Embryonic Heart Progenitors and Cardiogenesis

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The mammalian heart is a highly specialized organ, comprised of many different cell types arising from distinct embryonic progenitor populations during cardiogenesis. Three precursor populations have been identified to contribute to different myocytic and nonmyocytic cell lineages of the heart: cardiogenic mesoderm cells (CMC), the proepicardium (PE), and cardiac neural crest cells (CNCCs). This review will focus on molecular cues necessary for proper induction, expansion, and lineage-specific differentiation of these progenitor populations during cardiac development in vivo. Moreover, we will briefly discuss how the knowledge gained on embryonic heart progenitor biology can be used to develop novel therapeutic strategies for the management of congenital heart disease as well as for improvement of cardiac function in ischemic heart disease.

The functional heart is comprised of diverse muscle and nonmuscle cell lineages: atrial and ventricular cardiomyocytes, endocardial cells, valvular components, and connective tissues, conduction system cells, as well as smooth muscle and endothelial cells of the coronary arteries and veins. Understanding the molecular mechanisms that drive the specification of these cell lineages from naïve progenitor populations into terminally differentiated cell types within the four-chambered embryonic heart is of fundamental importance to elucidate the pathological mechanisms underlying congenital heart disease and to improve management of ischemic heart disease (Olson 2004; Srivastava 2006). Three spatially and temporally distinct sources of heart cell precursors have been identified in the embryo: cardiogenic mesoderm cells (CMCs), the proepicardium (PE), and cardiac neural crest cells (CNCCs) (Fig. 1A–C).

The cardiogenic mesoderm, which harbors the so-called first and second heart fields (FHF and SHF), forms the major proportion of the ventricular, atrial, and outflow tract (OFT) myocardium. Additionally, these progenitors contribute cells to the endocardium, the conduction

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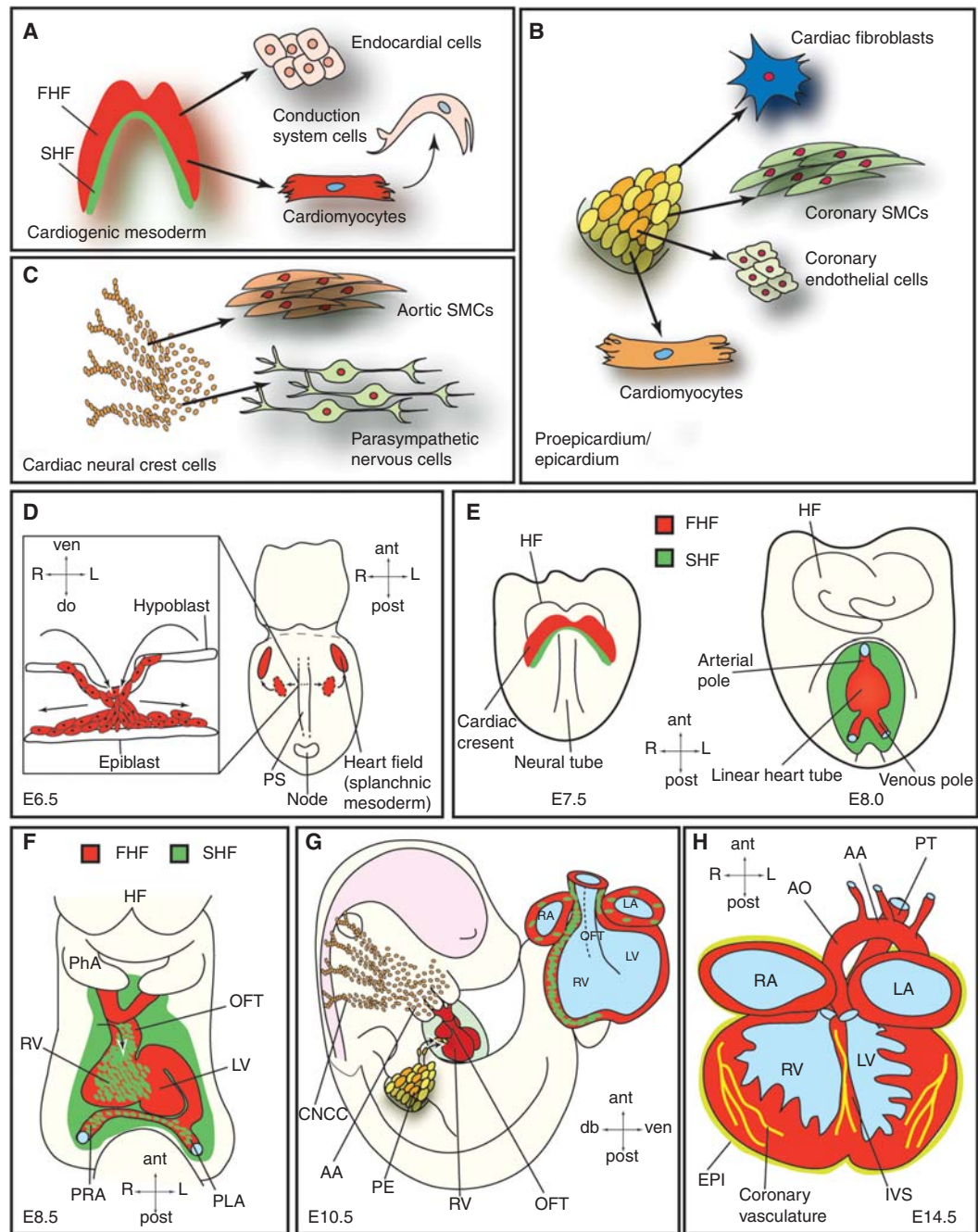
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Figure 1. Embryonic heart progenitor contributions to different cardiac compartments and cell types during heart morphogenesis in mouse development. (*A*) Cardiogenic mesoderm, (*B*) proepicardium/epicardium, and (*C*) cardiac neural crest cell lineage diversification. (*D*) At E6.5, mesodermal progenitors ingress through the primitive streak (PS) and migrate away from the PS (illustrated in the box showing a transverse section) to form the heart fields located in the splanchnic mesoderm. (*E*) At E7.5, the first and second heart fields (FHF and SHF) are discernible. The first heart fields fuse at the midline thereby forming the cardiac crescent caudal to the headfolds. FHF progenitor cells start to differentiate. (*Legend continues on facing page.*)

system, and the aortic and pulmonary cushions (Fig. 1A).

Progenitors originating from the PE comprise the epicardium and differentiate into interstitial fibroblasts embedded in the myocardium, vascular smooth muscle cells and endothelial cells of the coronary vessels, and some myocytes, mainly in the atrioventricular septum. Additionally, interaction of the epicardium with the underlying myocardium is crucial for chamber maturation and ventricular muscle growth (Fig. 1B).

Finally, the CNC progenitors give rise to the distal smooth muscle cells of the OFT and the aorticopulmonary ridge as well as to the autonomous nervous system of the heart. Cardiac valve development and septation of the heart is also critically dependent on proper CNC development (Fig. 1C).

Here we will describe "the lives" of these different cardiac progenitors, from their embryonic specification to their differentiation into mature cells of the heart, and we will discuss how this knowledge could inform the future development of novel therapies for cardiovascular heart disease.

THE CARDIOGENIC MESODERM PROGENITORS

Cardiogenic Mesoderm Progenitors in Early Vertebrate Cardiogenesis

The embryonic heart is the first organ to function during development. Fate mapping studies in mouse and chicken embryos have shown that time and location of cell ingression through the primitive streak (PS) (Fig. 1D section detail in box) determines the fate of gastrulating cells.

Consequently, cardiac mesoderm progenitors are among the first cells to ingress through the PS during gastrulation. These naïve cardiogenic mesoderm cells migrate away from the PS and coalesce in an anterior lateral region relative to the streak, the so-called splanchnic mesoderm (Fig. 1D), where they form the cardiac crescent (at mouse embryonic day E7.5, corresponding roughly to week 2 of human gestation); at this stage, their commitment to a cardiac fate remains plastic (Fig. 1E) (Buckingham et al. 2005). At E8, or 3 weeks in human gestation, the cardiac crescent fuses at the midline and gives rise to the FHF-derived linear heart tube (Fig. 1E), which subsequently commences beating and undergoes rightward looping and rapid growth (mouse E8.5, 4 weeks in human development) (Fig. 1F) (Zaffran et al. 2004). As development proceeds, the linear heart tube expands, essentially by two mechanisms: (i) cell proliferation, and (ii) recruitment of additional cells. These additional cells, which are added to the arterial and venous poles of the linear heart tube, originate from the pharyngeal mesoderm located caudal to the pharynx, and represent the SHF (Mjaatvedt et al. 2001; Waldo et al. 2001; Buckingham et al. 2005; Kelly 2012; Moorman et al. 2013). SHF progenitors mainly contribute to the OFT, the right ventricle, and a large portion of the inflow region (atria), whereas the left ventricle mainly derives from the FHF (Fig. 1F). The septated four-chambered fetal heart is subsequently generated involving intricate interactions of cardiomyocytes with epicardial and endocardial cells as well as CCNCs. At day E10.5 in mice (day 32 in human) the developing heart shows well-defined chambers (Fig. 1G), which are fully septated and connected to the pulmo-

Figure 1. (*Continued*) At E8.0, the cardiac crescent forms the beating, linear heart tube. SHF progenitors will gradually migrate into the linear heart tube and differentiate then. (*F*) At E8.5, the linear heart tube undergoes rightward looping. (*G*) At E10.5, cardiac neural crest and proepicardial cells contribute to the heart, which already shows a defined four-chamber morphology. (*H*) At E14.5, the heart shows four fully septated chambers and a septated outflow tract connected to the pulmonary trunk and the dorsal aorta. AA, Aortic arch; ant, anterior; AO, dorsal aorta; CNCC, cardiac neural crest cells; do, dorsal; EPI, epicardium; FHF, first heart field; HF, headfolds; IVS, interventricular septum; L, left; LA, left atrium; LV, left ventricle; OFT, outflow tract; PE, proepicardium; PhA, pharyngeal arch; PLA, primitive left atrium; post, posterior; PRA, primitive right atrium; PS, primitive streak; PT, pulmonary trunk; R, right; RA, right atrium; RV, right ventricle; SHF, second heart field; SMCs, smooth muscle cells; ven, ventral.

nary trunk and aorta at E14.5 ($\sim$7 weeks in human gestation) (Fig. 1H).

From Mesoderm Induction to Cardiogenic Mesoderm Progenitors

Mesoderm induction, a quintessential prerequisite for heart development, is evolutionarily conserved and regulated by numerous signaling pathways. Key players are Nodal and bone morphogenetic protein (BMP) signals as well as Wnt and fibroblast growth factors (FGF) (Kimelman 2006; Noseda et al. 2011). Expression of the T-box transcription factor Brachyury/T (Bry), a direct target gene of Wnt/β-catenin signaling, marks mesodermal cells ingressing through the PS (Showell et al. 2004). Commitment of nascent Bry$^+$ mesodermal progenitors toward a cardiogenic fate requires inhibition of canonical Wnt/β-catenin signaling and activation of noncanonical Wnt signaling (Gessert and Kuhl 2010). Moreover, intricate interactions between the mesodermal germ layer and the endoderm are additionally necessary for the induction of a common primordial cardiovascular progenitor population from Bry$^+$ mesodermal precursors, which later give rise to both first and second heart field progenitors (Figs. 1 and 2) (Abu-Issa et al. 2004; Buckingham et al. 2005). Tracing cardiogenic fate back to a Bry$^+$ population sparked the idea of a clonal model of heart lineage diversification analogous to hematopoiesis, in which a single hematopoietic stem cell can generate all blood-cell lineages (Weissman 2000). According to this hierarchy model, diverse muscle and nonmuscle lineages arise from a single-cell-level decision of multipotent, primordial cardiovascular stem cells, which in turn give rise to downstream intermediates representing tissue-restricted precursors of fully differentiated heart cells (Fig. 2). First, data supporting this hypothesis have been gained by lineage-tracing studies, and marker gene expression analysis in mice showing that the endocardial endothelium and the myocytic lineage develop from an intermediate mesodermal population that expresses vascular endothelial growth factor receptor 2 (VEGFR2, Flk-1, KDR) (Motoike et al. 2003; Ema et al. 2006). These initial findings were corroborated by additional in vivo and in vitro studies. During development as well as during in vitro differentiation of mouse and human embryonic stem cells, two distinct Bry$^+$ cell populations arise (Fig. 2). First, a Bry$^+$/Flk-1$^+$ hemangioblast precursor population contributes cells to the hematopoietic system and the developing vasculature. During subsequent development, Bry$^+$/Flk-1$^-$ mesodermal progenitors initiate a new wave of Flk-1 expression and constitute cardiovascular progenitors (Fig. 2) (Kouskoff et al. 2005; Kattman et al. 2006; Yang et al. 2008). Later, this primitive Bry$^+$/Flk-1$^+$ cardiogenic mesoderm population down-regulates Bry and activates, upon T-box transcription factor Eomesodermin action, expression of the mesoderm posterior 1 (Mesp1) gene (Fig. 2) (Bondue and Blanpain 2010; Costello et al. 2011). Mesp1$^+$ mesodermal progenitors contribute to paraxial mesoderm and skeletal muscle of the head as well as cardiac muscle (Saga et al. 2000; Scott 2012). By turning on the core cardiogenic transcriptional network, Mesp1 has been shown to further restrict these primitive mesodermal precursors toward a cardiovascular and anterior mesodermal fate (Bondue et al. 2008; Martin-Puig et al. 2008; Bondue and Blanpain 2010).

Differentiation of Early Cardiogenic Mesoderm Progenitors—Formation of the Cardiac Crescent and the Linear Heart Tube

After ingression through the PS, cardiogenic progenitor cells migrate to an anterior lateral position caudal to the headfolds and form the so-called cardiac crescent. At this time in development, the first and second heart fields can be distinguished (Moorman et al. 2013). Although the FHF progenitors already differentiate at this stage, cells within the SHF remain in a proliferative, undifferentiated progenitor state until they enter the heart tube at a later time point (Kelly 2012). In fact, because of their position in the crescent (more anterior and lateral in respect to SHF progenitors) (Fig. 1E), FHF progenitor cells are exposed to cytokines of the BMP (Schultheiss et al. 1997) and FGF (Reifers et al. 2000) families as well as to inhibitors of the
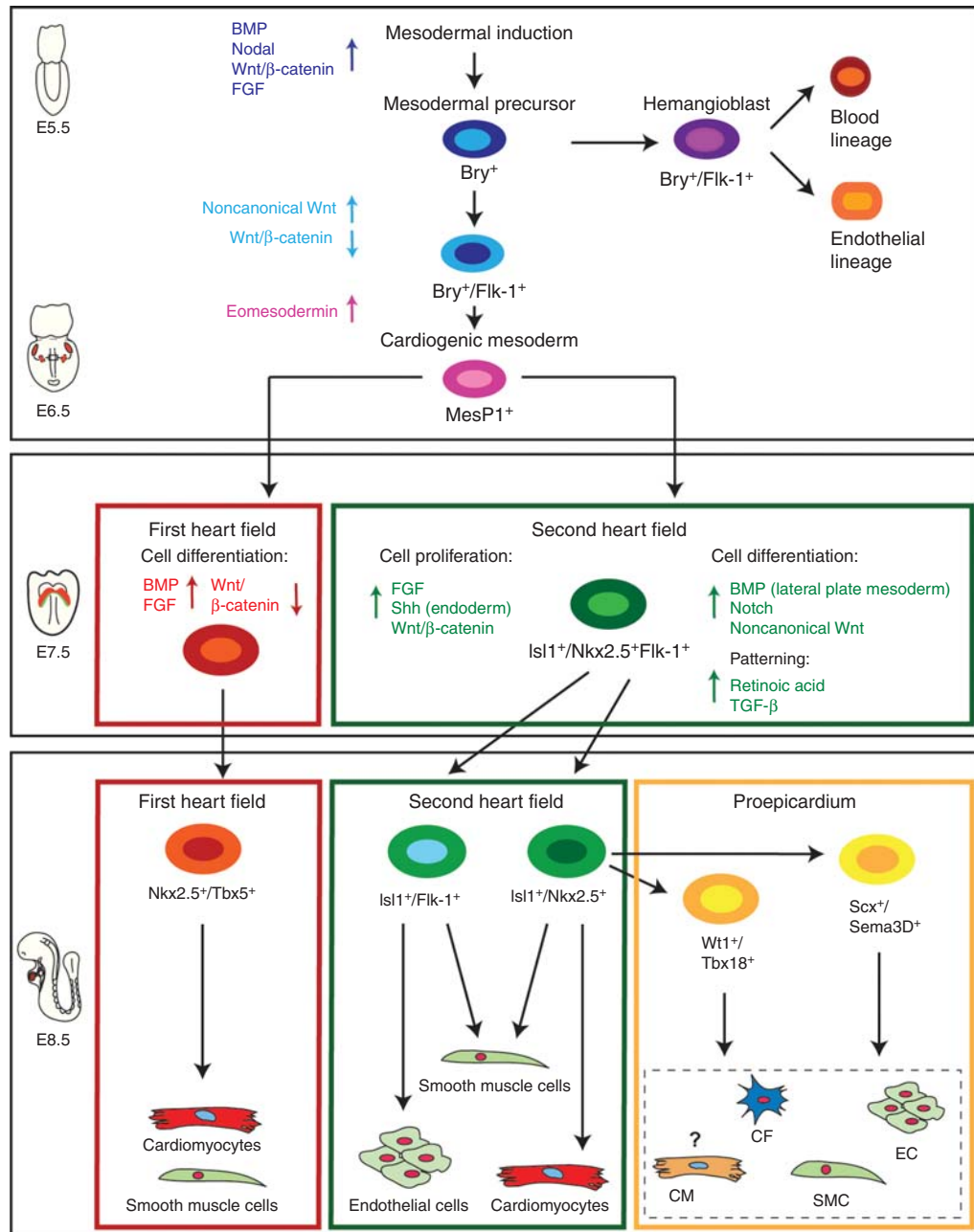
Figure 2. Cellular hierarchy of cardiac progenitor cells and their lineage specification. Several signaling pathways (BMP, Nodal, Wnt/β-catenin, FGF) interact to induce mesoderm; Brachyury (Bry) positive mesodermal precursors first differentiate through Bry⁺/Flk-1⁺ hemangioblasts toward endothelial and blood-cell lineages (around E5.5 during mouse development). Slightly later during development after down-regulation of Wnt/β-catenin signaling and induction of noncannonical Wnt signals a second wave of Bry⁺/Flk-1⁺ mesodermal progenitors appears. Eomesodermin signaling drives cardiogenic mesoderm specification from these primitive mesodermal precursors. Cardiogenic mesoderm is marked by the expression of mesoderm posterior 1 (Mesp1) (around E6.5 in mouse embryogenesis). (*Legend continues on following page.*)

Wnt pathway (Marvin et al. 2001; Schneider and Mercola 2001; Tzahor and Lassar 2001; Noseda et al. 2011), resulting in the onset of cardiac differentiation marked by the expression of key regulators of the lineage, namely, Nkx2.5 (Lints et al. 1993), Gata-4 (Arceci et al. 1993; Kelley et al. 1993; Heikinheimo et al. 1994; Zeisberg et al. 2005), and Tbx5 (Harvey 2002). Myocytic lineage commitment is associated with expression of contractile proteins including myosin light chain-2a (MLC2a) (Kubalak et al. 1994) and sarcomeric myosin heavy chain (MHC) first in the cardiac crescent and then throughout the linear heart tube.

Although no genes uniquely expressed in early FHF progenitors have been identified yet, SHF precursors are marked by the LIM-homeodomain transcription factor Isl-1. Although several studies suggest Isl-1 to be a very early pancardiac marker, after formation of the linear heart tube, Isl-1 clearly demarcates the SHF and its expression is absent in differentiated FHF derivatives (Prall et al. 2007; Kelly 2012). Isl-1 expression is dependent on canonical Wnt signaling (Tzahor 2007; Cohen et al. 2008), and its function is required for survival, proliferation, and migration of into the primitive heart tube of the SHF progenitors. Isl-1 expression is extinguished as progenitor cells reach the developing heart and differentiate (Cai et al. 2003). Several studies have shown that the molecular signature Isl-1$^+$/Nkx2.5$^+$/Flk-1$^+$ marks a specific pool of primitive SHF progenitors that are multipotent and give rise, by further lineage restriction, to downstream tissue-specific intermediates generating both myocytic and vascular cells (Fig. 2) (Kattman et al. 2006; Moretti et al. 2006; Wu et al. 2006; Bu et al. 2009). In particular, Isl-1$^+$/Nkx2.5$^+$ descendants (that have lost Flk-1 expression) are committed to form cardiomyocytes and smooth muscle cells (SMCs), extensively contributing to the proepicardium/epicardium (Cai et al. 2008; Zhou et al. 2008a,b), whereas the Isl-1$^+$/Flk-1$^+$ subset (in which Nkx2.5 expression has ceased) differentiates to form endothelial cells and SMCs (Fig. 2) (Moretti et al. 2006). The fate of SHF progenitors is controlled by many different signaling pathways. Among them, FGF signaling within the SHF promotes progenitor cell proliferation; Shh-mediated signals from the endoderm and canonical Wnt signaling from the midline (neural tube) are important for the maintenance of a proliferative state and inhibition of differentiation (Kelly 2012). On the contrary, BMPs secreted from lateral plate mesoderm as well Notch and noncanonical Wnt signals promote cardiac differentiation of SHF progenitors (Vincent and Buckingham 2010). Furthermore, mechanisms of epigenetic control (e.g., miRNA, lncRNA) are also important regulators of the progression of progenitors to terminally differentiated cardiac muscle and nonmuscle lineages (Liu and Olson 2010).

Taking together two decades' worth of research data, we can now construct a core transcriptional and signaling network regulating early cardiac development. This highly com-

Figure 2. (*Continued*) Early mesoderm-derived cardiac precursors undergo further lineage restriction and differentiate into progenitor pools that populate the FHF and SHF, respectively. At this stage (E7.5 mouse development) FHF progenitors start to differentiate upon BMP and FGF action toward cardiomyocytes and smooth muscle cells, whereas Wnt/β-catenin, FGF, and endodermal Shh signaling keeps SHF progenitors in a proliferative state. These SHF progenitors are defined by the molecular signature Isl-1$^+$/Nkx2.5$^+$/Flk-1$^+$. SHF progenitors are now gradually added to the looping heart tube and get further restricted in their differentiation potential (E8.5). Two subpopulations of SHF progenitors can be distinguished. One population marked by the expression of Isl-1 and Flk-1 differentiates into endothelial cells and smooth muscle cells, whereas a second pool of Isl-1$^+$/Nkx2.5$^+$ SHF precursors provides smooth muscle cells and cardiomyocytes as well as contributing to the proepicardial lineages (Wt1$^+$/Tbx18$^+$ and Scx$^+$/Sema3D$^+$ populations), which later form cardiac fibroblasts (CF), smooth muscle cells (SMCs), endothelial cells (EC), and cardiomyocytes (CM), with the latter contribution being still unclear. These distinct SHF progenitor populations differentiate upon BMP signals from the lateral plate mesoderm as well as Notch and noncanonical Wnt signals. SHF patterning is governed by RA and TGF-β signals.

plex network of spatiotemporal interactions of growth factors and transcriptional regulators (e.g., BMP, Wnt, FGF, Nkx2.5, and Gata4) governs both FHF and SHF development (Kelly 2012; Harvey 2013; Moorman et al. 2013). Importantly, our current knowledge supports a new paradigm for cardiovascular development involving the divergence of myocardial, smooth muscle and endothelial cell lineages from a common precursor.

THE PROEPICARDIUM

The early embryonic heart tube developing from the FHF and SHF progenitors consists only of two cell layers, the endocardium and the myocardium. The epicardium, the outermost layer of the heart, and the epicardium-derived cells (EPDCs), which are found in the mature heart, such as cardiac fibroblasts and cells of the coronary vasculature, are not present at the tubular heart stage. These cell lineages arise later (between E9.5 and E11.5 in mouse development) (Fig. 1) and are derived from a cluster of cells, which is known as the proepicardium (PE).

Induction of the Proepicardium and Epicardium Formation

The transitory structure of the PE arises from the coelomic mesenchyme of the septum transversum in close proximity to the venous pole of the linear heart tube at E 8.5 (d21 in human gestation) (Manner et al. 2001). PE induction, growth, and maintenance depends, among others, on opposing interaction between FGF signaling, which induces a proepicardial fate in the posterior splanchnic mesoderm, and BMP signaling, driving myocardial differentiation of this cell population (Kruithof et al. 2006; van Wijk et al. 2009; Torlopp et al. 2010; Schlueter and Brand 2012). PE progenitors are marked by the expression of Tbx18 and Wt1 (Kraus et al. 2001; Cai et al. 2008; Zhou et al. 2008b) as well as by FHF and SHF markers Nkx2-5 and Isl-1 (Zhou et al. 2008b). One important question concerns heterogeneity of PE progenitors, which could represent early-segregated progenitor pools. Earlier studies in chicken embryos showed at least two morphologically different proepicardial cell populations: (1) an external mesothelial epithelium, and (2) an internal extracellular matrix (ECM)-rich mesenchymal core (Nahirney et al. 2003). Although no morphological differences have been described in mammalian PE cells, a very recent lineage-tracing study in mouse analyzing semaphorin3D$^+$ (Sema3D$^+$) and scleraxis$^+$ (Scx$^+$) PE progenitors revealed that these cells show a distinct differentiation potential from earlier described Tbx18$^+$ and Wt1$^+$ proepicardial cells, confirming the data on PE heterogeneity evident from studying the avian system (Katz et al. 2012). In mammals, freely floating PE cell vesicles are released from the PE anlage, which flatten and spread out on contact to the naked myocardium, thereby forming the epicardium between E9.5 and E11.5 in mouse development (Fig. 3A). Genetic evidence from mouse embryos suggests a crucial role for cell adhesion molecules (vascular cell adhesion molecule [VCAM] and b4-a1-integrin) in this process (Kwee et al. 1995; Yang et al. 1995).

The Epicardium- and Epicardial-Derived Cells Govern Chamber Maturation and Coronary Vessel Formation

After the epicardium has been formed, a whole array of interacting signaling pathways drive (1) epicardial epithelial to mesenchymal transition (EMT) and formation of EPDCs, (2) differentiation of EPDCs into different cell lineages, (3) induction of compact zone growth, as well as (4) establishment of coronary vessel formation (Fig. 3B,C). Once the epithelial cell sheet of the epicardium is formed, directed proliferation parallel or perpendicular to the cellular basement membrane leads to the expansion of the epicardium in the former case or the induction of EMT and subsequent migration and differentiation of EPDCs in the latter. Wt1 signaling is a key factor for normal progression of epicardial EMT as well as EPDC differentiation and myocardial growth (Martinez-Estrada et al. 2010; von Gise et al. 2011; Lim and Thiery 2012).

EPDC differentiation and myocardial compact zone growth depend on a network of recipro-
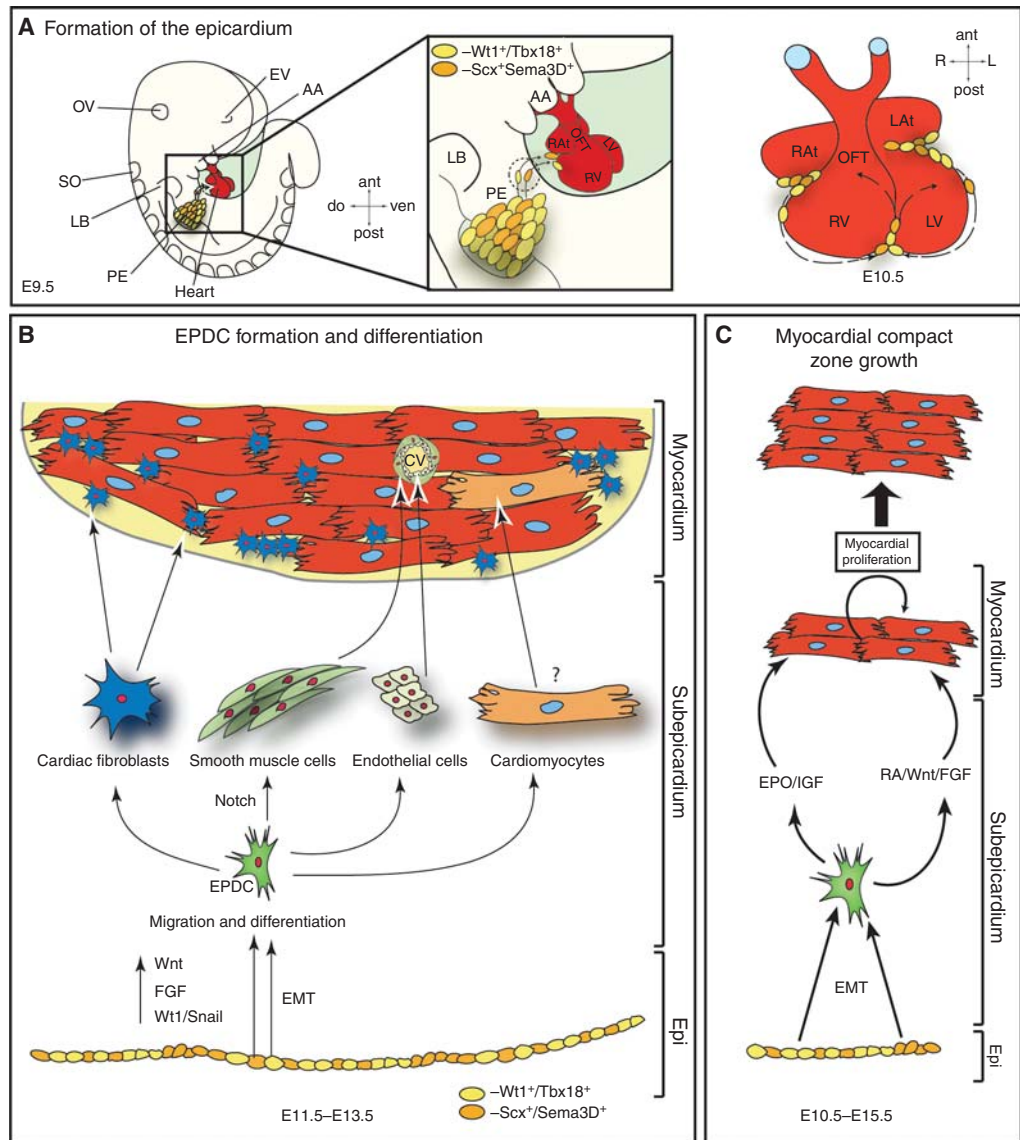
Figure 3. Origin and fates of the proepicardium. (*A*) Formation of the epicardium. Starting around E9.5 of mouse development, freely floating proepicardial cell vesicles attach to the naked myocardium, starting in the atrioventricular canal region. The whole heart will be covered by an epicardial epithelial sheet by E11.5. (*B*) Formation of epicardium-derived cells (EPDCs). At E11.5–13.5, epicardial cells undergo an epithelial-mesenchymal transition (EMT) (mediated by Wnt, FGF, and Wt1/Snail signals) and EPDCs emerge in the subepicardial space. EPDCs then differentiate toward various cell types, as indicated, and contribute to the coronary vasculature (cv) and cardiac fibroblast. EPDC differentiation toward the myocytic lineage is still under thorough investigation. (*C*) Epicardium/EPDCs are secreting important factors that induce myocardial proliferation and thereby myocardial compact zone growth. AA, Aortic arches; ant, anterior; cv, coronary vessels; do, dorsal; EMT, epithelial to mesenchymal transition; EPDC, epicardial-derived cells; Epi, epicardium; EPO, erythropoietin; EV, eye vesicle; IGF, insulin growth factor; L, left; LAt, left atrium; LB, limb bud; LV, left ventricle; OFT, outflow tract; OV, otic vesicle; PE, proepicardium; post, posterior; R, right; RA, retinoic acid; RAt, right atrium; RV, right ventricle; SO, somites; ven. ventral.

cal epicardium-myocardium signaling of largely unknown nature (Fig. 3C) (Perez-Pomares and de la Pompa 2011). There is still extensive discussion about the potential contribution of EPDCs to different cell lineages (Gittenberger-de Groot et al. 2012). Although it is consensus in the field that EPDCs can differentiate toward a cardiac fibroblast and vascular smooth muscle cell fate, formation of coronary endothelial cells and cardiomyocytes by EPDCs is less clear (Fig. 3B). Tabin and colleagues have shown for the first time that the mammalian epicardium consists of distinct cell populations that can give rise to endothelial cells (Katz et al. 2012). The vast majority of cardiomyocytes, on the other hand, seem to be derived from other sources (Gittenberger-de Groot et al. 2012). Signaling molecules with a prominent role in EPDC differentiation and compact zone growth are FGFs, Notch and retinoic acid (Sucov et al. 2009). Two different RA signaling networks have been proposed. One study showed that an RA stimulus is necessary to induce hepatic erythropoietin (Epo) production. Epo in turn induces epicardial IGF-2 secretion, which then acts as a proliferative factor on compact zone myocytes (Brade et al. 2011; Li et al. 2011). Other studies placed Wt1 upstream of epicardial Raldh2 (a key molecule in RA metabolism) expression (Guadix et al. 2011). RA signaling in turn has been described earlier to spearhead a signaling network that includes cross talk with Wnt and FGF signaling molecules (Fig. 3C) (Merki et al. 2005; Lavine et al. 2006; Zamora et al. 2007).

Formation of the coronary vessels is tightly linked to compact zone growth and relies on overlapping signaling molecules (Olivey and Svensson 2010; Perez-Pomares and de la Pompa 2011). Mesenchymal cells in the subepicardial space and in the myocardial wall (most of them if not all are EPDCs) will coalesce and differentiate, thereby forming the primitive coronary plexus around E11.5 during mouse development (Reese et al. 2002). Primary coronary vessels spread over the entire ventricle until E13.5; afterward, intricate remodeling and arterial and venous differentiation takes place. Important signaling molecules driving these processes include the aforementioned FGFs and

RA signaling as well as ligands belonging to the Hedgehog and VEGF families and the TGF-β superfamily (Lavine and Ornitz 2008; Olivey and Svensson 2010; Perez-Pomares and de la Pompa 2011). Moreover, epicardial Notch signaling is required for vSMC differentiation, whereas it is dispensable for EMT or migration and myocardial growth (del Monte et al. 2011; Grieskamp et al. 2011).

CARDIAC NEURAL CREST PROGENITORS

The third distinct embryonic heart progenitor population that is involved in cardiogenesis consists of the CNCCs. CNCCs comprise a noncardiac cell type important for proper patterning of the aortic arteries, for OFT development and septation, and normal myocardial function. Although CNCCs are differentiating into ectomesenchyme and aortic smooth muscle cells as well as forming the aorticopulmonary septum, which divides the arterial outlet of the heart, it seems that the main role of CNCCs in the abovementioned developmental processes is to provide signals rather than actively contribute differentiated cells to the specific cardiac structures. That said, there is a direct pivotal cellular contribution of the crest to the heart valves and they provide all parasympathetic innervation of the heart (Fig. 1C).

Signaling Pathways Governing Cardiac Neural Crest Cell Induction and Migration

CNCCs are a subpopulation of the cranial neural crest cells and arise from the dorsal neural tube between the mid-otic placode and the posterior border of somite 3 (Fig. 4A) (Keyte and Hutson 2012). The crest cells delaminate from the neural tube and migrate on preset routes toward the heart, reaching the pharyngeal arches 3, 4, and 6 (Fig. 4B). Signals important for the induction of the CNC progenitors belong to the BMP/TGF-β superfamily of growth factors as well as to the FGF, Wnt/β-catenin, and retinoic acid signaling pathways. These different families of secreted molecules control key aspects of neural crest biology, starting from the establishment of the neural plate border up to the cytosekeletal
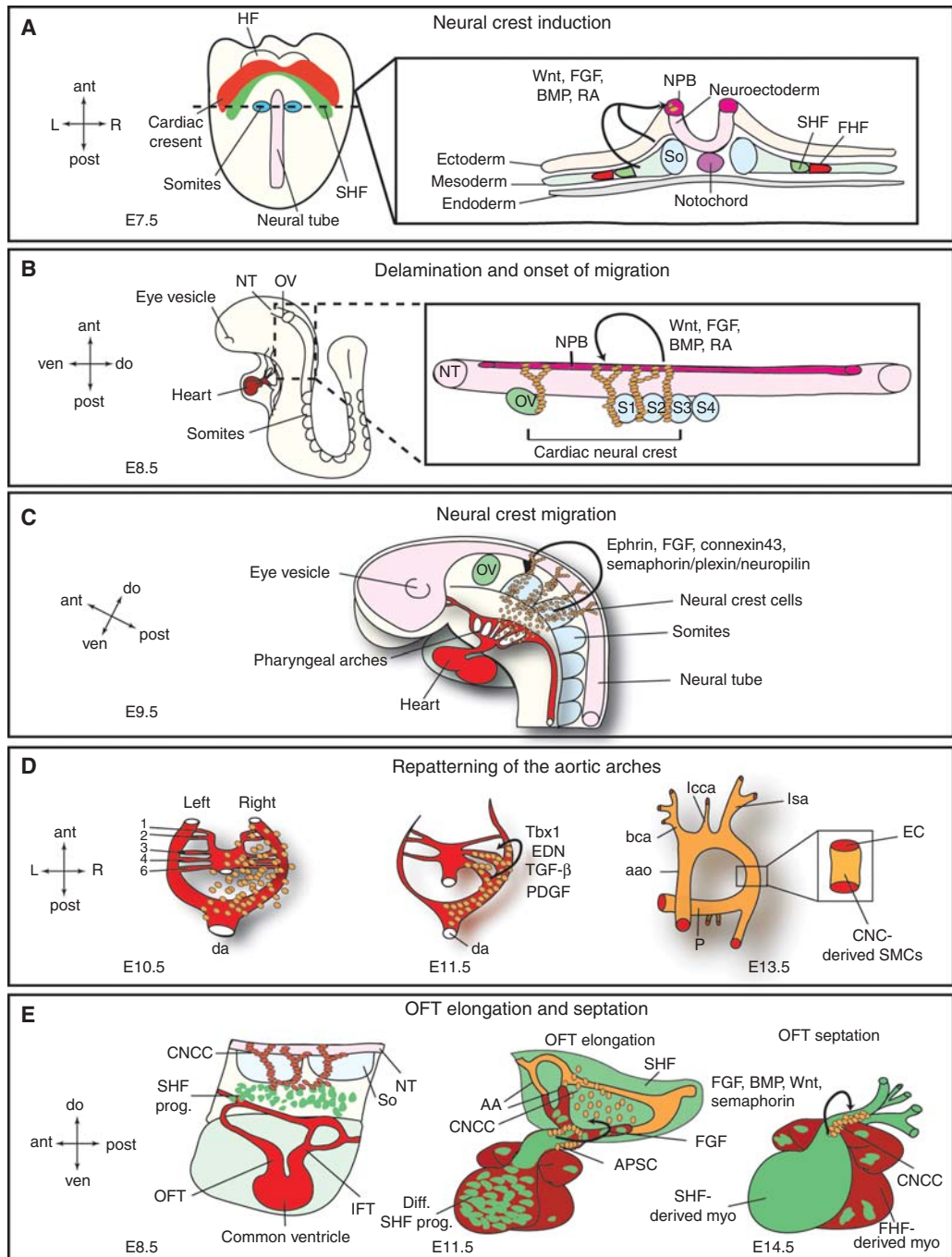
Figure 4. Cardiac neural crest. (*A*) Neural crest induction. Illustrated is a ventral view of an E7.5 embryo and a transverse section through the first somite pair. The section detail shows the different germ layers and factors originating in the ectoderm and mesoderm that are required for neural crest cell induction in the neural plate border (NPB). Ant, anterior; post, posterior; R, right; L, left; HF, headfolds; FHF, first heart field; SHF, second heart field; So, somite. (*B*) Delamination and onset of migration of cardiac neural crest cells. (*Legend continues on facing page.*)

rearrangements required for delamination of neural crest cells from the neural tube and onset of migration (Fig. 4A,B) (Scholl and Kirby 2009).

After induction and delamination the crest cells migrate along specific routes guided by chemical attractant and repellent cues toward the heart (Fig. 4C). Important molecules for this process belong to the ephrin family of ligands and receptors that ensure specific migration patterns alongside FGF signaling molecules, semaphorins and connexin 43 (Kuriyama and Mayor 2008). Semaphorin/plexin/neuropilin signaling is suggested as a targeting cue to the OFT cushions and Cx43 seems to facilitate proper migration and survival. Intricate interactions along their path between the pharyngeal ectoderm and endoderm and continuous proliferation ensure that the CNC progenitors reach their target areas in the heart in sufficient numbers (Xu et al. 2006; Keyte and Hutson 2012).

Signaling Pathways Instructing CNC Cells during Aortic Arch Artery Patterning and OFT Septation

Once the crest cells reached the pharyngeal arches, endothelin, TGF-β, and PDGF signaling pathways are crucial for the CNCCs to exert their role in patterning the aortic arch arteries (Fig. 4D). Endothelin (END) signals are important for the maintenance of the instructive cues of CNCCs during this process and, although downstream effectors are not well understood yet, Hand2 seems to process endothelin signals in the aortic arches. TGF-β signals are thought to provide important antiapoptotic effects, and PDGF signals ensure proliferation and maintenance (Hutson and Kirby 2007). T-box transcription factors (namely, Tbx1) also play a key role in neural crest biology. Although not expressed in CNCCs themselves, Tbx1 regulates the expression of the guidance molecule Slit in the pharyngeal endoderm. Loss of Slit function has been shown to prevent CNCC migration into aortic arch 4 and thereby leading to interrupted aortic arch. Besides that, Tbx1 also interacts with FGF-8, whose levels need to be regulated in a very confined manner for CNCC survival as well as for timing and targeting of the migrating progenitors to the pharynx (Abu-Issa et al. 2002; Sato et al. 2011).

Additional to their role in aortic arch patterning CNCCs exert another important function in OFT development and septation (Fig. 4E). One of the earliest detectable defects after CNCC ablation is defective looping because of a shortened OFT. This phenotype is caused by altered SHF progenitor addition to the developing OFT. In CNCC ablated chick embryos altered FGF-8 levels keep the SHF progenitors in a proliferative state, precluding proper migration and

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Figure 4. (*Continued*) At E8.5, cardiac neural crest cells delaminate from the NPB between the mid-otic vesicle (ov) and the posterior border of somite 3 (S3) and start on their migratory path toward the heart. These processes are mediated by Wnt, FGF, BMP, and RA signals. Please see text for more details. ant, anterior; post, posterior; ven, ventral; do, dorsal; S1,2,3,4, somite 1,2,3,4. (*C*) Neural crest migration. By E9.5, the CNCCs reach the aortic arches and are proceeding toward the OFT. Signals targeting the CNCCs to the aortic arches are indicated. ov, otic vesicle. (*D*) The CNCCs are vital for the repatterning of the aortic arches. There are initially six symmetrical arteries attached to the parallel dorsal aorta (da). Sequentially these arteries are remodeled and give rise to a separate ascending aorta (aao) and pulmonary trunk (P) with two pulmonary arteries attached. Additionally, the cardiac neural crest cells are differentiating into aortic smooth muscle cells and envelope the nascent endothelial cells forming the aortic arches. Required factors for both processes are indicated (END, endothelin); see text for details. bca, Brachiochepalic artery; lcca, left common carotid artery; lsa, left subclavian artery. (*E*) Interaction of CNCCs and SHF progenitors leads to outflow tract (OFT) elongation and septation. At E11.5, cardiac neural crest cells condense and form the U-shaped aorticopulmonary septation complex (APSC); see text for details. CNCC, Cardiac neural crest cells; SHF prog., second heart field progenitors; NT, neural tube; So, somites; IFT, inflow tract; diff. SHF prog., differentiated second heart field progenitors; AA, aortic arches; FHF derived myo., first heart field-derived myocardium; SHF derived myo, second heart field-derived myocardium.

eventually initiation of myocardial differentiation (Waldo et al. 2005; Ilagan et al. 2006; Scholl and Kirby 2009). Furthermore, upon CNCC ablation myocardial function is deranged, which is indicated by disrupted Ca^{2+} handling and depressed ejection fraction (Hutson et al. 2006). Further down the developmental road, CNCCs govern OFT septation. In the avian model system, the CNCCs reach the OFT cushions subendocardially and submyocardially where they form together with mesenchymal cells the so-called aorticopulmonary septation complex, which will initiate the division of the systemic and pulmonary blood flow (Keyte and Hutson 2012). Multiple signaling pathways are implicated to instruct CNCCs during this process, the major players being BMP, Wnt, and semaphorin signaling (Fig. 4E). The cellular contribution of the CNCCs to the cardiac conduction system and the cardiac valves remains an ongoing debate but development of both structures is obscured upon loss of neural crest function, indicating that at least signals from CNCCs are required for their normal development (Hutson and Kirby 2007).

EMBRYONIC CARDIAC PROGENITORS AND CONGENITAL HEART DISEASE

Congenital heart disease (CHD) is one of the most prevalent forms of human birth defects, affecting 0.4%–5% of live births, depending on which level of severity of CHD is included in the statistics. Moreover, around 30% of prenatal loss is owing to heart malformations (Hoffman 1995; Bruneau 2008). CHD most commonly arises from defective cardiac morphogenesis. In the last few decades, with our growing knowledge of normal cardiac development and of cardiac precursor origin and regulation, the role of the different embryonic cardiac progenitor populations (cardiac mesoderm progenitors, epicardial progenitors, and CNCCs) in the etiology of CHD has begun to gain increasing interest. Although disruption of embryonic cardiac progenitor biology (specification, proliferation, migration, lineage-specific differentiation) has not yet been assigned to be causative for any human CHD, identification of transcriptional programs

that are impaired in CHD patients and control key aspects of cardiac progenitor lineage diversification and fate strongly suggests compromised cardiac progenitor biology at the root of at least some human CHDs. Mutations in Nkx2.5 (e.g., atrial septal defects [ASD] and tetralogy of Fallot), Tbx5 (e.g., Holt-Oram syndrome, ASD, and ventricular septal defects [VSD]), and GATA-4 (e.g., ASD and VSD) point to cardiogenic mesoderm progenitor involvement, whereas mutations in the Tbx1 gene (DiGeorge syndrome, 22q11 deletions) implicates CNCCs. The involvement of epicardial progenitors in CHD is not as straightforward, but a role in hypoplastic left heart syndrome has been suggested along with contributions to the left ventricular noncompaction phenotype and valve malformations (Bruneau 2008; Musunuru et al. 2010; Gittenberger-de Groot et al. 2012; Keyte and Hutson 2012).

The possibility of derailed embryonic cardiac progenitors contributing to the developing heart as a causative factor for human CHD enables intriguing explanations for some unresolved questions in CHD etiology. It is, for example, unclear why most CHDs are polygenic and have variable penetrance and disease severity even within one family (Fahed et al. 2013). Given the complex networks of cytokines, transcription factors as well as physical stimuli (e.g., cell–cell interactions, hemodynamic flow, cell migration) that are regulating cardiac progenitor development, it becomes feasible that a plethora of variables dictates the phenotypic characteristics of a certain CHD, which undoubtedly can vary even between siblings. Another puzzling question is how the same morphological phenotype is caused by seemingly unrelated genetic causes (Lage et al. 2012). This could be explained if we consider progenitor development gone awry as a basis for the cardiac malformation at hand.

CARDIAC STEM AND PROGENITOR CELLS IN REGENERATIVE MEDICINE

Ischemic heart disease and heart failure are the leading causes of death in the western world. Even if patients survive an ischemic event such

as acute myocardial infarction (MI) their prognosis can be very poor. Diminished cardiac function owing to a massive loss of cardiomyocytes induces hypertrophic remodeling and eventually heart failure. Novel therapeutic avenues aiming at disrupting this vicious circle by renewal of lost cardiac muscle are greatly needed to improve our means to manage ischemic heart disease. Unfortunately, the regenerative capacity of the heart is extremely limited. Until recently, it was believed that the mammalian heart cannot regenerate at all owing to the inability of terminally differentiated cardiomyocytes to reenter the cell cycle and proliferate. Studies of the last few years have now provided evidence for cardiomyocyte turnover in the adult human heart. Although this process occurs at a low rate during normal homeostasis and aging of the heart, it seems to be elevated after injury (Bergmann et al. 2009; Parmacek and Epstein 2009; Kajstura et al. 2010; Senyo et al. 2013). It has been shown that mice can regenerate lost heart muscle within their first week of life, similarly to adult zebrafish, and that most of the new cardiomyocytes are mainly derived by cell division from preexisting cardiomyocytes (Jopling et al. 2010; Kikuchi et al. 2010; Porrello et al. 2011). Recently, a role of the epicardium and EPDCs in cardiac regeneration has been suggested. The epicardium seems to contribute mainly paracrine factors for myocardial proliferation and neovascularization as well as nonmyocytic lineages, therefore recapitulating embryonic development (Perez-Pomares and de la Pompa 2011; Kikuchi and Poss 2012).

Alternative options for improving cardiac function after MI arise from the purification of cardiac progenitors or cardiomyocytes differentiated from human embryonic or induced pluripotent stem cells (Burridge et al. 2012; Aguirre et al. 2013; Chien and Mummery 2013; Mummery 2013) and from direct in vivo reprogramming approaches. In recent years, a plethora of differentiation protocols driving hPSCs toward a cardiogenic fate have been established (Burridge et al. 2012; Mummery et al. 2012). However, clinical applications of hPSC-derived cells have so far been hampered by safety issues and low engraftment after injection in the injured heart (Aguirre et al. 2013). An exciting hope in the field of cardiac repair has come from the recent success in direct in vivo reprogramming of cardiac fibroblasts into cardiomyocytes using overexpression of Tbx5, Mef2c, and Gata4 in combination with Hand2 or only using miRNAs (miRs-1/133/208/499) (Song et al. 2010; Jayawardena et al. 2012; Qian et al. 2012). These studies have shown improvements of cardiac function after direct reprogramming in experimental rodent models of MI, suggesting the potential of this new technology as a viable strategy for cardiac regeneration.

Although a lot of problems still need to be tackled, activation of endogenous regenerative processes, along with stem cell-based technologies, is holding great promise for possible future clinical applications. In the past 20 years we improved the path to be traveled to reach this goal and further efforts of many scientists and clinicians around the world will provide a silver lining in the battle against the world's biggest life threat (see also Chien and Mummery 2013 and Mummery 2013 for more details).

CONCLUDING REMARKS

The different embryonic heart progenitor lineages all rely on a great variety of spatiotemporal signals that tightly regulate their terminal differentiation. But it is noteworthy that recurring genetic regulatory networks are now emerging. For induction/specification of progenitor populations EMT is indispensable. Here factors of the TGF-β superfamily play a vital role. Once the multipotent progenitor cell state is reached, this population needs to proliferate and maintain an undifferentiated state to ensure enough terminally differentiated cells for the developing organ. Here canonical Wnt and FGF signaling factor as well as RA are used, among others. Differentiation toward specialized cell types is then achieved by BMP growth factors (myocardium) and Notch signaling (vascular cell types). These genetic loops are fine-tuned by transcription factors and noncoding RNAs, gradually restricting the differentiation potential of the specific lineage. To transfer our knowledge of cardiac development to the bedside, the field faces new chal-

lenges within the coming years. One task would be to decipher definitive molecular signatures that mark cardiac precursors in a spatiotemporally defined manner and enables researchers to specifically isolate and analyze distinct progenitor populations in greater detail. Although we have elucidated some marker genes for embryonic heart progenitors, the segregation of distinct progenitor populations with defined differentiation potential remains incomplete, and known marker genes are so far not exclusive for a given population. Additionally, molecular signatures comprised by cell surface molecules rather than transcription factors would greatly help to better analyze precursor biology in vitro, which would give us tools at hand to further delineate factors needed for survival and differentiation. Great progress has been made in our understanding of the embryonic development of the heart. But so far we have only understood the broad concept of induction, proliferation/maintenance, and differentiation. It will require further efforts in filling the gaps to fully comprehend complex etiologies of human CHDs and to modulate embryonic developmental processes in vitro and in vivo to restore myocardial function in ischemic heart diseases.

ACKNOWLEDGMENTS

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