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Review

Pluripotent stem cell derived cardiovascular progenitors – A developmental perspective

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ABSTRACT

Human pluripotent stem cells can now be routinely differentiated into cardiac cell types including contractile cardiomyocytes, enabling the study of heart development and disease *in vitro*, and creating opportunities for the development of novel therapeutic interventions for patients. Our grasp of the system, however, remains partial, and a significant reason for this has been our inability to effectively purify and expand the intermediate cardiovascular progenitor cells (CPCs) equivalent to those studied in heart development. Doing so could facilitate the construction of a cardiac lineage cell fate map, boosting our capacity to more finely control stem cell lineage commitment to functionally distinct cardiac identities, as well as providing a model for identifying which genes confer cardiac potential on CPCs. This review offers a perspective on CPC development as understood from model organisms and pluripotent stem cell systems, focusing on issues of identity as well as the signalling implicated in inducing, expanding and patterning these cells.

Introduction

Progenitor cells have conventionally been distinguished from stem cells by their limited ability to replicate *in vivo* or *in vitro* and their restricted differentiation capacity. Their differentiation state is often considered more advanced, their fate being determined by both their parent cell and the niche in which they are found. For some tissues, like the intestine and stomach, this concept changed with the observation that in adults, there is a progenitor cell population that behaves like a stem cell in that it can divide indefinitely (Barker et al., 2010). Like the hematopoietic niche in the bone marrow to produce blood, these endodermal tissues have high turnover rates to replace cells lost daily so that an active stem cell population is not unexpected. The heart and brain, however, are relatively quiescent organs that were long regarded as "post-mitotic", with no ability to repair. This again, has turned out to be incorrect and both organs show cell turnover and replacement after damage, albeit at low rates (Bergmann et al., 2009; Jessberger and Gage, 2014; Waring et al., 2014). Accordingly, neural progenitors that can be expanded in culture have been described for adult and foetal brain (Gage and Temple, 2013). For the heart, this has been more contentious: cardiovascular progenitors with various identities have been observed *in situ* (Bearzi et al., 2007; Beltrami et al., 2003; Laugwitz et al., 2005; Martin et al., 2004; Messina et al., 2004; Moretti et al., 2006; Oh et al., 2003; Wu et al., 2006), and isolated as proliferative cell cultures that should ordinarily be restricted in differentiation to the three cardiac cell types: cardiomyocytes, smooth muscle cells and endothelial cells. In contrast to the various endodermal and neural progenitor populations, putative progenitor cells from the heart have not shown stem cell features of prolonged proliferation. If expansion of these multipotent cardiovascular progenitor cells (CPCs) were achieved, it would be useful in a wide range of cardiac-related research areas, from basic developmental enquiry through to cardiac disease modelling, tissue engineering and cell therapy (Mercola et al., 2011; Mummery and Lee, 2013; Thavandiran et al., 2013), through either enhancing endogenous CPC numbers *in vivo* or isolating, expanding and transplanting cultured cells in the heart. While efforts continue towards making practical use of primary or endogenous CPC populations in the heart (not covered extensively here, but recently reviewed by Van Berlo and Molkentin (2014)), increasing attention is being directed to using self-renewing human pluripotent stem cells (PSC) for generating cardiac cells. Differentiation protocols have advanced tremendously over the last few years, most particularly those based on defined media using small molecular inhibitors and growth factors identified through developmental biology logic (Kempf et al., 2014; Lian et al., 2013). PSC-derived cardiomyocyte-studies are burgeoning, particularly in the area of cardiac disease modelling and drug safety pharmacology (Doherty et al., 2013; Drawnel et al., 2014; Matsa et al., 2014). Combined advances in induced pluripotent stem cell (iPSC) and genome editing technologies now permit study of large numbers of genetic changes on isogenic backgrounds (Li et al.,

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2014; Wang et al., 2014a; Zhang et al., 2014), where multiple disease mutations can be introduced into a single PSC line. This also presents an opportunity for mutation-specific drug screening and the development of novel treatments for cardiac disease, which is currently a major global cause of morbidity and mortality and for which treatment options are currently inadequate (Adler et al., 2009; Lloyd-Jones et al., 2010).

Yet in terms of developing therapies for the heart, some fundamental challenges remain which are still restraining full exploitation of this powerful PSC technology. These include: 1) maintenance and expansion of PSC-derived CPCs as pure populations in a stable state, 2) efficient direction of PSCs/CPCs to ventricular, atrial and nodal cardiomyocyte subtypes, or vascular cells and 3) maturation of these cardiomyocytes to stages beyond those resembling the early foetal heart.

For several lineages for which the existence of progenitors with stem cell properties has been demonstrated in primary (adult) tissue, the isolation and maintenance of PSC-derived tissue specific progenitors is now achievable (Cheng et al., 2012, 2013; Darabi et al., 2012; Nakamura et al., 2014; Okabe et al., 1996; Ran et al., 2013; Reubinoff et al., 2001; Sneddon et al., 2012), whereas less progress has been made with CPCs. If this could be mastered in much the same way as for PSC-derived neural progenitors, which now serve as a stable and renewable source of multiple neural cell types, it would significantly enhance the options for research and accelerate progress towards new cardiac therapeutics. One of the principal restraints has been the uncertain molecular identity of CPCs; during development; putative populations in the heart; and also those derived from PSCs. This review discusses these issues and highlights some of the existing knowledge which could guide researchers in addressing the challenge of isolating, expanding and differentiating PSC-derived CPCs *in vitro*.

Cardiovascular progenitor cells: an identity crisis?

While CPCs can easily be defined functionally by their tripotency in clonal analysis, their molecular identity during development is only partially understood. The best evidence comes from classical lineage tracing in mice, although detailed examination of protein and gene expression during development has provided surrogate information. These studies have shown that although stable states may exist for certain periods of heart development, the molecular identity of these populations (*i.e.* their chromatin/epigenetic signature and gene and protein expression profiles) may not be static for long, and defining a spatiotemporal map of any changes as they occur during development may be critical for mimicking this *in vitro*. Conversely, defining these stable or metastable states and progression to differentiated cell types at the clonal level using *in vitro* models may help us better understand heart development (Fig. 1). This is particularly relevant in studies of human heart where lineage tracing is not an option.

The earliest markers of the cardiac lineage can be seen in the gastrulating mesoderm and include Mesp1/2 and Fgf8 (Saga et al., 1999; Sun et al., 1999). Mesp1-driven Cre activity can be detected in all cells of the mouse heart of mesoderm origin, and while Mesp1$^+$ cells contribute broadly to a number of mesodermal derivatives, a subset appear to be dedicated cardiac progenitors (Devine et al., 2014). These proteins are expressed transiently at this stage but have an important role in the migration of cells to the lateral plate mesoderm where the population takes on a crescent shape; this is described initially as cardiogenic mesoderm as it is the site of the first cardiac-specific gene expression. The transcription factor *Nkx2-5*, the vertebrate *tinman* homologue of the *Drosophila* fruitfly, is an important early gene in this hierarchy and within the heart field marks definitive cardiac progenitors. Unlike *tinman* mutant flies in which cardiac lineage commitment is completely compromised so that the flies have no hearts, *Nkx2-5* deficient mice do initially form hearts, although expression of many myocardial genes is reduced and heart morphogenesis is abnormal (Lyons et al., 1995). This implies that Nkx2-5 is high in the hierarchy of cardiac transcription factors.

In considering CPC populations during heart development, it is important to be aware that an early lineage segregation takes place into what is termed the first heart field (FHF), which forms the entire left ventricle as well as other parts of the heart, and the second heart field (SHF), which contributes to part of the right ventricle and atria and forms all of the outflow tract (see review by Buckingham et al. (2005)). Mesp1$^+$ cells may already be fixed in their lineage fate, and common precursors may in fact exist only prior to gastrulation (Devine et al., 2014; Lescroart et al., 2014; Meilhac et al., 2004). The FHF differentiates within the cardiac crescent and this field forms the early heart tube. The second field, located medially to the crescent at the point of first differentiation, and then behind the forming heart tube, adds onto the heart tube during its later differentiation. This SHF encompasses the anterior heart field marked by Fgf8/10 and is marked more widely by the LIM homeobox transcription factor Islet 1 (Isl1) (Cai et al., 2003; Watanabe et al., 2010).

Whereas this lineage distinction is important during development, in stem cell models that lack location-dependent diversity, the definition of lineage may be less relevant than that of differentiation potential. At present it is also unclear if and how precisely the progenitors of the two heart fields are different at the molecular level. Support for a more comparable identity comes from a study in mice using a sensitive Cre-activated reporter based on *GATA4*, which was activated by *Isl1*^{Cre} and *Nkx2-5*^{Cre} in very similar domains in all four cardiac chambers (Ma et al., 2008). This suggests that differences among CPCs, at least in terms of *Isl1* and *Nkx2-5* expression, may be quantitative rather than qualitative. The transient and/or limited expression of *Isl*1 in rapidly differentiating FHF progenitors may be due to the fact that on differentiation to cardiomyocytes *Nkx2-5* expression increases and may act to downregulate *Isl*1 as well as other progenitor genes such as *Fgf10* in the first heart field (Prall et al., 2007; Watanabe et al., 2012). In *Nkx2-5* null embryos, expression of these genes can be detected in the cardiac crescent. Yet in a recent molecular analysis of Mesp1-marked derivatives, early regional differences have been suggested to exist (Lescroart et al., 2014).

The knowledge that Nkx2-5 and Isl1 mark multipotent CPCs during development prompted investigations into their possible presence in postnatal hearts. This led to the discovery of Isl1$^+$ cells in mouse and human neonatal heart, which when isolated from mouse based on the *Isl*1-Cre/R26R directed expression of lacZ or YFP, and cultured on cardiac mesenchyme, differentiated to cardiomyocytes or smooth muscle cells (Laugwitz et al., 2005; Moretti et al., 2006). However, their number is few at birth and they are undetectable in the adult. A variety of putative CPCs have been identified in adult hearts based on alternative markers expressed by stem cells in other tissues (Bearzi et al., 2007; Beltrami et al., 2003; Martin et al., 2004; Messina et al., 2004; Oh et al., 2003). Particular interest has focused on a population expressing the stem cell factor (SCF) receptor c-kit, which at least at the neonatal stage encompasses a population with some evidence of cardiomyogenic potential (Beltrami et al., 2003; Jesty et al., 2012; Zaruba et al., 2010). However, using a carefully validated *c-kit* lineage reporter mouse it was later shown that $<0.03\%$ of cardiomyocytes derived from a *c-kit* expressing population during development, and $<0.01\%$ in the first 6 months of postnatal life (Van Berlo et al., 2014). The population in the heart marked by the *c-kit* lineage reporter instead included 18% haematopoietic cells (CD45$^+$) and 77% endothelial cells (CD31$^+$). So while c-kit$^+$ cells do contribute to the developing myocardium of the heart this new evidence suggests they may do so extremely rarely; similarly in the adult. *In vitro* differentiation of heart-derived c-kit$^+$ cells can result in the upregulation of cardiac

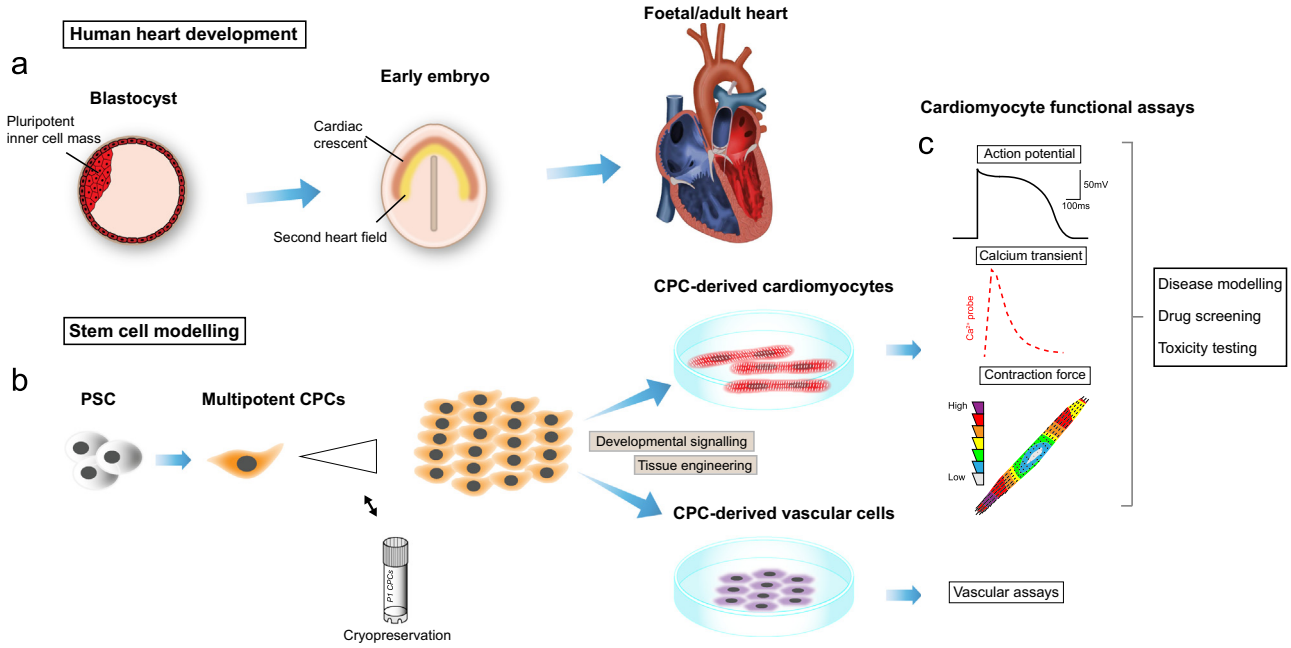

Fig. 1. Modelling human heart development and disease using pluripotent stem cell (PSC)-derived CPCs. (a) Human heart development from CPCs within the cardiac crescent and second heart field. (b) Deriving, expanding and banking CPCs from PSCs and using these cells to produce differentiated cardiomyocytes or vascular cells to study developmental processes, or (c) to apply experimental assays.

markers, but this also occurs infrequently and the derivation of contracting cells is seldom reported. c-kit does not therefore appear to be relevant to the CPC population observed during development and endogenous c-kit$^+$ lineage cells may also not be the primary cell source for cardiomyocyte renewal in adults. The latter conclusion, however, remains contentious and further replication of these studies is probably needed to reach consensus (Nadal-Ginard et al., 2014). More specific markers are clearly required to identify any stem cells/progenitors in the heart with genuine myocardial differentiation capacity (see review by Van Berlo and Molkentin (2014)), and will also be important to understand how they relate to developmental CPC populations as well as those from PSCs.

In terms of trying to define the molecular nature of CPC states, an important question is that of when cardiac fate and therefore the earliest CPCs are determined *in vivo* and therefore similarly during PSC differentiation. Fig. 2 illustrates our simplified model for these transitions. Although the earliest Isl1$^+$/Nkx2-5$^+$ cells in the lateral plate mesoderm clearly represent CPCs, are they merely a descendent of a pre-determined CPC population? It is important to note that a large part of the future SHF myocardium may be lacking *Nkx2-5* expression at least at the anterior lateral plate mesoderm stage (Wu et al., 2006), and these cells may already be pre-determined to the cardiac lineage (Scott, 2012). Definitive earlier markers of CPCs do not exist, but it may be critical to identify them, as once Nkx2-5 is expressed a negative-feedback loop may compromise their self-renewal and endothelial differentiation capacity may become restricted or even lost (Prall et al., 2007; Wu et al., 2006). So which other genes might define this population? As noted earlier, *Mesp*1 is one of the earliest markers of the cardiac lineage, but its expression is undetectable after gastrulation at the time when the cardiac transcription factor *Nkx2-5* becomes detectable. This has also been seen in a recently generated dual colour *MESP1*$^{mCherry/w}$-*NKX2-5*$^{eGFP/w}$ hESC cardiac reporter line (Den Hartogh et al., 2014). So which other genes might mark this intermediate population and be required to regulate CPC development? GATA factors have been suggested, as these are expressed early in gastrulating mesoderm and have an important role in myocardial gene expression. Although there is redundancy between the members, combined knockout of *GATA4* and *GATA6* completely blocks myocardial development in the mouse (Zhao et al., 2008). *GATA4* is the earliest expressed family member in the mouse cardiac lineage and may have a particularly important role in CPCs. Although apparently unable to facilitate cardiac gene induction when transfected alone into a gastrula-stage embryo, when GATA4 is combined with the SWI/SNF chromatin remodelling complex subunit Baf60c, remarkably they have this ability (Takeuchi and Bruneau, 2009). In zebrafish, overexpression of Baf60c and GATA5 (GATA4 homologue in zebrafish) promotes the induction of a primitive CPC-like state in a cell-autonomous, position-independent manner and these cells are capable of differentiating to cardiomyocytes, endothelium and smooth muscle (Lou et al., 2011). Mechanistically, Baf60c was shown to recruit GATA4 to target genes to initiate CPC specification, which is analogous to the role of Baf complexes in MyoD recruitment in skeletal muscle (Serna et al., 2005). *Baf60c* expression was shown to precede *Isl1* in the *Mesp1*^{Cre}-derived lineage and occur coincidently with activation of the *Mef2cAHF*, which was previously thought to be only activated later (Devine et al., 2014). The transcription factor MEF2C may therefore represent another important component of early CPC identity (Lin et al., 1997). It will be important to determine how cardiac Baf complex recruitment occurs and what the early target genes are, and whether these cells can somehow be arrested in this early state by regulating these signals.

Other putative CPC markers have been suggested from a study of *Nkx2-5* mutant mice and include platelet-derived growth factor receptor alpha (Pdgfrα), insulin-like growth factor-binding protein 5 (Igfbp5), and the transmembrane protein Odz4 (Prall et al., 2007). The VEGF receptor 2 (Flk-1/KDR) was previously shown to be co-expressed with Pdgfrα in cardiogenic mesoderm and so may represent another early marker (Kataoka et al., 1997). Expression of HCN4 (hyperpolarization-activated cyclic nucleotide-gated channel 4) is also evident in cardiogenic mesoderm and the cardiac crescent and marks cells seemingly restricted to a myocardial fate (Garcia-Frigola et al., 2003; Liang et al., 2013; Später et al., 2013). During human PSC differentiation, the cell-surface protein signal-regulatory protein (SIRPA) has also been shown to be expressed in CPCs prior to the onset of *NKX2-5* expression, although its early

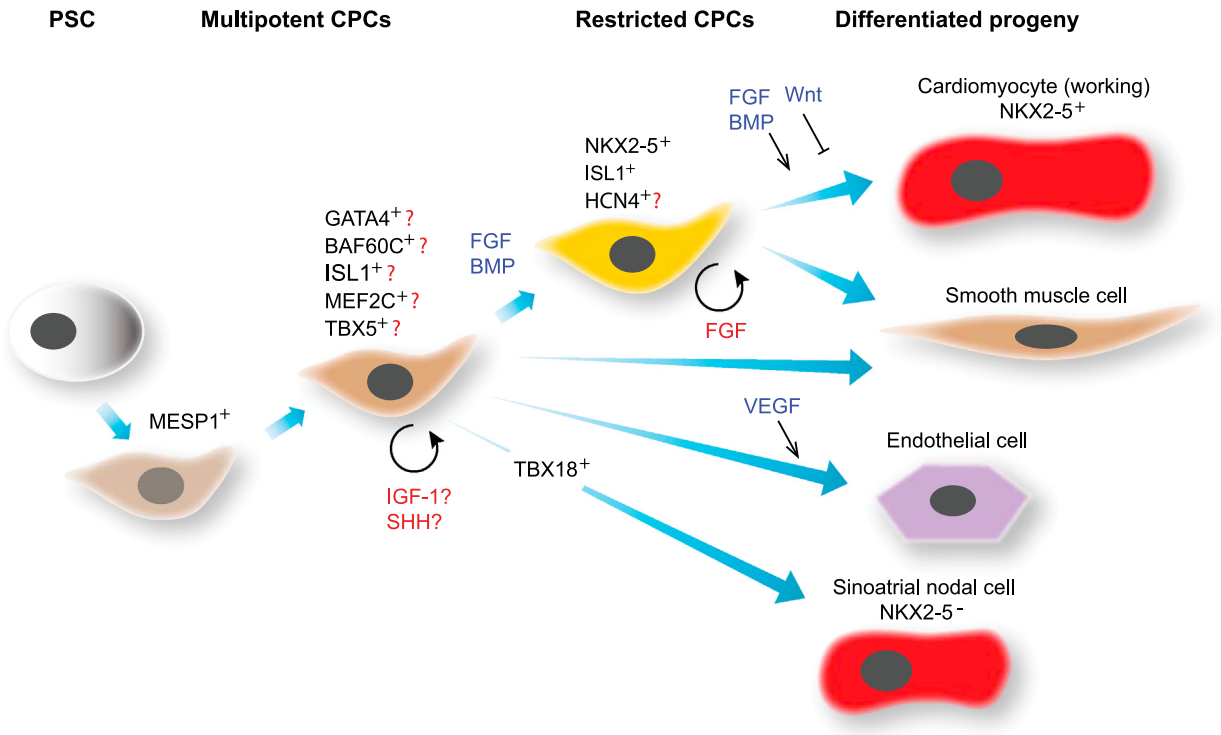

Fig. 2. Developmental progression and differentiation of cardiovascular progenitors. Genes predicted to be expressed in multipotent or bipotent CPCs are shown, as are the factors predicted to be involved in their self-renewal or progression/differentiation.

developmental expression *in vivo* is unknown (Dubois et al., 2011; Elliott et al., 2011). Whether any of these particular genes are functionally significant for CPCs remains to be determined.

Use of PSC models may be useful for determining how this range of markers, some of which may be expressed in CPCs prior to *NKX2-5*, reflect progression in the cardiac lineage and which genes are determinants of CPC function. If pure populations of these cells could be isolated and expanded, functional screening assays could be envisaged to identify these genes, applying current targeted gene deletion technology (Shalem et al., 2014; Wang et al., 2014b).

In continuing this discussion we will first outline the signalling events that induce the earliest cardiogenic populations in the embryo.

Lessons from embryonic heart development – signalling important for induction, patterning, expansion and differentiation of CPCs

Signalling regulating the induction and patterning of CPCs

During gastrulation, the future CPCs are among the first cells to migrate out of the primitive streak, where they were situated within anterior mesoderm. Adjacent populations of cells, particularly endoderm, play an important inductive role in the cardiac lineage by secreting instructive protein factors or morphogens, creating concentration gradients that pattern progenitor populations and regulate their differentiation. Conveniently, these early events appear to be so well conserved between species that the same factors and pathways identified in model organisms such as chick and zebrafish are so far proving to operate very similarly even during *in vitro* differentiation of human and mouse PSC systems, which means that the details of past and future developmental biology studies will no doubt prove indispensable for further development of PSC-derived CPC methodologies.

The three families of factors with recurring roles during mesoderm formation and cardiogenesis are the transforming growth factor- β (TGF-β) superfamily including bone morphogenic proteins (BMPs) and Nodal, Wnt proteins and fibroblast growth factors (FGFs). BMPs and canonical Wnts play highly conserved roles in inducing early primitive mesoderm. Nodal is essential in mouse embryos for positional information; it induces gastrulation but has a graded influence with intermediate doses inducing cardiogenic specification. FGF signalling may act in parallel to nodal signalling as a competence factor for mesoderm induction and it has important roles later alongside BMP in CPC specification (Bertocchini et al., 2004). As suggested from many of the model systems and now corroborated in PSC investigations, BMP, Nodal and Wnt may all have an antagonistic effect on cardiac differentiation after gastrulation or the equivalent stage in the stem cell system (Cai et al., 2013; Naito et al., 2006; Yuasa et al., 2005). Protocols to induce cardiac differentiation from PSCs have moved from serum-based culture media in simple cell aggregates or inductive cell co-culture towards defined, serum-free systems, involving the sequential activation or inhibition of the above pathways. A standard protocol involves exposing PSCs to BMP4, Activin A (a nodal substitute) and Wnt3a (or a GSK3 inhibitor) for 3 days followed by exposure to a Wnt signalling inhibitor. Using such a system, MESP1$^+$ cells appear at day 3–4 of differentiation, PDGFRα expression increases to a peak at around day 5–6, and NKX2-5 expression is clearly detectable by day 8, as illustrated in Fig. 3 (Elliott et al., 2011; Den Hartogh et al., 2014; Kattman et al., 2011). The progression of *differentiation* from the MESP1$^+$ population through to the point of *NKX2-5* induction is of significant interest as it encompasses the progressive development of CPCs through transitional states with increasing levels of cardiac commitment, of which the specific molecular identities remain obscure (Fig. 2). There is increasing focus on the regulation of chromatin remodelling induced by endoderm-derived signals. From the *Mesp*1-expressing stage, it was shown that inhibition of nodal and BMP can increase the expression of *GATA4*, *Tbx5* and *Baf60c*, which facilitates chromatin remodelling at cardiogenic loci and primes the cell for further cardiac differentiation (Cai et al., 2013; Lickert et al., 2004). However, we know from development that BMP signalling, possibly in concert with FGF signalling, is important

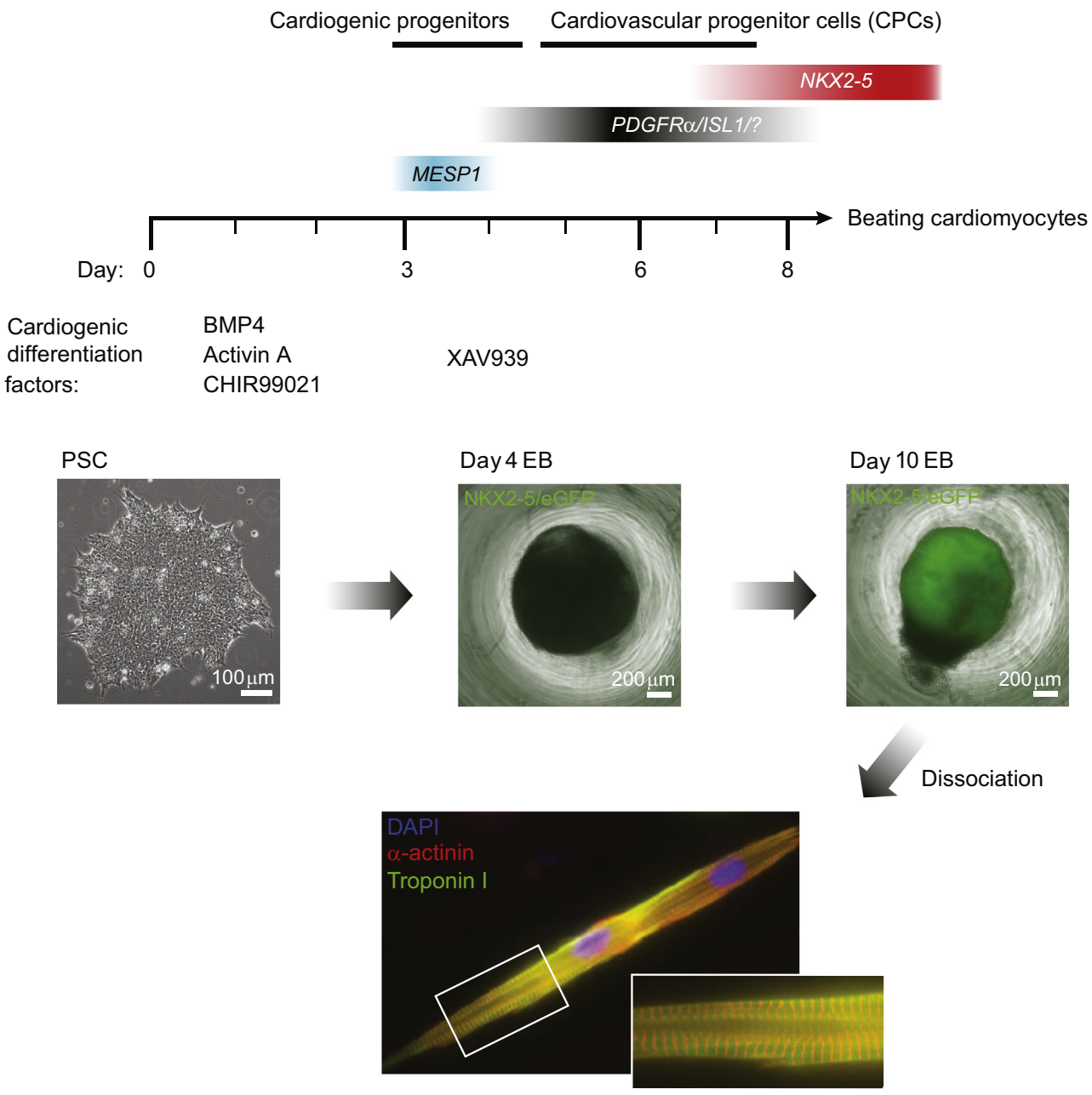

Fig. 3. Cardiovascular progenitor cell development and differentiation to cardiomyocytes during human pluripotent stem cell differentiation. A time-course of cardiac differentiation using a protocol based on defined factors, highlighting the expression kinetics of known markers of cardiac progenitors. Images show a representative embryoid body from *NKX2-5*$^{eGFP/w}$ hESC reporter cells, imaged on day 4 and again on day 10 of differentiation, at which time the EB would be beating and rich in cardiomyocytes. CHIR99021 = GSK3-inhibitor; XAV939 = Wnt pathway inhibitor.

for the induction of cardiac gene expression including *Nkx2-5* (Alsan and Schultheiss, 2002; Keren-Politansky et al., 2009; Liberatore et al., 2002; Lien et al., 2002; Reifers et al., 2000). It could be that *GATA4*/*Tbx5*/*Baf60c*-expressing cells are optimally poised to respond to this next wave of BMP and FGF signalling, thereby efficiently upregulating *Nkx2-5* and beginning the process of more definitive cardiac differentiation.

Signalling regulating proliferation of CPCs

In the mouse embryo it has been estimated that the myocardium is derived from a population of around 140 cardiac founder cells at around the time of gastrulation, which contribute two-thirds/one-third to the first/second heart lineage (Meilhac et al., 2004). The doubling time of these early cells has been calculated as approximately 7 h up to E8.5, and later in early myocardial cells up to E10.5 as approximately 15 h (Meilhac et al., 2003). Therefore proliferation at both the progenitor stage and after differentiation to cardiomyocytes is central to heart development. The primary pathways driving proliferation of CPCs are thought to be: FGF, canonical Wnt, IGF-PI3K and sonic hedgehog (SHH). As SHF progenitors exist in a dividing, undifferentiated state for longer, this population has been the focus of studies on proliferation.

FGF signalling exerts a pro-proliferative effect mostly by dose-dependently inducing ERK phosphorylation, but signalling via PI3K/Akt can also enable proliferation. Fgf8 is produced within the SHF but also by endoderm and ectoderm of the pharyngeal arches. In mice, conditional or hypomorphic *Fgf8* mutants must be used to ensure gastrulation, and these mutants show that Fgf8 signalling is critical for the proliferation of the anterior SHF (Ilagan

et al., 2006; Macatee et al., 2003; Park et al., 2006). Fgf10 is also expressed in the SHF, and while *Fgf10* mutants seem to have a defect of cardiac morphogenesis rather than in the SHF, heterozygous loss of *Fgf*10 exacerbates a cardiac-specific *Fgf*8 mutant and leads to severely disturbed anterior heart development (Marguerie et al., 2006; Watanabe et al., 2010). These results suggest that FGF dosage is critical for anterior SHF proliferation and deployment.

Wnt/β-catenin signalling can induce proliferation through the regulation of cell-cycle controlling target genes, including upregulation of *c-Myc*, *Cyclin D* and *c-jun*, and downregulation of *p*21 (Vlad et al., 2008). Wnt signalling may also stimulate FGF production and signalling within CPCs, where *Fgf*10 was shown to be a direct target (Cohen et al., 2007). While negatively modulating cardiac specification from mesoderm, numerous reports suggest a pro-proliferative effect of canonical Wnt signalling on established CPCs (Cohen et al., 2007; Klaus et al., 2007; Qyang et al., 2007). However, overactivation of Wnt/β-catenin signalling can lead to a loss of SHF CPCs, and non-canonical Wnt ligands Wnt5a and Wnt11 are required to inhibit Wnt/β-catenin signalling and maintain this population (Cohen et al., 2012). As well as inhibiting *Isl*1 expression, in some models Wnt/β-catenin signalling was shown to lead to a downregulation in GATA gene expression and other genes involved in cardiac development (Afouda et al., 2008; Kwon et al., 2009; Martin et al., 2010; Novikov and Evans, 2013; Palpant et al., 2013). So it seems that while some Wnt/β-catenin signalling is necessary for CPC proliferation, the signalling must be tempered to prevent loss of CPC identity.

Evidence suggesting a role for IGF signalling in CPCs is less direct but can be inferred from a number of studies. IGF signalling is involved in general embryonic growth, with surviving *Igf*-1 null mice becoming 30% of normal weight as adults (Baker et al., 1993). More specifically for the heart, *Nkx2-5Cre*/*Igf1r*/*Insr* conditional null embryos have a significant defect in myocardial proliferation (Li et al., 2011), while cardiac specific overexpression of nuclear Akt enhances CPC proliferation (Gude et al., 2006). PI3K and Akt are also essential for hedgehog signalling (Madhala-Levy et al., 2012; Riobó et al., 2006), which is another signalling pathway implicated in CPC proliferation as well as the maintenance of many stem cell/progenitor populations (Beachy et al., 2004). SHH is produced by the endoderm and its signalling activity was observed in the posterior SHF of mice and has been shown to be necessary for SHF proliferation in chick (Hoffmann et al., 2009). Increasing hedgehog signalling was also shown to stimulate proliferation in SHF explants (Dyer et al., 2010).

Cardiomyocyte subtype specification through CPC patterning

The mature four-chambered heart consists of cardiomyocytes that can be classified broadly as “working” myocardium (atrial and ventricular) and the conduction system population. Working myocardium is characterized by its low automaticity and efficient contractile function whereas the conduction system is characterized by the opposite.

In terms of working myocardium, the FHF contributes both atrial and ventricular myocytes, although atrial markers are not detectable in the cardiac crescent of the mouse and are only distinguishable later, where as ventricular markers such as Mlc2v are already present in ventricular myocytes at this stage. The SHF also contributes to both populations and is clearly patterned along its anteroposterior axis with anterior progenitors ($Isl1^+$/$FGF10^+$) contributing to right ventricular myocardium and posterior progenitors ($Isl1^+$/$FGF10^-$) contributing solely to the atria (Galli et al., 2008). An important signalling factor defining cardiac anteroposterior patterning is retinoic acid, which inhibits *Fgf8* and *Nkx2-5* gene expression in the cardiac progenitor field and is also important for the induction of atrial specific genes (Drysdale et al., 1997; Sirbu et al., 2008). It was demonstrated that retinoic acid can also promote differentiation of human embryonic stem cells (hESCs) towards an atrial fate (Zhang et al., 2011). BMP may also influence chamber assignment in development, with higher levels favouring atrial over ventricular fate (Marques and Yelon, 2009). However, little else is known in terms of atrial *versus* ventricular signalling determinants.

The conduction system consists of the sinoatrial node (SAN), the atrioventricular (AV) node, bundle of His and Purkinje fibres. Much research has focused on the origin and molecular identity of the SAN, the pacemaker of the heart, because its dysfunction is a common cardiac disorder. The SAN has a molecular signature distinct from the surrounding atrial myocardium, including maintained expression of the hyperpolarization activated pacemaker channel HCN4, which is important for pacemaker activity, and low levels of Cx40 and Cx43 gap junction proteins to ensure slower propagation rates of current than the working myocardium (Christoffels et al., 2010). Another key molecular feature of the primordial SAN is the early absence of *Nkx2-5* expression. The population develops from $Isl1^+$ SHF progenitor cells which have never expressed *Nkx2-5* but which have mostly expressed *Tbx*18, a gene which is first detected caudal/lateral to the cardiac crescent (Mommersteeg et al., 2007; Wiese et al., 2009). This absence of the Nkx2-5-driven working myocardial programme may be a key requirement for SAN development. Therefore in terms of SAN specification signalling, protection from *Nkx2-5*-inducing factors (such as FGF and BMP) may be important, or else early signalling events might render them subsequently resistant to these *Nkx2-5*-inducing signals. By contrast, the AV node expresses relatively high levels of *Nkx2-5*. Considerable efforts are being directed at trying to differentiate PSCs to pacemaker cells, for disease modelling purposes and also with the intention of making biological pacemakers in the future. Promising results have been shown through genetic means by overexpressing transcription factors important in SAN development, namely Tbx3 and Shox2 during mouse ESC differentiation (Ionta et al., (2015); Jung et al., 2014). In hESCs, inhibition of neuregulin (NRG)-1β/ErbB signalling was shown to promote the differentiation of nodal-type cells and inhibit induction of working-type cardiomyocytes (Zhu et al., 2010).

Clearly more needs to be learnt about the signalling and genetic determinants of lineage specification from PSCs towards either working myocardium or pacemaker cells to help facilitate the efficient generation of these functionally distinct populations.

Signalling regulating differentiation of CPCs

The CPCs of the FHF and SHF will ultimately differentiate to make up the vast majority of the heart's cardiomyocytes, and additionally make contributions to the heart's vasculature, and perhaps also to the endocardial inner lining of the heart. The cardiac neural crest and the proepicardium are the other important contributors to the heart's vascular and interstitial cells. So after the proliferative expansion of CPCs and their appropriate patterning, a new configuration of signalling must lead to their terminal differentiation, which could be into cardiomyocytes, smooth muscle or endothelial cells depending on the progenitor's identity and location.

Cell cycle exit may not necessarily be coupled to differentiation, as even myocytes continue proliferating in the early (or neonatal) heart (Porrello et al., 2011), but a reduction in proliferative signals may be a key differentiation-inducing event, with a changing balance of FGF and BMP signalling possibly at the crux of this determination for the myocardial fate. As the SHF CPCs approach the primary heart tube they are exposed to increasing BMP and non-canonical Wnt signals and these are thought to be primary drivers of myocardial differentiation.

In the mouse, *Fgf10* expression becomes downregulated in myocardium in response to increasing Nkx2-5-mediated repression and loss of Isl-1-mediated activation, and this is permissive for myocardial differentiation (Watanabe et al., 2012). Likewise, in the

absence of epicardial- and endocardial-derived FGF signals, differentiation to cardiomyocytes occurs prematurely (Lavine et al., 2005). BMP has been shown to be continually required during myocardial differentiation and it is actively repressed in differentiating cardiomyocytes (Pater et al., 2012). Wnt/β-catenin may also have a considerable fate determining influence, as its activity has been shown to promote an endothelial over a myocardial fate (Noack et al., 2012; Novikov and Evans, 2013; Palpant et al., 2013). By contrast, non-canonical Wnt signalling may have a positive role on cardiomyocyte differentiation, with Wnt11 having been shown to be important for normal myocardialization and outflow-tract morphogenesis via an upregulation in TGF-β2 expression (Zhou et al., 2007). In support of this, TGF-β markedly improves the cardiomyocyte differentiation of human heart-derived progenitors (Goumans et al., 2008). However, data suggest that this may be a late effect as earlier exposure inhibits myocardial commitment (Willems et al., 2012), and its inducing effect may relate at least partially to its anti-proliferative role. New data suggest that increased mitochondrial reactive oxygen species (ROS) may be a key mediator of cardiomyocyte cell cycle arrest (Puente et al., 2014); ROS were also previously shown to be elevated early in hESC-derived cardiomyocytes (Birket et al., 2013), and as TGF-β is known to increase mitochondrial ROS, which is also important for its gene regulatory effects, there could be a mechanistic connection involving mitochondria (Jain et al., 2013). Finally, roles for both SHH and IGF in cardiomyocyte commitment have been suggested by some models and also merit further exploration (D'Amario et al., 2011; Goddeeris et al., 2007; Thomas et al., 2008).

The signalling mechanisms more specifically guiding the differentiation of putative multipotent CPCs to vascular cells have been less studied, but may also be diverse. VEGF is known to be a critical factor for the differentiation and propagation of endothelial cells and has been used to facilitate endothelial cell differentiation from Isl1$^+$ CPCs of embryonic or ESC origin (Moretti et al., 2006). TGF-β, PDGF and retinoic acid signalling pathways have been implicated in the differentiation and proliferation of smooth muscle cells from a variety of progenitor sources but their action on CPCs has yet to be explored (Sinha et al., 2014). In explanted chick arterial pole progenitors, it was shown that FGF signalling promotes proliferation and later smooth muscle differentiation, where as BMP signalling promotes myocardial differentiation (Hutson et al., 2010). Likewise, inhibiting BMP signalling in zebrafish anterior heart field progenitors favours smooth muscle over myocardial differentiation (Hami et al., 2011).

Pluripotent stem cell-derived cardiac progenitor cells

PSCs offer a unique opportunity to recreate the process of cardiac development *in vitro*, to follow the development and progression of CPCs, and to study the competence of clonal populations in a controlled environment. The system is a valuable platform for testing hypotheses that have emanated from research on model organisms, and presents a rare opportunity to work in a human cell context. In this section we will review the studies that have attempted to isolate, expand, and differentiate PSC-derived CPCs. These have mostly focussed on the markers: VEGFR2, Isl1 or Nkx2-5, identified using surface staining (VEGFR2), or through the use of genetic reporter lines (Isl1 and Nkx2-5). A common feature of these studies is the limited evidence of expandability and so focus has mostly been on the differentiation capacity of these cells in terms of defining uni- bi- or tripotency.

The cardiac lineage arises from VEGFR2$^+$ (Flk1/KDR) mesodermal progenitors during development in mouse, and a number of studies have suggested that this protein marks the same population during ESC differentiation (Kattman et al., 2006; Motoike et al., 2003; Yang et al., 2008). Yang et al. (2008) demonstrated that during cardiac differentiation of hESCs, low KDR expression at around day 6 could mark a tripotent clonal CPC population. It was later shown that this ESC-derived population also expresses PDGFRα, equivalent to the *in vivo* situation (Kataoka et al., 1997; Kattman et al., 2011). However, in hESCs, den Hartogh et al. did not find enrichment of KDR expression in cardiac progenitors derived from MESP1$^+$ cells, which were enriched for PDGFRα, raising some doubt about the human VEGFR2 being a CPC marker *in vitro* (Den Hartogh et al., 2014). The surface makers ROR2, CD13 and SIRPA may also overlap with early PSC-derived CPCs at different stages, with SIRPA also being a useful marker of early cardiomyocytes derived from human PSCs (Ardehali et al., 2013; Dubois et al., 2011; Skelton et al., 2014). The cardiac progenitor marker *Isl1* becomes enriched within this PDGFRα$^+$ population and these putative CPCs could be isolated from *Isl1-nLacZ* knockin ESCs (Moretti et al., 2006). These cells showed some capacity to expand as colonies on cardiac mesenchyme, but still differentiated spontaneously, with colonies showing indications at the gene expression level of differentiation to cardiomyocytes, smooth muscle cells and endothelial cells. Likewise, in a human model, using transgenic *ISL1-cre DsRed* hES cells, DsRed$^+$ cells were enriched for *NKX2-5* expression and were capable of forming all 3 lineages, albeit with low efficiency (4% CMs, 3% ECs, 44% SMCs) (Bu et al., 2009). The non-cardiac marker c-kit was shown to mark a proportion of early Nkx2-5$^+$ cells differentiating from mESCs and to be associated with clonogenicity (Wu et al., 2006), where as in the human system the CPC population is c-kit$^-$ at day 6 of differentiation which is more consistent with lineage tracing data (Van Berlo et al., 2014; Yang et al., 2008).

Studies using these PSC systems suggest that a developmental loss of tripotency (loss of endothelial capacity) may be concomitant with, or occur around the time of *NKX2-5* upregulation. For example, in the Nkx2-5$^+$ population of mESC reporter lines only bipotency to cardiomyocytes and smooth muscle could be achieved (Moretti et al., 2006; Wu et al., 2006), although in one *NKX2-5$^{eGFP/w}$* hESC reporter line early NKX2-5$^+$ cells could also give rise to rare endothelial cells; although tripotent differentiation from single cells could not be demonstrated (Elliott et al., 2011). The onset of HCN4 expression may also occur concomitantly with progression to a more restricted myocardial progenitor (Liang et al., 2013; Später et al., 2013). Together these studies suggest that with the onset of NKX2-5 and/or HCN4 expression, CPCs may quickly lose endothelial differentiation capacity. It remains unclear how important VEGFR2 expression is in this regard, as endothelial cells could also be derived from KDR$^-$ cells marked by *ISL1* (Bu et al., 2009). The overlapping expression of other VEGFR family members might explain some of this heterogeneity (Nsair et al., 2012). Interestingly, in two colour *Isl1* and *Nkx2-5* reporter mESCs, cardiomyocytes could also be produced from Nkx2-5$^-$ cells, albeit at low efficiency ($<20\%$), but there was no mention of whether they remained Nkx2-5$^-$ similar to cells of the SAN (Domian et al., 2009; Gittenberger-De Groot et al., 2007; Mommersteeg et al., 2007).

A striking feature of all these studies is the limited proliferation shown by these CPC populations. Of the 11 publications summarized in Table 1, proliferation has only been shown over one or two passages and in no case has stability been shown across multiple markers to confirm that the cells remain unchanged even for this period. This raises the question of whether the requisite growth conditions have simply not been met, or whether the cells at this stage are in a transitional (quasi-stable) state by nature and are actually not amenable to stable expansion but are instead irredeemably fixed on the path of further differentiation.

Strategies to improve the utility of PSC-derived CPCs

Due to the challenges of isolating and expanding PSC-derived CPCs, the standard approach for generating cardiomyocytes from PSCs remains directed differentiation of one state to the other without the isolation of an intermediate cell population. While this

Table 1
A summary of literature describing the expansion and differentiation of PSC-derived CPCs. Abbreviations: CM=cardiomyocyte; SM=smooth muscle cell; EC=endothelial cell; ND=not described; CMC=cardiac mesenchymal cells; KSR=knockout serum replacement; LI-BPEL=low insulin, BSA, PVA, essential lipids; IQ1=wnt modulator.

Publication	Progenitor cell type/marker (s)	Differentiation potential	Expandability (*in vitro*)	Culture conditions	Clonality	Species
Moretti et al., 2006	Isl1$^+$	CM, SM, EC	Single expansion	CMC+DMEM/F12+B27+EGF	Yes	Mouse
Kattman et al., 2006	Flk1$^+$	CM, SM, EC	Passaged once	StemPro34+VEGF+bFGF+BMP4+DKK	ND	Mouse
Wu et al., 2006	NKX2-5$^+$	CM, SM	ND	ND	Yes	Mouse
Qyang et al., 2007	Isl1$^+$	CM, SM	Single expansion	Wnt3a-producing feeders or GSK inhibitor	ND	Mouse
Yang et al., 2008	KDR^{low}	CM (50%), SM, EC (30%)	Single expansion	StemPro34+VEGF+DKK+(bFGF)	Yes	Human
Bu et al., 2009	Isl1$^+$	CM, SM, EC	Single expansion	Wnt3a-producing feeders+DMEM/F12+5% KSR+B27+bFGF+EGF	Yes	Human
Domian et al., 2009	Isl1$^+$/NKX2-5$^+$	CM, SM	4-fold	ND	ND	Mouse
Moretti et al., 2010	Isl1$^+$	CM, SM, EC	ND	RPMI/B27+VEGF	ND	Human
Elliott et al., 2011	NKX2-5$^+$	CM, SM, EC	Single expansion	LI-BPEL+PDGFα+PDGFβ+Wnt3A+VEGF+bFGF+BMP4	Yes	Human
Nsair et al., 2012	Flt1$^+$/Flt4$^+$	CM, SM, EC	Passaged once	ESGRO+IQ1+ROCK inhibitor	Yes	Mouse
Ardehali et al., 2013	KDR$^+$/PDGFRα$^+$/ROR2$^+$/CD13$^+$	CM, SM, EC	Single expansion	StemPro34+FGF8+Wnt11+ROCK inhibitor	Yes	Human

strategy has served researchers well and allowed differentiation protocols to be improved and better understood, the approach can be variable in its efficiency due to the demanding number of specifying steps which must be made each time. Line-to-line variability can also be problematic. The option to work with lineage-restricted progenitors would have many advantages and efforts should continue with trying to make this feasible. For example, to define signalling pathways important in determining CPC fate choice, a pure population of CPCs would have the advantage of offering a defined extracellular environment in which to identify candidate agonists or antagonists. In terms of genetic manipulation, introducing transgenes or shRNA constructs in PSCs frequently leads to silencing during differentiation, whereas starting at the CPC stage should permit more consistent expression and could also allow the selection and expansion (and banking) of the best population. For disease modelling studies, having a bank of well characterized CPCs derived from mutant and control iPSCs/ESCs could greatly improve consistency in downstream measurements.

So what might be the factors preventing the expansion/maintenance of CPCs? One factor might be the developmental state. Most studies have focused on *Isl*1 and *Nkx2-5*-expressing cells, and it may be that cells at this stage are already engaging cell cycle arrest mechanisms and be naturally and inevitably progressing towards differentiation. Both of these genes regulate structural myocardial genes and so one would expect that their expression might at least need to be kept low to permit stable CPC proliferation. Supporting this hypothesis is data showing a negative feedback loop on CPC gene expression initiated by Nkx2-5 and a negative effect for Isl1 on CPC proliferation (Kwon et al., 2009; Prall et al., 2007). Indeed, the pro-proliferative effect of Wnt/β-catenin signalling on CPCs has been suggested to partly act through downregulation of *Isl*1 (Kwon et al., 2009). So looking at the proliferative potential of earlier developmental states may be more productive, although the lack of definitive markers for these stages as discussed earlier currently makes this challenging.

Identifying the optimal culture conditions will be important, and insufficient attention has been paid so far to the mitogens and morphogens already studied in the heart development field, as outlined in the section above, principally concerning FGF, BMP, SHH, IGF and Wnt. A combination of mitogenic stimulation and repressed differentiation signalling will be required to maintain cells in this transitional state. The correct extracellular matrix proteins may be important, just as it is for pluripotent cells, and the substrate stiffness can also be an important factor in regulating cell identity (Wells, 2008). Culture at physiological oxygen tension can improve the maintenance of stem cells and low oxygen conditions have already been shown to improve heart derived progenitor function (Ezashi et al., 2005; Van Oorschot et al., 2011).

To facilitate the maintenance of primary neural stem/progenitor cells, conditional overexpression of a *Myc* transgene has been employed (Kim et al., 2011), and one could envision a similar system being helpful for CPC maintenance at least to aid in the identification of the required culture conditions. Alternatively, specific cell cycle arrest mechanisms could be identified and these genes manipulated directly. Such a pragmatic approach could at least help to create a defined system to better understand CPC identity and help take us towards practical uses for these enigmatic yet potentially highly valuable cells.

Summary

PSC-derived CPCs have so far remained a transient and ill-defined cell population and this has hampered a thorough characterization of developmental patterning and cardiac cell fate decisions, as well as the practical opportunities for the system. Enormous benefit would be gained from the development of a human cardiac cell lineage fate map, with markers to identify cells at each stage, and a clear understanding of the signals and mechanisms regulating each transition. In this review we have aimed to highlight the wealth of knowledge from developmental studies in model organisms that should be used to help us address this challenge. However, the underlying message is that the exact genes conferring cardiac potential on CPCs still remain unknown and it is likely that some critical regulators of cardiac lineage commitment have not yet been identified. The human PSC system offers a tractable platform for identifying or confirming the human determinants, which should advance our control of cardiomyocyte differentiation and take us one step closer to therapeutic interventions for heart disease.

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