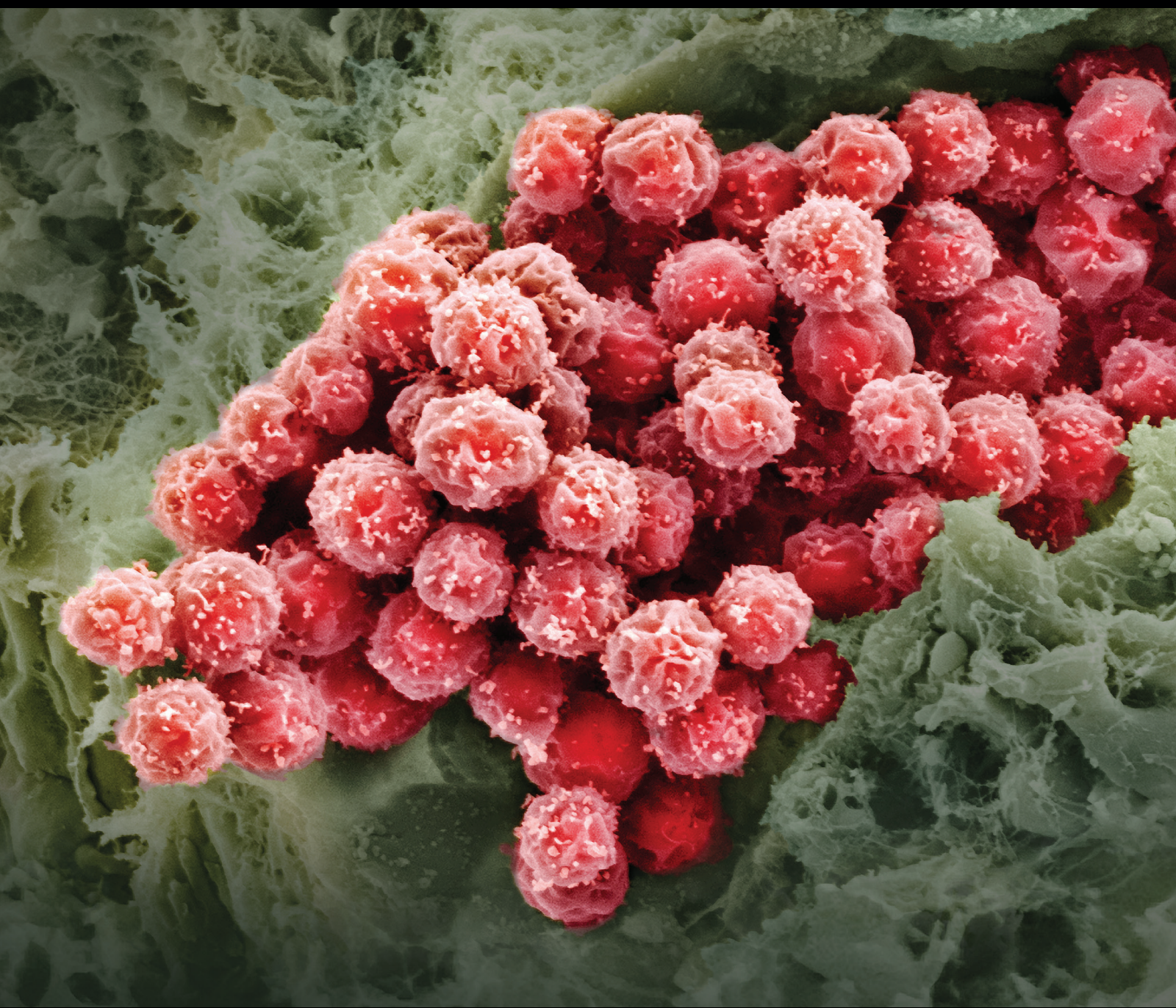
Stem Cells International

Cardiovascular Regeneration: Biology and Therapy

Guest Editors: Changwon Park, Kiwon Ban, Rebecca D. Levit, Wenbin Liang, Hun-Jun Park, and Mary Wagner



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Editorial Board

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Editorial

Cardiovascular Regeneration: Biology and Therapy

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Despite a multitude of advancements and efforts, morbidity and mortality rates of patients with ischemic cardiovascular diseases (CVDs) remain high. Recent progress in the fields of cardiovascular biology and stem cell and tissue engineering not only provides a detailed insight into the cardiovascular pathology but also opens opportunities to develop novel therapeutic approaches. Accordingly, this special issue provides an in-depth and up-to-date insight into cardiac regeneration covering key signaling pathways, stem cell-based or cell-free therapeutic strategies and generation of human cardiac myocytes for cell therapy.

Fueled by a rapid development of experimental techniques, stem cells such as embryonic stem cells, induced pluripotent stem cells, and adult stem cells have gained an extensive attention. In the article "Therapeutic Potential of Stem Cells Strategy for Cardiovascular Diseases" by C. Y. Lee et al., the authors thoroughly documented the efficacy and safety of stem cells and stem cell-derived extracellular vesicles for the treatment of CVDs. Also, they briefly touched limitations of these current approaches.

The review article by T. Simard et al. specifically discussed the latest information of endothelial progenitor cells (EPCs), which have been considered as one of the most promising cell sources for arterial repair. The authors described the details of the current definitions of EPCs, their sources, and the suggested underlying mechanisms of EPC-mediated vascular repair. Finally, they discussed the possibility of the use of EPCs as therapeutic options, focusing on endogenous augmentation and transplantation. Improved understanding of the fundamental biology of EPCs, which are well described in this article, will advance the use of EPCs as a valuable therapeutic option for vascular repair.

One of the most challenges of cell therapy for CVDs is to obtain cardiomyocytes, which can function to improve or cure the diseases without unexpected detrimental effects upon transplantation. In the article "De Novo Human Cardiac Myocytes for Medical Research: Promises and Challenges" by V. Hamel et al., the authors comprehensively reviewed the latest advances in the generation of human cardiac myocytes from pluripotent stem cells or by direct reprogramming. Due to the species difference, animal study data cannot be directly translated to human. Thus, human cardiac myocytes are of utmost importance for scientific research. This article also discusses the difficulties of using de novo human cardiac myocytes, such as their immature phenotype and heterogeneity. Moreover, advancements in addressing these challenges are described in this article.

Further, L. Wang and colleagues raised a fundamental question regarding a significantly impaired capability of cardiac regeneration in mammalian and discussed important

players that can contribute to therapeutic cardiac repair in the article entitled "Repair Injured Heart by Regulating Cardiac Regenerative Signals." The article covered functional significance of key extracellular, intracellular signals and cardiac transcription factors.

The authors of the articles published in this special issue have discussed the current status of cardiovascular diseases and related research with particular emphasis on therapeutic strategies for cardiac regeneration. Combined and continued endeavor between basic, translational, and clinical research will generate an innovative and efficient means of treating or curing the devastating disease.

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Review Article

De Novo Human Cardiac Myocytes for Medical Research: Promises and Challenges

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The advent of cellular reprogramming technology has revolutionized biomedical research. *De novo* human cardiac myocytes can now be obtained from direct reprogramming of somatic cells (such as fibroblasts), from induced pluripotent stem cells (iPSCs, which are reprogrammed from somatic cells), and from human embryonic stem cells (hESCs). Such *de novo* human cardiac myocytes hold great promise for *in vitro* disease modeling and drug screening and *in vivo* cell therapy of heart disease. Here, we review the technique advancements for generating *de novo* human cardiac myocytes. We also discuss several challenges for the use of such cells in research and regenerative medicine, such as the immature phenotype and heterogeneity of *de novo* cardiac myocytes obtained with existing protocols. We focus on the recent advancements in addressing such challenges.

1. The Need for Human Cardiac Myocytes in Biomedical Research

Despite extensive research, heart disease remains the number 1 killer worldwide leading to >17.3 million deaths in 2013, accounting for 31% of all cause deaths [1]. The economic burden for heart disease is estimated to be US$863 billion in 2010 and expected to increase to US$1,044 billion in 2030 [2]. On the other hand, market withdrawal of new drugs due to unexpected cardiotoxicity significantly contributes to the rising costs for drug developments [3]. One of the major reasons is that direct studies in human patients or healthy volunteers are limited by ethical concerns, and it is difficult to obtain and maintain primary human cardiac myocytes from patients for *in vitro* experiments. Accordingly, most heart research has been performed with experimental animal models and cultured animal cells. These animal studies are useful, but their values to understand human heart disease are compromised by profound species difference, such as in the composition of ion channels within the myocardial tissue [4], as well as differences in the size and beating rate of the hearts (500 beats/min in mouse versus 70 beats/min in human). Therefore, human cardiac myocytes that can be readily obtained without ethical limitations are needed to boost the fidelity of biomedical research.

2. Methods for Generating *De Novo* Human Cardiac Myocytes

Last decades have seen exciting advancements in biotechnology that make it possible to generate *de novo* human cardiac myocytes from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), or somatic cells via direct cellular reprogramming. Here, we provide a brief review on this topic. For more details, the readers are referred to recent excellent reviews [5, 6].

2.1. Cardiac Differentiation of ESCs and iPSCs. The first human ESC line was established from surplus human embryos by Dr. Thomson's group in 1998 [7], which is 17 years after the successful isolation of mouse ESC line from the inner cell mass of early mouse embryos in 1981 [8], because of the different characteristics and growth requirement between human and mouse ESC cells. Derived from early embryos, ESCs exhibit two important properties: (1) they can divide and self-renew indefinitely when kept in proper conditions; (2) they are pluripotent and thus have the potential to differentiate into all cell types of the three germ layers, including cardiac myocytes [9, 10]. The iPSC technique was invented in 2006 by Takahashi and Yamanaka [11] who discovered that ESC-like, pluripotent stem cells can be derived from differentiated fibroblasts by viral expression of four transcription factors—Oct4, Sox2, Klf4, and cMyc—that are important for maintenance of ESC pluripotency. Like ESCs, human iPSCs can give rise to all cell types found in the human body, including cardiac myocytes [12, 13]. Unlike ESCs, iPSCs are generated without compromising human embryos, thus minimizing the ethical concerns. In addition, iPSCs can be derived from individual patients, making it possible to generate patient-specific iPSCs (and iPSC-derived cardiac myocytes) for personalized medicine research.

Most protocols for cardiac differentiation were established for ESCs and have been found to be largely applicable for iPSCs as well [13–15]. The traditional embryoid body (EB) method employs serum-driven spontaneous differentiation of ESCs and iPSCs into different germ layer cells, in which cardiac myocytes can be found [16]. This protocol does not require expensive recombinant protein factors but has a low efficiency for generating cardiac myocytes (<5%). The second generation of cardiac differentiation protocols employ recombinant protein factors for stage-specific activation/inhibition of signaling pathways, to recapitulate the temporal pathway activities in embryonic heart development. Specifically, Activin A and BMP4 (bone morphogenetic protein 4) are included in cell culture at the early stage to induce mesoderm, which is followed by addition of Dkk1 (a Wnt inhibitor) and VEGF (vascular endothelin growth factor) to promote cardiac lineage specification [17–19]. These directed cardiac differentiation protocols are highly efficient and capable of generating >70% cardiomyocytes (based on $cTnT^+$ cells) but require expensive recombinant proteins, show significant batch-to-batch variation, and are labor-intensive especially when the EB formation is involved in the early stage [18]. The third generation of cardiac differentiation protocols only require the temporal modulation of the Wnt signaling pathway: activation at the early stage (e.g., with CHIR-99021) and inhibition (e.g., with Wnt-C59) at the late stage [20–22]. These protocols are based on monolayer cell culture, do not require expensive protein factors, and generate cardiomyocytes at a high efficiency with small molecules alone.

2.2. Direct Cellular Reprogramming. In 2010, Dr. Srivastava's group discovered that *de novo* cardiomyocytes can be generated by direct reprogramming of cultured mouse fibroblasts with 3 transcription factors (Gata4, Mef2c, and Tbx5) without the iPSC step [23]. Subsequent studies demonstrated direct cardiac reprogramming in animal hearts and such *in vivo* reprogramming has a higher efficiency than in cultured cells [24, 25]. Wang et al. investigated the effect of factor stoichiometry (Gata4, Mef2c, and Tbx5) on cardiac reprogramming and demonstrated that a higher level of Mef2c in combination with lower levels of Gata4 and Tbx5 increases reprogramming efficiency and promotes myocyte maturation [26]. A recent study by Zhou et al. identified Bmi1 as a key epigenetic barrier to cardiac reprogramming; inhibition of Bmi1 increases reprogramming efficiency and enhances the functional maturation of induced cardiomyocytes; Bmi1 inhibition can also substitute for Gata4 during the cardiac reprogramming [27]. Reprogramming of human fibroblasts into cardiomyocytes has been demonstrated, but additional factors (ESRRG and MESP1) are required [28]. In addition, different combinations of transcription factors and microRNAs have also been reported for direct reprogramming of somatic cells into cardiomyocytes with improved efficiency [29–32]. For example, Song et al. showed that a combination of four transcription factors (Hand2, Gata4, Mef2c, and Tbx5) reprograms mouse tail-tip and cardiac fibroblasts into functional cardiomyocytes in cell culture [30]. Jayawardena et al. demonstrated that transient transfection of microRNA-1, -133, -208, and -499 is also capable of reprogramming fibroblasts into cardiomyocyte *in vitro* [29].

Most recently, Dr. Ding's group has established a protocol that does not require transcription factors but uses only small molecules to reprogram human fibroblasts to cardiomyocytes [33]. This study is based on a cell activation and signaling-directed (CASD) reprogramming paradigm. The authors treated fibroblasts with chemicals to induce a progenitor fate in them and then applied cardiogenic molecules to differentiate the cells into mature cardiomyocytes. In sharp contrast to the transcription factor-mediated cardiac reprogramming that directly converts fibroblasts into cardiomyocytes, this method involves an early progenitor stage and the subsequent differentiation process. The advantage of direct cardiac reprogramming is that it does not involve the pluripotent stage and thus minimizes the risk of tumorigenesis after cardiomyocyte transplantation into host hearts. In addition, direct reprogramming can be performed *in situ* (inside the hearts) [6] and thus can avoid the issues of low survival, retention, and integration with host tissue, which are concerns for the cell transplantation strategy when iPSC- or ESC-derived cardiomyocytes are used. However, the efficiency of cardiomyocyte generation with direct reprogramming is much lower than that of cardiac differentiation of ESCs or iPSCs with current methods; the self-renewal property of ESCs and iPSCs also allows large scale cardiomyocyte generation.

3. Maturation of *De Novo* Human Cardiac Myocytes

Mature and immature cardiomyocytes have distinct properties in size, morphology, metabolism, and physiology. Although recent advances in technology have markedly improved the efficiency for *de novo* cardiomyocyte generation

Table 1: Strategies to promote cardiomyocyte maturation.

Strategies	Approaches	Findings	References	Pros/cons
Prolonged cell culture	3-month culture	Increased I_{to1} and I_{K1}	[37]	Simple protocol but time-consuming
	30-day culture	Increased conduction velocity	[38]	
	80–120-day culture	More adult-like morphology and subcellular structure	[39]	
Electrical stimulation	3–6 Hz pacing in 3D cultured hESC-CMs	Adult-like structure, calcium handling and electrical properties	[40]	Requires special equipment and optimization of stimulation protocol
	1 Hz in monolayer culture	Prolonged action potential, but no effects on resting membrane potential	[41]	
	2 Hz in 3D tissue	Increased contractile force	[42]	
Mechanical stretch	Cyclic stretch	Increased cell size, better sarcomere structure	[43]	Requires special equipment and optimization of stimulation protocol
	Cyclic stretch	Cell elongation, increased connexin expression, more mature calcium handling	[44]	
Adrenergic receptor stimulation	Phenylephrine, 48 h	Increased cell size, better sarcomere structure	[43]	Simple protocol, easy to apply
Thyroid hormone stimulation	mESC-CMs: T3 for 7 days	Adult-like action potential profile and calcium handing	[46]	Simple protocol, easy to apply
	hiPSC-CMs: T3 for 7 days	Increased contractile force and faster calcium transient kinetics	[47]	
MicroRNA overexpression	miR-1	Hyperpolarized resting E_m, faster Ca^{2+} transient kinetics	[48]	Requires introduction of foreign substance into cells

from pluripotent stem cells, the obtained cells exhibit an immature phenotype when maintained in standard culture [34, 35]. For example, hESC-derived cardiomyocytes typically have a small size (membrane capacity 18 pF versus 150 pF in adult cardiomyocytes) and a circular or irregular shape (versus rod-shape in adult), exhibit no T-tubules (present in adult and critical for excitation-contraction coupling), and use glucose as the primary metabolic substrate (versus fatty acid in adult), a more depolarized resting membrane potential (−60 mV versus −90 mV in adult), and a much slower action potential upstroke maximum velocity (50 V/s versus 250 V/s in adult) [35]. A study of the transcriptional landscape of cardiomyocyte maturation revealed marked shifts in cardiac gene expression during maturation and suggested that the immaturity of ESC-derived cardiomyocytes may result from dysregulation of critical transcription factors [36]. This immature phenotype represents a major challenge for the use of *de novo* cardiomyocytes in heart research, especially for disease modeling and drug testing for adult-onset heart disease. Therefore, much effort has been made to find ways to induce maturation of *de novo* cardiomyocytes (Table 1).

3.1. Prolonged Cell Culture. Maturation is a natural development process, and therefore increasing the culture duration has become an obvious strategy to promote maturation of *de novo* cardiomyocytes. Sartiani et al. reported that maintaining hESC-derived cardiomyocytes in culture for 3 months promoted electrical maturation of the cells, such as increased current densities for I_{to1} (transient outward potassium current) and I_{K1} (inward rectifier potassium current) [37]. In addition, Kadota et al. demonstrated a time-dependent increase in the conduction velocity in a monolayer of hESC-derived cardiomyocytes cultured for 30 days [38]. Lundy et al. showed that long-term culture (80–120 days) of hESC- and hiPSC-derived cardiomyocytes induced a more adult-like morphology and subcellular structure, including increased cell size, increased myofibril density, a better-organized sarcomere structure, and higher percentage of multinucleated cells [39]. Moreover, these long-term cultured hESC- and hiPSC-derived cardiomyocytes also exhibit more mature calcium handling and electrophysiological properties [39]. In summary, maintaining cardiomyocytes in a prolonged culture is a proven strategy for cell maturation, but it is both time- and labor-intensive. Therefore, alternative approaches to accelerate cardiomyocyte maturation have been explored.

3.2. Electrical Stimulation. hESC- and hiPSC-derived cardiomyocyte cultures can beat spontaneously. Nunes et al. showed that forcing hESC-derived cardiomyocytes in 3-dimensional culture to beat faster at 3–6 Hz (180–360 beats/min) with electrical pacing markedly promoted cardiomyocyte maturation, with organized myofibril structure and improved conduction velocity, as well as more adult-like calcium handling and electrophysiological properties [40]. Chan et al. reported that electrical stimulation of hESC-derived cardiomyocytes at 1 Hz increased expression of cardiac-specific genes (such as *SCN5A, MLC2V*, and *Kv4.3*), and a higher percentage of cardiomyocytes showed longer action potential duration, but electrical stimulation did not affect resting membrane potential or action potential upstroke velocity [41]. Hirt et al. reported that pacing heart tissues engineered with hESC-derived cardiomyocytes at 2 Hz led to enhanced contractile force [42].

Table 2: Strategies for cardiac subtype myocytes generation.

Subtype cardiomyocytes	Approaches	Findings	References
Atrial myocytes	Noggin and retinoic acid	95% atrial-like cardiomyocytes	[51]
	Retinoic acid	Increased atrial-specific gene expression; 85% atrial myocytes in cell product	[52]
Ventricular myocytes	Noggin and retinoic acid pathway inhibitor	83% ventricular-like cardiomyocytes	[51]
	ROCK inhibitor, BMP4, Actvin A, and IWR-1	93–100% ventricular-like cardiomyocytes	[53]
Pacemaker myocytes	Tbx18-mediated direct programming of ventricular myocytes.	Pacemaker-like phenotype	[54, 55]
	Tbx3 overexpression in mouse ESCs	Increased yield of pacemaker-like cells	[56]
	Shox2 overexpression in mouse ESCs	More pacemaker-like cells, with enhanced biological pacemaker function	[57]

3.3. Mechanical Stretch. Földes et al. reported that exposure of hESC-derived cardiomyocytes to cyclic mechanical stretch for 24 hours led to a 1.6-fold increase in cell size, increased mRNA levels of cardiac-specific genes (*αMHC, βMHC*, and *ANF*), and better-organized sarcomere structure [43]. Mihic et al. showed that cyclic stretch of 3D cultured hESC-cardiomyocytes increased percentage of $cTnT^+$ cells, led to greater cell elongation, and enhanced connexin expression at cell junctions, as well as leading to more mature calcium handling [44].

3.4. Adrenergic Receptor Stimulation. Földes et al. reported that treatment of hESC-derived cardiomyocytes with phenylephrine (an α-adrenoceptor agonist) for 48 hours led to a 1.8-fold increase in cell size and better-organized sarcomere structure [43].

3.5. Thyroid Hormone Stimulation. Thyroid hormone plays a critical role in cardiac development [45]. Lee et al. reported that triiodothyronine (T3) treatment of mouse ESC-derived cardiomyocytes for 7 days increased expressions of cardiac-specific genes (such as *Nkx2.5* and *MLC2v*) and led to a more adult-like action potential profile and calcium handing [46]. Similarly, Yang et al. found that T3 treatment of human iPSC-derived cardiomyocytes for 7 days resulted in increased cell size, a 2-fold increase of single cell contractile force associated with faster calcium transient kinetics [47].

3.6. MicroRNA Overexpression. Fu et al. reported that overexpression of microRNA-1 (miR-1, which is highly expressed in adult cardiomyocytes) in hESC-derived cardiomyocytes led to a more mature-like (more hyperpolarized) resting membrane potential associated with increased potassium currents, as well as more mature calcium transient kinetics [48].

3.7. Summary. All the above studies have limitations: no single strategy can solve all the aspects of cell immaturity (Table 1). For example, electrical stimulation can improve functional maturation (action potential, calcium transient, and contraction force), while mechanical stretch increases cell size and sarcomere structure. Therefore, it seems that combination of such strategies may be a better solution for generation of mature cardiac myocytes.

4. Cardiac Subtype Myocyte: Heterogeneity of *De Novo* Cardiac Myocytes

The heart is composed of different cardiomyocytes (ventricular, atrial, and pacemaker myocytes) with distinct properties corresponding with their function in different parts of the cardiac chambers [49]. Pluripotent stem cells (ESCs and iPSCs) have the potential to give rise to all types of cardiomyocytes, making them a good model system to study cardiac diseases that affect different parts of the heart. However, this also poses a major challenge, because *de novo* cardiomyocytes obtained with existing protocols from ESC, iPSCs [21], and direct reprogramming [33] are a mixture containing all types of cardiomyocytes. Cell transplantation into the patient ventricles for regenerative therapy of heart failure would require a pure population of ventricular myocytes, and the contamination of pacemaker cells in the cell product will bring the concern for cardiac arrhythmias after transplantation [50]. On the other hand, studies of atrial disease, such as atrial fibrillation, would need a pure population of *de novo* atrial myocytes for disease modeling and drug testing. A pure population of pacemaker myocytes is required for biological pacemaker therapy of bradycardias. Therefore, development of new methods that allow enrichment of specific subtypes of cardiomyocytes from ESC, iPSCs, or direct reprogramming has been a hot research topic (Table 2).

4.1. Atrial Myocytes. Zhang et al. reported that 95% of the cardiomyocytes from differentiating hESCs after treatment with Noggin and retinoic acid (RA) had atrial-like action potentials [51]. Devalla et al. reported that treatment of differentiating hESCs with retinoic acid (1 μM) from day 4 to day 7 (after the mesoderm formation stage but before the cardiac progenitor stage) increased atrial-specific

gene expression (such as *KCNA5, KCNJ3*, and *NPPA*) but decreased expression of ventricular-specific genes (such as *MYL2, IRX4*, and *HEY2*) [52]. In addition, RA treatment increased the percentage of atrial myocytes (based on action potential profiles) from 20% in control untreated group to 85% [52].

4.2. Ventricular Myocytes. Zhang et al. found that treatment of differentiating hESCs with Noggin and a RA inhibitor increased the expression of ventricular-specific genes (*IRX4* and *MLC2v*), and 83% of the obtained cardiomyocytes had ventricular-like action potentials [51]. Weng et al. reported a protocol that generates 93–100% ventricular myocytes from different hESC and hiPSC lines based on electrophysiological properties of the cardiomyocyte population [53]. This protocol employs the sequential additions of a ROCK inhibitor, BMP4, Activin A, and IWR-1 (a Wnt inhibitor) in the cell culture, and the resulting ventricular-like cardiomyocytes exhibited typical ventricular ionic currents (I_{Na}, $I_{Ca,L}$, I_{Kr}, and I_{KATP}) [53].

4.3. Pacemaker Myocytes. Kapoor et al. demonstrated that *de novo* cardiac pacemaker myocytes can be obtained by direct reprogramming of rodent ventricular myocytes via viral delivery of *Tbx18*, a transcription factor required for embryonic pacemaker tissue development [54]. The translational potential of this work is suggested by preclinical studies carried out with pigs demonstrating that this strategy can be translated to large animals [55]. Jung et al. reported that overexpression of *Tbx3* in mouse ESCs promoted their differentiation into pacemaker cells, and in combination of cardiac-specific promoter antibiotic selection, >80% of the obtained cardiomyocytes exhibited a pacemaker-like phenotype [56]. Similarly, Ionta et al. found that overexpression of *Shox2*, a transcription factor critical for embryonic pacemaker tissue development, in differentiating mouse ESCs resulted in upregulation of pacemaker-specific genes [57]. The obtained pacemaker-like cells exhibited enhanced automaticity and are able to pace adult rat hearts *in vivo* [57].

5. Summary

Techniques that allow the generation of *de novo* human cardiac myocytes have revolutionized cardiac research. It is now possible to study heart disease mechanisms and perform drug testing on *de novo* human cardiac myocytes in culture dishes. Current efforts are focused on improving existing technologies for efficient generation of mature cardiomyocytes that only contain the desired subtype myocytes. With the improved methodologies, *de novo* human cardiac myocytes would be a powerful tool for medical research.

Competing Interests

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

Authors' Contributions

Veronique Hamel and Kang Cheng contributed equally to this work.

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Review Article

Progenitor Cells for Arterial Repair: Incremental Advancements towards Therapeutic Reality

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Coronary revascularization remains the standard treatment for obstructive coronary artery disease and can be accomplished by either percutaneous coronary intervention (PCI) or coronary artery bypass graft surgery. Considerable advances have rendered PCI the most common form of revascularization and improved clinical outcomes. However, numerous challenges to modern PCI remain, namely, in-stent restenosis and stent thrombosis, underscoring the importance of understanding the vessel wall response to injury to identify targets for intervention. Among recent promising discoveries, endothelial progenitor cells (EPCs) have garnered considerable interest given an increasing appreciation of their role in vascular homeostasis and their ability to promote vascular repair after stent placement. Circulating EPC numbers have been inversely correlated with cardiovascular risk, while administration of EPCs in humans has demonstrated improved clinical outcomes. Despite these encouraging results, however, advancing EPCs as a therapeutic modality has been hampered by a fundamental roadblock: what constitutes an EPC? We review current definitions and sources of EPCs as well as the proposed mechanisms of EPC-mediated vascular repair. Additionally, we discuss the current state of EPCs as therapeutic agents, focusing on endogenous augmentation and transplantation.

1. Introduction

Coronary artery disease (CAD) remains a leading cause of morbidity and mortality [1]. Coronary revascularization (restoring blood flow to the myocardium) remains the standard treatment for obstructive CAD and can be accomplished by either percutaneous coronary intervention (PCI) or coronary artery bypass graft (CABG) surgery. PCI has become the most frequently performed means of revascularization, aided by considerable advances in the field. What started as simple balloon angioplasty has now evolved through many generations of vascular scaffolds, from bare-metal stents (BMSs) to first and then second generation drug-eluting stents (DESs) [2–5]. While early technologies in PCI were fraught with high rates of complications including abrupt vessel closure, subsequent advances have mitigated much of this risk. Nevertheless, numerous challenges remain, including in-stent restenosis (ISR) and stent thrombosis (ST), collectively occurring in 5–8% of cases per year [6, 7]. Moreover, our understanding of the vessel wall's response to injury continues to evolve offering further opportunities for therapeutic intervention as our understanding of the impact that progenitor populations play has crystallized. Over the last decade, characterizing progenitor cell populations, understanding their role in vascular homeostasis, and harnessing their therapeutic potential by modifying them at a biological level remain areas of intense investigation.

The vessel wall's response to injury (e.g., plaque disruption and/or stent deployment) depends on many interdependent factors, including the insult itself in combination with the dynamic nature of the vessel wall-stent interface (including the cellular composition of the vessel wall and stent material and dimensions), vascular tone, and circulating signals. Indeed, PCI itself prompts endothelial denudation

and disruption of vascular homeostasis given the high pressure balloon inflation and subsequent force transmitted by stent struts to the endothelial layer extending damage through the underlying medial and adventitial layers [8]. In response to injury, the endothelium secretes a variety of molecules that influence vascular tone, inflammatory cell invasion, thrombus formation, and smooth muscle cell proliferation [9]. Indeed, stent-induced endothelial injury leads to platelet activation and thrombus propagation which alone can result in stent thrombosis [10]. As well, injury mediated inflammation is driven by activation of hypoxia-induced factor (HIF), which has a downstream effect on a number of factors including stromal cell derived factor-1 (SDF-1), angiopoietin, interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and c-Kit ligand to name a few [11]. Collectively, this activation stimulates the influx of inflammatory cells (neutrophils, monocytes, and macrophages) into the vascular wall, while also stimulating smooth muscle cells (SMCs) and myofibroblasts leading to neointima (NI) formation and subsequent ISR [12]. Paradoxically, this process, including VEGF secretion in particular, provides a stimulus for mobilizing endothelial progenitor cells (EPCs) to the site of injury and mediating vascular repair [13], the mechanisms of which are reviewed in detail below.

EPCs represent an attractive therapeutic option given their purported ability to promote vascular repair after stent placement. Accordingly, an inverse correlation between circulating EPC numbers and both atherosclerotic burden and probability of future cardiovascular risk has been noted [14, 15]. Furthermore, patients with ISR also have lower numbers of circulating EPCs and impaired EPC function [16, 17]. Given the potential benefits of EPC activity at sites of vascular injury, means of augmenting their number and/or function has been pursued and bolstered by clinical and animal studies suggesting improved outcomes following administration of EPCs [18, 19]. Furthermore, evidence suggests that EPCs are mobilized in patients with acute coronary syndromes, suggesting the presence of an endogenous mechanism that, if harnessed, could potentially improve outcomes [20, 21]. This work has stimulated interest in the importance of EPC signaling, number, and function in modulating vascular homeostasis with the potential for therapeutic benefit by capitalizing on EPC biology, particularly in the setting of PCI [22]. Herein, we review the biology of EPCs and their role as marker, modulator, and therapeutic agent for vascular repair.

2. Endothelial Progenitor Cells

Vascular progenitor cells were first observed in animal models when implanting Dacron grafts or silastic tubes [23–29]. These experiments first indicated the existence of a "pseudointima" composed primarily of endothelial cells and vascular smooth muscle cells, generating interest as to the origin of these cells [30]. This origin was finally discovered in 1997, when Asahara isolated EPCs from peripheral blood, describing them as CD34+ cells circulating in the human vasculature which were subsequently implicated in vascular homeostasis and endothelial repair [5, 31–34]. Subsequent studies described the incorporation of bone marrow-derived EPCs into blood vessels following hind limb ischemia [35] and myocardial infarction [36] as well as at wound [37] and tumour [38] sites. EPCs are believed to promote vascular repair by homing to sites of vascular injury where they act via [1] paracrine signaling to neighbouring cells (now believed to represent the predominant mode of action) and [2] transdifferentiating to mature endothelial cells and undergoing angiogenesis to form new blood vessels [5, 39] (Figure 1). In this way, EPCs are believed to hold therapeutic promise, though harnessing this potential has proven challenging.

2.1. The Evolving Definition of EPCs. Since their discovery, the precise definition of EPCs remains the subject of considerable debate and represents a major hindrance in understanding the biology and application of these unique cells. This is believed to be a driving force behind conflicting results noted in both preclinical and clinical trials, where differing methodologies in cell sources, cell purification, detection methods, and animal models or assays have been published [40]. Thus far, EPCs have been identified through two main methods: (1) cell-surface markers and (2) colony forming units (CFUs) and cultured/circulated angiogenic cells (CACs) (Figure 2). However, while these methods allow for the physical identification of EPCs, they are unable to characterize the in vitro activity of these cells nor the phenotype they express in physiologic conditions. We herein briefly discuss two common methods used to define EPCs.

2.1.1. Identification by Cell-Surface Markers. EPCs can be classified based on the expression of specific cell-surface antigens via flow cytometry (Table 1). Mature endothelial cells (ECs) express multiple surface antigens including CD34, VE-cadherin, von Willebrand factor (vWF), kinase insert domain-containing receptor (KDR) or VEGFR2, and E-selectin [41]. However, surface antigen expression varies between mature ECs depending on the specific vessel, organ, and activation state of the endothelium. To distinguish between EPCs and mature ECs shed after vascular injury, CD133 (AC133) was utilized, given that it is typically lost during maturation of EPCs into mature ECs. Surface markers CD34, VEGFR2, and CD133 have been widely accepted by researchers for defining EPCs, though many studies typically employ two of the three receptors [40, 41]. Other studies of EPCs have employed CD45dim/CD34/CD133/CD117, with CD117 playing a role in mobilization of hematopoietic stem cells [32, 42, 43]. In 2007, Case et al. isolated EPCs from adult peripheral blood by targeting CD34+ VEGFR2+ and CD133+ cells which did not contribute to the formation of mature endothelial cells in vitro, further challenging the existing models of progenitor cells [44]. Despite controversy, cell-surface marker-based definitions of EPCs (namely, CD34+/KDR+) have been demonstrated to be predictive of cardiovascular outcomes in patients, lending support to this approach [15]. Further work, including in vivo and in vitro assessment of endothelial differentiation capacity, is required to better define this subpopulation of progenitors and ensure future studies and therapies focus on a homogeneous population.

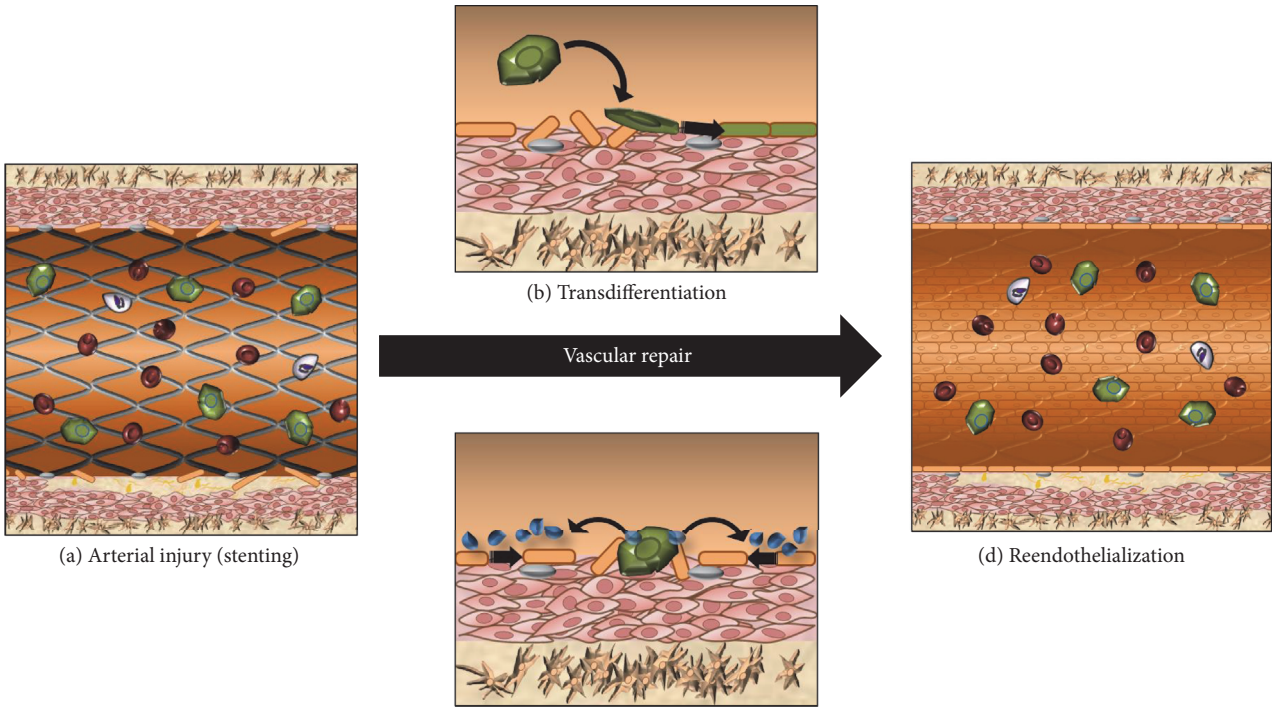

Figure 1: Mechanisms of EPC-mediated vascular repair: (a) coronary stenting resulting in arterial injury. Placement of coronary stent to treat obstructive coronary artery disease (CAD) with plaque being pushed into vessel wall and denudation of endothelial cells (ECs) causing vascular injury. Circulating EPCs then home to the site of vascular injury and mediate repair via either transdifferentiation or paracrine effects. (b) Transdifferentiation of EPCs: EPC transdifferentiates into ECs (black arrows) to repair denuded endothelium. (c) Paracrine effects of EPCs: EPCs secrete numerous chemokines (blue) which stimulate surrounding cells to proliferate and migrate towards site of injury to regenerate the EC layer (black arrows). (d) Reendothelialization: endothelial layer is restored with coronary stent underlying regenerated EC layer. Adventitia (brown), media (pink), endothelial cells (orange), EPCs (green), erythrocytes (red), and leukocytes (white).

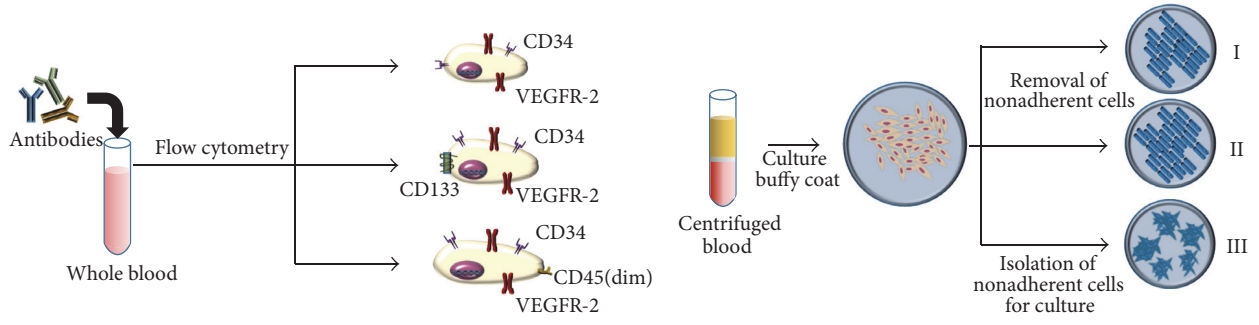

Figure 2: Definition of endothelial progenitor cells (EPCs). (a) Flow cytometry: analysis of whole blood samples by flow cytometry utilizing various combinations of antibodies tagged with fluorophores raised against surface antigens such as CD34+/VEGFR-2+, CD34+/VEGFR-2+/CD133+, or CD34+/VEGFR-2+/CD45(dim) to isolate populations. (b) Culture assay: obtained via Ficoll-based centrifugation of whole blood to isolate and culture the buffy-coat layer composed of peripheral blood mononuclear cells (PBMCs). I and II use adherent cells after removal of nonadherent cells. I, EPC culture on fibronectin plate; II, culturing these cells on collagen plate forming endothelial colony forming cells; III, isolation of nonadherent cells for culture to generate colony forming units (CFUs).

2.1.2. Identification by Culture-Based Techniques. EPCs can also be isolated from peripheral blood by plating these cells in various cell culture assays and assessing their colony forming ability (Figure 2). Asahara et al. first identified these CD34+ EPCs based on cluster-forming cells [31]. Colony counts were used as a predictor of the number of circulating progenitors while the spindle-shaped cells surrounding each colony were identified as EPCs. In 2003, Hill et al. isolated peripheral blood mononuclear cells (PBMCs) from venous blood by Ficoll gradient centrifugation. To minimize contamination by mature ECs, PBMCs were plated for 48 hours onto a fibronectin plate and then the nonadherent cells were replated onto a fibronectin-coated plate to quantify EPC-derived colonies. Colonies were then characterized by

Table 1: Evolution of cell-surface markers for endothelial progenitor cell isolation (2014–2016).

		CD34	VEGFR-2/KDR	CD133	CD31	CD45
2014	Liao et al. [51]	Yes	Yes			CD45(dim)
2014	Chi et al. [52]	Yes		Yes		
2015	Shim et al. [53]	Yes			Yes	CD45(dim)
2015	Martí-Fàbregas et al. [54]	Yes	Yes	Yes		
2015	Wang et al. [55]	Yes		Yes		
2015	Tam et al. [56]	Yes	Yes			
2015	Sandra et al. [57]	Yes	Yes	Yes		
2016	Ricottini et al. [58]	Yes	Yes	Yes		CD45(dim)
2016	Lanuti et al. [59]	Yes	Yes	Yes		CD45(dim)
2016	De Ciuceis et al. [60]	Yes	Yes	Yes		
2016	Qin et al. [61]	Yes		Yes		
2016	Niederseer et al., early EPC [62]	Yes				Yes
2016	Niederseer et al., late EPC [62]	Yes	Yes			CD45(dim)
2016	Kung et al. [63]	Yes	Yes	Yes		
2016	Liu et al. [64]	Yes	Yes	Yes		

thin, flat cells which radiated from a centre of clustered cells. The assay was commercialized and these colonies were referred to as colony forming unit-Hill (CFU-Hill). The CFU-Hill assay demonstrated an inverse relationship between the circulating CFU-Hill concentration and Framingham cardiovascular risk score in humans [14]. However, later work called into question the reliability of these units for quantifying EPCs and instead noted they may actually represent cells of macrophage/monocyte and/or T-cell origin [45]. Nonetheless, while CFU-based definitions of EPCs may remain imprecise, they have been shown to serve as a marker of clinical endpoints [14].

Identifying the relationship between these two different isolation methods for EPCs is vital to ensuring a uniform definition of EPCs. One study examined the relationship of both methods and revealed that CFU numbers did not correlate to numbers of either CD34+/KDR+ or CD34+/KDR+/CD133+ cells [46]. In 2007, $CD34^+/VEGFR2^+/CD133^+$ cells were isolated using a cell sorter and assayed using two different endothelial culture systems and hematopoietic colony assays. No endothelial colonies formed and the isolated cells expressed the hematopoietic cell marker, CD45 [44]. One possible explanation for this is that sorted cells may lose critical signaling molecules in the process, which impact the ultimate endothelial phenotype observed in other trials, though this phenomenon is poorly understood. In addition, surface markers used to define EPCs are also found in myeloid-monocytic cells, which may result in isolation of impure EPC populations [47]. Hence, the ideal methodology for isolating and defining an EPC remains the subject of debate, hampering therapeutic advancements.

2.2. Sources of EPCs

2.2.1. Bone Marrow. Asahara et al. described the first EPCs as originating in the bone marrow [31]. The bone marrow cells used expressed LacZ (β-galactosidase), which was transcriptionally regulated by an endothelial-specific promoter (Tie2 or Flk-1). The group identified LacZ-positive cells in the developing neovasculature in all the models [41]. Since then, several other animal models of ischemia have supported the role of bone marrow-derived mononuclear cells in vascular development [48, 49]. Moreover, several clinical trials have employed bone marrow-derived progenitor cells following revascularization for acute myocardial infarction with varying levels of success in terms of mortality and ventricular recovery [50].

2.2.2. Kidney. Renal progenitor cells (RPCs) were isolated from normal adult human kidney cortical parenchyma with cell-surface markers CD133+ and PAX-2+ (embryonic renal marker). These RPCs were capable of self-replication and could be differentiated in vitro into epithelial or endothelial cells [65]. "Nephrospheres" formed by these cells in culture form the basis of a functional assay used for stem cell isolation. One group utilized the PKH26 tracer to identify CD133+/CD24− cells that differentiated into cells with epithelial, endothelial, or podocytic features [66]. It is thought that these resident RPCs may play a role in renal repair in adults though there is limited data to support this [67, 68].

2.2.3. Blood Vessel Wall. Most models of EPC-mediated vascular repair invoke the homing capability of bone marrow-derived EPCs that are circulating in the blood. It is believed that human aortic endothelial cells (HAECs) or human umbilical vein endothelial cells (HUVECs) from vessel walls are terminally differentiated into mature ECs [69]. However, when HAECs were isolated to examine the hierarchy of ECs based on their proliferative capacity, they were able to be passaged in vitro for >40 population doublings using a single-cell deposition assay, thereby suggesting the existence of resident high proliferative potential endothelial colony forming cells (HPP-ECFCs) line, which may assist in vascular repair [69]. This finding suggested that angiogenesis may in fact be mediated by EPCs [70]. However, increasing evidence

suggests that resident ECs actually repair the damaged endothelium, challenging the previous premise that this ability was exclusive to circulating EPCs. Hagensen et al. compared the contribution of circulating and resident cells to vascular repair by transplanting wire-injured carotid artery segments from wild-type mice into Tie2-GFP mice, which expressed GFP in ECs. This model found that the vascular endothelium that formed in response to injury likely arose via migration of surrounding ECs rather than from circulating progenitor cells [71]. Further work is ongoing to identify and characterize the regenerative resident cells within arterial systems.

Recently, in addition to circulating progenitor cells, the existence of resident vascular progenitor cells within the adventitia of blood vessels has been demonstrated. ApoE$^{-/-}$ mice were used to identify adventitial progenitor cells stained by Sca-1, CD34, and Flk-1 [72]. Indeed, Sca-1+ cells were able to differentiate into both ECs and smooth muscle cells (SMCs), thereby potentially contributing to both reendothelialization and/or NI formation, respectively [73]. In 2007, Pasquinelli et al. identified two separate progenitor cell populations between the media and adventitia in human femoral arteries and thoracic aortas [74]. They isolated CD34+ and c-kit+ cells that acquired EC properties when cultured in the presence of VEGF. In 2010, Campagnolo et al. isolated CD34+/CD31− cells from human saphenous veins in CABG patients. These progenitor cells could differentiate into adipocytes, pericytes, and SMCs, stimulated angiogenesis, and improved blood flow when injected into ischemic hind limbs of mice [75].

2.2.4. Adipose Tissue. Adipose tissue was identified as a source of EPCs when Planat-Benard et al. isolated human adipocytes and dedifferentiated them into cells capable of then differentiating into adipocytes or ECs under appropriate conditions [76]. Isolating EPCs from peripheral blood is time consuming whereas adipose tissue may represent an alternative source from which to isolate EPCs. These adipose tissue-derived EPCs (ADEPCs) were capable of enhancing HUVEC capillary-like tube formation on Matrigel [77] and participated in neovascularization when transplanted into rat models of traumatic brain injury [78]. Recent work to characterize the surface markers of ADEPCs has demonstrated the presence of many described EPC markers including CD34, Stro-1, VEGFR-2 (KDR), eNOS, and CD31 [77].

2.2.5. Spleen. Several studies have confirmed an important role for the spleen in EPC mobilization and transplantation [79]. Most hematopoietic stem cells (HSCs) migrate to sinusoids in the bone marrow and spleen and communicate with the endothelium within them, an interaction that seems to be important for their maintenance [80]. Transplantation of spleen-derived mononuclear cells restored endothelium-dependent vasodilation in atherosclerotic ApoE mice, suggesting a splenic source of EPCs for vascular repair [81]. Also, EPCs isolated from spleen homogenate improved reendothelialization and reduced neointima (NI) formation in a model of carotid artery endothelial injury [82].

2.3. EPC-Mediated Vascular Repair

2.3.1. Mobilization. The predominance of evidence supports bone marrow-derived EPCs as the most likely source for arterial repair and accordingly we will focus on their mobilization, reviewing various chemokines responsible for guiding EPCs to sites of vascular injury. VEGF, an effective mobilizer and activator of angiogenesis, is believed to induce proliferation, differentiation, and chemotaxis of ECs following vessel injury [83]. Accordingly, augmenting VEGF levels via an adenovirus that expresses VEGF promoted recruitment of EPCs to sites of injury and promoted neovascularization in mice models. This effect is potentially mediated by matrix metallopeptidase-9 (MMP-9) which is converted to the soluble survival factor sKitL, thereby enhancing VEGFR2+ progenitor cells and facilitating transport from osteoblast-rich locations to vascular areas, promoting movement into the circulation [84]. Interestingly, nitric oxide (NO) appears to play a major role in the expression of MMP-9 in its inactive and active form. Studies of endothelial nitric oxide synthase 3 knockout mice (NOS3 KO) have shown reduced expression and activity of MMP-9 even after stimulation with VEGF. These same mice demonstrated reduced neovascularization with induced ischemia and reduced EPC mobilization to ischemic areas [85]. In contrast, AVE9488 (eNOS upregulator) increased NO expression in EPCs and enhanced their migratory capacity [86]. Thus, it appears that NO and MMP-9 play a critical role in facilitating VEGF-mediated angiogenesis.

Secondarily, SDF-1 is a chemokine of growing interest, a constitutively expressed protein that is upregulated by inflammatory mediators, extracellular matrix changes, mechanical forces, and hypoxia [87]. Platelets secrete SDF-1 upon being activated at sites of injury, which may provide the local signal needed to recruit EPCs to the site of injury. In fact, SDF-1 seems to only induce neovascularization in the presence of ischemic injury [88]. In NOS3 KO mice, SDF-1-associated neovascularization and EPC mobilization were silenced, suggesting that VEGF/eNOS signaling pathways likely play important roles in this vascular homeostasis [89]. Additional factors with similar function to SDF-1 include estrogen, statins, and erythropoietin. These factors improve EPC mobilization and neovascularization and inhibit neointima hyperplasia but are similarly silenced in NOS3 KO mice [90–93].

2.3.2. Homing. Understanding the mechanism by which EPCs home to sites of vascular injury is critical to harnessing their therapeutic potential. This process is thought to be similar to the rolling and adhesion behaviour exhibited by leukocytes in the setting of inflammation. EPC homing involves an interaction between EPC surface molecules and associated ligands expressed on dying ECs. P- and E-selectin are believed to play critical roles in this process. Foubert et al. have shed light on this process through their studies on the erythropoietin-producing human hepatocellular carcinoma (Eph) receptor and its associated ephrin ligands, which are regulators of vascular development. The group has shown that Eph4B activation by ephrin-B2-Fc chimeric protein increases the angiogenic potential of human EPCs in a hind limb

ischemia mouse model and that this response is blunted with EphB4 siRNA treatment. Eph4B appears to elevate P-selectin glycoprotein ligand-1 expression and EPC adhesion, an effect that can be abrogated with utilizing neutralizing antibodies to E- and P-selectin [94]. β2-Integrins on the surface of EPCs mediate migration and adhesion of EPCs to the damaged endothelium as well as neovascularization [95]. High motility group box 1 (HMGB1) has been shown to activate β1- and β2-integrins on the surface of EPCs by binding to HMGB1 receptors RAGE (receptor for advanced glycation endproducts) and TLR2 (toll-like receptor 2). Interestingly, HMGB1 is released into the extracellular space upon necrosis, but not apoptosis, and was associated with improved homing and adhesion of EPCs to the site of injury [96].

Next, intercellular adhesion molecule-1 (ICAM-1) upregulation in ischemia has been noted to increase EPC recruitment to ischemic limbs [97]. As a hypoxia-responsive gene, integrin-linked kinase (ILK) regulates ICAM-1 expression. Its overexpression is thus associated with increased expression of ICAM-1 as well as SDF-1 in ECs, two key molecules involved in the recruitment of EPCs during vasculogenesis [98]. The synergy between EPC surface proteins and endothelial and/or extracellular ligands is therefore essential for EPC homing to sites of injury (Figure 1).

Apart from EPC migration to sites of vascular injury, EPC invasion of injured tissue is critical for organ repair and function. Gene expression profiling of EPCs and mature ECs has identified the protease Cathepsin L (CathL) as being highly expressed. CathL was shown in vitro to be crucial for matrix degradation and EPC invasion. A CathL-deficient hind limb ischemia mouse model characteristically shows poor limb recovery. As well, when CathL-deficient progenitor cells are infused, impaired homing to sites of injury and impaired neovascularization are observed [99]. EPCs lacking MMP-2 similarly show reduced invasiveness and proliferative capabilities [100]. Hence, numerous molecules have been implicated in the homing of EPCs via a rolling and adhesion model, with additional protease-mediated invasion as required.

2.3.3. Paracrine Effects. It is unlikely that EPC transdifferentiation into mature ECs represents a meaningful reparative mechanism. Rather, an increasing number of studies have shown that these circulating cells may facilitate arterial repair through paracrine influence on neighbouring cells (Figure 1). Many of the cells previously identified as EPCs have more recently been shown to derive from a monocytic lineage, suggesting that they may contribute to vascular repair by releasing paracrine factors such as VEGF [101]. For example, treatment of HUVECs and coronary artery endothelial cells (CAECs) with cultured media (CM) from EPCs or mature ECs demonstrated that EPC-derived CM has higher levels of IL-8 and it demonstrated a stimulatory effect on HUVEC and CAEC proliferation [102].

Paracrine signaling appears to be an important aspect in both early and late EPCs. Early EPCs are derived from a monocytic cell lineage (CD14+) to form spindle-shaped cells after 7 days of culture. In contrast, late EPCs are derived from a CD34+ lineage after 14–28 days in culture and resemble ECs [39]. Early EPCs have a modest contribution to the direct incorporation into the endothelium relative to late EPCs [103] but secrete proangiogenic stimulatory cytokines such as VEGF, fibroblast growth factor (FGF), IL-8, placental growth factor, SDF-1, and platelet derived growth factor (PDGF) [104, 105]. Additionally, the manipulation of the VEGF-Akt pathway could stimulate cell survival and proliferation [106]. These signals are critical as they contribute to endothelial regeneration by recruiting resident endothelial and cardiac progenitor cells to improve endothelial regeneration. Late EPCs have a direct role in neovascularization but a reduced role in paracrine signaling compared to early EPCs. However, they still have been shown to secrete proangiogenic factors including VEGF, IL-8, and PDGF [107]. They suggest that further subsets of EPCs could be defined based on their secretion profile, while modulation of their chemokine profile may help improve their inflammatory profile [108].

More recently, EC-derived microparticles (EMPs) or microvesicles (MV) have been identified as vehicles for different paracrine modes of action. EMPs are complex vesicles shed from activated or apoptotic ECs which play a major role in endothelial function and angiogenesis. EMPs are highly functional molecules with several surface molecules and contain DNA, RNA, or microRNA [109]. These EMPs are taken up by target cells, such as mature ECs, to stimulate endothelial regeneration [47]. Injection of EMPs has been associated with attenuated kidney injury in rats by enhancing proliferation and decreasing apoptosis. This effect was reduced after treating EMPs with RNase or miRNA depletion, implicating a potential function of RNA within EMP for vascular repair [110]. Others have shown that treatment with EMPs accelerates endothelial repair after carotid artery injury in mouse models and that miRNA-126 within EMPs plays a critical role in regulating EC mobilization, proliferation, and regeneration [111]. As we continue to decipher the underlying biology of progenitor cells, the impact of paracrine signaling for vascular repair appears to grow evermore important, presenting both obstacles but also more potential therapeutic targets.

3. Therapeutic Potential

Preclinical trials involving EPCs have shown promising results, with EPCs demonstrating the ability to specifically incorporate into the site of injury and mitigate neointima formation [112]. Our group isolated cultured angiogenic cells from patients with CAD and showed that improving CACs could improve reendothelialization [42]. Other substances, including estrogen, G-CSF, and leptin have been demonstrated to mobilize EPCs and enhance arterial repair after vascular injury in animal models [113–115]. Accordingly, endothelial dysfunction is thought to portend cardiovascular disease. Thus, circulating EPC levels may serve as a potential biomarker of endothelial function and integrity, while augmenting EPC levels may represent a viable therapeutic target. Understanding and augmenting endogenous EPCs have attracted considerable attention thus far, so does the transplantation of EPCs directly into sites of injuries. Indeed, these two therapeutic themes represent the majority of applications of EPCs in a therapeutic role to date. However, clinical trials augmenting EPC levels and activity have great

variation in EPC isolation techniques and definitions, likely contributing to conflicting results and hindering the advancement of EPCs in the therapeutic realm.

3.1. Augmenting Endogenous EPCs

3.1.1. Antihypertensive Therapy. Some common antihypertensive (i.e., blood pressure lowering) agents include angiotensin converting enzyme inhibitors (ACEi), angiotensin II receptor blockers (ARB), and calcium channel blockers (CCB). All of these agents have been extensively studied and yielded an improvement in EPC number and function by differing mechanisms of action. ARBs have improved numbers and function of EPCs by inhibiting oxidative stress [116], while ACEi yielded augmented EPC number and function when given to patients with stable CAD [117]. ACEi have also demonstrated stimulation of nitric oxide activity and diminished oxidative stress in human cells [118]. CCBs such as nifedipine improved EPC function and provided a greater resistance to oxidative stress and apoptosis [119]. Hence, considerable evidence supports the role for antihypertensive agents in improving EPC number and function.

3.1.2. Statin Therapy. Statins were originally developed to modify lipid profiles in patients by reducing LDL and triglyceride levels to improve cardiovascular outcomes. However, clinical studies involving statin therapy in patients with CAD showed higher levels and mobilization of EPCs [120, 121]. These studies showed the initial promise of potentially enhancing endogenous EPCs to improve clinical outcomes. The mechanism behind statin's effect on EPCs is a dose-dependent augmentation of Akt phosphorylation within minutes, which yields an increase in mobilization, migration, proliferation, and survival of EPCs [120]. Elevated EPC levels have also been observed with statin therapy in patients with CAD, acute myocardial infarction, and post-CABG [122–124]. From a vascular repair perspective, patients who develop ISR have also been shown to have functionally impaired EPCs [16, 17]. In a previous review, we demonstrated that statin therapy resulted in a significant rise in circulating EPCs over control, with a median increase of 70.9% (range from 25.8% to 223.5%) [43]. The considerable variance in EPC augmentation following statin therapy most likely reflects differences in the definition of EPCs employed as well as varying isolation techniques.

3.1.3. GSK-3β. Glycogen synthase kinase (GSK-3β) is a serine/threonine kinase that phosphorylates β-catenin to negatively regulate the Wnt signaling pathway [125]. The Wnt signaling pathway plays an essential role in mobilization of EPCs and enhancement of neovascularization [126]. This pathway produces multiple secreted glycoproteins which regulate many cell processes, including hematopoiesis and stem cell function. Our group showed for the first time in an in vitro experiment that the inhibition of GSK-3β was associated with increase in EPC levels and reduction of EPC apoptosis. In vivo, we observed that GSK-3β inhibition resulted in an improvement in reendothelialization and reduction of neointima formation following injury [42].

Patients with diabetes mellitus (DM) have reduced EPC levels and increased rates of apoptosis. In addition, diabetics have shown higher level of GSK-3β activity that resulted in higher levels of phosphorylated β-catenin. DM-EPCs are associated with reduced mobilization, migration, and homing to sites of injury [127]. Our group has shown that treatment with GSK-3β inhibitors reduced apoptosis, increased VEGF production, and enhanced EPC invasive capacity in vitro. Proteomics analysis of DM-EPC versus normal EPC revealed 37 uniquely regulated proteins. Cathepsin B was identified as the protein that mediates enhanced invasive capacity of EPCs after GSK-3β inhibition [128]. Although the direct mechanism of action remains unclear, other studies appear to support our findings. Activation of Wnt/β-catenin pathway during human mesenchymal stem cell differentiation was associated with upregulation of Cathepsin B [129]. Although more studies are required on the application of GSK-3β inhibition to enhance EPC function, it appears to be a promising avenue for future therapeutic development.

3.1.4. Stents. As discussed, coronary stents have markedly advanced from simple metal scaffolds to sophisticated drug-delivery systems to facilitate arterial healing [5]. Drug-eluting stents (DESs) routinely used for PCI are coated with antiproliferative and anti-inflammatory agents, with first-generation DESs utilizing sirolimus and paclitaxel. Paclitaxel was originally developed as a treatment for ovarian cancer, preventing cellular proliferation via inhibition of microtubule regulation during mitosis [130]. Subsequent work then demonstrated its affinity for inhibiting smooth muscle cell and neointima formation, leading to its development for DESs [131]. Sirolimus (rapamycin) was originally developed as an antifungal agent, but its use was limited due to its immunosuppressive properties. However, it was also noted to suppress NI formation lending itself well to DES implementation. It enacts its effects via blocking G_1 to S phase progression in cell cycle via inhibition of mammalian target of rapamycin (mTOR) kinase [132]. As the role of EPCs in vascular healing became apparent, novel technologies then focused on discovering means of increasing EPC recruitment and proliferation specifically at the site of stent deployment and subsequent endothelial injury [133].

The first stent to harness the healing capacity of EPCs was the Genous monoclonal anti-human CD34 coated stent [134]. This EPC-capture stent consisted of a stainless steel scaffold covered in a covalently coupled polysaccharide polymer with CD34 antibodies. The Healing-FIM trial compared EPC-capture stent versus bare-metal stents, demonstrating similar NI hyperplasia between the two cohorts and suggesting that the EPC-capture stent failed to inhibit NI hyperplasia [134]. While initially a promising concept, subsequent randomized trials have actually revealed increased ISR with the Genous stent compared to the paclitaxel-eluting stent, paradoxically suggesting increased NI hyperplasia with this technology [135]. These disappointing results are felt to be related to three factors: (i) the stent scaffold itself was an early generation device (thicker struts without any antiproliferative medication), (ii) CD34 is a surface antigen common to various progenitors and may result in nonspecific binding

to the stent thereby promoting NI hyperplasia, and (iii) patients with CAD are known to have reduced number and functionality of EPCs [5, 15]. Moreover, the HEALING II registry reported that patients with normal levels of CD34+KDR+ EPC titers had lower rates of ISR compared to patients with reduced levels of EPCs – supporting the notion that augmentation of endogenous EPCs may be key to this success [136]. Hence, attempting to harness rare and dysfunctional cells is likely of little benefit. Accordingly, future trials tried to address the state of the endogenous EPCs available by combining statin therapy with Genous stenting to improve reendothelialization [137]. In a recent 5-year follow-up study of patients treated with Genous stent target lesion revascularization, stent thrombosis, and ISR were stabilized within 12–24 months and up to 5 years [138]. Further data is needed to better understand the role of EPC-capture stent technology in conventional PCI.

To further refine this technology, the EPC-capture stent has been combined with a paclitaxel-coated balloon with intent of reducing the noted NI proliferation and ISR that plagued first-generation Genous stents given its antiproliferative properties. The Perfect Stent study studied 120 patients with CAD who were treated with EPC-capture stent followed by paclitaxel-eluting balloon after dilatation. This approach was successful in reducing restenosis [139] and may well represent the future direction of this technology. Similarly, the Combo stent (OrbusNeich Medical, Fort Lauderdale, USA) is composed of EPC capturing technology combined with a sirolimus-eluting stent to minimize NI formation. The total concentration of sirolimus is half of what is found in a standard DES, but it is released in the same fashion. Preclinical trials in porcine models showed reduced NI thickness in the Combo stent compared to the standard sirolimus-eluting stent, low-dose Combo stent, and everolimus (sirolimus-analog) eluting stent [140]. In 2013, Haude et al. published the prospective, multicenter, randomized evaluation of an abluminal sirolimus coated bioengineered stent (REMEDEE) trial, comparing the Combo stent with the paclitaxel-eluting Taxus Liberte stent and demonstrate that the Combo stent was superior to the paclitaxel stent at 9-month angiographic follow-up because of in-stent late lumen loss with values of 0.39 ± 0.45 mm and 0.44 ± 0.56 mm, respectively [141]. This demonstrated the initial safety and efficacy of Combo stents in comparison to first-generation DESs, but further data is still required to establish this technology. While augmenting and maximizing endogenous EPCs show some promise, an alternative approach involves the direct administration of exogenous EPCs.

3.2. Transplantation of Exogenous EPCs. Stem cell therapies have been developed as new treatment regimen for patients suffering from acute myocardial infarctions since the early 2000s. The first of these trials involved the injection of bone marrow-derived mononuclear cells (BMMNC) in 10 patients at the site of infarct following balloon dilatation [142]. Since then, numerous trials injecting BMMNC have been conducted following revascularization by both PCI and CABG to assess myocardial recovery [143–145]. This success has led to large randomized controlled trials of progenitor cell transplantation worldwide [146–148], including myocardial infarction [149].

The procedure involves harvesting bone marrow under general anesthesia from the bony pelvis. CD34+ or CD133+ hematopoietic progenitor cells (similar surface markers as the EPCs defined earlier) are isolated from the BMMNC and then cultured for 2–4 weeks to obtain a sufficient yield of progenitor cells. These cells are then injected directly into the patient's heart at the site of injury during PCI. The timing of stem cell injection varies in studies from first 24–48 hours to four weeks after PCI. Also, the dose of cells that were administered varied considerably within these large trials anywhere from 10^6 to 10^{10} cells [146–149]. Overall, major trials showed no significant mortality difference between those who received stem cell transplant and those who did not receive cells in the short term. In addition, a Cochrane review on stem cell treatment for acute myocardial infarction revealed no significant reduction in cardiovascular mortality or major adverse cardiovascular events after cell therapy in short- and long-term follow-up [50]. While certainly intriguing and promising, this technology is still in its infancy, and considerable advancements are needed for this to establish itself as a legitimate therapeutic option.

4. Conclusion

Our understanding of the complex environment that is the vessel wall remains incomplete. Certainly, the discovery of EPCs holds great potential for both the monitoring and therapy of vascular disease, particularly in repairing the endothelial injury incurred by modern interventions. Be it by augmentation and exploitation of endogenous cells or direct transplantation of cells to areas of need, both approaches show promise. However, despite considerable efforts over the past few decades, our understanding of the definition, signaling, and differentiation of these enigmatic progenitor cells remains imperfect, hampering further developments. Only once these fundamental components are clarified will progenitor cells be able to advance and emerge as a legitimate therapeutic option.

Disclosure

Trevor Simard and Richard G. Jung shared first authorship.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Research Article

Isolation of an ES-Derived Cardiovascular Multipotent Cell Population Based on VE-Cadherin Promoter Activity

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Embryonic Stem (ES) or induced Pluripotent Stem (iPS) cells are important sources for cardiomyocyte generation, targeted for regenerative therapies. Several in vitro protocols are currently utilized for their differentiation, but the value of cell-based approaches remains unclear. Here, we characterized a cardiovascular progenitor population derived during ES differentiation, after selection based on VE-cadherin promoter (Pvec) activity. ESCs were genetically modified with an episomal vector, allowing the expression of puromycin resistance gene, under Pvec activity. Puromycin-surviving cells displayed cardiac and endothelial progenitor cells characteristics. Expansion and self-renewal of this cardiac and endothelial dual-progenitor population (CEDP) were achieved by Wnt/β-catenin pathway activation. CEDPs express early cardiac developmental stage-specific markers but not markers of differentiated cardiomyocytes. Similarly, CEDPs express endothelial markers. However, CEDPs can undergo differentiation predominantly to cTnT$^+$ (~47%) and VE-cadherin$^+$ (~28%) cells. Transplantation of CEDPs in the left heart ventricle of adult rats showed that CEDPs-derived cells survive and differentiate in vivo for at least 14 days after transplantation. A novel, dual-progenitor population was isolated during ESCs differentiation, based on Pvec activity. This lineage can self-renew, permitting its maintenance as a source of cardiovascular progenitor cells and constitutes a useful source for regenerative approaches.

1. Introduction

Regeneration of ventricular myocardium has been at the center of research efforts during the past decade. Embryonic Stem (ES) or induced Pluripotent Stem (iPS) cells are important cellular sources towards this aim. Reproduction of the sequential stages of cardiac differentiation has been established during pluripotent ESCs differentiation under appropriate conditions in vitro [1]. In general ES-derived cells are isolated either as terminally differentiated cardiomyocytes [2–4] or as cardiovascular progenitor populations left to further differentiate after transplantation in vivo [5, 6]. The therapeutic potential of such isolated cardiogenic progenitors, even limited, has been reported in numerous studies [7–9]. Despite the advent of various protocols utilized for cardiomyocyte generation, the value of cell-based approaches for cardiac regeneration remains unclear. Specifically, the homing properties, survival, proliferation, and maturation of transplanted cells in the environment of myocardium are challenges that remain to be addressed [10–12].

Isolation and expansion of novel multipotent cardiovascular progenitors with limited differentiation potential could present a valuable tool towards this goal. Genetic-based ESCs differentiation systems take advantage of developmental stage

specific activity of promoters for selection of cell populations. VE-cadherin is an adhesion molecule that contributes to adherens junctions formation between endothelial cells. VE-cadherin promoter (Pvec) has been previously characterized as endothelial specific in vivo and in vitro [13–16]. However, its transient activation was also detected in hemopoietic progenitor populations called "hemogenic endothelium" [17–19]. In our laboratory, we have previously analyzed Pvec activation during ESCs differentiation and found evidence of such activation in a subset of early Isl1$^+$ cardiovascular progenitors (Maltabe et al., submitted). Isl1 belongs to a group of lineage-specific transcriptions factors expressed in early cardiogenesis [20]. Particularly, Isl1$^+$ cells have been characterized as multipotent cardiovascular progenitors, because they differentiate further to cardiomyocytes, endothelial, endocardial, and smooth muscle cells [21].

In the present study, we aimed to generate and isolate a novel cardiovascular progenitor population derived from ESCs, based on genetic selection strategy. Towards this aim we used Pvec activity to drive an antibiotic resistance gene expression during ESCs differentiation and we provide evidence that a cardiovascular progenitor population can be isolated by this strategy. We further show that this population has the capacity to self-renew and differentiate to cardiac and endothelial cells under specific cell culture conditions. Moreover, these cells survived and differentiated after direct intramyocardial transplantation in the left ventricle of adult rats.

2. Materials and Methods

2.1. Plasmids

2.1.1. Pvec. An ~2.5 kb fragment containing mouse VE-cadherin promoter elements and the first nontranslated exon was derived by PCR using primers AGCAGAAACAAGGTCCTCTGGAAGAG (sense) and TCACTTACCTTGTCCGTGAGC (antisense) from a mouse BAC library as template, further subcloned in Topo-XL vector (Invitrogen).

2.1.2. Ppvec-puro. The following subcloning steps were performed: Construct A, the chimeric gene and stuffer fragment of pPyCAGIP (an episomal vector, kind gift from Professor A. Smith, Wellcome Trust Centre for Stem Cell Research, University of Cambridge, UK), was inserted in the Topo-XL vector downstream of the mouse Pvec by SchI/EcoRI-blunt ligation. The SpeI/XhoI fragment from construct A was ligated to pPyCAGIP. Finally, a hygromycin resistance gene was inserted at NdeI blunt/SalI.

2.1.3. Ppvec-puro-EGFP. EGFP coding sequence (from pEGFP-N) digested with Xho/NotI was ligated to the same sites of pPvec construct.

2.2. Cell Culture. E14T Embryonic Stem Cells (ESCs) were kindly provided by Professor A. Smith and Dr. I. Chambers (MRC Centre for Regenerative Medicine, Edinburgh, UK). They were propagated on gelatin (0.1% swine skin), in high glucose Glasgow modified Eagle's medium (Sigma) supplemented with LIF conditioned medium, 15% FBS (Biochrom), 1 mM Sodium Pyruvate (Invitrogen), 2 mM L-Glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 0.05 mM β-mercaptoethanol (Sigma), 100 u/mL penicillin, and 0.1 mg/mL streptomycin (Invitrogen).

2.3. Stable ES Cell Lines. 5×10^6 ESCs were electroporated with 20 μg of DNA in 600 μL PBS at 200 V and 960 uF in a 0.4 cm cuvette using BTX-ECM600 electroporator (Harvard Apparatus). After 24 h and for the following 14 days, cells were selected with hygromycin (150 ug/mL to 120 ug/mL). Resistant clones were isolated and propagated individually.

2.3.1. ESCs Differentiation

(a) In Mass Culture. 1×10^6 ESCs were seeded on 100 mm bacteriological Petri dishes in differentiation medium DM1. DM1 is Iscove's modified Dulbecco's medium (IMDM), supplemented with 15% FBS, 2 mM L-Glutamine, 100 u/mL penicillin, 0.1 mg/mL streptomycin, 5 ng/mL human VEGF (ImmunoTools), 30 ng/mL human bFGF (ImmunoTools), and 5 μg/mL Ascorbic Acid (Sigma). Briefly, ESCs were trypsinized and suspended in DM1 medium, and cells were cultured for three days in a 37°C humidified incubator with 5% CO^2.

(b) By "Hanging Drops." ESCs were seeded at 500 cells per 20 μL drop in DM1 and cultured in hanging drops for 2 days. EBs formed were collected and plated on bacterial Petri dishes for further differentiation.

2.3.2. Puromycin Selection during Differentiation. EBs were formed in mass culture for three days in culture medium DM1 that supports endothelial and cardiac differentiation. At day 3 EBs were dissociated by mild trypsin treatment and seeded on fibronectin coated tissue culture plates. For selection, puromycin (0.75 ug/mL to 1.5 ug/mL) was added at day 4.5 to day 8.

2.3.3. Differentiation of CEDPs. CEDPs were trypsinized and suspended in DM2. DM2 is Iscove's modified Dulbecco's medium (IMDM), supplemented with 15% FBS, 2 mM L-Glutamine, 100 u/mL penicillin, 0.1 mg/mL streptomycin, 10 ng/mL human VEGF (ImmunoTools), 30 ng/mL human bFGF (ImmunoTools), and 5 μg/mL Ascorbic Acid (Sigma). Spheres formed in low attachment plates were cultured in a 37°C humidified incubator with 5% CO^2.

2.3.4. Differentiation of CEDPs in Alginate (Pronova, Oslo, Norway). CEDPs were resuspended at a density of 1×10^6 cells/mL in 1.1% gelatinized alginate solution. This solution was added dropwise in 1 M $CaCl_2$ pH7.4 through an insulin syringe and the encapsulated cells were washed in 0.9% NaCl, resuspended in DM2 medium. To increase viability of CEDPs, they were mixed with gelatinized alginate solution and were injected in the left ventricle of rats in vivo. Under these conditions, alginate solution is known to cross-link with endogenous calcium ions, quickly forming hydrogel [22].

2.3.5. In Vivo Study Population and Ethics. The in vivo experiments were conducted on 15 Wistar rats (all male, 17–20 weeks of age, weighing 280–320 g). The animals were housed in plexiglas-chambers in groups of two or four, with free access to water and standard rodent pellet-diet. The housing facilities at the University of Ioannina adhere to international guides and offer stable conditions, in terms of temperature (20–22°C), humidity (60–70%), and light-to-dark cycles (12 : 12 h). The experimental procedures followed the guiding principles of the Declaration of Helsinki, regarding ethical conduct of animal research, and conformed to European legislation (European Union directive for the protection of animals used for scientific purposes 609/1986, revised in 2010/63/EU). The study protocol was approved by the Department of Agricultural Economy and Veterinary Medicine, Ioannina, Prefecture of Epirus, approval number 6003, 19/04/2013.

2.4. Implantation Protocol. Following anesthesia induction with isoflurane-inhalation via mask, the rats were intubated and mechanically ventilated using a rodent apparatus (model 7025, Ugo Basile); anesthesia was maintained with a mixture of oxygen and 2.5% sevoflurane.

Via left lateral thoracotomy, the heart was exposed, and the pericardium was removed; implantation was performed by intramyocardial injections, as described previously [23]. In brief, the heart was exteriorized and slight traction was applied via a 6-0 suture, passed through the apex, thereby facilitating manipulations and providing support during injections. A total of 0.2 mL of normal saline ($n = 5$) or alginate-hydrogel with CEDPs ($n = 10$) was administered by six intramyocardial injections in the anterolateral LV wall, as in previous experiments [24] occasional bleeding stopped after light pressure was applied locally. The incision was closed in three layers and pneumothorax was evacuated. For analgesia, a single intraperitoneal injection of an opioid-analgesic (buprenorphine, 0.05 mg/kg) was administered postoperatively.

2.5. Immunosuppression Protocol. To prevent allograft rejection, low-dose immunosuppression was administered, as outlined previously [25, 26]. Specifically, cyclosporine (10 mg/kg) was administered orally by gavage, starting from the day prior to implantation, until the end of the experiment.

Heart specimens were harvested three ($n = 3$), seven ($n = 3$), and 14 days ($n = 4$) after implantation. The animals were anesthetized (as described above), and the site of previous thoracotomy was reopened. The aorta, pulmonary artery, and superior and inferior vena cava were clamped; the heart was excised and quickly immersed in normal saline. Subsequently, hearts were processed for immunocytochemistry or RNA isolation.

2.6. Immunocytochemistry. EBs and spheres were allowed to attach on gelatinized glass coverslips for 2 days before staining. Cells were fixed in 4% formaldehyde for 10 min at RT. Subsequently, they were incubated with 3% BSA containing 0.2% Triton-X100 for 30 min and primary antibody labeling was performed at 4°C O/N, followed by incubation with the secondary antibody for 1 h. For microscopy, rat hearts were fixed in 4% formaldehyde for 2 h and then 30% sucrose overnight and then embedded in OCT, sectioned and stained using standard protocols. In brief, frozen tissue sections were permeabilized with 100% ice-cold methanol for 10 minutes at −20°C and rinsed in PBS for 5 minutes. Antibody labeling was carried out as above, with the exception that primary antibody was diluted in 0.2% fish skin gelatin and labeling was performed for 1 h at room temperature.

2.6.1. Antibodies. For immunocytochemistry, the following antisera were used: rat monoclonals against VE-cadherin (11D4.1, BD Biosciences), PECAM-1 (MEC 13.3, Santa Cruz), and E-Cadherin (DECMA-1, Santa Cruz), mouse monoclonals against cardiac Troponin T (CT3, Iowa Hybridoma Bank), Isl1 (39.4D5, Iowa Hybridoma Bank), Oct3/4 (C-10, Santa Cruz), SMA (Neomarkers), N-cadherin (clone 3B9, Invitrogen), MyHC (MF20, Iowa Hybridoma Bank), and a-actinin (Clone BM-75.2, Sigma), goat polyclonals against GATA4 (C-20, Santa Cruz) and Isl1 (GT15051-100, Acris Antibodies), rabbit monoclonals against MEF2c (D80C1) and VEGF receptor 2 (Flk1) (55B11) from Cell Signaling, and rabbit polyclonals against EGFP (kindly provided from Dr. Charalambia Boleti, Pasteur Institute, Athens), Desmoplakin 1/2 [27], and DSC2 (DSC2, RDI Research Diagnostics, Inc.).

2.7. Confocal Microscopy. Confocal images were taken in a Leica confocal microscope (LCS SP5) using the LAS AF Lite software. Pictures were further manipulated with Fiji (NIH Image) and/or Adobe Photoshop (Adobe) software.

2.8. RNA Isolation, rt-PCR, and Quantitative rt-PCR. RNA was isolated using TRIzol reagent according to manufacturer's protocol (Invitrogen). To synthesize cDNA 1 μg of purified RNA was used in 20 μL reaction, using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara). Quantitative real time PCR analysis was performed with one-twelfth or one-sixth of the cDNA reaction as template, using KAPA SYBR® FAST qPCR Kit Master Mix (Kappa) in Bio-Rad CFX96 for 45 cycles. All samples were analyzed in triplicates. All values were normalized with respect to GAPDH and β-actin expression levels, translated to relative values. Analysis was performed by qBase plus software (Biogazelle). Primer sequences are shown in Supplementary Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/8305624.

2.9. FACS Analysis. Cells during selection from differentiating cultures were trypsinized using 0.25% trypsin-EDTA and EGFP expression was analyzed by FACS (Partec CyFlow Space). Data obtained were analyzed with FCS Express 4 software (Flow Research Edition).

2.10. Cell Growth Analysis. 1×10^6 Pvec$^+$ cells were seeded in a 30 mm tissue culture plate. Upon reaching approximately 80% confluence, the cells were treated with 0.05% trypsin-EDTA solution (Gibco) for detachment and counted before reseeded in a new tissue culture plate and cultured until the next passage. This process was repeated up to ten passages.

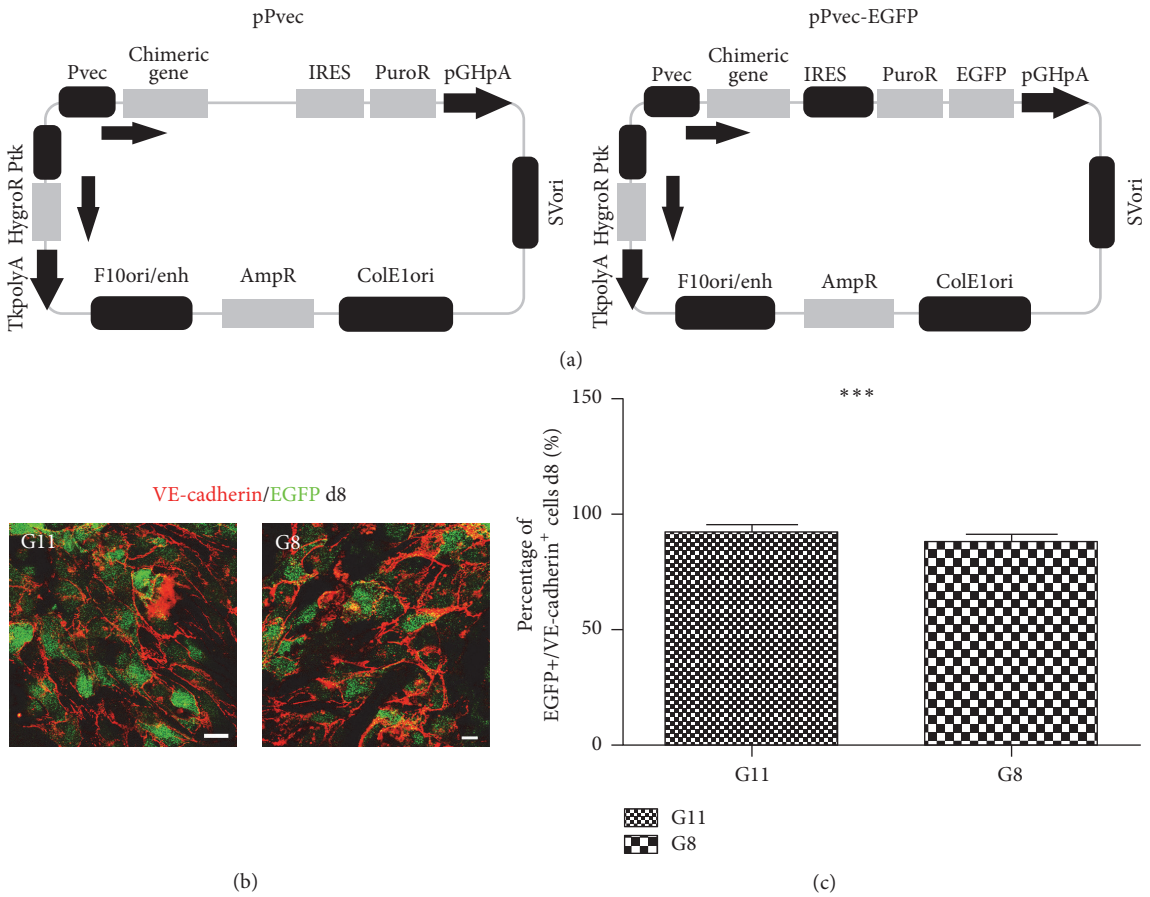

Figure 1: Generation and characterization of genetically modified ES clones expressing puromycin resistance gene under Pvec. (a) Schematic representation of episomal constructs pPvec and pPvec-EGFP, used for generation of ES clones. (b) The majority of VE-cadherin$^+$ cells express EGFP in EBs derived from clones G11 and G8 at d8. (c) Statistical analysis of the percentage of VE-cadherin$^+$ cells coexpressing EGFP at d8 from three independent experiments. For each experiment ~600 VE-cadherin$^+$ cells were counted. $^{***}P < 0.001$. Scale bar: 20 μm.

The doubling time was calculated using Doubling Time Computing [28].

2.11. Statistical Analysis. Statistical Analysis was performed with GraphPad Prism 5 Software. Data represent the mean ± SD from three independent experiments. The statistical significance of difference was determined by one-way ANOVA followed by Tukey's Multiple Comparison Test. Probability values $P < 0.05$ were considered significant. For calculating the total cell numbers in heart tissue (Figure 7(e)) the computer algebra system Maple 18 and package Curve Fitting were used.

3. Results

3.1. Isolation of Cells with Cardiac and Endothelial Phenotypes during ES Cells Differentiation by Selection Based on Pvec Activity. In order to isolate ES-derived Pvec$^+$ cells we genetically modified E14T ESCs. E14T cells were electroporated with an episomal plasmid containing the genes for puromycin resistance under Pvec and hygromycin resistance under Thymidine Kinase promoter (Figure 1(a)). Hygromycin resistant clones A11 and A12 (Pvec-ESCs) were isolated and expanded. In similar experiments, EGFP-expressing clones G8 and G11 under Pvec were also isolated (Pvec-EGFP ESCs) (Figure 1(a)). Clones were positive for pluripotency markers Oct4, Sox2, and E-cadherin and their differentiation properties were examined (Supplementary Figure S1). Tissue-specific Pvec activity was assessed during in vitro differentiation of G8 and G11 by EGFP expression (Figure 1(b)). EBs formed at d8 were double-stained for VE-cadherin and EGFP and the percentage of double-positive cells was calculated. More than 90% of EGFP$^+$ cells were also VE-cadherin$^+$, an indication of high promoter specificity (Figure 1(c)). VE-cadherin was found predominantly in the plasma membrane, as well as in the cytoplasm, forming nascent adherens junctions at this early developmental stage (Figure 1(b)).

Next, we analyzed the surviving products of Pvec- and Pvec-EGFP ESCs during differentiation in puromycin selection DM1 medium (Figure 2(a)) (see Section 2). Pvec- and Pvec-EGFP-ESCs resistant cells were observed at d10, in contrast to wtESCs-derived cells that were eliminated (Figure 2(b)). FACS analysis for EGFP$^+$ cells showed that they were 26% of the total cells at d4.5 and increased to 40% at d6 and 60% at d7 and d8 (Figures 2(c) and 2(d)).

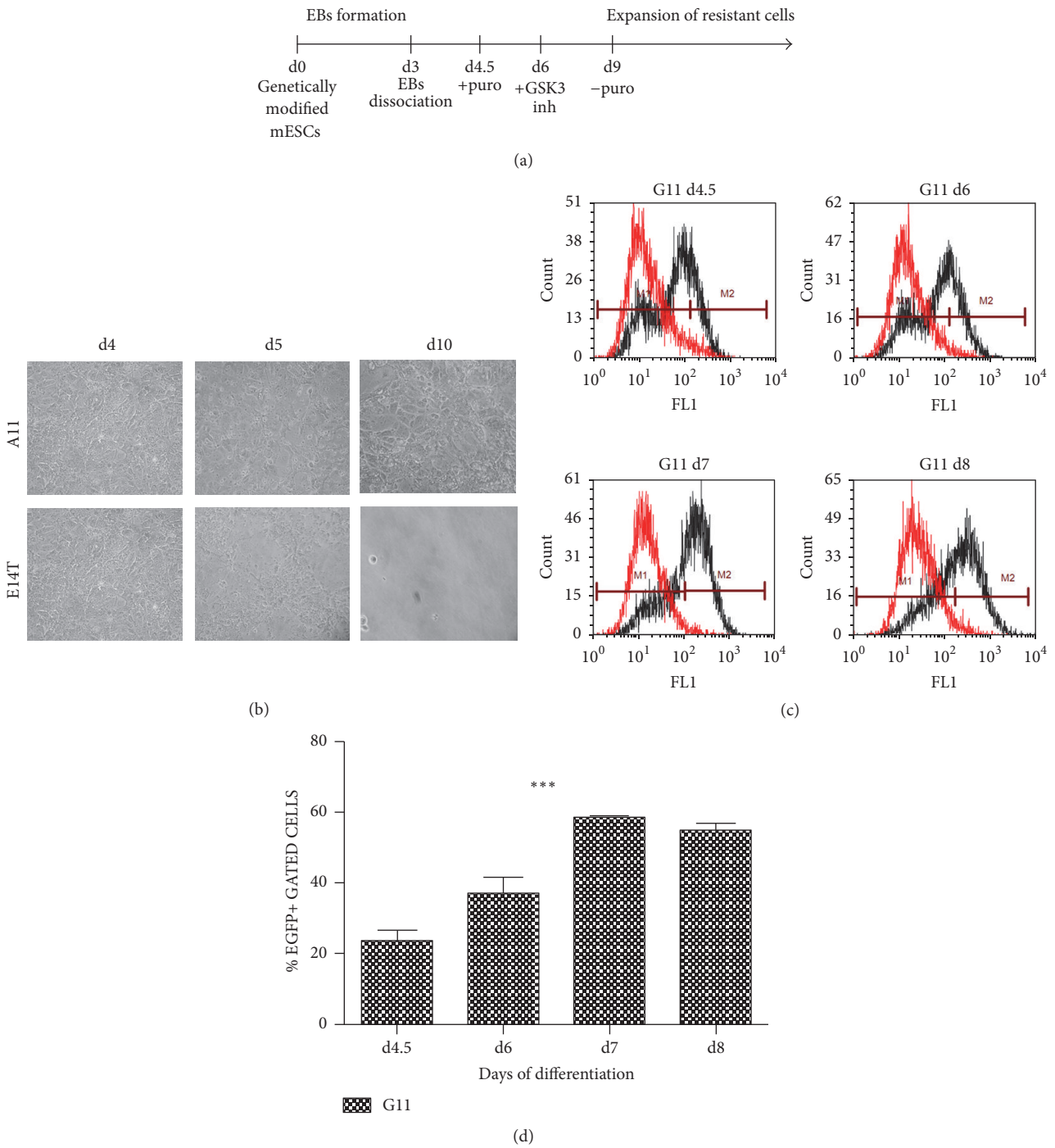

Figure 2: Continued.

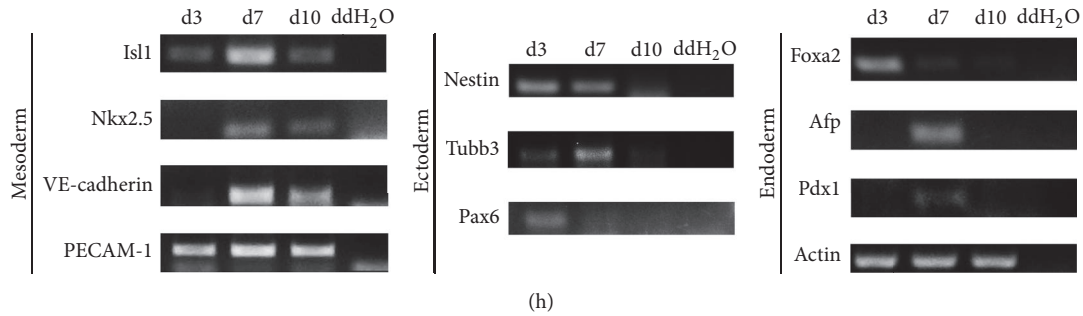

Figure 2: Selection of cells with Pvec activity during differentiation. (a) Schematic representation of selection strategy during differentiation of genetically modified ESCs to isolate Pvec$^+$ cells. (b) Optical microscope images of clone A11- and E14T-derived cells, during selection at d4 (before the addition of puromycin), d5, and d10 (selected cells). (c) Quantification of EGFP$^+$ cells during clone G11 differentiation/selection at d4.5, d6, d7, and d8 by FACS analysis (representative results are shown). (d) Analysis of FACS data from three independent experiments. $^{***}P < 0.001$. (e–g) G11-derived EGFP$^+$ selected cells at d7 coexpress VE-cadherin, Isl1, and GATA4. Note that EGFP was detected at low Isl1 and GATA4 expressors. (h) Expression of mesodermal, endodermal, and neuroectodermal markers during selection of clone A11 at d3, d7, and d10 by RT-PCR analysis.

Surviving cells were further analyzed for cardiac and endothelial specific markers expression by immunofluorescence at d7. They expressed endothelial VE-cadherin and PECAM-1 (Figures 2(e) and 2(h)), as well as cardiac developmental stage-specific markers Isl1, GATA4, and Nkx2.5 (Figures 2(f), 2(g), and 2(h)). Interestingly, VE-cadherin-mediated adherens junctions were compromised in the presence of puromycin. No endodermal or neuroectodermal specific markers were detected by RT-PCR at d3, d7, and d10 (Figure 2(h)). As control, we performed differentiation and selection experiments of clone A11 under the same conditions with hygromycin instead of puromycin. Since the episomal vector Pvec contains hygromycin resistance under the ubiquitous Thymidine Kinase (TK) promoter (Figure 1(a)), A11 cells survived and differentiated efficiently to neuroectodermal, endodermal, and mesodermal lineages (Supplementary Figure S2).

3.2. Activation of Wnt/β-Catenin Pathway Induces Propagation and Self-Renewal of Selected Pvec$^+$ Cells. We observed that VE-cadherin$^+$ and Isl1$^+$ surviving cells could not grow further after d14 in DM1 medium. In an attempt to expand Isl1$^+$ cells, we induced Wnt/β-catenin, a signaling pathway known to support self-renewal of Isl1$^+$ cardiac cells (Figure 3(a)). When SB-216763 [29], a GSK3 inhibitor, was added at days 5–8, significant expansion of Isl1$^+$ cells was observed at d12 (Figure 3(b)).

The percentage of Isl1$^+$ cells calculated by cell-counting was found to exceed 60% of total cells (Figure 3(c)). These properties were maintained for at least eight passages up to 30 days and multiple freeze and thaw cycles. Afterwards their growth declined substantially. The doubling time of Pvec$^+$ cells in the presence of SB-216763 calculated after growth curve generation between day 1 and day 28 was ~4.5 days (Figure 3(d)).

Cells were examined for self-renewal by cardiomyocyte differentiation stage-specific markers expression. We analyzed for cardiac progenitor markers GATA4 and Mef2c expression and found that Mef2c, a marker of the AHF (anterior heart field), is coexpressed with Isl1 and GATA4 in the majority of cells (Figures 3(e)–3(g)). During SB-216763 induced propagation it became evident that endothelial cells also survived, proliferated efficiently, and formed extensive VE-cadherin-mediated adherens junctions and PECAM-1 junctions, as shown by immunofluorescence staining (Figures 3(h)–3(j)). Isl1, VE-cadherin, and GATA4 expression levels were quantified and compared between Pvec$^+$ cells expanded in SB-216763 and Pvec$^+$ cells after selection by qPCR. In SB-treated cultures Isl1 was found upregulated 2.7- and 4.7-folds, GATA4 was upregulated 1.5- and 3.6-folds, and VE-cadherin was upregulated 1.8- and 3-folds in two biological independent selection/expansion experiments (Figure 4(a)).

Further cardiac differentiation was inhibited as they were negative for MLC2v, MLC2a, and cTnT, although they coexpressed Desmin and Isl1 (Figures 4(b), 4(c), and 4(d)). This is consistent with the role of Wnt/β-catenin signaling pathway known to induce proliferation and inhibit Isl1$^+$ cells differentiation.

Endothelial cells also did not differentiate further, as vWF and CD39 markers of mature endothelium were not expressed (Supplementary Figure S3). Interestingly, expression of Nfatc1 and Nrg1 was detected, implying the presence of endocardial cells (Figure 4(d)). In control experiments pluripotency markers Nanog and Sox2 were not expressed in these cells, shown by RT-PCR analysis (Figure 4(d)).

Thus, under the protocol described above propagation of a cardiac/endothelial dual-progenitor population (called CEDPs) was achieved.

3.3. CEDPs Differentiation to Cardiac and Endothelial Cells In Vitro. We examined next the potential of CEDPs to differentiate further towards cardiac and endothelial cell types. For this purpose, CEDPs were cultivated in differentiation medium DM2 in the presence or absence of SB-216763 in low adhesion plates. SB-216763 inhibited differentiation, evident by small spheres formation with no beating activity. In contrast, in the absence of SB-216763 approximately 3-4-fold

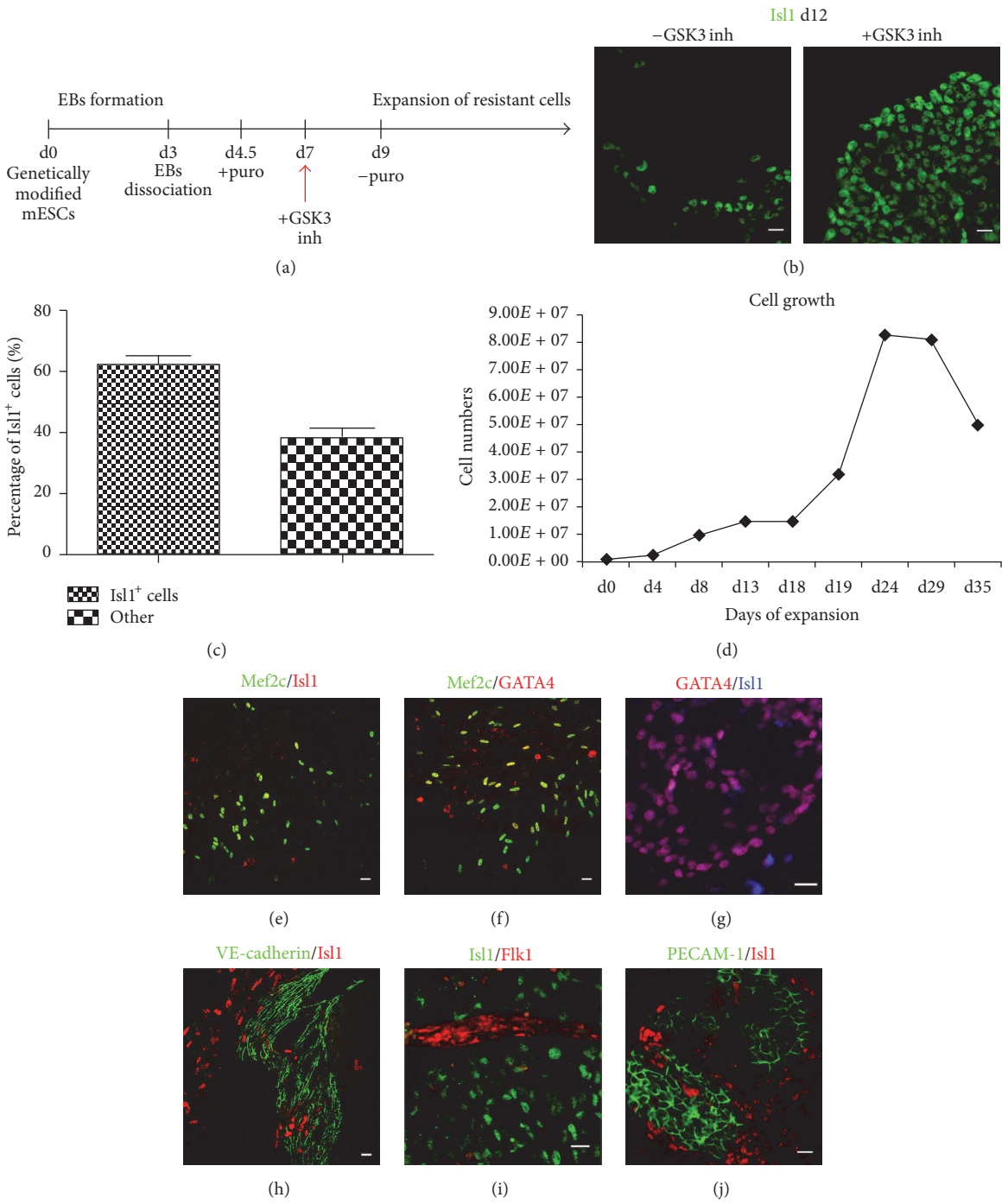

Figure 3: Expansion of Pvec$^+$ cells. (a-b) Schematic representation of expansion strategy and propagation of Pvec$^+$ cells. Addition of GSK3 inhibitor (SB-216763) induces propagation of Pvec$^+$ cells expressing Isl1 at d12. (c) Pvec$^+$/Isl1$^+$ cells percentage in SB-216763 from three independent experiments. For each experiment ~1000 cells were counted. (d) Pvec$^+$cell growth curve. (e–j) Propagated cells consist of cell populations expressing markers of cardiac (Isl1, Mef2c, and GATA4) and/or endothelial (Flk-1, PECAM-1, and VE-cadherin) progenitors.

larger spheres with beating phenotype were formed after 10 days (Figure 5(a) and Supplementary Figure S4). Spheres contained extensive areas of cTnT$^+$ or MyHC$^+$ cells coexpressing adhesion molecules Desmoplakin and Desmocollin 2, an indication of intercalated disk structures formation between cardiomyocytes (Figures 5(c), 5(d), and 5(e))). MLC2a and MLC2v markers of differentiated cardiomyocytes were also detected in such cultures by RT-PCR analysis, in contrast to CEDPs (Figures 5(f) and 4(d)). Induction of endothelial markers vWF and CD39 and VE-cadherin$^+$ cobblestone structures formation observed during differentiation of CEDPs indicate maturation of endothelial cells (Figures 5(g)–5(i)). Interestingly, cells expressing progenitor markers Isl1 and Mef2c could be detected in cardiac and endothelial cells,

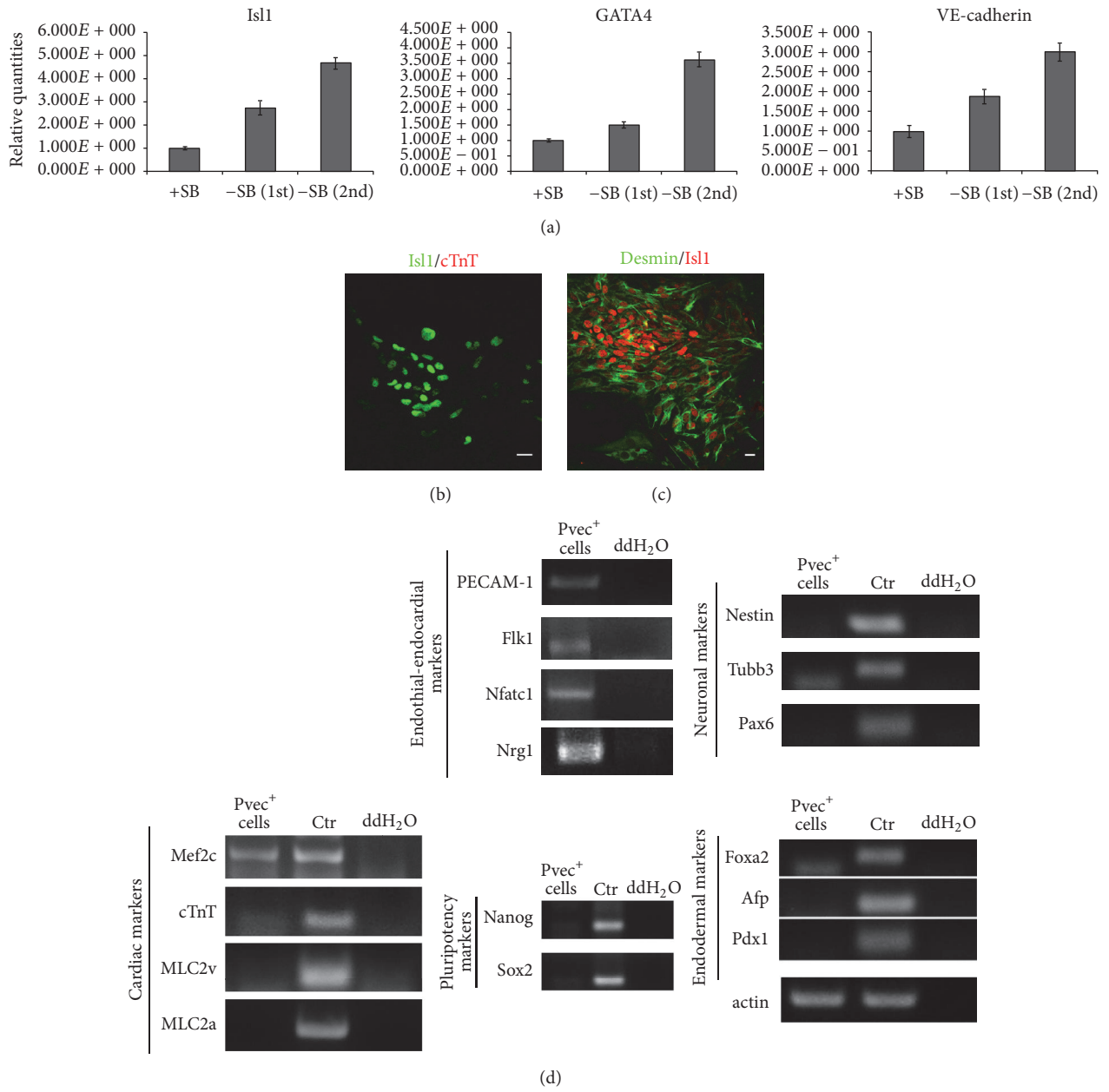

Figure 4: Characterization of Pvec$^+$ cells. (a) Isl1, GATA4, and VE-cadherin mRNA levels quantification in Pvec$^+$ cells in the presence or absence of SB-216763 by real time qPCR. Results in -SB first and -SB second referred to two independent selection/differentiation experiments. (b-c) Isl1$^+$ cardiac progenitors express desmin but not cTnT, a marker of differentiated cardiomyocytes. (d) RT-PCR analysis of expanded Pvec$^+$ cells showed expression of cardiac, endothelial, and endocardial but not neuroectodermal, endodermal, or pluripotency markers. As positive controls (ctr) mRNA from E14T ESCs differentiation was used. Note the lack of cTnT, MLC2v, and MLC2a expression in expanded Pvec$^+$ cells. Scale bar: 20 μm.

respectively, during differentiation (Figures 5(j) and 5(k)). In addition, SMA$^+$ cells could also be observed (Figure 5(l)).

Cardiac and endothelial cells derived during CEDPs differentiation at day 12 were quantified by counting cTnT$^+$ and VE-cadherin$^+$ cells in three independent experiments (total of 10417 cells). We found that 47% of cells were cTnT$^+$, 28% were VE-cadherin$^+$, and the remaining nonendothelial, noncardiac cells (approximately 25%) were positive for smooth muscle actin or vimentin (Figure 6(a) and Supplementary Figure S5), possibly representing a cell population related to cardiac mesenchyme. cTnT, VE-cadherin, Isl1, GATA4, Nkx2.5, and Flk1 expression levels were quantified and compared in CEDPs and in CEDP-derived differentiation cells at day 12 by qPCR. We observed upregulation of cTnT, Nkx2.5, VE-cadherin, and Flk1 and downregulation of Isl1 and GATA4 (Figure 6(b)). These results show that CEDPs can differentiate further to cardiomyocytes.

In control experiments differentiation towards endoderm and neuroectoderm was not observed in spheres, since lineage-specific markers (FoxA2, AFP, and Pdx1 for endoderm

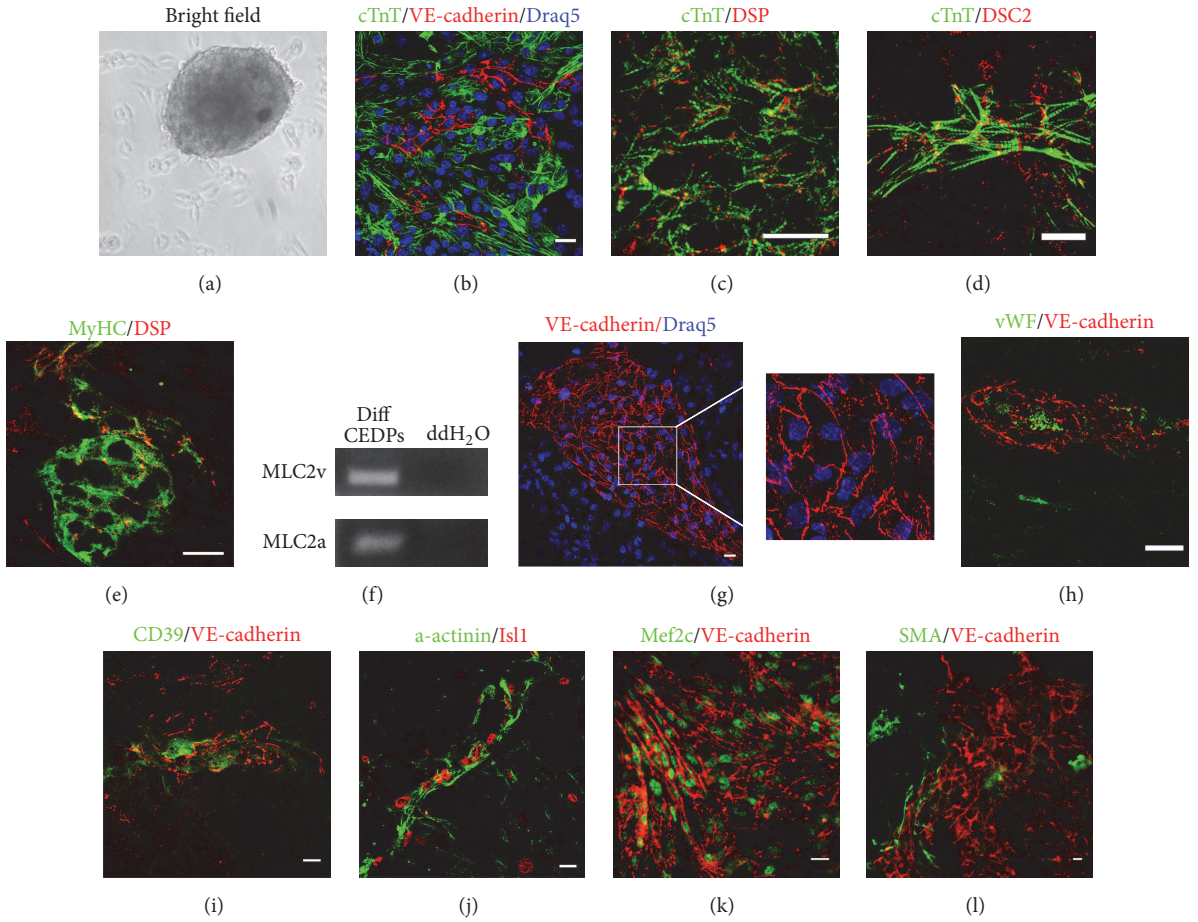

Figure 5: CEDPs differentiation potential. (a) Optical microscope image of a sphere formed in the absence of SB-216763 after 5 days of CEDPs differentiation. (b) cTnT$^+$ cells formation after immunostaining with anti-cTnT and anti-VE-cadherin. (c–e) Formation of intercalated disk structures in beating spheres after 10 days of CEDPs differentiation, shown by double-IF-staining with anti-cTnT/anti-Desmoplakin (DSP), anti-cTnT/anti-Desmocollin-2 (DSG2), and anti-MyHC/anti-Desmoplakin. Note the striations of the sarcomeric cTnT staining and the punctate desmosomal staining in areas connecting adjacent cardiomyocytes. (f) Expression of MLC2v and MLC2a after 10 days of CEDPs differentiation by RT-PCR analysis. (g) VE-cadherin$^+$ cells in CEDPs-derived differentiated cells. Note extensive adherens junctions formation between endothelial cells. Magnification corresponds to marked area. (h-i) Expression of vWF and CD39 in VE-cadherin$^+$ endothelial cells after 10 days of CEDPs differentiation. (j-k) Expression of Isl1 and Mef2c progenitor markers in a-actinin$^+$ and VE-cadherin$^+$ cells, respectively, at day 10 of CEDPs differentiation. (l) Expression of SMA during CEDPs differentiation. Draq5 counterstained DNA (b, g). Scale bar: 20 μm.

and Tub-b3, nestin, and Pax6 for neuroectoderm) were not detected by RT-PCR analysis (Supplementary Figure S6).

CEDPs differentiation properties imply that they could potentially represent a progenitor population useful for cardiac regeneration. Therefore, we examined their differentiation efficiency in alginate, a hydrogel biomaterial commonly used as a scaffold in transplantation experiments [30]. CEDPs were encapsulated in alginate and cultured in DM2 medium as above (Supplementary Figure S7). Spheres were formed and remained up to 10 days in hydrogel when the biodegradable material started to disintegrate. We observed that CEDPs differentiated in alginate in a similar manner compared to liquid DM2 media, evident by cardiac beating, observed after 12 days.

3.4. Survival and Differentiation of CEDPs after Transplantation. Survival and differentiation of CEDPs were examined in vivo after transplantation in the left ventricle (LV) of immunosuppressed rats. Distinction between CEDPs-derived and endogenous cardiac cells was based on cardiac progenitor marker Isl1 present only in CEDPs and their progeny but not in the LV of the adult heart. For this purpose, the LV areas from CEDPs or saline recipient rats were dissected and examined after 3, 7, and 14 days by immunocytochemical and RT-PCR analysis for survival and differentiation of CEDPs. Immunostaining of frozen sections demonstrated in all cases that Isl1$^+$ cell populations appeared specifically in CEDPs- but not in saline-injected animals. RT-PCR analysis also showed that Isl1$^+$ expression could not be detected in this heart portion (Figure 7(a)). Expression of pluripotency markers Oct4 and Nanog was not detected in transplanted animals examined (Supplementary Figure S8).

Differentiation of CEDPs was monitored by RT-PCR for MLC2v, a differentiation marker not expressed in CEDPs using a mouse-specific primer set (Figure 4(a) and Supplementary Figure S9).

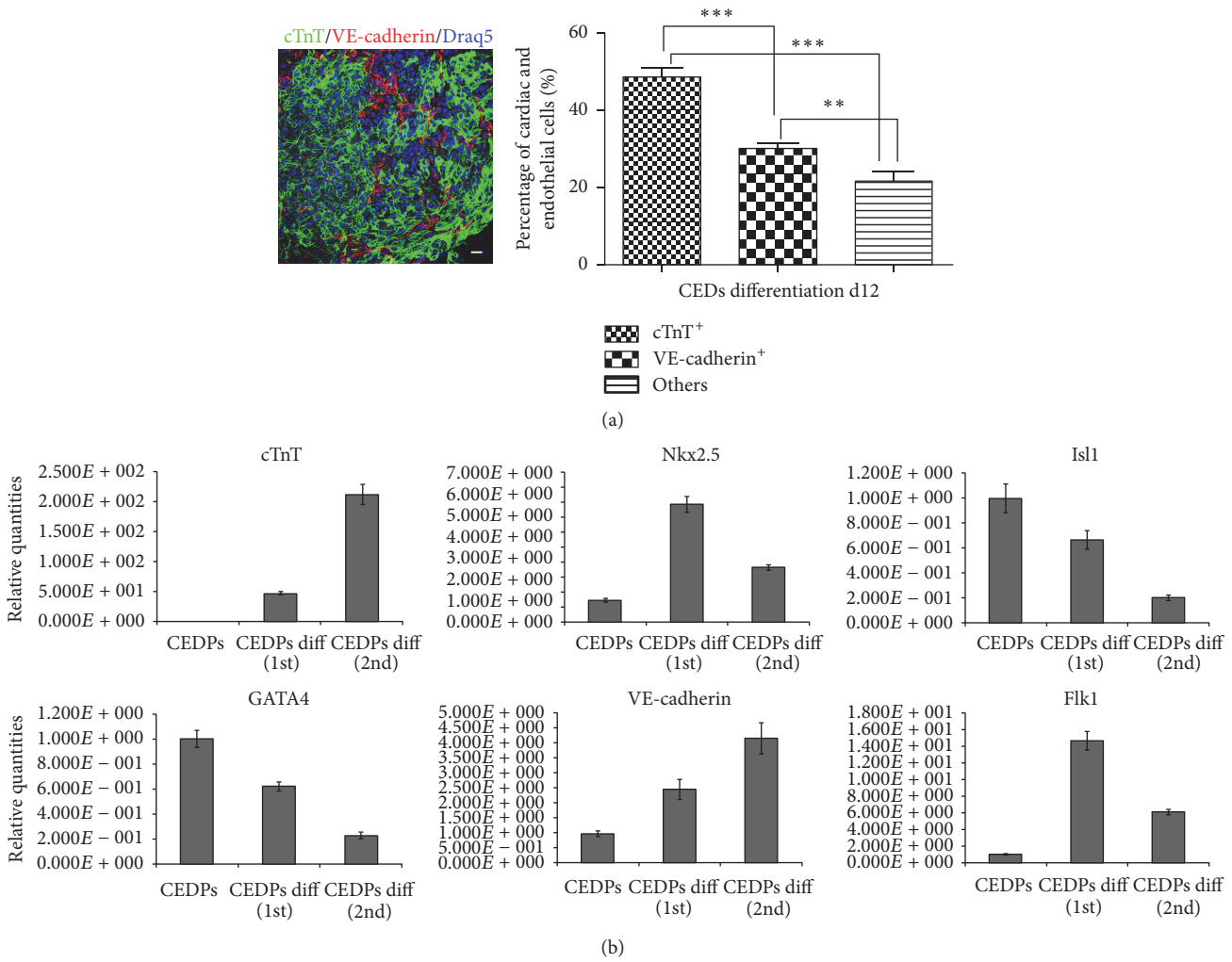

FIGURE 6: Characterization of CEDPs-derived products. (a) Statistical analysis of cTnT$^+$ and VE-cadherin$^+$ cells in CEDPs-derived differentiated cells at d12. The total number of cells counted was 10417 in three independent experiments. $^{***}P < 0.001$ and $^{**}P < 0.01$. Cells from random fields were photographed (as in representative image left) and counted by Fiji cell-counter. Scale bar: 20 μm. (b) cTnT, Nkx2.5, Isl1, GATA4, VE-cadherin, and Flk1 mRNA levels quantification in CEDPs-derived differentiated cells at d10 by real time qPCR. Results in CEDPs differentiation first and CEDPs differentiation second referred to two independent differentiation experiments.

CEDP-derived cells were detected on the 3rd, 7th, and 14th day after transplantation (Figures 7(a)–7(d)). In vivo differentiation of CEDPs was induced 3 days after transplantation shown by MLC2v expression on the 3rd, 7th, and 14th day after transplantation (Figure 7(a)).

The numbers of Isl1$^+$ cells that survived 7 days after transplantation were quantified in dissected LVs. For this purpose, a 4 mm area was sectioned (400 sections, 10 μm each). Six different planes were chosen (at 1.4 mm, 1.8 mm, 2.4 mm, 2.8 mm, 3.4 mm, and 3.7 mm) and ten sections around each plane were stained with anti-Isl1. Isl1$^+$ cells were found between 1.4 and 3.7 mm (280 sections, approximately 2.8 mm) and counted. Based on Isl1$^+$ cells counted on two nonsequential sections/planes (Supplementary Table S2) we produce a piecewise object of interpolating splines and plot this expression. Then we evaluate the total number of cells by taking the integral of the function in the interval [110, 370] and found that Isl1$^+$ cells were approximately 1,31 $\times$ 10^5 (Figure 7(e)). Considering that Isl1$^+$ cells percentage is ~60% of CEDPs (Figure 3(c)); surviving Isl1$^+$ cells represent 21.8% of the initially injected.

4. Discussion

Genetic strategies based on tissue-specific activity of promoters are established to isolate ES-derived cell types. Cardiac specific promoters α-MHC and MLC2v were used to isolate highly purified cardiomyocytes during differentiation of genetically modified pluripotent ESCs [31–36]. In addition, the activity of developmental stage-specific promoters like Flk1, Isl1, and Nkx2.5 was used to isolate cardiac precursor cells from ES or iPS cells [21, 37–43].

4.1. Main Findings. We provide evidence that a novel population can be isolated by Pvec activity during ESCs differentiation. The endothelial specific activity of the (−2486, +24) fragment of mouse VE-cadherin promoter has been

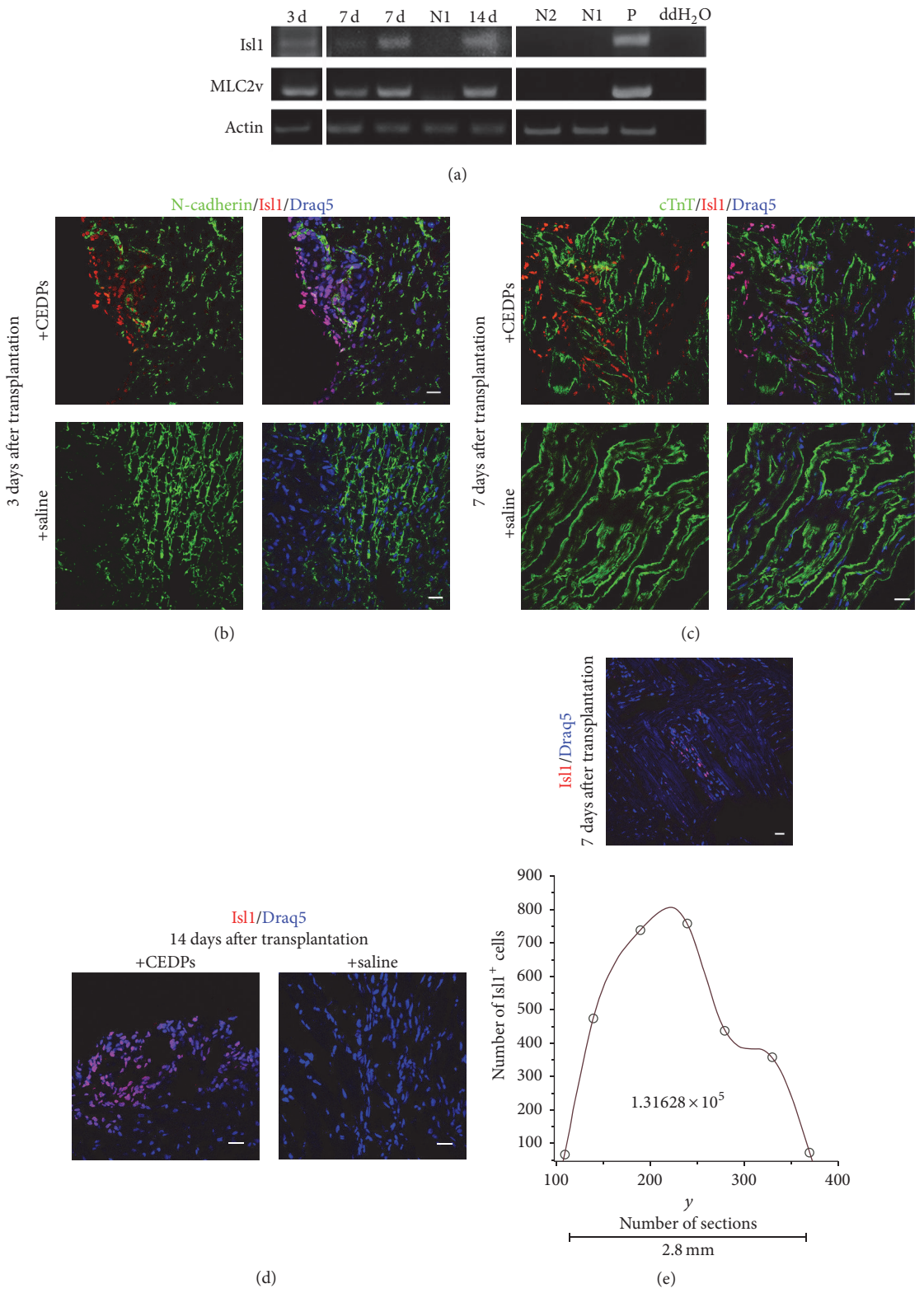

Figure 7: Transplantation of CEDPs in adult rats' heart. (a) Expression of Isl1 and MLC2v after 3, 7, and 14 days of transplantation by RT-PCR analysis. mRNA from saline-injected immunosuppressed (N1) or untreated (N2) LV of adult rats was used as negative controls and mRNA from E12 mouse embryos (P) as positive control. (b–d) Clusters of $Isl1^+$ cells derived from CEDPs were detected in frozen sections of LVs isolated 3, 7, and 14 days after transplantation. Sections were stained for DNA with Draq5 and with anti-N-cadherin (b) and anti-cTnT (c). Sections from isolated LVs of saline-injected immunosuppressed rats were used as control for each time-point. (e) All $Isl1^+$ cells from two nonsequential sections were photographed (as in representative upper image) and counted with Fiji cell-counter. Draq5 used for DNA staining. The total $Isl1^+$ cell number found in 2.8 mm tissue was calculated after spline interpolation. Scale bar: 20 μm.

demonstrated in transgenic animals and during ESCs differentiation [13]. It has been further utilized for endothelial lineage tracing, endothelial specific knock-out mutant mice generation, and isolation of ES-derived endothelial cells [14, 16]. However, transient activation of Pvec was also found in hemogenic endothelium, a progenitor population that differentiated to endothelial and definitive hemopoietic cells lineages [19]. This demonstrates that precise timely evaluation of Pvec activity is crucial for selection experiments.

We have previously studied in detail the Pvec activity pattern during ESCs differentiation. Perhaps surprisingly, we found that it was transiently activated in a subset of $Isl1^+$ multipotent cardiovascular progenitors between days 4 and 5; based on this finding, we selected cells surviving at this time-window. This genetically based approach resulted in isolation of $Pvec^+$ cell population with endothelial- and cardiac-progenitor phenotypes but not endodermal or neuroectodermal phenotypes, an indication of specific selection. In our tissue culture system, utilizing activation of Wnt/β-catenin signaling resulted in propagation of the $Pvec^+$ selected progenitor cells. Genetic approaches utilized Pvec to isolate endothelial cells. In these cases Pvec activity combined with FACS selection at day 8 of ES differentiation was used resulting in predominantly endothelial cell populations cells [14, 44]. Isolated CEDPs on the other hand is a complex cell population that has the ability for self-renewal for at least 30 days and is comprised from endothelial, cardiac, endocardial, and mesenchymal cell types. Therefore, it clearly represents a different and novel cell population. The most striking difference between our study and others resulting in cardiac progenitor cells (CPCs) isolation is that we chose a strategy allowing simultaneous isolation and expansion of endothelial and cardiac progenitors. For this purpose, our strategy was based on two steps: initially $Pvec^+$ cells selection and subsequently $Isl1^+$ cells expansion. Wnt/β-catenin induced expansion of $Isl1^+$ cells was in agreement with previous reports [45]. However, quite unexpected self-renewal driven by Wnt/β-catenin pathway activation was observed for endothelial progenitors as well, a finding that requires further investigation. Thus, two independent populations with self-renew capacity were isolated, in contrast to previous studies, where endothelial cells were derived only after CPCs differentiation [21, 41]. Endothelial progenitors could improve their differentiation and viability properties of cardiac progenitors in a synergistic manner.

4.2. Wnt Signaling. Wnt signaling is pivotal for progenitor cells proliferation in a variety of tissues, such as the skeletal muscle [46] and the neuronal [47, 48], hemopoietic [49], and cardiac [50, 51] tissues. Its role during cardiac differentiation has been studied in detail and its activation was found to be essential for proper cardiac specification, progenitor expansion, and myocardial growth. Specifically, activation of Wnt/β-catenin at the emergence of $Isl1^+$ cells resulted in the clonal expansion of these cardiovascular progenitors [45, 50]. At a later stage, when Wnt/β-catenin is downregulated, $Isl1^+$ cells differentiated to cardiac and endothelial cells [21].

We report that, in addition to cardiac, proliferation of endothelial cells was achieved under our culture conditions in $Pvec^+$ selected cells. This finding is important, given the inconclusive results of previous studies on the role of Wnt signaling on endothelial progenitors [52]. Whether positive regulation of endothelial progenitors by GSK3 inhibition is the result of Wnt activation on a subset of $Pvec^+$ cells or, alternatively, whether they derive from $Isl1^+$ cardiovascular progenitors remains to be seen.

4.3. Self-Renewal of Cellular Populations. Isolation of progenitor cells with cardiovascular potential, able to self-renew, is particularly interesting, since their differentiation is restricted to cardiac, endothelial, and smooth muscle cell types. These cells could provide a source for cardiac regeneration upon transplantation [39, 53, 54]. In the present study, we used the VE-cadherin promoter activity pattern to isolate a novel, dual-progenitor cell population. These cells could self-renew, under Wnt/β-catenin pathway stimulation and differentiate further to endothelial, cardiac, and smooth muscle cells. Dual differentiation to endothelial cells and cardiomyocytes can be viewed as an advantage, since it results in formation of vascularized transplants, with enhanced survival potential after transplantation [55].

4.4. Cell-Survival In Vivo. Survival of mouse origin transplanted cells in rat recipient animals was based on Isl1 expression as a positive marker. The $Isl1^+$ cell population declines during mammalian embryogenesis and can be detected predominantly in sinoatrial node in the adult heart but not in the left ventricle [56]. Weinberger et al. reported $Isl1^+$ cells presence in the sinoatrial node of the adult heart, a subpopulation derived from embryonic $Isl1^+$ cells. In our experiments, we described the isolation of the $Isl1^+$ population that emerges during cardiovascular development and differentiates to form a major part of the adult heart (left ventricle, part of atria, and the outflow tract endothelium). Future studies will show its differentiation potential to specific cardiac subpopulations.

In our study, we transplanted CEDPs in the left ventricle and all immunocytochemical and PCR analysis was performed in isolated left ventricle portion of the heart. Therefore, we believe that $Isl1^+$ cells represent a genuine CEDPs-derived population. Based on this feature, we showed that transplanted cells survive and differentiate in the adult heart for at least 14 days, which underlines their potential in cell-based therapy for myocardial infarct.

The concept of a novel cardiac and endothelial population described in this manuscript could be potentially valuable for the field of cardiac regeneration when followed in the human system with necessary modifications. The strategy for clinical exploitation would require establishment of culture conditions of human ESCs (or iPSCs) towards cardiac differentiation in defined, serum-free medium. The $VE\text{-}cadherin^+/Isl1^+$ cell population can then be isolated and used further based on early VE-cadherin expression between days 4 and 5 in human by FACS sorting [57].

5. Conclusions

We isolated and characterized a novel cardiovascular progenitor cell population that has the capacity to self-renew

and further differentiate to endothelial, cardiac, and smooth muscle cells in vitro and in vivo. These cells can be used in a cell-based therapy for myocardial infarct and in drug screening. Further characterization is needed, focusing on myocardial structures, such as the T-tubule system, the sarcolemma complex, and gap junctions.

Competing Interests

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

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Review Article

Therapeutic Potential of Stem Cells Strategy for Cardiovascular Diseases

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Despite development of medicine, cardiovascular diseases (CVDs) are still the leading cause of mortality and morbidity worldwide. Over the past 10 years, various stem cells have been utilized in therapeutic strategies for the treatment of CVDs. CVDs are characterized by a broad range of pathological reactions including inflammation, necrosis, hyperplasia, and hypertrophy. However, the causes of CVDs are still unclear. While there is a limit to the currently available target-dependent treatments, the therapeutic potential of stem cells is very attractive for the treatment of CVDs because of their paracrine effects, anti-inflammatory activity, and immunomodulatory capacity. Various studies have recently reported increased therapeutic potential of transplantation of microRNA- (miRNA-) overexpressing stem cells or small-molecule-treated cells. In addition to treatment with drugs or overexpressed miRNA in stem cells, stem cell-derived extracellular vesicles also have therapeutic potential because they can deliver the stem cell-specific RNA and protein into the host cell, thereby improving cell viability. Here, we reported the state of stem cell-based therapy for the treatment of CVDs and the potential for cell-free based therapy.

1. Introduction

Cardiovascular diseases (CVDs) are a major cause of morbidity and mortality that have a significant impact on health care systems and financial and social consequences. It is estimated that CVDs will be responsible for 23.3 million global cardiovascular deaths worldwide in 2030 [1]. CVDs include diseases that have different causes and characteristics [2]. There is a state of acute conditions such as myocardial infarction and chronic diseases induced by genetic mutations [3]. Classic treatments of CVDs, such as physical surgery and medicinal treatment, are not sufficient to recover damaged cardiovascular tissue and instead only delay the progression of CVDs.

Stem cells, including embryonic stem cells (ESCs), adult stem cells, and induced pluripotent stem cells (iPSCs), are useful in the field of regenerative medicine because they have pluripotency and self-renewal. In addition, stem cells have beneficial effects such as paracrine effects [4, 5], anti-inflammatory activity [6, 7], and immunomodulatory capacity [8]. However, transplantation of stem cells for treatment of heart disease is hampered by their potential for differentiation into host cell types, such as cardiac cells or blood vessel cells, to hinder cardiac function by causing arrhythmia [9]

and their low therapeutic effects and viability under the harsh conditions of damaged tissue [10]. To overcome these problems, establishment of new strategies is needed for priming of stem cells.

Interestingly, it has recently been reported that miRNA, small molecules, and extracellular vesicles can modulate the biological activity of stem cells, such as their survival [11, 12], migration [13], and differentiation [14–16]. According to reports, they have been attracting attention owing to their potential to overcome the limitations associated with stem cell therapy. Moreover, a previous study of miRNA small molecules and extracellular vesicles demonstrated that they were effective in the treatment of CVDs. Based on these studies, the stem cell therapy can contribute to treatment of CVDs.

In this review, we describe the state of stem cell therapy for the treatment of CVDs and evaluate the potential for the use of miRNA or stem cell-derived extracellular vesicles.

2. Cardiovascular Diseases and Stem Cells

2.1. Cause of CVDs. CVDs include a range of major clinical disease conditions, such as cardiomyopathy, hypertensive heart failure, valvular heart disease, peripheral arterial disease, and coronary artery disease [25, 26]. Senescence and male typically raise hazards of CVDs and are primary criteria used to classify risk evaluation [27, 28]. CVDs are strongly connected to lifestyle, age, and gender, with alcohol and tobacco consumption, physical inactivity, psychosocial factor, diet and obesity, and increased blood pressure being major factors [28]. In addition, psychosocial risk elements including low socioeconomic status, deficiency of social support, stress, depression, anxiety disorder, and hostility contribute to development of CVDs [28–34]. Familial prevalence of CVDs is another major risk factors [28]. Nutrient deficiency including low vitamin D levels also plays a role in CVDs pathogenesis [35]. Several studies have shown that vitamin D affects heart function through regulation of hormonal systems including the parathyroid hormone and renin-angiotensin system [36, 37]. Vitamin D has also been shown to affect the cell cycle of cardiomyocytes, to inhibit cardiac cell proliferation, and to protect the structure and function of cardiomyocytes [35, 38, 39]. Hypertension is the most common CVD, leading to a growing risk of stroke, myocardial infarction, and heart and renal failures [40]. Previous clinical tests have indicated that decreased blood pressure reduces the outbreak of CVDs such as stroke and heart attack [40]. There have been various efforts to overcome CVDs in the past few decades [41]. Despite advanced medical and surgical trials, there are still no effective therapies for treatment of CVDs [42, 43]. Unlike other organs, the recuperative ability of heart is limited for treatment of injured cardiac tissue [43]. Heart transplantation can be utilized as a last resort to treat CVDs, but the approach is expensive and extremely limited for patients because of their comorbidities or poor supply of donor organs [43].

2.2. Embryonic Stem Cell Therapy for CVDs. Recent studies have suggested that stem cell therapies target cardiac regeneration in CVDs [42, 43]. Application of stem cells to therapeutic devices and methods may lead to effective and rapid myocardium regeneration and eventually affect cardiovascular morbidity and mortality [44]. There are different types of stem cells such as embryonic stem cells, adult stem cells, and induced pluripotent stem cells for treatment of CVDs (Table 1). Previous studies have demonstrated therapeutic effects of ESCs differentiated into cardiomyocytes [17] and endothelial cells [18] for myocardial infarction, umbilical-cord-blood-derived MSCs for dilated cardiomyopathy [19], bone-marrow-derived-MSCs for cellular cardiomyoplasty [20], and iPSCs [21] and iPSCs-derived cardiovascular progenitor cells [22], endothelial progenitor cells (EPCs) [23], and cardiac stem cells [24] for myocardial infarction.

2.2.1. Therapeutic Characteristics of ESCs. ESCs are pluripotent cells derived from the inner cell mass and infinitely replicate without senescence, maintaining their undifferentiated state [45]. This cell population is called ESCs and follows irreversible process of differentiation to become specialized [44, 46]. ESCs have pluripotency, with the capacity of differentiation into approximately 210 different cell types, making them an up-and-coming stem cell source for cell-based tissue engineering [44]. ESCs can be differentiated into cardiomyocytes, endothelial cells, and vascular smooth muscle cells [17, 18, 47, 48]. Owing to their potential for use in the treatment of CVDs, efforts for stem cell therapy have been concentrated on the differentiation of human ESCs into the cardiac lineage directly [44, 49].

2.2.2. Using ESCs for Myocardial Regeneration Therapy. In previous studies, undifferentiated ESCs or ESCs differentiated into cardiomyocytes, endothelial cells, or vascular cells were directly injected into animal CVDs models [17, 18, 50]. Treatment with those ESC-derived cells showed beneficial effects such as improvement of cardiac regeneration and remodeling and increased myocardial performance [1, 17, 18, 50]. Therapeutic effects of the differentiated cells are mediated through cell engraftment and proliferation and paracrine effects [1, 51, 52]. Recent studies have suggested that genetic and epigenetic regulations of cardiomyocytes differentiation are new approaches to inducing a cardiac lineage for stem cell therapy [43]. Deletion or knockdown of microRNA (miRNA), a small regulator of gene expression, brings about dysregulation of morphogenesis, electrical conduction and hypertrophy of heart, and the cell cycle of cardiocytes [14, 43, 53]. Ivey et al. demonstrated that miR-1 and miR-133 can control the ability to differentiate into the cardiac lineage in mouse and human ESCs [53]. Moreover, epigenetic modification can regulate ESCs differentiation and genetic control [43]. Weber et al. suggested that histone deacetylation is involved in cardiovascular development through target regulation of hey bHLH as major effector in Notch signaling [54]. Although ESCs have considerable potential for direct differentiation into cardiac lineage in various models, several limitations hinder their clinical applications [41]. The greatest limitation is that research using ESCs is hampered by ethical issues that prevent their actual clinical applications [41, 55].

Table 1: Application of stem cells for therapy of CVDs.

Disease model	Stem cell type	Delivery route	Dose and follow-up duration	Outcomes	Reference
Rat MI	ESC-CMs	Intramyocardial transfer	1.5×10^6 8 weeks	Observation of grafted cardiomyocytes survival, proliferation, maturation, alignment, and forming gap junctions with host cardiac tissue	[17]
Mouse MI	ESC-ECs	Intramyocardial transfer	1×10^6 8 weeks	Appropriate patterns of endothelial gene expression, functional vessels formation *in vivo*, and cardiac function improvement	[18]
Mouse DCM	UCB-MSCs	Intramyocardial transfer	1.5×10^6 4 weeks	Improvement of cardiac function by antiapoptosis, anti-inflammation, and proangiogenesis	[19]
Mouse cellular cardiomyoplasty	BM-MSCs	Intramyocardial transfer	$5–10 \times 10^6$ 4–8 weeks	Engrafted hMSCs from adult BM in the myocardium to differentiate into cardiomyocytes	[20]
Mouse MI	iPSCs	Intramyocardial transfer	1×10^5 2 weeks	Improved iPSCs maintenance through improved function and cell proliferation in infarcted myocardium	[21]
Mouse MI	iPSC-CPCs	Intramyocardial transfer	2×10^5 2–4 weeks	Exertion of protective effect on LV remodeling by paracrine effects through enhanced angiogenesis and augmented networking in the infarcted milieu	[22]
Rat MI	EPSs	Intramyocardial transfer	1×10^6 7 weeks	Increase of regional wall motion and decrease of ventricular dimension in left ventricle	[23]
Rat MI	CSCs	Intramyocardial transfer	5×10^6 2–4 weeks	Reduction of ejection fraction, fractional shortening, and infracted size of the left ventricle	[24]

BM: bone marrow; CMs: cardiomyocytes; CPCs: cardiovascular progenitor cells; CSCs: cardiac stem cells; DCM: dilated cardiomyopathy; ECs: endothelial cells; EPCs: endothelial progenitor cells; ESC: embryonic stem cell; h: human; iPSC: induced pluripotent stem cell; MI: myocardial infarction; MSCs: mesenchymal stem cells; and UCB: umbilical cord blood.

Moreover, treatment with ESCs poses the risk of immunorejection because of differences in genome information among patients [41, 56].

2.3. Adult Stem Cell Therapy for CVDs

2.3.1. Therapeutic Characteristics of MSCs. In adult bodies, tissues and organs contain a small cell subpopulation with the capacity for self-maintenance through the potential to proliferate indefinitely and the ability to form an extended family of daughter cells [57, 58]. These cells are widely known as adult stem cells [7]. Mesenchymal stem cells (MSCs) are found in most of adult tissues, including bone marrow and adipose tissues [58, 59]. The nonhematopoietic cells can be differentiated and modified *in vitro* to present phenotypes of cardiomyocytes and vascular endothelial cells [58]. In addition, MSCs are able to produce and secrete a broad variety of cytokines, chemokines, and growth factors for enhancing neovascularization, attenuating fibrosis in heart, and recovering cardiac functions [4, 5, 60]. Accordingly, it is possible that MSCs could be a therapeutic cell source with the capacity to repair injured tissue in CVDs.

2.3.2. Treatments of Myocardial Diseases by Using MSCs. Bone-marrow-derived MSCs (BM-MSCs) have been widely reported as a promising therapeutic strategy for CVDs [61]. These cells can differentiate into cardiomyocytes and endothelial cells [20, 61]. Many studies have indicated that BM-MSCs possess therapeutic effects in heart diseases such as myocardial infarction, diabetic cardiomyopathy, and dilated cardiomyopathy [61–63], and BM-MSCs are now considered one of the most attractive adult stem cell populations for cardiovascular repair [61, 64]. Cai et al. showed that BM-MSCs cocultured with neonatal rat ventricular cardiomyocytes prevented isoproterenol-induced typical hypertrophic characteristics of cardiomyocytes in *in vitro* and *in vivo* studies [61]. Moreover, interplay of BM-MSCs with cardiomyocytes produced synergistic effects on VEGF secretion [61]. Today, many studies showed that paracrine factors, such as VEGF, bFGF, and IGF-1, play an essential therapeutic role [4]. Tang et al. demonstrated that autologous BM-MSCs transplantation in rat MI model improved vascular regeneration and cardiac performance through paracrine effect of VEGF, bFGF, and SDF-1 [5]. Ohnishi et al. also found conditioned medium of BM-MSCs affected the antiproliferation of cardiac fibroblasts via expressing paracrine antifibrotic effects of MMPs [60]. Adipose tissue-derived MSCs (AD-MSCs) have become an attractive therapeutic cell source [65] because they are easily expanded *in vitro* and express the same cell surface markers as BM-MSCs [65, 66]. Moreover, injected AD-MSCs have been shown to differentiate toward a cardiogenic phenotype [67] and reduce the infracted size, exhibiting a powerful and persisting angiogenic potential [65, 68]. Siciliano et al. reported on plasticity of human AD-MSCs and their phenotypical modification in cardiac-specific microenvironments [65]. They also indicated that human AD-MSCs cocultured with cardiospheres-conditioned media changed toward a cardiac/endothelial/muscular-like phenotype in response to regulation of the expression of cardiogenic markers and induction of the activation of intracellular survival signaling pathways [65]. Umbilical-cord-blood-derived MSCs (UCB-MSCs) are a new xenogeneic stem cell therapy source for CVDs [19]. The newly proposed cell source may be optimum for CVDs because they have a low immunogenicity and a large change of cardiomyocyte reprogramming of UCB-MSCs in comparison with xenogeneic stem cells [19, 69–71]. In addition, UCB-MSCs are easily obtained through low-invasive surgery without raising ethical issues, demonstrating their promising clinical application [19]. Gong et al. demonstrated that intramyocardial grafts of human UCB-MSCs promote cardiac function via mechanisms of antiapoptosis, anti-inflammation, and proangiogenesis in cardiomyopathy of $cTnT^{R141W}$ transgenic mice [19]. They also found that UCB-MSCs derived conditioned medium protects H9C2 cells from apoptosis in hypoxic condition by paracrine effects *in vitro* [19].

2.3.3. Treatments of Myocardial Diseases by Using Other Stem Cells (CPCs, EPCs). The tissue-specific stem cells were found in several tissues, such as skeletal muscle, brain, fat, liver, gastrointestinal tract, and epidermis. These stem cells are differentiated into specific tissue cells and contribute to maintaining tissue homeostasis. Interestingly, the tissue-specific stem cells were found in heart [72]. Heart was not known to be able to self-regenerate before the establishment of cardiac progenitor cells (CPCs). Since then, cardiac progenitor cells have been reported on therapeutic potential in CVDs.

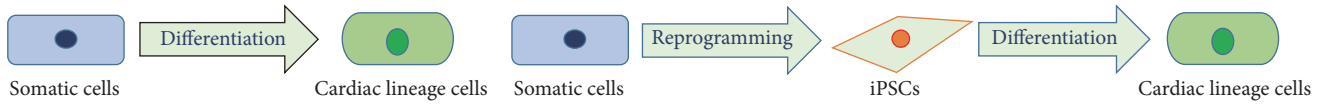
Beltrami et al. reported on Lin^{-} $c\text{-}Kit^{POS}$ cells in CPCs. They are self-renewing and multipotent and have colony forming ability [72]. Injected CPCs into the ischemic heart can be differentiated into cardiomyocytes, reconstruct heart, and induce new blood vessel. They suggest that CPCs to repair the heart provide a new opportunity. However, CPCs present in very small amounts in the heart and require *in vitro* expansion of a few weeks [73].

In 1997, Asahara et al. reported that isolated $CD34^{POS}$ cells are endothelial progenitor cells which are separate from peripheral blood [74]. EPCs also have capacity of differentiation into endothelial cells and angiogenesis. For that reason, EPC was noted in the study for treating various ischemic injury. Kawamoto et al. studied effects of heart regeneration by transplantation of EPCs into rat MI heart in 4 weeks after transplantation [23]. According to this paper, the effect of regeneration is caused by angiogenesis which is promoted by transplanted EPCs into ischemic damage area.

Another strategy is reported based on the stimulation of the EPCs *in vivo* for treating ischemic disease. Oikonomou et al. reported that 26 patients with heart disease have improved blood vessel function after administration of atorvastatin for 4 weeks by increasing circulated EPCs [75].

Transplantation of EPC has benefit for treatment of disease, but we need to concern about immune rejection in the allograft because therapy using EPCs is based on the autograft method. Therefore, we need to overcome this limitation for transplantation into damaged area.

2.4. iPSCs as a Source of Cell Therapies for CVDs. In 2006, iPSCs were established by Takahashi and Yamanaka by



(a) Direct differentiation (b) iPSCs reprogramming

Category	Direct differentiation	iPSC reprogramming
Differentiation capacity	Limited-fibroblast self-renewal	Easily scalable
Time consuming for differentiation	Short	Long (over 10 days)
Cell fate stable	Unclear	Stable
Tumor risk	Unclear tumor risk during reprogramming	Teratoma and overgrowth
Clinical application	Lack of differentiated cells for transplantation	Various results *in vivo* and *in vitro*
Limitation	Low conversion efficiency, heterogeneous cell population, and risk of viral action in clinical trial	Proliferation capacity, cell type diversity, senescence, and limited cell type diversity

Figure 1: Strategies for differentiation from somatic cells into cardiac lineage cells.

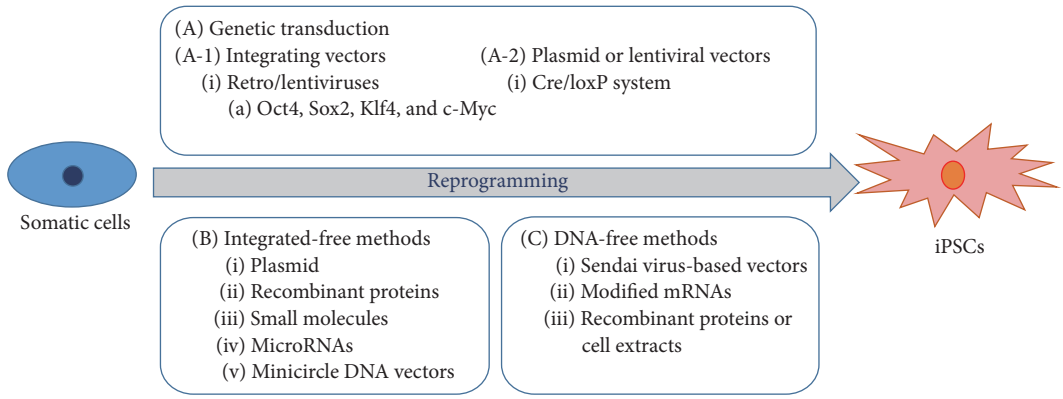


Figure 2: Two ways for inducing pluripotent cells from somatic cells.

reprogramming mouse fibroblasts through overexpression of four specific transcription factors: Oct3/4, Sox2, c-Myc, and Klf4 [76]. Since this breakthrough, various opportunities for application have been reported in cell therapy, modeling of new diseases, and studies of complex genetic features and allelic variations, as substrates for drug, in toxicity, differentiation, and regenerative medicine therapies, and in therapeutic screening [77, 78]. In addition, using the iPSCs, it is possible to differentiate into patient- and disease-specific cell types. Thus, using iPSC is one of the tools for treatment of the patients [79, 80]. However, many considerations, including the optimal materials for reprogramming, high cost, safety, and efficient derivation, still remained regarding using iPSCs for clinical applications [81]. Thus, it is essential to understand the following events after the activation of reprogramming factors for transplantation of iPSCs for a safe way.

2.4.1. Methods for Differentiation from iPSCs to Cardiomyocytes. Recently various efficient methods for inducing differentiation into cardiac lineage cells using iPSCs or direct reprogramming (Figure 1). iPSCs have been tried to use genetic transduction or integrated-free methods. Using viral vector for reprogramming has higher efficiency than using integrated-free methods, but safety is lower than using integrated-free methods (Figure 2) [82]. Another method for differentiation into cardiac lineage cells is direct differentiation. One of the protocols for direct differentiation is overexpression of combined cardiac-specific transcription factors such as GATA4, Mef2c, and Tbx5 (GMT) [83, 84] or GMT and Hand2 [85]. Another method is using miRNAs or small molecules without lineage-specific transcription factors: overexpression of miRNAs 1, 133, 208, and 499 is effective to increase the capacity of direct differentiation into cardiac lineage cells [86]: chemically defined medium (CDM) containing three components is also reported that can induce differentiation into cardiomyocytes [87]. Recently, Hou et al. also reported that treatment of seven small molecules into cells can generate iPSCs [88]. Direct differentiation into cardiac-specific lineage may provide the therapeutic strategy for cardiac regeneration; however, it needs to improve the low efficiency of differentiation into the cardiac lineage cells [84].

2.4.2. Treatment of CVDs by Transplanted iPSCs. iPSCs have characters like embryonic stem cells (ESCs)—pluripotency and self-renewal—so that iPSCs can be provided to be transplanted into damaged tissue or organ [89]. Masumoto et al. reported cardiac regeneration by transplantation of engineered human iPSC as a cardiac tissue sheet (hiPSC-CTSs) into rat heart [90]. They checked that heart function was improved and survival of transplanted cells was over 40% cells. Rojas et al. performed transplantation of iPSCs with fibrinogen into heart of myocardial injury model [21]. Although heart function was recovered by transplantation of iPSCs into damaged heart, they suggested transplantation of iPSC-derived cardiomyocytes is more relevant for clinical trial. In addition, the problems of transplantation of iPSCs including tumorigenicity, immunogenicity, and genomic instability have been reported [88, 91–94]. Recently, transplantation of differentiated cells into tissue-specific lineage is reported to overcome the aforementioned problems; Funakoshi et al. reported transplantation of iPSC-derived cardiomyocyte and they tried to optimize condition for transplantation [95]; and Ja et al. showed the effects of the transplantation of iPSC-derived human cardiac progenitor cells improved heart function [22]. They suggested that the improvement of heart function is caused by angiogenesis and interstitial networking of damaged heart.

2.5. Cell-Free Therapeutic Strategy for CVDs. Extracellular vesicles have been reported to modulate a variety of biological actions in the cells, such as proliferation, migration, apoptosis, and differentiation. Stem cell-derived extracellular vesicles especially have potent cardiac protection, regeneration, and angiogenic properties. In addition to affordable benefits, it is well known the vesicles can be used to communicate with other cells and control level of protein expression. Thus, transplantation of extracellular vesicles has the potential as a novel cell-free therapy for treatment of CVDs, which has various advantages to overcome the limitations related to the cell-based therapeutics.

2.5.1. Treatment of CVDs by Extracellular Vesicles-Derived Stem Cells. Various reports have emphasized the effectiveness of secretion factors during treatment of heart disease using stem cells. Paracrine effect is one of the benefits by transplantation of stem cells into the damaged area causing secretion of various cytokines and growth factors [4, 5]. Recently, many studies have investigated cell-derived extracellular vesicles, and they have been shown to exert positive effects on a variety of cellular activities such as antiapoptosis, migration, differentiation, and cell recruitment [96]. In fact, extracellular vesicles have a biologic function of communication between the cells and recipient cells. Several studies have reported that the extracellular vesicles contained various factors including nucleic acids, cytokines, growth factor, and miRNAs [97–99]. Lai et al. reported that extracellular vesicles can promote repair of damaged heart by myocardial ischemia/reperfusion injury [100]. They have demonstrated that the exosome was contained in the stem cell conditioned media so that it contributed to the cardiac protection. Lee et al. demonstrated that mesenchymal stromal cell-derived exosomes (MEX) inhibited vascular remodeling and hypoxic pulmonary hypertension [101]. They also demonstrated that MEX inhibit the inflammatory response and cell proliferation of vascular pulmonary hypertension in animal models. They suggested that the reason of the advantages was based on the downregulation of miR-17 cluster and stat3 signaling in MEX-treated vessel cells. Khan et al. reported that ESC-derived exosome promoted the repair of ischemic myocardium [102]. Therapeutic potential of ESC-derived exosome stimulates CPCs activity including survival, cell cycle progression, and proliferation, by overexpression of ESC-specific miR-294. Furthermore, therapeutic effects of CPCs-derived exosomes have been reported [103]. Vrijsen et al. reported that CPCs-derived exosomes promote cell migration *in vitro* wound assay [104]. According to their report, CPCs-derived exosomes include EMMPRIN and MMP, to induce endothelial cell migration. Chen et al. reported a myocardial infarction protective effect of the CPCs-derived exosome [105]. CPCs-derived exosome contains higher level of miR-451 so it can protect H9C2 cells from oxidative stress by inhibiting caspase-3 and caspase-7 activation.

Although there are a lot of experiments that have studied transplantation of extracellular vesicles which have beneficial effect in treating CVD *in vitro* and *in vivo*, we still need to consider side effects on other organs, appropriate amount for transplantation, and compatibility for clinical practices in human. Therefore, more researches which targeted safety to use for therapeutic approaches using extracellular vesicles are required in future clinical trials.

2.6. Limitations of Stem Cell and Cell-Free Therapy Strategies for CVDs. Stem cell therapy should be more considered for efficient clinical application in CVDs. Despite the many positive efforts for stem cell therapy, it has still some problems, such as low efficiency, immune rejection, and difficulty in control of stem cell behavior *in vivo*. In addition, administered stem cells often do not show effective integration or persistence in the heart tissues and trigger tumor formation [106].

On the other hand, the cell-free therapy was proposed as a means to avoid such a problem which can occur in stem cell therapy. However, the short half-life is a problem of the cell-free therapy because the proteins and nucleic acids are rapidly biodegradable *in vivo* as a main component [107–109]. For this reason, cell-free therapy must be administered more frequently. Moreover, disease specificity, biodistribution, and persistency of the cell-free factors must be validated before clinical application [110].

3. Conclusions

In this review, we focus on the strategy for treatment of CVDs by transplantation of stem cells. Transplantation of stem cells including ESCs, adult stem cells, and iPSCs can promote tissue regeneration of damaged area. Stem cells have specific characters such as self-renewal and pluripotency. In addition, stem cells secrete paracrine factors, so transplantation of which shows beneficial effects. Stem cell-derived vesicles can stimulate the cell activity by transferring beneficial

materials, including the stem cell-specific transcription factor and miRNA to the damaged tissue. Thus, transplantation of vesicle can contribute to recovery in damaged area. In addition, treatment of additional materials such as miRNA or small molecules for promoting differentiation of stem cells into specific cell types can improve therapeutic effects for treatment of CVDs.

Although stem cell therapy has problems such as low survival rate after transplantation into harsh condition and still needs to overcome these problem, stem cell therapy and cell-free based therapy have potential to improve heart function after CVDs.

Competing Interests

The authors report no conflict of interests in this work.

Authors' Contributions

Chang Youn Lee, Ran Kim, and Onju Ham contributed equally to this work.

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Review Article

Repair Injured Heart by Regulating Cardiac Regenerative Signals

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Cardiac regeneration is a homeostatic cardiogenic process by which the sections of malfunctioning adult cardiovascular tissues are repaired and renewed employing a combination of both cardiomyogenesis and angiogenesis. Unfortunately, while high-quality regeneration can be performed in amphibians and zebrafish hearts, mammalian hearts do not respond in kind. Indeed, a long-term loss of proliferative capacity in mammalian adult cardiomyocytes in combination with dysregulated induction of tissue fibrosis impairs mammalian endogenous heart regenerative capacity, leading to deleterious cardiac remodeling at the end stage of heart failure. Interestingly, several studies have demonstrated that cardiomyocyte proliferation capacity is retained in mammals very soon after birth, and cardiac regeneration potential is correspondingly preserved in some preadolescent vertebrates after myocardial infarction. There is therefore great interest in uncovering the molecular mechanisms that may allow heart regeneration during adult stages. This review will summarize recent findings on cardiac regenerative regulatory mechanisms, especially with respect to extracellular signals and intracellular pathways that may provide novel therapeutics for heart diseases. Particularly, both *in vitro* and *in vivo* experimental evidences will be presented to highlight the functional role of these signaling cascades in regulating cardiomyocyte proliferation, cardiomyocyte growth, and maturation, with special emphasis on their responses to heart tissue injury.

1. Introduction

The mammalian heart is generally considered a circulatory pump with poor reparative and regenerative capabilities due to the cell-cycle withdrawal and mitosis cessation of cardiomyocytes soon after birth. A recent study identified a few time windows in which cardiomyocytes can proliferate in preadolescent mouse hearts [1]. Indeed, the cardiomyocytes population expansion was observed during postnatal days 1–4 (P1–4) and days 14–18 (P14–18), with a 40% increase in cardiac cell number during each period. Interestingly, it is possible to achieve 100% regeneration in mouse hearts exposed to myocardial infarction at P1–4, but only 30% when treatment is delayed until P14–18, suggesting this proliferative burst actually improves reparative capability to counteract and compensate for heart injuries created in a mouse model [2]. Further fate mapping studies have also revealed that both extra- and intracellular mechanisms contribute to this temporary enhancement in proliferative ability. This review aims to highlight the most recent findings illustrating the causal mechanism links between cardiac regenerative signals and burst of cardiomyocyte proliferation.

2. Extracellular Signals

Myocardial extracellular space provides a microenvironment for tissue regeneration wherein cardiogenesis can be directly differentiated from cardiac progenitors and induced by a variety of secretory factors. It was reported recently that triiodothyronine (T3), neuregulin-1 (NRG1), follistatin-like 1 (Fstl1), and TWEAK regulate cardiogenesis and cardiac regeneration in a temporally or spatially dependent pattern (Table 1).

Table 1: Extracellular signals for cardiac regeneration.

Extracellular signals	Receptor	Signal pathway	Biological effects		Clinical effects on HF patients
			In vitro	*In vivo*	
Triiodothyronine (T3)	TR1α (thyroid hormone receptor α1)	① AMPK↑ [10] ② Regulating IGF1/IGF1-R/AKT [13] ③ ERK↑ [11]	CM growth↑ [10], CM maturation↑ [4], myofilament proteins expression↑ [12], L-type Ca^{2+} channel↓ [6]	CM proliferation and cardiac regeneration↑ [2], cardiac ischemia-reperfusion injury↓ [15–17], cardiac remodeling↓ [16, 18, 19]	Ventricular performance↑ [23], cardiac index↑ [24], cardiac function not improved [25],
Neuregulin-1 (NRG1)	ErbB receptor	① ERK1/2→MAPK↑ [34] ② PI3K→AKT↑ [35, 36] ③ FAK↑ [37]	CM survival↑ [36], calcium handling↑ [39], CM communication↑ [40, 41], myofibrillar structural damage in response to ErbB inhibition [38]	Development of cardiac conduction system↑ [45, 46], CM dedifferentiation and proliferation↑ [42, 43], myofilament organization↑ [44]	cardiac output↑, LVEF↑, systemic vascular resistance↓, ESV↓ EDV↓ [50, 51]
Follistatin-like 1 (Fstl1)	DIP2A (disconnected interacting protein 2 homolog A)	① AMPK↑ [57] ② p-AKT↑ [58] ③ ERK1/2↑ [55]	Cell apoptosis↓ [58]	Cardiac rupture↓ [55], CM proliferation↑, heart performance↑ [56]	N/A
TNF-related weak inducer of apoptosis (TWEAK)	Fn14 receptor	① TRAF→NF-κB↑ [68] ② ERK [70] ③ PI3K→AKT↑ [70]	CM cell cycle reentry↑ [70], cardiomyocyte proliferation↑ [69], CM-derived proinflammatory cytokines↑ [68], fibroblast proliferation and myofibroblast differentiation↑ [71]	Cardiac hypertrophy↑ [72], myocardial inflammatory responses↑ [73], heart performance↓ [74]	N/A

CM: cardiomyocyte; ↑: increase or intensify; ↓: decrease; LVEF: left ventricular ejection fraction; ESV: end-systolic volume; EDV: end-diastolic volume; N/A: not available.

2.1. Triiodothyronine. Triiodothyronine (T3), a thyroid hormone derived from its prohormone thyroxine (T4) through biocatalysis induced by a thyroid-stimulating hormone, is released from pituitary gland. After it is released from the thyroid body, T3 is transported into the myocardium through circulation distribution. There are two spiked T3 concentrations that appear in mouse serum during preadolescent ages. The first of these occurs at birth and drops on postnatal day 7 (P7), while another T3 peak value appears between P10 and P12 causing a 5.6-fold increase compared to basal conditions. Interestingly, pharmacological inhibition of T3 biosynthesis using propylthiouracil (PTU) significantly suppressed cardiac growth during P14-P15, a crucial period during which the population of mononucleated cells increases 2.6-fold associated with a 36-fold increase in mitosis events in the left ventricle [2]. These results indicate that T3 promotes both cardiac growth and cardiomyocyte proliferation. Studies previously revealed that T3 can promote cardiomyocyte cell-cycle withdrawal [3] and enhance cardiomyocyte maturation with downregulation of cell proliferation [4]. *In vitro* experiments in fetal sheep have indicated that the population of terminally differentiated multinucleated cells expands in association with enlarged cardiomyocyte size after T3 treatment [3]. Correspondingly, T3-dependent alterations in cardiomyocyte maturation are accompanied by changes in expression of contractility-regulating proteins, heart pacemaker, and calcium handling proteins. Particularly, abundance and maturation of multiple adrenergic receptors in differentiated cardiomyocytes are considered to be tuned up by the presence of T3. Although the molecular mechanism has not been elucidated for T3-induced cardiomyocyte proliferation, the signaling pathways that account for T3-induced cardiomyocyte maturation have been extensively studied. Thyroid hormone receptor α (TRα), a nuclear hormone receptor, has been demonstrated as the major protein molecule that receives T3 signaling. Upon T3 stimulation, TRα can bind to distal or proximal promoter elements to regulate cardiac gene expressions during myocardial growth. Indeed, TRα activation increased transcriptional activity of promoters, which subsequently regulate gene expressions of Na^+-H^+ exchanger (NHE) [5], voltage-gated potassium channel, L-type calcium channel [6], and phospholamban [7]. A recent study revealed that T3-induced cardiomyocyte growth can be abrogated by muscle ring finger-1 (MuRF-1), a muscle-specific ubiquitin ligase [8]. Such a noncanonical ubiquitination mechanism, along with the process of SUMOylation [9], posttranscriptionally modified the lysine residues and conformationally altered the ligand-binding domain (E/F) region of TRα, consequently inhibiting T3-induced TRα activation. Nongenomic effect of T3 was also revealed in adult cardiomyocyte, as evidenced by the rapid increase in phosphorylation of several kinases, AMPK [10], ERK1/2 [11], PKCdelta, p38-MAPK, and AKT, associated with the upregulation of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and α and β-myosin heavy chain (MHC). Interestingly, this activation can be inhibited when T3's binding to cell membrane was pharmacologically antagonized, indicating that this TH effect is mediated through a cell membrane-initiated mechanism [12] (Figure 1). A current study also claims that T3 coordinates with the IGF-1 signaling pathway to mediate these cardiogenesis and maturation effects, which are responsible for the key structural and functional changes of the postnatal heart [13, 14].

Intriguingly, T3/TRα is also involved in cardiogenesis and cardioprotection in the presence of ischemic injury [15–17]. In an experimental model of acute myocardial infarction, T3 treatment has been shown to improve heart performance while decreasing cardiac remodeling [16, 18, 19], favorably improving cardiomyocyte shape and the geometry of left ventricular cavity [20]. The important role of T3 in maintaining heart performance has also been demonstrated by clinical investigation. Indeed, serum concentration of T3 was significantly reduced in patients with idiopathic dilated cardiomyopathy [21], and the mortality was significantly increased in cardiac disease patients with thyroid dysfunction [22]. These clinical data indicated that administration of T3 may provide a therapeutic opportunity for heart failure and myocardial repair. Actually, beneficial effects of short-term synthetic L-T3 replacement therapy have been revealed in dilated cardiomyopathy patients with low T3 syndrome, as evidenced by the remarkable improvements in heart remodeling, the enhanced resting cardiac output, and the reduced systemic vascular resistance [23, 24]. However, these therapeutic effects were not displayed when long-term L-T3 treatment was performed on chronic and stable heart failure patients with low serum T3 level [25]. Such an ineffective response might be therapeutic timing in relation to the course of the disease. Moreover, undesirable outcomes, such as arrhythmias, myocardial ischemia, or hemodynamic instability may be encountered during T3 therapy, and an excess of triiodothyronine administration may be associated with weight loss, increased heart rate, fatigue, reduction in serum cholesterol, and suppressed TSH- all signs and symptoms associated with thyrotoxicosis.

2.2. Neuregulin-1 (NRG1). Neuregulin-1 is a 44-KD glycoprotein that serves as a direct ligand for ERBB tyrosine kinase receptors resulting in increased phosphorylation on tyrosine residues. In past few years, the major progress has been made in understanding the biological functional role of NRG1-ErbB axis in the regulation of neurodevelopment, synaptic plasticity, and synaptic transmission [26]. Indeed, NRG1 has shown the essential role in controlling rapid impulse conduction in the central nervous system through determining the myelination of an individual axon [27], and the disorders in NRG1-ErbB signaling have been etiologically implicated in schizophrenia [28], Parkinson's disease [29], Alzheimer's disease [30], and Hirschsprung disease [31]. Importantly, administration of NRG1 displayed protective effects in experimental model of neuron injury [32], and this trophic factor can significantly attenuate cognitive function and improve behavioral performance [33].

It is well accepted that a variety of different isoforms can be generated from the NRG1 gene by alternative splicing process. There are fifteen NRG1 isoforms that have been identified so far, and these isoforms are distinguished based on their N-terminal sequence, receptor affinity-determined

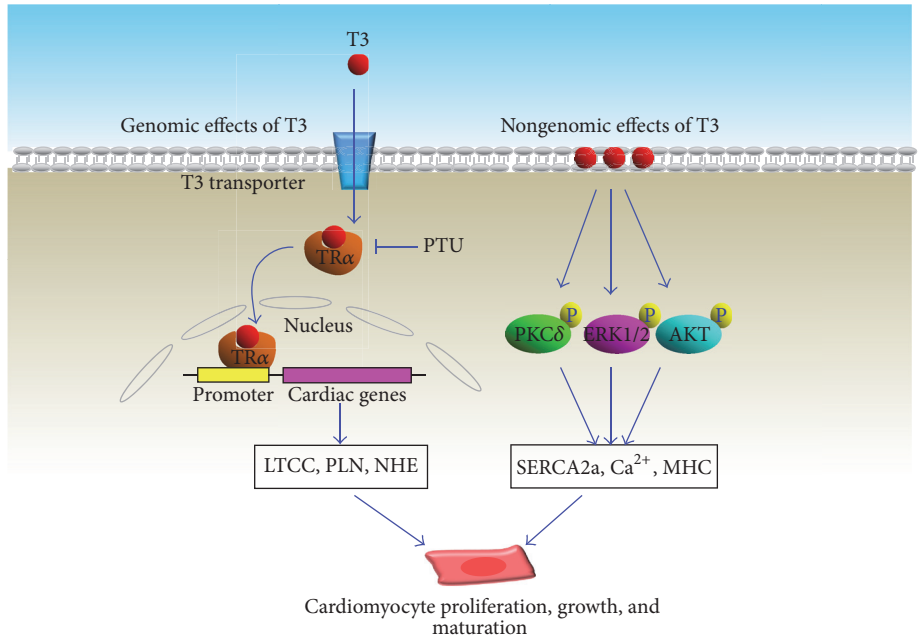

Figure 1: T3 promotes the proliferation, growth, and maturation of cardiomyocytes. LTCC: L-type calcium channel; PLN: phospholamban; NHE: Na^+/H^+ exchanger; MHC: myosin heavy chain.

region (EGF-like domain), and a membrane-associated synthetic site. Upon proteolytic cleavage by transmembrane proteases, neuregulin-1 is activated and released, and EGF-like domains will then bind covalently with ErbB receptors. Particularly, the binding of neuregulin-1 to the extracellular ligand-binding domain of ErbB receptors induces a structural conformational change and subsequently results in the homodimerization of these receptors, by which ErbB receptor intracellular kinase domains phosphorylate their dimerization partner's C-terminus. These alterations start serial downstream signaling pathways, including ERK/MAPK [34], PI3K/AKT [35, 36], and FAK [37], which are responsible for heart development, cardiac structure maintenance [38], and functional integrity of heart muscle [39–41] (Figure 2).

The importance of NRG1-ErbB signaling in regulating cardiac development has been researched primarily using cardiomyocyte-specific ErbB gene knockout mouse, conditional ErbB transgenic mouse, and zebrafish models [42, 43]. The formation of ventricular trabeculations and atrioventricular cushions stagnated in ErbB knockout mice, leading to death during midembryogenesis. This similar lethal phenotype also occurred in NRG1-deleted mouse during embryogenesis. Although the severe fatal phenotype was not observed in ErbB deficient zebrafish, myofibril disarrangement jeopardized structural maintenance by altering the spatiotemporal organization of cardiomyocytes [44]. Developmental biological evidence revealed that trabeculation was delayed in ErbB deficient zebrafish, associated with the decreased heart contractile function. In addition protein synthesis, F-actin organization, and physiological hypertrophic responses were reinforced in rat neonatal cardiomyocytes after exposure to NRG1, while pretreatment with rapamycin blocked these effects, suggesting that activation of phosphatidylinositol 3 kinase (PI3K)/p70S6K signal cascade contributes to NRG1-induced myosin protein synthesis and fibril organization.

Interestingly, NRG1-ErbB signaling has also been implicated as having a key role in regulation of cardiac conduction system development. An initial study using an *in vivo* gene reporter system demonstrated that NRG1 could convert murine contractile cardiomyocytes isolated from 9.5-day postcotium embryos into conduction cells, and expression of these conduction system genes increased in a dose-dependent pattern [45]. A further study indicated that NRG1 treated hearts demonstrate enhanced electrical conduction and integration based on evidence that cardiac impulses propagated from the atrioventricular (AV) canal not only along the dorsal aspect of the ventricles, but also along the ventral aspect of the ventricle from the AV canal region [46]. Research on NRG1-ErbB signaling has contributed to stem cell therapeutics regulating cardiac development. When exposed to NRG1 on days 1–3 after differentiation, embryonic stem cells were more likely to differentiate into pacemaker cardiomyocytes. However, cells were more likely to differentiate into contractile cardiomyocytes when ESCs were treated NRG1 on days 5–9 after differentiation, suggesting that the temporal expression pattern of NRG1 is crucial in determining differentiation termination of heart cells. Unfortunately, these results somewhat contradict *in vivo* reports in which the cardiac conduction system developed only after the formation of heart trabeculation and compaction [47].

Regulation of cardiomyocyte proliferation during adult stages has received enormous attention in regenerative medical research, and some intriguing hints have been implied from comparative biological analysis among hearts from

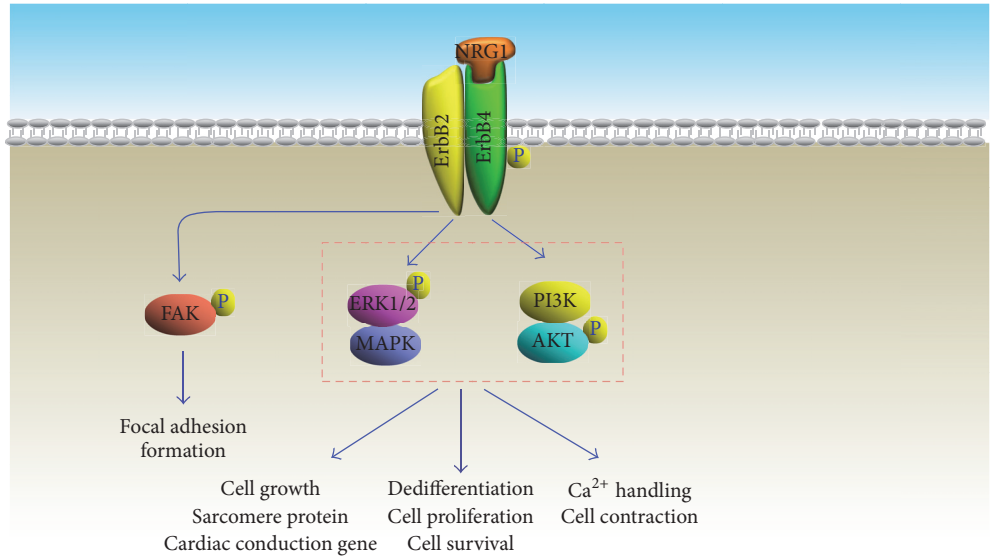

Figure 2: Signal pathways contributing to NRG1-induced cardiac regenerative effects.

mammals, newts, and zebrafish. For instance, turnover of both mouse and human cardiomyocytes occurs at a rate of 1% per year, while the regeneration rate of adult cardiomyocytes can reach 30% in adult zebrafish heart within a few months of the injury. Importantly, NRG1-ErbB signaling has been identified as a mechanism contributing to cardiomyocyte proliferation and regeneration in zebrafish hearts [43], which is a tantalizing goal for those investigating this signal pathway on mammal hearts. D'Uva et al. provided experimental evidence that the ErbB receptor is required for cardiac cell growth in mice just after birth, and NRG1-induced cardiomyocyte proliferation diminished, owing to the reduced expression of ErbB, at 1 week after birth, the same time point as cardiomyocytes lose proliferation ability [42]. However, specific induction of ErbB in juvenile or adult cardiomyocytes extended the heart regenerative capacity into adulthood, restoring cardiac regenerative ability. In addition, the disassembled muscle contractile apparatus was observed in mature cardiomyocyte in response to overexpression of activated ErbB, indicating that ErbB can promote cardiomyocytes to partially dedifferentiate to a less-specialized phenotype, and this morphological alteration may facilitate cardiomyocyte proliferation in hearts of juvenile and adult mice.

In light of these findings, NRG1 treatment has been studied on several experimental heart injury models. Polizzotti et al. simulated myocardial infarction on the hearts of newborn mouse pups through localized freezing, and recombinant NRG1 was administrated to stimulate regeneration of heart muscle cells [48]. As predicted, heart tissue scarring and reduced heart function were exhibited in wild type hearts, whereas heart performance and cardiomyocyte proliferation were improved in NRG1 treated newborn mice. However, these beneficial effects did not emerge when NRG1 treatment was initiated later than 4 days after birth, suggesting that NRG1 treatment efficacy is largely dependent on the individual age of those receiving therapy. This limitation may be owing to the postnatal loss of ErbB expression [42], which has been correspondingly addressed in human heart regeneration studies. After isolation and purification of cells from biopsies of diseased human hearts, cardiomyocytes were cultured *in vitro* and then exposed to NRG1. The cardiomyocytes from heart biopsies of newborns displayed robust proliferation capacity after NRG1 treatment, but this effect was significantly reduced in cardiomyocyte biopsy samples obtained from 6-month-old hearts. Therefore, identification of suitable NRG1 therapeutic windows might improve the success rate for heart regenerative treatments and cardiac reconstructive surgeries. Clinical trials have been designed and performed to evaluate the efficacy and safety of recombinant human NRG1 (rhNRG-1), a 61 amino acid peptide, for treating stable chronic heart failure (CHF) since 2010 [49]. In phase I clinical trial, favorable acute and chronic hemodynamic effects were observed in patients with stable CHF after parenteral administration of rhNRG-1 [50]. Excitingly, a randomized, double-blind, placebo-controlled, and multicenter-performed phase II study, which is based on standard therapy, has demonstrated that rhNRG-1 can improve the cardiac function of patients with NYHA class II and III heart failure, as evidenced by the increased LVEF%, the reduced ESV, and EDV compared with pretreatment [51].

It has been authorized to perform phase III clinical trials in US by FDA at the end of 2013, and these promising clinical trial results support moving this program forward aggressively (http://www.zensunusa.com/clinicaltrials/index.aspx).

2.3. Follistatin-Like 1 (Fstl1). Fstl1 is a secreted glycoprotein which was originally identified and cloned from mouse osteoblastic cell line. This protein is highly conserved among mouse, rat, and human, as reveled by more than 92% amino acid sequences identity. Although this follistatin-related protein is expressed predominantly in the cells of the mesenchymal lineage and widely involved in regulating development of several organs such as lung, ureter, and skeletal and central nervous system, recent studies have demonstrated this mesenchymal-derived factor also participates in immune response, carcinogenesis, and tumor metastasis through a paracrine manner [52].

An early study on AKT-inducible transgenic mouse indicated that Fstl1 protein and transcript expression are increased in hearts in response to AKT activation, and Fstl1 can be secreted from cardiomyocytes to produce cardioprotective effects through a paracrine mechanism [53]. This secretory protein is composed of 4 functional structural domains: (1) follistatin module containing 10 conserved cysteine residues; (2) an extracellular segment called Kazal-type serine protease inhibitor domain; (3) 2 EF-hand calcium-binding domains; (4) a Von Willebrand factor type-C domain. Interestingly, this multiple-functional secretory protein participates in a variety of physiological and pathological processes including heart remodeling, cardiogenic regulation, cardiomyocyte proliferation and division, and cardiac regeneration.

Enhanced Fstl1 expression in heart tissue and increased serum Fstl1 levels were observed in heart failure mouse models [54]. The experimental research on genetically modified mice demonstrated that neither Fstl1 ablation nor overexpression affected the heart size or contractile function under basal conditions, but the detrimental effects appeared in Fstl1 deficient mouse hearts in response to overload pressure. Specific disruption of cardiac *Fstl1* gene led to exacerbated cardiac hypertrophy and left ventricular dysfunction after thoracic aortic constriction (TAC) injury, indicating that Fstl1 functions as a negative regulator of cardiac growth under stress conditions. Interestingly, Fstl1 treatment attenuated epinephrine-induced cardiomyocyte hypertrophic response and protected against cardiac fibrosis in the presence of pathological hypertrophic stimuli. Coincidentally, the therapeutic impact of Fstl1 has also been observed in ischemia-reperfusion (I/R) injuries in both small and large preclinical models [55, 56]. Administration of Fstl1 protein significantly attenuated I/R-induced myocardial infarct areas associated with reduced apoptosis and decreased detrimental immune responses. Mechanistically, Fstl-1-induced cardiac protective effect is mediated by several independent signaling cascades. As a receptor of Fstl-1, expression of Dip2a (disconnected interacting protein 2 homolog A) has been identified on the cell surface of cardiomyocytes and endothelial cells. Along with AMPK activation-induced cell protective effects [57], upon binding with Fstl1, Dip2a can upregulate AKT phosphorylation to protect cell from hypoxia/reoxygenation-induced apoptosis and promote neovascularization by regulating endothelial cells' migration and differentiation [58]. During the early stage response to injury, Fstl1-induced ERK activation can activate fibroblasts to protect the heart from rupture and is considered as the essential cellular and molecular mechanisms for acute repair of the infarcted myocardium [55]. Despite that Fstl1-induced proinflammatory effects in immune cells have been demonstrated both *in vitro* and *in vivo* [52], detrimental myocardial inflammatory response was indeed alleviated in the presence of Fstl1. Such an opposite response may be attributed to the receptor competitive effects between Fstl1 and bone morphogenetic protein 4 (BMP4). Actually, Fstl1 can play as antagonist of bone morphogenetic protein receptor type II (BmpRII) and directly negatively regulate Smad1/5/8 phosphorylation [59], which consequently abolished BMP4-induced cell programmed death and BMP4-dependent induction of mediators [57] (Figure 3).

Most recent studies have identified Fstl1 as an epicardial cardiomyogenic factor. Enhanced mature cardiomyocyte features were observed when mouse embryonic stem cell- (ESC-) derived cardiomyocytes were cocultured with epicardial mesothelial cells (EMCs). Importantly, EMC-derived conditioned medium increased the number of beating colonies and promoted rhythmic calcium transients in ESC-derived cardiomyocytes. Interestingly, the incidence of aurora B kinase positive signals was doubled in ESC-derived cells, indicating that cell proliferation capacity was intensified after exposure to EMC-derived conditioned medium. Among 1596 mass spectrometry-recognized bioactive proteins in EMCs-derived conditioned medium, Fstl1 was identified as the major contributor to these cardiomyogenic and proliferative effects [56]. Finally, implantation of recombinant human Fstl1 via epicardial cell patch stimulated cell-cycle entry and promoted the division of preexisting cardiomyocytes, attenuating cardiac dysfunction and improving survival in mouse and porcine myocardial infarction models [56].

Despite showing promising therapeutic effects in preclinical experiments, it remains elusive whether the contributions of this protein are beneficial or detrimental to the development of human cardiovascular diseases. For instance, heart failure patients with increased Fstl1 levels in serum and the myocardium maintained the highest risk of mortality, suggesting that Fstl1 may serve as a biomarker in chronic systolic heart failure [60]. In addition, plasma Fstl1 levels were elevated in patients with acute Kawasaki disease, a major cause of acquired coronary aneurysm in childhood [61].

2.4. Tumor Necrosis Factor Ligand Superfamily Member 12 (TNFSF12). TNFSF12, also called TNF-related weak inducer of apoptosis (TWEAK), is a multifunctional cytokine that is encoded by the *TNFSF12* gene. TWEAK is a type II transmembrane protein that belongs to the TNF ligand family. This protein is composed of four major domains, an N-terminal cytoplasmic domain, a transmembrane domain followed by an extracellular furin-identified stalk region, and the C-terminal tumor necrosis factor (TNF) homology domain (THD). Furin can function as an endoprotease that targets and cleaves TWEAK extracellular stalk peptide sequences,

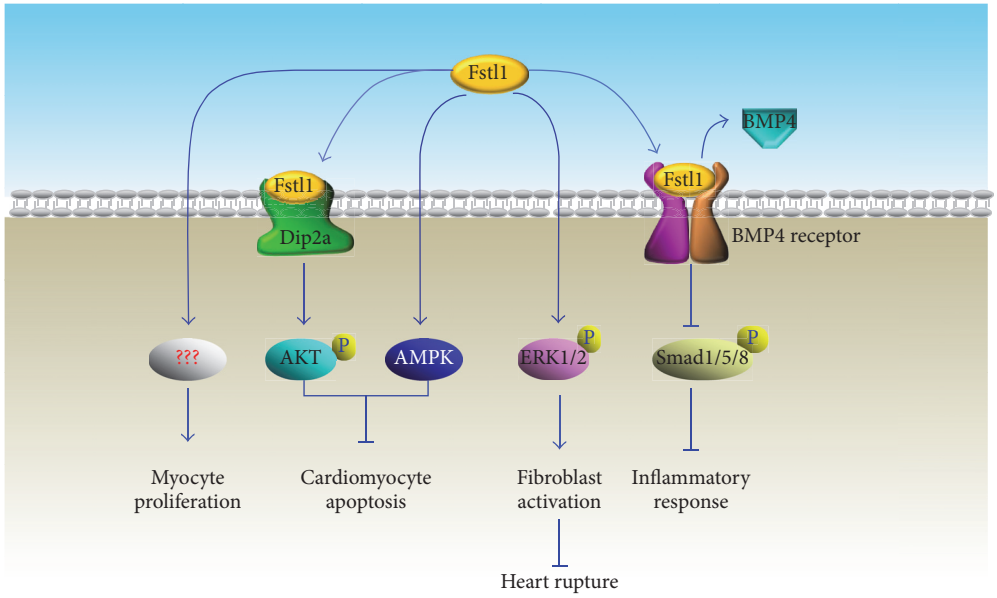

Figure 3: Fstl1-induced effects on cardiac regeneration and repair.

resulting in a soluble ligand containing the THD. Notably, both membrane-bound and soluble TWEAK (mTWEAK and sTWEAK) can be trimerized as a homogeneous complex to fully function and regulate similar cellular biological functions by binding to fibroblast growth factor-inducible 14 (Fn14) receptors, the smallest member of the tumor necrosis factor receptor (TNFR) superfamily. Specifically, site-mutagenesis studies have demonstrated that TWEAK can covalently bind to the single cysteine-rich domain (CRD) in the extracellular ligand-binding region of Fn14 receptor, which is determined by 3 evolutionarily conserved amino acid residues (ASP, Lys, and Met) in this domain [62]. Subsequently, a short cytoplasmic tail of Fn14 receptor will recruit TNFR-associated factor (TRAF) to regulate multiple cellular activities including apoptosis, migration, differentiation, proliferation, angiogenesis, and inflammation [63–66].

Activation of the TWEAK/Fn14 pathway in leukocyte subsets is thought to be a mediator of tissue response under pathological conditions. Activation of this pathway in non-hematopoietic tissue cell types also actively contributes to shaping of the tissue repair process, including the inflammatory response, tissue fibrosis, and functional components of regeneration [67]. Indeed, *in vivo* experimental evidence has shown that both TWEAK and Fn14 expression levels were significantly upregulated in remodeling cardiac tissue after myocardial infarction [68]. Further studies have also indicated that cardiomyocytes serve as a cell source reserve for the increased TWEAK and Fn14 cells under stressful conditions, an induction mediated by the Rho/ROCK pathway [68]. Although TWEAK/Fn14 activation can promote NF-κB nuclear translocations that trigger inflammatory responses in injured heart tissue, proproliferative effects have also been observed in mammalian cardiomyocytes exposed to TWEAK treatment [69]. TWEAK can stimulate DNA synthesis in neonatal cardiomyocytes in a dosage-dependent manner associated with increased expression of proliferative markers cyclin D2 and Ki67, while reducing the amount of endogenous cell-cycle inhibitor p27/KIP. These studies demonstrated that TWEAK-induced cell-cycle reentry, mitosis, and cytokinesis are mediated by activating extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3 kinase (PI3K). Conversely, TWEAK-induced proliferation was not detected in adult cardiomyocytes, which may be due to the decreased Fn14 expression during postnatal stage. This hypothesis was partially verified in isolated murine cardiomyocytes response to Fn14 inhibition. Indeed, TWEAK-induced neonatal cardiomyocytes DNA synthesis and proliferation were significantly inhibited when endogenous Fn14 was specifically blocked by a neutralizing antibody or siRNA. A recent study on cardiomyocytes using coimmunoprecipitation and proximity ligation assays revealed the interactions between endogenous Fn14 and fibroblast growth factor receptor-1 (FGFR-1), which serve an essential role in promoting cell proliferation. This interaction becomes more pronounced in the presence of TWEAK or FGF-1, and the synergistic interaction can stimulate cell-cycle reentry of >40% adult cardiomyocytes [70].

Due to TWEAK/Fn14's modulatory effects in tissue response and repair, an attempt has been made to investigate whether TWEAK administration can provide therapeutic options for patients after myocardial infarction. Unfortunately, heart damage effects have been revealed in several

preclinical experiments. Along with fibroblast proliferation and myofibroblast differentiation, Fn14 overexpression strengthened hypertrophic responses in cultured adult rat cardiomyocytes, which may lead to pathological remodeling in response to pressure overload stimulation [71, 72]. Correspondingly, treatments of mice with a recombinant human serum albumin conjugated-TWEAK induced myocardial healing defects after MI, associated with an exaggerated neutrophil infiltration into the myocardium [73]. In another study, systemic administration of TWEAK displayed maladaptive effects after MI, as revealed by worsened left ventricular function and the enhanced late mortality. Further molecular analysis revealed that expressions of PGC-1α and oxidative phosphorylation-regulating genes were significantly suppressed in cardiomyocytes, indicating such detrimental responses are exerted most likely via direct effects on cardiomyocytes [74] (Figure 4). TWEAK-induced maladaptive effects were partially supported by a clinical investigation that soluble TWEAK serum level was increased to a higher level in chronic heart failure with reduced ejection fraction (HF-REF) compared to healthy subjects [83]. Therefore, more preclinical *in vivo* experiments are necessary in order to elucidate the role of TWEAK in cardiac repair procedure.

3. Intracellular Signals

Signals within intracellular compartments not only participate in the regulation of cell bioactivities under physiological conditions, but also receive and transmit warning signals that coordinate stress responses and tissue repair after injury. Several intracellular signal pathways including PI3K-AKT, Hippo-Yap, cardiogenic transcription factors, and microRNAs have been identified as prominent regulators in processing cardiac development and regeneration.

3.1. PI3K-AKT Pathway. The PI3K-AKT pathway has been extensively studied and is recognized as a prominent intracellular signaling pathway in regulating a diverse selection of cellular functions and processes such as glucose uptake, energy metabolism, cell-cycle progression, apoptosis, and gene transcriptional regulation. Class 1A PI3K is a heterodimeric complex composed of a p110 catalytic subunit and a p85 regulatory subunit located on the plasma membrane. Upon activation by a variety of stimuli including growth factors, attachment of extracellular matrix, and oncogene products, PI3K can phosphorylate phosphatidylinositol 4,5-bisphosphate (IP2) into phosphatidylinositol 3,4,5-trisphosphate (IP3), which serves as a principle intracellular lipid second messenger to recruit downstream signals [84]. As an important downstream effector of PI3K, protein kinase B (PKB, also known as AKT) can employ its pleckstrin homology (PH) domain to bind IP3 and subsequently undergo a conformational change triggering PKB activation through 3-phosphoinositide-dependent protein kinase-1- (PDK1-) induced phosphorylation [85].

PI3K-AKT activation is required and linked to restriction point progression for G1-to-S transition, which is determined primarily by cyclin D-dependent kinase- (CDK-) induced phosphorylation of pRb. Cyclin D1, whose expression can be induced by c-Myc, can interact with CDK to promote pRb phosphorylation. Unfortunately, both cyclin D1 and c-Myc are proteins with short half-lives, because these signals are susceptible to proteolytic degradation when exposed to glycogen synthase kinase 3 beta (GSK-3β). Indeed, nuclear translocation of cyclin D1 will be blocked when GSK-3β-induced phosphorylation occurs at residue threonine-286 (T286), resulting in ubiquitin-mediated proteolytic degradation in the cytoplasm [86]. Constitutive expression of active PKB can prolong the half-life of cyclin D, while pharmacological inhibition of PI3K speeds up cyclin D1 degradation, indicating that PKB-mediated GSK-3β inhibition can stabilize cyclin D1 [87]. Additionally, PKB can also upregulate expression levels of cyclin D and c-Myc at both the transcriptional and translational levels. The positive cell-cycle regulatory effects of cyclin D can be counteracted by a group of inhibitory proteins including p15, p21, and $p27^{Kip1}$. Among them, $p27^{Kip1}$ is a principle inhibitor required for maintaining cell quiescence, whereas reduction is critical for cell-cycle reentry [88]. It has been established that the stability of p27 is regulated by the PI3K-AKT pathway, since activation of this pathway decreased both p27 expression level and the accumulation of p27 observed in cells as a response to AKT inhibition [89].

A well accepted study revealed the role of PI3K in regulating the efficiency of G2/M phase progression [90]. The number of neonatal cardiomyocytes in the mitotic and cytokinesis phase was increased 50~100%, associated with enhanced CDK7 expression, after activation of PI3K-AKT signals by C3orf58 (a kind of hypoxia), and AKT-induced stem cell factor (HASF). CDK7 is identified as a subunit of the general transcription factor IIH (TFIIH) and a member of intricate network of CDKs to promote mitosis and cell division, ensuring the genetic materials are accurately and equally segregated between two daughter cells [91]. Interestingly, it was recently reported that AKT activation could dramatically accelerate and amplify the transcriptional reprogramming of mouse cardiac fibroblasts, a process in which functional cardiomyocytes can be induced and generated from autologous fibroblasts. Spontaneous beating occurred in approximately 50% of reprogrammed fibroblasts after 3 weeks of induction in the group that was treated with AKT plus cardiac transcription factors. The result was more mature cardiomyocytes features, including enhanced polynucleation, cellular hypertrophic gene expression, and metabolic reprogramming in the reprogramed cells [92].

3.2. Hippo-Yap Pathway. The Hippo signaling complex is composed of a cluster of cytoplasm-located protein kinases and two major transcription factors associated with corresponding regulators. These pathway components are enriched with WW domains and their cognate proline-rich interacting motifs provide an efficient signaling mechanism to sense upstream input and start off the downstream output [93]. Briefly, mammalian sterile 20-like kinase (Mst) activation can be initiated when exposed to diverse stress signals such as extracellular matrix stiffness, mechanic stress, cytoskeletal rearrangement, contact inhibition, and anoxemia [94]. This signal activation can directly mediate mitochondrial function to affect energy metabolism. Subsequently,

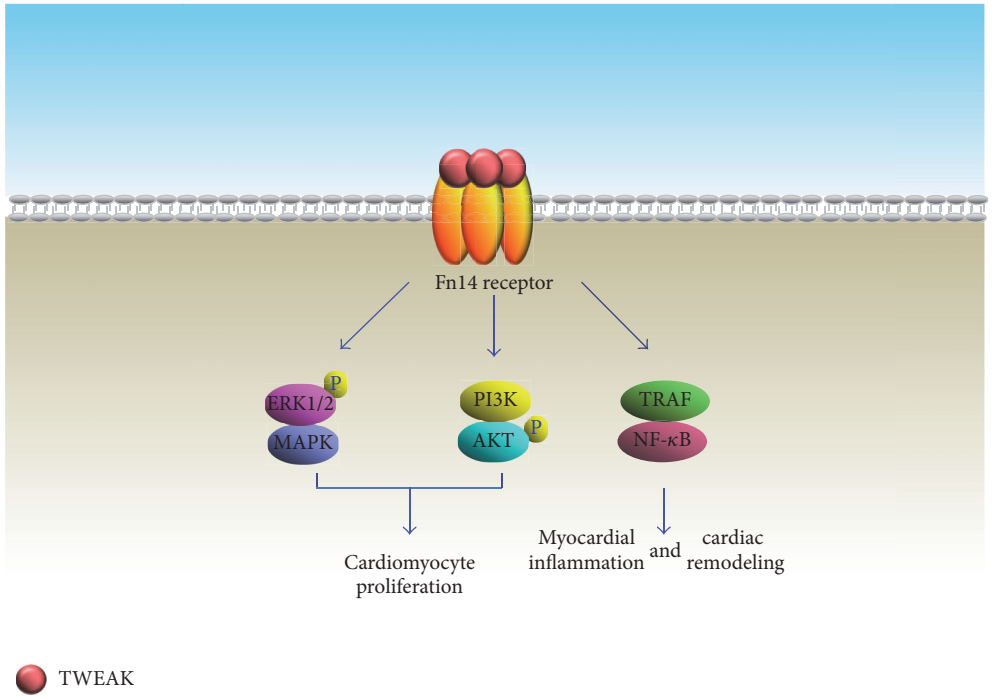

Figure 4: Involvement of TWEAK-Fn14 signaling pathways in cardiac regeneration.

Mst can transduce this activation to large tumor suppressor kinase-1/2 (Lats1/2) to phosphorylate Yap. Spatial alteration of Yap, which serves as a transcriptional coactivator, is principally determined by the phosphorylations at serine residues 127 and 379. Accumulating *in vitro* experimental evidence has shown that Yap is subjected to cytoplasmic retention and ubiquitin-dependent degradation upon Lats kinase-induced phosphorylation, whereas unphosphorylated Yap will emerge in the nucleus. This intensified Yap signal can be observed in the nucleus when residue serine 127 is changed into Alanine (S127A), a mutation that can keep this residue from phosphorylation. Correspondingly, Yap-induced biological effects (including cell proliferation) are also enhanced to a greater extent in experimental S127A mutation Yap subjects when compared with those with wild type Yap. Nuclear-located Yap cannot identify or interact with DNA-binding domains *per se*. When combined with TAZ, this Hippo effector can act as a coactivator to modulate the DNA-binding activity of TEAD, a critical transcriptional factor that initiates proliferative and prosurvival gene progression programs. TEAD owns a C-terminal protein-binding domain, characterized by an immunoglobulin-like beta-sandwich fold with two extra helix-turn-helix inserts. This structure enabled TEAD to precisely recognize and covalently bind to the TEAD-binding domain of Yap [95]. Accordingly, Yap wraps around the globular structure of TEAD and forms extensive interactions via three highly conserved interfaces [96]. TEAD also contains an N-terminal TEA-domain, a DNA-binding module that can interact with canonical M-CAT elements to regulate target gene expression. M-CAT sequence motif (5′-TCATTCCT-3′) has been identified in several gene promoters and is a decisive DNA region for the regulation of cell growth, differentiation, and epithelial-mesenchymal transition. Notably, the enhanced protein-protein interaction between Yap and TEAD has been identified as a molecular mechanism contributing to oncogenesis and metastasis, especially hepatocellular carcinoma and gastric cancer [97, 98], and pharmacological blockade of the Yap-TEAD complex formation may foster the development of novel therapy strategies for inhibiting tumor growth [99].

The importance of Yap in regulating heart development has been documented in several studies using gain- and loss-of-function approaches. Slower heartbeat and a decreased number of cardiac Troponin-positive cardiomyocytes were observed, consequently resulting in embryonic death in inducible Yap gene mutant embryos. Although cardiac looping or chamber formation was not affected, deletion of Yap led to a significant reduction in ventricular myocyte number compared with the wild type littermates due to the diminished proliferation of cardiomyocytes. Correspondingly, forced expression of YapS112A (a Yap mutant form that is constitutively active and localized to the nucleus) significantly promoted the proliferation of cardiomyocytes in the hearts of transgenic embryos, and YapS112A transgenic mice displayed an abnormally thickened myocardium and expanded trabecular layer compared with those of Yap transgenic mice [100]. This similarly compromised cellular phenotype was also observed in Mst deficient embryonic bodies ($Mst^{-/-}$ EBs), in which beating cell clumps disappeared and the expressions of cardiac progenitor markers

such as Nkx2.5, Tbx5, Mesp1, Isl1, and Baf60c were significantly suppressed. Further studies have revealed that Mst is involved in cardiogenesis through regulation of noncanonical wnt ligands. Indeed, expression and secretion of several noncanonical Wnt ligands such as Wnt2, Wnt2b, and Wnt5a were reduced in $Mst^{-/-}$ EBs, whereas canonical Wnt ligand gene expression was not affected [101]. Numerous studies have provided evidence to support that Yap is a nexus of multiple signaling pathways in governing cardiac growth and survival. Yap builds up interlink among the Hippo pathway, Wnt pathway, and the IGF pathway to regulate β-catenin signaling and precisely control cardiac development [100].

In the process of cardiac differentiation, maturation of cardiomyocyte morphology is characterized by enhanced myofibril density and alignment, associated with visible sarcomeres under bright-field microscopy [102], while functional maturation is characterized by increased ion channel expression in the cell membrane, enhanced calcium storage capacity in the sarcoplasmic reticulum, high density distribution of adrenergic receptors, and robust contractility [103]. There is no evidence to date showing that the Hippo-Yap signal directly regulates the functional maturation of electrophysiology and calcium handling during cardiomyocyte differentiation. However, a most recent study implicated this signal pathway is involved in actin cytoskeletal remodeling with protrusion formation using the *Salvador* gene knockout (*Salv* KO) mouse model and chromatin immunoprecipitation sequencing (ChIP). Mst1 activation is dependent on the interaction with Salvador. Ablation of Salvador will inhibit the kinase activity of whole Hippo signals leading to accumulation of nonphosphorylated Yap in the nucleus. For example, Yap-Chip sequencing and mRNA expression profiling in *Salv* KO hearts revealed that Yap is involved in gene transcription and regulation of Sarcoglycan and Talin2, which composes the plasmalemmal complexes that link the actin cytoskeleton to the extracellular matrix. Importantly, this was confirmed in mouse ischemic hearts after left anterior descending artery ligation. The greater extent of cytoskeleton rearrangement was observed in Hippo kinase-compromised cardiomyocytes than in their wild type counterparts, enabling the migration of cardiomyocytes into the infarct border-zone. Upregulation of Sarcoglycan and Talin2 help *Salv* KO cardiomyocytes extends sarcomere-filled protrusions into scar tissue in the region of myocardial injury, as demonstrated by the appearance of costameres linking ECM to actin through the integrin-vinculin-talin complex, an essential cellular event for heart regeneration [104]. Interestingly, recent study demonstrated that Hippo-Yap function coordinates with PI3K-AKT pathway to promote cardiomyocyte proliferation and survival. Indeed, the p110 catalytic subunit of PI3K is encoded by gene, *Pik3cb*, which is the direct target of Yap. Through cooperation with its transcriptional partner TEAD, Yap can enhance *Pik3cb* expression, which subsequently induces AKT activation [105] (Figure 5).

3.3. Cardiogenic Transcription Factors. Cardiac transcription factors are protein molecules that can bind to specific DNA domains, such as promoters and enhancers, to specifically regulate expressions of cardiac genes. The biological functions of several cardiac transcription factors have been revealed in human congenital heart defects and confirmed in transgenic mice expressing dominant-negative mutant alleles. Also, downstream targets of these transcription factors have been demonstrated using next-generation RNA sequencing techniques. This part will highlight recent discovery in three cardiogenic transcription factors, especially their potentials for heart regeneration (Table 2).

3.3.1. T-Box Transcription Factor 20 (TBX20). The relevance of the TBX20 gene in maintaining heart development has been revealed in various forms of congenital heart diseases and in many animal models [106]. This gene encodes transcription factor TBX20, which is essential for development of the interatrial septum. A septal defect has also been observed in TBX20 mutants, resulting in deoxygenated blood flowing into the left atrium and left ventricle. TBX20 is characterized by the presence of a 180-residue DNA-binding domain (T-box), a highly conservative β-sheet structure among T-box protein family members. A palindromic consensus sequence 5′-T(G/C)ACACCTAGGTGTGAAATT-3′ has been identified using site-selection experiments, named the T-site, that has been recognized as T-box-targeted DNA region, and the half site of this segment 5′-AGGTGTGA-3′ (also named as T/2 site) was identified as a core motif that can efficiently and sufficiently interact with transcription factors [107].

Increased TBX20 gene expression was observed in endomyocardial biopsies from idiopathic dilated cardiomyopathy patients and animal models of cardiomyopathy that are associated with compromised heart performance [108], and upregulation of this gene is considered an adaptive response of a stressed heart. In the loss-of-function experiments, chamber dilation was observed in TBX20 knockout mouse hearts, and cardiomyocyte-specific ablation of this transcription factor resulted in animal lethality within 15 days [75]. Conversely, cardiac-specific overexpression of TBX20 significantly improved animal survival, increased heart contractile function, and reduced myocardial infarction after ischemia injury [76]. Genome-wide ChIP analysis of the TBX20-binding motif in the adult mouse heart indicated that many cardiac genes meant for encoding critical transcription factors, ion channels, and cytoskeletal/myofibrillar proteins are direct downstream targets of TBX20 [75]. Correspondingly, prolonged QRS duration and heart blockage were detected in TBX20-deficient mice, and electrophysiological disorders also appeared in isolated TBX20-null cardiomyocytes as evidenced by decreased levels of L-type calcium current, inactivation of potassium current, and compromised calcium cycling [75]. Ongoing studies have revealed that TBX20 overexpression can activate multiple cell proliferation signaling pathways (including PI3K-AKT, Hippo-Yap, and BMP/Smad1/5/8) in the myocardium of adult mice, while repressing cell-cycle inhibitory genes P21 and Meis1 to induce cardiomyocyte proliferation under both basal and detrimental stress conditions [76].

3.3.2. GATA4. GATA4 is a member of the GATA protein family, which are transcription factors evolutionarily conserved in the context of structure and function. At the

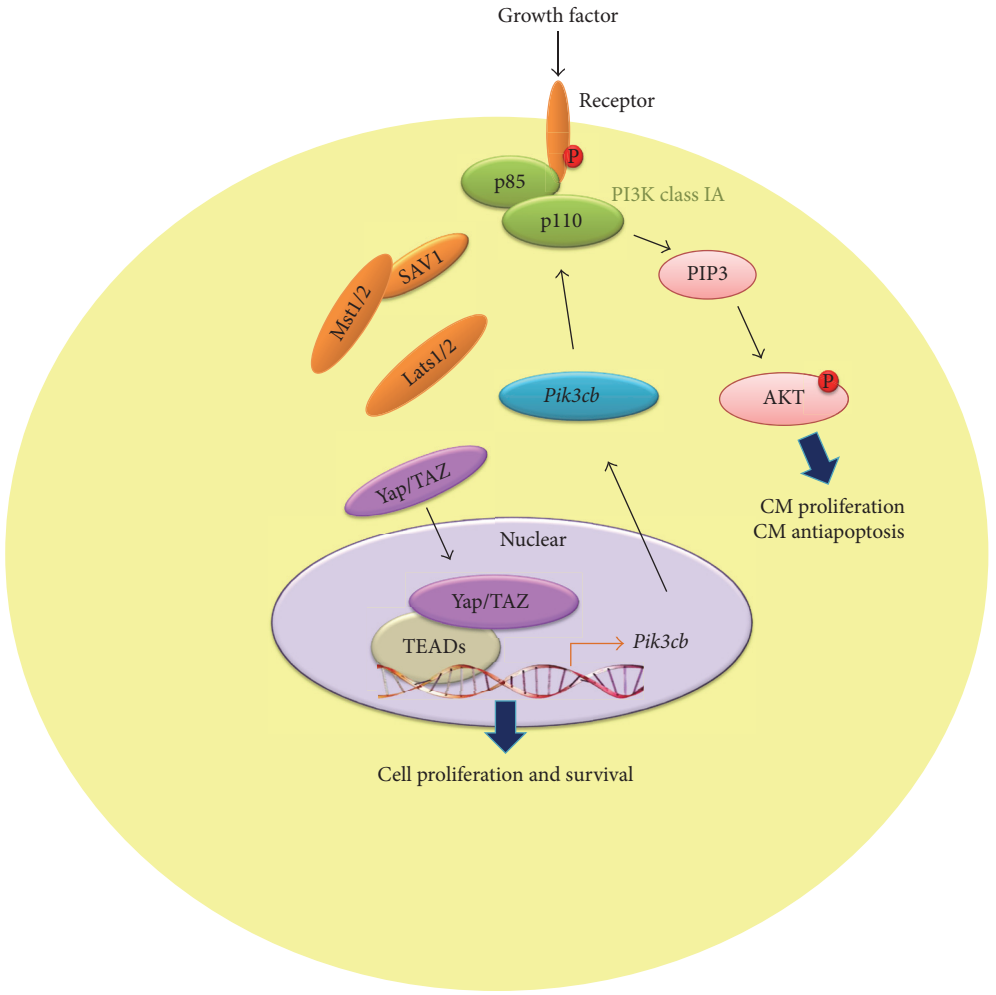

Figure 5: Pik3cb as a crucial direct target of Yap that links Hippo-Yap to PI3K-AKT signaling activation and regulates cardiomyocyte proliferation and survival. Dephosphorylated Yap/TAZ translocates into nuclear and directly activates target gene Pik3cb expression with several transcription factors (TEA-domain sequence-specific DNA-binding proteins). Pik3cb activation downstream of Yap promotes cardiomyocyte proliferation and survival by stimulating AKT activation.

structural level, GATA4 is characterized by two highly conserved type IV zinc fingers located at the N-terminal and C-terminal. These two functional domains coordinate the specific binding of GATA4 to the A/TGATAA/G conserved region inside the DNA. Specifically, C-terminal zinc finger (along with the adjacent domain) is adequate and essential for specific covalent binding, while the N-terminal counterpart functions as an equalizer in stabilizing the protein-DNA interaction [109]. A recent report demonstrated that GATA4 transcriptional activity is mediated by cGMP-PKG signaling [77]. GATA4 is preferentially expressed in heart tissue, and the binding motifs have been identified within the promoter of genes that regulate cardiogenic differentiation, cell proliferation, and cell fate determination. A previous investigation revealed that a heterozygous G296S missense mutation of GATA4 is associated with an increased incidence of atrial and ventricular septal defects and pulmonary valve stenosis in humans [110]. In line with these findings, GATA4-deficient mice displayed embryonic lethality, a thin myocardium with reduced cardiomyocyte proliferation, and atrioventricular septum defects [111], and GATA-4 inactivation caused severe cardiac dysfunction [80]. Cyclin-dependent kinases (CDKs), especially CDK2 and CDK4, are the direct targets of GATA4. Dysfunction of GATA4 leads to the reduction of these CDKs and contributes to cardiac intraseptal defects in humans [78]. In addition, GATA4 can also promote cardiomyocyte replication through an indirect paracrine mechanism. A recent study demonstrated that a conserved region of the Fgf16 second intron can be identified and bound by GATA4, and the activation of this enhancer domain led to an increased expression and secretion of FGF16, which subsequently promoted cardiomyocyte proliferation and cardiac regeneration [79].

3.3.3. NF-κB. NF-κB is a well-known transcription factor that regulates a variety of cellular biological effects including inflammation, cell proliferation, cell survival, and tissue growth. Under resting conditions, NF-κB is sequestered and

Table 2: Cardiogenic transcription factor.

Cardiogenic transcription factor	Functional domain	Upstream signal	Direct target (gene)	Cardiac phenotype	
				Knockout	*Knock-in*
TBX20	T-box (β-sheet structure)	BMP/Smad	Ion channels, cytoskeletal proteins; myofibrillar proteins [75]	Ventricular dilation, long QRS wave, heart arrest [75],	Heart performance↑, CM proliferation↑ [76],
GATA4	Zinc fingers (type IV)	PKG-1α [77]	Cyclin-dependent kinases [78] FGF16 [79]	embryonic lethality [78], severe cardiac dysfunction [80]	heart function↑, cardiomyocyte replication↑, heart regeneration↑ [79]
NF-κB	Rel homology domain (RHD)	N/A	Cardiac hypertrophic genes ANF, β-MHC↑ [81], GATA4 [82]	Zebrafish heart regeneration↓ [82]	N/A

↑: increase or intensify; ↓: decrease.

insulated by IκB within the cytoplasm. Under stressful stimuli, proteasome-induced degradation of IκB releases NF-κB to induce gene expression after nuclear translocation. In the event of pressure overload, NF-κB activation increases cardiac hypertrophic response, characterized by increased cardiomyocyte size and enhanced expression of fetal genes [81]. It was previously reported that cardiomyocyte-specific NF-κB activation (which is induced by IκB kinase (IKK) overexpression) can induce myocarditis, inflammatory dilated cardiomyopathy, and muscle fiber atrophy with robust interferon type 1 (IFN-1) responses resulting in heart failure [112]. However, a recent study using the zebrafish model system indicated that myocardial NF-κB activation is essential for heart regeneration. The pronounced spatiotemporal correlation between NF-κB activity and heart tissue regeneration was exhibited following injury, while myocardial blockade of NF-κB activity suppressed cardiac regeneration with pleiotropic effects, including reduced cardiomyocyte proliferation and a blunted epicardial injury response. Interestingly, the signals of NF-κB activation colocalize with GATA4 activation during the myocardial regenerative process, and GATA4-induced regulatory sequences can be prevented when NF-κB signaling is antagonized, indicating that NF-κB is an important signal for triggering a heart regeneration program. These studies imply that NF-κB acts as a crucial node between cardiac injury and tissue regeneration, and precise spatiotemporal regulation of NF-κB activity is an issue that determines the quality of heart tissue repair [82].

4. Conclusion and Perspective

Intracellular and extracellular molecular mechanisms both contribute to the complicated repair and renewal process of cardiac regeneration. Although textbooks suggest that the lost or damaged tissue cannot be replaced by adult mammalian heart regeneration, several studies have described evidence that neonatal mammalian hearts have some regenerative capacity, though it is lost beyond 7 days of age. This capability has encouraged the search for alternate effective ways to stimulate cardiac regenerative signal pathways promoting cardiac myocyte proliferation and differentiation. Current findings on cardiac regenerative signals will thus likely provide new therapeutic opportunities for the treatment of heart failure.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

Acknowledgments

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