experimental staphylococcal toxic shock syndrome," *BMC Immunology*, vol. 15, p. 1, 2014.

[27] H. Ou, S. Zhao, Y. Peng et al., "Comparison of bone marrow tissue- and adipose tissue-derived mesenchymal stem cells in the treatment of sepsis in a murine model of lipopolysaccharide-induced sepsis," *Molecular Medicine Reports*, vol. 14, no. 4, pp. 3862–3870, 2016.

[28] L. Pedrazza, A. Lunardelli, C. Luft et al., "Mesenchymal stem cells decrease splenocytes apoptosis in a sepsis experimental model," *Inflammation Research : Official Journal of the European Histamine Research Society ... [et al.]*, vol. 63, no. 9, pp. 719–728, 2014.

[29] Y.-H. Chao, H.-P. Wu, K.-H. Wu et al., "An increase in CD3 +CD4+CD25+ regulatory T cells after administration of umbilical cord-derived mesenchymal stem cells during sepsis," *PLoS One*, vol. 9, no. 10, article e110338, 2014.

[30] F. Alcayaga-Miranda, J. Cuenca, A. Martin, L. Contreras, F. E. Figueroa, and M. Khoury, "Combination therapy of menstrual

derived mesenchymal stem cells and antibiotics ameliorates survival in sepsis," *Stem Cell Research & Therapy*, vol. 6, p. 199, 2015.

[31] Y. Wang, L. Tan, J. Jin et al., "Non-cultured dermal-derived mesenchymal cells attenuate sepsis induced by cecal ligation and puncture in mice," *Scientific Reports*, vol. 5, article 16973, 2015.

[32] D. Andaluz-Ojeda, V. Iglesias, F. Bobillo et al., "Early levels in blood of immunoglobulin M and natural killer cells predict outcome in nonseptic critically ill patients," *Journal of Critical Care*, vol. 28, no. 6, pp. 1110.e7–1110.e10, 2013.

[33] D. Andaluz-Ojeda, V. Iglesias, F. Bobillo et al., "Early natural killer cell counts in blood predict mortality in severe sepsis," *Critical Care*, vol. 15, no. 5, p. 243, 2016.

[34] G. Giannikopoulos, A. Antonopoulou, G. Kalpakou et al., "The functional role of natural killer cells early in clinical sepsis," *Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, vol. 121, no. 4, pp. 329–336, 2013.

[35] W. Liu, Y. Gao, H. Li et al., "Intravenous transplantation of mesenchymal stromal cells has therapeutic effects in a sepsis mouse model through inhibition of septic natural killer cells," *The International Journal of Biochemistry & Cell Biology*, vol. 79, pp. 93–103, 2016.

[36] J. C. Sepúlveda, M. Tomé, M. E. Fernández et al., "Cell senescence abrogates the therapeutic potential of human mesenchymal stem cells in the lethal endotoxemia model," *Stem Cells*, vol. 32, no. 7, pp. 1865–1877, 2016.

[37] K.-H. Wu, H.-P. Wu, W.-R. Chao et al., "Time-series expression of toll-like receptor 4 signaling in septic mice treated with mesenchymal stem cells," *Shock*, vol. 45, no. 6, pp. 634–640, 2016.

[38] C. Vaure and Y. Liu, "A comparative review of toll-like receptor 4 expression and functionality in different animal species," *Frontiers in Immunology*, vol. 5, p. 316, 2014.

[39] M. Matejovic, J. Chvojka, J. Radej et al., "Sepsis and acute kidney injury are bidirectional," *Contributions to Nephrology*, vol. 174, pp. 78–88, 2011.

[40] H. Gomez and J. A. Kellum, "Sepsis-induced acute kidney injury," *Current Opinion in Critical Care*, vol. 22, no. 6, pp. 546–553, 2016.

[41] C. J. Luo, F. J. Zhang, L. Zhang et al., "Mesenchymal stem cells ameliorate sepsis-associated acute kidney injury in mice," *Shock*, vol. 41, no. 2, pp. 123–129, 2014.

[42] J. M. Condor, C. E. Rodrigues, R. Sousa Moreira et al., "Treatment with human Wharton's jelly-derived mesenchymal stem cells attenuates sepsis-induced kidney injury, liver injury, and endothelial dysfunction," *Stem Cells Translational Medicine*, vol. 5, no. 8, pp. 1048–1057, 2016.

[43] P.-H. Sung, C.-L. Chang, T.-H. Tsai et al., "Apoptotic adipose-derived mesenchymal stem cell therapy protects against lung and kidney injury in sepsis syndrome caused by cecal ligation puncture in rats," *Stem Cell Research & Therapy*, vol. 4, no. 6, p. 155, 2013.

[44] K. Tsoyi, S. R. R. Hall, J. Dalli et al., "Carbon monoxide improves efficacy of mesenchymal stromal cells during sepsis by production of specialized proresolving lipid mediators," *Critical Care Medicine*, vol. 44, no. 12, pp. e1236–e1245, 2016.

[45] P. H. Sung, H. J. Chiang, C. H. Chen et al., "Combined therapy with adipose-derived mesenchymal stem cells and ciprofloxacin against acute urogenital organ damage in rat sepsis syndrome induced by intrapelvic injection of cecal bacteria," *Stem Cells Translational Medicine*, vol. 5, no. 6, pp. 782–792, 2016.

[46] H. H. Chen, K. C. Lin, C. G. Wallace et al., "Additional benefit of combined therapy with melatonin and apoptotic adipose-derived mesenchymal stem cell against sepsis-induced kidney injury," *Journal of Pineal Research*, vol. 57, no. 1, pp. 16–32, 2014.

[47] S. Krishnagopalan, A. Kumar, J. E. Parrillo, and A. Kumar, "Myocardial dysfunction in the patient with sepsis," *Current Opinions in Critical Care*, vol. 8, pp. 376–388, 2002.

[48] Y. Wu, J. Zhou, L. Bi et al., "Effects of bone marrow mesenchymal stem cells on the cardiac function and immune system of mice with endotoxemia," *Molecular Medicine Reports*, vol. 13, pp. 5317–5325, 2016.

[49] B. R. Weil, M. C. Manukyan, J. L. Herrmann et al., "Mesenchymal stem cells attenuate myocardial functional depression and reduce systemic and myocardial inflammation during endotoxemia," *Surgery*, vol. 148, pp. 444–452, 2010.

[50] C. L. Chang, S. Leu, H. C. Sung et al., "Impact of apoptotic adipose-derived mesenchymal stem cells on attenuating organ damage and reducing mortality in rat sepsis syndrome induced by cecal puncture and ligation," *Journal of Translational Medicine*, vol. 10, p. 244, 2010.

[51] M. C. Manukyan, B. R. Weil, Y. Wang et al., "Female stem cells are superior to males in preserving myocardial function following endotoxemia," *American Journal of Physiology*, vol. 300, pp. 1506–1514, 2011.

[52] B. R. Weil, J. L. Herrmann, A. M. Abarbanell, M. C. Manukyan, J. A. Poynter, and D. R. Meldrum, "Intravenous infusion of mesenchymal stem cells is associated with improved myocardial function during endotoxemia," *Shock*, vol. 36, pp. 235–241, 2011.

[53] X. Wang, H. Gu, D. Qin et al., "Exosomal miR-223 contributes to mesenchymal stem cell-elicited cardioprotection in polymicrobial sepsis," *Science Reports*, vol. 5, article 13721, 2015.

[54] J. Cohen, J. L. Vincent, N. K. Adhikari et al., "Sepsis: a roadmap for future research," *The Lancet, Infectious Diseases*, vol. 15, no. 5, pp. 581–614, 2015.

[55] E. S. Sena, B. H. v. d. Worp, P. M. W. Bath, D. W. Howells, and M. R. Macleod, "Publication bias in reports of animal stroke studies leads to major overstatement of efficacy," *PLoS Biology*, vol. 8, no. 3, article e1000344, 2014.

[56] J. Seok, H. S. Warren, A. G. Cuenca et al., "Genomic responses in mouse models poorly mimic human inflammatory diseases," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 9, pp. 3507–3512, 2013.

[57] J. Peired, A. Sisti, and P. Romagnani, "Mesenchymal stem cell-based therapy for kidney disease: a review of clinical evidence," *Stem Cells International*, vol. 2016, Article ID 4798639, 22 pages, 2016.