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Inducible Pluripotent Stem Cells as a disease model to study inherited cardiovascular syndromes

Human pluripotent stem cell-derived cardiovascular progenitors for heart regeneration

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During normal development, cardiac progenitor cells (CPCs) in the pharyngeal mesoderm migrate and contribute to formation of the heart tube. Characterization of the signals that maintain, expand and regulate migration and differentiation of CPCs is essential for understanding the etiology of congenital heart diseases and the potential to differentiate pluripotent stem cells (PSCs) into CPCs for cardiac repair. Although the intricate mechanisms of cardiogenesis are being gradually unraveled, recent clinical and preclinical research studies underscore that full restoration of myocardial structure and function following pathological injuries or aging remains a daunting challenge. Here, we discuss the innate capacity for cardiac regeneration in zebrafish, the types of progenitors driving development in the mammalian heart and how to empower CPCs or myocytes derived from human PSCs to survive, engraft and improve function in the hostile microenvironment of the post-ischemic heart.

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Introduction

According to the World Health Organization, ischemic heart disease was a leading cause of mortality in 2011, accounting for 12.8% of deaths worldwide. One of its most common forms is myocardial infarction (MI), in which a vascular occlusion leads to insufficient perfusion of the heart muscle. A single occurrence of MI can kill as much as 25% of the two to four billion left ventricular cardiomyocytes (CMs) within a few hours [1]. Although various lines of evidence suggest that the human CM compartment undergoes some kind of regeneration 15 times in women and 11 times in men from 20 to 100 years of age [2], this innate ability to regenerate is considered limited and insufficient to cause any therapeutic benefit. As an immediate response after MI, collagen deposition by fibroblasts leads to scar formation but such a non-contractile structure fails to restore cardiac output and may even contribute to post-ischemic arrhythmias.

Unlike mammals, lower vertebrates such as zebrafish demonstrate a natural capacity for heart regeneration throughout life. Understanding their underlying cellular and molecular mechanisms of cardiac regeneration has the

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potential of enlightening future therapeutic development. Zebrafish CMs show a much higher degree of basal cell-cycle activity compared to mammalian cells even under uninjured conditions [3]; pulse-labeling experiments demonstrate that approximately 3% of CMs in uninjured adult zebrafish hearts incorporate bromodeoxyuridine (BrdU), with this proportion increased by ten-fold two weeks after amputation of the cardiac apex [3]. Genetic fate-mapping study further shows that adult zebrafish CMs are replaced by the proliferation of pre-existing CMs rather than differentiation from cardiovascular progenitor cells (CPCs) (Fig. 1a) [4]. More recently, zebrafish have been demonstrated to undergo complete regeneration even after destruction of over 60% of the ventricular myocardium [5]. Such a massive myocardial loss stimulates all major cardiac cell types to rapidly regenerate, ameliorating symptoms of disrupted electric conduction and signs of severe cardiac failure within several days [5].

Cardiac regeneration seems to be evolutionarily conserved among adult non-mammalian vertebrate species [3,4,6] and, to some extent, in murine embryonic [7] and neonatal hearts [8]. In one-day-old neonatal mice, amputation of the left ventricular apex results in complete replacement with normal myocardial tissue that shows normal contractile function by 8 weeks [8]. This regenerative capacity is not observed in 7-day-old mice. Intriguingly, CMs lost in older mice get replaced via the proliferation of pre-existing CMs [8] (Fig. 1b). Indeed, CM proliferation is the predominant mechanism for cardiac growth throughout the first few days of life in the rodent model system, and ceases in juvenile and adult hearts [9]. During normal aging or after MI, although CM replacement in adult mice occurs at very low rates, it is mediated via *de novo* differentiation of endogenous CPCs rather than proliferation of pre-existing CMs [10] (Fig. 1c). Unfortunately, robust regeneration in mammalian hearts occurs only in early life, while patients with heart failure and the greatest clinical need for replacement of CMs are often over 60 years of age [11]. Current therapeutics can only delay the progression of heart failure until potential orthotopic heart transplantation is available. A growing body of studies suggests that the developmental and neonatal repair programs can be recapitulated by pluripotent stem cell (PSC)-based therapy or 're-awakening' by activating endogenous regenerative mechanisms. In this review, we discuss the relevant aspects of human cardiogenesis and how heart regeneration can be promoted by the use of human PSCs (hPSCs).

Cardiovascular progenitors

Recent discoveries of several populations of relatively rare but multipotent resident CPCs in the mammalian heart represent an invaluable potential source of cells for cardiac regenerative therapies. These CPCs are typically active during development and become largely quiescent in adulthood. In principle, these cells could be harnessed to reverse aging or combat pathophysiological processes. To this end, the following populations have been frequently investigated for their potential in cardiac regeneration (see also [12–14] for the discovery of other populations of cardiovascular progenitors).

Beltrami and colleagues have reported that cells marked by the stem cell surface protein c-kit make up a small subset of the rat myocardium (~0.01%) and are self-renewable, clonogenic and multipotent *in vitro* [15]. *In vivo*, c-kit$^+$ cells labeled with BrdU develop organized sarcomeric structures, gap junctions and contractile activity comparable to the native myocardium. More recently, Bearzi and colleagues further demonstrate that human c-kit$^+$ cells injected into an infarcted rat heart results in a chimeric myocardium, not only consisting of CMs as the majority derivatives but also endothelial cells (ECs) and smooth muscle cells (SMCs) [16]. The same group has also used c-kit$^+$ cells additionally selected for vascular endothelial growth factor receptor 2 (VEGFR2, or KDR) to expand a new sub-class of clonogenic coronary vascular progenitors. Not only are they capable of self-renewing and differentiating primarily into ECs and SMCs, but can also become CMs [17]. Taken together, these studies suggest that the two progenitor populations might be exploited separately or in concert for regenerating muscle (from c-kit$^+$/KDR$^-$ cells) and vessel (c-kit$^+$/KDR$^+$ cells).

Oh and colleagues have reported another subset of CPCs, the Sca1$^+$/c-kit$^-$ cells [18]. These cells are isolated from the murine hearts, and can be cultured and differentiated into cells expressing cardiac genes upon treatment with the demethylating agent 5-aza-deoxycytidine. Using additional supplementation (e.g. oxytocin [19], FGFs [20]), other groups have reported similar results. *In vivo*, Sca1$^+$ cells administered intravenously home to the infarcted mouse myocardium, where they appear to differentiate into CMs, thereby attenuating adverse remodeling and leading to improved cardiac functions. Later experiments further suggest that the Sca1$^+$ cells may also work through a paracrine cascade [20,21], in which the secretion of soluble VCAM-1 [21] and other cytokines induces EC migration and limits oxidative stress. Unfortunately, the absence of Sca1 protein in humans limits their potential clinical applications.

Another progenitor population, found in the second heart field, is marked by the LIM homeodomain transcription factor Islet-1 (Isl1). Isl1$^+$ cells give rise to both atria, right ventricle and outflow tract of the heart during development, and have also been observed in postnatal mouse, rat and human myocardium [22–24]. Isl1$^+$ cells can be isolated, and have been shown to self-renew and differentiate into all three lineages of the heart, both *in vitro* and *in vivo*.

The potential for other CPC subsets to give rise to replacement CMs is an area of active research. Recent work has shown that in addition to canonical epicardial lineages, proepicardial progenitor cells expressing Wilms tumor 1 (Wt1) [14] and Tbx18 [12] also contribute to myocytes in

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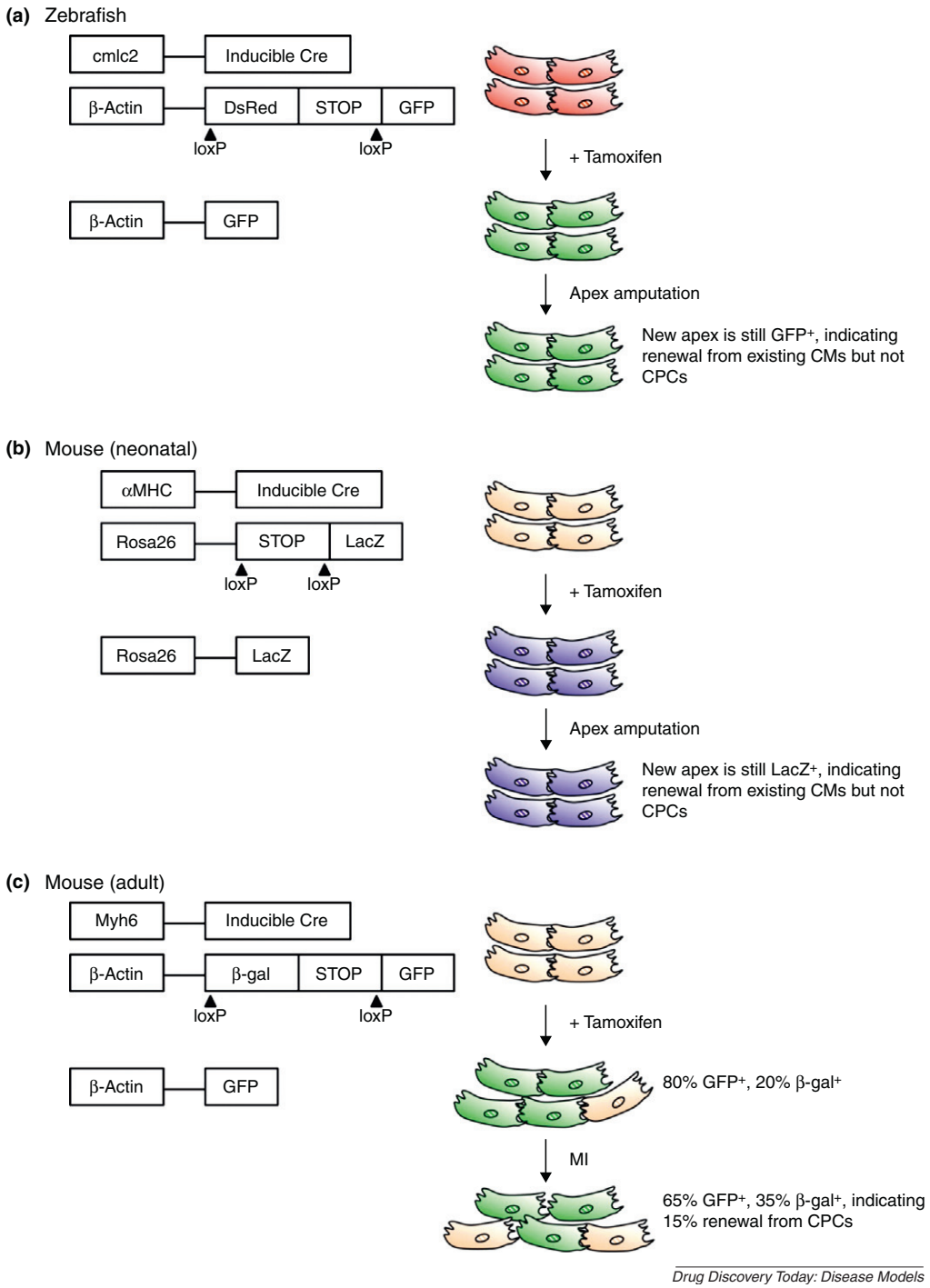

Figure 1. Genetic fate-mapping experiment in transgenic zebrafish and murine hearts. **(a)** In zebrafish, all cells were labeled as $DsRed^{+}$ (driven by β-gal promoter). Upon tamoxifen treatment, CMs were labeled as GFP^{+} (driven by inducible Cre under the control of CM-specific cml2 promoter) while CPCs remained $DsRed^{+}$. Following amputation of the cardiac apex, damaged CMs were quickly replaced by GFP^{+} cells but not $DsRed^{+}GFP^{-}$ cells, suggesting replacement via proliferation of the preexisting GFP^{+} CMs rather than differentiation from $DsRed^{+}GFP^{-}$ CPCs. **(b)** In murine one-day-old neonatal hearts, upon tamoxifen treatment, all CMs were labeled as $LacZ^{+}$ (driven by inducible Cre under the control of CM-specific αMHC promoter) while CPCs remained unlabeled. Following amputation of the cardiac apex, damaged CMs were quickly replaced by $LacZ^{+}$ cells but not unlabeled cells, suggesting replacement via proliferation of the preexisting $LacZ^{+}$ CMs rather than differentiation from unlabeled CPCs. **(c)** In murine adult hearts, upon tamoxifen treatment, all CMs were labeled as GFP^{+} (driven by inducible Cre under the control of CM-specific Myh6 promoter) while CPCs remained unlabeled. Following MI, there was a reduction in the proportion of GFP^{+} CMs, indicating dilution by the unlabeled CPC-derived CMs, albeit with very low efficiency. Therefore, heart regeneration in postnatal or adult mammalian hearts depends on differentiation of CPCs rather than proliferation of pre-existing CMs, as demonstrated in neonatal murine or zebrafish hearts.

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the ventricular septum and chamber walls during development. Following injury such as MI, Wt1 is found to be upregulated in the epicardial cells of adult mice [25,26] and $Wt1^+$ cells give rise to epicardium-derived cells (EPDCs) which secrete paracrine factors to promote angiogenesis [26]. The EPDCs-conditioned medium is found to reduce infarct size and improve cardiac functions after MI [26]. More recently, it has been shown that, in the presence of thymosin-β4, $Wt1^+$ cells differentiate into islands of functional CMs which structurally and functionally integrate with host cardiac muscle. Therefore, stimulating the existing adult progenitor pool represents a significant step toward cell-based therapy in human ischemic heart disease.

Signaling pathways in cardiogenesis

Embryonic cardiogenesis is controlled spatially and at discrete stages of development by a panoply of growth and transcription factors that regulate migration, proliferation, specification and maturation of cardiac cell lineages. Both first and second heart fields develop in response to inductive and repressive signals arising from fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), Sonic hedgehog (Shh), Wnt and Notch pathways (see [27] for review). Additional signals from the epicardium (e.g. retinoic acid [28]) and endocardium (e.g. neuregulin [29]) play an important role in heart field specification and cell differentiation. Signaling cascades are not only complex but dynamic: Wnt signaling, for example, is required for mesoderm formation, repressed for cardiac mesoderm commitment, and subsequently activated to induce progenitor expansion and CM proliferation [27]. In addition to epigenetic and transcriptional regulation, cardiogenesis is also controlled at the level of translation by small noncoding RNAs including microRNAs (miRs), which bind to and either inhibit or degrade mRNAs, thereby fine-tuning protein expression [30].

A major goal of regenerative cardiology is to selectively exploit these intricate developmental pathways for the purpose of directing differentiation from resident adult progenitors into cells lost during aging or diseases. With the advent of CPC lineage tracing models, it is now possible to purify and clonally expand specific CPC populations (e.g. $Flk\text{-}1^+$ [31], $Isl1^+$ [22,24], $Nkx2.5^+$ [32], $Tbx18^+$ [12], $Wt1^+$ [14,25]) derived *in vitro* from PSCs. PSC-derived multipotent populations closely resemble their physiological counterparts in the developing heart, and can readily differentiate into CMs (PSC-CMs), smooth muscle (PSC-SMCs) or endothelial cells (PSC-ECs).

In vitro cardiac differentiation protocols can broadly be categorized into embryoid body (EB) and two-dimensional (2D) approaches. EB approaches [13,26,33] rely on suspension culture, which recapitulates the developmental environment with more fidelity than 2D monolayers, but suffers from inherent heterogeneity and thus comparatively poor reproducibility. Two-dimensional culture methods include coculture with the visceral endoderm-like (END-2) stromal cells [33] or monolayer differentiation [34,35] using stage-specific growth factors and inhibitors. With each method, results are subject to variations between the specific PSC lines and batches, and protocols often need to be laboriously optimized independently for each line.

Despite these hurdles, differentiation protocols share a basis in the pathways that govern fetal cardiogenesis. Using an armamentarium of small molecules, growth factors and other proteins, researchers can systematically induce lineage commitment and specification. For example, approximately 60% beating EBs can be generated by isolating the KDR^{low}/$c\text{-}kit^-$ CPCs from EBs and culturing them in the presence of BMP4 (d0–d1), Activin-A (d1–d4), FGF2 (d1–d4 and d8–d14), VEGF-A (d4–d14) and DKK1 (d4–d14) [13]. In general, culturing EBs in hypoxic conditions with both BMP4 and FGF2 can yield 64–89% cardiac troponin I^+ CMs from a wide range of hESC and hiPSC lines [36]. Monolayer differentiation methods have variously employed Hedgehog [37], L-ascorbic acid [38], prostaglandin I2 [39], retinoic acid [35], Notch signaling inhibitor (e.g. gamma-secretase inhibitor) [40] and the MAP kinase inhibitor SB203580 [41]. Many of the growth factors or inhibitors are time-specific for their effectiveness; with some promoting formation of cardiogenic mesoderm at earlier stage but repressing subsequent specification to CMs (see [42] for review).

Differentiation from pluripotent cells is delineated by distinct marker expression at each stage: mesoderm (brachyury/T), cardiogenic mesoderm (Mesp1), early cardiac mesoderm (Nkx2.5, Mef2c), and early (Isl1) and mature (αMHC (MYH6), βMHC (MYH7, ANF, cTnT) CMs. Enrichment of CMs is now possible without genetic manipulation by targeting surface markers such as EMILIN2 [43], SIRPα [44] and VCAM [45], and purifying specific populations with FACS or magnetic bead sorting. Other approaches include the use of a mitochondrial dye, tetramethylrhodamine methyl (TMRM) ester percholate [46], which labels more mature CMs based on their higher mitochondrial density [44]; or optical and second harmonic generation (SHG) from sarcomeric myosin to enrich hPSC-CMs [47].

From progenitors to cell therapy

In 2001, severe arrhythmic complications following transplantation of skeletal myoblasts into the myocardium of patients with ischemic heart failure raised concerns over the safety of adoptive cell transfer therapy [48]. Alternative strategies swiftly emerged, calling chiefly on peripheral blood stem cells or bone marrow-derived stem cells, including mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs) or endothelial progenitor cells (EPCs) (see [49] for review). These approaches generally offer an excellent safety profile, but with a comparably poor record of efficacy. Allogeneic or autologous stem cell transplantation appears to

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induce, at best, a very modest (2.5–14%) improvement in left ventricular ejection fraction [49]. Several clinical trials have failed to demonstrate any statistically significant differences compared with controls. Regardless of the initial effect, any functional improvements appear to be temporary and have been largely attributed to a poorly understood paracrine effect [20,21,50].

A parallel search for progenitor cells with innate cardiomyogenic potential eventually presented another avenue for experimental regenerative therapeutics. With the discoveries of pluripotent hESCs in 1998 [51] and hiPSCs in 2007 [52,53], scientists can now generate an expandable source of human CMs in a 'patient-specific' manner. With advances in generating the lineage tracing ESC lines with a transgenic reporter system driven under the control of a specific CPC marker, such as Flk-1/cre [31], Isl1/cre [22,24], Nkx2.5/cre [32], Tbx18/cre [12] or Wt1/cre [14,25], it is now feasible to purify and clonally expand a specific type of CPCs, which resemble developmental progenitors in the embryonic heart. These PSC-derived multipotent progenitors can be differentiated into CMs, SMCs or ECs for performing *in vivo* heart regeneration studies. To date, there is no consensus regarding which cell type (e.g. CPCs, ECs, CMs, CMs + CPCs, CMs + ECs, or CMs + mesenchymal cells) is ideal for cell therapy. Most published studies suggest that transplanted hPSC-CMs yield only short-term improvements in cardiac function after MI, if any [54,55].

Various lines of recent evidence further suggest that it is possible to transdifferentiate scar-forming fibroblasts directly to CMs within the infarct zone *in vivo*. Exogenous expression of Gata4, Tbx5 and Baf60c has been shown to reprogram murine non-cardiogenic mesoderm to CMs *in vivo* [56]. Similarly, murine dermal fibroblasts have been directly reprogrammed to CMs via exogenous expression of Gata4, Mef2c and Tbx5 (GMT), resulting in the expression of cardiac cTnT in 4% of the initial fibroblasts which spontaneously beat in culture and display a gene expression pattern similar to that of murine neonatal CMs [57]. More recently, the same cocktail has been used to directly reprogram cardiac fibroblasts into CMs following coronary ligation *in vivo* [58]. Analysis of single cells demonstrates ventricular CM-like action potentials. The treated hearts also exhibit a reduction in infarct size with modestly attenuated cardiac dysfunction up to 3 months after GMT delivery. In another study, murine embryonic fibroblasts are reprogrammed to CMs by an initial expression of the pluripotency-inducing factors (Oct4, Sox2, Klf4 and c-myc), followed by exposure to the cardiogenic factor BMP4 [59]. Intriguingly, approximately 40% of the differentiated cells express cTnT by day 18 after induction. Despite these promising findings in mice, further studies are pending to demonstrate the direct reprogramming or transdifferentiation of somatic cells into CMs in the human system.

Creating a better microenvironment to facilitate cell therapy

Although PSCs are widely hailed as the harbinger of a revolutionary era in regenerative medicine, their derivatives do not engraft nor survive long-term after implantation [60,61], at least in the experimental settings that have been commonly employed and reported. Only marginal benefits have been observed in large-animal studies [55]. In a recent human trial, only moderate improvements are seen 4 months after subretinal transplantation of hESC-derived retinal pigment epithelium (RPE) in a patient with Stargardt's macular dystrophy and a patient with dry age-related macular degeneration [62]. In the genotoxic and inflammatory environment of the infarcted heart, implanted cells are forced to preserve in the presence of reactive oxygen species, inflammatory cytokines and chemical substances that compromise their regenerative potential and may even lead to DNA damage and mitochondrial DNA destruction [54,63]. In one study, *in vivo* bioluminescence imaging demonstrates that 90% of the implanted hESC-CMs die in the infarcted myocardium within 3 weeks in immunodeficient mice [60,61]. Although it is possible to isolate and expand potentially therapeutic cells from a variety of human PSC sources with up to 98% purity via temporal modulation of the canonical Wnt signaling [64], it is clear that implanted cells must also be primed to survive against the apoptotic, inflammatory, ischemic and necrotic signals within diseased and damaged tissues. Strategies using prosurvival factors, proliferative signals and paracrine factors via preconditioning with growth factors, pharmacological agents, biomaterials, or genetic engineering could stimulate survival signaling cascades and, therefore, improve the regenerative potential of implanted cells.

Prosurvival factors

Evidence suggests that targeting a single pathway (e.g. by overexpressing the antiapoptotic Bcl-2 and Akt in graft cells), or delivering a single prosurvival growth factor (e.g. insulin-like growth factor 1, IGF-1) does not improve graft survival or cardiac function [34]. Some successes have been seen in studies that deploy multiple interventions simultaneously. Laflamme and colleagues have reported the use of a prosurvival cocktail to prolong survival of hESC-CMs after injection into the infarcted rat heart. This cocktail includes Matrigel to prevent anoikis, a Bcl-$_{XL}$-associated peptide to block mitochondrial apoptotic pathways, cyclosporine A to attenuate cyclophilin D-dependent mitochondrial pathways, pinacidil to open ATP-dependent K^+ channels and thereby mimic ischemic preconditioning, IGF-1 to activate Akt pathways and the caspase inhibitor ZVAD-fmk [34]. In another study, overexpressing silent information regulator 1 (Sirt1) is found to be important in regulating expression of prosurvival factors such as manganese superoxide dismutase, thioredoxin-1,

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Table 1. Biomaterials that enhance stem cell function in the heart

Biomaterials	Cell types	Host species	Benefits	Protective mechanisms	References
Matrigel	ESCs	Mouse	Fractional shortening	n/a	[79]
Collagen	MSCs	Rat	Fractional shortening	n/a	[80]
	ESC-CMs	Rat	n/a	Enhanced engraftment	[81]
Fibrin	MSCs	Rat, Pig	n/a	Angiogenesis; Retention	[82,82]
PLCL	MSCs	Rat	Ejection fraction	Reduction in infaract size	[84]
Self-assembling peptides	MNCs	Pig	Ejection fraction	Angiogenesis; Retention	[83]
	CSCs	Mouse	Fractional shortening	Angiogenesis	[85]
Alginate	MSCs	Rat	n/a	Reduction in infract size; Enhanced cell survival	[86]

and Bcl-$_{XL}$ [65]. Sirt1 also represses proapoptotic molecules such as Bax and cleaved caspase-3 [65].

Proliferative signals

Some *in vivo* studies have shown that overexpression of cell-cycle activators (e.g. cyclin D2 [66]) or introduction of paracrine factors (e.g. neuregulin 1/NRG1 [67], thymosin β4 [25]) can induce proliferation of CMs. In one study, the transgenic expression of cyclin D2 is found to improve cardiac functions and reduce scar formation after MI [6]. In another study, injection of NRG1, which induces CM proliferation *in vitro*, improves cardiac functions and reduces scar size by 46% after 12 weeks of treatment post-MI [67]. The functional improvement is accompanied and presumably mediated by a 4.4-fold increase in CM proliferation after 15 weeks of treatment. More recently, it has been demonstrated that Wt1$^+$ epicardial progenitors proliferate after MI [14,25]. Lineage-tracing experiments further suggest that the Wt1-expressing cells normally contribute to cardiac fibroblasts, SMCs or perivascular cells in the epicardium [14,25]. However, upon stimulation by thymosin β4, Wt1-expressing cells differentiate into islands of CMs after MI, albeit with a low efficiency [25].

Paracrine factors

One of the mechanisms by which cell therapy modulates the damaged myocardium is paracrine signaling. Bone marrow derived c-kit$^+$ cells, for instance, stimulate endogenous CPCs and cardiac repair through secretion of paracrine factors [68]. In addition, evidence suggests that post-MI signaling (e.g. mediated by thymosin β4) stimulates proliferation and differentiation of the Wt1$^+$ epicardial progenitors into EPDCs, which secrete paracrine factors promoting angiogenesis, reducing infarct size and improving cardiac function [26]. In the presence of thymosin β4, *de novo* differentiation of the Wt1$^+$ epicardial progenitors into functional CMs which facilitate improvement in cardiac functions has also been reported [25]. Nevertheless, in the adult myocardium, non-myocardial cells such as ECs outnumber CMs by 3:1. These cells are in close proximity with every CM, and not only form blood vessels and provide oxygen and nutrients, but also exert trophic and inotropic effects [54,69]. Indeed, it has been shown that endothelial-derived angiocrine signals induce and sustain regenerative lung alveolarization [70]. Examining the effects of paracrine or angiocrine factor signaling in the heart (Table 1) or related organ systems may reveal a cardioprotective cascade for treatment of the injured heart.

Biomaterials

Extracellular matrix (ECM) is also important in supporting successful cellular engraftment in the heart. Relatively immature cells such as stem cells or progenitor cells, for example, have high differentiation potential but do not produce sufficient ECM, and are less likely to be engrafted following epicardial implantation [71]. Biomaterials are, therefore, essential for developing stable tissue engineering to support cell engraftment and survival following implantation. Various injectable biomaterials have been identified to deliver stem cells or progenitor cells to the heart, including Matrigel, collagen, fibrin, polylactic-coglycolic (PLCL) acid, self-assembling peptides and alginate (Table 2). Biomaterials can also be used as a source of naturally occurring ECM, delivering inotropic stimuli (e.g. proteins, genes or RNAs) to prevent inflammation, promote angiogenesis and differentiation of CPCs, and accelerate electromechanical integration of implanted CPCs or newly formed CMs into the host myocardium (for review, see [71]). For example, it has been shown that delivery of IGF-1 together with CPCs leads to the development of more mature CMs and greater improvement in cardiac function, compared to delivery of cells alone in the post-infarcted hearts [72].

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Table 2. Paracrine factors that enhance stem cell function in the heart

Factors	Delivery methods	Cell types injected	Host species	Protective benefits	References
Ang-1	Adenoviral transduction in MSCs	MSCs	Rat	Reduction in infarct size; Angiogenesis	[87]
Atorvastatin	Oral	MSCs	Pig	Reduction in infract size; Improved perfusion; Enhanced cell survival	[88]
Erythropoietin	Intracardial injection	n/a	Rat	Reduction in infarct size; Improved LV functions; Angiogenesis; SC homing	[89]
HGF	Intravenous injection	n/a	Mouse	Reduction in infarct size; Improved LV functions	[90]
IGF-1	Anenoviral transduction in MSCs	MSCs	Mouse	Reduction in infarct size; Improved LV functions; Angiogenesis; SC homing	[91]
SDF-1	Via GCSF or IGF induction	Cardiac fibroblasts, MSCs	Mouse, Rat	Reduction in infarct size; Improved LV functions; Angiogenesis; SC homing	[91,92]
VEGF	Adenoviral transduction of preconditioning of MSCs	MSCs	Mouse, Rat	Reduction in infract size; Angiogenesis; SC homing	[93,94]

Perspectives

From zebrafish to neonatal mice, heart regeneration seems to depend on a source of newly formed CMs, derived either from pre-existing CMs or CPCs, as well as a cardiac niche containing nonmyocardial tissues and signals facilitating repair after injury [6]. A wide range of research interests have converged to make progress toward this goal, but several significant hurdles remain. No single cell source has been identified as optimal for transplantation. The efficiencies for differentiation, purification and maturation still need to be optimized *in vitro* to generate sufficient and functional CPCs or CMs for transplantation (see [73] for review). One major caveat of cell-based systems lies in extrapolation from *in vitro* to *in vivo* improvement, especially because the implanted cells may not survive, engraft, integrate and/or function long-term alongside the host tissues. Therefore, it is important to modulate the microenvironment within the infarct zone to support cell growth and function following transplantation. Indeed, although hESC-CMs have been shown to engraft within the infarct zone in various animal models, most of the cells die within 3 weeks, even in immunocompromised recipients [60,61]. Recent studies suggest that direct intramyocardial injection of PSC-CMs should be performed in the border zone between viable and nonviable tissue, but not directly into the infarct zone [55,71] because the ischemic and inflammatory microenvironment may be less hostile in the peri-infarct area. Nevertheless, longer-term functional studies are needed to validate this finding and to identify optimal strategies for improving microenvironmental conditions.

Another challenge lies in the safety of transplanting hPSC-derived cells *in vivo*. Preliminary results from the first hESC clinical trial shows that there are no signs of hyperproliferation, abnormal growth or immune-mediated transplant rejection in either of two patients during the first 4 months following hESC-RPE implantation [62]. Concerns about immunogenicity apply not only to the allogeneic hESC- but also extend to autologous hiPSC-derived cells, which are vulnerable to incomplete reprogramming or genetic abnormalities accumulated during derivation [74]. Therefore, interdisciplinary fields draw from advances in areas such as stem cell biology, developmental biology, materials engineering and immunology to ensure safety and create a new PSC-derived myocardium that is electrically and mechanically integrated for cardiac repair.

More recently, evidence from direct reprogramming cardiac fibroblasts into CMs within the infarct zone *in vivo* [58] raises the intriguing possibility of transdifferentiating scar-forming fibroblasts directly to functional CMs after myocardial injury. However, further studies are needed to demonstrate whether long-term functional improvement can be achieved with this approach and whether other parallel interventions to induce angiogenesis or modulate the local environment are necessary for sustained beneficial effects. Apart from cardiac repair, it should be noted that derivation

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of hPSC-CMs can also generate new paradigms to model cardiogenesis or cardiovascular diseases 'in a dish' [75–78] for studying epigenetic and genetic regulation following injuries or aging. Moreover, such an *in vitro* model can utilize high-throughput assay systems for pathway discovery and small molecule drug screening using hiPSC-CMs derived from individual patients. Combined with more direct efforts to develop cell-based treatments for heart failure patients, these approaches may one day fulfill the promise of personalized medicine and finally bring regenerative therapeutics from benchside to the clinic.

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