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Review

Cardiac progenitors and paracrine mediators in cardiogenesis and heart regeneration

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ABSTRACT

The mammalian hearts have the least regenerative capabilities among tissues and organs. As such, heart regeneration has been and continues to be the ultimate goal in the treatment against acquired and congenital heart diseases. Uncovering such a long-awaited therapy is still extremely challenging in the current settings. On the other hand, this desperate need for effective heart regeneration has developed various forms of modern biotechnologies in recent years. These involve the transplantation of pluripotent stem cell-derived cardiac progenitors or cardiomyocytes generated *in vitro* and novel biochemical molecules along with tissue engineering platforms. Such newly generated technologies and approaches have been shown to effectively proliferate cardiomyocytes and promote heart repair in the diseased settings, albeit mainly preclinically. These novel tools and medicines give somehow credence to breaking down the barriers associated with re-building heart muscle. However, in order to maximize efficacy and achieve better clinical outcomes through these cell-based and/or cell-free therapies, it is crucial to understand more deeply the developmental cellular hierarchies/paths and molecular mechanisms in normal or pathological cardiogenesis. Indeed, the morphogenetic process of mammalian cardiac development is highly complex and spatiotemporally regulated by various types of cardiac progenitors and their paracrine mediators. Here we discuss the most recent knowledge and findings in cardiac progenitor cell biology and the major cardiogenic paracrine mediators in the settings of cardiogenesis, congenital heart disease, and heart regeneration.

1. Introduction

Since the human heart has a significantly limited ability to repair itself following injury, heart regeneration has long been sought after yet remains extremely hard to accomplish and thus in high demand for cardiovascular researchers and clinical cardiologists. Currently, standard of care treatment options offer little hope for a wide variety of severe forms of heart diseases including ischemic cardiomyopathy following myocardial infarction (MI) in adults and congenital cardiac birth defects in children. Thus, there is an urgent need to develop novel therapeutic approaches to better treat severe heart disease and improve the quality of life for the affected patients. In this regard, recent advances in stem cell biology and biotechnologies have helped us to gain a deeper understanding of the cellular and molecular mechanisms in heart formation and development. In addition, these new findings and the updated knowledge in this field hold great promise for cardiac regenerative medicine [1,2].

The human heart is a complex organ system and composed of highly diverse cell types, which are originally derived from mesodermal precursors and multipotent cardiac progenitors at early embryogenesis [3–6]. From the molecular viewpoint, multiple signaling pathways, as well as transcription factors and other mediators sequentially play essential roles during cardiogenesis [7,8]. Identifying various cardiac progenitor subpopulations and paracrine mediators is critically important to understand heart development and also the pathogenesis of congenital heart disease (CHD) in humans. New knowledge and discoveries in these areas may lead to novel strategies for heart regeneration and perhaps new treatment such as cell-based or cell-free regenerative therapy [1,2,9,10]. In this review, we describe the most recent notions in cardiac progenitor cell biology and the cardiogenic

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paracrine mediators in the settings of cardiogenesis and CHD, and thereafter discuss novel strategies for therapeutic heart regeneration.

2. Cardiac progenitors in cardiogenesis and heart regeneration

2.1. Embryonic cardiac progenitors

The vertebrate heart forms a complex three-dimensional structure in the early embryonic stages by a wide variety of cell types: cardiomyocytes (CMs), conductive cells (CCs), vascular smooth muscle cells (SMCs), endothelial cells (ECs), and cardiac fibroblasts. These cell types are derived from multipotent cardiac progenitors, which are self-renewing clones defined by their spatiotemporal presence and potential to differentiate into these specific lineages. Various types of mesodermal precursors and the earliest cardiac progenitors are present before commitment to either of the first or second heart field (FHF or SHF). Brachyury (Bry), a transcription factor and a member of the T-box family of genes has been shown to define the mesoderm during gastrulation and to be critical for mesoderm formation, thereby representing a mesodermal precursor marker [11–14]. Bry$^+$cells have the capacity to differentiate into Isl1 and Tbx5 expressing cells in humans, and further they have been shown to differentiate *in vitro* into the major cardiac cell populations: CMs, and vascular SMCs and ECs [12,15,16]. Another T-box transcription factor Eomes is also a critical intrinsic factor that initiates mesoderm differentiation and patterning of the primitive streak [11]. Eomes induces expression of mesoderm posterior 1 (Mesp1) as a downstream target [17,18]. Mesp1 is an essential regulator of cardiac mesoderm commitment in mammals and thus, marks the earliest cardiac progenitors within the primitive streak from embryonic day 6.25 (E6.25) to E7.25 in mice [19,20]. Mesp1$^+$cells can be identified preceding the separation into the FHF and SHF, where it has been shown that early FHF and SHF progenitors express a transcription factor NK2 homeobox 5 (Nkx2-5) at E7.5 in mice [19,21,22]. Another early cardiac progenitor population in humans has been defined by the expression of stage-specific embryonic antigen-1 (SSEA-1). SSEA-1$^+$cells have been shown to express markers of both the FHF and SHF and differentiate into CMs, SMCs and ECs [23]. They have even been used as a sorting marker for cardiac progenitors in a heart failure clinical trial [24]. Similar to SSEA-1, vascular endothelial growth factor type 2 receptor Flk-1, also known as kinase insert domain protein receptor (KDR), and platelet derived growth factor receptor alpha (PDGFR-α) are shown to be one of the earliest cardiac progenitor cell surface markers in mice and humans, as the Flk-1 (KDR)$^+$or PDGFR-α$^+$cells were demonstrated to give rise to CMs, SMCs and ECs *in vitro*and *in vivo* [12,15,25,26].

The FHF derives its name from harboring the first differentiated myocardial cells which specifically express the ion channel hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) [27]. The majority of CMs in the left ventricle and a small population of CMs in the right ventricle are derived from the HCN4$^+$progenitors, together with parts of the atria, and CCs from both the sinoatrial and atrioventricular nodes, and the ventricular conduction system [27,28]. The transcription factor Islet1 (Isl1) marks the SHF progenitors, which migrate from the pharyngeal mesoderm to the posterior side of the heart tube where morphological extension and looping occur [4,29–31]. Through contributions to several cardiac lineages, the SHF forms the majority of the right ventricle and parts of the atria and the outflow tract [4,31,32]. Besides the major contributions of the FHF to the left ventricle and the SHF to the right ventricle, field-specific progenitor cell populations have also been shown to support a minor contribution to the opposing ventricles as well [3,7].

The proepicardial organ (PEO) is a mesodermal precursor-derived transient structure which eventually forms the epicardium [33]. A murine linage tracing study suggested that Nkx2.5$^+$ and/or Isl1$^+$ cardiac progenitors contribute to PEO formation, and that Nkx2-5, but not Isl1, is functionally required for PEO development [34]. The PEO comprises two subpopulations, namely Wilms tumor-1 (Wt1) and T-box 18 (Tbx18)-positive cells, which mainly contribute to the SMC and cardiac fibroblast populations, and the semaphorin 3D (Sema3d) and scleraxis (Scx)-positive cells which additionally contribute to the EC population [35–37]. After migration of the PEO derivatives over the entire surface of the heart and formation of the epicardium, a subepicardial mesenchyme is formed by epithelial to mesenchymal transformation of epicardial cells overlying the atrioventricular groove [38,39]. Of note, Wt1$^+$ epicardial progenitors contribute to not only SMC and cardiac fibroblast formation but also cardiac endothelial cell formation within the myocardial ventricular layer of the developing heart in mice and humans [40,41]. It is a point of current debate if these epicardium-derived cells can contribute to the CM lineage [35–37,42].

A great number of these murine studies mentioned above and below employ fate mapping strategies with Cre/Lox technologies. It is important to note several limitations with this technology including: 1) The promoter driving Cre expression may be expressed at low levels in some untargeted cell types and induce accidental recombination (*e.g.*, Wt1 is also expressed in some myocardial cells and this might possibly lead to misinterpretations on epicardium giving rise to myocardium); 2) If a non-inducible Cre is used, no conclusion on the cell fate can be ascertained as a positive signal might represent Cre expression at the time of analysis; and 3) A good control on leakiness is needed (*e.g.*, CreERT2 may be active in the absence of tamoxifen).

The majority of the developed heart is composed of cells derived from the FHF and SHF progenitors, yet some components consist of cells derived from the cardiac neural crest cells (CNCCs). CNCCs originate from the dorsal neural tube with expression of Wnt1, Pax3 and Sox10 and migrate through the posterior pharyngeal arches to the arterial pole of the heart tube at around E9.5 in mice [43–46]. CNCCs and their derivatives give rise to SMCs of the pharyngeal arch arteries and cardiac cells of the outflow tract. They are involved in the formation of the cardiac valves, the parasympathetic nerve system and outflow tract patterning and septation [44,47–49].

2.2. Newly identified cardiac progenitors in cardiogenesis

Recent advances of biotechnologies involving multi-color lineage tracing, single-cell RNA and DNA sequencing, CRISPR-CAS genome editing, *etc.*, have identified previously unknown cardiac progenitor populations that would play certain roles in cardiogenesis. For example, an elegant study by Cui et al. demonstrated how single cell transcriptomics provides new techniques to identify and map out human developmental cardiogenesis [50]. Taken together, these new findings and advances in technologies enable us to understand the cellular and molecular mechanisms in cardiogenesis in a spatiotemporal manner more deeply. The novel cardiac progenitor populations may also be relevant for pathogenesis of CHD and/or serve as therapeutic tools for heart regeneration.

Lee et al. have recently reported a cardiac progenitor population identified by the G protein-coupled cell surface receptor latrophilin-2 (Lphn2) [51]. Deletion of *Lphn2* in murine embryonic stem cells (ESCs) significantly decreased their ability to express the cardiac lineage-related genes such as *Gata4*, *Nkx2-5*, *Tbx5* and *Isl1* during cardiac differentiation. The decline in cardiomyogenic gene expression subsequently resulted in a significantly decreased number of cardiac troponin T (Tnnt2)-positive cells that emerged after 10 days of differentiation. *In vivo* these impairments were validated and resulted in defective formation of the right ventricle, atrium and outflow tract, eventually causing a small and single ventricle ultimately leading to embryonic lethality in *Lphn2*$^{-/-}$ mice [51]. *Lphn2*$^{-/-}$ embryos also showed markedly reduced expression of *Gata4*,*Nkx2.5*,*Tbx5*, *Isl1* and *Tnnt2* genes, although the detailed mechanisms to clarify a role and function of Lphn2 on induction of these cardiogenic genes were unknown.

The majority of cardiac progenitors have been identified in mice, though gene expression profiles differ significantly between human and

rodent species [50]. Utilizing population and single-cell RNA sequencing in human ESC and embryonic/fetal heart derived cardiac cells, our group has recently described a human specific cardiac progenitor population [52]. This population is marked by the expression of the leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), involved in the Wnt signaling pathway. LGR5 has previously been shown to mark stem cells in various other organs, including intestin, colon, kidney, hair and follicle [53,54]. Intriguingly, deletion of *LGR5*in human ESCs using the CRISPR-Cas9 technology confirmed less efficient cardiomyocyte induction or differentiation through impaired expansion of Isl1$^+$Tnnt2$^+$ intermediates [52]. *In vivo*, LGR5$^+$cells were found in the proximal outflow tract (cono-ventricular) region in the early stage, *i.e.*, at 4–5 weeks of human fetal development. Importantly, LGR5$^+$cono-ventricular progenitors appear to be specific in humans as they are not found in murine embryonic hearts, suggesting that the population would be associated with human-specific mechanisms of cardiogenesis and also the pathology underlying CHD where mechanisms of defective development are largely unknown.

Recently, another zinc-finger transcription factor, *Spalt-like gene 1 (Sall1)*has been reported to mark undifferentiated heart precursors in both heart fields and thereby represent a unique subset of the early cardiac progenitors giving rise to both left and right ventricles in mice [55]. *Sall* genes are the vertebrate homologs of the *Drosophila* homeotic gene, *spalt*, and have been shown to play pertinent roles in the embryonic development of the limb [56,57]. The team showed *Sall1* was transiently expressed in pre-cardiac mesoderm contributing to development of the FHF and SHF and its expression was maintained in the SHF but not in FHF or differentiated cardiac cells [55]. *In vitro*, high levels of Sall1 protein at mesodermal stages enhanced cardiomyogenesis, whereas its continued expression suppressed cardiac differentiation, indicating the role of *Sall1*as a regulator of cardiac progenitor maintenance and cardiac differentiation.

Other interesting and novel cardiac progenitor populations include the Hopx$^+$, Foxa2$^+$ and Gfra2$^+$cells, which are described elsewhere [9]. In brief, *Hopx* (homeodomain-only protein homeobox) expression initiates shortly after the emergence of both heart fields (Nkx2-5$^+$), and Hopx$^+$ cells are fully committed to CMs distributed across all the adult heart chambers and essential for cardiac development in mice [58]. Interestingly, a recent single-cell RNA-seq study of the *in vitro* cardiac differentiation has also shown that *Hopx*is a key regulator of CM hypertrophy and maturation *in vitro* [59]. Bardot et al. have recently reported that Foxa2 (forkhead box protein A2)$^+$ cells are specified during gastrulation when they transiently express Foxa2 and that those cells represent a cardiac progenitor population with ventricular specification, giving rise primarily to CMs of the ventricles and only a few atrial cells of the differentiated heart in mice [60]. On the other hand, Ishida et al. have shown that Gfra2 (Glial Cell Line Derived Neurotrophic Factor Family Receptor Alpha 2) identifies a specific cardiac progenitor population functioning for cardiac compaction [61].

2.3. Adult cardiac progenitors and their potential for heart regeneration

The human heart possesses the ability to renew CMs, albeit very limited. Bergman et al. elegantly showed, using ^{14}C labelling, that 0.5 %–1 % of CMs renew yearly, resulting in a total of 40 % of CMs being replaced after a lifespan [62]. Although the origin of the newly generated CMs is of current debate, these findings suggest the presence of either a dividing population of preexisting CMs, differentiation of local resident cardiac progenitors, or both in the adult heart [63–66]. A more recent study investigated turnover of several cell-types in the heart more thoroughly, confirming CM exchange is highest in the peri-natal period, while interestingly, endothelial cells and mesenchymal cells continue to turn-over at much higher rates throughout life [67]. Several populations of endogenous cardiac progenitor cells in the adult mammalian hearts have been identified; however, it is likely that these "adult" cardiac progenitors reported to date contribute only rarely to direct cellular generation of new CMs in the adult setting, as described below [68].

Cells expressing the hematopoietic stem cell marker tyrosine kinase receptor c-kit have been isolated from the adult human heart and used as the adult cardiac progenitor marker [69,70]. The cardiac c-kit$^+$cells were reported to result in improved cardiac function when clinically injected in patients with ischemic cardiomyopathy [71], although that clinical trial (SCIPIO) was highly controversial and recently retracted [72]. In addition, the mechanisms underlying these debated effects are unknown as recent reporter and lineage tracing studies in mice have shown that c-kit$^+$ cells do not, or insignificantly, contribute to new CM formation during normal ageing or following injury such as MI *in vivo* [73–75]. Instead, *c-kit* predominantly labels a cardiac endothelial cell population in developing and adult hearts with or without injury [73], which is consistent with the recent study reporting that the majority ($\approx$90 %) of the resident c-kit$^+$cells in the rodent heart are blood/endothelial lineage-committed cells (CD45$^+$CD31$^+$) [76]. Collectively, their potential cardioprotective effects might be mainly due to secreted factors acting in a paracrine fashion rather than direct cellular contributions to newly generated heart muscle [77–79].

Besides their essential roles during cardiac development, undifferentiated Isl1$^+$ cells are present in very low numbers in the adult heart in mammals, mainly located in the atria [29,80]. However, their potential role for heart regeneration is questioned as they do not proliferate postnatally, even when CMs are still dividing [80].

Flk-1 (KDR)$^+$ cardiovascular progenitors were shown to contribute to the embryonic formation of the CM, EC and vascular SMC lineages [12,15], while their presence and/or potential roles in the adult hearts are not well determined. A recent study has reported that in adult rats, Flk-1 (KDR)$^+$ cells were detected in the pericardial adipose tissue and capable of giving rise to both myogenic and angiogenic precursors *in vitro*. It was further shown that after purification and transplantation of these Flk-1 (KDR)$^+$ cells *in vivo*, they could reconstitute the damaged heart in rats by the neoformation of microvasculature and of CMs, although these effects were mainly derived from the paracrine effects but not from the direct cellular contributions of the injected cells [81].

To isolate more putative and suitable cardiac progenitors in the adult heart, several groups used additional markers from mouse hematopoiesis, such as Stem cell antigen-1 (Sca-1) that is a mouse specific progenitor cell marker [82]. Other teams have used the ability to grow putative cardiac progenitors in three-dimensional clusters called cardiospheres [83]. Although the initial reports indicated the cardiomyogenic potential of the Sca-1$^+$cells [84–86], recent lineage tracing studies in mice have demonstrated that Sca-1$^+$cells exhibit endothelial but not myogenic contribution to the murine heart [87,88]. Cardiosphere-derived cell populations are heterogenous and their composition depends on the age of the subject they are derived from, with cells derived from neonatal hearts harboring the strongest regenerative capacity [83,89,90]. Injection of autologous cardiosphere-derived cells has been shown safe and beneficial in many preclinical models and the CADUCEUS clinical trial [83,91,92]. In the CADUCEUS trial, 17 patients with left ventricular dysfunction after MI received autologous cardiosphere-derived cells through intracoronary infusion in the infarct related artery. Cells were obtained from endomyocardial biopsies 2–4 weeks after infarction and administered within an average time of 36 days after biopsy. Significant decreases in scar size and increases in viability and regional function (but not global function) were observed after 1 year [91,92]. Since the engraftment rates are low, paracrine effects, and more recently exosomes and micro-RNAs have been identified as the putative cardioprotective mechanisms of cardiosphere-derived cell therapy [79,93–96].

Chong et al. isolated a cell population, termed cardiac colony forming units–fibroblasts (cCFU-F), resembling mesenchymal stem cells using FACS enrichment for Sca1$^+$PDGFRα^+CD31$^-$ cells from adult murine hearts [97,98]. These cells had a proepicardial origin and could give rise to a wide range of cell types, mainly cardiac fibroblast and

stromal cells, but with the right chemical cues they could also generate low amounts of CMs [98]. In a human setting, PDGFRα$^+$ cells were found in the interstitial cells of the epicardium, myocardium, and endocardium, as well as the coronary smooth muscle cells in the adult heart [25]. Only rare ECs and CMs in the heart expressed PDGFRα, although their presence increased in diseased hearts. *In vitro*, these PDGFRα$^+$ cells did not differentiate into CMs using the 5-azacytidine protocol, but large numbers of SMCs and ECs could be obtained [25]. Of further interest was a recent finding that observed a novel resident cardiac mesenchymal stem cell (MSC) niche identified as CD44$^-$CD44$^+$DDR2$^+$ which became pro-proliferative following MI in rats [99]. Of note, the team demonstrated a promoting role by erythropoietin in the stimulation of cardiac mesenchymal proliferation, which showed the newly identified cardiac MSCs exerted cardiomyogenic and angiogenic properties. Furthermore this newly identified MSC population also accelerated a healing process through transforming growth factor β (TGF-β) and wingless-int (Wnt) signaling pathways [99]. Very recently, Valente et al. identified another population of immature cardiomyocytes marked by cell surface markers, heat stable antigen (HSA) and CD24 in both embryonic and adult hearts in mice [100]. The team has found that the HSA/CD24$^+$ CM subset actively proliferated up to 1 week of age and engrafted cardiac tissue upon transplantation. Interestingly, in the adult heart following MI injury, a 3 fold increase in HSA/CD24$^+$ mononucleated CMs with modest Ki67 expression was observed around the areas of MI [100].

Other subpopulations of the epicardium, such as Wt1 and Tbx18 expressing cells have been of interest for their ability to differentiate into CMs following injury [36,37]. Wt1$^+$ cells in mice were successfully mobilized and differentiated into CMs as well as ECs with the use of a single injection of modified mRNA encoding vascular endothelial growth factor A [101]. Finally, side population (SP) cells from neonatal rat hearts have been reported to home to the heart after injury and differentiate into CMs, SMCs and ECs [102]. The SP cells are a heterogenous population of cells representing 0.02–2 % in adult murine hearts [103,104]. As this population is defined by their ability to efflux the DNA-binding dye Hoechst 33342 from their nucleus, lineage tracing experiments that require a specific marker cannot be performed, which makes it difficult to interpret the results of the studies employing the SP cells, mechanistically [103].

3. Cardiogenic paracrine mediators in cardiogenesis and congenital heart disease

Multiple signaling pathways play critical roles during cardiogenesis in a sequential and coordinated fashion. The major signaling pathways involved in cardiac development include the TGF-β superfamily, Wnt, fibroblast growth factors (FGFs), Hedgehog, Notch and Retinoic acid pathways [1]. These signaling pathways, in concert with transcription factors and epigenetic regulators, control cardiac progenitors' specification, proliferation and differentiation into diverse cardiac cell lineages and contribute to building the entire heart. Below, we offer detailed descriptions of these major signaling pathways and their importance in normal cardiac development (Section 3.1) and in the pathogenesis of CHD (Section 3.2). In addition, in Section 3.3, we focus on more details of each paracrine mediator and describe how these factors exert cardiomyogenic and/or vasculogenic effects and how they have been applied in a regenerative context.

3.1. Cardiogenic signaling pathways in cardiogenesis

3.1.1. TGF-β superfamily signaling pathway

The TGF-β superfamily members contain more than 30 structurally related polypeptide growth factors including TGF-βs, bone morphogenetic proteins (BMPs), activin and nodal [105]. TGF-β signals *via* their protein kinase receptors and downstream mediators, Smads, which regulate a plethora of biological processes. BMPs are indispensable for gastrulation and primitive mesoderm formation in mammals. Previous studies showed that deletion of *Bmp4* or a BMP type I receptor (*Bmpr1a*) in the germline system caused embryonic death before E9.5 in mice [106,107]. Further, conditional deletion of *Bmp4* or *Bmpr1a* under the mesodermal and cardiomyogenic Cre drivers such as *Mesp1-Cre*, *Nkx2-5-Cre*, or *Tnnt2-Cre* mouse lines results in abnormal cardiac morphogenesis, respectively, highlighting the essential roles of BMPs for cardiac specification and development [108–111]. Interestingly, conditional deletion of *Bmpr1a* using the *Isl1-Cre* mice caused right ventricle and outflow tract hypoplasia with an increased number of undifferentiated Isl1$^+$ cells, indicating that the activation of BMP signaling is important for the second heart field (SHF) progenitors' differentiation and myocardium maturation [108,112]. Recently, the single-cell RNA-seq analysis using wild type and Mesp1-knockout (KO) murine embryos has revealed that among Mesp1$^+$ mesodermal precursors, *Bmp4* could distinctly mark the cardiomyocyte (CM)-committed population at E7.25 without co-localized expression of an endothelial cell marker *Sox7* [113,114].

Activin and nodal are also important regulators of gastrulation, primitive streak and mesoderm/endoderm formation, left-right asymmetry of the body axis, and positional patterning in early embryos and later for cardiomyogenesis [115,116]. Interestingly, a recent study has revealed that the genes encoding the activin A subunit *Inhbaa* was critical for organization of atrioventricular canal (AVC)-localized extracellular matrix (ECM), facilitating migration of epicardial progenitors onto the developing heart tube in zebrafish [117].

Smad4 is a core transcription factor of the TGF-β signaling pathway. Loss of the *Smad4* gene has no effects on the self-renewal of human ESCs (hESCs), but causes a subsequent complete loss of CM induction during the *in vitro* hESC cardiogenesis, suggesting an essential role of *Smad4* for the formation of cardiac mesoderm [118].

3.1.2. Wnt signaling pathway

The Wnt signaling pathway participates in multiple developmental events during embryogenesis. The Wnt family has 19 different Wnt proteins and 10 types of Frizzled receptors [119]. These Wnt and Frizzled receptors can be divided into two major classes based on their primary functions, the canonical and non-canonical Wnt pathways [120]. The function of the canonical Wnt pathway is exerted through the active β-catenin/TCF transcriptional complex in the nucleus. The canonical Wnt ligands include Wnt1, Wnt2a, Wnt3a, and Wnt8 [121,122], while the non-canonical Wnt ligands such as Wnt5a, Wnt4 and Wnt11 act through the Wnt/calcium and Wnt/JNK pathways [123]. Before gastrulation, the canonical Wnt signals are involved in the formations of primitive streak, mesoderm and endoderm [124]. But, after gastrulation, a secreted Frizzled-related protein (sFRP) and Dickkopf1 (Dkk1) secreted from the adjacent endoderm inhibit these signals. This spatiotemporal inhibition of the canonical Wnt signaling is essential for further cardiac specification in the mesoderm [125]. These biphasic effects of the canonical Wnt signals are also recapitulated in cultured mouse and human pluripotent stem cells (PSCs) *in vitro*. The active Wnt/β-catenin signals promote mesoderm and endoderm formation in the early phase of the PSC differentiation yet inhibit cardiac myogenesis after the mesoderm has been once established [126–129]. The canonical Wnt signaling also plays an important role at later stages of embryonic cardiogenesis, which involves both the proliferation and maintenance of the SHF progenitors and the prevention of their differentiation [130]. Conditional deletion of the *β-catenin* gene using the *Mef2c-Cre* mouse line led to right ventricular and outflow tract hypoplasia with a dramatic reduction in the number of the Isl1$^+$ SHF progenitors, while enhanced β-catenin expression in the Isl1$^+$ SHF progenitors led to right ventricular enlargement and hyperplasia with an increase in the number of Isl1$^+$ cells [131,132]. Interestingly, recent studies have revealed that Alpha Protein Kinase 2 (ALPK2) is the promising candidate for negative regulators of the Wnt/β-catenin signaling pathway and promotes cardiac differentiation and maturation in hESCs

and zebrafish analyzed by antisense knockdown and CRISPR/Cas9 mutagenesis [133]. Furthermore the canonical Wnt signaling specifically regulates specification of the SHF, but not the FHF, since the addition of Wnt3A in pre-cardiac organoid models resulted in a further increase in the SHF markers' expression and a reduction in the FHF markers' expression [134].

The non-canonical Wnt signaling is also required for cardiac specification and differentiation. *Wnt5a*- or *Wnt11*-null mice showed impaired pharyngeal artery patterning and outflow tract defects [135,136]. Inversely, overexpression of *Wnt11* promoted cardiac specification and differentiation of the cardiac progenitors derived from murine ESCs *in vitro* [128].

3.1.3. FGFs signaling pathway

The FGF signaling pathway includes more than 20 ligands and 4 transmembrane receptor tyrosine kinases [137,138]. Four members of them (FGF11–14) are intracellular proteins that do not interact with FGF receptors (FGFRs) [139–141]. The FGFR-like 1 (FGFRL1) protein lacks an intracellular tyrosine kinase domain [142]. Most members of the FGF family play important roles as paracrine or endocrine signals in heart development and disease [143]. During differentiation of human PSC (hPSCs), FGF2 specifically promotes mesoderm-committed precursors' formation [144]. *Fgf8* is expressed in the early posterior dorsal mesoderm, and the *Fgf8-KO* mice died at the gastrulation stage due to the lack of embryonic mesoderm-derived structures [145]. Conditional deletion of the *Fgf8* gene using the *Tbx1-Cre* mice led to impaired outflow tract morphologies, suggesting that mesodermal *Fgf8* expression is essential for formation of the SHF-derived structures [146]. In fact, *Fgf8* regulates the expression of the SHF marker genes *Isl1* and *Mef2c* in mice [147]. *Fgf9* and its relatives *Fgf16* and *Fgf20* are expressed in both murine endocardium and epicardium at mid-gestation and contribute to myocardial proliferation and maturation [148]. Indeed, the proliferative capability of *Fgf9*-mutant CMs was significantly diminished [148]. FGF10 promotes CM differentiation and proliferation *in vitro* and *in vivo*, and over expression of the *Fgf10* gene in transgenic mice induced the cell-cycle re-entry of adult CMs [146,147]. The embryonic hearts of the *Fgf10-KO* mice showed impaired right ventricular morphology [149]. Similarly, conditional deletion of the FGFR type 1 (*Fgfr1*) and type 2 (*Fgfr2*) genes using the ventricle-specific driver *Mlc2v-Cre* mice caused severe ventricular defects [148].

3.1.4. Hedgehog, Notch, and retinoic acid signaling pathways

In mammals, there are three Hedgehog (Hh) proteins: Sonic Hh, Indian Hh, and Desert Hh [150]. The Hh ligands bind to patched 12-span transporter-like receptors that inhibit the function of Smoothened (Smo) serpentine receptors in the absence of ligands [151]. In zebrafish, the Hh signaling promoted CM formation [152], whereas in mice, it has been shown to be involved in the establishment of left and right asymmetry, coronary vasculature, atrial septation and outflow tract morphogenesis [153–155].

Notch signaling is associated with a wide range of developmental processes and cell-fate decisions in various cell lineages [156]. In mammals, there are four Notch receptors (Notch1–Notch4) and five structurally similar Notch ligands (Delta-like 1 [DLL1], DLL3, DLL4, Jagged1, and Jagged2) [157]. During embryonic cardiogenesis, the Notch signaling controls right ventricle and outflow tract formation, vascular smooth muscle development, chamber specification and trabeculation [158–161]. The SHF-specific deletion of *Notch1* using the *Isl1-Cre* mice promoted proliferation of $Isl1^+$ progenitors and caused overexpression of β-catenin in the SHF, indicating that the Notch signaling interferes with the canonical Wnt signaling in the SHF and inhibits proliferation of the SHF progenitors, thereby promoting their differentiation [151,162]. Intriguingly, it has been shown in zebrafish embryos that differentiated atrial CMs could transdifferentiate into ventricular CMs through activation of the Notch signaling [163].

Retinoic acid (RA), a biologically active metabolite of vitamin A (retinol), is produced by retinaldehyde dehydrogenase 2 (Raldh2) [164]. The RA signaling regulates the patterning of the SHF derivatives along the anterior and posterior axes [165]. The developing hearts of the *Raldh2*-KO murine embryos failed to undergo left-right (LR) looping morphogenesis at E9.5 along with the abnormal expression of the anterior SHF marker genes such as *Tbx1* and *Fgf8/10*, and died at mid-gestation (E10.5) [166,167]. Thus, the RA signaling is essential for normal development of embryonic outflow tract and atria [166,168,169]. Further, a recent study has revealed that RA signaling at the mesoderm stage of development is required for atrial specification and promotes differentiation into atrial-like CMs at the expense of ventricular CMs in the *in vitro* hPSC cardiogenesis [170].

3.2. Genetic disorder of cardiogenic signaling pathways and transcription factors in congenital heart disease

CHD is a serious issue of structural and functional deficits of the developing heart and the most common malformation with high morbidity in children, affecting 1/100 live births [171]. The most common types of congenital heart defects are: ventricular septal defect (VSD), atrial septal defect (ASD), tetralogy of Fallot (TOF), single ventricle defects (SVD) (*e.g.*, hypoplastic left heart syndrome [HLHS] and pulmonary atresia [PA]), double outlet right ventricle (DORV), common arterial trunk (CAT), pulmonary valve stenosis (PVS), patent ductus arteriosus (PDA), transposition of the great arteries (TGA) and aortic valve stenosis (AVS). Many factors that are classified into genetic and environmental categories are associated with the etiology of CHD. Normal cardiac development is depending on multiple signaling pathways spatiotemporally regulated, as noted above. If any of these pathways are disrupted or incorrectly function, the specific cardiac defects would be emerged as a form of syndromic or isolated CHD (Table 1) [172]. Here we describe several important genes relating to the cardiogenic signaling pathways, which have been identified as rare causes of CHD. Further, various embryonic cardiac progenitor populations would also be most likely involved in the pathogenesis of CHD. However, it is still an unaddressed question how the fundamental cellular and/or molecular defects in cardiac progenitors lead to each of specific morphologic phenotypes of CHD, which is currently under investigation [52,173].

3.2.1. Mutations in the TGF-β superfamily signaling pathway associated with CHD

Disruption of individual genes in the TGF-β superfamily signaling pathway often lead to embryonic lethality in mice [174]. Due to their critical roles in cardiac development, mutations within the TGF-β superfamily genes are also detected in human CHD [175,176]. Loeys-Dietz syndrome (LDS) is one example of the TGF-β signaling malfunction-related CHD and an autosomal dominant genetic connective tissue disorder [177]. The key feature of LDS is an enlarged aorta or an aortic aneurysm, often detected in children. The aortic aneurysm may undergo sudden dissection in the weakened layers of the aortic wall, leading to greater risk for dying, and thereby, surgical repair of aneurysms is required for treatment [177]. These features of LDS are overlapped with those of Marfan's syndrome (MFS), which is caused by mutations in the *Fbn1* gene and an autosomal dominant genetic disorder of the connective tissue [178,179]. There are five types of LDS which are distinguished by their genetic cause as follows: type I (*TGFβR1*), type II (*TGFβR2*), type III (*SMAD3*), type IV (*TGFβ2*) and type V (*TGFβ3*) (Table 1) [180–183]. The type I and II are the most common forms of LDS. Mutations of these five genes encoding the TGF-β signaling pathway-associated factors cause dysfunction of connective tissue proteins (*e.g.*, collagen), resulting in a wide variety of the phenotype of LDS, including arterial tortuosity, long limbs and fingers, hypertelorism, split uvula, abnormal skin scars, aortic aneurysms, and ASD [177,180].

Animal experiments, especially in mice, have been used to confirm

Table 1
Genes of the signaling pathways and transcription factors associated with CHD.

Gene	Signaling pathway	Cardiac phenotype	Syndrome	References
TGFβR1	TGF-β superfamily signaling pathway	Aortic root aneurysm, Arterial tortuosity, ASD	Loeys-Dietz	[177]
TGFβR2	TGF-β superfamily signaling pathway	Aortic root aneurysm, Arterial tortuosity, ASD	Loeys-Dietz	[177]
SMAD-3	TGF-β superfamily signaling pathway	Aortic root aneurysm, Arterial tortuosity, ASD	Loeys-Dietz	[180,181]
TGFβ2	TGF-β superfamily signaling pathway	Aortic root aneurysm, Arterial tortuosity, ASD	Loeys-Dietz	[180,182]
TGFβ3	TGF-β superfamily signaling pathway	Aortic root aneurysm, Arterial tortuosity, ASD	Loeys-Dietz	[180,183]
ACVR1	TGF-β superfamily signaling pathway	AVSD		[172]
ACVR2B	TGF-β superfamily signaling pathway	TGA, DORV		[187]
GDF1	TGF-β superfamily signaling pathway	TGA, TOF, DORV		[189]
LEFTY2	TGF-β superfamily signaling pathway	AVSD, CoA, IAA		[187]
NODAL	TGF-β superfamily signaling pathway	VSD, ASD, TOF		[185,186]
SMAD6	TGF-β superfamily signaling pathway	BAV		[234]
TDGF1	TGF-β superfamily signaling pathway	VSD, TOF		[235]
JAG1	Notch signaling pathway	TOF, PVS, AVSD, AVS	Alagille	[193,194]
NOTCH1	Notch signaling pathway	AVS, BAV, CoA, TOF, VSD	Adams-Oilver	[199,200]
NOTCH2	Notch signaling pathway	TOF	Alagille	[191,192]
ALDH1A2	Retinoic acid pathway	TOF		[201]
STRA6	Retinoic acid pathway	VSD, ASD	Matthew-Wood	[202]
SOS1	RAS-MAPK pathway	PVS, ASD, VSD	Noonan	[211]
SHOC2	RAS-MAPK pathway	PVS, ASD, VSD	Noonan	[172]
PTPN11	RAS-MAPK pathway	PVS, ASD, hypertrophic cardiomyopathy	Noonan Leopard	[210]
Raf1	RAS-MAPK pathway	TOF, hypertrophic cardiomyopathy	Noonan Leopard	[212,213]
TAB2	IL-1 signal transduction pathway	OFT defects		[236]
VEGF	Vascular Endothelial Growth Factor (VEGF) Signaling Pathway	CoA, OFT defects		[237]
FLT4	Vascular Endothelial Growth Factor (VEGF) Signaling Pathway	TOF		[238]

Gene	Transcription Factors	Cardiac phenotype	Syndrome	References
GATA4	GATA-binding TF	ASD, PVS, TOF		[226,227,228]
GATA6	GATA-binding TF	OFT defects		[239]
NKX2-5	Homeobox TF	TOF, ASD, VSD, atrioventricular conduction defects		[240]
NKX2-6	Homeobox TF	TFO, DORV, VSD		[240]
TBX1	T-box TF	TOF, VSD, PTA, IAA	DiGeorge	[231]
TBX5	T-box TF	ASD, VSD, conduction defects	Holt–Oram	[232]
TBX20	T-box TF	ASD, TOF, aberrant valvulogenesis		[233]
TFAP2B	AP-2 TF	PDA	Char	[241]
ZIC3	Zinc finger TF	DORV, TGA, AVSD		[242]

*This table includes representative genes associated with CHD, and the more comprehensive lists of them were excellently reviewed elsewhere [172,215].
ASD, Atrial septal defect; AVS, aortic valve stenosis; AVSD, Atrioventricular septal defect; BAV, bicuspid aortic valve; CoA, coarctation of aorta; DORV, double outlet right ventricle; IAA, Interrupted aortic arch; PDA, patent ductus arteriosus; PTA, persistent truncus arteriosus; PVS, pulmonary valve stenosis; TOF, tetralogy of Fallot; TF, transcription factor; TGA, transposition of the great arteries; VSD, ventricular septal defect.

human disease genes and to gain further insight into the cellular and molecular mechanisms behind CHD. For example, *TGFβ2*-null mice showed reduced muscularization of the outflow tract septum and incomplete ventricular septation of the hearts [184]. Interestingly, using the *Wnt1-Cre/TGFβR1* floxed mice, specific loss of *TGFβR1* in the neural crest led to 100 % penetrance of a persistent truncus arteriosus (PTA) phenotype [185], or endothelial cell-specific loss of *TGFβR1* using the *Tie2-Cre* mice led to severely reduced cellularity of the atrioventricular cardiac cushion [186]. These animal studies further shed light on the essential role and molecular signatures of the TGF-β signaling pathway on cardiogenesis.

Nodal signaling, which also belongs to the TGF-β superfamily, is a critical component in establishing left-right asymmetry of the body-axis and determining organ laterality in early embryogenesis [187]. *Nodal*, *Lefty2*, *ACVR2B* and *GDF1* are all associated with the laterality signaling pathway [188–192], and mutations of these genes result in a variety of cardiac laterality defects, including TGA, atrioventricular septal defects (AVSD), DORV and TOF (Table 1).

3.2.2. Mutations in the Notch signaling pathway associated with CHD

Similarly to the TGF-β superfamily signaling pathways, disruption of individual genes in the Notch signaling pathway often results in a human genetic disease with cardiac phenotypes. Alagille syndrome is a genetic multisystem disorder that can affect the heart, liver, kidneys, eyes and other parts of the body. More than 90 % of patients with Alagille Syndrome have cardiovascular anomalies, such as TOF, PVS, AVSD and pulmonary arterial stenosis [193]. The majority (> 90 %) of patients with Alagille Syndrome are caused by mutations in *Jagged1* (*Jag1*), encoding a Notch signaling ligand, while a small number of cases are caused by mutations in *Notch2*, encoding a Notch receptor (Table 1) [194–197]. Homozygous *Jag1*-null ($Jag1^{-/-}$) mice die from hemorrhage possibly due to vascular defects during early embryogenesis [198], while heterozygous $Jag1^{-/+}$ mice display eye defects but do not exhibit other phenotypes such as cardiovascular anomalies [199]. In humans, however, Alagille Syndrome is caused by haploinsufficiency of *Jag1* [200].

Notch1 signaling is important at endothelial-to-mesenchymal transformation, an early process in cardiac valve formation, which is required in forming endocardial cushions from migratory mesenchyme cells [201]. Mutations in *Notch1* have been identified in patients with isolated CHD, often presenting malfunctions of the aortic valve (Table 1) [202,203].

3.2.3. Mutations in the retinoic acid signaling pathway associated with CHD

The RA signaling is essential for primitive heart tube formation. Mutations in the *Stra6* and *Aldh1a2* genes associated with the RA signaling have been linked to CHDs [204,205]. RA is synthesized from vitamin A (retinol) that is transported to cells by retinol binding protein *via* Stra6, a membrane protein involved in the metabolism of retinol. In humans, mutations in the *Stra6* gene are associated with Matthew

Wood Syndrome, which has a broad spectrum of malformations including CHD such as ASD and VSD, anophthalmia, diaphragmatic hernia, alveolar capillary dysplasia, lung hypoplasia and mental retardation [205], although *Stra6*-null mice did not have overt cardiac defects [206]. Aldh1a2 protein is an enzyme that catalyzes the synthesis of RA from retinaldehyde and is responsible for production of almost all RAs during early development [207]. In mice, deletion of *Aldh1a2* caused heart defects with poor development of atria and sinus venosus [166], while in zebrafish, deletion of *Aldh1a2* caused emergence of the enlarged heart with increased CM number [208]. Mutations in *Aldh1a2* display TOF in humans (Table 1) [204].

3.2.4. Mutations in the Ras-MAPK signaling pathway associated with CHD

The Ras-mitogen activated protein kinase (MAPK) signaling pathway activates cell proliferation, differentiation, maturation, survival and metabolism. Mutations in the genes related to this pathway cause a wide range of multisystem anomalies, including CHD [209]. Noonan syndrome and related disorders are causally linked to germline mutations in the Ras-MAPK signaling-associated genes [210]. Approximately 85 % of patients with Noonan syndrome have a variety of cardiac defects, most commonly including PVS, ASD, and hypertrophic cardiomyopathy [210]. Thus, Noonan syndrome is the second most common genetic syndrome of CHD [211]. *Ptpn11* is the first identified causal gene of Noonan syndrome, accounting for 40–60 % of the cases [210]. Subsequently, mutations in many of other genes were reported to cause Noonan syndrome and Noonan-like phenotypes. Patients with mutations in the *Raf1* or *Rit1* genes have hypertrophic cardiomyopathy [212,213]. A minority of the cases with *Raf1* mutations have TOF [213]. Germline gain-of-function mutations in *Sos1* can cause Noonan syndrome, in which PVS is emerged more frequently in individuals with *Sos1* mutations (Table 1) [214].

3.2.5. Mutations in cardiac transcription factors associated with CHD

Cardiogenic signals, as noted above, are transmitted to multi-sequential transcriptional circuits that spatiotemporally regulate gene expression during normal heart development. These transcriptional networks rely on the functions of core transcription factors, many of which can cause syndromic or isolated CHD when genetically mutated (Table 1). Here we briefly describe only several important transcription factors about their function and molecular signatures associated with CHD, as they were reviewed more comprehensively elsewhere [215].

In humans, mutations in the homeodomain protein gene *Nkx2-5* result in a plethora of CHDs, including ASD, VSD, TOF, DORV and atrioventricular conduction defects [216,217]. *Nkx2-5* is expressed in both the FHF and SHF [218], and *Nkx2-5*-null mice exhibit embryonic lethality due to faulty cardiac looping and insufficient myocardial differentiation during chamber formation [219,220], along with lack of the primordium of atrioventricular node [216]. It has also been reported that Nkx2-5 interacts with other cardiogenic transcription factors Gata4 [221,222] or Tbx5 [223,224] within cardiac promoters, cooperating in the transcriptional activation of cardiac target genes. Recently, analysis of three missense single-nucleotide variants in the *Mkl2*, *Myh7*, and *Nkx2-5* genes in murine hearts and human induced pluripotent stem cell (iPSC)-derived CMs confirmed the *Nkx2-5* variant's contribution as a key genetic modifier [225]. Further, the triple-heterozygous mice exhibited deep trabeculation in the left ventricular walls that were similar to those seen in patients with left ventricular non-compaction (LVNC) [225].

Gata4 is another important transcription factor during heart development. In humans, mutations in the *Gata4* gene can cause isolated CHDs, including cardiac septal defects, PVS and TOF [226–228]. *Gata4*-null mice exhibit embryonic lethality at E10.5 due to failure to establish a primitive heart tube, while mice heterozygous for *Gata4* mutations develop CHD phenotypes, such as septation and endocardial cushion defects [229].

The T-box transcription factors are also essential cardiac transcription factors, which function in cardiac developmental processes, such as the formations of outflow tract, heart chambers, and the conduction system [230]. Haploinsufficiency of *Tbx1* is the primary cause of CHD in patients with DiGeorge syndrome whose cardiac phenotypes are commonly conotruncal malformations, including interrupted aortic arch, persistent truncus arteriosus, TOF and VSD [231]. Mutations in *Tbx5* cause Holt–Oram syndrome, which display upper limb defects and heart defects, primarily septal and conduction defects [232]. Patients that have mutations in *Tbx20* have aberrant valvulogenesis, septal defects, TOF and cardiomyopathy [233]. Other CHD-associated genes, *e.g.*, transcription factor AP-2 beta (TFAP2B) and a zinc finger transcription factor ZIC3, and their cardiac phenotypes when mutated are shown in Table 1 [234–242].

3.3. Paracrine factors for cardiomyogenesis and vasculogenesis

Induction of myocardial repair *via* revascularization and/or proliferation of CMs using growth factors (GFs) or other mediators has aroused much interest within cardiovascular regenerative medicine. Such GFs have the ability to quickly induce direct actions on a multitude of cellular properties capable of enhancing reparative mechanisms including cell growth, proliferation, migration, trans-differentiation and others. The human body naturally expresses many GFs after injury, but the expression is often too low and transient to induce tissue repair. Therefore, the overexpression of certain GFs *via* gene or protein therapies in pre-clinical and clinical studies is intensely being investigated as a treatment regime to treat ischemic cardiomyopathy and prevent the progression of heart failure. Below we briefly highlight some of the most essential GFs and other mediators as the paracrine factors involved in mammalian vasculogenesis and cardiomyogenesis, which are being applied in regenerative medicine (Table 2). For a more comprehensive review of GF therapies in heart repair and regeneration we refer the readers to the following reviews [243,244].

3.3.1. Vasculogenic growth factors

In the case of cardiac injury such as MI, therapeutic vasculo-/angiogenesis in myocardium is a promising mechanism for ischemic tissue salvage. To date, some of the most encouraging angiogenic factors capable of inducing regenerative mechanisms in the diseased setting include, but are not limited to: vascular endothelial growth factor (VEGF), FGF, stromal-derived factor-1alpha (SDF-1α), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), and TGF-β. The exogenous administration of a vast majority of these angiogenic factors have been shown to protect the myocardium at the onset of hypoxia/ischemia injury and as such have been coined cardioprotective GFs. The mechanisms and signaling pathways by which these factors act in order to protect CMs are reviewed elsewhere [245–247]. Several of these GFs have been explored clinically and we briefly summarize a few relevant results below.

SDF-1 (CXCL12) and its receptor CXCR4 have been shown to play key roles in cardiac development. Mutant rodent models with misregulated CXCL12/CXCR4 give rise to VSD or truncated and irregular coronary artery development [248,249]. SDF-1 is a chemotactic factor that has been shown to recruit stem cells to sites of injury from the bone marrow in order to help grow new blood vessels as well as prevent cell death and reduce scar sizes when administered to the damaged heart [250]. More recently SDF-1 has gained appeal for clinical use in patients with heart disease. The STOP-HF was a double blinded, placebo controlled clinical study that tested the safety and efficacy of plasmid SDF-1. In this study the administration of SDF-1 to ischemic heart patients reported increased left ventricular ejection fraction (LVEF) compared with placebo groups at 12 months and the study concluded no adverse effects were seen from SDF-1 treatment [251].

HGF and its receptor c-Met, a transmembrane tyrosine kinase, are transiently expressed in CMs in early developmental stages of the rodent heart [252]. HGF has been identified as a marker of acute MI and

Table 2
Paracrine factors for cardiomyogenesis and vasculogenesis.

Growth factor family or other mediators	Receptor/Pathway	Action on cell types	Main mechanisms of action	References
VEGF	VEGFR1-2	EC	Angiogenesis, reduced fibrosis	[101,256,257,258,259,260,261,262,263,264,265,266,396,397,398]
FGF	FGFR1-4	CPC, CM, SMC, EC	Cardiomyogenesis, angiogenesis, anti-apoptosis	[137,138,139,140,141,142,143,144,145,146,147,148,267,268,269,270,271,289,290,291,292,293,294]
BMP	BMPR1A, BMPR1B, BMPR2	CPC, CM	Cardiomyogenesis	[106,107,108,109,110,111,112,113,114]
SDF-1	CXCR4	EC, SMC, CM, BMC/HSC	Angiogenesis, BMC/HSC mobilization, reduced fibrosis	[248,249,250,251]
IGF	IGF1R, IGF2R	CM, CPC	Cardiomyogenesis	[272,273,274,275,276,277,278,279]
IGFBP	Insulin signaling	CM	CM differentiation	[280,281,282,283,284]
HGF	c-Met	EC	Angiogenesis	[252,253,254,255]
PDGF	PDGFR	EC, SMC	Angiogenesis, reduced fibrosis	[302]
Ang-1	Tie1-2	EC, CM	Angiogenesis, Cardiomyogenesis, anti-apoptosis	[301]
NRG-1	EGFR; ErbB pathway	CM	Cardiomyogenesis	[285,286,287,288]
Periostin	PI3K/AKT, ERK pathways	CM	Cardiomyogenesis	[295,296,297,298]
YAP	Hippo/YAP pathway	CM	Cardiomyogenesis	[314,315,316,317,318,319]
Agrin	Hippo/YAP, ERK pathways	CM	Cardiomyogenesis, reduced fibrosis	[321]

Ang-1, angiopoietin-1; BMC, bone marrow-derived cell; BMP, bone morphogenetic protein; CM, cardiomyocyte; CPC, cardiac progenitor cell; EC, endothelial cell; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; HSC, hematopoietic stem cell; IGF, insulin-like growth factor; NRG-1, neuregulin-1; SDF-1, stromal cell-derived factor-1; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor.

has gained value as a potential angiogenic factor [253,254]. Intracoronary administration of HGF and IGF (*as described below*) in pre-clinical large animal models have been shown to activate endogenous cardiac stem cells which foster the generation of new myocardium and improved ventricular function [255].

VEGF is a major endothelial cell mitogen capable of driving angiogenesis and vasculogenesis [256]. VEGF and its receptors play essential roles in many different aspects regarding the developmental formation of the cardiovascular circuitry [257]. Early reparative studies employing VEGF-A administration through plasmid and recombinant protein delivery systems documented angiogenic stimulation and enhanced cardiac repair in large pre-clinical animal models of heart disease [258,259]. However, studies employing VEGF-A gene and protein therapies in patients with coronary artery disease have been predominantly negative and did not demonstrate significant clinical benefits [260–262]. In contrast to these studies, some Phase I/II clinical cardiac studies have reported positive results from VEGF-A protein or gene therapy [263–265]. On-going gene therapy trials and explanations of discrepancies in these studies are described elsewhere [266]. More recently VEGF-A has been administered to the diseased myocardium in the form of a therapeutic mRNA agent with much success, as described in detail below (see Section IV).

FGF-2 (basic FGF) and FGF-4 are also known as pro-angiogenic factors [267]. They have pleiotropic roles in various cell types and act as mitogenic, angiogenic and survival factors that are involved in cell proliferation and differentiation [267,268]. FGF-4 likely plays a role in more mature blood vessel formation and has been shown to have an additive role in the induction of VEGF synthesis [269]. In a series of AGENT clinical trials, it has been indicated that there was a significant and gender (women)-specific beneficial effect of intracoronary adenovirus-containing FGF-4 treatment in patients with coronary artery disease and chronic stable angina [270,271].

3.3.2. Cardiomyogenic growth factors

In recent years, the concept of the adult heart being a terminally differentiated organ has been challenged [62]. This work and others has prompted another concept for restoring large lost cell masses often seen with infarction injuries; namely by employing GFs to trigger CM cell cycle entry to induce proliferation and expansion of new functioning populations of CMs. To date several promising GFs and secreted peptides have been shown to activate CM cell cycle entry, which include IGF, neuregulin-1 (NRG-1), acidic FGF (FGF-1), and periostin (Postn).

IGF-1 is a small signaling peptide that shares 50 % homology with insulin and regulates cellular growth and metabolism in the heart [272,273]. IGF-1 signaling in cardiac muscles involves activation of both MAP-kinase and phosphatidylinositol 3-kinase (PI 3-kinase) pathways [274,275]. IGF-1 has been shown to prevent long-term left ventricular remodeling in large animal models of cardiac injury through mechanisms involving stem/progenitor cell activation, differentiation and enhanced viability/survival [276]. Recent studies also showed that epicardium secretes IGF-2, which activates IGF-1 receptors and subsequently ERK signaling in CMs to induce proliferation [277]. More recently IGFs have been shown to play an emerging role in CPC proliferation, expansion and induction into CMs, a valuable asset for PSC-derived cell therapies, which are discussed in Section IV [278,279]. Interestingly, IGF proteins are heavily regulated by IGF binding proteins (IGFBPs), which can positively regulate IGF stability by increasing the biological half-life of the proteins in circulation; or negatively regulate insulin signaling by blocking IGF receptors and/or insulin receptors [280,281]. There are six highly conserved IGFBP family members expressed in vertebrates [282]. A number of the IGFBP family members have been reported to display cardiomyogenic effects. For example, a previous report affirmed a role for IGFBP-4 as a cardiogenic GF where it enhanced CM differentiation *in vitro* and acted as a molecular link between IGF and Wnt signaling [283]. More recently circulating IGFBPs have emerged as potential biomarkers for cardiovascular

diseases [284]. A more thorough understanding of the mechanisms of actions driving and resulting from IGF-IGFBP interactions in the normal heart as well as in the diseased cardiac settings are needed.

NRG-1 is a member of the epidermal growth factor (EGF) family of membrane-tethered ligands and functions as a cardioactive GF released from cardiac ECs, which has been shown to induce adult CMs to enter the cell cycle, proliferate CMs, and improve cardiac function following injury in rodent models *via* ErbB signaling [285,286]. More recently and quite interestingly, the interplay between nerve growth factor-β (NGF-β) and NRG-1 were reported to regulate CM proliferation and thus enhance cardiac regeneration and repair in zebrafish and rodent models [287]. Clinical studies that capitalize on patient puncture biopsies in order to elucidate the effects of NRG-1 on human cardiac tissue are underway (NCT02820233). Indeed, a completed Phase II clinical study successfully confirmed a tolerable and safe dose of recombinant human NRG-1 (rhNRG-1) that was capable of enhancing heart function and reverse-remodeling the left ventricle in patients with chronic heart failure [288]. An additional clinical study is now aiming to evaluate the functional efficacy in reducing death rates of heart failure subjects receiving different doses of rhNRG-1 (NCT03388593).

FGF-1 is a multifunctional peptide involved in a multitude of different cellular functions that are mediated through interactions with the four FGFRs [289]. In the heart, FGF-1 is secreted by a number of different cell types including CMs, ECs, macrophages and fibroblasts and plays a role in cardiac developmental-morphogenesis [290]. FGF-1 secretion intensifies under physiologically stressed conditions including hypoxia or ischemic injury [291,292], and has been shown to reduce apoptosis in CMs following vascular injuries in the heart [293]. The underlying mechanisms regulating these pathways are not yet fully understood. More recently the use of rhFGF-1 is being explored in regenerative cardiovascular medicine. In preclinical studies of ischemia reperfusion the delivery of FGF-1 (and NRG-1) using the NOGA MYOSTAR™ injection catheter was met with improved cardiac function and decreased transmural infarct sizes [294]. More recently the application of rhFGF-1 is being considered in clinical studies for no-option heart patients with coronary artery disease (NCT00117936).

Postn expression stems from, predominantly mesenchymal-related cell types where it has been reported to play paramount roles in the development and morphogenesis of cardiac valves [295,296]. Postn is a non-structural extracellular membrane (ECM) protein, which has been reported to be a major regulator of cell-matrix interaction, cell fate determination, migration and proliferation in the developing heart [297]. Using rat-derived mature CMs Kuhn et al. showed Postn treatment significantly increased DNA synthesis in CMs *in vitro* [298]. This team further explored the role of Postn to induce CM cycling *in vivo*, where Postn stimulated and sustained CM cell-cycle activity thus improving cardiac functional parameters and decreased scar sizes following myocardial injury. However, some conflicting studies report no effect of Postn on CM cell-cycle entry [299] or confirmed the administration of Postn was capable of enhancing CM proliferation, but was associated with negligible side effects of increased fibrosis in a large animal model of heart disease [300]. Thus, further investigations are required to confirm the authentic effects and mechanisms of Postn on cardiomyogenesis.

3.3.3. Growth factor cocktails for cardiac repair

In order to stimulate effective repair to the damaged heart following ischemic injury, medical research teams have begun to employ combinatorial cell survival and regenerative GFs to co-stimulate cardiomyogenesis and vasculogenesis. In a large animal model of left-anterior descending artery (LAD)-ligation, Tao et al. employed adeno-associated viruses (AAVs) expressing VEGF and another angiogenic peptide, namely angiopoietin-1 (Ang-1), which were co-administered to infarct and peri-infarcted regions [301]. Co-expression of both factors were met with higher vascular densities, increased CM proliferation and activation of pro-survival pathways that decreased apoptosis in CMs when compared to control animals. Another study employing a rat MI model demonstrated the sequential delivery of two angiogenic factors, one potent mediator of neo-angiogenesis and another that importantly should help stabilize sprouting neovessels [302]. In this study, VEGF-A and platelet-derived growth factor-BB (PDGF-BB) were sequentially injected intramyocardially following injury through a fibrin gel system. The beneficial effects of sequential delivery of VEGF-A and PDGF-BB recombinant proteins were improved cardiac function, improved ventricular wall thickness and reduced inflammatory/fibrotic responses, as compared to controls. Thus, a GF cocktail strategy rather than a single GF approach appears to hold promise for cardiomyogenesis and vasculogenesis to the damaged hearts, but successful treatment strategies involving multiple GFs need to involve effective routes of administration. For example, in a study by Hwang et al. the team administered a cocktail of four GFs as soluble factors: FGF-2, SDF-1, IGF-1 and HGF that were delivered intraperitoneally in rats following cardiac injury – the results of which had no effect on cardiac functional improvement, reduction in scar sizes or improved microvasculature [303]. Controlling the expression kinetics of a GF cocktail through dose, timing and methods of administration could go someway to help mitigate the injury induced by MI and add value to cardiovascular regenerative programs.

3.3.4. Alternative mediators of cardiomyogenesis

In recent years, investigational studies aiming to employ cell-cycle phase activators/mediators as a potential source for regenerative repair in the heart after injury have dramatically been increased. In mammalian CMs, cyclins and cyclin-dependent kinases (CDKs) help orchestrate cell-cycle state and transitions [304]. The mechanisms which underlie the CM cell-cycle in development and regeneration are discussed in detail elsewhere [305]. In general, the expression of these cell-cycle regulators to induce adult CM division has been met with several caveats including nuclear division without proliferation/cytokinesis or instability following cellular division [306–308]. Yet induction of the cell cycle continues to emerge as a possible intervention for inducing heart repair. One previous study showed the transgenic expression of cyclin D2 improved survival rates in a mouse model of transverse aortic constriction (TAC) [309]. The TAC-treated mice presented with hypertrophy, which was met a 50 % increase in CM number. In another study cyclin D2 lentiviral-mediated gene transfer increased cell proliferation in human iPSC (hiPSC)-derived CMs [310]. The same team went on to show beneficial effects in mice following MI injury, where the cyclin D2 transfected hiPSC-derived CMs increased in cell number between 1 and 4 weeks after implantation, which improved cardiac function and reduced infarct size. Also of interest was a recent study that extended proliferation of adult CMs through miR-128 deletion [311]. The deletion had an effect on suppressing p27 (a cyclin-dependent kinase inhibitor) and subsequently activated cyclin E and CDK2. Together the stimulation of cell cycle re-entry of adult CMs reduced fibrosis and cardiac dysfunction in response to MI. Of further interest was a large animal study that revealed a regenerative response by employing adenoviral delivery of cyclin A2 following catheter-based MI [312]. Six weeks after treatment, MRI based assessment revealed nearly a 20 % increase in cardiac contractile function and histological evaluation determined a substantial increase in CM mitoses and decreased fibrosis. These data provide compelling evidence for continued development of cell-cycle regulation for cardiac regenerative therapies.

In line with the studies from above, a more recent publication elegantly identified pertinent cell-cycle mediators expressed during fetal cardiomyogenesis and subsequently used adenovirus to over-express them in rodent and human adult CMs, resulting in the induction of adult CM cell division *in vitro* [313]. Interestingly, when the team administered a discrete cocktail comprising of CDK1, CDK4, cyclin B and cyclin D1 to an acute or sub-acute cardiac injury model, substantial and effective *in vivo* CM proliferation was noted, up to 15–20 %, a response that was ultimately capable of driving functional cardiac improvement.

The Hippo signaling pathway controls the size of several organs during development [314] and inhibits CM proliferation through inactivating the transcriptional coactivator Yap, which is a terminal effector of the Hippo pathway [315,316]. Yap has robust CM mitogenic activity linked to the PI3K-AKT pathway, as forced expression of constitutively active Yap in CMs strongly stimulated their proliferation in both neonatal and adult murine hearts [317,318]. Thus, the Hippo/Yap pathway has become a therapeutic target for stimulating CM proliferation and heart regeneration following MI injury. Indeed, a recent study using AAVs to overexpress activated Yap in adult CMs has demonstrated that this treatment could improve myocardial function and survival after MI in mice [319].

Heart pathologies after MI highlight extensive remodeling of the extracellular matrix (ECM) resulting in formation of fibrotic scars and reduced cardiac function. Although the role of the ECM during heart regeneration was largely unknown, a recent elegant study has uncovered a new mechanism of Yap inhibition by the dystrophin-glycoprotein complex (DGC) and its component dystroglycan 1, which directly binds to Yap to block CM proliferation in neonatal mice [320]. Notably, Bassat et al. have identified the ECM protein Agrin that disassembles the DGC through mediating Yap and ERK signaling pathways, and thereby induces the full regenerative capacity of neonatal mouse hearts [321]. Importantly, the team has also shown that a single administration of Agrin promotes heart regeneration in adult mice after MI, highlighting fundamental roles of the ECM in cardiac repair.

Similarly to the Hippo pathway and the DGC-Yap interaction to inhibit CM proliferation, cumulative evidence suggests that polyploidization and/or binucleation of CMs, which occur naturally in the post-natal mammalian heart, create a barrier to heart regeneration [322,323]. Several recent studies have revealed that simply increasing DNA content and/or promoting polyploidization in the highly regenerative zebrafish CMs *via* knockdown of the cytokinesis inducer Ect2 or the Tnni3k interacting kinase, are sufficient to suppress their proliferative potential during regeneration, respectively [324,325]. This suggests that blocking the emergence of polyploidy and binucleated CMs and in turn, increasing the number of diploid and mononucleated CMs by manipulating the Ect2-mediated or other cytokinesis-associated pathways (*e.g.*, Tnni3k), could offer a new therapeutic target for heart regeneration.

Other signaling networks capable of influencing cell-cycle stimulation in adult CMs and currently being heavily explored include Jak/Stat, Wnt/β-catenin, p38 and Notch, and these pathways are discussed in detail in the above sections or in a recent review [304].

4. Novel strategies of therapeutics for heart regeneration

Patients suffering from a vast majority of cardiovascular diseases are treated with medications that help to alleviate symptoms and decelerate disease progression, however such drugs fail to restore and/or repair damaged, necrotic tissue. Ischemia-related cardiomyopathies following the events of MI, for example, often lead to chronic heart failure with high morbidity in patients where the progressive loss of functional CMs is replaced by a non-functional fibrotic scar tissue [326]. This results in architectural remodeling and eventually adds strain to the muscle, limiting the pumping capacity of the heart.

To date, there are multiple different approaches to promote cardiac regeneration in the damaged hearts in humans (Fig. 1; Table 3). These therapeutic strategies that are classified into cell-based or cell-free therapy have been or are currently being investigated for their authentic efficacies and potentials for heart regeneration in the settings of pre-clinical and early clinical trials, as extensively reviewed elsewhere [1,9,327]. Here, we shortly discuss the latest discoveries and notions in these attempts. Further, we would like to mention that there is a body of knowledge spanning decades of work regarding how “lower vertebrate” species including aquatic salamanders and teleost fish, but not mammals, naturally regenerate their hearts following cardiac injuries. Understanding the natural mechanisms of heart regeneration in those species has attracted much interest for researchers over the decades. To go into detail on such studies, however, would greatly outweight the scope of this review. For an up-to-date overview on these model systems including some descriptions of molecular and cellular networks driving naturally occurring regeneration, we direct the reader to the following informative reviews [328,329].

4.1. Cell-based therapies

Stem cell therapies are emerging as the potential "white knights" for cardiac repair, showcasing considerable promise for the generation of new cardiac muscle *in situ* and improving heart function. The development of cell-based therapies for heart regeneration have emerged from many exogenous sources that include somatic stem/progenitor cells such as bone marrow-derived cells (BMCs), hematopoietic stem cells (HSCs), MSCs, and adult cardiac progenitor cells (CPCs); or the directed differentiation of hPSCs into CMs and/or CPCs (Fig. 1; Table 3) [1,9,327]. Here, due to space limitations, we concisely focus on the most recent advancements in novel cardiac cell therapies, namely those involving transplantation of hPSC-derived cardiac cell types to repair damaged myocardial tissue. Therapeutic potentials of adult CPCs are discussed in Section 2, and for the recent and on-going clinical trials of adult CPC therapies for heart regeneration, see [9]. Much controversy and scientific misconducts surrounding the c-kit$^+$ CPC research are reviewed elsewhere [66]. We also refer readers to other excellent reviews that describe other somatic stem cell therapies including BMCs, HSCs and MSCs for heart regeneration [330–332]. Of interest, the results obtained from many of these reported clinical trials employing adult somatic stem cells (BMCs, *etc.*) are quite contradictory and show at best only modest therapeutic efficacy. These contradictory outcomes may stem from a combination of methodological/technical processing techniques used by different research teams, the parameters of the study design, or even inherent limitations and/or various weak points involved in the stem cell therapy itself, the details of which are discussed in recent reviews [333,334].

Another exciting strategy for heart regeneration is direct reprogramming of nonmyocytes such as fibroblasts to CM-like cells (induced CMs), which is achieved by transduction of nonmyocytes with a cocktail of cardiac transcriptional regulators for myocardial transdifferentiation [335,336]. This strategy is still in development, but results have been promising, as described elsewhere (*see review* [337,338]).

4.1.1. Pluripotent stem cell-derived cardiomyocytes

Human ESCs are derived from the inner cell mass of the blastocysts. They can indefinitely self-renew and are capable of differentiating into all the major cell types of all 3 germ layers [339]. Human ESC-derivatives have great therapeutic potential for treating cardiovascular disease (CVD) yet continue to provoke ethical concerns [340]. In 2006 Takahashi and Yamanaka reported the remarkable story of generating an iPSC from somatic cells using 4 defined transcription factors, the characteristics of which were remarkably similar to ESCs [341]. Over the years many protocols have been established which induce cardiomyogenic differentiation from either human ESCs or iPSCs, namely hPSCs [342–346]. Such technology holds much promise yet remain hampered by several technical challenges, which often include the production of immature CMs in a heterogenous population as well as limitations in regards to generating these cells in large-scale quantities. Regardless, numerous paramount studies have made significant strides towards the realization of clinically translating hPSC-derived CMs for cardiovascular diseases.

In an elegant study from the Murry lab, human ESC-derived CMs (hES-CMs) were generated in clinical scale large batches and transplanted in diseased non-human primate (NHP) hearts following injury [347]. Ischemia-reperfusion injury was met with extensive remuscularization (from the hES-CMs) in infarcted regions of the heart as

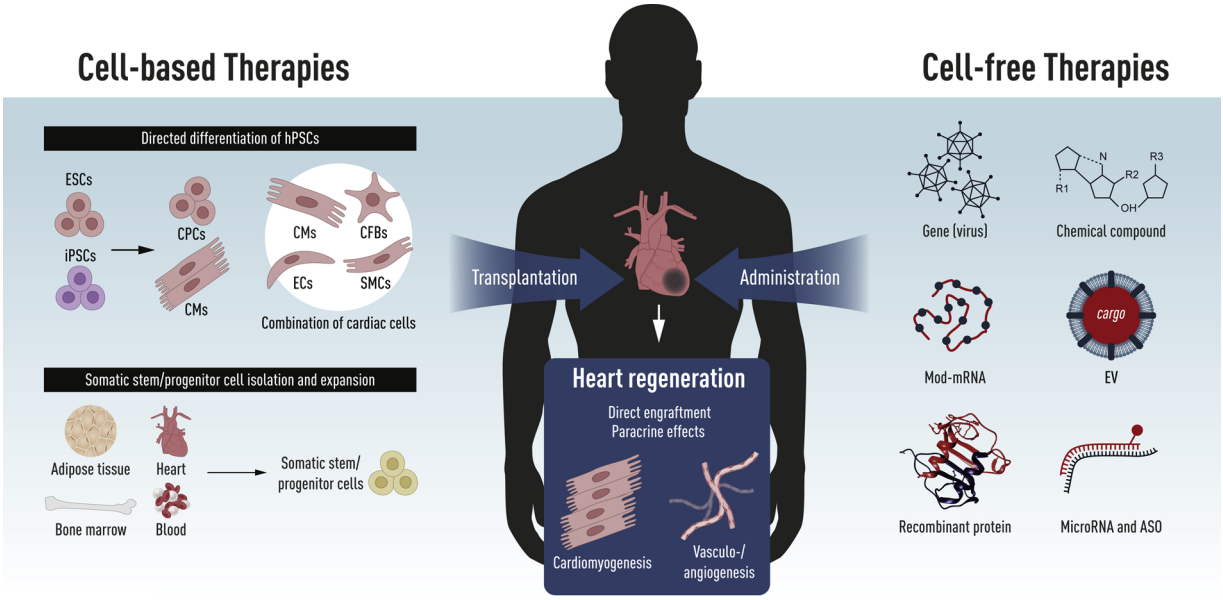

Fig. 1. Schema of potential therapeutic strategies for heart regeneration.
Novel therapeutic strategies for cardiac regeneration are classified broadly into cell-based (*left*) or cell-free therapies (*right*). The cell-based therapies involve transplantation of human pluripotent stem cell (hPSC)-derived cardiac progenitor cells (CPCs), cardiomyocytes (CMs), or multiple cardiac cells (combination of some among CMs, endothelial cells [ECs], smooth muscle cells [SMCs], cardiac fibroblasts [CFBs], *etc.*), or somatic stem/progenitor cells isolated and expanded from adult organs and tissues. The cell-free therapies are conducted by administrating some cardiomyogenic and/or vasculogenic factor(s) in the forms of gene (plasmid or mainly, viruses), modified mRNA (Mod-mRNA), or recombinant protein. Recently, small non-coding RNAs (*i.e.*, microRNAs or antisense oligonucleotides [ASOs]) and extracellular vesicles (EVs) such as exomes have attracted much attention as novel non-cellular strategies for heart regeneration (*also see* Table 3). hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells.

early as 2 weeks post-transplantation. The engrafted human cells gave rise to patchy muscle grafts that ranged in size, none-the-less the authors revealed some evidence that supports electromechanical coupling between the host and graft, however frequent arrhythmias were reported which could be explained by either re-entry circuits or spontaneous automaticity of the graft. Shortly following the study by Chong et al., another team published some pioneering work employing NHP-derived iPSC-CMs (iPS-CM) as a major histocompatibility complex (MHC)-matched allogeneic transplantation model in NHPs [348]. In this way, the team was able to reduce the risks of immune mediated

Table 3
Chart of potential therapeutic strategies for heart regeneration.

Classification	Methodology/category	Cell or vector type	Details/examples	References
Cell-based therapies	hPSC differentiation	CPC	SSEA1$^+$ISL1$^+$	[24,360]
			HVP	[359]
		CM		[347,348,349,350,351,352]
		Cell combination	CM/EC	[366,367]
			CM/EC/SMC	[364]
			CM/MSC	[365]
	Somatic stem/progenitor cell isolation/expansion	BMC/HSC	CD34$^+$, CD133$^+$	*see review* [330,331,332]
		MSC		*see review* [330,331,332]
		MSC/CPC	Flk1$^+$	[81]
		Adult CPC	c-kit$^+$	*see review* [66]
			CDCs	[83,91,92]
	Direct reprogramming	Induced CPC		[355,357]
		Induced CM		*see review* [337,338]
Cell-free therapies	Gene (DNA)	Plasmid	VEGF, SDF-1	[250,251,258], *also see review* [391,392,393]
		Adenovirus	FGF4, VEGF, HGF, CDKs	[270,271,313,384], *also see review* [391,392,393]
		AAV	SERCA2a, VEGF/Ang-1, YAP	[301,319,386,387,388,389,390], *also see review* [391,392,393]
		LV	Kit ligand	[382,383]
	RNA	Mod-mRNA	VEGF-A	[396,398]
		MicroRNA	"Myo-miRs" miR1, miR21, miR199	[370], *also see review* [368,369,374]
		ASO	miR-25	[373], *also see review* [372,374]
	Protein	Recombinant protein	HGF, IGF1, VEGF, NRG-1, FGF-1, Periostin, PDGF, SDF-1, FGF-2	[255,263,265,288,294,298,300,302,303]
	Exosome	EV	Derived from: ESCs, iPSCs, UC-MSC, CDC, CM, PBMCs	*see review* [378]

AAV, adeno-associated virus; ASO, antisense oligonucleotide; BMC, bone marrow-derived cell; CDC: cardiosphere-derived cell; CDK, cyclin-dependent kinase; CM, cardiomyocyte; CPC, cardiac progenitor cell; EC, endothelial cell; ESC, embryonic stem cell; EV, extracellular vesicle; HSC, hematopoietic stem cell; hPSC, human pluripotent stem cell; HVP, human ventricular progenitor; iPSC, induced pluripotent stem cell; Mod-mRNA, modified mRNA; MSC, mesenchymal stem cell; LV, lentivirus; PBMC, peripheral blood derived mononuclear cell; SMC, smooth muscle cell; UC-MSC, umbilical cord derived mesenchymal stem cell.

graft rejection and demonstrated iPS-CMs endure long-term survival in infarcted hearts. In addition, as shown by the Murry lab, histological evaluation by Shiba et al. also demonstrated the iPS-CM transplantation partially re-muscularized myocardium in patch-like islands. Furthermore, the transplantation significantly improved cardiac contractile function, as shown by echocardiogram assessment in as little as 4 weeks post-infarct and transplantation. The team was also able to demonstrate electrical coupling between the host and graft, and this integration likely explains the improved force generation, although several studies describe similar functional improvements from paracrine-mediated effects [349,350].

Quite recently, several other studies investigated the physiological basis for ventricular arrhythmias associated with hPSC-derived CM engraftment in infarction models using NHP or porcine models, respectively [351,352]. The first study concluded the basis for ventricular arrhythmias associated with hPSC-derived CM grafting is likely to not stem from reentry but arise from pulse generation from an ectopic activation source. In the second report the study from the Laflamme team point to focal automaticity driving ventricular tachycardia rather than a reentrant phenomenon. Whether high rates of arrhythmia stem from reentrant pathways caused by electrical instability between graft and host, increased sympathetic innervation, or impurities within the transplanted grafts themselves, requires more attention. It has become genuinely accepted that gap junction proteins like connexin-43 (Cx43) are essential for electromechanical integration and serve to protect against electrical instability [353,354]. Thus, engineering cells to inherently form gap junction proteins could perhaps increase electromechanical coupling and help curb the occurrence of irregular arrhythmias.

4.1.2. Cardiac progenitor cells

A major interest in innovative stem cell therapies has recently involved employing the adult or hPSC-derived CPCs for cardiovascular regenerative therapies [9]. In regards to regenerating infarcted heart tissue, transplanted CPCs may have more favorable mechanisms than immature CMs, as it is speculated the progenitor cells are more capable of *in situ* proliferation, migration, and differentiation into multiple lineages of cardiac cells as well as exert beneficial effects through the release of robust paracrine factors. In fact, several reports have recently demonstrated successful engraftment from administration of CPCs that were induced with cell reprogramming from nonmyocytes and expanded *in vitro*, resulting in decreased scar sizes in murine models of heart disease [355–357]. Of further interest, one study directly compared the differential regenerative effects between the hESC-derived CM and CPC populations when administered in a nude rat MI model [358]. The results revealed profound beneficial effects stemming from both cell populations and the lack of any significant advantages of employing one cell type over the other, however further studies should be performed in order to address the ideal cell types, cell dose and timing of administration to the damaged hearts.

Recent work from our lab demonstrated the potential for the hESC-derived $Isl1^+$ CPC population, termed as “human ventricular progenitors (HVPs)” that are generated using a CM differentiation protocol based on Wnt signaling modulation, to self-assemble into a functionally mature ventricular muscle patch *in vivo*. Here the newly formed muscle patch was capable of preserving myocardial function following MI injury [359]. Most recently, the hESC-derived $SSEA1^+Isl1^+$ CPC has been evaluated in the first clinical series of the human setting, where six patients with ischemic cardiomyopathy and severe heart failure (median age, 66.5 years; median LVEF, 26 %) received a median dose of 8.2 million of these CPCs [24,360]. After the 1-year follow-up, it was shown that there was a modest decrease of LV volumes and an increase in LVEF, which rose from 26 % (interquartile range [IQR]: 22–32 %) at baseline to 38.5 % (IQR: 33.5–41 %) [360]. Notably, no evidence of teratoma or adverse arrhythmia events was reported. Thus, this study gives the rationale for setting the grounds for adequately powered efficacy studies (Transplantation of Human Embryonic Stem Cell-derived Progenitors in Severe Heart Failure [ESCORT] trial; NCT02057900).

Several additional papers highlight novel approaches to cardiac regeneration by employing novel cardiac stem/progenitor cell types. For example, Oldershaw et al. derived a cardiac specific MSC-like cell population from human patients that displayed multipotentiality by giving rise to CMs and ECs *in vitro* [361]. In addition, these cells were shown to be secretory and as such may offer cardioprotective and beneficial effects when applied to myocardial injury models. In another example, the Chaudhry lab has identified multipotent fetal-derived $Cdx2^+$ cells from placenta, which were capable of driving cardiac repair in murine hearts following MI injury [362]. In brief, when administered intravenously to infarcted mice, labeled fetal-derived $Cdx2^+$ cells robustly homed to the dameged heart and differentiated to CMs and blood vessels, resulting in significant improvement in contractility. As such, these recent studies call attention to novel cellular therapeutic advances and potential for cardiac cell therapy.

Despite these positive outcomes, some ethical and technical hurdles are still impeding the clinical translation of the CPC-therapy for treating CVD. For the clinical translation of CPCs to be fully realized, more functional and safety studies are needed. Mechanistic feasibility studies that explore cell dosage, surgical technologies involving the routes of administration and surgical devices should be investigated in large animal models, as the logistics and results are more easily extrapolated to humans.

4.1.3. Combined cells therapy

More recently, the new concept that transplantation in combination with multiple cardiac cell types, such as CMs, vascular ECs and SMCs, and cardiac fibroblasts (CFBs), would have better outcomes for heart regeneration following injury rather than injection of a single type of cells (*e.g.*, CMs alone) has attracted more interest in this field [363]. This is due to the fact that the myocardium is a complicated multicellular organ and as such, multiple cell types are building up the entire heart in a coordinated fashion. A logical corollary would be to enhance the degree of therapeutic repair in the setting of the diseased heart; complex multi-cellular populations capable of interaction and communication with one another will be needed for regenerating the heart to a fuller extent.

In line with this, a recent report highlighted the efficacy of a trilineage cell transplantation incorporating human iPSC-CMs, ECs and SMCs which significantly improved left-ventricular cardiac function and decreased scar sizes following MI in a porcine model [364]. Of significance, the hPSC-CMs were capable of integrating within the host myocardium and the ECs and SMCs were further able to contribute to the host vasculature. This study, and other similar studies which have either combined ESC derivatives with MSCs in order to enhance preservation of transplanted cell types [365]; or studies which are establishing conditions capable of wielding multiple cardiac cell types from single differentiation protocols [366,367] are paving the way forward towards advancing cardiac cell therapies.

4.2. Cell-free therapies

4.2.1. Noncoding RNAs, ASO, extracellular vesicles

The quest for discovery around novel bioactive drug therapies for more effective and safer treatments for CVD has led to a multitude of non-cellular strategies. Small non-coding RNAs such as microRNAs (miRNAs) have been shown to fine-tune many biological processes involved in heart development, disease and repair and are reviewed elsewhere [368,369]. In a recent report by Gabisonia et al., the research team assessed the effects of miR-199a in pig hearts after MI [370]. One month following injury and treatment, the pigs treated with miR-199a showed robust and significantly improved regional and global cardiac function. The over-expression of miR-199a induced rapid and

uncontrollable CM turnover through de-differentiation and proliferation, resulting in arrhythmic deaths to the pigs, despite increased muscle mass and reduced fibrotic injury. This study provides novel insights to expanding endogenous CMs following MI injury, however the dosage of the therapy along with safe and efficacious routes of administration are needed before bringing this miRNA therapy to light.

An alternative noncoding RNA, antisense oligonucleotides (ASOs), are chemically active, short, single-stranded DNAs that contain complimentary sequences to their RNA counterparts. ASO technology supports a variety of chemical modifications, all of which alter pharmokinetic (PK) properties in a tissue specific manner [371]. ASO therapies are emerging in a growing field of cardiovascular medicine, as their precision could be used to restore cardiac function due to defective hereditary conditions, several examples of which are discussed in a recent review [372]. On the other hand, ASO technology could also be employed to treat conditions of the chronic failing heart. For example, a recent elegant study was able to employ antisense oligonucleotides against miR-25, a suppressor of intra-cellular calcium handling during heart failure, which unequivocally improved cardiac contractility [373]. It is likely that ASO therapies will continue to advance technologically for applications in wide-spread cardiovascular disease treatments. Employing non-coding RNAs as therapeutic tools that target the cardiovascular system show much promise, but many challenges ensue which are discussed elsewhere [374]. Extracellular vesicles (EVs) such as exosomes are one way these small noncoding particles traverse and communicate through intercellular trafficking [375]. Exosomes have been shown to transport RNAs, lipids and proteins even among cardiac cells [376,377]. In fact, many biological sources of [378,379] exosomes for myocardial repair have been reported and include stem cells, body fluids and somatic cell types (*see* Table 3). Exosomes may mediate cardiac repair after injury and the therapeutic role for exosomes in cardiac repair are reviewed elsewhere [378].

4.2.2. Gene therapy

Although gene therapies were once touted to be a potential treatment option for cardiovascular diseases, at the time of this review very few results report clinical significance [266]. Vehicles used in cardiovascular gene therapy include non-viral (naked plasmid DNA) and viral vectors. DNA plasmids are often met with low transfection efficiency [380] and as such various kinds of viral vectors have gained momentum for applications in gene therapy [381]. Adenoviruses (AVs), AAVs and lentiviruses (LVs) all have the capability to deliver genetic material to cardiac cell types and as such have become of great interest to researchers. A previous study employed LVs to express Kit ligand (KL) in mice undergoing MI [382]. Here the team showed injection of the LV-KL at the time of MI later attenuated ventricular remodeling and improved post-MI survival rates. It was speculated that the over-expression of the KL may have enhanced bone marrow cells homing to the infarcted heart where they play a role in repair through paracrine/autocrine effects and/or transdifferentiation and engraftment. Of note, LVs act by stably integrating into the host genome of target cells making them a harbinger for insertional mutagenesis and thus have not been used in the clinical trials for patients with heart failure. It is speculated that in understanding site selections and mechanisms driving interactions with chromatin, LVs may become accepted for gene therapy [383].

AVs are non-integrating and have been shown to achieve high levels of transduction in CMs, the success of which, however, may require direct IM injection [384]. AV therapies carry the weight of being worldly known for the death of a young man who developed a systemic inflammatory response to AV [385], which lead to organ-wide failure. As such enthusiasm for exploring AVs in gene therapy studies have become grandly lost.

AAVs seem to be the current vector of choice in a number of cardiovascular gene therapy clinical trials [386]. Multiple strains of AAVs have been identified, where AAV1, AAV6, AAV8 and AAV9 are genuinely accepted as the most cardiotropic serotypes (that is, they have a specificity for infecting CMs in particular) [387,388]. Gene therapies employing AAVs could potentially replace deficient proteins in order to protect against the progression of dilated cardiomyopathy in patients with heart failure. A recent Phase1/2 clinical trial delivered AAV1/SERCA2a, which is a gene that regulates CM contraction and relaxation by transporting Ca^{2+} from the cytosol into the sarcoplasmic reticulum during diastole, *via* intracoronary infusion in patients with advanced heart failure, the results of which showed limited safety concerns and marginal cardiac improvements [389]. However, the results in the larger Phase 2b trial involving 250 patients with moderate-to-severe heart failure revealed that at the 1-year follow-up, the mortality rate was similar between the placebo and AAV1/SERCA2a-treated groups and the time to recurrent events such as hospitalization was not significantly improved in AAV1/SERCA2a group [390].

Regardless, continued improvements in gene delivery methods and control of transgene expression are heavily required if gene therapy trials are to be successful. Discussions concerning updated concepts that include novel vector design and various gene delivery methods are discussed in detail elsewhere [391]. Further technological shortcomings including patients' immunological responses to these treatments, which are causing clinical conflicts or failures, are reviewed elsewhere [392,393]. In addition, we briefly introduced several cases of recent promising gene therapy, as well as recombinant protein therapy, in the field of cardiac regenerative medicine in Sections 3 and 4.

4.2.3. Synthetic chemically modified mRNAs

Messenger RNAs (mRNAs) containing synthetic modified nucleosides (modRNA) have become a technology gaining wide recognition in novel therapeutic platforms including genetics, immunotherapy and cancer [394,395]. More recently, the full *in vivo* therapeutic potential of modRNA technology has been realized in the cardiovascular field. Following cardiac injury, direct intramyocardial injections of modRNA encoding the VEGF-A gene was shown to enhance neoangiogenesis leading to improved cardiac function in small and large animals where administered in lipid nanoparticles (LNPs) or a biocompatible citrate formulation [101,396,397]. Notably, due to its adequate pharmacokinetics and/or other reasons, there have been no adverse effects in the VEGF-A modRNA therapy, such as aberrant vascular growth, weak neovessel formation with considerable extravasation, and cardiac edema, which were often reported in the previous preclinical and clinical studies testing the efficacies and safety of the VEGF-A recombinant protein or gene therapy for cardiovascular disease [244,392,393].

On the basis of this evidence together with the ease of production and expected safety profiles associated with modified mRNA technology, a Phase I first time in human (FTIH) study has already been reported [398]. This study successfully demonstrated the safety tolerability and efficacy of dermal administrations of modRNA encoding the VEGF-A gene in diabetic patients. Thus, it is expected that modRNA technologies will continue to gain widespread attention and the spectrum of clinical conditions that could benefit from this technology will expand further.

4.2.4. Other synthetic particles

In line with the production of synthetic chemical modRNA for cardiac therapies, alternative novel bioengineered synthetic particles have begun to gain momentum as novel treatment regimes for cardiac indications. For example, a recent paper by Vandergriff et al. elegantly employed a synthetic targeting peptide which was capable of inducing the homing of intravenously infused exosomes into infarcted rodent hearts [399]. By targeting exosomes to the heart with a synthetic construct, the rodents had reduced fibrotic injuries and scar sizes along with improved cardiac function and improved angiogenesis following myocardial injury. In another study, Tang et al. employed cell-mimicking microparticles (CMMP), which exerted reparative effects

similar to those effects seen by paracrine secreting adult stem/cardiac cells [400]. Interestingly, in a mouse model of MI, these CMMPs exerted cardioprotective outcomes and promoted myocardial repair, while failing to stimulate T-cell infiltration indicating their safety tolerability. Further testing in large animal models highlighting efficacy and clinical delivery routes need to be addressed. However, the combination of synthetic bio-engineered technology platforms holds promise in the future of regenerative medicine.

4.3. Future perspectives

Recent discoveries and advanced knowledge of the cardiac progenitors and the cardiogenic paracrine mediators hold great promise for heart regeneration through stimulating CM proliferation and blood vessel growth in the damaged hearts. Yet multiple issues, including inefficient induction of CM proliferation even by using novel approaches, increased cancer risk in noncardiomyocytes due to activating cell cycle, teratoma risk by transplanted immature stem cells, and incomplete electrical coupling between transplanted cells and host cardiac tissue need to be fully addressed before clinically applying these cell-based or cell-free strategies for heart disease. In regard to the cancer risk, for example, both NRG-1 and Yap signals have oncogenic potentials and are involved in some types of cancers [401,402], requiring precise targeting of mitogenic stimuli to CMs in order to minimize oncogenic risk. One of the keys to success for heart regeneration is to recapitulate the developmental paradigm of paracrine mediator cues acting on the specific cardiac progenitors to build the heart, routinely observed in embryogenesis. In this regard, a combinatorial approach that brings together cell-based and cell-free therapies will attract much interest in future and promising regenerative medicine for heart disease. For example, we envision the combined administration of VEGF-A modRNA and HVPs in the damaged hearts as an interesting direction to stimulate both vasculogenesis and cardiomyogenesis. Collectively, all of the ongoing efforts by cardiac/stem cell biologists and clinical cardiologists will someday open the door and novel paths to groundbreaking advances in the establishment of heart regeneration and thereby in the treatment of congenital and acquired heart diseases in children and adults.

Declaration of Competing Interest

The authors declare no conflict of interest.

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