# Heart regeneration with human pluripotent stem cells: Prospects and challenges

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## ARTICLE INFO

## ABSTRACT

Cardiovascular disease, ranging from congenital heart disease to adult myocardial infarction, is the leading cause of death worldwide. In pursuit of reliable cardiovascular regenerative medicine, human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), offer plenty of potential cell-based applications. HPSCs are capable of proliferating indefinitely in an un-differentiated state, and are also pluripotent, being able to differentiate into virtually any somatic cell types given specific stepwise cues, thus representing an unlimited source to generate functional cardiovascular cells for heart regeneration. Here we recapitulated current advances in developing efficient protocols to generate hPSC-derived cardiovascular cell lineages, including cardiomyocytes, endothelial cells, and epicardial cells. We also discussed applications of hPSC-derived cells in combination with compatible bioactive materials, promising trials of cell transplantation in animal models of myocardial infarction, and potential hurdles to bring us closer to the ultimate goal of cell-based heart repair.

## 1. Introduction

Cardiovascular diseases (CVD) remain the number one cause of global death nowadays, claiming increasing number of lives and bringing huge burden in human health and economics. As reported in the fact sheet from World Health Organization (WHO) updated in 2017, CVD caused estimated 17.9 million deaths in 2016, taking up 31% of all global deaths. 85% of these cases were caused by heart attack and stroke. In regional prevalence, three quarters of deaths from CVD occurred in low- and middle-income countries. Data from medical expenditure panel survey of 2013–2014 also pointed out that the direct or indirect cost of CVD per year in the United States was estimated to be $329.7 billion [1]. Therefore, despite decades of efforts to treat CVD, curative therapies are still in great demand in this pandemic context of heart failure.

### 1.1. Subdivisions of CVD

In concept, CVD covers a group of disorders related to heart and blood vessels. Based on inheritable factors, they are divided into acquired CVD and congenital cardiovascular defects that arise from structural malformation of the heart or major blood vessels before birth. These inherited defects range from abnormal tiny septal connections, to major chamber deformity that is in need of multiple surgical procedures in infancy, at times even leading to in-utero demise. Birth prevalence (the incidence of disorders present before or at birth) of congenital heart defects is reported around 8 per 1000 live births in the United States, 6.9 per 1000 births in Europe and 9.3 per 1000 births in Asia [2,3]. In recent decades, improved surgical procedures and medical management extend the aging of patients with congenital heart defects, leading to a significant decline of related mortality rate and substantial expansion of this patient group [1]. Overall, congenital cardiovascular defects have a nonnegligible impact on the morbidity, mortality and healthcare costs in both children and adults.

Based on location of nidus, CVD are divided into cerebrovascular disease, peripheral arterial disease, heart diseases (coronary or rheumatic), deep vein thrombosis, pulmonary embolism and others. Accounting for the majority of CVD, heart diseases refer to the issues and deformities in the heart itself, including congenital heart defects, heart attack, arrhythmia, coronary artery disease, myocardial infarction (MI), heart failure, hypertrophic cardiomyopathy, mitral regurgitation, valvular diseases and others. Except for genetic determinant, multiple

risk factors account for CVD occurrence. The most familiar ones include unhealthy diet, physical inactivity and abuse of tobacco and alcohol. Commonly, non-ideal lifestyles tend to induce high level of blood cholesterol and buildup of plaque in the arteries, which advances hardening and narrowing of arteries, termed atherosclerosis. After blood blockage, the delivery of oxygen and nutrients will be stopped, causing the death of surrounding cardiomyocytes. Loss of cardiomyocytes leads to MI, heart failure, heart arrhythmias or heart attack.

### 1.2. Current therapies: advantages and drawbacks

Currently, there are several therapeutic approaches for CVD. Donor hearts from carcass serve as the primary source for heart replacement. However, donor hearts are greatly limited by lack of donors and a series of post-transplantation immune rejection issues. For local fixation of ischemia and MI, heart bypass surgery or angioplasty is considered as common options for adult patients. Heart bypass surgery is recommended for patients who get one or more cardiac blood vessels blocked. By performing open chest surgery, the surgeon removes a blood vessel from somewhere else in the patient's body without affecting original blood flow, and uses it to create a detour or bypass around the blockage. Overall, it is a complicated procedure that takes huge preparation and recovery time, but it is rated as one of the most effective weapons against blocked arteries. Another option is angioplasty, during which an inflatable balloon or stent is delivered through a catheter along the artery, push the plaque and stretch the artery open in blocked regions. Compared with heart bypass surgery, angioplasty is relatively easy-to-perform, and the recovery time post-surgery will be shorter.

There are also other methods targeting specific symptoms. For instance, artificial pacemakers are applied to send electrical impulses to the heart muscle for keeping up a suitable heart rate and rhythm. Left ventricular assist device (LVAD) functions as a mechanical heart, helping pump the oxygen-rich blood throughout the body. Valve disease treatment involves traditional surgical procedures or balloon valvuloplasty with reduced cutting wounds. And implantable cardioverter defibrillator (ICD) is leveraged for treatment of abnormal heart rhythms.

### 1.3. Human pluripotent stem cells (hPSC) technology

In 1998, James Thomson derived the first five human embryonic stem cell (hESC) lines using human blastocysts donated with in vitro fertilization procedures. This revolutionized the regenerative medicine field and started a new era of stem cell technology, triggering abundant research on hPSC carried out all over the world [4]. Unlimited proliferation, self-renewal capacity and ability to derive somatic cell lineages of all three germ layers, have endowed hPSC with unprecedented promise to study human development, perform disease modeling, predictive toxicology and drug screening, as well as develop cell therapies for a variety of degenerative diseases. In less than one decade, contemporaneous report of human induced pluripotent stem cell (hiPSC) derivation from somatic cells by the Yamanaka and Thomson group further bypassed the ethical controversy of hESC and introduced the concept of cellular reprogramming, renewing the application potential of stem cell technology [5,6].

## 2. hPSC derived cardiomyocytes (CMs)

Accounting for 70–85% of the total volume of the mammalian heart [7–10], CMs play a fundamental role in pumping blood throughout our circulatory system by orchestrated contraction and relaxation. In structure, CMs are connected end-to-end by gap junctions and intracellularly, they are highly organized with the contractile apparatus, sarcomere, which is composed of fundamental protein like myosin, actin, tropo-myosin and troponin complex. Fluctuation of intracellular

$Ca^{2+}$ leads to interactions between these proteins, followed by occurrence of ATP hydrolysis, changes in physical-chemical dynamics and eventually tension formation in the myocytes [11,12]. Located in the upper part of the wall of the right atrium, there is a specialized group of natural pacemaker cells, termed sinoatrial node (SA node), initiating electrical impulses and responsible for controlled rhythmic beating of the whole heart [13].

Limited turnover rate of CMs have been revealed by research leveraging integration of nuclear bomb test-derived $^{14}C$. In contrast to high renewal rate of endothelial cells throughout life (more than 15% annually) and lower turnover rate of mesenchymal cells (less than 4% per year for adults), CM exchange is highest in childhood and exponentially decreases with age, presenting < 1% turnover rate per year in adults [14,15]. As a consequence, endogenous capacities of regeneration or repair of CMs fail to make up for cell death arising from apoptosis, necrosis and autophagy, resulting in development of chronic or acute heart diseases like MI in the long term [16]. Given this, cell substitutes for CM regeneration are in great demand, where hPSC derived CM lineages hold tremendous promise.

As the most studied cell types in heart, encouraging results have been reported in multiple research for efficient generation of hPSC-CMs (Table 1). Early studies pointed out the vital role of Activin/Nodal/TGF-β, Wnt, and BMP signaling pathways in embryonic cardiac specification [17–19], which inspired multiple directed differentiation methodologies for generating hPSC-CMs by mimicking these developmental signaling cues [20–24]. The exploration started with the discovery of spontaneous beating areas in serum-based hESC-EB culture, with 2%–70% of the beating areas consisting of cardiomyocytes [25–27]. Despite the enhancement of differentiation by adding the demethylating agent 5-aza-deoxycytidine [25], wide variation on batches or cell lines and insufficiency of quantitative data made it difficult to maintain the consistency of these protocols. To overcome these limitations, the Graichen group described the positive effects of serum-free medium conditioned by the mouse visceral endoderm-like cells (END-2), and a specified molecule SB203580, a P38 MAP kinase inhibitor from it, to generate approximately 2.5-folder higher CM efficiency than controls [28]. With subsequent applications of Activin A, BMP4, FGF2, VEGF, dickkopf homolog 1 (DKK1) [29,30], Kattman et al. defined a stage-specific and dosage-dependent role of these factors in cardiac specification by monitoring the co-expression of cardiac progenitor-markers KDR/PDGFR-α [22]. It highlighted the necessity of optimization of pivotal signaling pathways to efficiently generate PSC-CM population from individual cell lines. However, this protocol was limited by the requirement of using FACS sorting to enrich cardiac progenitors, which was difficult for scaling up. To enable robust and efficient CM differentiation in multiple cell lines, Lian et al. investigated the role of canonical Wnt signaling in cardiac specification and developed a small-molecule-based, chemically-defined protocol to robustly produce a high yield of virtually (up to 98%) pure functional human CMs from multiple hPSC lines in 2012 [20]. Sequential treatment of hPSCs with a glycogen synthase kinase 3 (GSK3) inhibitor followed by a chemical inhibitor of Wnt signaling was employed to efficiently generate CMs from hPSCs and thus this protocol was termed GiWi protocol. In 2014, Burridge et al. further optimized the GiWi protocol and suggested a chemically defined xeno-free medium consisting of just three components: the basal medium RPMI 1640, L-ascorbic acid 2-phosphate and recombinant human albumin, to support CM differentiation in the presence of GSK3 inhibitors and Wnt inhibitors. This provided a simplified medium system for CM differentiation [24]. To summarize, during the past decades, the efficiency of hPSC-CM differentiation has dramatically increased from 1 to 10% in the form of embryoid bodies (EB) [21] to over 90% in monolayer [24,31].

To avoid the contamination of undifferentiated hPSCs and other undesired lineages, several strategies have been developed for CM purification, broadening the range of potential applications of hPSC-CMs and facilitating progress toward hPSC-based cardiac regenerative

**Table 1**
Key advances in hPSC-CM differentiation.

| Initial cell type | Bioactive factors | Culture | Key advances | Refs |
|---|---|---|---|---|
| hESC | dimethyl sulfoxide (DMSO), all-trans retinoic acid (RA), or 5-aza-2'-deoxycytidine (5-aza-dC) | EB | CM differentiation can be enhanced by adding 5-aza-dC, not DMSO or RA, generating 70% CMs after Percoll density centrifuge. | 2001, Kehat et al. [25] |
| hESC | SB203580, P38 MAP kinase inhibitor, serum-free medium conditioned by END2-CM | EB | Generated 2.5-folder higher CM efficiency than controls | 2008, Graichen et al. [28] |
| hESC | Activin A, BMP4, FGF2, VEGF, dickkopf homolog 1 (DKK1), etc. | EB | Defined a stage-specific and dosage-dependent role of these factors in cardiac specification and identified a human cardiovascular progenitor population. | 2007, 2008, Kattman et al. [29,30] |
| hESC/iPSC | CHIR99021, IWP2/IWP4 | monolayer | Investigated the role of canonical Wnt signaling in cardiac specification and developed small-molecule-based GiWi protocol to produce up to 98% functional human CMs from multiple hPSC lines. | 2012, Lian et al. [20] |
| iPSC | CHIR99021, Wnt-C59 | monolayer | Used xeno-free medium to generate up to 95% CMs. | 2014, Burridge et al. [24] |

therapy [32] (Table 2). One way is to introduce CM-specific fluorescent reporters or drug selectable elements into hPSCs, allowing CM enrichment with fluorescent-activated cell sorting (FACS) or appropriate drug selection [33–35]. But the major drawback of this approach is that, genomic insertion of reporter constructs leads to genetically modified CMs, limiting their utility for clinical applications. Another approach using a Percoll gradient centrifuge capitalized on the specific density of CMs [25,29], but due to its crudeness, the maximal purity of the cells was only 53% [36]. With the identification of CM-specific surface markers, such as signal-regulatory protein alpha (SIRPA) [37] and vascular cell adhesion molecule 1 (VCAM1) [38], FACS-based approach serves as a reliable tool to enrich CM and exclude non-myocyte populations. In addition to cell surface markers, Ban et al. also designed fluorescent molecular beacons targeting CM-specific mRNA, specifically MHC1 mRNA, identifying up to 99% functional CMs in combination with FACS [39]. Fluorescent mitochondrial dye tetramethylrhodamine methyl ester perchlorate (TMRM) [38,40] has also been used to purify CMs because mature CMs have high mitochondrial density. However, TMRM's reaction with undifferentiated hPSCs and failure to detect most immature CMs restrict the sensitivity and specificity of this method. Based on the remarkable biochemical differences in glucose and lactate metabolism between CMs and non-CMs, Tohyama et al. developed a metabolic screening method with glucose-depleted culture medium containing abundant lactate, leading to exclusive survival of CM population with up to 99% purity [41].

Multiple in vivo transplantation experiments have been conducted using hPSC-CMs to treat cardiac diseases, showing promising effects of heart regeneration in both small animals like mice [42], rats [29], guinea pigs [43], and large animals like pigs [44,45] or non-human primates [44,46]. Specifically, hPSC-CMs show progressive maturation over months after transplantation and generate remuscularization of infarcted heart. Perfusion in graft by host vasculature, electro-mechanical junctions and coupling between graft and host myocytes have been observed post engraftment. No teratoma formation ensures clinical safety to some degree [45]. Nevertheless, in contrast to results from small animals [42,43], ventricular arrhythmias observed in transplanted large animals remains to be overcome [44,47].

## 3. hPSC derived endothelial cells

Constituting the interior surface of blood vessels, endothelial cells (EC) are required for angiogenesis and vasculogenesis. These cells are estimated to take up 24% in the human heart revealed by stereological and flow cytometric methods [15], whose abundance in the adult heart and proximity to CMs endow ECs with a crucial role of regulating heart functions and responding to pathophysiological stress [48].

Early studies of murine embryogenesis revealed developmental knowledge about EC differentiation from mouse embryonic stem cells, including maturation steps, molecular events, and growth factor involvement [49–51]. This information has been utilized to derive ECs from hESCs by the Levenberg group, marked with expression of EC marker platelet endothelial cell-adhesion molecule-1 (PECAM1, also called CD31), correctly organized cell junctions, high metabolism of acetylated low-density lipoprotein (LDL), formation of in vitro tube-like structures, as well as microvessel integration with mouse blood cells in transplanted immune-deficient mice [52]. However, the efficiency of such spontaneous hESC-EC differentiation was as low as 2% PECAM1 + cells, limiting scaling up and further therapeutic or research applications. Furthermore, utilization of mouse embryonic fibroblast (MEF) feeders during this differentiation may cause xeno contamination from animal cells. To address these drawbacks, researchers reported to include more factors to enhance EC differentiation (Table 3). These factors included stromal cell-derived factor-1 (SDF-1)/CXCR4 signaling [53], vascular endothelial growth factor (VEGF) [54], basic fibroblast growth factor (bFGF) [53,55,56], bone marrow protein-4 (BMP-4) [56], TGFβ signaling [57,58], microRNA-21 [59], and WNT signaling [60].

**Table 2**
Purification approaches of hPSC-CMs.

| Procedure | Key advances or drawbacks | Refs |
|---|---|---|
| Introduce CM-specific fluorescent reporters or drug selectable elements, combined with FACS or drug selection. | Genomic insertion of reporter constructs leads to genetically modified CMs, limiting their utility for clinical applications | 2007, Huber et al. 2007, Anderson et al. 2011, Ritner et al. [33–35] |
| Percoll gradient centrifuge capitalized on the specific density of CMs. | Maximal CM purity was only 53%. | 2002, Xu et al. 2007, Laflamme et al. 2011, Tulloch et al. [25,29,36] |
| FACS based on CM-specific surface markers like SIRPA or VCAM1. | Able to separate live CMs from non-CMs. | 2011, Dubois et al. 2011, Uosaki et al. [37,38] |
| FACS based on fluorescent molecular beacons for MHC1-mRNA. Fluorescent mitochondrial dye TMRM | Identify up to 99% functional CMs. Reaction with undifferentiated hPSCs and failure to detect most immature CMs restrict its sensitivity and specificity. | 2013, Kiwon et al. [39] 2011, Uosaki et al. 2009, Hattori et al. [38,40] |
| Metabolic flow screening based on glucose and lactate metabolism of CMs and non-CMs. | Enable non-genetical large-scale CM purification to up to 99% purity. | 2013, Tohyama et al. [41] |

Recently, scientists developed new feeder-free 2D protocols for differentiating hPSCs into ECs [56,61] and generated functional hPSC-EC populations with high efficiency. For example, Lian et al. developed a rapid, small molecule based protocol for differentiating hPSCs to become CD34 $^+$ CD31 + VE-cadherin + endothelial progenitors with the efficiency of over 50% by temporary activation of WNT signaling alone, without using any exogenous growth factors [60]. Based on previously reported role of TGFβ signaling for specifying smooth muscle cells or ECs [57], further treatment of these endothelial progenitors with a TGFβ inhibitor SB431542 leads to generation of functional EC populations. The populations show advanced proliferating rate [57], express typical EC markers (CD31, CD144, von Willebrand Factor (vWF), ID1). HPSC-ECs also produce nitric oxide (NO), migrate across a wound, form lumenized vessels on scaffolds, and show ICAM-1/VCAM1 upregulation in response to TNFα [54,57,60,62]. Based on shared signaling pathway in cardiogenic or hemogenic mesoderm, Palpant et al. reported a common platform regulated by Activin A, BMP4 or other small molecules, for directed differentiation of CMs and endothelial subtypes from hPSCs. The protocol yields functional endothelial populations with higher efficiency [63].

Heterogeneity of hPSC-derived EC populations is another issue. hPSC-ECs purified based on CD31 expression consist of arterial, venous, and lymphatic EC subtypes. These EC sub-populations can be further specified by modulating concentrations of VEGF-A, VEGF-C, angiopoietin-1, and 8-bromoadenosine-3':5'-cyclic monophosphate, or purification based on specific surface markers [66,67]. HPSC-ECs are also functional after transplantation in vivo. These ECs survived post implantation, revascularized tissues and promoted vascular regeneration without evidence of arrhythmias [62,66–68], highlighting the potential of utilizing hPSC-ECs for cardiac repair.

## 4. hPSC derived vascular smooth muscle cells (VSMC)

Another major component of blood vessel is smooth muscle cells. They have striated spindle shapes and are under involuntary control. In addition, they also have contractile and factor-release functions. Besides heart, smooth muscle cells are also located in the walls of hollow visceral organs like intestines and stomach. Since they hold the ability to proliferate, secrete angiogenic factors like NO, FGF or VEGF [69–71], and are also easily obtained and cultured, early studies have worked on the effects of transplanted smooth muscle cells for cardiac repair. In vivo results indicated that transplanted smooth muscle cells restored tension and elasticity of heart wall, minimized dilatation, preserved cardiac functions and improved MI in animal models, making this cell population another promising candidate for heart repair [72,73].

Multiple protocols [74–87] have been developed to differentiate pluripotent stem cells into VSMCs, by manipulating WNT, TGFβ, or BMP4 signaling pathways in either EB or monolayer culture, followed by purification of cells of interest (Table 4). Purified VSMC populations are commonly characterized by expression of specific markers for mature contractile VSMCs like smooth muscle myosin heavy chain (SM-MHC) and smoothelin (SMTN), as well as functional properties such as the ability to contract in response to vasoactive agents like carbachol, endothelin-1 or KCl.

## 5. hPSC derived epicardial cells

As the outmost epithelial layer of heart, the epicardium functions as a cell source to generate multi-lineage descendants and secrete paracrine trophic signals to the myocardium and coronary vessels. During cardiac development, the epicardial population undergo epithelial-to-mesenchymal transition (EMT) and invade the myocardium to produce cardiac fibroblasts, coronary smooth muscle cells and endothelial cells [88–91]. Cells of the adult epicardium are typically quiescent, but mounting evidences indicate that injury of the adult heart tends to reactivate the developmental program in the epicardium mediated by core Hippo pathway effectors. After activation, epicardial cells may facilitate neovascularization and myocardial repair [92–96] via secretion of paracrine factors like retinoic acid, FGF9, insulin growth factor-2. Therefore, adult epicardium might be utilized for promoting myocardial regeneration.

To generate epicardial cells from hPSCs, the Lyer group and the Witty group reported differentiation protocols using FGF2, BMP-4, Activin A, VEGF and WNT3A in medium containing animal proteins [97,98]. Subsequently, scientists developed chemically defined and xeno-free protocols by temporarily modulating canonical WNT signaling, leading to generation of 80–85% epicardial-like cell populations [99–101] (Table 5). These cells present characteristic epicardial cell morphology post passaging, express epicardial specific marker genes TBX18 and WT1. More importantly, these cells hold the ability to differentiate into functional SMCs and cardiac fibroblasts. In a recent study, Bargehr and colleagues reported the role of hPSC-derived epicardial cells in advance CM-driven heart regeneration [102]. Co-transplantation of epicardial cells together with CMs resulted in doubled CM proliferation rate, increased graft size and augmenting host vascularization, compared with grafted CM or epicardial cells only. Moreover, systolic function presented upregulation post co-transplantation of CM plus epicardial cells, demonstrating the promise of epicardial cells to be applied as an adjuvant therapeutic for cardiac repair.

Because epicardial cells exhibit spontaneous EMT in culture [103], it is important to develop an efficient method to prevent EMT for long-

**Table 3**
Key advances in hPSC-EC differentiation.

| Initial cell type | Bioactive factors | Culture | Key advances | Refs |
| --- | --- | --- | --- | --- |
| hESC | Spontaneous differentiation in medium without LIF and bFGF; Purified PECAM1 + cells. | EB | Isolated PECAM1 + cells display characteristics like vessel endothelium in vitro or in vivo. | 2002, Levenberg et al. [52] |
| hESC | FBS | EB | Role of SDF-1 and CXCR4 in initial vessel formation. | 2007, Chen et al. [53] |
| hESC | FBS | monolayer | A scalable two-dimensional method avoiding an embryoid-body intermediate. | 2007, Wang et al. [55] |
| hESC | BMP-4, VEGF, bFGF | monolayer | Role of BMP-4, VEGF and bFGF in hemangioblast differentiation. | 2008, Lu et al. [56] |
| hESC | VEGF, bFGF | EB | An extracellular matrix culture system for xeno-free hESC-ECs. | 2009, Li et al. [61] |
| hESC | TGFβ inhibitor, BMP-4, Activin A, FGF-2, VEGF-A | EB | Role of TGFβ inhibition and Id1 for endothelial cell commitment. | 2010, James et al. [57,58] |
| iPSC | microRNA-21, TGFβ-2, VEGF | monolayer | MicroRNA-21 and TGF-β2 signaling pathways regulate iPSC differentiation to endothelial lineage. | 2014, Bernardini et al. [59] |
| hESC, iPSC | CHIR99021 | monolayer | Role of WNT/β-catenin in generating endothelial progenitors. | 2014, Lian et al. [60] |
| hESC, iPSC | BMP4, Activin A, CHIR99021, VEGF, SB431542 | monolayer | Described efficient generation of ECs and pericytes from hPSCs under defined conditions and two assays for functional hPSC-EC evaluation. | 2014, Orlova et al. [64] |
| hESC, iPSC | BMP-4, VEGF-A | monolayer | Produced mature ECs with 80% efficiency in six days and purification to 99% via surface markers. | 2015, Patsch et al. [65] |
| hESC, iPSC | Activin A, BMP4, CHIR99021, VEGF, bFGF | monolayer | First protocol that provides a common platform for directed differentiation of cardiomyocytes and endothelial subtypes from hPSCs, with > 90% EC efficiency. | 2016, Palpant et al. [63] |
| hESC, iPSC | FGF, VEGF, BMP, SB431542 | monolayer | Identified pathways for regulating arterial EC differentiation via single-cell RNAseq and derived hPSC-arterial EC that improved cardiovascular function. | 2017, Zhang et al. [66] |

**Table 4**
Key advances in hPSC-VSMC differentiation.

| Initial cell type | Bioactive factors | Culture | Key advances | Refs |
| --- | --- | --- | --- | --- |
| hESC | TGF-β1 | EB | First demonstration of role of TGF-β1 in hESC-SMCs. | 2004, Sinha et al. [76] |
| hESC | VEGF-releasing dextran/HA hydrogels | encapsulation in hydrogel | Protocols describing EC or VSMC derivation from hESCs encapsulated in VEGF-releasing hydrogels. | 2010, Gerecht et al. [77] |
| hESC | PDGF-BB, TGF-β1 | monolayer | A simple protocol for the efficient derivation of highly purified functional SMCs from hESCs. | 2010, Vo et al. [80] |
| hESC, iPSC | SB431542, FGF2, LY294002, BMP4, PDGF-BB, TGF-β1 | monolayer | A chemically defined protocol to derive neuroectoderm, lateral plate mesoderm or paraxial mesoderm, from which origin-specific SMCs were derived, displaying contractile ability in response to vasoconstrictors and invested perivascular regions in vivo. | 2012, Cheung et al. [82] |
| hESC | Myocardin | monolayer, EB | Myocardin can increase the development and maturation of SMC-like cells from human embryonic stem cells despite not activating the full repertoire of SMC genes. | 2012, Raphel et al. [85] |
| hESC, iPSC | PDGF-BB, TGF-β1 | monolayer | HPSC-derived VSMCs displayed mechanotransduction in response to tensile strain. Mechanical stimulation of hPSC-derived VSMCs in vessel constructs using uniaxial and circumferential strain may potentially modulate derived VSMC function. | 2015, Wanjare et al. [86] |
| iPSC | PDGF-BB, TGF-β1 | Monolayer + 3D scaffold + bioreactor | A novel differentiation approach to enhance elastic fiber expression and maturation in vascular smooth muscle tissue derived from hiPSCs. | 2017, Eoh et al. [87] |

**Table 5**
Key advances in hPSC-epicardial differentiation.

| Initial cell type | Bioactive factors | Culture | Key advances | Refs |
|---|---|---|---|---|
| hESC, iPSC | rhBMP4, rhActivin A, rhbFGF, rhDKK1, SB431542, rhVEGF, CHIR99021, XAV-939, PD-173074 | EB, monolayer | Identified BMP and WNT as key regulators of the epicardial lineage in vitro. | 2014, Witty et al. [98] |
| hESC, iPSC | FGF2, LY294002, BMP4, WNT3A. RA, IWP2 | monolayer | Described a chemically defined method for generating epicardium and epicardium-derived smooth muscle cells and cardiac fibroblasts from hPSCs through an intermediate lateral plate mesoderm (LM) stage. | 2015, Iyer et al. [97] |
| hESC, iPSC | CHIR99021, IWP2, A83-01 or SB431542 | monolayer | Chemically-defined, xeno-free method of generating epicardial cells from hPSCs by modulating Wnt signaling. And TGF-β inhibition allows long-term expansion of hPSC-derived epicardial cells. | 2016, Bao et al. 2017, Bao et al. [99,101] |
| hESC, iPSC | CHIR99021, IWR1, RA | monolayer | A chemically defined, immunogen-free, small molecule-based method for generating TBX18 + /WT1 + epicardial-like cell populations with 80% homogeneity from hPSCs by modulation of the Wnt and retinoic acid signaling pathways. | 2017, Zhao et al. [100] |

term maintenance of epicardial cells. To address this issue, Bao et al. reported that treatment of TGFβ inhibitors permitted long-term self-renewal of hPSC-derived epicardial cells, leading to over 25 population doubling of WT1 + cells in homogenous monolayers [99]. Taken together, these findings contribute to our understanding of epicardial development and points to potential epicardial cell-based therapies.

## 6. Combined applications

Promising results not only come from in vitro differentiation or in vivo transplantation with hPSC derived cells from a single lineage, but also have been reported in preclinical tests of animal models combining multiple cardiovascular lineages with biomaterials. Here, the biomaterials derived from either natural or synthetic origins, play an important role primarily in two aspects: one is to provide physical support for cells to attach or aggregate, another is to deliver bioactive factors like growth factors or small molecules to enhance the differentiation efficiency or functional maturation from hPSCs. By adjusting the ingredients, structure or attachment of various functional group of these materials, a microenvironment with specific mechanical strength, proper degradability and biocompatibility can be created for a certain cell type [77,104–107]. For instance, to address the effects of transplanting hPSC-derived cardiovascular cells on treating MI, Xiong et al. have examined functional improvement by combining hESC derived ECs and SMCs with a fibrin 3D porous scaffold biomatrix in both murine and swine acute MI models. Using cardiac magnetic resonance imaging (MRI) and bioluminescent imaging (BLI), these experiments demonstrated significant beneficial effects in left ventricular (LV) remodeling post engraftment, underscoring the therapeutic potential of hESC-derived vascular cell types along with patch delivery [108]. In addition, the Ye group delivered hiPSC-derived tri-lineage cells (CMs, ECs, and SMCs) with a 3D fibrin patch loaded with insulin growth factor (IGF)-encapsulated microspheres in a porcine model of MI [109]. Functional integration of engrafted CMs into host myocardium, formation of organized sarcomeric structures and contribution of EC and SMC to host vasculature have been observed. Furthermore, this treatment also significantly improved LV function, myocardium metabolism and arteriole density, with reduction of infarct size, ventricular wall stress, and apoptosis without inducing ventricular arrhythmias. Notably, contributions of IGF-1-loaded patch are remarkable, in comparison to cell-only delivery, with significantly better retention and survival of transplanted cells (~8.97% in cells plus patch and ~4.2% in cells only) [110]. These progresses in a preclinical large animal model of MI takes us one step closer to the potential applications of hPSC-derived cardiovascular cells in clinical trial.

## 7. Discussion

To move one step closer to clinical applications, several challenges in developing cardiovascular cell populations from hPSCs as cell therapy deserves consideration, some of which have been addressed in recent studies. One obstacle is heterogeneity in differentiated cell populations and contamination from undesired cell lineages. To solve this, enrichment or purification of desired subtypes have been studied. As discussed above (Table 2), it can be realized by FACS or drug selection based on lineage-specific markers (either in protein level or mRNA level), as well as separation based on difference in metabolic activities, cell intensity, or mitochondria density. And those procedures avoiding genetical insertion or function disturbance in cell products hold greater potential for clinical applications. Another way is to identify key regulators of lineage bifurcation and modify protocols to derive more specific subtypes. The second challenge is the scale-up to derive large number of cardiovascular cells of specific types from hPSCs for transplantation. Among the reported protocols, a common trend is to develop chemically defined, small molecule-based procedures with higher efficiency and shorter time, which attempts to reduce the production

cost to the maximum degree and makes it more stable and easier to scale up. More importantly, scale-up of cell production requires re-adjustment of parameters of all aspects to transfer monolayer-based protocols to suspension culture in bioreactors, involving in distinct changes in bioactive molecule delivery, mechanical factors, and oxygen release, which all have a great impact on differentiation efficiency from hPSCs. For in vivo trials, challenge lies in consistent cell performance like survival, maturation, integration and function post transplantation in animal models like mice, guinea pigs, swine or primate models.

In a nutshell, to seek optimal treatments for CVD, which is one of the most common causes of death worldwide, research on generating cardiovascular cell lineages from hPSCs is in full swing and brings tremendous promise. Here in this review, we discussed the groundbreaking progress in developing differentiation protocols for various cardiac cell types in vitro, encouraging in vivo outcome of cell transplantation in animal MI models, explorative combinations with bioactive materials, as well as potential challenges in future clinical applications. Overall, the hPSC-derived cardiovascular cells present a rapidly developing technology with exciting applications, and with further refinements it will pave the way for the development of reliable regenerative therapies for CVDs.

## Author Contribution Statement

Yuqian Jiang: Writing – Original draft preparation. Xiaojun Lance Lian: Supervision, Writing – Reviewing and Editing.

## Declaration of competing interest

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2020.01.003.

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