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Biology of *Isl1*$^{+}$ cardiac progenitor cells in development and disease

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Abstract. The LIM-homeodomain transcription factor Islet-1 (*Isl1*) marks a cell population which makes a substantial contribution to the embryonic heart. *Isl1* expression is downregulated as soon as the cells adopt a differentiated phenotype, suggesting that this transcription factor delineates a cardiogenic progenitor cell population. Taking advantage of this developmental lineage marker, we have identified in the postnatal heart a novel cardiac cell type, which is capable of self-renewal and readily differentiates into mature cardiomyocytes. Utilization of embryonic stem (ES) cells that harbour knock-ins of reporter genes into the endogenous *Isl1* locus will enable us to isolate *Isl1*$^{+}$ cardiac progenitors from mouse and human ES cell systems during *in vitro* cardiogenesis. These genetic cell-based systems should allow the direct identification of signalling pathways which guide formation, renewal and diversification of *Isl1*$^{+}$ cardiogenic progenitors into distinct heart cell lineages, and would complement *in vivo* studies in the mouse embryo during cardiac development.

Keywords. Cardiac development, embryonic stem cells, isl1+ cardiac progenitors, second heart field, heart lineages.

Introduction

The formation of a functional heart from cardiac precursor cells is a complex process that is tightly regulated. Expansion and migration of cardiogenic progenitor cells must be temporally and spatially coordinated to result in cardiac lineage specification and morphogenesis [1, 2]. A main goal in developmental cardiology has been to define signalling pathways and developmental environments required for specification and differentiation of cardiac precursors. Congenital heart disease most commonly arises from structural defects caused by errors in morphogenetic programs regulating heart and outflow tract development [3, 4]. In the past decade, classical gene targeting techniques have been invaluable in identifying genes that are involved in critical aspects of cardiogenesis, including *Nkx2.5* [5], *Gata4* [6-8], *Mef2* [9], *Tbx5* [10] and *Hop* [11, 12]. However, to unravel the logic of congenital heart disease, a deeper understanding of mechanisms governing key steps in the specification of diverse cardiac lineages will be required. The challenge will be to study these complex phenomena during cardiac development in the intact embryo *in vivo* and in cell-based systems *in vitro* to decipher the blueprint for building a heart.

Heart fields as sources of myocardial cells in the developing heart

A key event in cardiogenesis occurs when cardiogenic plate mesoderm responds to signals from adjacent tissues to adopt a cardiac fate [13]. Cardiac precursors in the cardiogenic crescent proliferate and migrate to

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the embryonic midline to form a linear heart tube. Rightward looping of the linear heart, followed by balloon-like growth of the outer curvature, generates the right and left ventricular chambers. In the last decade, a segmental model of heart development was proposed, in which progenitors of each segment of the heart, including the outflow tract, right ventricle, left ventricle and atrial chambers, were present at the early heart tube stage in an anterior to posterior arrangement [14]. However, earlier lineage studies utilizing radiolabelled tracers had demonstrated that outflow tract progenitors were not present in the linear heart tube, but appeared at later developmental stages [15]. More recent studies in both chick and mouse have revealed that a second population of cardiac progenitor cells exists in anterior pharyngeal regions [16, 17]. Called the secondary heart field because of its timing of differentiation, or the anterior heart field because of its location, anterior and dorsal to the linear heart tube, cells from this region were observed to contribute to the cardiac outflow tract. Examination of an Fgf10-lacZ transgene which marked pharyngeal mesoderm, outflow tract and right ventricle suggested that the anterior heart field might also contribute to the right ventricle [18].

Soon after the discovery of the secondary/anterior heart field, analysis of a mouse mutant for the LIM homeodomain transcription factor *Isl1* revealed an absence of outflow tract and right ventricle in *Isl1* null mice [19]. RNA transcript analysis of *Isl1* demonstrated expression in the pharyngeal endoderm and mesoderm, but not within the heart itself. These observations suggested a role for *Isl1* in the secondary/anterior heart field, which was confirmed by lineage studies utilizing *Isl1*-cre and R26R indicator mouse lines [20, 21]. Lineage tracing was facilitated by the fact that *Isl1* was not co-expressed with atrial myosin light chain 2 (*Mlc2a*), a marker of early cardiomyocyte differentiation, and demonstrated that *Isl1*$^{+}$ precursors contributed most or all cells to the outflow tract and right ventricle, consistent with *Isl1* marking the secondary/anterior heart field. Surprisingly, *Isl1* descendents were also observed to contribute a majority of cells to the atria, and a subset of cells within the left ventricle, suggesting that *Isl1* expression marked a broad range of undifferentiated cardiac progenitors, including but not restricted to those of the secondary/anterior heart field.

Subsequent, retrospective clonal analysis in the mouse embryo investigated lineage relationships between different compartments of the heart. This study confirmed the existence of two populations of myocardial precursor cells and suggested a common clonal origin before the crescent stage [22]. Buckingham and colleagues proposed that the two cardiac progenitor populations be termed the primary and second heart lineage, derived from the primary and second heart fields, respectively. Thus, the secondary/anterior heart field represents a subset of the second heart field. The early segregation of the two lineages and their distinct regional contribution to the heart could provide a potential explanation for cardiac abnormalities in humans and model organisms in which specific segments are underdeveloped or completely deleted, leaving the remainder of the heart unaffected. These discoveries put our understanding of congenital heart disease in a new perspective; heart malformations can be thought of as a cellular defect in a cardiac progenitor cell population, rather than of a defect in a specific gene or transcription factor [23].

Molecular aspects of *Isl1* in early cardiogenesis

A great deal of work in invertebrate and vertebrate models, including *Drosophila*, *Xenopus*, zebrafish, chick and mouse, has provided many insights into early cardiogenesis with many pathways being evolutionarily conserved. Heart formation requires precise control of **a**) specification of cardiac precursors within the mesodermal germ layer, **b**) self-renewal or proliferation of these cardiac committed cells and **c**) subsequent activation of genes responsible for differentiation and lineage specification into diverse cell types of the heart. Specific signalling molecules and tissue-specific transcription factors known to be required for these processes have recently been comprehensively reviewed [24–26]. Readers are directed to these reviews for a more in depth description of molecular pathways involved in cardiogenesis. Our intention here is to focus on the role of *Isl1* in early cardiac development and the potential use of this transcription factor as a marker to establish *in vitro* systems of cardiac progenitors for studying cardiac lineage specification and differentiation.

Isl1 was first reported to control various aspects of motoneuron identity in early embryogenesis [27-29]. Aside from its expression in embryonic motoneurons, *Isl1* is widely expressed in other cell lineages during embryogenesis. Consistent with its identification as an insulin enhancer binding protein, *Isl1* is expressed in a variety of cell lines of pancreatic endocrine origin [30]. *Isl1* knockout mice have been generated and studied for defects both in motoneuron formation and pancreatic development [31, 32]. Homozygous mutants for *Isl1* exhibit growth retardation around embryonic day (ED) 9.5–10, and die at approximately ED10.5–11. Heterozygous mice survive and have no obvious phenotype. Histological analysis of mutant hearts between ED9.0 and ED9.5 showed a misshapen single

ventricle as cause of death (Fig. 1a) [19]. Expression analysis for atrial myosin light chain 2 (*Mlc2a*), which marks all myocardial cells at this stage [33] and ventricular myosin light chain 2 (*Mlc2v*), which specifically identifies ventricular cells [34], demonstrated a single ventricular chamber and a severe reduction in atrial tissue. Using probes for *Tbx5* and *Hand1*, the ventricular portion of the *Isl1* mutant hearts was shown to have left ventricular identity. Additionally, the lack of expression of Fgf10 and Wnt11 in homozygous knockout hearts at ED9.5 compared with wildtype littermates substantiated the absence of the outflow tract and the right ventricle [18]. These observations, along with cell-lineage tracing experiments using Cre mice, which express the Cre recombinase under the *Isl1* promoter [20] and the reporter strain R26R [21], demonstrated that *Isl1* marks undifferentiated cardiac progenitors that contribute substantially to the embryonic heart, comprising cells of the outflow tract, right ventricle, both atria and a subset of cells within the left ventricle. The *Isl1*-expressing progenitors appear to correspond to the cells of the second heart field which will contribute to the second heart lineage. In addition to marking cells of the second heart field, *Isl1* seems to be required for their survival, proliferation and migration.

These studies showed that *Isl1*+ progenitors are located throughout the anterior-posterior extent of splanchnic mesenchyme, dorsal to the embryonic heart region (Fig. 1b). The first projecting segments of cardiogenic mesoderm at ED8.0–8.5, deriving from the first lineage of myocardial progenitors, do not appear to express *Isl1*. Since early cardiac crescent stages, *Isl1*+ progenitors migrate from splanchnic mesoderm adjacent to anterior endoderm into the forming heart from dorsal and medial regions, and at later stages from anterior and posterior poles, progressively differentiating as they join the cells of the first myocardial precursor lineage, which have already differentiated. Thus, the first and second lineages represent a continuum of differentiating cells of the original heart field: one population of myocardial precursors differentiates first to facilitate the pumping function of the heart early on, and will be later incorporated mainly into the ventral wall of the left ventricle and of the left atria; the second population contributes not only to anterior segments of the heart, the right ventricle and the outflow tract, but also to posterior portions, the atria and the dorsal wall of the left ventricle (Fig. 1b).

Isl1 transcription is turned off as precursor cells differentiate, leading to the hypothesis that *Isl1* remains expressed and is required in cells which need to continue to proliferate, expand and migrate into the forming heart. Since its expression demarcates undifferentiated and differentiated progenitor states, *Isl1* represents an excellent lineage tracer for cardiac mesodermal cells during embryogenesis.

Cardiac developmental pathways involving *Isl1*

The morphogenetic analyses of cardiac phenotypes thus far have identified many factors that are imperative to proliferation, migration, lineage specification and differentiation within cardiac progenitor cell populations. The cardiac phenotype of *Isl1* knockout mice suggests that primary and second myocardial lineages are governed by distinct cardiogenic programs and that the functional role of *Isl1* is more critical in the second myocardial lineage compared with the first. A transcription factor network in the second heart field is beginning to emerge with analysis of other mutant mice with defects in outflow tract or right ventricle development.

Embryos mutant for the transcription factors forkhead box H1 (*Foxh1*) or myocyte enhancer factor 2C (*Mef2c*) share similar phenotypes of reduced outflow tract and defective right ventricle formation [35]. The *Mef2c* gene has been shown to be a direct target of *Isl1* and *Gata4*, with consensus sites for these transcription factors in its enhancer elements [36]. Foxh1, on the other hand, requires physical interaction with *Nkx2.5* to activate a transforming growth factor-β (TGFβ)-responsive enhancer element in the *Mef2c* gene [35]. In addition, T-box 20 (*Tbx20*) synergizes with *Isl1* and *Gata4* to activate *Mef2c* and *Nkx2.5* enhancers [37]. *Mef2c* itself directly regulates the SET-domain protein *Bop*, which in turn mediates *Hand2* expression during development of the second heart lineage, implying that Bop is an indirect downstream target of *Isl1*/*Gata* and *Foxh1*/*Nkx2.5* factors [38]. Thus, these factors appear to act at the top of a cascade of cardiac transcription factors in the second myocardial lineage.

Conditional gene targeting using lineage-specific Cre lines would add additional insights into how these key factors relate to each other in a broader context. However, to define the precise role of a gene in these complex signalling networks, an *in vitro* cell-based system which could mimic the embryonic environment would provide an ideal complement to *in vivo* animal models. Decreasing the complexity of the developing embryos, *in vitro* cell systems offer the advantages of higher experimental flexibility and better control conditions.

Combined with lentiviral expression systems, cell-based systems can overexpress or 'knock down' the gene of interest with high efficiency, allowing for the assessment of its effect on myocyte specification and differentiation. Not only are cell-based systems suited

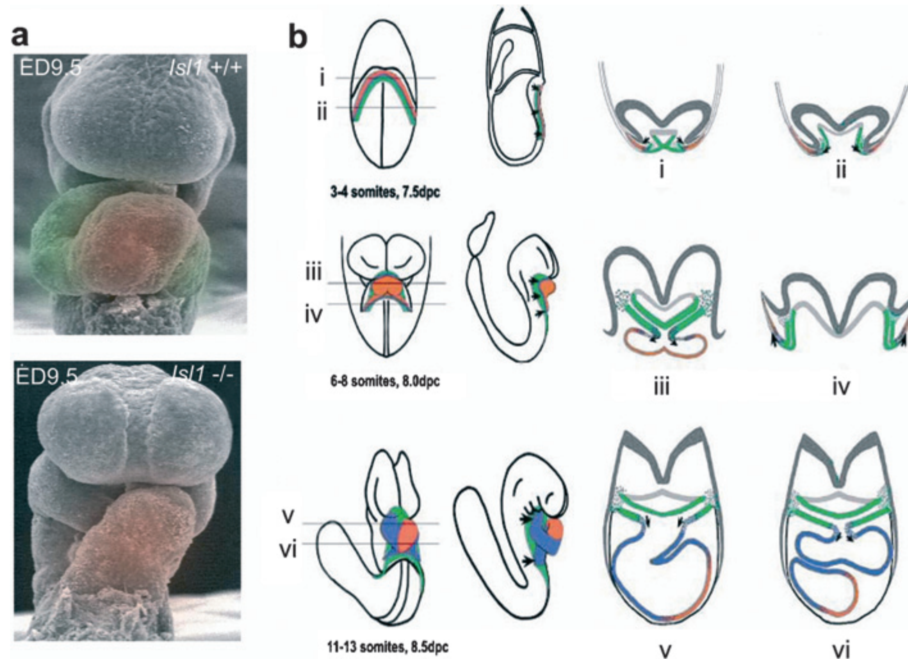

Figure 1. Contribution of *Isl1*$^+$ cardiac progenitors to the developing heart. **a**) Electron microscopy on wildtype (*Isl1* +/+) and *Isl1* knockout (*Isl1*–/–) mouse embryos at ED9.5. Highlighted in green in the wild-type embryo are the outflow tract, right ventricle, and the atria, regions that are missing in the *Isl1*–/– embryo. The red region designates cardiac structures which are derived from the primary heart field lineage. **b**) Embryos are shown in frontal (first column) and lateral (second column) views. Lines drawn through the frontal views indicate the anterior-posterior position of the corresponding sections (i–vi). *Isl1*-expressing cells are shown in green and *Isl1*$^+$-derived myocytes are marked in purple. Myocytes derived from non-*Isl1*-expressing progenitors are shown in red. Starting at ED7.5, *Isl1*$^+$ progenitors migrate from the splanchnic mesoderm into the forming heart from dorsal and medial regions and at later stages from anterior and posterior poles to give rise to the outflow tract, right ventricle, atria and a subset of cells in the left ventricle. Reproduced with the permission from reference [19].

for defining which external growth factors or specific cell-cell interactions are required for self-renewal, specification or differentiation, they offer the opportunity to use high-throughput technologies to perform these experiments in a manner that is faster, cheaper and more thorough. Additionally, *in vitro* systems of cardiac progenitors could open the possibility of studying the transcriptional and signalling networks regulating cellular events in human cells.

However, the isolation of specific cardiac progenitor cell populations has remained a major challenge directly related to the lack of available molecular markers that define the progenitor stage and distinguish it from the differentiating progeny.

Isolation, self-renewal and differentiation of *Isl1*$^+$ cardiac progenitor cells of the second lineage

Utilizing *Isl1* as a developmental lineage marker for undifferentiated cardiogenic precursor cells of the second heart field, we have identified in post-natal rat, mouse and human hearts a novel cardiac cell type whose distribution within the heart matches the defined contribution of *Isl1*$^+$ embryonic precursors (Fig. 2) [39]. Tamoxifen-inducible Cre/lox technology enables selective marking of this progenitor cell population, including its progeny, at a defined time, and purification to relative homogeneity. A cardiac mesenchymal feeder layer drives progenitor self-renewal, maintaining their ability to subsequently differentiate. Co-culture studies with neonatal myocytes indicate that *Isl1*$^+$ cells represent endogenous cardiac precursors that display highly efficient conversion to a mature cardiac phenotype with stable expression of myocyte markers and intact Ca^{2+} cycling in the absence of cell fusion. Electrophysiological analysis of *Isl1*$^+$ progenitor-derived cardiomyocytes showed in subsets of cells action potential characteristics of atrial, ventricular or conduction cells, suggesting the ability of *Isl1*$^+$ myocardial precursors to enter these lineages (Fig. 2).

A series of studies demonstrated the existence of *Isl1*$^+$ progenitors in the atrial septum of patients with diverse forms of congenital heart disease [39]. This finding suggests a potential role of *Isl1*$^+$ precursor cells in the formation of the atrial septum and perhaps in other remodelling events in the immediate post-natal physiological window.

Recent work from several groups suggests that the heart may contain other resident populations of

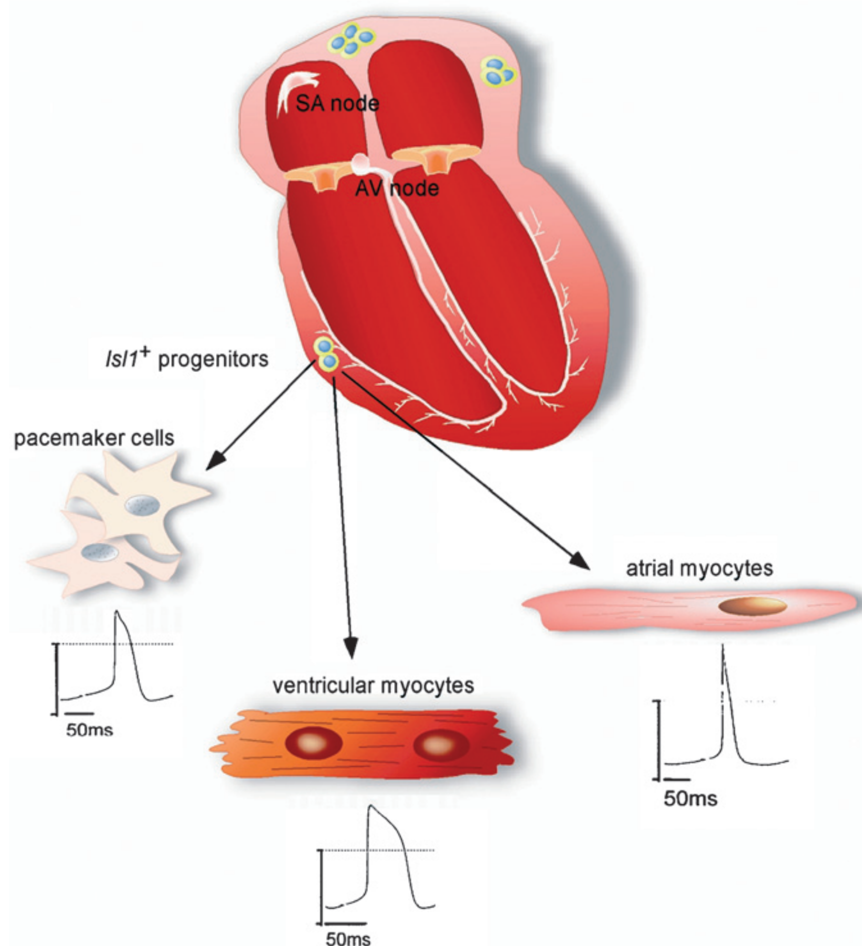

Figure 2. Model for lineage diversification into ventricular, atrial and conduction system lineages of postnatal *Isl1*+ cardiac progenitor cells. During *in vivo* cardiogenesis the LIM homeodomain transcription factor *Isl1* marks a population of cardiac progenitor cells. A rare subset of *Isl1*+ progenitors persists in the outflow tract myocardium, atria and myocardial wall of the right ventricle in rodent and human postnatal hearts. These cells have the potential to differentiate into atrial, ventricular and conduction system cells with their contractile and electrical properties.

progenitor cells with cardiomyogenic potential. *Sca1*- [40], c-*Kit*- [41] and *Abcg2*- [42] expressing cells have been isolated from the rodent heart, induced to activate cardiomyocyte-specific genes *in vitro* and to differentiate into cardiac muscle cells after injection into the injured myocardium *in vivo*. However, the expansion of these cells *in vitro* without loss of cardiogenic potential is very challenging, and their efficiency for entering the fully mature cardiac muscle phenotype in the absence of cell fusion is limited. The origins and developmental relationships of the different newly identified resident cardiac stem/progenitor cell populations needs to be further investigated. Various possibilities can be considered: **a**) persistence as undifferentiated remnants of the first and second heart field lineage, **b**) hematogenous origin from bone marrow or sites of early hematopoiesis (extra- and intra-embryonic blood-forming islands) or **c**) the proepicardial organ. To address these issues, genetic strategies of cell-fate mapping using different Cre reporter strains have to be employed during cardiac development.

Since *Isl1*+ progenitors can be isolated from post-natal and embryonic hearts and renewed on cardiac mesenchymal feeder layers, they represent a powerful cell-based system which allows rapid and direct characterization of signalling pathways controlling formation, renewal and lineage specification of cardiac progenitors of the second heart field (Fig. 3) [39]. The extension of this technology to chemical libraries and high-throughput screening opens the possibility of discovering small molecules that could regulate proliferation or differentiation of post-natal *Isl1*+ precursors resident in the heart. Furthermore, isolation of *Isl1*+ cells from patients or mouse models with diverse forms of congenital heart disease offers the opportunity to identify key signalling and transcriptional events that direct the differentiation program of *Isl1*+ progenitors into distinct cardiac lineages (Fig. 3). This cell-based model, in which genetic and developmental aspects of heart disease can be controlled, will provide an important scientific tool that could lead to a deeper mechanistic understanding of the cause of congenital heart defects and consequently to new treatment strategies.

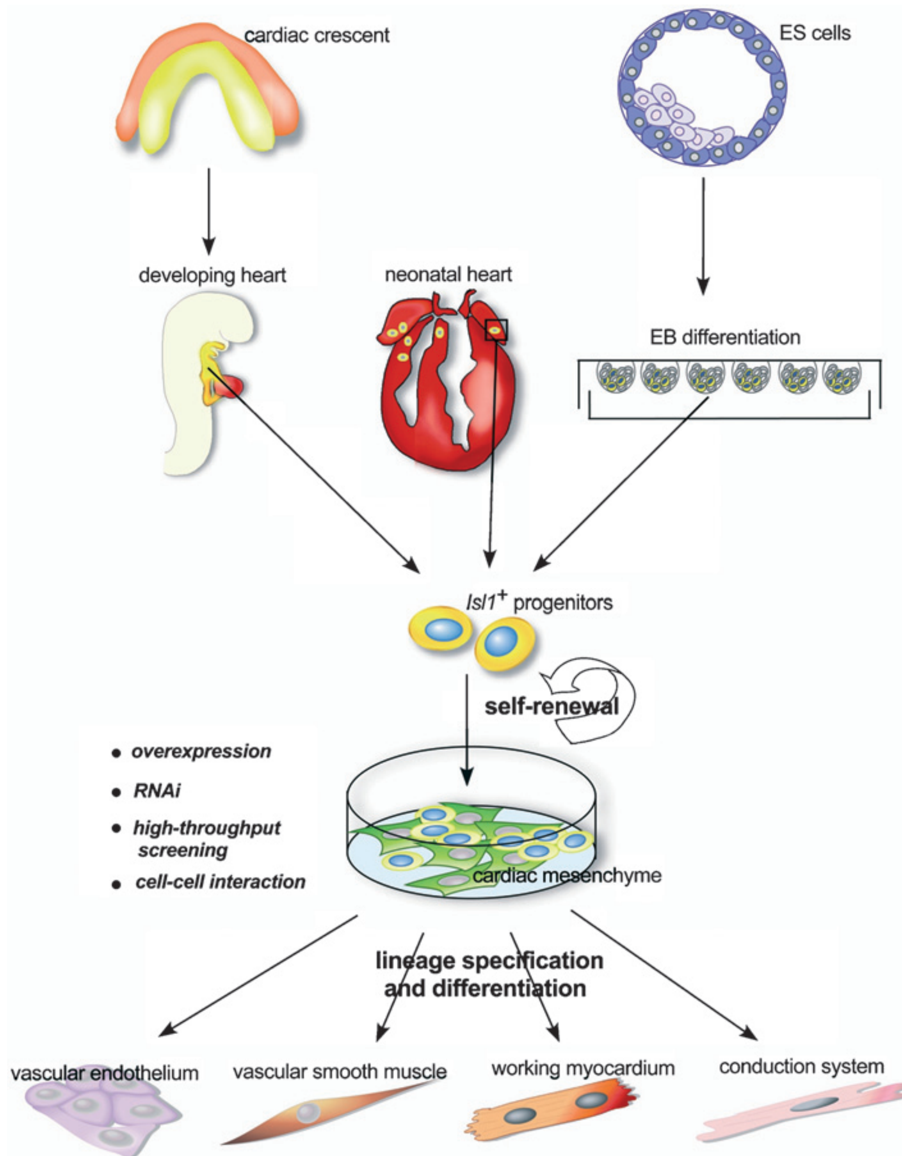

Figure 3. Outline of potential use of *Isl1*$^+$ progenitor to study signalling pathway for self-renewal, lineage specification and differentiation. *Isl1*$^+$ progenitor cells can be isolated from developing embryos, post-natal hearts, or ES cells of wild-type or genetically manipulated animals. High-throughput screening and inducible genetic systems can be utilized to study gain or loss of function of gene products to decipher signalling pathways for cellular function of *Isl1*$^+$ second heart field precursors.

In vitro cardiogenesis and the ES cell system

Pluripotent ES cells, when allowed to differentiate in culture, generate embryo-like cell aggregates called embryoid bodies (EBs) that contain lineage derivatives of all three germ layers [43, 44]. The ability to generate a wide spectrum of differentiated cell types from ES cells in culture represents a powerful tool for studying cell lineage formation, specification, maturation and differentiation. Several studies using murine ES cells demonstrated that molecular and cellular events associated with establishment of lineages, including hematopoietic [45], endothelial [46], neural [47] and skeletal muscle [48] present striking similarities between EBs and early embryos. These findings support the ES cell differentiation system as a valid *in vitro* model to study embryonic development. The recent successful isolation of human ES (hES) cells [49, 50] has opened the possibility of similar forays to those performed with their murine counterparts.

In the last decade, the ES/EB system has proved its significance in the cardiovascular field. Mouse and human ES/EBs allow the isolation of spontaneously contracting cardiomyocytes [51–53]. When compared with their *in vivo* counterparts utilizing gene expression analysis and action potential characterization, cardiomyocytes obtained from mouse ES cells closely resemble fetal stage cells [54, 55]. Similarly to mouse, human ES cell-derived cardiomyocytes show the expected molecular, structural and electrophysiological properties of nascent embryonic myocardial cells [56]. However, the efficiency of spontaneous cardiogenesis during EB differentiation of human ES cells varies with precise culture conditions as well as with the specific ES cell line employed [56, 57].

Although significant efforts have been made to optimize culture conditions for the generation of diverse cell types from ES cells, the identification and efficient isolation of specific, committed progenitor cells for different lineages within these cultures remains a challenge. To investigate the developmental processes that regulate mesoderm induction and specification, ES cell lines with green fluorescent protein (GFP) targeted to the *brachyury* locus have been generated [58]. *Brachyury* is expressed in all nascent mesoderm and downregulated as these cells undergo patterning and specification into the derivative tissues, including skeletal muscle, cardiac muscle and blood [59]. Analysis of GFP and Flk1 after EB differentiation of the genetically tagged cells led to the identification of a developmental progression of pre-mesodermal cells to hemangioblasts.

To track cardiac progenitor cells during *in vitro* cardiogenesis, a GFP knock-in into the genomic *Nkx2.5* locus has been developed [60]. Results obtained with this ES cell line demonstrate that *Nkx2.5*/GFP$^+$ cells possess the potential to differentiate into various myocyte cell types of the heart.

Isl1 represents an ideal marker for cells with early cardiac mesodermal commitment and could be utilized in the ES cell system to identify cardiac progenitors during *in vitro* cardiogenesis. Cardiac mesenchymal cells have been shown to maintain *Isl1* expression in the progenitor population and promote their expansion in culture without differentiation. If the *Isl1* renewal pathway is operative in ES cell-derived progenitors under the same culture conditions, it would open the possibility of obtaining clonal cardiac progenitor cell lines from mouse and human and allow the multipotentiality of *Isl1*$^+$ progenitors to be explored. Moreover, established progenitor cell lines will be most useful in gene targeting experiments to delineate signalling pathways involved in lineage specification, proliferation and differentiation (Fig. 3).

An important caveat of cell-based systems is extrapolation from the *in vitro* situation to *in vivo* cardiovascular development in the embryo: the pheno- and geno-typical identification of cells in differentiating ES cell cultures does not necessarily imply that the cells will function as anticipated *in vivo*. However, we believe that the cell-based systems mentioned above should prove to be complementary to each other as well as to animal models. *In vitro* data obtained with primary cultured *Isl1*$^+$ cardiac progenitors can be tested in ES cell-derived cardiac precursors by means of drug treatment, altered culture conditions or gene targeting. Furthermore, the *in vivo* contribution of the 'manipulated' ES cell-derived progenitors during embryonic development can be examined by blastocyst injection or embryo aggregation chimeras.

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