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Multipotent Embryonic *Isl1*$^+$ Progenitor Cells Lead to Cardiac, Smooth Muscle, and Endothelial Cell Diversification

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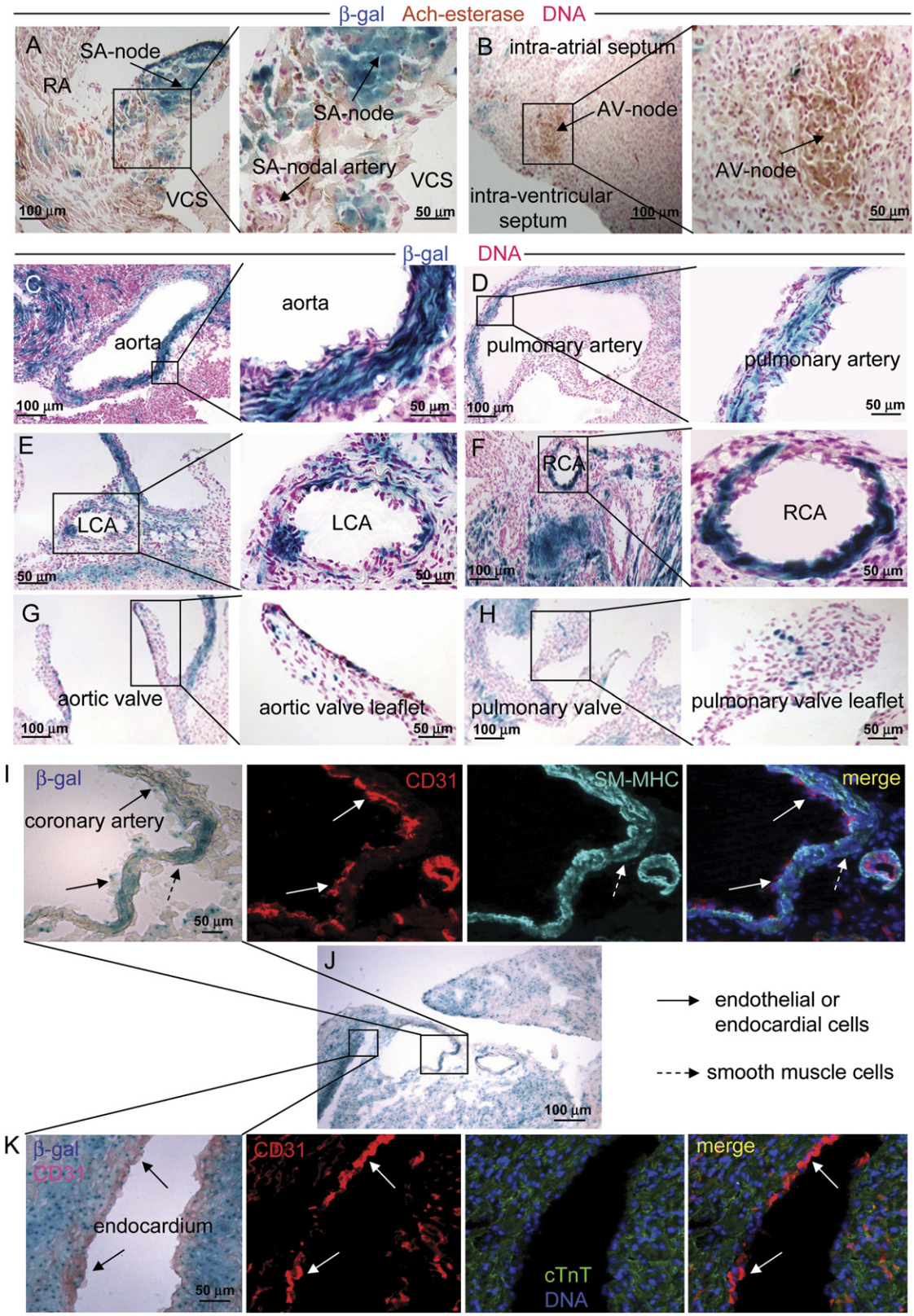
SUMMARY

Cardiogenesis requires the generation of endothelial, cardiac, and smooth muscle cells, thought to arise from distinct embryonic precursors. We use genetic fate-mapping studies to document that *isl1*$^+$ precursors from the second heart field can generate each of these diverse cardiovascular cell types in vivo. Utilizing embryonic stem (ES) cells, we clonally amplified a cellular hierarchy of *isl1*$^+$ cardiovascular progenitors, which resemble the developmental precursors in the embryonic heart. The transcriptional signature of *isl1*$^+$/*Nkx2.5*$^+$/*flk1*$^+$ defines a multipotent cardiovascular progenitor, which can give rise to cells of all three lineages. These studies document a developmental paradigm for cardiogenesis, where muscle and endothelial lineage diversification arises from a single cell-level decision of a multipotent *isl1*$^+$ cardiovascular progenitor cell (MICP). The discovery of ES cell-derived MICPs suggests a strategy for cardiovascular tissue regeneration via their isolation, renewal, and directed differentiation into specific mature cardiac, pacemaker, smooth muscle, and endothelial cell types.

INTRODUCTION

The formation of cardiac, smooth muscle, and endothelial cell lineages in the heart has largely been ascribed to a set of nonoverlapping embryonic precursors derived from distinct origins. Cardiac neural crest, the proepicardium, and the cardiac progenitors of the two heart fields are thought to follow separate parallel pathways for sequential lineage maturation (Mikawa and Gourdie, 1996; Manner et al., 2001; Waldo et al., 2001; Kelly and Buckingham, 2002; Stoller and Epstein, 2005). The discovery of several heart lineage-restricted genes suggests that the generation of different cardiac cell types might be driven by a unique combinatorial subset of transcriptional networks operating within distinct cardiovascular precursors (for review, see Srivastava and Olson, 2000). Nevertheless, an alternative possibility exists that diverse muscle and nonmuscle lineages arise from a single cell-level decision of multipotent, primordial cardiovascular stem cells, which in turn give rise to a hierarchy of downstream cellular intermediates representing tissue-restricted precursors for the fully differentiated heart cells. This clonal model of heart-lineage diversification would be analogous to hematopoiesis, in which a single hematopoietic stem cell can generate all of the blood-cell lineages (Morrison and Weissman, 1994; Weissman, 2000).

The recent identification of a second source of embryonic myocardial precursors has begun to modify the classical view of heart formation (Mjaatvedt et al., 2001; Waldo et al., 2001). The LIM-homeobox transcription factor islet-1 (*isl1*) delineates this second cardiogenic progenitor field (Cai et al., 2003; Laugwitz et al., 2005). In this regard, we have recently reported that after birth the mammalian heart harbors a rare subset of *isl1*$^+$ precursors in the atria, outflow tract, and right ventricle. The postnatal *isl1*$^+$ murine cells can be renewed on cardiac mesenchymal feeder layers and triggered into fully differentiated muscle cells, thereby fulfilling the criteria for endogenous


β-gal Ach-esterase DNA
A
SA-node
RA
VCS
100 µm
SA-node
SA-nodal artery
VCS
50 µm
B
intra-atrial septum
AV-node
intra-ventricular septum
100 µm
AV-node
50 µm
β-gal DNA
C
aorta
100 µm
aorta
50 µm
D
pulmonary artery
100 µm
pulmonary artery
50 µm
E
LCA
50 µm
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50 µm
F
RCA
100 µm
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G
aortic valve
100 µm
aortic valve leaflet
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H
pulmonary valve
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pulmonary valve leaflet
50 µm
I
β-gal
coronary artery
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CD31
SM-MHC
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J
100 µm
endothelial or endocardial cells
smooth muscle cells
K
β-gal
CD31
endocardium
50 µm
CD31
cTnT
DNA
merge

cardioblasts that are developmental remnants of the second heart field lineage (Laugwitz et al., 2005). Fate-mapping experiments have demonstrated that *isl1* and *Nkx2.5* can mark cell populations that contribute to myocardial cells, subsets of endocardium, and aortic endothelium (Cai et al., 2003; Stanley et al., 2002). Furthermore, Cre-mediated lineage tracing of *flk1*$^+$ cells have shown that both vascular endothelium and cardiac muscle arise from *flk1*$^+$ mesodermal progenitors during development (Motoike et al., 2003; Coultas et al., 2005). In both murine and avian systems, the smooth muscle layer of the proximal outflow tract originates from the second heart field lineage, while only the more distal regions of the aorta and pulmonary artery are derived from cardiac neural crest (Waldo et al., 2005; Verzi et al., 2005). Thus, there is a possibility that *isl1* marks a multipotent primordial cardiovascular progenitor, which gives rise to distinct cell lineages within the heart components known to originate from the second cardiogenic field (Buckingham et al., 2005).

Herein, by employing genetic fate-mapping techniques, we identify a subset of primordial multipotent *isl1*$^+$ cardiovascular progenitors (MICPs), which can indeed generate diverse cardiovascular cell types during in vivo embryonic heart development. The transcriptional signature of *isl1*$^+$/*Nkx2.5*$^+$/*flk1*$^+$ defines embryonic stem (ES) cell-derived cardiovascular precursors, which are multipotent and give rise to cardiac muscle, smooth muscle, and endothelial cells in vitro. Analogous triple-positive and dual-positive progenitors were identified in the developing embryo in vivo, which can be clonally amplified and differentiated similar to the ES cell-derived MICPs. These studies report the discovery of a novel subset of multipotent, embryonic *isl1*$^+$ progenitors that contribute to a majority of muscle cells and a subset of nonmuscle cells in the heart and suggest a new paradigm for cardiogenesis employing similar principles of stem/progenitor cell hierarchies as the hematopoietic system. Since MICPs can be isolated and selectively expanded from a renewable ES cell-based source, the findings point to a new strategy for cardiovascular tissue regeneration via the directed differentiation of ES cell-based MICPs into discrete cardiac, pacemaker, smooth muscle, and endothelial cell lineages.

RESULTS

isl1$^+$ Cells of the Second Heart Field Contribute to Smooth Muscle, Endothelial, Pacemaker, and other Nonmuscle Cell Lineages in the Postnatal Heart

In this study, we used *isl1*-IRES-Cre/R26R double-heterozygous animals to define the contribution of *isl1*$^+$ precursors to other cardiac lineages besides myocytes in the postnatal and adult heart (Figure 1). Histochemical analysis of β-galactosidase (β-gal) and acetylcholinesterase (Ach-esterase) activities revealed a remarkable contribution of *isl1*$^+$ progenitors to the sino-atrial (SA) node (Figure 1A), while only a few cells of the atrial-ventricular (AV) node seem to derive from *isl1*$^+$ precursors (Figure 1B). Additionally, β-gal expression was observed throughout the proximal aorta (Figure 1C), the trunk of the pulmonary artery (Figure 1D), and the stems of the main left and right coronary arteries (Figures 1E and 1F). β-gal$^+$ cells were detected in connective tissue structures of the aortic and pulmonary valve leaflets (Figures 1G and 1H), thereby indicating that components of the conotruncal cushions, which have an endocardial origin, are derived from *isl1*$^+$ progenitors. Coexpression of the genetic marker lacZ with endothelial and smooth muscle cell specific proteins, such as CD31, VE-cadherin, and smooth muscle myosin heavy chain (SM-MHC), demonstrated that *isl1*$^+$ precursors are capable to give rise to vascular lineages (Figures 1I and 2) and to cells forming the endocardium, the endothelial cell lining of the heart, in vivo (Figure 1K). To examine isl1-IRES-Cre-directed lacZ expression in vascular lineages in greater detail, we isolated cells from the endothelium and muscular layer of the aorta of *isl1*-IRES-Cre/R26R double-heterozygous mice and assayed β-gal directly by immunohistochemistry using an anti-β-gal antibody (Figures 2F–2M). Costaining for β-gal and specific endothelial and smooth muscle markers was observed in a significant proportion of cells, confirming a contribution of *isl1*-expressing cells to endothelial and smooth muscle lineages during outflow tract development. Although indications exist that some cells of the endocardium originate from the second heart field progenitors (Cai et al., 2003; Verzi et al., 2005), our results represent the first evidence that vascular endothelium arises from *isl1*$^+$ precursors.

Figure 1. Genetic Marking of *Isl1*$^+$ Progenitors and Their Progeny via Cre/Lox Technology

Frozen sections of hearts were obtained from isl1-IRES-Cre/R26R adult mice.

(A and B) β-gal (blue) and acetylcholinesterase (Ach-esterase, brown) activities in sections of the sino-atrial (SA) node (A) and the atrioventricular (AV) node (B) at low and high magnification. Nuclei (red) are counterstained with hematoxylin. RA, right atrium; VCS, vena cava superior.

(C–H) Low and high magnification of sections of the proximal aorta walls (C), the trunk of the pulmonary artery (D), the stems of the main left (E) and right (F) coronary arteries, and the aortic (G) and pulmonary (H) valves after X-gal staining (blue) and Nuclear Red counterstaining (red).

(I–K) LacZ reporter gene expression (blue) in a heart section showing coronary vessels and endocardium regions at low magnification (J). Higher magnification of a coronary artery portion (I) after X-gal stain (blue, left panel) and immunohistochemical staining on the following section for the endothelial marker CD31 (red) and the smooth muscle marker SM-MHC (cyan) are shown. Higher magnification of endocardium (K) after X-gal (blue) and CD31 (red) costain (left panel) and immunofluorescence analysis on the following section for CD31 (red) and the cardiac muscle marker cTnT (green) are shown. In the fluorescence images nuclei are detected by Hoechst 33258 (blue).

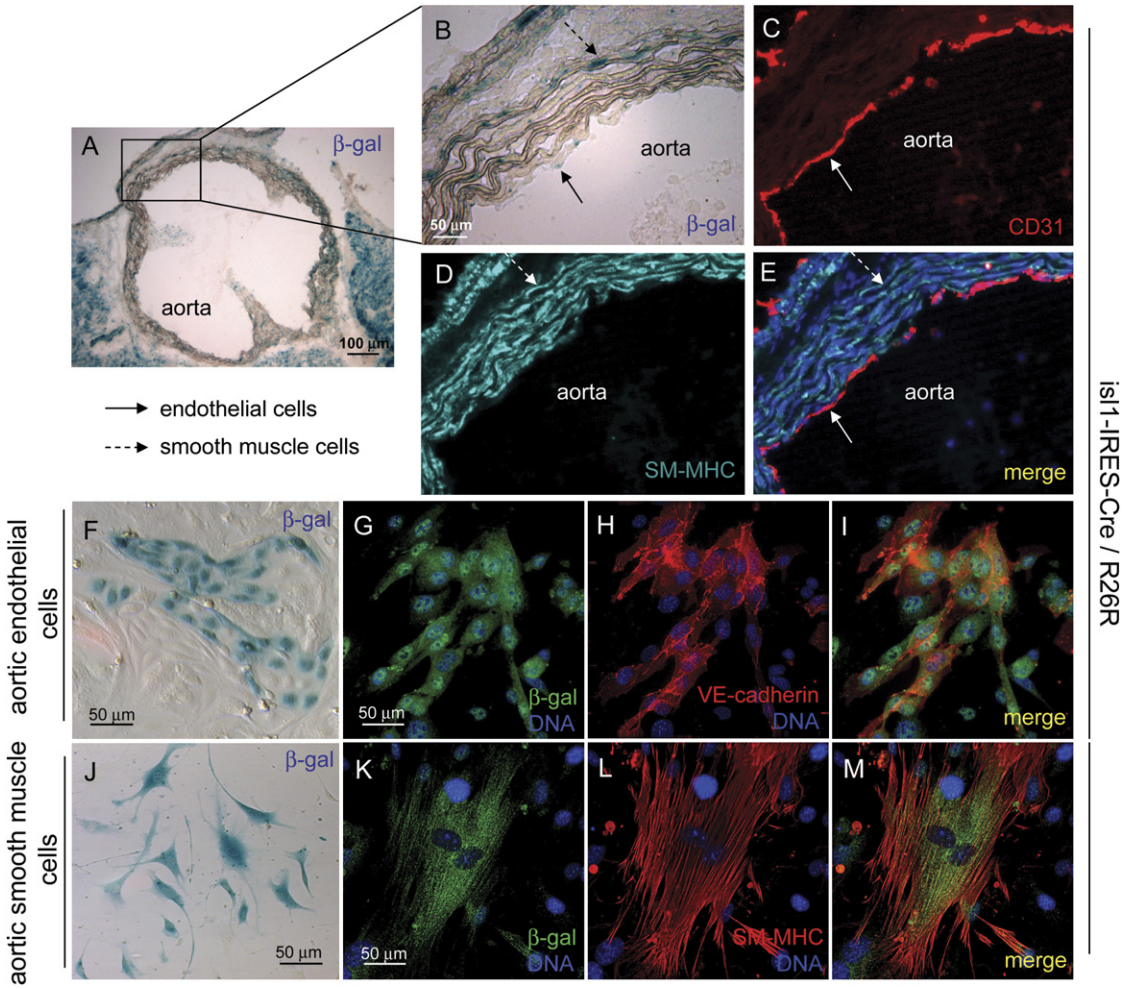

Figure 2. In Vivo Lineage Tracing and Fate Studies of Isolated Endothelial and Smooth Muscle Cells from Isl1-IRES-Cre/R26R Double-Heterozygous Mice

Endothelial and mural cells were isolated from aortas of adult mice and maintained for 3 days in culture, prior to β-gal expression and immunohistochemical analyses.

(A–E) Representative section of aorta at lower (A) and higher (B) magnification after X-gal stain. High magnification immunofluorescence images of the following section stained for the endothelial marker CD31 (red, C) and the smooth muscle marker SM-MHC (cyan, D) are shown. Nuclei are detected by Hoechst 33258 dye.

(F) β-gal$^+$ cluster of isolated endothelial-like cells detected by X-gal stain.

(G–I) Coexpression of β-gal and endothelial markers in isolated aortic cells, assessed by immunofluorescence using anti-β-gal (green, G) and anti-VE-cadherin (red, H) antibodies. Nuclei were visualized by Hoechst 33258 (blue).

(J) Isolated β-gal$^+$ cells with smooth muscle-like morphology.

(K–M) Coimmunostaining for β-gal (green, K) and SM-MHC (red, L) in isolated aortic cells. Nuclei were labeled with Hoechst 33258 (blue).

Cardiac neural crest also contributes to smooth muscle cells in the more distal regions of the outflow vessels, while smooth muscle in the proximal outflow tract are derived from the second heart field lineage (Epstein et al., 2000; Waldo et al., 2005; Verzi et al., 2005). Coronary vessels and the epicardium have a common developmental origin in the proepicardial organ, although its exact extent to the coronary tree remains to be determined.

A semiquantitative analysis of the in vivo lineage tracing results is presented in Table S1. Around 80%–90% of right ventricular myocardium and 50%–70% of the atria from double-heterozygous hearts displayed coexpression of β-gal and specific sarcomeric markers. In the conduction system the majority of genetically marked cells were detected in the SA nodal region. The contribution of *isl1*$^+$ cells to the endothelial and smooth muscle 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. Our genetic fate-mapping results clearly demonstrate that *isl1* marks a population of

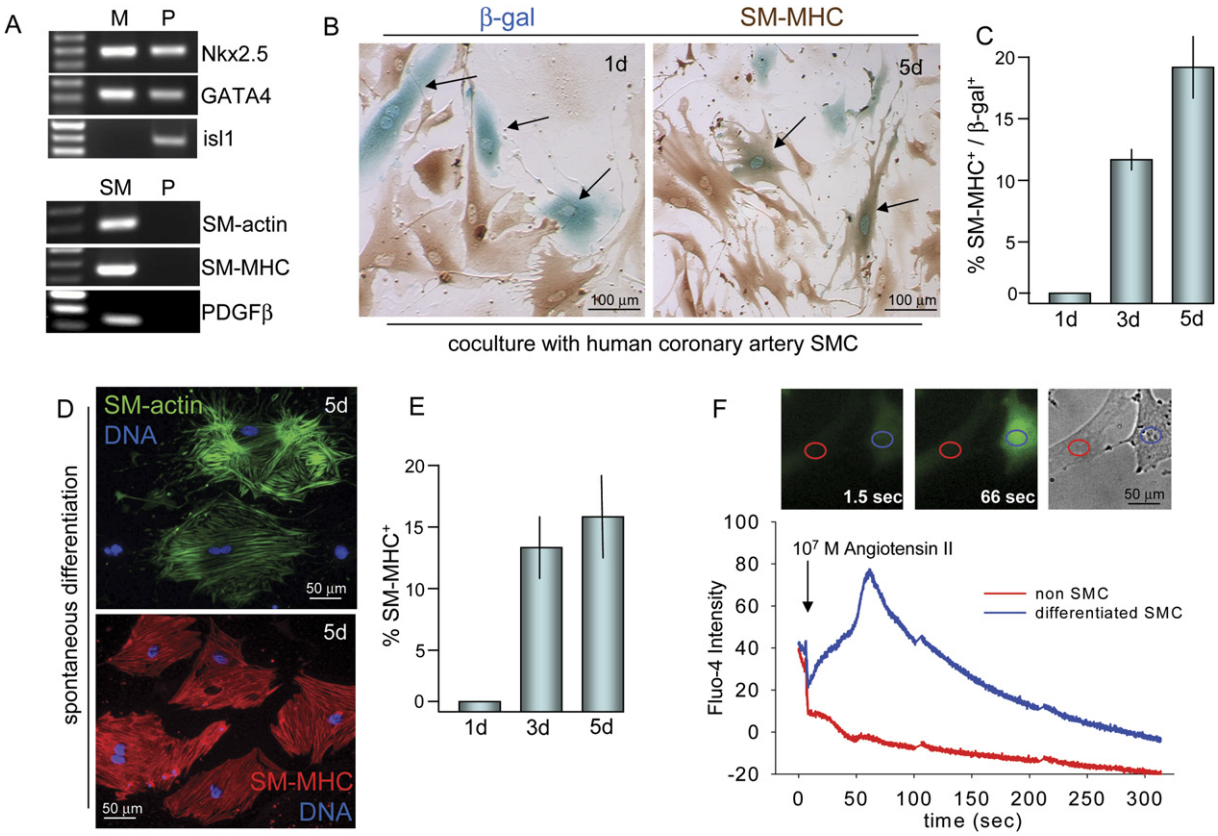

Figure 3. Cell Fusion-Independent Differentiation of *Isl1*$^+$ Postnatal Progenitor Cells into the Smooth Muscle Lineage

CMC fractions isolated from isl1-mER-Cre-mER/R26R double-heterozygous hearts were treated with 4-OH-TM and β-gal$^+$ precursors were purified by FACS sorting at 10 days in culture.

(A) RT-PCR analysis for smooth muscle and progenitor markers in FACS-sorted progenitors (P), neonatal myocytes (M), and smooth muscle cells (SM).

(B) Immunohistochemistry for SM-MHC (brown) after X-gal stain (blue) in coculture of β-gal$^+$ precursors and hca-SMC. Arrows indicate β-gal$^+$ cells before (1 day coculture) and after (5 days coculture) conversion into SMC. Costain for β-gal and SM-MHC results in a dark olive-green color.

(C) Quantification of differentiation events over time in coculture. Mean values ± SEM from three experiments (n = 1000 cells per group).

(D) Spontaneous conversion of *isl*$^+$ progenitors into SMC in vitro, assessed by expression of SM-actin (green) and SM-MHC (red) at 5 days in culture. Nuclei are detected with Hoechst 33528 (blue).

(E) Frequency of spontaneous differentiation of β-gal$^+$ progenitors into SM-MHC-expressing cells over time in culture. Mean values ± SEM from three experiments (n = 1000 cells per group).

(F) $[Ca^{2+}]_i$ measurements after Angiotensin II stimulation in a representative *isl1*$^+$ progenitor, which spontaneously converted into an SMC (blue trace) and in one that did not acquired the SMC phenotype (red trace). Fluorescence images show fluo-4 intensity immediately after Angiotensin II application (1.5 s, left panel) and at the peak of the calcium response (66 s, middle panel). Bright field image of the two measured cells (right panel). Circles indicate the regions of interest used for measuring fluo-4 intensity.

precursors, which give rise to a subset of working cardiac muscle, pacemaker, endothelial, and smooth muscle cells in multiple heart-tissue compartments.

Spontaneous, Cell Fusion-Independent Differentiation of *Isl1*$^+$ Progenitors into the Smooth Muscle Lineage

To assess the differentiation potential of postnatal *isl1*$^+$ progenitors into vascular cell lineages, we isolated β-gal$^+$ precursors from *isl1*-mER-Cre-mER/R26R animals, as previously described (Laugwitz et al., 2005). After exposure of the culture to 4-hydroxytamoxifen (4-OH-TM) to induce specific marking of *isl1*-expressing cells, we purified the β-gal$^+$ progenitors by fluorescence-activated cell sorting (FACS) using the fluorogenic β-gal substrate C_{12}FDG, and performed coculture experiments with low-passage human coronary artery smooth muscle cells (hca-SMC). As shown in Figure 3A, FACS-sorted precursors expressed *isl1* and the early specification markers for cardiac mesoderm, *Nkx2.5* and *GATA4*, while lacking transcripts of mature smooth muscle cells. After 5 days in coculture, ~18% of the β-gal$^+$ cells colabeled with SM-MHC in a staining pattern similar to that of the hca-SMC (Figures 3B and 3C). Interestingly, even in the absence of the coculture environment a significant proportion of β-gal$^+$ progenitors converted spontaneously in vitro into functional

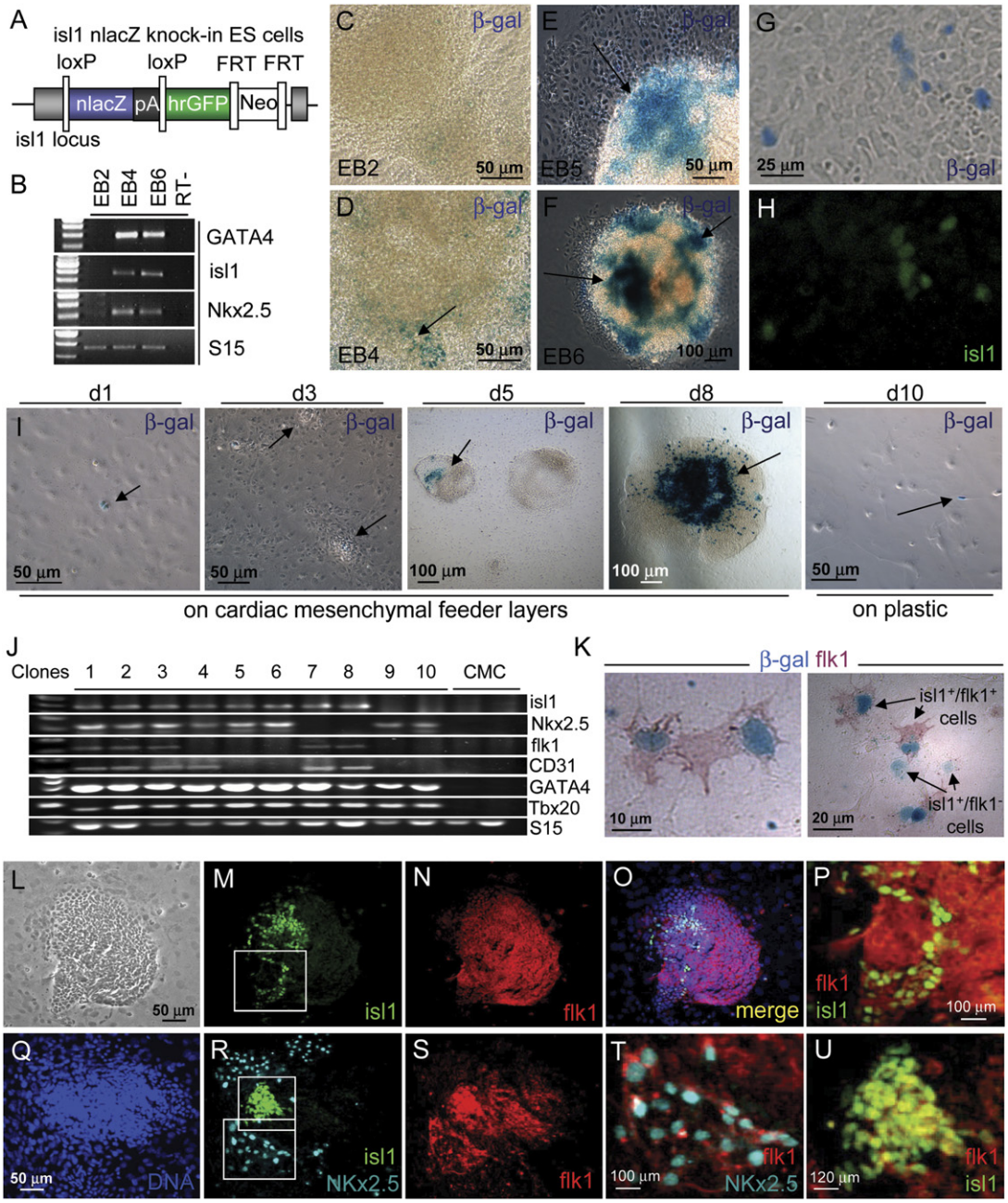

Figure 4. ES Cells as a Source for *Isl1*$^+$ Cardiac Precursor Cells

(A) Schematic diagram of the *isl1* targeted locus in the isl1-nLacZ knockin ES cell line.

(B) Expression analysis of *isl1* and other cardiac progenitor markers by RT-PCR in EBs from isl1-nLacZ knockin ES cells at the indicated days of differentiation.

(C–F) LacZ reporter gene expression assessed by X-gal stain in EBs from isl1-nLacZ knock-in ES cells at days 2 (C), 4 (D), 5 (E), and 6 (F) of differentiation.

(G and H) β-gal activity correlates with *isl1* expression in EBs from isl1-nLacZ knockin ES cells. β-gal$^+$ nuclei after X-gal stain (blue, G) costaining for *isl1* protein (green fluorescence, H).

(I) Selective amplification of ES cell-derived *isl1*$^+$ progenitors on CMC feeder layer. EBs from isl1-nLacZ knockin ES cells were dissociated at 5 days differentiation and single cells were plated on CMC feeder layer or plastic. β-gal activity was tested at day 1, 3, 5, and 8 on the CMC co-culture and at day 10 on plastic.

(J) Expression analysis of cardiovascular specification genes in 10 representative clones grown on CMC for 7 days (lanes 1–10) and in control CMC (last two lanes). Clones can be classified by RT-PCR profile into 4 main groups: *isl1*$^+$/*Nkx2.5*$^+$/*flk1*$^+$ (clones 1–3), *isl1*$^+$/*Nkx2.5*$^+$/*flk1*$^-$ (clones 4–6), *isl1*$^+$/*Nkx2.5*$^-$/*flk1*$^+$ (clones 7 and 8), and *isl1*$^-$/*Nkx2.5*$^+$/*flk1*$^-$ (clones 9 and 10).

smooth muscle cells, as demonstrated by the expression of smooth muscle-specific markers (Figures 3D and 3E) and by the response to the vasoactive hormone Angotensin II (Figure 3F). In 4 of 25 measured cells, exposure to Angiotensin II induced a progressive cytosolic $[Ca^{2+}]_i$ increase, which reached the maximum at ~70 s and diminished thereafter, analogous to the agonist-induced vascular SMC $[Ca^{2+}]_i$ transients (Figure 3F).

Thus, postnatal *isl1*$^+$ cardiac progenitors can adopt the functional properties of smooth muscle cells in the absence of cell fusion in vitro. An experimental strategy was then designed to assess whether single cell-derived clones of *isl1*$^+$ progenitors might generate cardiac, smooth muscle, and endothelial cell lineages.

ES Cells as a Source for *Isl1*$^+$ Cardiac Precursors

In order to establish an induction and purification system for cardiac *isl1*$^+$ precursors from ES cells, we generated *isl1*-nlacZ knockin ES cells in which a loxP-flanked nuclear lacZ gene, followed by hrGFP, was targeted to the genomic *isl1* locus (Figure 4A). When allowed to differentiate in culture, ES cells generate embryoid bodies (EBs) that contain a broad spectrum of cell types representing derivatives of the three germ layers (Smith, 2001). We analyzed the time course of *isl1* expression in developing EBs from *isl1*-nlacZ knockin ES cells by RT-PCR and β-gal staining (Figures 4B–4F). In undifferentiated ES cells and early EBs, *isl1* expression was not detected on mRNA and protein level (Figures 4B and 4C). Within 4–6 days of EB differentiation, ES cell-derived progenitors expressing *isl1* arose, as demonstrated by transcript detection and β-gal activity (Figures 4B and 4D–4F). Immunohistochemistry using a monoclonal anti-*isl1* antibody revealed coexpression of *isl1* and β-gal proteins, indicating that *isl1* gene expression can be monitored by lacZ staining (Figures 4G and 4H).

ES Cell-Derived *Isl1*$^+$ Cardiac Progenitors Can Be Clonally Expanded on Feeder Layers of Cardiac Mesenchyme

Several markers of early cardiogenic progenitors, including *Nkx2.5*, *GATA4*, and *GATA6*, continue to be expressed in differentiated cardiomyocytes and thus do not allow one to distinguish between progenitors of the crescent stage and differentiated cardiomyocytes (Buckingham et al., 2005). *Isl1*, a cellular marker of the second myocardial lineage, is downregulated as soon as the cardiac progenitors enter a differentiation program. This feature makes it a suitable marker for isolation of cardiac precursors from mammalian ES cell systems. However, *isl1* is broadly expressed in many cell lineages during embryogenesis (Karlsson et al., 1990; Thor et al., 1991). We previously established a cardiac mesenchyme culture system that allows the maintenance of *isl1* expression in the postnatal cardiac progenitor population and promotes their renewal in culture without differentiation (Laugwitz et al., 2005).

To test whether the mesenchyme environment could support expansion of *isl1*$^+$ cardiac precursors arising during EB differentiation, we dissociated EBs from *isl1*-nlacZ knock-in ES cells at day 5 into single cells and plated them at low density on feeder layers of cardiac mesenchymal cells (CMC) and mouse embryonic fibroblasts (MEFs) (Figure 4I). After 1 day, we observed single or dividing β-gal$^+$ cells in the CMC coculture, but none were detected on MEFs (data not shown). Within 5 days, clones with a distinct morphology were visible exclusively on top of the CMC feeders, and around 40% ± 10% presented β-gal activity in a characteristic focal pattern, reflecting that the clones originated from a single expanding β-gal$^+$ cell. Mock treatment by plating dissociated cells from day 5 EBs on plastic or gelatin resulted in attachment and survival of a small number of cells without any clone formation (Figure 4I).

Transcriptional profiling of 80 clones following expansion on CMC feeder layers revealed that all of them express early cardiac specification markers *GATA4*, *Tbx20*, and either *isl1* and/or *Nkx2.5* (Figure 4J). Interestingly, in a proportion of *isl1*-expressing clones we detected the transcript for *flk1*. *Flk1* is the type-2 receptor for the vascular endothelial growth factor (VEGF) (Yamaguchi et al., 1993), and one of the earliest common mesodermal differentiation markers for vascular endothelial and hematopoietic cells (Millauer et al., 1993; Shalaby et al., 1995; Shalaby et al., 1997). However, recent evidence suggests that *flk1*$^+$ cells also exhibit a differentiation potential for other mesodermal lineages such as cardiac muscle during development (Motoike et al., 2003; Ema et al., 2006). Immunohistochemical analysis revealed *flk1* protein on the extracellular membrane of single β-gal$^+$ cells growing on the CMC feeder layers (Figure 4K), suggesting that *isl1*-expressing precursors derived from ES cells could have the potential to differentiate into the endothelial lineage. After clone expansion, examination of *isl1*, *Nkx2.5*, and *flk1* expression demonstrated the existence of subsets of double-positive cells within a clone: *isl1*$^+$/*flk1*$^+$, *isl1*$^+$/*Nkx2.5*$^+$, and *flk1*$^+$/*Nkx2.5*$^+$ cells were found in different proportions depending on the time in culture (Figures 4L–4U). These results indicate that CMC feeders act as a prespecification matrix toward an early cardiac precursor state and open the possibility to investigate whether the multipotentiality of *isl1*$^+$ cardiac progenitors is based on a single cell-level decision.

(K) Immunohistochemistry for *flk1* (purple) after X-gal stain (blue) in representative ES cell-derived *isl1*$^+$ cardiac precursors on CMC at day 2.
(L–U) Representative clones of ES cell-derived cardiac progenitors on CMC at day 6, after immunofluorescence analyses for the transcription factors *isl1* (green) and *Nkx2.5* (cyan) and the surface marker *flk1* (red). Nuclei are visualized by Hoechst 33528 (blue). A clone containing cells costaining positively for *isl1* and *flk1* is shown at 20× (L–O) and at 63× magnification (P). Low magnification images (Q–S) of a different clone presenting double positive *isl1*/*flk1* and *Nkx2.5*/*flk1* cells, which are marked in (R) and shown at 63× in panels (T) and (U).

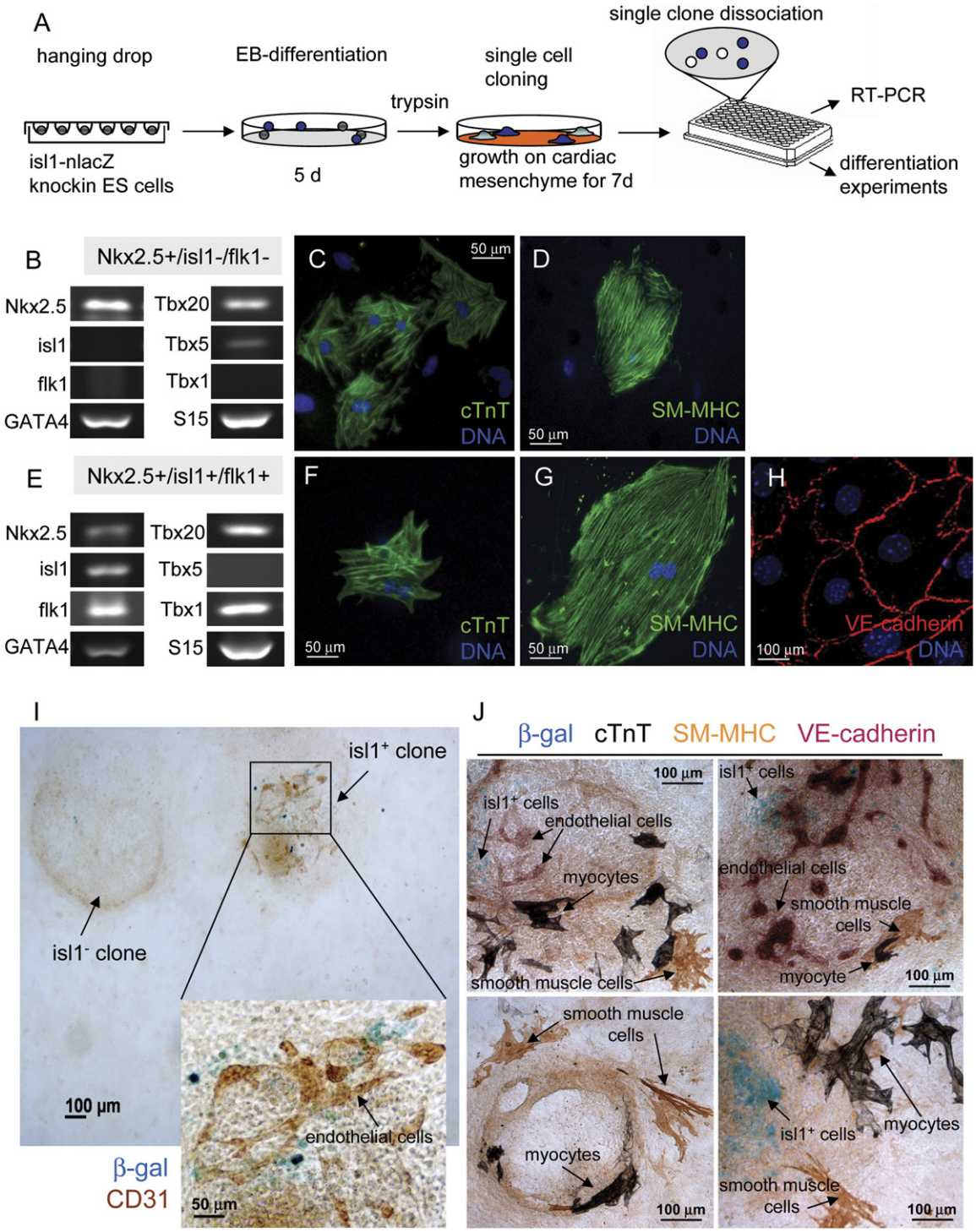

Figure 5. Clonal Differentiation Analysis of Cardiac Precursors Derived from *Isl1*-nLacZ Knockin ES Cells after Expansion on cardiac CMC

(A) Schematic representation of the experimental procedure used for generating clones of cardiac precursors derived from *isl1*-nlacZ knockin ES and for their clonal analysis.

(B–D) RT-PCR profile (B) of a representative progenitor clone, which differentiated into cells expressing the myocytic marker cTnT (green fluorescence, C) and the smooth muscle marker SM-MHC (green fluorescence, D).

(E–H) RT-PCR profile (E) of a representative progenitor clone, which differentiated into all the three cardiovascular lineages, giving rise to cells positive for cTnT (green fluorescence, F), SM-MHC (green fluorescence, G), and VE-cadherin (red fluorescence, H).

Clonal Differentiation Analysis of ES Cell-Derived Cardiac Precursors after Expansion on Cardiac CMC

Cardiac progenitors arising from isl1-nlacZ knockin ES cells during EB differentiation were clonally expanded on CMC feeder layers. After 7 days coculture, clones were picked, dissociated into single cells, and subjected to gene-expression profiling and differentiation experiments in vitro (Figure 5A). We tested the differentiation potential of each clone (n = 207) into the three cardiac lineages: cardiomyocytes, endothelial cells, and vascular smooth muscle. After 4 days in specific culture conditions (see Experimental Procedures), 12% of the clones differentiated into all three lineages, as demonstrated by the appearance of cells expressing cardiac troponin T (cTnT), SM-MHC, and VE-cadherin (Figures 5F–5H). In these progenitor clones transcripts of *isl1*, *Nkx2.5*, *flk1,* and/or *CD31*, *GATA4,* and *Tbx20* were detected (Figure 5E, Table S2). Two cell lineages originated from ~30% of the clones, the most common being cardiomyocytes-SMC (22.7%) obtained from clones that expressed either *Nkx2.5* only or *Nkx2.5*/*isl1* ± *flk1* (Figures 5B–5D, Table S2). All clones that converted into myocyte-endothelial cells or SMC-endothelial cells showed expression of *isl1* and *flk1*/*CD31* regardless of *Nkx2.5* expression. Differentiation into only one lineage was observed in ~33% of the clones, the least abundant being endothelial cells, triggered in clones that were all positive for *isl1*, *flk1*, and *Nkx2.5* (Table S2). The requirement of cells expressing *isl1* and *flk1*/*CD31* for the transition of cardiac progenitor clones into the endothelial lineage was confirmed by analyzing the spontaneous differentiation pattern of the isl1-nlacZ knockin ES cell-derived clones on CMC. By 10 days in coculture, we observed that a proportion of cells within the clones undergo spontaneous differentiation into myocytes, smooth muscle, and/or endothelial cells. Cardiac troponin T and SM-MHC-expressing cells were detected in both β-gal$^+$ and β-gal$^-$ clones (Figure 5J), while only clones presenting β-gal activity contained endothelial-like cell structures staining positively for CD31 or VE-cadherin (Figures 5I and 5J).

By employing a second independent ES knockin cell line, in which eGFP is targeted to the *Nkx2.5* locus, we also have documented that ES cell-derived cardiac progenitors can be clonally amplified on CMC feeders and that *Nkx2.5* is sufficient for cardiac and smooth muscle specification (Supplemental Results, Figure S1).

Taken together, these results suggest that *isl1* and *flk1*/CD31 expression is important for the conversion of cardiac precursors into endothelial cells, while *Nkx2.5* plays a pivotal role in the specification into the myocytic lineage. Moreover, our findings indicate that a single ES cell-derived MICP possesses the potential to serve as "cardiovascular progenitor cell" in vitro, being able to give rise to cell types of the working myocardium and the heart vasculature.

Identification, Isolation, and Clonal Amplification of an *Isl1*$^+$ Common Precursor for Cardiovascular Lineages in the Embryo

To establish the existence of MICPs in vivo, we investigated the expression of *isl1*, *Nkx2.5*, and *flk1* during early stages of mouse heart development. Immunohistochemical analysis in embryos between ED8.0 and ED8.5 revealed a rare subset of triple positive cardiogenic precursors within splanchnic mesenchyme comprising the mesocardium (Figure 6A). The majority of *isl1*$^+$ cells detected in this region coexpressed *flk1*. In the mesenchyme adjacent to foregut endoderm, we observed a partially overlapping expression gradient of *isl1* and *Nkx2.5*, with *isl1* being mainly expressed in splanchnic mesoderm and foregut endoderm while *Nkx2.5* being upregulated in differentiating myocardial precursors (Figure 6B). Beside the endocardium, *flk1* was detected on the membrane of a subset of *Nkx2.5*$^+$ cells of the forming heart tube (Figure 6C).

To investigate the differentiation potential of these embryonic cardiac precursors in vitro, we isolated cells from the heart regions of embryos at ED8.0 and ED8.5 and plated them at low density as single cells on CMC feeders (Figure 7). Similarly to the ES-derived cardiac progenitors, the CMC environment allowed the expansion of the embryonic isolated cells with maintenance of their cardiac precursor phenotype. Cells coexpressing *isl1*, *Nkx2.5*, and *flk1* were detected in small growing clones after 3–5 days coculture. At this stage the majority of embryo-derived cardiac progenitors displayed double positivity for two of the three markers, being *isl1*$^+$/*Nkx2.5*$^+$, *isl1*$^+$/*flk1*$^+$, or *Nkx2.5*$^+$/*flk1*$^+$ (Figure 7A–7D). With increasing time in culture, cells lost *flk1* expression, and most of the clones contained *isl1*$^+$ and/or *Nkx2.5*$^+$ precursors (Figure 7E–7H). Transcriptional profiling and triggered differentiation of cells from single clones picked after 7 days on the CMC showed that 24% (34/144) of the clones maintained the potential to convert into both myocytic and smooth muscle lineages while expressing *isl1* and/or *Nkx2.5* (data not shown). Differentiation into smooth muscle cells was observed in 36% of the clones (52/144), whereas specification into the cardiac muscle lineage was less abundant (8%, 11/144). Transition into the three major cardiac cell types (endothelium, myocytes, and smooth muscle) occurred spontaneously in a rare number of clones differentiating on the CMC after 10–12 days in culture (Figure 7I).

Taken together, our findings demonstrate that *isl1*$^+$/*Nkx2.5*$^+$/*flk1*$^+$ cardiovascular progenitors (MICPs) exist in

(I) Immunohistochemical analysis on progenitor clones at 10 days coculture with CMC for CD31 (brown). Blue stain corrisponds to β-gal activity. Inset represents a magnification of the areas of interest.

(J) Triple immunoperoxidase stain for markers of differentiated endothelial cells (VE-cadherin, purple), cardiomyocytes (cTnT, black), and smooth muscle cells (SM-MHC, brown) on progenitor clones after X-gal stain (blue) at day 10 on CMC.

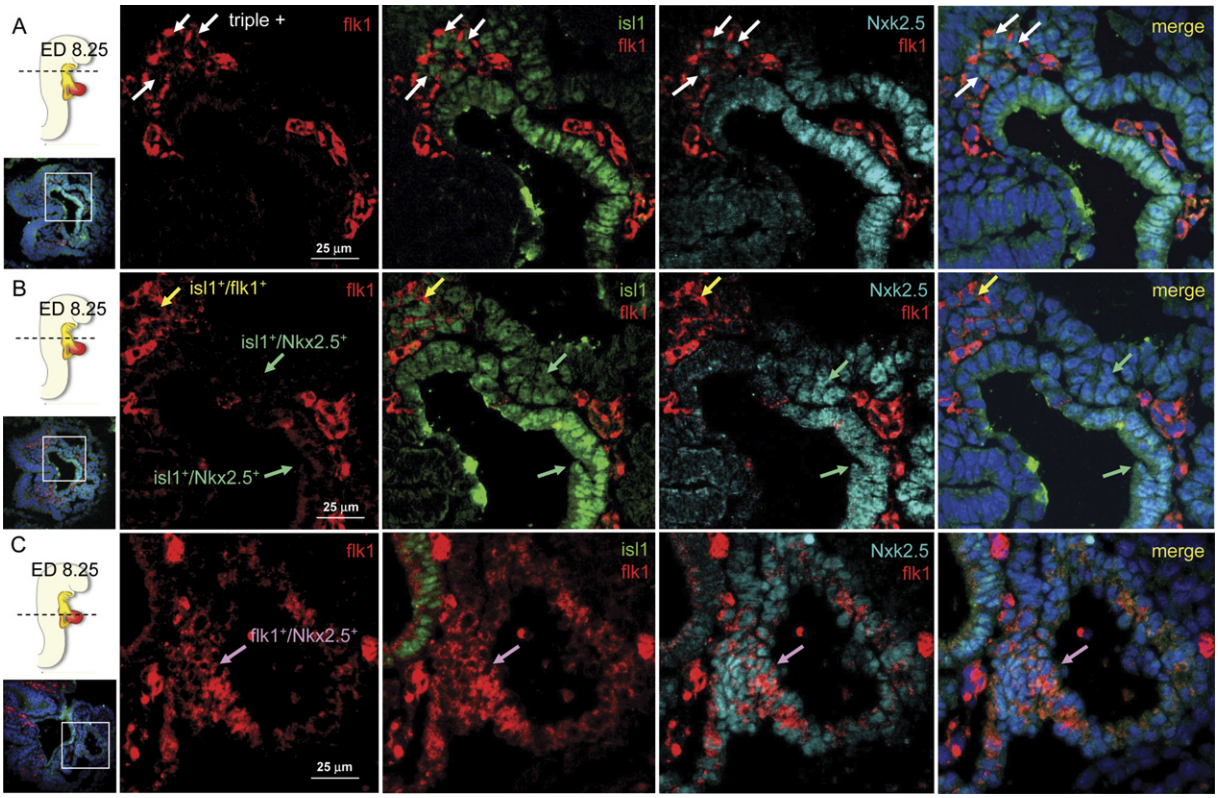

Figure 6. Existence of *Isl1*$^+$/*Nkx2.5*$^+$/flk1$^+$ Cardiac Precursors in the Developing Embryo In Vivo

Immunofluorescence analysis for the transcription factors *isl1* (green) and *Nkx2.5* (cyan) and the surface marker *flk1* (red) in transverse cryosections of embryos at ED8.25. Sections correspond to the position indicated by the lines drawn through the adjacent embryo view and are shown at 20× magnification in the small left panels and at 63× magnification of the area of interest in the big panels.

(A) Triple-positive cells in splanchnic mesoderm are indicated by white arrows.

(B) Yellow arrows mark double *isl1*/*flk1*-labeled cells and green arrows cells of the foregut endoderm and adjacent mesoderm, which costain positively for *isl1* and *Nkx2.5*.

(C) Cells coexpressing *Nkx2.5* and *flk1* are found in differentiating myocardium and are indicated by pink arrows.

the developing embryo, can be isolated, and maintain their multipotentiality after clonal amplification in vitro.

A Single *Isl1*$^+$ Progenitor Gives Rise to Three Distinct Cardiovascular Cell Lineages

Based on the genetic fate mapping of embryonic *isl1* heart progenitors, as well as the multilineage differentiation and transcriptional profiling of postnatal, ES-, and embryo-derived *isl1* cardiac precursors, we propose a working model for a hierarchy of *isl1*$^+$ cells that control lineage specification in the second heart field (Figure 7J). A multipotent *isl1*$^+$ progenitor cell (MICP) serves as a common cardiovascular precursor for the three main lineages of the developing heart. Toward cell-fate determination, this cell expresses *flk1* and *Nkx2.5*. Dual *isl1*$^+$/*flk1*$^+$ cells could represent a subset of "vascular" downstream progenitors, being able to convert into endothelial and smooth muscle cells. Cardiac or smooth muscle lineages arise from *Nkx2.5*-expressing cells, which can be either *isl1*$^+$/*flk1*$^-$ or *isl1*$^-$/*flk1*$^+$. Thus, both *isl1*$^+$/*Nkx2.5*$^+$/*flk1*$^-$ and *isl1*$^-$/*Nkx2.5*$^+$/*flk1*$^-$ populations would serve as more restricted "muscle" progenitors.

DISCUSSION

Spatial and Temporal Control of the Formation of Endothelial, Cardiac, and Smooth Muscle Cell Lineages for Diverse Heart Structures Occurs within a Subset of *Isl1*$^+$ Progenitors in the Second Heart Field

Two distinct pools of cardiac progenitors originate from the cardiogenic plate, the primary lineage, which differentiates first, and a later differentiating population of cardiac precursors, called the second lineage. The more dorsal/medial localized second lineage takes a distinct migratory path, which results in later arriving cells entering the forming heart region from dorsal positions at the anterior and posterior poles (Mjaatvedt et al., 2001; Waldo et al., 2001; Brand, 2003). *Isl1* marks this second population (Cai et al., 2003), which is a major source of the cardiac progenitors that contribute to the outflow tract, the right

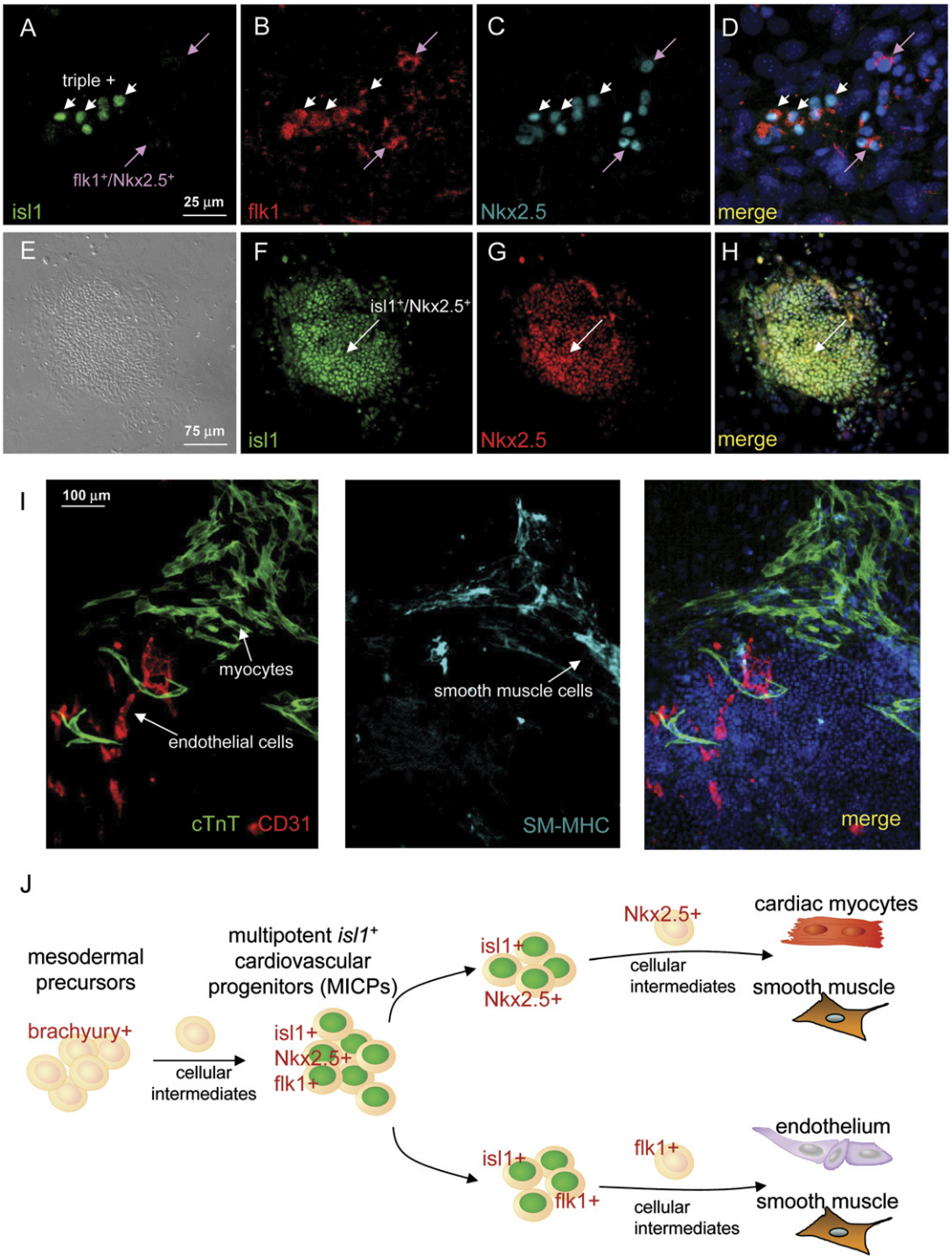

Figure 7. Clonal Amplification of Embryo-Derived Cardiac Precursors on Cardiac CMC and Their Differentiation Analysis

(A–D) Representative clone of embryo-derived cardiac progenitors on CMC at day 3, after immunofluorescence analysis for the nuclear proteins *isl1* (green) and *Nkx2.5* (cyan) and the surface marker *flk1* (red). Nuclei are visualized by Hoechst 33528 (blue). Short white arrows indicate triple-positive cells and long pink arrows cells costaining positively for *Nkx2.5* and *flk1*.

(E–H) Representative clone of embryo-derived cardiac progenitors after 6 days on CMC. The majority of the cells coexpress *isl1* (green, F) and *Nkx2.5* (red, G).

(I) Triple immunofluorescence stain for markers of differentiated endothelial cells (CD31, red), cardiomyocytes (cTnT, green) and smooth muscle cells (SM-MHC, cyan) on progenitor clones after 12 days in culture on CMC. Nuclei are detected by Hoechst 33528 (blue).

(J) Model of cellular hierarchy of cardiovascular progenitors and their lineage specification. See Discussion for details.

ventricle, portions of the left ventricle, and the atria, anatomical structures, which are absent in mutant hearts of *isl1* homozygous knockout mice. In contrast, the first population of cardiac precursors give rise to the left ventricle and parts of the atria (Buckingham et al., 2005). Retrospective clonal analysis in the mouse indicated that the first and the second cardiogenic lineages may segregate from one common primordial cell before the crescent stage (Meilhac et al., 2004).

The early segregation of the two lineages, the different time course of differentiation, and the distinct regional contributions to the embryonic heart support the idea that the two populations may have discrete properties. In addition, the two pools of cardiac progenitors appear to be governed by different genetic programs of self renewal and differentiation, which are in turn regulated by distinct transcriptional networks, with *Nkx2.5* as a critical transcription factor in the first lineage and *isl1*, along with *Foxh1*, *GATA factors*, and *Hand2*, as key regulators in the second heart progenitor field (von Both et al., 2004; Dodou et al., 2004). Lineage tracing studies indicate that the first progenitor population has a more restricted potential, generating the left ventricle and parts of the atria, while the second lineage contributes to a greater diversity of heart structures (Cai et al., 2003; Buckingham et al., 2005; Verzi et al., 2005).

The question arises as to whether the initial specification of the three major cell types within the heart, endothelial, cardiac, and smooth muscle, actually originates in the second heart lineage, and which are the cell populations that act as cellular precursors for these distinct lineages.

The finding that *isl1*$^+$ precursors in the second heart field can contribute to muscle and nonmuscle cell lineages has important implications for unraveling the pathways that guide cardiac organogenesis (Ward et al., 2005). Previous cell fate-mapping studies in avian systems have revealed a common myogenic precursor that gives rise to both working myocardium (atrial and ventricular muscle) and the Purkinje cells of the conduction system (Gourdie et al., 1998). The current study suggests that this precursor is within the cellular hierarchy of *isl1*$^+$ progenitors and that it may be possible to isolate a unique subset of precommitted SA nodal myocyte precursors from the pool of *isl1*$^+$ cardiogenic cells. In regard to the aorta and pulmonary artery, it now emerges that *isl1*$^+$ cells within the second heart field lineage can account for a major portion of the smooth muscle cells in the most proximal outflow tract and their contribution appears to be independent from the known requirement of the migrating cardiac neural crest derivatives (Verzi et al., 2005; Waldo et al., 2005). With respect to the coronary arterial system, the current study indicates that the proepicardial organ is not the only source of precursor cells originating this vascular structure, as a significant portion of the proximal region seems to be derived from the *isl1*$^+$ progenitors within the second heart field. Finally, our results document that the pool of second heart field *isl1*$^+$ precursors contributes to the endothelial cells within all of the heart components (endocardium, aorta, pulmonary, and coronary artery). This would indicate a distinctly different origin for cardiac endothelial lineages versus those of the peripheral vasculature, which appear to be largely derived from hemangioblast precursors within the hematopoietic cell hierarchy (Choi et al., 1998; Kouskoff et al., 2005).

A Model for Clonal Heart Cell Lineage Diversification via Multipotency of Individual *Isl1*$^+$ Cardiovascular Progenitors

Since *isl1*$^+$ progenitors in the second heart field can give rise to endothelial, cardiac, and smooth muscle lineages, we sought to determine if there might be a primordial *isl1*$^+$ cardiovascular stem cell that can account for all three lineages. Previous evidence for a common vascular progenitor arose from experiments with *flk1*$^+$ mouse ES cells, which were shown to differentiate into vascular smooth muscle and endothelial cells upon injection into the chick embryo or when analyzed under clonal conditions in vitro (Yamashita et al., 2000; Ema et al., 2003). Retrospective clonal studies utilizing an nlacZ reporter targeted to the α-cardiac actin gene have documented in vivo a common origin for precursor cells in the dorsal aorta and in the myotome, which can differentiate into smooth muscle and endothelium during development (Esner et al., 2006). Furthermore, transcriptome-wide analysis of cardiac progenitors FACS-purified from transgenic Nkx2.5$_{enhancer}$-eYFP mouse embryos revealed an enriched expression of *flk1* and *tie2* transcripts, suggesting a shared identity between cardiac crescent stage progenitors and vascular/hematopoietic (hemangioblasts) cell types (Masino et al., 2004). In concordance, recent work in mouse ES cells identified *flk1*$^+$ precursors capable of differentiating into endothelium and cardiac muscle in vitro (Iida et al., 2005; Yamashita et al., 2005).

In the current study, we describe the discovery of single-cell derived clones of *isl1*$^+$/*Nkx2.5*$^+$/*flk1*$^+$ precursors (MICPs) that can spontaneously convert into endothelial, cardiac, and smooth muscle cells. Taken together, these findings suggest a working model for the initial framework of the cellular hierarchy that governs lineage specification in the second heart field (Figure 7J). In this model, *isl1*$^+$ cells that eventually express *Nkx2.5* and *flk1* serve as primordial cardiovascular progenitors, which can give rise to all three lineages, cardiac muscle, smooth muscle, and endothelium. Since endothelial cells arose only from clones containing precursors, which expressed *isl1* and *flk1*, the dual *isl1*-*flk1* positive cells may represent a subset of downstream progenitors that are more restricted in their differentiating potential, being able to convert into endothelial and smooth muscle cells. These results are in concordance with previous studies suggesting that ES cell-derived *flk1*$^+$ cells can give rise to the two major vascular cell types (endothelial and mural cells) in vitro and in vivo (Yamashita et al., 2000). The *isl1*$^+$/*Nkx2.5*$^+$ are akin to the postnatal *isl1*$^+$ progenitors and could ultimately generate either cardiac or smooth muscle lineages. Both muscle lineages can also arise from cells that are only *Nkx2.5*$^+$.

This finding and the recent study by Wu et al. (2006), in combination with the evidence that the differentiation event is associated with *isl1* downregulation (Cai et al., 2003: Laugwitz et al., 2005), suggest that the decision to enter either the cardiac or smooth muscle cell program is made at the level of precursors expressing *Nkx2.5*$^+$.

This model of clonal heart-lineage diversification in *isl1*$^+$ cardiovascular progenitors would be analogous to the hematopoietic stem-cell (HSC) model proposed by others (Kiel et al., 2005), where a primordial single HSC can generate all of the blood-cell lineages. The single cell-level decision also implies that there will be critical molecular networks that not only act as positive regulators toward a certain lineage but also repress entry into alternative cell phenotypes.

Isl1$^+$ Cardiovascular Progenitors and Molecular Pathways for Congenital and Adult Heart Disease

Previous studies have shown that congenital heart disease due to mutations in the *Nkx2.5* gene (Schott et al., 1998) arises as a result of defects in cardiac lineage specification (Pashmforoush et al., 2004). Accordingly, a subset of congenital heart diseases associated with abnormalities in pivotal genes expressed within *isl1*$^+$ cardiovascular progenitors might be due to selective defects in the self renewal, differentiation, and lineage specification of distinct tissue-restricted precursors. The availability of clonal-based assay systems to study the generation of endothelial, cardiac, and smooth muscle cell lineages from primordial *isl1*$^+$ cardiovascular progenitors is extremely valuable in sorting out these pathways at a single cell level.

Recent experimental and clinical studies have underscored the need to identify the optimal cell type to drive robust cardiac muscle regeneration for the failing heart (for review, see Schwartz, 2006; Chien, 2004, 2006). While embryonic stem cells have been suggested to be a renewable source of cardiac muscle cells for transplantation, this has been problematic given the difficulty in generating sufficient amounts of homogenous cardiac myocytes and the dangers associated with the risk of teratomas (Laflamme and Murry, 2005; Murry et al., 2005; Rubart and Field, 2006). In this regard, the current study suggests an alternative strategy for achieving the regeneration of distinct heart components that are affected in diverse forms of degenerative heart disease. The delivery of specific clonally derived cardiac progenitors from ES cells, which represent tissue-restricted precursors limited in their differentiation potential, may allow regeneration of specific heart structures without the risks and limitations of other ES cell-based systems.

EXPERIMENTAL PROCEDURES

Isolation and Cell-Culture Conditions of Mouse Postnatal Cardiac Progenitors and CMC

To isolate cardiac progenitors, we used 40–60 hearts from 1–5 day-old pups, which were double heterozygous for isl1-mER-Cre-mER and R26R alleles and cultured the CMC, containing the majority of β-gal$^+$ progenitor cells, as previously described (Laugwitz et al., 2005). 4-OH-TM (Sigma) was applied in culture 1 day after cell plating at a concentration of 1μM and maintained for 2 days. CMC were isolated from CD1 wild-type mice and used as a mitomycin-treated feeder layer for ES cells.

Differentiation of Postnatal Cardiac Progenitors into Smooth Muscle Cells

For coculture, hca-SMC cells were plated at a density of 10^4/cm^2 on fibronectin-coated permanox chamber slides, using SMBM medium (Cambrex). Twenty-four hours later, CMC from isl1-mER-Cre-mER/R26R animals were FACS sorted after C_{12}FDG labeling and β-gal$^+$ cells were added to hca-SMC (5 × 10^3cells/cm^2). After 1–5 days, cells were stained for LacZ and SM-MHC. For spontaneous differentiation, FACS-sorted β-gal$^+$ cells were plated at a density of 10^4 cells/cm^2 on fibronectin-coated permanox chamber slides and cultured in Dulbecco's minimal essential media (DMEM)/F12 containing B27 supplement, 2% fetal bovine serum (FBS), and 10 ng/ml EGF (DMEM/F12 complete) for 1–5 days, prior to immunostain for smooth muscle markers.

ES Cell Culture and Differentiation

Isl1-nLacZ knockin ES cells were generated by insertion of a loxP-flanked nuclear lacZ SV40 pA cassette, followed by hrGFP and a neoselectable marker flanked by ferrotransferrin sites into Exon 1 of the genomic isl1 locus (Y. Sun and S.M. Evans, submitted). ES cells were maintained on mitomycin-treated embryonic feeders in DMEM medium supplemented with 15% FBS (Hyclone), 2mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 μg/ml LIF. Cells were differentiated for 5 days as EBs formed in hanging drops of ES cell medium without LIF. Five day EBs were dissociated into single cells with 0.25% trypsin for 5 min at 37°C. For the isl1-nLacZ knockin ES cells, dissociated cells were plated as single cell on top of mitomycin-treated CMC or embryonic feeders at a density of 10^3 cells/cm^2 in DMEM/F12 complete medium. Growing clones from single cells plated on CMC were picked after 6–7 days coculture and trypsinized. Half of the cells from each clone were used for RNA extraction and the rest was plated into 3 wells of a 384-well plate for differentiation experiments. Differentiation was triggered into myocytes, on fibronectin by using DMEM/M199 (4:1 ratio) medium containing 10% horse serum and 5% FBS; SM cells, on fibronectin by using DMEM/F12 complete medium; and endothelial cells, on collagen IV by using DMEM supplemented with 10% FBS and 50 ng/ml mouse VEGF (R&D systems).

Isolation, Culture Amplification, and Differentiation of Embryo-Derived Cardiac Precursor Cells

Tissue from the heart region of 60–100 wild-type embryos ED8.0–8.5 was digested with 470 U/ml collagenase type II (Worthingthon) in HBSS for 30 min at 37°C, followed by a 5 min treatment with 0.25% trypsin. Dissociated cells were filtered through a 40 μm cell strainer and plated on top of mitomycin-treated CMC at a density of 10^3 cells/cm^2 in DMEM/F12 complete medium. For triggered differentiation experiments and transcriptional profiling by RT-PCR, growing clones from single cells plated on CMC were picked after 6–7 days coculture, trypsinized, and processed as described above for the ES cell-derived progenitor clones.

Supplemental Data

Supplemental data include Supplemental Experimental Procedures, Supplemental Results, one figure, and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/127/6/1151/DC1/.

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