Islet1 cardiovascular progenitors: a single source for heart lineages?

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The creation of regenerative stem cell therapies for heart disease requires that we understand the molecular mechanisms that govern the fates and differentiation of the diverse muscle and non-muscle cell lineages of the heart. Recently, different cardiac cell types have been reported to arise from a common, multipotent Islet1 (Isl1)-positive progenitor, suggesting that a clonal model of heart lineage diversification might occur that is analogous to hematopoiesis. The ability to isolate, renew and differentiate Isl1\textsuperscript{+} precursors from postnatal and embryonic hearts and from embryonic stem cells provides a powerful cell-based system for characterizing the signaling pathways that control cardiovascular progenitor formation, renewal, lineage specification and conversion to specific differentiated progeny.

“Is myogenesis controlled by a single master gene? Possibly, combinatorial schemes and cell lineage are both used to generate positional identity in the embryo for each individual cell.”

Hal Weintraub and colleagues (Davis et al., 1987)

Introduction

Heart is muscle. Or so it would seem to the outside observer. From this vantage point, the discovery of MyoD almost 20 years ago by Weintraub, Lassar and colleagues (Davis et al., 1987) represents one of the seminal advances in our understanding of myogenesis, not only in skeletal muscle, but also in cardiac muscle. Subsequent studies by many investigators (for a review, see Srivastava and Olson, 2000; Olson, 2006) have underscored this point and have documented the conservation of the pathways and principles of myogenesis across muscle types, spanning the evolutionary spectrum from invertebrates to humans. This beautiful body of work has had major implications for our understanding of both development and disease, and has uncovered a host of genetic regulatory circuits that contribute to the control of cardiogenesis. As such, the molecular paradigm for cardiogenesis has largely been based on studies of skeletal myogenesis, an approach that has yielded spectacular advances in the field of cardiovascular science and medicine.

But is heart more than muscle? And if so, might there be another major paradigm that accounts for the generation of these diverse cell types, which include endothelial, smooth muscle and conduction system cells? Early cell lineage-tracing studies in avian systems indicated that a common muscle cell precursor exists for both working myocardium (atrial and ventricular myocardium) and the conduction system (Mikawa, 1999). At the same time, other studies identified a common progenitor, the hemangioblast, for endothelial and blood cell lineages, a finding which indicated that a common precursor might also exist for endothelial cells in the heart (Choi et al., 1998; Fehling et al., 2003; Kouskoff et al., 2005). The recent discovery of multipotent Islet1-positive (Isl1\textsuperscript{+}) progenitors in several species, including mouse, rat and human, and in different regions of the embryonic and adult heart, implies that there might be a stem cell paradigm for the generation of diverse cell lineages in the heart (Moretti et al., 2006). Unexpectedly, a growing body of evidence from multiple independent laboratories now suggests that, with respect to lineage diversification, the heart could be like blood, an organ in which a single stem/progenitor cell is able to generate all of the major cell types of the system (Kattman et al., 2006; Wu et al., 2006). The concept of progressive lineage restriction is well accepted for hematopoiesis but has not been established in such detail in the development of solid organs such as the heart. In this review, we explore this idea further by focusing on the Isl1 cardiovascular progenitor story, placing it into the context of the generation of diverse cardiovascular lineages, and discussing its implications for cardiovascular development and disease. The reader is also directed to reviews on the potential general role of progenitors in cardiogenesis and disease (see Buckingham et al., 2005; Chien and Karsenty, 2005; Srivastava, 2006; Black, 2007).

Cardiovascular cell lineages arise from discrete embryonic precursors

The heart is composed of diverse muscle and non-muscle cell lineages: atrial/ventricular cardiac myocytes, conduction system cells, smooth muscle/endothelial cells of the coronary arteries and veins, endocardial cells, valvular components and connective tissue (Fig. 1). During cardiogenesis, the differentiation of these multiple heart lineages is under tight spatial and temporal control, resulting in the coordinated formation of the distinct tissue components of the heart, including the four specialized chambers, diverse structures of the conduction system, the endocardium, the heart valves, the coronary arterial tree and the outflow tract (Harvey, 2002; Brand, 2003). Understanding how this diversity of heart cell lineages arises is a fundamental question that has major implications for understanding and treating both congenital and adult heart diseases, a subset of which have recently been shown to be due to defects in the pathways involved in heart lineage specification (Schott et al., 1998; Benson et al., 1999; Garg et al., 2003; Pashmforoush et al., 2004) (Table 1). In this regard, three major sources of heart cell precursors have been identified in the embryo: the cardiogenic mesoderm, the cardiac neural crest and the proepicardial organ (see Fig. 1A,B and glossary, Box 1). Each of these represents a spatially and temporally distinct pool of embryonic heart progenitors, which are known to give rise to distinct cardiac structures and cell components. The cardiogenic mesoderm forms the linear heart tube (see Box 1) and ultimately the bulk of the working myocardium in the ventricular
and atrial chambers. The cardiac neural crest migrates into the heart at a later stage of cardiac development, and gives rise to the vascular smooth muscle of the aortic arch, ductus arteriosus (see Box 1) and the great vessels; additionally, neural crest contributes to essential components of the cardiac autonomic nervous system (Kirby et al., 1983; Epstein and Buck, 2000). The mesenchymal portion of the developing heart and the majority of epicardial cells are derived from the proepicardium (Dettman et al., 1998; Moore et al., 1999; Manner et al., 2001). The epicardial mantle begins to envelop the myocardium at the same time that coronary precursor cells first appear in the heart tube (Mikawa and Gourdie, 1996) and retroviral genetic labeling experiments have shown that single vasculogenic cells of the proepicardium differentiate into solitary vessel-associated clusters that consist of three cell types: endothelial, smooth muscle and perivascular connective tissue cells (Mikawa and Gourdie, 1996). This evidence suggests that the coronary vasculature might be partially derived from mesodermal cells in the proepicardium, although the contribution of the proepicardium to the endothelial lineage is still controversial (Poelmann et al., 2002). Taken together, these studies on the contribution of the primordial heart field, cardiac neural crest, and of the proepicardium to specialized structures during cardiogenesis indicate that lineage diversification in the heart can arise, in part, via distinct pools of cardiovascular progenitors that are spatially and temporally segregated in the developing embryo (see Fig. 1B).

**Isil identifies the second heart field lineage**

One of the earliest steps in cardiogenesis is the formation of the cardiac crescent (Fig. 2 and see Box 1), which is derived from cells of the mesoderm that become instructed to adopt a cardiac fate in response to signals from adjacent tissues (Harvey, 2002; Srivastava and Olson, 2000). Recent studies have revealed that the cardiogenic
mesoderm in fact consists of two populations or fields (see Box 1) of cardiac precursor cells that contribute to different parts of the heart. The earliest population of cardiac progenitors, referred to as the first heart field, originates in the anterior splanchnic mesoderm, gives rise first to the cardiac crescent, later to the linear heart tube, and ultimately contributes to parts of the atrial chambers and the left ventricular region. The second cardiogenic region, known as the second heart field, lies anterior and dorsal to the linear heart tube and is derived from the pharyngeal mesoderm medial to the cardiac crescent (see Fig. 2A and Box 1). Cells from this second heart lineage are added to the developing heart tube and give rise to the outflow tract, the right ventricular region and the main parts of the atrial tissue (reviewed by Kelly and Buckingham, 2002; Buckingham et al., 2005). The discovery of the second heart field

Table 1. Gene mutations in human non-syndromic congenital cardiovascular malformations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Inheritance</th>
<th>Cardiac malformations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nkx2.5 (NK2 transcription factor related, locus 5)</td>
<td>Autosomal dominant</td>
<td>ASD, conduction system defects</td>
<td>(Schott et al., 1998; Benson et al., 1999; Goldmuntz et al., 2001; Elliott et al., 2003; McElhinney et al., 2003)</td>
</tr>
<tr>
<td>Gata4 (GATA binding protein 4)</td>
<td>Autosomal dominant</td>
<td>ASD, VSD</td>
<td>(Garg et al., 2003)</td>
</tr>
<tr>
<td>Tbx20 (T-box 20)</td>
<td>Partial penetrance</td>
<td>ASD, VSD, valve abnormalities, cardiomyopathy</td>
<td>(Kirk et al., 2007)</td>
</tr>
<tr>
<td>Jag1 (jagged 1)</td>
<td>Partial penetrance</td>
<td>TOF</td>
<td>(Krantz et al., 1999)</td>
</tr>
<tr>
<td>CrelD1 (cysteine-rich with EGF-like domains 1)</td>
<td>Partial penetrance</td>
<td>AVSD</td>
<td>(Robinson et al., 2003)</td>
</tr>
<tr>
<td>Notch1</td>
<td>Autosomal dominant</td>
<td>BAV, calcification</td>
<td>(Garg et al., 2005)</td>
</tr>
<tr>
<td>Myh6 (myosin heavy chain 6)</td>
<td>Autosomal dominant, partial penetrance</td>
<td>ASD</td>
<td>(Ching et al., 2005)</td>
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ASD, atrial septal defect; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; TOF, tetralogy of Fallot; VSD, ventricular septal defect.

**Fig. 2. First and second heart fields and their contributions to the developing heart.** (A) The upper drawings show the relative position, movement and contribution of the second heart field progenitors (green) relative to the first heart field cells (red) from the cardiac-crescent through to the looping stages of mouse heart development. The dashed lines indicate the position of the corresponding sections shown in the lower panels. (B) Location (upper) and contribution (beneath) of the second heart field progenitors (blue) to the outflow tract in the chick embryo. Cranial (Cr)-caudal (Ca), right (R)-left (L), and dorsal (D)-ventral (V) axes are indicated. DOFT, distal outflow tract; HFs, head folds; LA, left atrium; LV, left ventricle; ML, midline; PhA, pharyngeal arches; OFT, outflow tract; POFT, proximal outflow tract; RA, right atrium; RV, right ventricle.
reads like a scientific detective story, in which independent pieces of evidence were brought together to ultimately identify a prime suspect.

The initial evidence that the outflow tract was not present in the linear heart-tube stage came from a series of in vivo lineage-tracing experiments performed in chick embryos by de la Cruz and co-workers in the 1970s, which indicated that the outflow tract myocardium originates from a progenitor population that is different from the cardiac crescent and is situated anterior to the heart tube (reviewed by de la Cruz and Sanchez-Gomez, 2000). Over the past few years, the source of the outflow tract and the right ventricle has been addressed by studies from three different laboratories, two performed in chick embryos and one in mouse embryos (Waldo et al., 2001; Kelly et al., 2001; Mjaatvedt et al., 2001). The lineage studies in chick demonstrated that the splanchnic mesoderm adjacent to the pharyngeal endoderm migrates in through the aortic sac to contribute cells to the outflow tract and the right ventricle of the heart, and that the flow of these progenitors along this path gives rise to distinct outflow tract regions at different times during cardiac development (Mjaatvedt et al., 2001; Waldo et al., 2001) (see Fig. 2B). In mouse embryos, a lacZ reporter gene integrated into the genomic fibroblast growth factor 10 (Fgf10) locus marked the outflow tract and the right ventricle of the developing embryonic heart (Kelly et al., 2001). This transgene was expressed at the cardiac-crescent stage of heart development at embryonic day (E) 7.5, and β-galactosidase (β-gal) activity was observed in splanchnic mesoderm that lies medial to the classical first heart field. As development progressed, β-gal+ cells were found in anterior splanchnic mesoderm adjacent to pharyngeal endoderm and were subsequently observed in branchial arch mesoderm proximal to the heart, in the outflow tract and in the right ventricle. Taken together, these studies demonstrated that cells comprising the earliest fusing myocardium do not contain all of the progenitors of the outflow tract and the right ventricle and that these heart structures derive, wholly or in part, from precursor cells of a second heart field, which are added to and supplement the myocardium that originates from the first heart field progenitors (Kelly and Buckingham, 2002). Furthermore, an elegant retrospective clonal analysis (see Box 1) in the mouse embryo suggested that the first and second lineages (see Box 1) of cardiac progenitors originate from a common precursor population that segregates prior to the cardiac-crescent stage (Meilhac et al., 2004). However, a clear delineation of the existence of the second myocardial lineage as a separate subset of cardiovascular precursors, and the ultimate identification of the heart components that it generates, would await the identification of a suitable genetic marker.

In this regard, recent studies have revealed that the expression of the LIM-homeodomain transcription factor Isl1 is a marker of the second myocardial lineage during mammalian cardiogenesis (Cai et al., 2003), a finding that ultimately allowed the isolation of the Isl1+ cardiovascular progenitors themselves (Laugwitz et al., 2005). LIM/HD proteins are a subset of homeodomain (HD)-containing transcription factors that are defined on the basis of a common LIM domain, which consists of a conserved cysteine- and histidine-rich structure of two tandemly repeated zinc fingers. The acronym of LIM is derived from the first identified members of this family, namely LIN-11 from Caenorhabditis elegans (Frey et al., 1990), Isl1 from rat (Karlsson et al., 1990), and MEC-3 from C. elegans (Way and Chalfie, 1988). Subsequent studies of LIM/HD proteins in embryonic motoneurons have led to the identification of a combinatorial code of several LIM/HD proteins, including the Isl1 gene, that controls various aspects of motoneuron identity (Tsuchida et al., 1994; Thor et al., 1999; Thaler et al., 2004). Aside from its expression in embryonic motoneurons, Isl1 is expressed in a variety of cell lineages of pancreatic endocrine origin during embryogenesis, as well as in normal adult islet cells (Karlsson et al., 1990).

**Isl1** knockout mice have been generated and studied for defects both in motoneuron specification and pancreatic development (Pfäff et al., 1996; Ahlgren et al., 1997). Homozygous Isl1 mutants exhibit growth retardation around E9.5-10, and die at approximately E10.5-11. Histological analysis of mutant hearts between E9.0 and 9.5 revealed that homozygous Isl1 mutants have a severe cardiac phenotype. Isl1-null hearts fail to undergo looping morphogenesis and appear to have a common atrium and a uni-ventricular chamber, whereas the right ventricle and the outflow tract are absent (Cai et al., 2003).
al., 2003). Genetic marker analysis for expression of *Thx5*, *Hand1* and *Fgf10* demonstrated that the remaining ventricular tissue had a left ventricular identity and confirmed the lack of the right ventricle and the outflow tract.

These observations suggested that Isl1 is expressed in cells of the second heart field, which contribute to both the venous and arterial poles of the heart. Lineage tracing of Isl1-expressing cells using the Cre-loxP strategy showed that these cells colonize the outflow tract, the right ventricle, part of the atria and a minor portion of the inner curvature of the left ventricle, confirming that this transcription factor marks cardiac progenitors of the second myocardial lineage (see Box 1). Furthermore, Isl1 seems to be required for the survival, proliferation and migration of these cells into the cardiac tube, and its transcription is turned off as the precursor cells differentiate.

Since Isl1 expression delineates undifferentiated and differentiated progenitor states, it represents an excellent lineage tracer for cardiac mesodermal cells during embryogenesis.

Is Isl1 expression restricted to the second heart field of cardiac progenitor cells or is it also transiently expressed in the first? Two lines of evidence support the latter notion. First, recent lineage-tracing experiments that used a highly efficient *Isl1*-Cre knock-in mouse line showed that the majority of cells in the left ventricle express the genetic marker β-gal (Park et al., 2006). However, it is possible that the inefficiency of the excision of the original *Isl1*-IRES-Cre allowed cells in which *Isl1* was expressed for a longer period of time to be preferentially detected (Srinivas et al., 2001; Cai et al., 2003).

The second piece of evidence comes from recent studies in mouse and *Xenopus*. Harvey and co-workers report that, in contrast to *Isl1* mRNA, *Isl1* protein is expressed at E7.5 throughout the anterior intra-embryonic coelomic walls and proximal head mesenchyme, regions that encompass both first and second heart fields in mouse (Prall et al., 2007). Similarly, during neurula stages in *Xenopus*, *Isl1* is co-expressed with *Nkx2-5* throughout the cardiac crescent, which is the first heart field in amphibians (Brade et al., 2007). These data suggest that Isl1 might be a pan-cardiac progenitor marker, but additional work is needed to clarify this issue. In this regard, the identification of specific markers for the first heart field would be extremely valuable. However, analyses of the cardiovascular phenotype of Isl1 knockout mice suggests that Isl1 does not play such an essential role in the first myocardial precursor lineage as compared with the second. So far, the earliest molecular pan-cardiac markers for both myocardial cell lineages are the transcription factors Mespl/2 (mesoderm posterior 1/2) and Fgf8, which are expressed transiently in cells of the newly formed mesoderm at the primitive-streak stage (see Box 1), the descendants of which colonize the whole myocardium (Saga et al., 2000; Kitajima et al., 2003; Ilagan et al., 2005).

**Is11: an early nodal point in cardiogenesis**

A comparison of the cardiovascular defects that arise in *Is11*-deficient mouse embryos with those of mice with mutations in other cardiac transcription factors has begun to uncover a genetic regulatory network that controls the fate of Is11+ cardiovascular precursors in the second heart field (Table 2; Fig. 3).

The first and second myocardial lineages seem to be governed by both shared and distinct genetic programs. Myocardial regulatory genes that are activated in both lineages include the zinc-finger-containing transcription factors of the GATA family (*Gata4*, 5 and 6) (Charron and Nemer, 1999) and NK2 transcription factor related, locus 5 (*Nkx2-5*) (Harvey, 1996). Recent studies have identified *MeF2c* (myocyte enhancer factor 2C; also known as RSRF, related to serum response factor) as a direct transcriptional target of *Is11* and GATA factors in the second heart field (Dodou et al., 2004). The forkhead box H1 transcription factor Foxh1 is also expressed in the second lineage of cardiac precursors, and *Foxh1* mutant mouse embryos, like *Is11* and *MeF2c*-deficient embryos, display defects in the right ventricle and in outflow tract formation (von Both et al., 2004). *Is11* and Foxh1 directly activate transcription of *MeF2c* by regulating two independent enhancer regions in collaboration with GATA factors and *Nkx2-5* (Arceci et al., 1993; Dodou et al., 2004). Thus, these genes appear to act at the top of a cascade of cardiac transcription factors in the second myocardial lineage.

The SET-domain protein Smyd1 (Bop) is a direct target of MeF2c and regulates *Hand2* (heart and neural crest derivatives expressed transcript 2) expression during second heart field development, implying that Smyd1 is an indirect downstream target of *Is11/GATA* factors and Foxh1/Nkx2-5 (Gottlieb et al., 2002; Phan et al., 2005). Recently, it has been shown that Forkhead factors are central components of additional regulatory circuits that intersect and reinforce the *Is11-GATA-Mef2c* main pathway in the second heart lineage transcriptional network. Foxa2, Foxc1 and Foxc2 can bind and activate in vitro a *Tbx1* enhancer that is sufficient to direct expression to the second heart field (Maeda et al., 2006). *Tbx1*, in turn, activates *Fgf8* (Hu et al., 2004), whose loss-of-function in the second heart field results in a reduction of *Is11* expression in the pharyngeal mesoderm and outflow tract (Park et al., 2006).

Taken together, the early segregation of the two lineages, the different time-course of myocytic differentiation and the distinct regional contributions to the embryonic heart support the idea that the two progenitor populations may have discrete properties and distinct transcriptional hierarchies for cardiac development, with *Nkx2-5* as the critical transcription factor in the first and second lineages, and *Is11*, along with *Foxh1* and GATA factors, as the key transcriptional regulators in the second heart progenitor field (Biben and Harvey, 1997) (see Fig. 3).

Alternatively, one might question the existence of two unbridgeable progenitor lineages regulated by distinct transcriptional networks whose descendents form distinct heart compartments, and instead propose the complex patterning of one primordial precursor field (Abu-Issa et al., 2004). Such a view carries the implication that cardiac precursors within this solitary field have the capacity to form different heart structures, depending on positional cues. Temporal microenvironmental stimuli and differing concentrations of diffusing morphogens could create cellular diversity that leads to a progressive restriction of developmental potency within a field that was initially homogenous (Moorman et al., 2007).

**Is11** progenitor cells in the embryonic and postnatal heart

The purification, renewal and differentiation of native Is11+ cardiac progenitors provides a means to unravel the steps for both cardiac lineage formation and regeneration, and to identify how these steps are linked to certain forms of congenital and adult cardiac diseases. Taking advantage of the fact that Is11 expression is downregulated in most cardiac precursor cells as they differentiate, recent studies have utilized inducible *Is11*-Cre and knock-in *Is11-nlacZ* mice to analyze the timing of Is11+ progenitor migration into the looping heart and the distinct subdomains of the heart that they colonize during embryonic development (Laugwitz et al., 2005; Sun et al., 2007) (Fig. 4).

Interestingly, a subset of Is11+ undifferentiated progenitors remains embedded in the embryonic heart after its formation and a few cells are still detectable after birth in the compartments that arise from Is11+...
second lineage precursors during cardiac development. Tamoxifen-inducible Cre-lox technology has enabled this novel postnatal Isl1+ cell population and its progeny to be selectively marked at a defined time and purified to relative homogeneity (Laugwitz et al., 2005). The ability of these cells to self-renew in vitro on a cardiac mesenchymal feeder layer and to be stimulated to differentiate into fully mature functional cardiomyocytes indicates that these cells represent native cardiac progenitors, remnants of the embryonic Isl1+ precursors (Laugwitz et al., 2005). Postnatal Isl1-expressing cells can be detected in mouse, rat and human myocardium and appear to be distinct from the previously reported cardiac c-Kit+ and Sca1+ cells (Sca1 is also known as Ly6a), which were reported to activate cardiomyocyte-specific genes in vitro and to differentiate into cardiac muscle cells in vivo (Beltrami et al., 2003; Oh et al., 2003).

The Isl1 cell-based in vitro system, which can use both mouse embryos and differentiating ES cells as a source of Isl1-expressing cells, should facilitate the rapid and direct identification of the signaling pathways that guide the formation, renewal and diversification of Isl1+ progenitors into distinct heart cell lineages.

**Isl1+ cardiovascular progenitors and heart lineage diversification**

In vivo cell lineage tracing using the Cre-loxP strategy in the mouse has been an invaluable tool for precisely delineating the specific cell types that derive from Isl1+ precursors, as well as for defining their specific locations in the components of the heart, vascular and conduction systems. To date, several separate Cre mouse lines have proven to be useful for the in vivo lineage tracing of cardiovascular

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**Table 2. Mutant phenotypes of genes involved in second heart field development**

<table>
<thead>
<tr>
<th>Gene</th>
<th>SHF expression</th>
<th>Mutant phenotype in the cardiac tube</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isl1</td>
<td>SHF progenitors; weak in distal OFT</td>
<td>Embryonic lethal at E10. Single atrial and ventricular (LV identity) compartments; no RV or OFT; atria at the venous pole are abnormal</td>
<td>(Cai et al., 2003)</td>
</tr>
<tr>
<td>Mef2c</td>
<td>Early SHF progenitors</td>
<td>Embryonic lethal at E10. OFT reduced; RV does not develop (Hand2 is downregulated); INF abnormalities</td>
<td>(Lin et al., 1997; Dodou et al., 2004)</td>
</tr>
<tr>
<td>Hand2</td>
<td>Both FHF and SHF progenitors at cardiac crescent stage</td>
<td>Embryonic lethal at E10.5. Defective looping with RV abnormalities</td>
<td>(Srivastava et al., 1997)</td>
</tr>
<tr>
<td>Hand1/2</td>
<td>Yes</td>
<td>No ventricles; only atrial chamber forms</td>
<td>(Yamagishi et al., 2001; McFadden et al., 2005)</td>
</tr>
<tr>
<td>Foxh1</td>
<td>SHF progenitors and pharyngeal mesoderm at E9</td>
<td>Embryonic lethal at E10. OFF reduced or absent; RV does not develop; disrupted expression of Mef2c, Hand2, Fgf8 and Tbx5</td>
<td>(von Both et al., 2004)</td>
</tr>
<tr>
<td>Foxc1/c2</td>
<td>SHF progenitors at E8 and splanchnic and pharyngeal mesoderm</td>
<td>Embryonic lethal at E10.5. Severe defects in RV and OFT formation; abnormal coronary vessels; disrupted expression of Fgf10, Fgf8 and Tbx1</td>
<td>(Kume et al., 2001; Seo and Kume, 2006)</td>
</tr>
<tr>
<td>Smyd1</td>
<td>Precardiac mesoderm at E7.5</td>
<td>Embryonic lethal. Defective looping and single ventricular chamber</td>
<td>(Gottlieb et al., 2002)</td>
</tr>
<tr>
<td>Nkx2-5</td>
<td>Both FHF and SHF progenitors</td>
<td>Single atrial and ventricular compartments; loss of ventricular tissue; no Hand1 expression</td>
<td>(Lyons et al., 1995; Tanaka et al., 1999; Yamagishi et al., 2001)</td>
</tr>
<tr>
<td>Tbx1</td>
<td>Pharyngeal mesoderm</td>
<td>Perinatal lethal. Defective OFT and ventricular septation</td>
<td>(Xu et al., 2004; Hu et al., 2004)</td>
</tr>
<tr>
<td>Tbx20</td>
<td>Cardiac crescent stage</td>
<td>Embryonic lethal at E11.5. Failed looping; chambers do not develop; no Hand1 expression; hypoplastic RV; OFT disrupted</td>
<td>(Takeuchi et al., 2005; Singh et al., 2005; Stennard et al., 2005; Cai et al., 2005)</td>
</tr>
<tr>
<td>Fgf8</td>
<td>Yes</td>
<td>OFT defects</td>
<td>(Abu Issa et al., 2002; Brown et al., 2004)</td>
</tr>
<tr>
<td>Fgf10</td>
<td>Yes</td>
<td>No early phenotype detected</td>
<td>(Kelly et al., 2001)</td>
</tr>
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FHF, first heart field; INF, inflow region; OFT, outflow tract; RV, right ventricle; SHF, second heart field.

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![Fig. 3. Regulatory networks within the second heart field lineage](image)
progenitors from the first and second heart field. The Isl1-IRES-Cre line (Srinivas et al., 2001) has the advantage of driving Cre expression only in undifferentiated cardiac progenitors, but the disadvantage of being widely expressed later on in embryonic development. The inducible Isl1-mER-Cre-mER line offers temporal control over Cre activity (Laugwitz et al., 2005). In this line, the presence of the two mutated oestrogen-receptors (mERs) results in Cre being sequestered in the cytoplasm. In the presence of Tamoxifen, the mER-Cre-mER protein undergoes rapid nuclear translocation, which allows Cre-mediated recombination to then occur in those cells that express Isl1. A transgenic Cre mouse line that allows gene inactivation to be restricted to the second heart field lineage has been recently described in which Cre is expressed under a specific Mef2c promoter/enhancer region (Verzi et al., 2005). Nkx2-5-Cre and Mesp1-Cre deleter lines have also been well characterized, and can be utilized to trace lineages from both the first and second heart fields (Moses et al., 2001; Kitajima et al., 2000). Fate-mapping experiments utilizing these different Cre lines have demonstrated that Mef2c, Nkx2-5 and Mesp1 can mark cell populations that contribute to myocardial cells and to subsets of myocardial cells.
endocardium (Verzi et al., 2005; Stanley et al., 2002; Kitajima et al., 2000). Furthermore, the Cre-mediated lineage tracing of cells that express Flk1 (also known as Kdr), one of the earliest mesodermal progenitor markers for vascular endothelial and hematopoietic lineages, interestingly, shown the potential of Flk1+ precursors to give rise to both cardiac and smooth muscle during development (Motoike et al., 2003; Coulas et al., 2005).

To define the contribution of Isl1+ precursors to cardiac cell lineages in the adult heart, Isl1-IRESCre/R26R double heterozygous mice have been generated and analyzed (Fig. 5) (Laugwitz et al., 2005; Moretti et al., 2006). In these mice, the Cre-mediated removal of a stop sequence in the cells that express Isl1 results in the expression/translation of lacZ, which is itself under the control of the endogenous Rosa26 (R26) promoter. Thus, this approach marks all the cells that once expressed Isl1 during development, and its use has revealed that a high proportion of the right ventricular myocardium and parts of both atria consist of cells that express lacZ and the myocytic protein sarcomeric α-actinin (Laugwitz et al., 2005). The histochemical analysis of β-gal and acetylcholinesterase expression has revealed that Isl1+ progenitors make a remarkable contribution to the cells of the conduction system, primarily to the sino-atrial node, the pacemaker of the heart. Additionally, β-gal staining was observed throughout the proximal aorta, the trunk of the pulmonary artery and the stems of the main left and right coronary arteries (see Fig. 5). The co-expression of lacZ with endothelial and smooth muscle-specific markers, such as CD31 (Pecam1), VE-cadherin (cadherin 5) and smooth muscle myosin heavy chain (SM-MHC; also known as myosin heavy chain 11), revealed that Isl1+ precursors can give rise to vascular lineages. Additional studies in the mouse embryo have confirmed that Isl1+ cardiovascular progenitors contribute to over two-thirds of all the cells in the embryonic heart, and to the major cell types in all of the cardiovascular compartments and conduction system, with the exception of the free left ventricular wall (Cai et al., 2003; Moretti et al., 2006; Sun et al., 2007).

Taken together, these results demonstrate that Isl1 marks cardiac precursors that give rise to working cardiac muscle, to the conduction system and to endothelial/smooth muscle cells in multiple heart tissue compartments during cardiogenesis (see Fig. 5). Furthermore, this evidence raises the question of what role Isl1 plays in the specification of each of these mesodermal lineages. Is Isl1 marking an early anterior mesodermal progenitor not yet committed to a cardiac fate? Or is Isl1 defining a multipotent primordial cardiovascular progenitor, which contributes to distinct cell lineages within heart components known to originate from the second heart field? Or do different lineage-restricted precursors exist, all of which independently express Isl1? The early onset of Isl1 expression is consistent with the first two possibilities, although previous retroviral marking experiments have shown, for the proepicardial progenitors of the coronary vasculature, that clones of precursors can give rise only to a single phenotype (for example, only to epicardial cells, endothelial cells, smooth muscle cells or fibroblasts) (Reese et al., 2002). A clear delineation of the developmental potency of Isl1+ cells during cardiogenesis awaits in vivo clonal differentiation analysis.

**Multipotent Isl1+ cardiovascular progenitors**

Understanding how embryonic precursors generate and control the formation of distinct endothelial, pacemaker, atrial, ventricular and vascular smooth muscle lineages, as well as how these cells become positioned to form the specific chambers, aorta, coronary arteries and conduction system of the heart, is of fundamental importance for understanding the developmental logic and molecular cues that

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**Fig. 5. Cell lineage tracing of Isl1+ cardiac progenitors and lineage contribution to the adult heart.** Lineage tracing utilizing the Isl1-IRESCre mouse crossed with the indicator R26R line to mark all cells that once expressed Isl1 during development. The boxes represent magnifications of different regions of the coronary tree (black boxes) and of the aorta/pulmonary artery (red boxes), where β-gal-positive cells have been detected. (A) Contribution of Isl1+ progenitors (blue) to the coronary vasculature, the valves and the pulmonary artery/aorta. The contribution of Isl1+ cells to the endothelial (purple) and smooth muscle (yellow) cell layers is limited to the proximal area of the great vessels and progressively declines from the proximal to the distal parts of the coronary tree. β-gal+ cells were also detected in connective tissue structures of the aortic and pulmonary leaflets, indicating that components of the conotruncal cushions, which have an endocardial origin, are derived from Isl1+ progenitors. (B) Contribution of Isl1+ progenitors (blue) to the atrial/ventricular myocardium (red), the conduction system (white) and cardiac ganglia (green). In the conduction system, most genetically marked cells were detected in the sino-atrial nodal (SAN) region. AS, atrial septum; AVN, atrioventricular node; CAS, coronary arteries; LA, left atrium; LCA, left coronary artery; LV, left ventricle; RA, right atrium; RCA, right coronary artery; RV, right ventricle; VS, ventricular septum.
underlie both cardiovascular development and disease. As noted above, the formation of cardiac, smooth muscle and endothelial cell lineages in the heart has largely been ascribed to a set of non-overlapping embryonic precursors that have distinct origins. The discovery of several heart lineage-restricted genes has led to the suggestion that the generation of different cardiac cell types could be driven by a unique combinatorial subset of transcriptional networks that operate within distinct cardiovascular progenitors (for a review, see Srivastava and Olson, 2000). An alternative possibility exists that diverse muscle and non-muscle lineages arise from multipotent, primordial cardiovascular stem cells, which give rise to a hierarchy of downstream cellular intermediates that represent the tissue-restricted precursors of fully differentiated heart cells (Fig. 6). This clonal model of heart lineage diversification is similar to that of hematopoiesis, which is initiated by a few multipotent hematopoietic stem cells that generate large numbers of differentiated progeny by a process of amplification and progressive lineage restriction (Morrison and Weissman, 1994; Weissman, 2000).

Recent work from our laboratory has indeed proven the existence of multipotent Isl1+ cardiovascular progenitors (MICPs), which are marked by the transcriptional signature of Isl1, Nkx2-5 and Flk1. These cells can generate the three major cell types of the heart: cardiac, smooth muscle and endothelial cells (Moretti et al., 2006). MICPs have been cloned from both mouse ES cells and mouse embryos, and can make the decision to enter the muscle or endothelial differentiation pathways at the single-cell level, hinting at a hematopoietic-like system for how the diverse cardiovascular lineages can be generated (Moretti et al., 2006). In support of this concept, a hierarchy of distinct Isl1+ cardiovascular progenitors has been uncovered (Laugwitz et al., 2005; Moretti et al., 2006) (see Fig. 6), including a rare subset of Isl1+ cardioblasts that persists until birth and can develop into fully mature cardiac and smooth muscle cells (Laugwitz et al., 2005; Sun et al., 2007). At the same time, independent in vivo and in vitro studies from other laboratories have also identified other multipotent and bi-potent cardiovascular precursors (Kattman et al., 2006; Moretti et al., 2006; Wu et al., 2006), which might also arise from the early heart fields. However, the multi-lineage potential of MICPs still needs to be confirmed by fate-mapping techniques in vivo. Uncovering the molecular pathways that control the formation, renewal and differentiation of cardiovascular progenitors into specific mature cellular progeny will be crucial for unlocking the potential of stem cell therapy for use in a myriad of cardiovascular degenerative diseases, such as heart failure, conduction system disorders and congenital heart disease (see Table 1).

**Wnt/β-catenin signaling controls renewal of Isl1+ cardiovascular progenitors**

The microenvironment provided by a stem cell niche plays a vital role in stem cell/progenitor maintenance (for a review, see Scadden, 2006). Previous studies have shown that cardiac mesenchymal cells serve in vitro as an effective microenvironment that allows embryonic-, ES cell- and postnatal-derived Isl1+ progenitors to renew in culture (Laugwitz et al., 2005; Moretti et al., 2006). Utilizing a chemical screen, recent work from our laboratory has identified the Wnt/β-catenin pathway as being a major component of the cardiac microenvironment.
Congenital heart disease (CHD), which is one of the most important and prevalent forms of human birth defect, is present in nearly 1 in 100 live births and is responsible for the vast majority of prenatal losses (Hoffman, 1995). Some congenital cardiovascular malformations occur as syndromes in which multiple organs are affected. Over the past decade, a number of single-gene mutations have been correlated with syndromic CHDs, such as the association of mutations in Tbx1 with DiGeorge syndrome and of Tbx5 with Holt-Oram syndrome (for reviews, see Gruber and Epstein, 2004; Ransom and Srivastava, 2007). Although these syndromes have helped researchers to elucidate some of the mechanisms of CHD, most CHD occurs in the absence of any other organ malformation.

In humans, 50% of these non-syndromic CHDs manifest as failed atrial/ventricular septation or outflow tract defects, and necessitate open-heart surgery to restore normal circulation. A subset of non-syndromic CHD is familial, and causative genes have been identified, most of which encode transcription factors that are part of a conserved regulatory network that controls cardiogenesis (see Table 1). Mutations in NKX2-5 have been identified in individuals with atrial/ventricular septal defects and with conduction system abnormalities, whereas mutations in GATA4 and TBX20 are found in patients with septation defects, valve abnormalities and cardiomyopathy (Gruber and Epstein, 2006; Schott et al., 1998; Garg et al., 2003; Kirk et al., 2007). However, most non-syndromic CHD cases are sporadic and multifactorial, with no single gene being wholly responsible. Therefore, a major challenge is to dissect the transcriptional and signaling pathways that regulate cardiogenesis and to better understand at a genetic and mechanistic level how a cardiac progenitor cell can develop into each of the specific cell lineages that form the heart.

The identification of two distinct populations of cardiac precursors, one that exclusively forms the left ventricle and the other that mainly forms the outflow tract, the right ventricle and most of the atria, hints at a new approach to understanding CHDs, not as a defect in a specific gene or transcription factor, but rather as a defect in the lineage decisions of a defined subset of cardiac precursors. In this manner, CHDs might be associated with alterations in the formation, expansion and differentiation of embryonic cardiac progenitor cells, which in turn form essential components of the heart, such as the atria, ventricles, coronary arteries and conduction system (Goldmuntz et al., 2001; Pashmforoush et al., 2004; Prall et al., 2007). The discovery of a unique Isl1+ cardiovascular progenitor that contributes to all of these structures in the heart has important implications for understanding the mechanistic origins of CHD. In this regard, a series of studies of the localization of Isl1+ progenitors in patients with diverse forms of CHD (atresia of the aortic and mitral valve, transposition of the great arteries, ventricular septal defects and an interrupted aortic arch) have added important new insights into the potential role of Isl1+ progenitors in the closure of the atrial septum, in remodeling events that occur in the latest stages of cardiac morphogenesis or in the immediate postnatal window when the right ventricle starts to pump blood into the pulmonary circulation (Laugwitz et al., 2005). Moreover, the recent discovery of the role of β-catenin pathways in regulating the renewal and differentiation of Isl1+ cardiovascular precursors in vivo (Cohen et al., 2007; Kwon et al., 2007; Lin et al., 2007; Yang et al., 2007) is likely to have a significant impact on our understanding of several forms of CHDs that involve outflow tract defects, especially those implicating mal-rotations or outflow tract dysplasia.

### Isl1+ progenitors and congenital heart disease

Whereas the pivotal role of Isl1+ cardiovascular progenitor cells in the formation of the major heart lineages is clear, the importance of these cells in endogenous programs of cardiovascular regeneration is still unknown. Cardiomyocytes rarely seem to enter the cell cycle after birth and, consequently, the heart has a very limited regenerative capacity following injury. Given that the number of Isl1+ cells within the heart is vanishingly small after the postnatal window, it is unlikely that they play a role in the regeneration of the adult working myocardium. However, there is a persistence of the postnatal Isl1+ cells within the cardiac autonomic nervous system in regions that intersect with the cardiac conduction system, raising the question as to their role in the maintenance of normal cardiac conduction (Moretti et al., 2006; Sun et al., 2007). The location of Isl1+ cells in the embryonic/fetal structures that are associated with CHDs (outflow tract, inter-atrial septum, etc.) suggests that these cells might participate in regenerative pathways in these tissues in response to genetic or environmentally induced injury in the hearts of newborns. Uncovering the stimuli that might lead to the in vivo mobilization of Isl1+ cardiovascular progenitor cells will be of importance for understanding their contribution to endogenous regenerative pathways.

The ability to isolate and clonally expand multipotent Isl1+ cardiovascular progenitors from mouse ES cells encourages the hope that the same could be achieved in human ES cells. If so, it could form the basis for the generation of models of human CHD and of adult forms of heart disease. The technology to genetically manipulate human ES cells is moving forward rapidly, as are technologies that will allow the generation of ES cells via somatic cell nuclear transfer (SCNT) without requiring the use of eggs (Hochedlinger and Jaenisch, 2002). Moreover, recent work from several independent groups has demonstrated the possibility of reprogramming in vitro, differentiated mouse adult fibroblasts into a pluripotent ES cell-like state through the ectopic expression of only a few defined factors: Oct3/4 (also known as Pou5f1), Sox2, c-Myc and Klf4 (Maherali et al., 2007; Oktata et al., 2007; Takahashi and Yamanaka, 2006; Weinberg et al., 2007). This finding represents an important achievement in controlling pluripotency and might allow patient-specific pluripotent ES-like cells to be created directly from somatic cells, which could be a powerful tool for studying
human disease in a ‘culture dish’. The human ES cell-based ISL1+ cardiovascular progenitor model system could prove to be extremely valuable in designing new human-based assay systems for screening the toxicity of new drugs during the early stages of cardiac development, as well as for identifying and validating new therapeutic targets in specific human cardiovascular cell types, such as in coronary vascular smooth muscle, pulmonary arterial smooth muscle, coronary endothelial cells and conduction system cells.

Conclusion

In the search for master cardiovascular genes, it has become increasingly apparent that the generation of the diverse cell lineages of the heart is likely to be due to the employment of combinatorial codes for specific cell types, akin to the theory proposed by Weintraub and colleagues almost 20 years ago (Davis et al., 1987). At the same time, recent studies of ISL1+ cardiovascular progenitors suggest that a key element of lineage diversification in the heart relates to the presence of a renewable, rare subset of multipotent cells, perhaps a master heart progenitor, that ultimately gives rise to over two-thirds of the heart and to the heart’s three major cell types: cardiac muscle, smooth muscle and endothelium. The generation of specific cardiovascular cell types might ultimately relate more to a series of decisions that result in a sequential increase in the restriction of multipotent cardiovascular progenitors to specific cellular intermediates and their differentiated derivatives, than to the functioning of dominantly acting genes that enforce lineage specification. As such, understanding cardiogenesis at the level of specific decisions that are made by discrete heart cell lineages requires that we identify the pathways that govern critical steps in the formation, renewal, specification and differentiation of the hierarchy of ISL1+ progenitors and their derivatives. The identification of specific markers that enable FACS purification of these intermediates, and genetic approaches to reveal key steps in their cell fate decisions, will be essential. In short, if heart is like blood, then approaching cardiogenesis as ‘cardiopoiesis’ might represent the road ahead.

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