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Probing Early Heart Development to Instruct Stem Cell Differentiation Strategies

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Abstract

Scientists have studied organs and their development for centuries, and along that path described models and mechanisms explaining the developmental principles of organogenesis. In particular, with respect to the heart, new fundamental discoveries are reported continuously, that keep changing the way we think about early cardiac development. These discoveries are driven by the need to answer long-standing questions regarding the origin of the earliest cells specified to the cardiac lineage, the differentiation potential of distinct cardiac progenitor cells and, importantly, the molecular mechanisms underlying these specification events. As evidenced by numerous examples the wealth of developmental knowledge collected over the years has had an invaluable impact on establishing efficient strategies to generate cardiovascular cell types *ex vivo*, from either pluripotent stem cells or via direct reprogramming approaches. The ability to generate functional cardiovascular cells in an efficient and reliable manner will contribute to therapeutic strategies aimed at alleviating the increasing burden of cardiovascular disease and morbidity. Here we will discuss the recent discoveries in the field of cardiac progenitor biology and their translation to the pluripotent stem cell model to illustrate how developmental concepts have instructed regenerative model systems in the past and promise to do so in the future.

Keywords

gastrulation; cell fate specification; cardiac; mesoderm; pluripotent stem cell differentiation; epicardium; cardiac maturation

INTRODUCTION

Our understanding of how the differentiated four-chambered heart develops has been revolutionized with data from the past 15 years that reveal a greater complexity of the early

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stages of heart development than had been previously anticipated (Vincent, Buckingham 2010, Meilhac et al. 2015). The major recent discoveries consist of the description of progenitor populations with distinct contributions to the heart, the elucidation of the clonal potential of individual progenitors as the heart develops, the examination of the underlying molecular mechanisms of cell fate specification, and lastly, the more formal demonstrations that specification to the cardiovascular lineages occurs very early during development.

Aside from the fascination with investigating concepts of development and organogenesis, in-depth comprehension of how the heart develops has the potential to contribute to therapeutically relevant strategies in several ways. First, cardiac development studies have opened the door to a better understanding of congenital heart defects (Bruneau 2008), which remain the most frequent congenital defects and are a large contributor to adult heart complications in the human population. Second, pluripotent stem cell (PSC) differentiation approaches, as well as direct reprogramming strategies, are largely based on developmental concepts that provide information about the critical regulatory mechanisms required to form cardiac cells and structures. Lastly, regenerative approaches in the adult heart are often instructed by the properties and mechanisms of the developing cardiac lineages (Xin, Olson & Bassel-Duby 2013). Since the human adult heart is one of the least regenerative organs in our body with a very limited capacity to renew cardiac tissue after injury, addressing the pressing burden of heart disease in patients will rely on readily available sources of cells that are able to increase the performance of the heart. With this objective, many cell types have been investigated in the past for their potential to improve the function of the heart after injury, including mesenchymal stem cells, hematopoietic cells, and myoblast cells. While these approaches have yielded various extents of functional improvement, most of the observed effects were reported to be transient, and primarily based on paracrine effects rather than an integration of new cells into the heart. PSC differentiation and direct reprogramming have emerged as promising technologies to address these problems; however, both approaches have hurdles to overcome before being beneficial to heart patients on a larger scale. While we will focus our discussion on the PSC differentiation system here, direct reprogramming, as well as regenerative approaches in the adult myocardium, are intriguing alternatives for generating new heart tissue, and these topics have been comprehensively reviewed in several reports (Qian, Srivastava 2013, Nam, Song & Olson 2013, Foglia, Poss 2016, Xiao et al. 2016).

The main challenges of the PSC system as of now include the generation, expansion, and maintenance of progenitor populations, the specification of distinct sub-types of cardiovascular cells and the maturation of cells in culture. We propose that answers to many of these questions can be found by understanding these processes *in vivo*, during normal cardiac development. Therefore, continuing to extend our knowledge of cardiac development will likely improve many of the current approaches in direct reprogramming and differentiation of PSCs into relevant cell types for therapy. In the present review, we will discuss how developmental concepts have instructed PSC differentiation strategies in the past, and how they may help to address the current challenges still remaining.

The pluripotent stem cell differentiation system: a brief overview of the accomplishments and current challenges

The potential of pluripotent stem cells (PSCs) to give rise to any cell type in our body has long been appreciated, but it has taken a collective effort from many laboratories to advance this model system to where it stands today. Within just a few years, the generation of human PSC-derived cardiomyocytes (hPSC-CMs) has evolved from the initial inefficient serum-based, proof-of-principle differentiation protocols to today's robust strategies that yield nearly pure cardiomyocyte cultures (Kehat et al. 2001, Yang et al. 2008, Kattman et al. 2011, Lian et al. 2013, Burridge et al. 2014, Karakikes et al. 2014, Aguilar et al. 2015, Hwang et al. 2015). Importantly, the critical advancements enabling the progress were based on the concept of reproducing development in culture by guiding cells along their natural developmental path in a time and signaling-dependent manner.

A thorough evaluation of the PSC-CM population has revealed that these cell populations are heterogeneous and immature in nature. Electrophysiological assessment suggests that the hPSC-CMs produced via current protocols consist of mixed populations of different cardiomyocyte subtypes (atrial, ventricular and nodal-like cells) (Maltsev et al. 1993) and that their characteristics remain similar to those of fetal cardiomyocytes. This is further supported by transcriptional comparison and functional assays illustrating that Ca2+-handling properties of PSC-CMs resemble those of human fetal cardiomyocytes (van den Berg et al. 2015) (16–18 weeks of gestation) and significantly differ from those of adult cardiomyocytes (Liu et al. 2009, Liu et al. 2007, Satin et al. 2008). Despite many efforts to refine current protocols, it is not fully understood how functionally mature cells of the four-chambered heart can be generated from PSCs. Overcoming these roadblocks will be critical for cell transplantation studies, where transplanting mixed PSC-derived cultures can lead to arrhythmias (Chong et al. 2014), as well as for disease modeling approaches from induced pluripotent stem cells (iPSCs), where cardiac phenotypes that manifest later in life may not be recapitulated in immature cells.

Generating cardiac progenitor populations from PSCs

One promising approach to address lineage specification from PSCs consists of investigating the origin of individual lineages and therefore their respective progenitor populations. This is of particular relevance in the light of the many recent discoveries in the cardiac progenitor field that provide a better understanding of the earliest stages of heart development. Progenitor populations that contribute to the developing heart over time consist of the cardiac mesoderm population (shortly after gastrulation, contributes to all cells of the heart), the first and second heart field cells (FHF/SHF, at the cardiac crescent stage; FHF: left ventricle, part of right ventricle, majority of both atria; SHF: most of right ventricle, part of both atria, outflow tract), the proepicardial organ (PEO, early heart tube stage, contributes to the epicardium and epicardium-derived cells), and the neural crest cells (NCC, early heart tube, contribute to valves and smooth muscle cells of the outflow tract). Identifying and generating the different cardiovascular progenitor populations *in vitro* promises the possibility of investigating the signaling pathways and molecular mechanisms at play during their specification to a defined cardiovascular lineage, as well as towards generating pure populations of the different cell types of the heart.

Mesoderm progenitor cells—*Mesp1* is a pivotal transcription factor broadly expressed in lateral plate mesoderm, from which the cardiac mesoderm and subsequently the majority of cardiovascular cells arise during development (Devine et al. 2014, Saga et al. 1999, Bondue et al. 2008, Lescroart et al. 2014). As such, it has been a key target in many past and present efforts at understanding the earliest cardiovascular lineage specification events. For instance, the first prospective labeling of cardiac precursors using genetic markers was conducted using Mesp1-driven lineage tracing (Saga et al. 1999). Expression of Mesp1, as determined by *Mesp1-lacZ*, was restricted to the primitive streak (PS) region and the newly formed mesoderm, and *Mesp1-Cre* lineage tracing labeled regions including the craniocardiac mesoderm. Fifteen years after that initial discovery, two independent studies further explored the specification and contribution of individual Mesp1 progenitor cells and concluded that they are temporally restricted during gastrulation to either the FHF or SHF lineage (Lescroart et al. 2014, Devine et al. 2014). While Eomes has been shown to directly induce Mesp1 expression in the presumptive cardiac mesoderm, the wide range of mesendodermal tissues derived from Eomes-expressing cells suggests that it does not act as an exclusive cardiac regulator (Costello et al. 2011). Given the early specification of Mesp1+ cells, they are likely not yet fully determined, which is supported by the observation that the visceral endoderm is necessary for the formation of beating foci from cultured mesoderm explants (Arai, Yamamoto & Toyama 1997).

These findings have greatly advanced our knowledge of the differentiation potential of mesoderm precursor cells and have provided the fundamental tools needed to explore downstream specification events. Work in the mESC system has greatly expanded on this knowledge and on the action of Mesp1 at the molecular level and has uncovered that Mesp1 induces expression of cardiac transcription factors while repressing positive regulators of other cell fates (Bondue et al. 2008, Bondue et al. 2011). In the human PSC system, MESP1+ cells have been isolated and characterized during differentiation using a dual *MESP1mCherry/NKX2-5eGFP* reporter line. Their derivatives consist of a population enriched for NKX2-5 and Troponin T expressing cells, indicating a preferential differentiation to cardiomyocytes. A small fraction of smooth muscle and endothelial cells was also obtained, consistent with data in the mouse demonstrating that Mesp1+ cells do contribute to these lineages (Den Hartogh et al. 2015, Saga et al. 1999).

Cardiac mesoderm progenitor cells—During and shortly after gastrulation, cardiac mesoderm populations are characterized by specific gene and cell-surface protein expression. The expression domains of Pdgfra, Kdr (also known as Vegfr2 or Flk1) (Yamaguchi et al. 1993), and cKit have been demonstrated to distinguish mesodermal subpopulations in the mouse. Kdr is one of the earliest common mesodermal differentiation markers for vascular endothelial and hematopoietic cells, and Pdgfra is expressed in most of the mesodermal cells of the embryo; however, only co-expression of Kdr and Pdgfra characterizes a mesoderm population specified to the cardiac lineage (Kataoka et al. 1997). Pdgfra+Kdr+ cells sorted from the mouse embryo or from mouse PSC differentiation cultures efficiently differentiate to cardiovascular cells, further supporting their commitment to the cardiac lineage (Kataoka et al. 1997, Kattman, Huber & Keller 2006, Kattman et al. 2011). This strategy was readily translated to human PSC differentiations, where KDR and

PDGFRA expression defines the cardiac mesoderm, which is the population that efficiently differentiates to cells of the cardiovascular lineage (Kattman et al. 2011, Yang et al. 2008). KDR+/PDGFRA+ PSC-derived cells are enriched in ISL1 expression and are multipotent, as evidenced by their tri-lineage differentiation potential to cardiomyocytes, endothelial, and vascular smooth muscle cells (Kattman et al. 2011). Recent studies have taken advantage of the ability to generate defined and developmentally early cardiac cell populations *in vitro* to study the epigenetic regulation of cardiac specification and have resulted in detailed gene regulatory networks for cardiovascular lineage commitment (Paige et al. 2012, Wamstad et al. 2012). These studies represent elegant examples that illustrate the power of having access to early cardiac populations in sufficient quantities to study the detailed molecular mechanisms of heart development.

The receptor tyrosine kinase-like orphan receptor family (ROR2) and aminopeptidase-N (CD13) were identified as additional cell surface markers that enrich for mesodermal progenitors (Drukker et al. 2012) and that increase the temporal resolution of lineage-committed precursors that emerge during hPSC cardiac differentiation. CD13 and ROR2 are expressed on MESP1+ cells, suggesting that the combination of these markers characterizes an early mesoderm population that likely precedes the KDR+PDGFRA+ cardiac mesoderm (Den Hartogh et al. 2015). When transplanted into the pig heart, CD13+ROR2+ cells differentiate to cardiomyocytes, fibroblasts, smooth muscle cells, and endothelial cells, thus possibly representing a cardiovascular progenitor population with multi-lineage differentiation potential (Skelton et al. 2016). Given their multi-lineage differentiation potential, early cardiovascular progenitor populations hold great promise as a potential cell source for regenerative cell replacement approaches, as supported by the successful transplantation of CD13+ROR2+ cells into large animals (Skelton et al. 2016).

ISLET1/NKX2-5 progenitor cells—After cardiac mesoderm cells exit the PS, they migrate to the anterior side of the embryo and organize into a structure called the cardiac crescent at E8.0 (Buckingham, Meilhac & Zaffran 2005, Camp, Munsterberg 2011, Zhen et al. 2012). In the last decade, work in multiple model systems has greatly advanced our knowledge of progenitor cells within the cardiac crescent, leading to a revision of the simple linear tube model and the definition of the first and second heart fields (FHF/SHF). Based on this model, the FHF and SHF cells give rise to the majority of the heart myocardium, with the left ventricle, most of the atria, and part of the right ventricle originating from the FHF, and SHF giving rise to the right ventricle, outflow tract, and part of the atria (Figure 1a) (Kelly, Brown & Buckingham 2001, Cai et al. 2003, Zaffran et al. 2004, Yamagishi et al. 2001, Srivastava et al. 1997, Hami et al. 2011, Yuan, Schoenwolf 2000).

The FHF cells are known to specifically express *Tbx5* and *Hcn4*, and lineage tracing using an *Hcn4-Cre* driver demonstrates specific contribution to the left ventricle (Bruneau et al. 2001, Spater et al. 2013, Liang et al. 2013). The SHF expresses high levels of Isl1, which has been used extensively for lineage tracing and analyses of SHF derivatives in multiple model systems (Cai et al. 2003, Yuan, Schoenwolf 2000, Hami et al. 2011). This data was further reinforced through the observation of an enhancer region of the *Mef2c* gene that is active in the SHF and can be induced by Isl1 (Dodou et al. 2004, Verzi et al. 2005). However, lineage tracing with *Isl1-Cre* and a more sensitive reporter system suggested a

wider expression of Isl1, possibly including FHF derivatives (Ma, Zhou & Pu 2008). Thus, it is possible that some of the molecular differences observed between the heart fields are a result of the limited spatiotemporal resolution inherent to embryological studies, and that the heart fields reflect transient steps during the transition from precursors specified during gastrulation to the differentiating cells of the heart tube. For these reasons, when attempting to recapitulate the developmental steps to generate FHF and SHF cells in culture, it is rather unlikely that they will serve as distinct progenitor populations but should rather be viewed as sequentially occurring populations during cardiac differentiation.

Consistent with this idea, aside from being a marker for SHF cells at the cardiac crescent stage, it is well known that Isl1 is highly expressed in proliferative cells and that it is downregulated as cardiac progenitor cells enter the heart tube and differentiate (Cai et al. 2003), suggesting that Isl1 regulates proliferation, expansion, and migration of cardiovascular precursors. More recent work demonstrates that Nkx2-5 directly binds to an *Isl1* enhancer and represses *Isl1* transcriptional activity (Dorn et al. 2015), suggesting a sequential requirement for these factors during progenitor expansion and differentiation. At the time of migration into the heart tube, a subset of cells gains expression of the atypical homeodomain protein Hopx, which commits the cells to the cardiomyoblast fate through the integration of BMP and Wnt signaling (Jain et al. 2015). Hopx+ cells expand briefly before differentiating to the cardiomyocyte fate and taking up residence in the myocardium. Due to their promising duality as lineage committed yet progenitor-like and proliferative cells, efforts in the PSC field have attempted to reproduce these intermediate populations in culture.

In relation to this goal, one of the first human PSC reporter cell lines generated was the NKX2-5-GFP line (Elliott et al. 2011). This reporter line has been instrumental for advancing the field and has supported the establishment of novel tools, differentiation strategies and cardiac physiological studies alike (Elliott et al. 2011, Skelton et al. 2014, Dubois et al. 2011, Kempf et al. 2014, Birket et al. 2013). Furthermore, it has facilitated the isolation and characterization of NKX2-5+ progenitor cells, as well as more differentiated cardiomyocytes from PSC differentiations, which represented the first opportunity to study PSC-derived human cardiomyocytes in appropriate detail.

In order to specifically generate SHF progenitor cells, an Isl1-nlacZ knock-in mES cell line was used to successfully isolate and expand Isl1+ cardiac precursors *in vitro*, and their clonal analysis revealed the multipotent capacity of these cells (Moretti et al. 2006). A similar population was isolated using an ISL1-βgeo bacterial artificial chromosome (BAC) or a lineage tracing hESC line. These ISL1+ cardiovascular progenitors give rise to cardiomyocytes, smooth muscle, and endothelial cell lineages *in vitro*, as expected (Bu et al. 2009).

Information from developmental model systems continues to highlight the importance of correctly establishing the early progenitor populations for proper heart development. As we have discussed here, several seminal studies have demonstrated that the various progenitor populations identified *in vitro* can be generated and isolated during PSC differentiation in culture, promising to help improve differentiation protocols for the generation of distinct

cardiovascular cell types. With respect to their clinical potential, expansion of the transiently occurring progenitor populations without affecting their differentiation potential has been the subject of intense study in the field.

Cardiac progenitor proliferation—Aside from generating pure and functional cell populations from PSCs, a parallel challenge consists of generating large numbers of cells for future cell replacement strategies. Differentiated cardiomyocytes from PSCs, similar to their counterparts in the heart, display very limited proliferative capacity. Therefore, proliferative progenitor cells that are lineage-committed represent an ideal target population for such strategies. Contrary to progenitor populations from the endoderm and ectoderm lineages (Cheng et al. 2012, Ziegler et al. 2014), protocols to expand defined cardiac progenitor cells have only been proposed very recently, and are largely based on signaling pathways identified to be relevant for cardiac progenitor proliferation *in vivo* (Zhang et al. 2016, Lalit et al. 2016, Birket et al. 2015, Bu et al. 2009).

In animal models, the Wnt/β-catenin signaling pathway is broadly involved in early cardiac development and growth (Bejsovec 2005, Clevers 2006, Tzahor 2007). In murine and human embryonic development, Wnt has highly stage-specific effects on mesoderm and cardiac progenitor specification and expansion. Wnt signaling is required for primitive streak formation (Haegel et al. 1995, Rivera-Perez, Magnuson 2005, Barrow et al. 2007), while later during development, Wnt inhibition is necessary for the specification of mesodermal progenitors to cardiac precursors (Schneider, Mercola 2001, Rivera-Perez, Magnuson 2005). Inactivation of β-catenin in multipotent Isl1 progenitor cells results in a partial loss of this population during embryonic development (E7.0-E9.5), while constitutive expression of β -catenin in these multipotent progenitors results in their expansion (Ai et al. 2007, Cohen et al. 2007, Kwon et al. 2007, Qyang et al. 2007, Bu et al. 2009). More recently, cardiomyocyte-specific β-catenin loss-of-function and gain-of-function mutants suggested that ventricular myocyte proliferation is controlled by β-catenin during development and the perinatal period (Buikema et al. 2013).

During mouse PSC differentiation, activation of the Wnt and JAK/STAT pathways (BIO, LIF), or use of the BACS cocktail (BMP4, Activin A, CHIR99021, and SU5402) was shown to promote expansion of induced cardiac progenitors without affecting their differentiation potential *in vitro* or *in vivo* (Zhang et al. 2016, Lalit et al. 2016). Similar results have been described using human PSC-derived cardiac progenitors. Expansion of ISL1+ cells was achieved by co-culturing cells with Wnt3a-secreting feeder cells or by treating cells with BIO (Bu et al. 2009), while mesodermal SSEA1 progenitor expansion was obtained by manipulating the Wnt, BMP, and Activin/Nodal pathways (Cao et al. 2013), again recapitulating developmental concepts described *in vivo*. While not reflecting an in vivo process, overexpression of the oncogene c-myc in the presence of IGF1, Hedgehog activators, TGF-β/Activin/BMP inhibitors, and bFGF was demonstrated to arrest cardiac progenitors at a pre–NKX2-5+ multipotent stage and facilitate their expansion (Birket et al. 2015). Collectively these data demonstrate that expansion of PSC-derived cardiac progenitor cells is possible and that strategies may vary depending on the developmental stage of the individual progenitor population. However, activation of the canonical Wnt signaling pathway appears to be a common critical factor required for all cardiac progenitor

expansion, concomitant with observations in various *in vivo* model systems. Additional mechanisms such as VCAM-1 (Kwee et al. 1995), α-integrin (Yang, Rayburn & Hynes 1995), and Jak2/Stat3 signaling (Marrero et al. 1998, Liu et al. 2015) have been suggested to impact cardiomyocyte proliferation and future combinatorial studies will demonstrate their implication in this process.

Alternatively to the proliferation of cardiac progenitor cells, one could anticipate taking advantage of the vast knowledge of cardiac regeneration in the adult myocardium in order to induce proliferation in differentiated cardiomyocytes. While this process promises to be less efficient in mouse and human cells, information from the highly regenerative zebrafish model may yield interesting candidates and approaches. Due to the magnitude of information these topics cannot be reviewed here, but are illustrated comprehensively elsewhere (Garry et al. 2005, Foglia, Poss 2016).

Generating cardiovascular lineages of the differentiated heart

One of the major promises of generating and studying cardiovascular progenitor cells in culture is their potential to differentiate to the multiple lineages of the heart (cardiomyocytes, smooth muscle cells, fibroblasts, endothelial cells, epicardium). Both for regenerative strategies and disease modeling approaches, it will be critical to generate pure, defined, and functional cells of the cardiovascular lineages. This applies to the safety and efficacy of transplanting defined and functional cells into the hearts of large animals and humans, but also to dissecting disease mechanisms in a cell-specific manner. Major progress has been made in recapitulating lineage-specific differentiation in culture, and the following section will summarize the milestones of that progress, as well as highlight open questions the field is currently investigating.

Myocardium—Generating contracting cells from PSCs in culture has become standard procedure in many laboratories, however, the heart is composed of different myocardial cell types including the atrial and ventricular cardiomyocytes and the conduction system cells.

In the working myocardium, atrial and ventricular cardiomyocytes differ in morphological, molecular, and physiological properties (Wagner et al. 2014, Volkova, Drapkina & Ivashkin 2006, Walden, Dibb & Trafford 2009), yet their specification mechanisms remain poorly understood and their origin cannot be ascribed exclusively to any of the previously characterized progenitor populations. The paucity of mechanistic insight into this process renders their specific generation in culture particularly challenging. Based on electrophysiological analysis, both atrial- and ventricular-like cells are present in cultures of current differentiation protocols, but their ratio varies and their respective isolation and characterization is not broadly established (Josowitz et al. 2014). While theoretically very useful, isolation of cardiomyocyte subtypes is challenging due to the lack of specific surface markers. New approaches have been developed to purify cells by targeting mRNA with molecular beacons. Using such an approach, ventricular cardiomyocytes were isolated via IRX4 molecular beacons (Ban et al. 2015). While this approach may not be ideal for clinical applications, such isolated ventricular cells can be used for disease modeling or drug discovery. In the following paragraphs, we will discuss various aspects of myocardial

development and specification that we deem most promising with respect to their translation to the PSC differentiation system and with the goal of generating specific myocardial cell types.

Cell type-specific gene expression may imply relevant mechanisms for myocardial sub-specification, however, most chamber-specific genes are expressed uniformly throughout the early heart tube and do not display region-specific expression at early stages of mammalian cardiogenesis (DeGroot et al., 1989; Sassoon et al., 1988; Lyons et al., 1990; Lyons, 1994). Irx4 and MLC2v expression is restricted to the ventricular segment of the linear heart tube prior to the formation of distinct cardiac chambers (O'Brien, Lee & Chien 1993, Bruneau et al. 2000, Christoffels et al. 2000). Irx4-deficient hearts suggest that while Irx4 is not necessary for ventricular chamber formation, it is required for the establishment of some of the components of a ventricle-specific gene expression program (Bruneau et al. 2001).

Mef2c and *Hand2* both play an important role in ventricular development. Both *Mef2c* and *Hand2* null embryos display single hypoplastic ventricular chambers at day E9.5 and die from heart failure by E10. However, *Mlc2v* and *Mlc2a* were appropriately localized and showed normal levels of expression, indicating unperturbed atrial-ventricular specification. Rather, *Mef2c* and *Hand2* are thought to control right ventricular development by specifying a subpopulation of cardiogenic cells within the cardiac tube and by regulating the expansion and survival of that population (Lin et al. 1997, Srivastava et al. 1997, Tsuchihashi et al. 2011). Controlled HAND1 expression in the left ventricle is also important for proper ventricular development, as evidenced by impaired interventricular septum formation and poor development of the myocardium compact zone in Hand1/eHand knock-in embryos. However, Hand1 is not a master regulator of ventricle/atrial chamber specification either (Togi et al. 2004).

In terms of signaling, the retinoic acid (RA) pathway was proposed to have chamber specific regulatory potential with respect to atrial-ventricular development (Xavier-Neto et al. 1999, Moss et al. 1998, Niederreither et al. 2001, Xavier-Neto et al. 2000). As such, RA signaling is necessary for atrial development, but not for the generation of ventricular cells in the embryo. Specifically, work using a RAREhsplacZ transgenic mouse, wherein lacZ is expressed in response to RA, demonstrated that retinaldehyde dehydrogenase 2 enzyme (RALDH2) expression colocalizes with endogenous response to RA in sino-atrial tissues from E8.25 to E12.5, while ventricular tissue remained devoid of RA metabolism up to E12.5. Based on this data it was determined that RALDH2 expression is a reliable readout of RA synthesis in the developing heart, and its early restriction to sino-atrial structures suggests that RA signaling is involved in the specification of cardiac cells (Moss et al. 1998). Moreover, blocking of RA synthesis at E7.5 results in hearts lacking atrial chambers, and inhibition of RA in ventricular precursors is essential for correct specification of the ventricles (Xavier-Neto et al. 1999).

Several studies have recently translated this concept to the PSC system, and have illustrated that exposing cells to RA during mesoderm formation does enrich for atrial cells in culture (Devalla et al. 2015, Zhang et al. 2011, Gassanov et al. 2008). Gene expression profiling of RA-exposed cells demonstrated up-regulation of atrial-specific transcripts such as *COUP-*

TFII, SLN, NPPA, and *PITX2* along with a down-regulation of ventricular transcripts such as *HAND1, HEY2, IRX4*, and *MYL2* (Devalla et al. 2015). Mechanistically, induction of the RA signaling pathway *in vitro* seems to alter the activity of Wnt signaling. Specifically, RA increases the expression of ligands and receptors of the non-canonical Wnt pathway (Wnt5a, 7a, Fzd2, and Fzd6), while concomitantly inhibiting the canonical pathway (Osei-Sarfo, Gudas 2014), which may act synergistically with Wnt inhibitors to induce cardiac mesoderm.

Manipulations of the RA and WNT pathways represent a promising start to cardiomyocyte subtype specification, however, additional signaling pathways or regulatory mechanisms are likely involved in the specification and differentiation process of chamber-specific myocardium. Studies in zebrafish have shown that inhibition of BMP signaling during cardiac progenitor specification stages results in a significant reduction in the size of the atria without affecting the ventricle, rendering BMP signaling a potential regulator of chamber proportionality. However, differential exposure to BMPs alone is again not sufficient to pattern a uniform progenitor field into atrial and ventricular populations (Marques, Yelon 2009). FGF signaling has been reported to promote proliferation of ventricular myocytes in zebrafish hearts (Marques et al. 2008, Reifers et al. 2000). Experiments using different conditional *Fgf8* mutant alleles further revealed a requirement for Fgf8 signaling at the crescent stage to support formation of the heart tube and the RV/OFT, as well as for looping. Early loss of Fgf8 affects both ventricle and atrium formation (Park et al. 2006). Consistent with these findings, application of exogenous FGF2 or FGF4 in chick embryos promotes ventricular myosin heavy chain 1 (*VMHC1*) gene expression and decreases atrial myosin heavy chain (*AMHC1*) expression (Lopez-Sanchez et al. 2002).

Shortly after looping of the heart tube, the ventricular chambers begin to expand and undergo trabeculation, a process necessary for development of a full-thickness ventricular wall and chamber formation. While relatively little remains known about the mechanisms of trabeculation, multicolor clonal analyses in fish suggest that the adult ventricular structure is determined by highly structurally regulated expansion of distinctly localized cardiac cells at a clonal level (Gupta, Poss 2012). Similar studies in the mouse confirm this concept and illustrate that the ventricular trabeculae are highly clonal and often discontinuous with the adjacent ventricular wall (Chabab et al. 2016, Gupta, Poss 2012). Understanding the underlying mechanisms of ventricular morphogenesis in detail may contribute to strategies for generating mature differentiated ventricular cardiomyocytes from PSCs.

Mechanistically, Notch signaling takes a prominent role during trabeculation by coordinating the interaction between ventricular endocardium and myocardium (Grego-Bessa et al. 2007). Active Notch1 signaling is detectable throughout the ventricular endocardium within 4 hours of initiation of contraction, and Notch1 activation induces the expression of its downstream effectors ephrin b2a (efnb2a) and neuregulin 1 (nrg1) in the endocardium (Samsa et al. 2015, Grego-Bessa et al. 2007). Mice lacking *ephrin-B2* present with heart defects at E10, specifically absence of myocardial trabecular extensions (Wang, Chen & Anderson 1998). NRG-1 can exert its effect via the ErbB family of tyrosine kinase receptors (ErbB2, ErbB3, and ErbB4) (Yarden 2001). Concordantly, mutations in *neuregulin-1* cause similar defects in the heart compared to ephrin-B2 loss of function hearts

(Meyer, Birchmeier 1995). Interestingly, ephrin-B2 and ephirin-B4 expression in the differentiated heart appears much higher in the atrium than in the ventricular endocardial cells lining the trabecular extensions of the myocardium (Wang, Chen & Anderson 1998), suggesting a role for ephrin signaling that is not chamber restricted. However, ErbB2 and ErbB4 are expressed in ventricular myocytes, and disruption of ErbB2 or ErbB4 in mice results in embryonic lethality at E9-E10 due to multiple defects, including lack of ventricular trabeculation (Gassmann et al. 1995, Lee et al. 1995). In contrast, ErbB3-null mice demonstrate normal trabeculation but display defects in the endocardial cushion formation, also leading to embryonic lethality at E13.5 (Erickson et al. 1997). Few studies have explored the role of ephrin or ephrin-related signaling mechanisms during PSC differentiation to date. Differentiation of EGFR-deficient ES cells results in the generation of cardiac and skeletal muscle as the predominant differentiated cell types *in vitro*, while other cell types are infrequent or absent, suggesting that EGFR activity is not necessary for cardiac and skeletal muscle or endoderm formation (Wu, Adamson 1996). However, it has also been suggested that NRG-1 promotes cardiomyocytes differentiation of ESCs and that the ErbB/PI3 K/Akt signaling pathway represents the underlying molecular mechanisms (Wang et al. 2009). In accordance with this, inhibition of the ErbB receptor in ESCs decreases the frequency of cardiomyocytes and the transcript levels of cardiac genes (Nkx2.5, β-MHC, cTnI, and MLC2a) (Kim et al. 2007).

As the myocardial lineage differentiates in the embryo, it gradually loses its proliferative potential. Understanding what mechanisms maintain the proliferative status of the early cardiomyocytes promises to be highly valuable when translated to *in vitro* differentiation systems, as this may enable expansion of PSC-derived cardiomyocytes prior to their terminal differentiation. Bmp10 appears to be critical for maintaining the proliferative activity of the embryonic cardiomyocytes in the developing ventricles at E8.75-E9.0, by preventing premature activation of the negative cell cycle regulator p57kip2 and by maintaining the required expression level of Nkx2-5 and Mef2c, (Chen et al. 2004). Moreover, mutant mice deficient in FK506 binding protein 12 (FKBP12) upregulate expression of Bmp10 in trabecular myocardium, rendering these mice nonviable due to overproduction of ventricular trabeculae (Shou et al. 1998). Interestingly, RBPJk and Notch1 mutant mice display reduced ventricular BMP10 activity and cells exit the cell cycle prematurely, demonstrating that Notch and Bmp10 interact during cardiogenesis (Grego-Bessa et al. 2007).

In addition to the endocardium, paracrine signaling from the epicardium is also required for proper cardiac morphogenesis. Specifically, mutations of the RXRα gene (RA receptor) in different cardiac cell lineages, including the neural crest, endothelium, ventricular myocytes, or a combination of both neural crest and endothelium do not result in cardiovascular phenotypes (Chen, Kubalak & Chien 1998, Tran, Sucov 1998, Merki et al. 2005). In contrast, mutation of RXRα in the epicardium results in thin-walled ventricles, suggesting that thickening of the myocardium is determined by retinoid-dependent signals originating in the epicardium (Stuckmann, Evans & Lassar 2003, Merki et al. 2005). Epicardium-derived mitogens responsible for the effect of the epicardium on compact muscle proliferation or compaction have been challenging to identify. Along the lines of previous discoveries, inhibition of either retinoic acid (RA) or erythropoietin (epo) signaling from the

epicardium inhibits cardiac myocyte proliferation and survival (Stuckmann, Evans & Lassar 2003, Merki et al. 2005).

In conclusion, chamber specification and maturation of the myocardial lineage have been investigated in numerous model systems, resulting in an in-depth understanding of many of these processes *in vivo*. The challenge of directing PSC differentiations to specific and functional myocardial cell types now consists in establishing an integrative approach to recapitulate the relevant aspects of cardiac cell fate specification and differentiation *in vitro*. In doing so, implying cell intrinsic as well as paracrine signals from the surrounding tissues will likely be important to generate PSC-derived cardiomyocytes comparable to their *in vivo* counterparts.

Conduction system—The cardiac conduction system (CCS) comprises several distinct cell types, including the sinoatrial and atrioventricular nodes (SAN/AVN), the atrioventricular (His) bundle, its right and left branches, and the network of Purkinje fibers in the ventricles (Sedmera, Gourdie 2014). Any of these cell types would harbor tremendous therapeutic potential if generated *in vitro*. This is particularly true for SAN cells, which have the potential to serve as biological pacemakers. Conversely, ridding cultures of pacemaker activity will be desirable to generate cardiac grafts without ectopic electrical activity (Chong et al. 2014).

Several reports have used expression of *Hcn4*, a marker restricted specifically to SAN cells after differentiation of the heart, to isolate and study nodal-like cells in culture. Hcn4+ cells isolated from E7.5-E8 *Hcn4CreERT2* lineage-tracing embryos and cultured *ex vivo* generate up to 8% of nodal like cardiomyocytes, and exclude smooth muscle or endothelial cells (Spater et al. 2013). Concomitantly, *in vitro* studies have demonstrated enrichment of nodal-like cells in Hcn4 overexpressing mESC lines (Saito et al. 2015). Furthermore, HCN4+ cells can be isolated from hESCs at early stages of differentiation using an antibody against HCN4. The HCN4+ population is enriched in *TBX5* and *NKX2-5* expression and gives rise to cardiac Troponin T+ cells (Spater et al. 2013). While this data confirms previously established *in vivo* principles of cell fate specification, one of the challenges of translating concepts from developmental biology to *in vitro* systems is to correlate temporally distinct events in the embryo with observations in cultured cells. For instance, Hcn4 is broadly expressed in the primitive heart tube and only later restricted to atrial and specifically SA nodal cells. Therefore, Hcn4+ cells emerging *in vitro* have to be thoroughly evaluated before being assigned to any of these structures.

Work in the mouse has identified podoplanin as a marker for the MLC-2a+NKX2.5- sinus venosus myocardial area derived from the posterior heart field (Gittenberger-de Groot et al. 2007). In a comprehensive study from the Mummery lab, podoplanin was reported to be highly expressed in cardiac mesoderm and was used in combination with NKX2-5 expression to isolate prospective pacemaker-like myocytes. NKX2-5^{-}Podoplanin^{high} progenitors exhibit higher expression of *TBX3, SHOX2, TBX18*, and *HCN4* than NKX2-5^{+}Podoplanin^{low} progenitors, as well as appropriate electrophysiological and contractile characteristics. However, while these are promising observations, the authors acknowledge that whether this population can develop a fully functional pacemaker

phenotype has yet to be investigated (Birket et al. 2015). Furthermore, the specific signaling pathways involved in the development of SAN cells remain to be uncovered.

During heart development, Purkinje cells, the conduction system cells in the ventricles, are recruited from contracting myogenic progenitors present in the tubular heart (Gourdie et al. 1995). During formation of the peripheral Purkinje fiber network, this specialized recruitment occurs in close correlation with the in-growth of cells derived from the epicardium, including progenitors of the coronary arterial tissues (Gittenberger-de Groot et al. 1998, Gourdie et al. 1995). Connexin 40 (Cx40) and contactin expression were previously utilized to identify Purkinje cells in the heart (Pallante et al. 2010). Using a dual *CCS:lacZ* and *Contactin2:egfp* reporter cell line, Purkinje-like cells can be isolated from mESC differentiation cultures (Maass et al. 2015). In a mechanistic follow-up of this work, sodium nitroprusside was found to promote the generation of Purkinje cells from cardiac progenitors initially expressing cardiac myosin heavy chain, via the activation of cyclic AMP signaling (Tsai et al. 2015). These studies illustrate yet another example of a faithful recapitulation of developmental concepts *in vitro*, and resulted in the discovery of novel cardiac development mechanisms in the process. Mechanistically, it is further well known that NRG-1/ErbB signaling is critical for the development of the cardiac conduction system (Rentschler et al. 2002) and activating Notch signaling can reprogram cardiomyocytes to a conduction-like phenotype (Rentschler et al. 2012).

Epicardium—The epicardium, consisting of a cell layer surrounding the entire heart, has long been known to influence the development and maturation of the myocardium and to contribute smooth muscle and fibroblast cells to the heart via epithelial-to-mesenchymal transition (Nakajima, Imanaka-Yoshida 2013). This process is regulated by PDGF (Smith et al. 2011), TGFβ (Bax et al. 2011) and RA (von Gise et al. 2011) signaling as has been discussed earlier in this review. More recent data suggest that the epicardium may also play an important role in regeneration after injury (van Wijk et al. 2012). Recent work has identified the epicardial secreted factor follistatin-like 1 (Fstl1) as a regenerative factor that is normally present in healthy epicardium, but lost upon myocardial infarction. Its restoration in the epicardium improves cardiac function by stimulating cell cycle entry and division of pre-existing cardiomyocytes (Wei et al. 2015). Not surprisingly, the epicardium has thus attracted great interest with respect to regenerative approaches.

Developmentally, the epicardium originates from the proepicardial organ (PEO) at E9.5, which forms from Nkx2-5 and Isl1 expressing lateral plate/splanchnic mesoderm progenitors (Zhou et al. 2008a). The PEO contains distinct sub-populations, and multiple lineage tracing studies suggest that PEO cells are able to give rise to vascular smooth muscle, myocardium, fibroblasts, and endocardium (Katz et al. 2012, Cai et al. 2008, Zhou et al. 2008b). Several signaling pathways, including Fgf8/Snai1 (Schlueter, Brand 2009), FGF (Torlopp, Schlueter & Brand 2010, Kruithof et al. 2006), and BMP (Kruithof et al. 2006, Schlueter, Manner & Brand 2006) are involved in regulating the formation of the epicardium in the chick embryo. However, these pathways remain less studied during mammalian epicardium development to date (Rudat et al. 2013, Nakajima, Imanaka-Yoshida 2013).

Importantly, epicardial cells can be generated from human PSCs by stage-specific activation of the BMP and WNT signaling pathways with very high efficiency and to high purity (Witty et al. 2014). While BMP signaling is required for the specification of both cardiomyocyte and epicardial lineages, development of the epicardial lineage is dependent on WNT signaling, while establishment of the cardiomyocyte lineage requires efficient inhibition of this pathway (Witty et al. 2014). In addition to BMP and WNT, RA signaling was found to be critical for the induction of the epicardial lineage from PSCs (Iyer et al. 2015). Both protocols efficiently generate cells expressing markers of the epicardial lineage that have the ability to undergo EMT toward smooth muscle–like and fibroblast-like cells in response to TGFβ, as they would *in vivo* (Iyer et al. 2015, Witty et al. 2014).

These milestone discoveries encourage and enable interesting future studies such as investigating the crosstalk between epicardial and myocardial cells *in vitro,* or dissecting the specific contribution of epicardial cells to myocardial growth, which are of major scientific and therapeutic relevance.

Endothelial, endocardial and smooth muscle cells—In addition to cardiomyocytes, the heart is composed of numerous supportive cell types, which strongly contribute to the functionality of the organ and are often involved or compromised in disease.

Endocardial precursors can be first observed in the embryo at the heart tube stage (Brutsaert et al. 1996), however, little is known about the development and embryonic origins of the progenitor cells that will give rise to the endocardium. Whether endocardium and myocardium share a common progenitor in cardiac mesoderm *in vivo* has remained controversial for a long time. Evidence from *in vitro* experiments suggests that endocardium and myocardium are derived from a common multipotent mesodermal progenitor that expresses Flk1 (Moretti et al. 2006, Misfeldt et al. 2009, Motoike et al. 2003, Yang et al. 2008, Kattman, Huber & Keller 2006), and *in vivo* evidence of such a common progenitor is only just emerging (Lescroart et al. 2014). The precise timing of specification mechanisms of these presumptive precursors remains poorly understood and no protocols have reported efficient generation of functional endocardial cells from PSCs to date.

In contrast, many protocols have been developed to generate endothelial cells from both mouse and human PSCs (Nourse et al. 2010, Choi et al. 2009, Li et al. 2007, Orlova et al. 2014, Kane et al. 2010, White et al. 2013), which closely resemble endocardial cells both functionally and phenotypically. The basic approach to generating endothelial cells relies on inducing mesoderm via BMP signaling, followed by specification of the endothelial lineages via high levels of VEGF. Such approaches result in efficient generation of CD31- and VE-Cadherin-positive populations with functional properties characteristic for endothelial cells.

While smooth muscle cells share many similarities with cardiac muscle, surprisingly few studies have reported their generation from PSCs. The current protocols consist of modifications of cardiac-specific protocols, with supplementation of PDGF-BB or VEGF and bFGF at the mesoderm stage to enhance differentiation to smooth muscle cells. Smooth muscle cells generated in such fashion are reported to be functionally indistinguishable from primary human coronary smooth muscle cells (Cheung et al. 2012, Steinbach, Husain 2015,

El-Mounayri et al. 2013, Paige et al. 2010). From the mouse embryo we know that Notch signaling is critical for the differentiation of vascular smooth muscle precursors, though this has not been reported *in vitro* to date (Doi et al. 2006, High et al. 2008).

Maturation of PSC-derived cells

One of the major challenges to determining the differentiation potential and lineage contribution of various progenitor populations from PSC differentiation is the broadly accepted notion that PSC-derived cells are immature, and do not correspond to cells from the fully differentiated heart. Specifically, cardiomyocytes derived from PSCs present different size, morphology, molecular composition, Calcium handling, and metabolism as compared to late fetal or adult cardiomyocytes. This represents a problem for numerous aspects of PSC biology and applications, such as the study of cardiac sub-types, the establishment of cardiac disease models that manifest after early fetal life, or the testing of drugs and discovery of novel treatment strategies for the adult heart. In order to address this current shortcoming of the PSC system, insights from development pertaining to the mechanisms of cardiac maturation may again lead the way to improving current PSC differentiation protocols.

One of the most significant changes that occur during cardiac development and maturation consists of the transition from anaerobic to oxidative metabolism (Sartiani et al. 2007, Kolwicz, Purohit & Tian 2013). The fetal heart is adapted to a low oxygen environment where levels of circulating fatty acids are low, and fetal cardiomyocytes are highly dependent on glycolysis to produce ATP. However, fatty acids are the predominant substrate used in the adult myocardium (Lopaschuk, Jaswal 2010, Alaynick et al. 2007). Several studies support the concept of cardiac metabolic flexibility that confers the advantage of adequately supplying ATP for continual cardiac contraction using diverse substrates when they become abundantly available under a variety of physiological conditions. (Wentz et al. 2010, Goodwin, Taegtmeyer 2000, Kaijser, Berglund 1992, Stanley et al. 2003, Schonekess 1997).

Efficient mitochondrial oxidative metabolism secures cardiac specification and excitation-contraction coupling. In support of this, manipulation of genes regulating mitochondrial fusion and fission results in mitochondrial maturation, while disrupting respiratory chain function prevents mitochondrial organization and causes deficient sarcomerogenesis and contractile malfunction (Chung et al. 2007). As cardiomyocytes mature, the number of mitochondria in each cell increases, the mitochondria become more uniform in size and present more densely packed cristae (Legato 1979, Artman, Graham & Boucek 1985). Comprehensive gene expression analysis on human adult and fetal heart samples, as well as 20-day and 1-year old cardiomyocytes derived from hESCs, revealed that the top genes differentially expressed in fetal versus adult hearts include many metabolic genes in lipid and fatty acid metabolism and oxidative phosphorylation (Ellen Kreipke et al. 2016). Current PSC differentiation protocols are entirely based on glucose-rich media, devoid of fatty acids. Given the major consequences of a metabolic switch during cardiac development, attempts to further differentiate and mature cardiac cells from PSCs may

benefit from adjusting metabolic substrates to more accurately reflect the *in vivo* developmental status being recapitulated.

Studies aimed at elucidating the molecular mechanisms of cardiac maturation have revealed that mature cardiomyocytes upregulate the let-7 family of microRNAs. The let-7 miRNA family targets genes involved in the PI3/AKT/insulin pathway, and their inhibition leads to decreased expression of markers of maturation, as well as decreased respiratory capacity and use of palmitate as an energy source (Kuppusamy et al. 2015). Furthermore, activation of integrin signaling and focal adhesion kinase has been reported as being essential for significant maturation of human PSC-derived cardiac monolayers *in vitro*. Such maturation of cardiomyocytes is achieved in a 1-week period and the cells harbor faster impulse propagation velocities, more mature action potential profiles and higher expression of key mature sarcolemmal (SCN5A, Kir2.1, and connexin43) and myofilament markers (cardiac troponin I) (Herron et al. 2016).

Another promising and well-appreciated research direction with respect to maturation of PSC-derived cells lies in the realm of bioengineering approaches. The collective goal of these studies is to provide the cells with a physiological environment with respect to extracellular matrix, directional contraction, contraction against resistance, and defined cellular tissue composition. These criteria stem from detailed studies of the developing heart, which forms in a spatially very defined and regulated manner.

Contact guidance through cell-cell interactions or cell-extracellular matrix (ECM) interactions may play an important role during maturation of cardiomyocytes and physical constraint of cardiac cells by extracellular matrix is an important regulator of myofibrillar organization (Bray, Sheehy & Parker 2008, Huang, Ingber 1999, Parker et al. 2008). In attempts to translate this information to PSC-derived cells, engineered alignment of cardiomyocytes on biomimetic grooves revealed longitudinal and transverse conduction velocities resembling the native human anisotropic ratio, and the total incidence of spontaneous and inducible arrhythmias is significantly reduced in aligned preparations but not in isotropic controls (Wang et al. 2013). Moreover, several studies have developed electrically stimulated systems for recreating the natural cardiomyocyte environment (You et al. 2011, Dvir et al. 2011, Nunes et al. 2013).

Diverse biomaterials and synthetic polymers help the cardiomyocytes organize into functioning tissues but the poor conductivity of these materials limits the patch from contracting strongly as a unit. PSC-derived cardiomyocytes contract most reliably on substrates where the rigidity matches the embryonic tissue rigidity (approximately 10 kPa), which is not surprising given the immature stage of these cells (Jacot et al. 2010, Ribeiro et al. 2015, Heras-Bautista et al. 2014). Major efforts and impressive work in the bioengineering community have provided the field with a series of tissue engineering model systems designed from various extracellular matrices, providing different scalability, functional read-outs, and tissue compositions. The field of tissue engineering for PSC-derived cardiomyocytes has expanded tremendously over the past years, and the topic has been reviewed comprehensively (Eschenhagen et al. 1997, Zimmermann et al. 2002, Hansen et al. 2010, Wang et al. 2013, Thavandiran et al. 2013, Hirt et al. 2014).

Maturation of PSC-derived cell types, particularly of the cells of the heart, remains a challenge in the field and will be a requirement for maximizing the potential of the PSC model system for disease modeling, drug testing and discovery, and for human developmental studies *in vitro*. Approaches to overcome these hurdles will likely consist in combinatorial strategies including bioengineering, metabolism, cell-cell interactions, and signaling parameters to generate more physiologically relevant and mature PSC-derived cardiac cells and tissues (Figure 1b).

PERSPECTIVES AND FUTURE DIRECTIONS

Knowledge of developmental processes has proven to be a reliable and effective instructor for *in vitro* differentiation systems, direct reprogramming approaches and adult regeneration strategies in the heart. This has led to a broad spectrum of discoveries using mouse and human pluripotent stem cells (PSCs), including the efficient production of cardiovascular populations and differentiated cell types, the uncovering of molecular mechanisms of heart development and the description of novel tools for *in vitro* tissue generation (Figure 1b). The field has moved quickly towards therapies and the first successful injections of PSC-derived cell products have been reported recently (Chong et al. 2014), with many more on the way. While these studies consist of impressive milestone achievements, they simultaneously illustrate the need for further improvement of the *in vitro* cell production strategies.

One promising avenue has long been thought to be the definition and isolation of cardiovascular progenitor populations with defined differentiation potential. Several different novel progenitor populations have recently been generated from human PSCs, and how their derivatives perform in *in vivo* models will be of the utmost interest. In order for these cells to convey therapeutic benefit they will have to be generated in large quantities and at high purities. Thus strategies supporting the expansion of progenitor populations without compromising their differentiation potential will be critical to develop. This has been done successfully for endoderm and neuronal lineages in the past (Cheng et al. 2012, Schwartz et al. 2008).

With respect to disease modeling and the study of differentiated cardiovascular cells, a major drawback of the current protocols is the consistently fetal phenotype of PSC-derived cells. This renders investigations of non-congenital, degenerative, or late onset disorders challenging to study and the need for more mature cardiomyocytes has populated the field with intriguing new studies, which we hope will provide new solutions to this important problem in the coming years.

Moving forward, we propose that answers to many of the outstanding questions can be found by investigating the respective processes during normal heart development and maturation. The promise of such discoveries is the establishment of efficient, reliable, and highly defined *in vitro* systems that will support the therapeutic approaches from regenerative biology to in-depth disease investigation.

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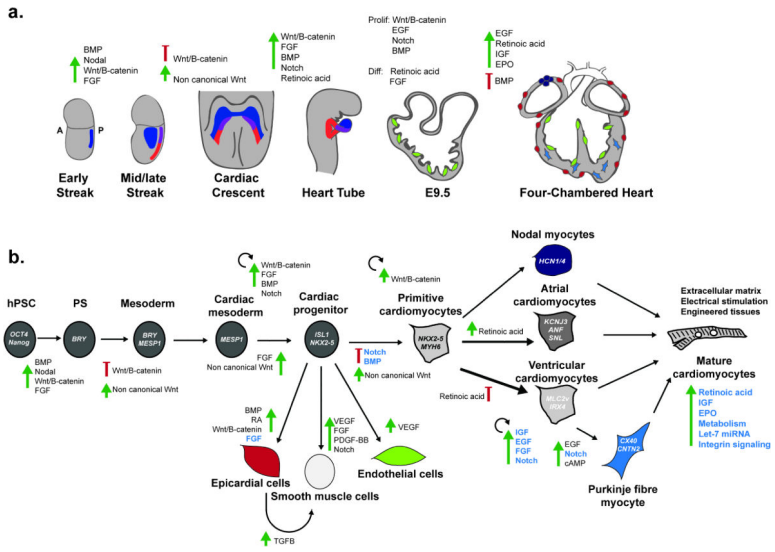

Figure 1. Schematic illustration of early heart development as translated to PSC differentiations to the cardiovascular lineages

a) Specification and differentiation of cardiac precursors during development of the mouse embryo. Cardiac progenitors are first specified at gastrulation in a manner that is both temporally and spatially relevant. The first population to ingress (blue) will migrate anteriorly and establish the cardiac crescent. The cells that migrate later can be divided in an anterior-posterior manner, with the anterior most (red) proceeding to the anterior region of the anterior heart field. Cells that ingress more posteriorly (purple) will form the posterior heart field. BMP, FGF and Wnt consist of the key signaling pathways that regulate early cardiogenesis. Upon formation of the primitive heart tube, the most anterior populations, which will have entered the tube first, will take up residence in the prospective ventricular areas, while the posterior heart field continues to feed cells into the heart tube. These cells will contribute to the atria and outflow tract of the fully differentiated heart. Endocardium and epicardial cells actively influence the process of specification, trabeculation and compaction of the myocardium mainly through activation of the Notch, BMP, EGF and retinoic acid signaling pathways.

b) PSC differentiation recapitulates cardiogenesis in the embryo. Most of the signaling pathways involved in heart development *in vivo* are equally critical *in vitro* to achieve generation of human cardiomyocytes. Manipulation of the signaling pathways in black have been described in the human system to be require to induce differentiation of PSCs to cardiac progenitors, cardiomyocytes, epicardial cells, smooth muscle cells and endothelial cells. Signaling pathways in blue represent observations made in the embryo but not yet studied in the PSC system.