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Cardiac Progenitor Cells and their Therapeutic Application for Cardiac Repair

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Abstract

Heart disease is the principal cause of death in humans. Stem cell-based therapy for heart regeneration has long been seen as a potential application since the heart lacks adequate intrinsic regenerative potential. In the cardiovascular field, clinical trials have already been carried out by implantation of both bone marrow-derived stem cells and cardiac resident progenitor cells derived from the adult heart tissue into the injured myocardium to restore the functionality of the heart after damage. However, before a robust stem and progenitor cell-based therapy for cardiovascular diseases can be applied in the clinical setting, more research is necessary to generate sufficient quantities of functional cardiomyocytes from stem cells and to understand behavior of cardiomyocytes upon transplantation. A comprehensive understanding of the developmental processes involved in cardiogenesis might support further investigations in more efficient cell-based regeneration therapies. This review discusses the molecular aspects of cardiogenesis during early development and links the insights with the *in vitro* generation of cardiac progenitor cells as well as functional cardiomyocytes. Furthermore, we discuss the advantages of cardiac progenitor cells and cardiomyocytes derived from pluripotent stem cells, cardiac resident stem cells in regenerative applications to cope with the damaged heart.

Keywords: Cardiac progenitor cells; Cardiomyocytes; Molecular mechanism; Cardiac resident stem cells; Heart regeneration

Introduction

Cardiovascular diseases are considered to be one of the largest health problems in developed countries by being the principal cause of death in humans with over 4 million deaths per year in Europe alone (http://www.escardio.org/about/what/advocacy/EuroHeart/Pages/2012-CVD-statistics.aspx). Furthermore, congenital heart disease as the most common birth defect in humans affects nearly 1% of newborns. With inclusion of spontaneous abortions, the incidence is exaggerated to 10% [1].

During early embryogenesis, the heart is the first functional organ that pumps blood to provide the growing organism with required metabolic demands like essential nutrients and oxygen. The development of the heart comprises complex processes of cell migration, proliferation, differentiation, specification and maturation throughout cardiogenesis. Although a persistent heart function is fundamental for development and survival of the organism, the heart lacks efficient, intrinsic regenerative potential to restore the functionality after cardiac damage. Previous studies show that the mitotic renewal rate in the human myocardium is very low and most cardiomyocytes will never be exchanged during a normal life span [2]. Experimental evidence suggests that the heart harbors a resident population of stem cells which are able to give rise to cardiomyocytes, smooth muscle and endothelial cells [3,4]. However, these populations have limited capacity to replace a large number of cardiomyocytes lost after a serious heart attack [5]. Therefore, a robust novel therapy to regenerate lost myocardium could help millions of patients every year.The limited regeneration capability of heart tissue has prompted methodological developments for creating *de novo* cardiomyocytes, both *in vitro* and *in vivo*. Newly generated cells are required to integrate electrically and mechanically in order to enhance the deficient pumping function of the heart.

The stem cell-based cellular therapy focusing on the possibility of re-growing heart muscle has been regarded as an innovative option in therapeutic approaches in the recent years [6,7]. Significant progress has already been made in this field [8,9]. Patient-specific induced pluripotent stem cells (iPSCs) may be a realistic cell type for providing a source of new functional cardiomyocytes [10]. Their therapeutic applications may circumvent the ethical concerns and immunogenic complications associated with human embryonic stem cells (ESCs). However, before the application of iPSCs in regenerative medicine, many aspects have to be addressed: for example, generation of sufficient quantities of functional cardiomyocytes, prevention of the massive amount of cell death after transplantation, induction of cardiomyocyte maturation derived from such pluripotent stem cells, and demonstration of safety and efficacy in animal models and pre-clinical trials.

During early cardiogenesis, the cardiac identity of the progenitor cells is regulated by tightly coordinated, spatially and temporally active signaling pathways and molecular mechanisms leading to the progressive restriction of undifferentiated progenitors to the different cardiovascular lineages. Dissecting the molecular basis of cardiac progenitor cell emergence, proliferation, diversification, specification, phenotypic modulation and subsequent maturation does not only help to understand the developmental processes of embryonic cardiogenesis and cardiovascular diseases but also facilitates investigations in stem cell-based regeneration therapies of the failing heart. A comprehensive knowledge would allow developing more extensive strategies in cardiomyocytes differentiation and their structural and functional

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maturation, which could provide the basis for the establishment of efficient therapeutic approaches in heart failure and disease modeling.

This review aims at highlighting the current knowledge of the developmental processes in cardiogenesis at molecular levels with special focus on the emergence and early differentiation of cardiac progenitor cells (CPCs). We emphasize the potential of CPCs and cardiomyocytes in regenerative approaches, pointing out recent advances in regenerative cell-based applications and therapies for cardiovascular diseases. Furthermore, we attempt to shed light on the discovery of cardiac resident stem cells in the adult heart and their potential in cardiac repair.

Cardiac Progenitor Cells: From the Developmental Prospective

The mammalian heart is comprised of a highly diversified set of both muscle and non-muscle cell types, including chamber-specific cardiomyocytes, pacemaker cells, the cardiac conduction system, endothelial cells, vascular smooth muscles, and endocardial cells [11]. These various cardiovascular cell lineages are formed from a closely related set of multipotent cardiac progenitor cells (CPCs) in the early embryonic heart [12,13].

The heart development and origin of CPCs

In vertebrates, the development of the heart is initiated during gastrulation by induction of the mesodermal cells located in the anterior lateral part of the primitive streak [14,15]. The early embryonic heart field can be divided into the primary or first heart field (FHF) and secondary heart field (SHF). The cardiac mesodermal cells from the posterior levels of the cardiac region of the primitive streak migrate cranially under the head folds of the embryo and form the FHF, also referred to as the cardiac crescent. The SHF, also known as the anterior heart field, lies medially and anteriorly to the FHF [14,16].

The FHF becomes the exclusive source of the left ventricular myocardium and partially contributes to the atrial myocardium, whereas the SHF contributes primarily to the right ventricular myocardium and the outflow tract [17,18]. However, it is believed that both of the myocardial progenitor lineages with distinct molecular signatures are derived from a common precursor population which segregates early at the onset of gastrulation [11,12,19-21]. In more advanced stages of cardiac development, the FHF-derived CPCs rapidly expand in size and fuse at the ventral midline to form the bilaterally symmetric primitive heart tube consisting of an inner layer of endocardial cells and an outer layer of myocardial cells, which becomes functional at day 21 in humans and begins to pump blood around the embryo [22-24]. As the first asymmetric event in development, the heart tube undergoes rightward looping by the anterior movement of the venous pole and the realignment of the future ventricles and atria [16,25]. The SHF located dorsally in the heart tube rearranges posteriorly and anteriorly upon looping, and the SHF-derived CPCs at the arterial and venous poles subsequently contribute to heart tube elongation and expansion [14,26,27]. Furthermore, cells from additional sources like the pharyngeal arches, the proepicardium or neural crest cell derivatives with neuroectodermal origin cooperate in the formation of the heart [18,28]. The four chambered heart develops by expansion, remodeling, subsequent septation and valve formation of the cardiac tissue which terminally differentiates and matures anatomically and functionally (week 4-10 in humans) [22,29,30].

Signaling pathways involved in formation and differentiation of early mesoderm into CPCs

A series of signaling mechanisms including members of the bone morphogenetic protein (BMP), nodal, activin, transforming growth factor-β (TGF-β), sonic hedgehog (Shh), fibroblast growth factor (FGF), Wnt, and notch proteins co-operate in complex positive and negative networks to induce cardiogenesis (Figure 1A) [14,25,31].

The initial formation of the early mesoderm during gastrulation is induced by canonical Wnt/β-catenin signaling in the posterior epiblast by driving expression of the mesendodermal markers Brachyury and Eomesodermin followed by up-regulation of genes involved in mesoderm patterning and epithelial-mesenchymal transition [31,32]. Brachyury and Eomesodermin prompt the expression of mesoderm posterior 1 (Mesp1) as one of the key regulators to induce the differentiation of early mesodermal cells into CPCs [33,34]. Consequently, the formation of the cardiac mesoderm is initiated, which further gives rise to the FHF, SHF, endocardium and proepicardium [18]. Since canonical Wnt/β-catenin signaling inhibits the cardiac lineage, the pathway is rapidly silenced in the prospective cardiogenic mesoderm by Wnt antagonists from adjacent tissues [35-37]. Secreted factors including BMP2/4, FGF2/4/8, Shh, non-canonical Wnts (Wnt5a, Wnt11) and canonical Wnt inhibitors (Dickkopf-1 homolog, Dkk1), which are released by the underlying anterior endoderm, promote the cardiac phenotype [38-45]. These signals together with cardiac inhibitors like canonical Wnts (Wnt3a/8) and BMP inhibitors (noggin, chordin) produced by the neural ectoderm and the notochord, respectively, play important roles in specification, maintenance and shaping of the cardiac precursor zone, a region characterized by high BMP and low Wnt activity (Figure 1A) [36,46-48].

Transcriptional regulation in formation and differentiation of CPCs

The transiently expressed Mesp1 together with Mesp2 are the earliest molecular markers of CPCs and key regulators of the cardiovascular cell fate. They are required for the primitive streak delamination and label all cardiac cells of mesodermal origin [49-51]. In the presence of the canonical Wnt inhibitor Dkk1 Mesp1 directly down-regulates pluripotency and early mesodermal genes as well as up-regulates the cardiac markers NK2 transcription factor related locus 5 (Nkx2-5), GATA4 and Islet Lim homeobox 1 (Isl1) [33,52,53].

Nkx2-5 as one of the first genes expressed in the early vertebrate embryo is crucial in the induction of cardiogenesis by directly and indirectly activating further transcriptional factors like GATA4, myocyte enhancer factor 2C (MEF2C), T-box 5 (Tbx5) or heart and neural crest derivatives expressed transcript 1 (Hand1) [54,55]. Upon induction of the cardiogenic program in the CPCs at primitive streak stage, a complex network of co-operating developmental key transcription factors, such as Nkx2-5, GATA4/5/6, MEF2C, Tbx5/20, Hand1/2 and their downstream targets subsequently establishes the cardiac phenotype [16,18].

The GATA-binding transcription factor GATA4 is involved in multiple steps of differentiation and morphogenesis in the developing heart [56-60]. GATA4 together with the chromatin remodeling component Baf60c has been shown to directly induce Nkx2-5 gene expression in mesodermal cells, where the expression of both GATA4 and Nkx2-5 is maintained in a positive feedback loop [61-63]. Furthermore, GATA4 associates in a complex with Nkx2-5 and Tbx5 to activate downstream cardiac genes [64-67]. In the progressive heart development Nkx2-5 is important in cardiac looping and in ventricular

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marker expressionand its mutations give rise to congenital heart disease in humans [68,69]. The transcription factor MEF2C plays a critical role in the determination and differentiation of the procardiogenic mesoderm and in cardiac looping [30,70,71]. Additionally, disrupted MEF2C function results in down-regulation of Hand1/2 and failing to properly develop to a right ventricle [72]. In humans, MEF2C mutants display non-syndronic congenital heart defects [73]. Tbx5 has been implicated in multiple processes throughout different stages of cardiogenesis including heart tube formation, cardiac looping and chamber septation accompanied by spatial and temporal expression changes in the CPC subpopulations [74-78]. Hand1 and Hand2 are expressed in the early cardiac development with significant roles in differentiation, rightward looping and normal ventricular development [79-82]. During the cardiac crescent stage the expression of the transcription factors in the early CPCs progressively changes with Hand1 being restricted to the FHF and later left ventricle, whereas Hand2 is confined in the SHF which further forms the right ventricular myocardium and the outflow tract [83,84].

Soon after cardiac induction, the CPC population segregates into two different lineages: the FHF maintains expression of markers like Nkx2-5, Tbx5 and Hand1, whereas the SHF is defined by Isl1-, Hand2- and FGF10-positive cells [22]. Isl1 has been regarded as a specific marker for the SHF [85]. Recent advances in dissecting the molecular mechanisms of cardiogenesis revealed that Isl1 is additionally expressed in the FHF and its derivatives [86-88]. However, Isl1 mutants do not display any defects in primitive heart tube formation, but have deficits in migration, proliferation and survival of SHF-derived CPCs [85,87,89]. Isl1 can be activated by canonical Wnt/β-catenin signaling [90] and is considered to be a key regulator of the SHF by directly and indirectly interacting with further transcription factors in a complex regulatory network including MEF2C, Nkx2-5, GATA, Fork head box (Fox) transcription factors, Tbx1/20 and FGF8/10. Therefore, it is crucial for the development of the right ventricle and the outflow tract [13,91-93]. Tbx1, which is activated by the Fox transcription factors, was shown to have a high impact in promoting the proliferation of SHF-derived CPCs with consequent impact on outflow tract myocardium expansion

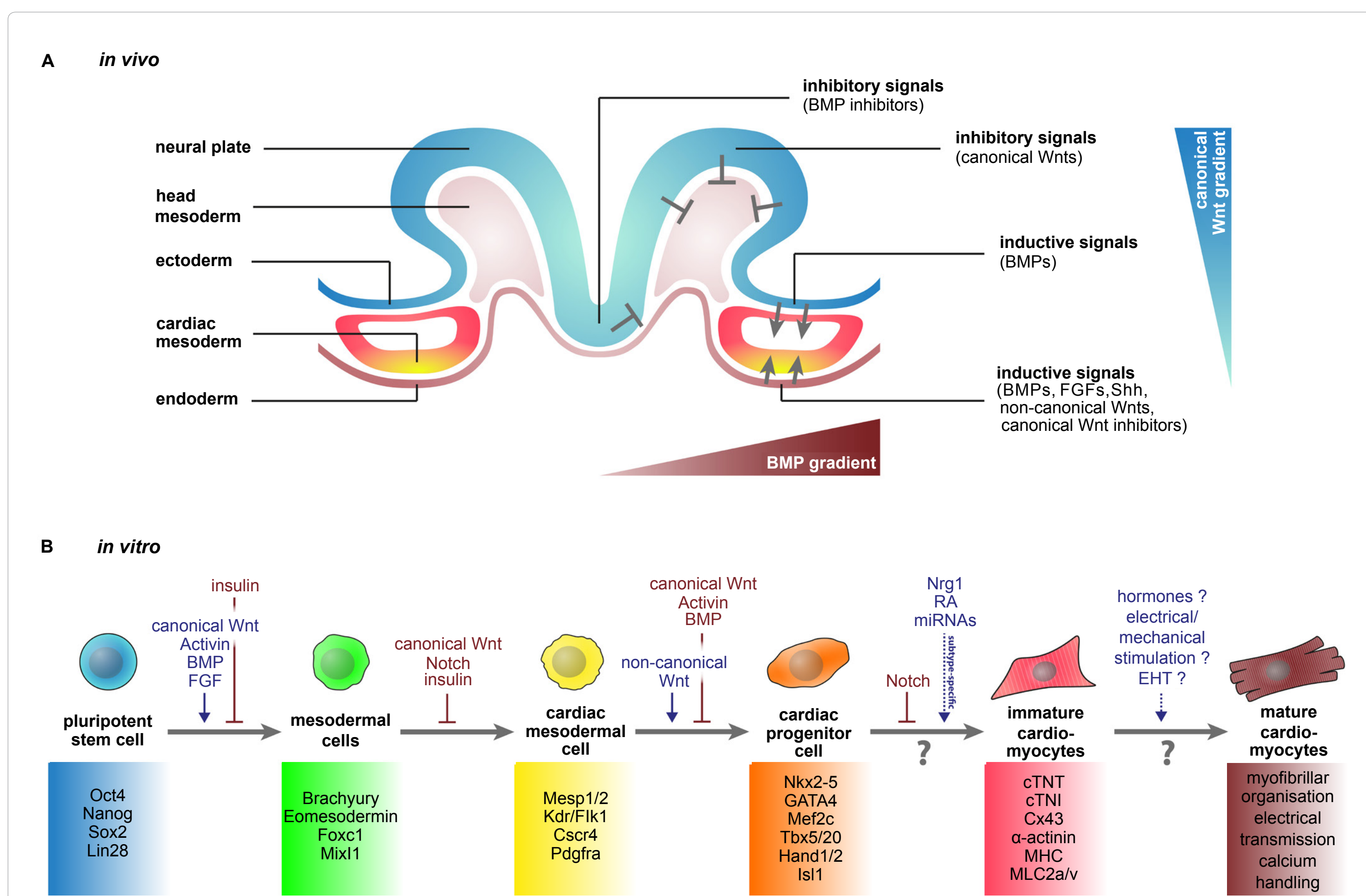

Figure 1: ***In vivo* and *in vitro* cardiogenesis. (A) Schematic illustration of a transverse section of an early stage mammalian embryo showing counteracting signals which shape the cardiac precursor zone *in vivo*.** Cardiogenic secreted factors for cardiac mesoderm formation are emitted by the underlying anterior endoderm and by the anterior ectoderm including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) sonic hedgehog (Shh), non-canonical Wnts and canonical Wnt inhibitors. These signals counteract with cardiac inhibitors like canonical Wnts and BMP inhibitors released by the neural plate. The cardiac mesoderm is defined by a region with high BMP and low Wnt activity. **(B) Sequential steps in cardiac differentiation *in vitro* from pluripotent stem cells to functional cardiomyocytes.** Positive (blue) and negative (red) acting signals influence cardiomyogenesis *in vitro* and display parallels to embryonic development. Early mesodermal cells are induced by canonical Wnt, activin, BMP and FGF signaling and inhibited by insulin. Diversification to cardiac mesodermal cells can be inhibited by canonical Wnts, notch and insulin signaling. Cardiac progenitor cell specification is promoted by non-canonical Wnts and negatively regulated by canonical Wnt, activin and BMP signaling. Notch signaling promotes cardiac progenitor cell differentiation and inhibits cardiomyocyte differentiation. Neurogenin 1 (Ngn1), retinoic acid (RA) and specific microRNAs (miRNAs) direct cardiomyocyte subtype-specific differentiation. Structural and functional maturation is not well understood. Maturation might be provoked by hormones, electrical and mechanical stimulation and organization in 3D engineered heart tissues (EHTs). Typical markers and characteristics for the different cell types are indicated.

[94-99]. Interestingly, both Isl1+ and Tbx1+ CPCs show the potential to differentiate into myocardial, endothelial and vascular smooth muscle cells [89,100-102]. Additionally, specific microRNAs like miR-1 were recently brought into focus of cardiogenesis [103].

Fine-tuned synergistic interactions between multiple signals encompassing the CPCs including canonical Wnt/β-catenin, non-canonical Wnt, FGF, BMP and notch pathways have been implicated in CPC proliferation versus cardiomyocyte differentiation, although the complex mechanisms are not yet fully understood [18,35,87,89,104-109]. Furthermore, these various signaling networks and numerous additional transcription factors are implicated in morphogenesis of the four-chambered heart and in the terminal maturation of cardiomyocytes, namely the expression of structural proteins (such as troponin I, T or C, myosins and α-actinin) and functional proteins (ion channels and gap junction proteins needed for electrical stimuli transmission, intracellular channels such as ryanodine receptor 2 responsible for calcium handling) for mechanoelectrical activity, calcium handling, myofibril organization or sarcomeric striations.

Cardiac Progenitor Cells: Derived From Pluripotent Stem Cells

Human pluripotent stem cells have been proven to constitute a promising source for a myriad of cell types. Diverse molecular insights into embryonic cardiogenesis have been transferred to stem cell biology by supporting the efficient generation of cardiomyocytes *in vitro*. Influencing important signaling cascades and molecular mechanisms throughout the differentiation of pluripotent stem cells under culture conditions can guide the cellular phenotype into a defined direction (Figure 1B). In recent years numerous protocols have been developed to produce cardiomyocytes from ESCs and iPSCs, which become increasingly sophisticated.

The initial strategy to obtain cardiomyocytes *in vitro* was the differentiation in embryoid bodies, in which stem cell clusters differentiate spontaneously into cells from all three germ layers including diverse multipotent cardiovascular cell lineages [110,111]. However, this spontaneous differentiation method yielded in very low amounts of cardiomyocytes. A first step toward targeted differentiation was the differentiation of human pluripotent stem cells on visceral endoderm-like cells which could artificially mimic the *in vivo* situation by providing the differentiating pluripotent cells with requisite signals for the cardiac phenotype [112]. The yield of cardiomyocytes via this method was increased by addition of cyclosporine A [113], addition of ascorbic acid and use of serum-free approaches [114].

Recently established protocols to control and direct differentiation of pluripotent stem cells into cardiomyocytes utilize our understanding of molecular mechanisms regulating early cardiogenesis. Induction of mesendodermal and cardiac mesodermal identities has been proven to be critical in the generation of cardiovascular cells from pluripotent stem cells. During *in vitro* differentiation of pluripotent stem cells, similar to *in vivo*, Brachyury induces the expression of Mesp1 [115] and Tbx5 associates with Nkx2-5 and synergistically promotes cardiomyocyte differentiation [116]. In addition, Nkx2-5 expressing CPCs or Isl1+ and Tbx1+ CPCs derived from pluripotent stem cells show the potential to differentiate into myocardial, endothelial and vascular smooth muscle cells [12,86,89,101].

Many groups focused their research in manipulating the signaling pathways identified in *in vivo* studies, and applied this in the *in vitro* differentiation system of pluripotent stem cells, including BMP, activin, FGF, canonical Wnt/β-catenin and notch signaling. Addition of BMP4 [117], BMP4 and activin A [118,119], BMP4 and FGF2 [120], activin A and FGF2 [121], canonical Wnt3a [122] or a mixture including BMP4, activin A and FGF2 [123] over the first days of differentiation greatly enhanced the formation of mesendoderm and subsequently generated a large amount of cells which expressed various cardiomyocyte markers in both embryoid bodies and monolayer culture conditions (Figure 1B). Furthermore, it was shown that notch [124,125] and insulin [126-128] signaling possess strong inhibiting effects on cardiac mesoderm formation during the initial stages of cardiogenesis. Interestingly, the time point, at whichthe cells were passaged before differentiation, greatly influenced differentiation efficiency [120,129].

Canonical Wnt/β-catenin signaling displays a biphasic role in embryonic heart development by inducing mesodermal commitment and inhibiting cardiac mesoderm specification (Figure 1A). Treatment with Wnt inhibitors at later stages of cardiogenesis under *in vitro* culture conditions could boost the cardiac differentiation. Negative regulation of canonical Wnt/β-catenin signaling after mesodermal diversification during *in vitro* differentiation of pluripotent stem cells by the Wnt antagonist Dkk1 [130,131] or the chemically produced inhibitors IWR-1, IWP-2, IWP-4 [129,132,133] enhanced the yield of cardiomyocytes up to 97%. Recent studies further reveal that inactivation of BMP pathways by the BMP antagonist noggin [134] or small molecule inhibitors dorsomorphin or dorsomorphin homologue-1 [135] during the cardiac mesoderm formation stage of *in vitro* differentiation of human pluripotent stem cells can promote cardiac differentiation. Activated notch signaling can promote the proliferation of CPCs by blocking the subsequent differentiation steps [136-139]. On the other hand, induction of non-canonical Wnts in pre-cardiogenic cells also results in higher CPC differentiation efficiency along with the typical expression of CPC markers, in both mouse and human pluripotent stem cells [42,140,141]. Treatment with factors like ascorbic acid results in expansion of the CPC population whereas induction of insulin-like growth factor (IGF) signaling influences the cardiomyocyte proliferation [142-145].

The current focus of research is the discovery of signals that play a role in later steps of cardiogenesis leading to the different subclasses of cardiomyocytes and their structural and mechanical maturation. The endogenous Neurogenin 1 (Nrg1) signaling, which is a well-established regulator in later cardiogenesis *in vivo*, has been shown to be involved in regulating cardiac subtype specification during *in vitro* differentiation of human pluripotent stem cells [146]. When endogenous Nrg1 signaling was inhibited, there was a substantial increase in the proportion of cardiomyocytes exhibiting the nodal phenotype [146]. In contrast, retinoic acid inhibits CPC differentiation into ventricular cardiomyocytes and promotes atrial phenotypes instead [147]. Further critical factors of *in vivo* heart morphogenesis and differentiation might also have an impact in *in vitro* differentiation and could provide new insights regarding more detailed strategies towards distinct cardiac subtypes. Additionally, microRNAs like miR-1 and miR-499 are considered to be a relevant compound in directed cardiogenic differentiation and maturation [148,149].

The *in vitro* generated cardiomyocytes possess an immature phenotype compared to adult cardiomyocytes [111,150]. Therefore, it is important to elucidate how maturation can be achieved under *in vitro* culture conditions. Recent studies reported that long-term culture protocols could enhance the cardiomyocyte maturation [151]. Mechanical stimulation facilitated by engineered heart tissues is expected to guide immature cardiomyocytes to more mature

phenotypes under *ex vivo* pseudo-physiological conditions [152]. Additionally, a myocardium-derived hormone, named relaxin, could be a potential candidate for the terminal maturation of cardiac cells [153].

Numerous protocols have been described enabling the production of a large number of cardiomyocytes in high purities, thereby paving the way toward cardiac regeneration by cardiomyocyte transplantation. However, the complex mechanisms of terminal maturation are far from being fully understood. In the future, it will be interesting to decipher more precisely, which additional factors provoke the differentiation towards distinct subclasses of cardiomyocytes and prompt a maturation towards adult-like cardiomyocytes.

Cardiac Resident Stem Cells in the Adult Heart

Although the adult heart lacks adequate intrinsic potential to regenerate itself after damage, experimental evidences show that the adult heart harbors a population of cardiac resident stem cells which are able to give rise to cardiomyocytes, smooth muscle and endothelial cells [3,4]. Different pools of cardiac resident stem cells have been classified according to their expression profiles and surface markers, namely side population (SP) cells, c-Kit$^+$, Sca-1$^+$, Isl1$^+$, cardiospheres and cardiosphere-derived CPCs.

Side population (SP) cells

Side population (SP) cells, first isolated from the bone marrow [154], can be identified by their ability to exclude Hoechst 33342 dye due to expression of ATP-binding cassette transporters ABCG2 and MDR1 [155]. Martin et al. have first isolated them from embryonic and adult mouse hearts [156]. In rodents, cardiac SP cells were reported to be positive for stem cell antigen-1 (Sca-1$^+$) and CD34 (CD34$^+$), negative for c-Kit (c-Kit$^-$), CD31 (CD31$^-$) and CD45 (CD45$^-$), as well as expressing cardiac specific transcription factors such as MEF2C, GATA4 and Nkx2-5, and have the potential to differentiate into cells expressing sarcomeric proteins troponin and cardiac α-actinin [157,158]. Their self-renewal properties were also demonstrated by expression of cell cycle regulators such as notch and TGF-β [156,159]. A recent study demonstrates that the adult human heart contains a pool of SP cells expressing the breast cancer resistance protein (BRCP) (Figure 2) [160]. Human cardiac BRCP$^+$ cells exhibit cardiac differentiation potential *in vitro* [160], and, as with mouse cardiac SP cells, are negative for c-Kit. Interestingly, Sca-1$^+$ SP cells in murine studies showed the highest potential of differentiating into cardiac lineages [158] in contrast to human cardiac BCRP$^+$ cells which are negative for Sca-1 [160]. Despite the *in vivo* potential of SP cells to be able to home to the injured heart and lead to an increase in neovascularization, cardiomyocyte regeneration, and improvement in cardiac function [161,162], absolute proof of the source of SP cells and its bona fide stem population defined by clonogenicity and self-renewal have not yet been reported.

c-Kit positive cells

c-Kit is a tyrosine kinase receptor for the stem cell factor initially used to isolate haematopoetic stem cells from bone marrow [163]. The existence of c-Kit$^+$ cells in the adult heart was first described in 2003 by Beltrami and colleagues where they managed to isolate c-Kit$^+$, Lin$^-$ and CD45$^-$ cells from the adult rat heart with significant potential to differentiate into cardiomyogenic lineages and regenerate the infarcted myocardium [3]. c-Kit$^+$ cells express cardiac-specific transcription factors Nkx2-5, GATA4 and GATA5. The long-term culture of c-Kit$^+$ cells has been established and exhibits self-renewal and clonogenic properties [164]. They have been proven to enhance therapeutic efficacy by pre-treatment with IGF1 and hepatocyte growth factor before injection into rodents with myocardial infarction [165]. Although c-Kit$^+$ cells show an apparent therapeutic utility, it has been pointed out that these subpopulations of cardiac resident stem cells are rare within the adult human myocardium [160], and the cardiomyocyte derivatives are structurally immature and deficient in calcium handling. However, a recent study reported that human cardiac c-Kit$^+$ progenitor cells are readily expanded and enriched using a single-cell culture protocol with enzymatic digestion [166], providing a promising tool for isolation of c-Kit$^+$ derivatives for cellular therapy.

Sca-1 positive cells

Mouse Sca-1 was first identified as a member of the Ly-6 antigen family, and is widely used as a marker for enrichment for haematopoetic stem cells [167]. These cells express CD31 but were negative for c-Kit, CD45, CD34 and Flk1. When treated with oxytocin, Sca-1$^+$ cells expressed genes encoding cardiac transcription factors and contractile proteins and showed sarcomeric structure with spontaneous beating [168]. In a study by Tateishi and co-workers, they demonstrated that cardiac stem cell proliferation and survival after direct cell-grafting into ischemic myocardium requires Sca-1 to up-regulate the secreted paracrine effectors that augment neoangiogenesis and limit cardiac apoptosis [169]. The importance of Sca-1$^+$ cells in cardiovascular regeneration is further emphasized by Sca-1 knockout studies in mouse models which demonstrate cardiac defects in myocardial contractility and repair consistent with impairment of cardiac resident stem cells proliferative capacity associated with altered canonical Wnt signaling [170]. Interestingly, although no Sca-1 homologue in humans have been identified, human Sca-1$^+$ cells from the heart isolated with the rodent antibody were able to form functional cardiomyocytes upon stimulation with TGF- [171].

Isl1 positive cells

Isl1 is expressed during development by the SHF and contributes to the right ventricle and outflow tract. Isl1$^+$ cells are found in the embryonic and postnatal mouse, rat and human heart [172]. Isl-1$^+$ cells are a distinct population of cardiac resident progenitors in that they do

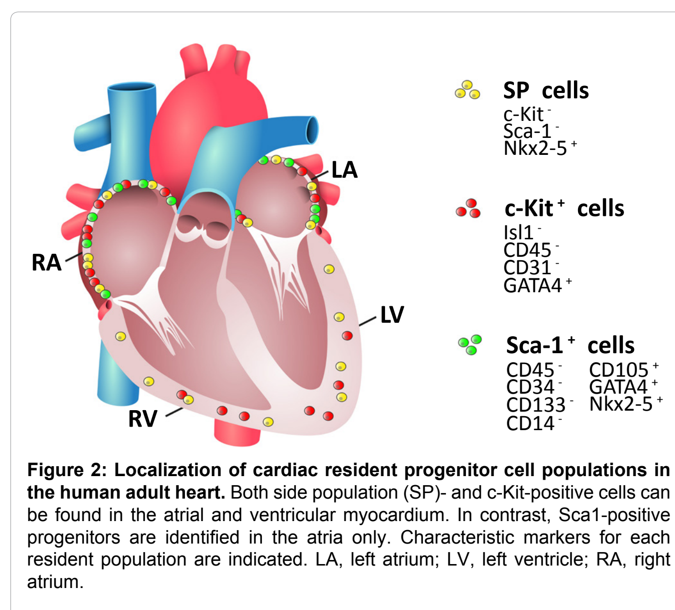

Figure 2: Localization of cardiac resident progenitor cell populations in the human adult heart. Both side population (SP)- and c-Kit-positive cells can be found in the atrial and ventricular myocardium. In contrast, Sca1-positive progenitors are identified in the atria only. Characteristic markers for each resident population are indicated. LA, left atrium; LV, left ventricle; RA, right atrium.

not exclude Hoechst 33342 dye and did not express c-Kit and Sca-1. It has been long regarded that Isl1+ cells are limited in their therapeutic potential as the progenitor population declines rapidly after birth. However, the expansion of Isl1+ cardiac progenitors could be promoted by using directed Wnt/β catenin signaling via the regulation of FGF ligands [35].

Cardiospheres and cardiosphere-derived cardiac progenitor cells

There has been a recent focus on a subpopulation of undifferentiated, self-adherent cell clusters termed "cardiospheres" [173]. Messina et al. first isolated them from subcultures of postnatal atrial or ventricular human biopsy specimens and from murine hearts [174]. They are highly clonogenic and express stem markers such as c-Kit, Sca-1, CD31 and CD34. Although there are controversies about the cardiospheres isolation protocols [175] in rodents where spontaneously beating clusters were suggested to be contaminating myocardial tissues rather than clonogenic, cardiomyogenic cardiac resident stem cells, *in vivo* studies via transplantation into myocardial infarction models have demonstrated the potential of cardiospheres in cardiac regenerative therapies [176].

Origin of cardiac resident stem cells

While the presence of cardiac resident stem cells seems promising, several questions still remain open, and these include the origin of cardiac resident stem cells and the mechanisms underlying their *in vivo* activation and role in tissue repair. The question of the origin of cardiac resident stem cells has been widely debated. One hypothesis portrays the fact that cardiac resident stem cells arise during embryonic cardiogenesis and that they are retained as undifferentiated remnants in putative stem cell populations concentrated mainly in the atria and apex of the heart (Figure 2) [3,177]. This is best supported by the organ distribution of Isl1+ cells in the postnatal heart, which match the contributions of its embryonic precursors [172,178]. Furthermore, SP cells have also been found to arise from neural crest cells in the embryonic heart and contribute to development and homeostasis of cardiac tissue [179]. The second hypothesis supported by many other studies suggest that cardiac resident stem cells stem from an extra-cardiac pool of circulating progenitors coming from the bone marrow and are able to home to the heart upon stimulation. This was first demonstrated by sex-mismatched bone marrow and cardiac transplantations in which recipients show a high level of chimerism in their hearts [180]. As previously described, cardiac resident stem cells have also been characterized by the expression of markers commonly used to isolate bonemarrow hematopoietic stem cells and have been shown by several groups to contribute to the pool of cardiac progenitors [162,181].

Stem Cell-Based Cellular Therapy for Heart Regeneration

There have been already several ongoing clinical trials for cardiac repair whereby bone marrow-derived cells or peripheral blood-derived endothelial progenitor cells were transplanted via intra-coronary cell infusion or direct intra-myocardial injection in patients after myocardial infarction [182-184]. Although the initial trials assure the safety and feasibility of using bonemarrow cell transplantation for cardiac therapy, the results are still somewhat ambiguous as demonstrated by the STEMI (ST-Elevation acute Myocardial Infarction) and the ASTAMI (Autologous Stem Cell Transplantation in Acute Myocardial Infarction) trials [184,185]. Challenges for bonemarrow related applications in cellular therapy still need to be overcome as the efficiency of regeneration to myocardial tissue is still insignificant.

Previous studies, which examined the use of fetal or neonatal cardiomyocytes as donor cells clearly established that exogenous cardiomyocytes can structurally and functionally integrate into the host myocardium [186]. However, there is no accessibility of fetal or neonatal sources for treating patients. The ethical and immune rejection concerns also make this therapy not practical. Therefore, recent focus has been placed on cardiac resident stem cells, or CPCs and cardiomyocytes derived from pluripotent stem cells.

Cardiac resident stem cells for cardiac repair

Previous studies showed that human cardiac resident stem cells could be activated within the cardiac niche to renew themselves after myocardial infarction [177], overcoming the problems of cell loss after cellular implantation. Cardiospheres or cardiosphere-derived cardiac progenitor cells have already found their way into clinical trials (CADUCEUS; cardiosphere derived autologous stem cells to reverse ventricular dysfunction) to demonstrate the applicability of these cells by intra-coronary injection into patients with ischemic left ventricular dysfunction and myocardial infarction [187]. However, the long-term clinical outcome needs to be further investigated. In conclusion, although cardiac resident stem cells remain an attractive candidate for the use in regenerative therapies in the treatment of heart failure, their functional significance under physiological and pathological states remains to be elucidated. Future studies on regulatory mechanisms determining self-renewal and differentiation of these cells would improve our understanding of cardiac biology and hopefully foster our knowledge in the development of cardiac resident stemcell-based therapy.

Selection and therapeutic potential of CPCs derived from pluripotent stem cells

Heart regeneration requires the formation of cardiomyocytes and coronary vessels, and it can most likely not be accomplished by a cell already committed to one specific lineage. Thus, it is necessary to use cells that are multipotent and can differentiate into functional cardiomyocytes as well as vascular smooth muscle and endothelial cells. As mentioned above, CPCs (Isl1+/Nkx2-5+) derived from pluripotent stem cells can satisfy the need for multi-lineage differentiation. For cellular therapy, however, one of prerequisites is to generate sufficient numbers of the desired cell type with high purity.The formation of teratomas after transplantation of undifferentiated pluripotent stem cells [188] implies that it is essential to purify pluripotent stem cell-derived CPCs before transplantation.

A recent study demonstrated that the combination of cell surface markers Flt1 and Flt4 could contribute to the enrichment for Isl1+/Nkx2-5+ CPCswith tri-lineage cardiovascular potentialin mouse embryonic hearts and differentiating iPSCs [189]. The purified CPCs could be propagated *in vitro* while simultaneously maintaining their multipotent differentiation potential in the presence of the β-catenin/p300 inhibitor IQ-1 and the ROCK-inhibitor Thiazovivin. Furthermore, the purified CPCs were successfully transplanted into the healthy heart and showed the capacity to engraft into the native tissue with robust differentiation ofcardiomyocytes which are morphologically and electrophysiologically mature [189]. In addition, these CPCs can also differentiate into smooth muscle cells in the normal heart [189]. As a proof of concept for cardiac repair, Mauritz et al. evaluated the

potential of mouse iPSC-derived Flk1+ CPCs to restore myocardial tissue and improve cardiac function after acute myocardial infarction in mice [190]. Flk1+ CPCs formed remarkable grafts only two weeks after cell transplantation *in vivo*, suggesting their rapid proliferation capacity, therefore raising questions as to whether such proliferation could be controlled *in vivo*.

CPCs have also been derived from human ESCs by the addition of activin A, BMP4, bFGF, VEGF and Dkk1 in a time- and dose-dependent manner during *in vitro* differentiation [130]. The KDR^{low}/c-Kit^{-} population expresses high levels of genes involved in cardiac development, including *Nkx2-5*, *Isl1* and *Tbx5*, and displays potential to differentiate into cardiac, endothelial and vascular smooth muscle cells *in vitro* [130]. In contrast, the KDR^{high}/c-Kit^{+} population expresses high levels of genes and proteins associated with vascular development [130]. Transplantation of KDR^{low}/c-Kit^{-} cells into normal and infarcted hearts of immunodeficient mice showed that these cells were able to differentiate into the cardiac, endothelial and vascular smooth muscle lineages and to improve cardiac function *in vivo*. In addition, no teratomas were observed in any of the transplanted animals [130]. Human iPSC-derived CPCs transplanted into the infarcted rat hearts revealed engraftment, differentiation into cardiomyocytes and smooth muscle, and persisted for at least 10 weeks post cell injection, thereby resulted in a non-significant trend toward protection from decline in function after ischemic damage [191]. Although such cell transplantation does improve function, the mechanisms mediating this effect are largely not known [192]. Future *in vitro* studies on expansion of human pluripotent stem cell-derived CPCs should also be performed to meet the needs of cellular therapy. In addition, the safety and effectiveness using CPCs should be assessed using large animals as preclinical models. The differentiation and maturation of these cells *in vivo* need to be studied. Methods for the cell administration into damaged hearts should be optimized. CPCs might be most suitable for tissue engineering approaches that will allow for directed differentiation as well as control of proliferation and maturation *in vitro* before transplantation.

Pluripotent stem cell-derived cardiomyocytes in transplantation studies

ESCs are the first pluripotent stem cell source that could reliably give rise to cardiomyocytes. The use of patient-specific iPSCs for generation of cardiomyocytes for cardiac repair could overcome the ethical and immunological complications associated with human ESCs. Transplantation of purified cardiomyocytes derived from pluripotent stem cells may limit the oncogenic risk of injecting undifferentiated iPSCs or ESCs. However, the ability to utilize cardiomyocytes derived from human iPSCs/ESCs therapeutically will require efficient induction of cardiomyocyte lineages from pluripotent stem cells, the identification of surface markers to enrich for cardiomyocytes without genetic manipulation, and elimination of any residual pluripotent stem cells.

As mentioned above, the efficient induction of cardiomyocytes from pluripotent stem cells *in vitro* can be achieved by manipulation of signaling pathways that play important roles during mesodermal and cardiac development using factors and small molecules. Numerous protocols have been described enabling the production of a large number of cardiomyocytes. Uosaki et al. provided an important tool for the purification of pluripotent stem cell-derived cardiomyocytes. They used a differentiation system to screen 242 antibodies for surface markers of cardiomyocytes and found VCAM1 as a suitable candidate. It has been shown that 95% of MACS-purified VCAM1-positive cells were positive for cardiac muscle troponin T [131].

A number of reports have shown that human ESC-derived cardiomyocytes proliferate after transplantation into infarcted rodent hearts, form human myocardial grafts, and enhance regional and global contractile function [118,193-195]. These first experiments showed that transplanted cardiomyocytes can survive in the recipient, that they can in principle couple to host cardiomyocytes and that they have at least the ability to attenuate myocardial infarction for certain time. However, a major problem identified in almost all cardiomyocytes transplantation studies in animal models, is the relatively poor survival of the engrafted cells, which impact the effectiveness of the transplantation of pluripotent stem cell-derived cardiomyocytes. Therefore, systematic approaches to prevent transplanted cell death in cardiac repair are still necessary [196].

Therapeutic potential of direct conversion of fibroblasts into cardiomyocytes

The path for an entirely different approach was paved in 2010, when Ieda et al. reprogrammed cardiac and tail-tip fibroblasts with the transcription factors GATA4, MEF2C and Tbx5 (GMT) to induced cardiomyocytes without passing through a pluripotent stage [197]. The induced cardiomyocytes expressed cardiac markers, showed well-defined sarcomeric structures and contracted spontaneously. Importantly, the fibroblasts after viral transduction could be also converted into cardiomyocytes *in vivo* [197]. Moreover, *in vivo* delivery of GMT into the myocardium after coronary ligation attenuated ejection fraction and stroke volume decline after three months. The scar area was significantly reduced and induced cardiomyocytes were detected in the scar area [198]. The addition of the transcription factor Hand2 to the GMT (GHMT) resulted inincreased generation of induced cardiomyocytes from both cardiac and tail-tip fibroblasts. When the GHMT retroviruses were delivered into the heart after myocardial infarction, the cardiac function was improved and the adverse ventricular remodeling was reduced. By comparison, functional improvement was accelerated and more complete with GHMT than GMT [199]. Jayawardena et al. used transient expression of miRNAs 1, 133, 208 and 499 in combination with JAK Inhibitor I to convert 14% of cardiac fibroblasts into α-MHC positive cardiomyocytes which exhibit spontaneous calcium oscillations and prominent striations [200]. Moreover, when delivered with a lentivirus in a ligated mouse heart, the miRNA combination induced trans-differentiation of fibroblasts to well-integrated, striated cardiomyocytes [200]. Taken together, these studies provide proof of principle for the possibility of *in vivo* trans-differentiation of cardiac fibroblasts into functional cardiomyocytes and may have broad and important implicationsformyocardial regeneration therapy.

Conclusion

If the massive cardiac cell loss resulting from myocardial infarction could be replaced by the functional cardiomyocytes and revascularization could occur, millions of patients' life can be rescued every year. Studies on patient-specific iPSCs prove that they are an excellent tool for disease modeling, drug discovery and cardiotoxicity screenings. Importantly, this technology also provides an invaluable source for generation of patient-specific cardiomyocytes, vascular smooth muscle and endothelial cells which can be used for regeneration of the damaged heart. However, extensive basic research and well-designed clinical trials have yet to be performed to better understand the biology of stem cell-based therapy, to develop more

efficient methods for the cell administration into damaged hearts, to improve the survival and integration of the transplanted cells, and to demonstrate the safety. Recently, cardiac tissue engineering has made rapid progress. By using different strategies, such as myocardial patches and whole organ decellularization-recellularization, stem-cell based cardiac therapy could be expanded to treat non-ischemic heart disease, such as congenital heart diseases and acquired heart defects. In addition, further studies are required to elucidate the safety and effectiveness of cardiac repair by reprogramming of endogenous non-cardiomyocytes towards a cardiomyocyte fate *in vivo* through forced expression of different transcription factor or microRNA combinations.

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