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Developmental Origin of a Bipotential Myocardial and Smooth Muscle Cell Precursor in the Mammalian Heart

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SUMMARY

Despite recent advances in delineating the mechanisms involved in cardiogenesis, cellular lineage specification remains incompletely understood. To explore the relationship between developmental fate and potential, we isolated a cardiac-specific Nkx2.5$^+$ cell population from the developing mouse embryo. The majority of these cells differentiated into cardiomyocytes and conduction system cells. Some, surprisingly, adopted a smooth muscle fate. To address the clonal origin of these lineages, we isolated Nkx2.5$^+$ cells from in vitro differentiated murine embryonic stem cells and found ~28% of these cells expressed c-kit. These c-kit$^+$ cells possessed the capacity for long-term in vitro expansion and differentiation into both cardiomyocytes and smooth muscle cells from a single cell. We confirmed these findings by isolating c-kit$^+$Nkx2.5$^+$ cells from mouse embryos and demonstrated their capacity for bipotential differentiation in vivo. Taken together, these results support the existence of a common precursor for cardiovascular lineages in the mammalian heart.

INTRODUCTION

The mammalian heart develops through a series of carefully orchestrated events and contains three major lineages—myocardial, endothelial, and smooth muscle cells. The developmental precursor for myocardial cells is most commonly ascribed to the cardiogenic mesoderm arising from cells in the primitive streak (Garcia-Martinez and Schoenwolf, 1993; Hatada and Stern, 1994). These cells migrate bilaterally into the anterolateral mesoderm to form the primary heart field, adopt a crescent shape, and fuse at the midline to produce a linear heart tube composed of an outer epithelial myocardium and an inner endothelium (Rosenquist and DeHaan, 1966; Srivastava and Olson, 2000). Subsequent morphogenetic events give rise to a mature four-chambered heart.

The origin of the endocardium is unclear. Earlier studies in chick support the existence of distinct progenitor cell populations within the primitive streak for myocardium and endocardium prior to their migration into the cardiogenic mesoderm (Coffin and Poole, 1988). More recent studies on gene expression and differentiation potential of mesodermal explants point to the existence of a bipotential precursor cell (Eisenberg and Bader, 1995). Using a retroviral labeling technique to tag single cells in early mesoderm, Cohen-Gould and Mikawa found no simultaneously labeled endocardial and myocardial cells at the linear heart tube stage (Cohen-Gould and Mikawa, 1996). Taken together, these studies support an early separation of endocardial and myocardial lineages in the developing chick heart, most likely prior to or during gastrulation. It remains to be seen whether early separation of these lineages occurs in the mouse embryo.

The embryonic development of vascular smooth muscle cells takes place later than that of myocardial or endocardial cells. As such, diverse tissue sources have been described to give rise to smooth muscle cells in different

organs (Stavros and Majesky, 1996). For example, aortic smooth muscle cells have dual origins in the neural crest and the lateral plate mesoderm (Le Lievre and Le Douarin, 1975; Waldo and Kirby, 1993; Hungerford et al., 1996). The proepicardial organ, a dorsomesodermal structure, gives rise to the epicardium, a tissue that undergoes epithelial-to-mesenchymal transformation to generate the endothelial and smooth muscle cells of the coronary arteries (Mikawa and Gourdie, 1996). It appears that smooth muscle cells have a less distinct developmental origin in general and are recruited locally from mesenchymal precursors and differentiate in response to specific tissue signals.

While these pioneering studies provide a conceptual framework for understanding the relative contributions to cardiac morphogenesis by cells of distinct origin, recent investigations suggest multipotential progenitors exist for cells of the mesodermal lineage. Using rare recombination events to genetically mark single cells in the developing embryo, Esner et al. have demonstrated a common precursor for skeletal muscle and aortic smooth muscle cells (Esner et al., 2005). Similarly, cells of the cranial pharyngeal mesoderm, a population that normally gives rise to head musculature, can contribute to the developing cardiac outflow tract (Tirosh-Finkel et al., 2006). Moreover, progenitor cells capable of differentiating into endothelial and smooth muscle cells have recently been described (Ema et al., 2003). Detailed lineage analyses of smooth muscle cells at the base of aorta and pulmonary artery have revealed an unexpected origin from the secondary heart field, a recently described cell population arising dorsomedial to the primary heart field and thought to give rise to only the myocardium of the outflow tract, right ventricle, and ventricular septum (Verzi et al., 2005; Waldo et al., 2005). Furthermore, these vascular smooth muscle cell progenitors express Nkx2.5, a lineage marker associated with myocardial progenitor cells. Indeed, lineage analysis of the progenies of Nkx2.5$^+$ cells using locus-specific Cre recombinase approaches revealed that multiple cell types (including outflow tract smooth muscle cells and yolk sac endothelial cells) express this gene (Moses et al., 2001; Stanley et al., 2002). Taken together, these studies raise the possibility that developmental multipotentiality is more pervasive than previously expected. None of these studies, however, attempted to define the identity of these putative progenitor cells by prospectively isolating and characterizing their multilineage differentiation.

To investigate the relationship between developmental fate and potential of embryonic cardiac progenitor cells, we isolated early Nkx2.5-expressing cells from the developing mouse heart and demonstrated their differentiation into both beating cardiomyocytes and smooth muscle cells. To address whether bipotential differentiation occurred at a single cell level, we isolated Nkx2.5$^+$ cells from in vitro differentiated embryonic stem (ES) cells and showed that a c-kit$^+$ subpopulation harbors enhanced capacity for in vitro expansion and bipotential differentiation from a single cell. To explore these ES cell-based findings in an embryological context, we isolated c-kit$^+$ Nkx2.5$^+$ cells from the developing mouse embryo and demonstrated their ability to undergo both myocardial and smooth muscle cell differentiation in vivo. Taken together, these data support the divergence of smooth muscle and myocardial lineages from a common cardiovascular precursor in the developing heart field.

RESULTS

Generation of Cardiac-Specific Nkx2.5-eGFP Transgenic Mice

Nkx2.5 is one of the earliest cardiac transcription factors expressed in the developing heart field. The expression domain of murine *Nkx2.5*, however, encompasses many tissues, including the thyroid, pharynx, stomach, and spleen (Moses et al., 2001). To achieve cardiac-specific eGFP expression, we utilized a 2.1 kb enhancer located 9.5 kb upstream of the translation start site of murine *Nkx2.5* (Lien et al., 1999) along with a 500 bp *Nkx2.5* base promoter to generate transgenic mouse lines expressing eGFP specifically in the developing heart (Figure 1A).

eGFP Expression in Nkx2.5-eGFP Transgenic Mice

The expression of eGFP in embryonic day 7.5 (E7.5) cells could only be observed by flow cytometry (data not shown) but not by whole mount fluorescence microscopy (Figure 1B). This expression, however, was easily detectable from E8.5 until E11.5 (Figure 1B). Immunohistochemical analysis revealed the presence of eGFP$^+$ cells precisely in the myocardial layer (M) but not the endocardial (E) layers (Figure 1C, b′ and f′ insets). At E8.5, the expression of eGFP (Figure 1C, b′ inset) overlapped partially with that of isl1 (Figure 1C, c′ and inset with arrows), which marks exclusively progenitor cells for the secondary heart field (Cai et al., 2003). eGFP expression overlapped completely with sarcomeric myosin heavy chain (Sarc. MHC) (Figure 1C, d′), an early myocardial marker in the primary heart field. At E10.5, a minor cell population in the outflow tract (OFT) beyond the endocardial cushion (EC) coexpresses Nkx2.5 (via eGFP) (Figure 1C, i′) and isl1 (Figure 1C, j′ and inset). The expression of isl1, however, is found predominantly in the dorsomesocardium and the neural crest at E9.5 in a nonoverlapping manner with Nkx2.5 (Figure S1, E9.5 isl1 and inset). The low number of coexpressed isl1$^+$ and Nkx2.5$^+$ cells can be explained by the fact that Nkx2.5$^+$ cells here are mostly derivatives of the primary heart field or that isl1 expression may have declined prior the onset of Nkx2.5 expression in the secondary heart field (Cai et al., 2003; Laugwitz et al., 2005).

Interestingly, coexpression of early myocardial (Sarc. MHC) and smooth muscle (smooth muscle actin-α [SMAα]) markers were found in looping heart tube at E9.5 (Figure S1). The expression of definitive myocardial genes such as cardiac troponin T (cTnT) was absent at these stages of cardiac development (Figure 1C, g′) and appeared only after E11.5 (Figure S1, E11.5). Likewise, expression of the definitive marker for smooth muscle

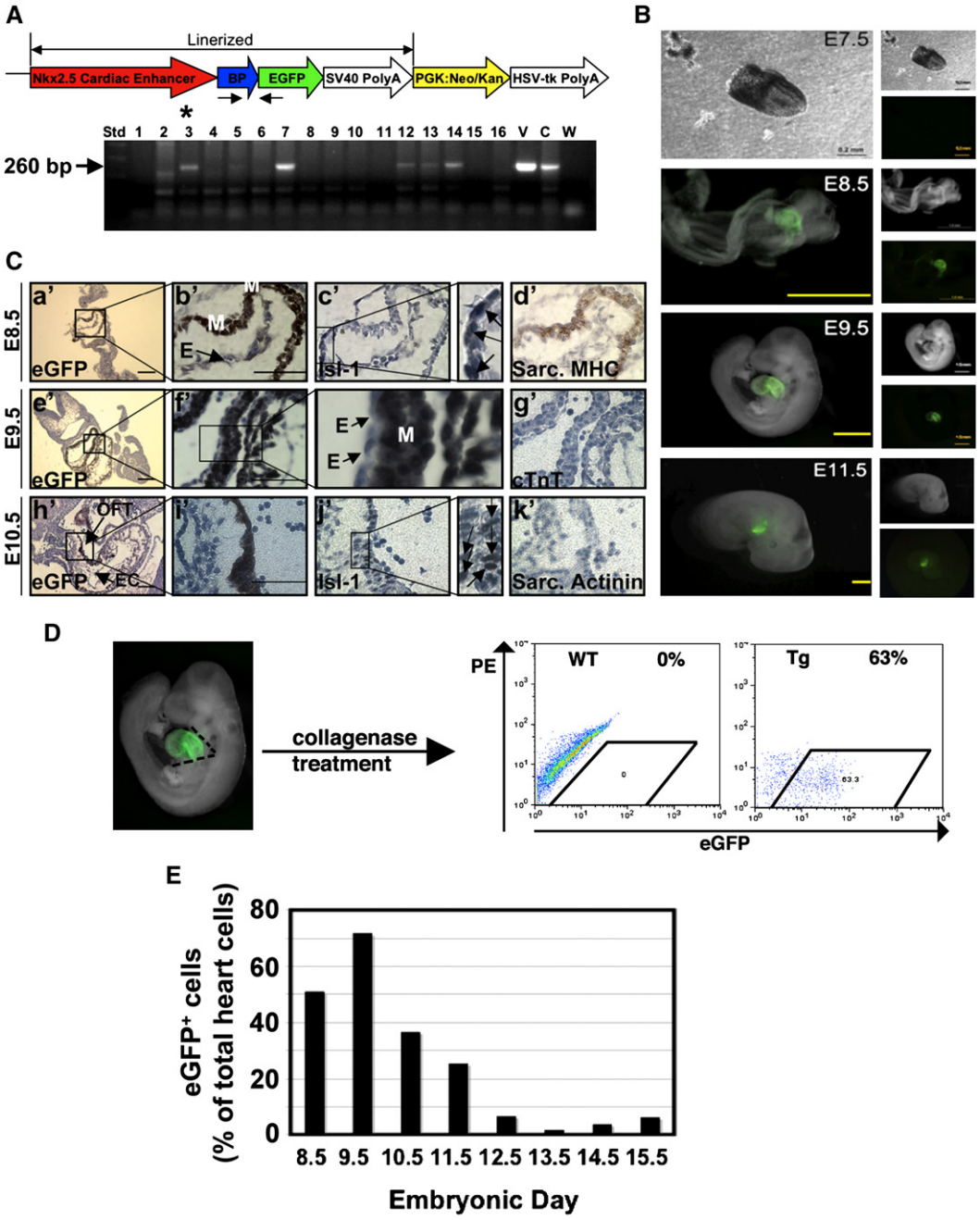


Figure 1. Analysis of Nkx2.5-eGFP Expression in Transgenic Embryos

(A) Construct diagram for the Nkx2.5 cardiac enhancer-eGFP plasmid (upper panel). Founder number 3 (asterisk), carrying the 260 bp transgene PCR product, was propagated (lower panel). Std, molecular weight standard; V, Nkx2.5-eGFP vector; C, positive tail control for transgene; W, H_2O negative control.

(B) Whole mount fluorescence analysis of eGFP expression in E7.5–E11.5 embryos. Bar, 1 mm.

(C) Immunohistochemical analysis of eGFP expression in serial sections of E8.5–E10.5 embryos. The expression of eGFP (black stain) at E8.5 (a′, b′), E9.5 (e′, f′, inset), and E10.5 (h′, i′) is restricted to the myocardial layer (indicated by M in b′ and f′ inset) but not the endocardial layer (indiated by E in b′ and f′ inset). Isl1 (c′ and inset, j′ and inset) (nuclear black stain), sarcomeric MHC (d′) (brown stain), cTnT (g′), sarcomeric actinin (k′).

(D) Flow cytometric analysis of dissected and collagenase treated E9.5 transgenic mouse heart cells. Compared with wild type, 63% of the transgenic heart cells were $eGFP^+$.

(E) Analysis of the percentage of $eGFP^+$ cells in the developing heart. Each bar represents mean of six $eGFP^+$ hearts.

lineage, smooth muscle myosin heavy chain (SM-MHC), was absent in myocardial cells at E9.5 and initiated in vascular smooth muscle cells of the thoracic cavity at E11.5 (Figure S1). The expression of eGFP diminished by E11.5 during myocardial maturation, in contrast with the expression of endogenous Nkx2.5 that is sustained throughout cardiac development (Lien et al., 1999). Analysis of E9.5 heart cells by flow cytometry showed a range of eGFP expression levels within each heart, consistent with the presence of a mixed population of progenitor cells and differentiating cardiomyocytes (Figure 1D). The percentage of eGFP$^+$ heart cells also decreases from E8.5 to E15.5 (Figure 1E). It appears that the expression of eGFP directed by this Nkx2.5 cardiac enhancer is specific for cells at the progenitor stage of development.

Differentiation Potential of Nkx2.5$^+$ Cells from the Embryonic Heart

To assess the potential of Nkx2.5$^+$ cells to spontaneously differentiate into chamber-specific myocytes and conduction system, we isolated eGFP$^+$ cells from E9.5 transgenic hearts by fluorescence-activated cell sorting (FACS) and cultured them in vitro. As expected, greater than 87% of these cells differentiated into cardiomyocytes and conduction system cells (Figure 2A and Video S1). Electrophysiological analysis at a single-cell level confirmed their maturation into conduction system cells as well as atrial and ventricular myocytes (Figure 2A). We observed that 92% ± 4% of the eGFP$^+$ cells survived the sorting procedure. Of these, ~12% were immature in appearance (arrows) and formed clusters when plated at low density (Figure 2B). Within these clusters, some cells differentiated into beating cardiomyocytes (arrowheads) and vascular smooth muscle cells (asterisks). The expression of differentiated myocardial (cTnT) and smooth muscle cell (SM-MHC) markers was detected by immunofluorescence microscopy (Figure 2C). Quantitatively, cells within each cluster possessed modest capacity for spontaneous differentiation in vitro into either myocardial (9.4%) or smooth muscle (15.3%) lineages (Figure 2D). The expression of endothelial cell markers (Flk1 and Tie-2) was not observed (data not shown), supporting an early separation of endothelial and myogenic lineages.

The capacity for the Nkx2.5$^+$ cells to undergo smooth muscle differentiation suggests either common expression of Nkx2.5 in two distinct lineages or the existence of a common progenitor for both cell types. To ascertain whether a single cell can undergo bipotential differentiation into both myocardial and smooth muscle cells, we dissected E7.5 and 8.5 mouse embryos and deposited FACS-purified single eGFP$^+$ cells into individual wells of a 96-well plate. However, we were unable to induce expansion or terminal differentiation of these cells at single-cell density despite repeated attempts.

Derivation of Nkx2.5-eGFP ES Cell Line

As an alternative approach, we isolated Nkx2.5-eGFP$^+$ cells from in vitro differentiated ES cells to address whether clonal differentiation into myocardial and smooth muscle cell lineages can occur from a single progenitor cell. ES cells can differentiate upon withdrawal of leukemia inhibitor factor to express lineage markers in a temporal pattern similar to that of the developing embryo (Figure 3A).

To generate an Nkx2.5-eGFP ES cell line, we introduced the Nkx2.5 cardiac enhancer-eGFP transgene into CJ7 ES cells and selected transgenic clones that expressed eGFP specifically in beating cell clusters at day 9 of differentiation (Figure 3B and Video S2). To confirm the cardiac specificity of eGFP expression in vivo, ES cell clones were introduced into E3.5 blastocysts to generate E10.5 chimeric embryos. These embryos express eGFP exclusively in the developing outflow tract and common ventricle (Figure S2A) mirroring that of the transgenic mice (Figure 1B).

Differentiation of ES Cell-Derived Nkx2.5$^+$ Progenitor Cells In Vitro and In Vivo

To assess the identity of these eGFP$^+$ cells, eGFP$^+$ and eGFP$^-$ cells were segregated by FACS from day 5 in vitro differentiation transgenic ES cells (Figure 3C, middle panel). The purity of the sorted eGFP$^+$ cells was confirmed by reanalysis of the eGFP$^+$ population (>93%) (Figure 3C, right panel). The segregation of cardiac from noncardiac cells was supported by expression analysis of lineage markers within each sorted population (Figure 3D). As expected, the expression of markers for noncardiovascular lineages (e.g., hematopoietic and skeletal muscle) was found predominantly in the eGFP$^-$ subpopulation. Consistent with this, eGFP$^+$ cells also preferentially differentiated into cardiomyocytes, as assessed by the presence of beating cell clusters (Figure 3E). Furthermore, the commitment of these eGFP$^+$ cells into a cardiogenic fate was supported by the inability of growth factor supplementation to direct these cells into neuronal, hematopoietic, or skeletal muscle fates when compared with undifferentiated ES cells (Figure S2B). These eGFP$^+$ cells exhibited high capacity for in vitro expansion as demonstrated by their ability to undergo >90 passages in culture. Analysis of the progeny of eGFP$^+$ progenitors by immunocytochemistry confirmed the presence of both cardiomyocytes and smooth muscle cells (Figure 3F).

To investigate the commitment of eGFP$^+$ cells for myogenic differentiation in vivo, we injected FACS-purified eGFP$^+$ cells from day 5 in vitro differentiated ES cells into the hindlimbs of syngeneic mice (1×10^6 cells/limb; N = 15) to achieve stable engraftment (Figure 4A). Control injections using undifferentiated transgenic ES cells (1×10^6 cells/limb, N = 5) were performed in parallel. As expected, undifferentiated ES cell-injected limbs formed progressively enlarging teratomas at 7, 21, and 35 days postinjection (Figure 4B). These teratomas were comprised of cells from all three germ layers, including neurons, glandular epithelia, blood, bone, and trachea. Injection of eGFP$^+$ cells failed to generate teratomas (0 of 15). Instead, they infiltrated existing skeletal muscle bundles at day 7

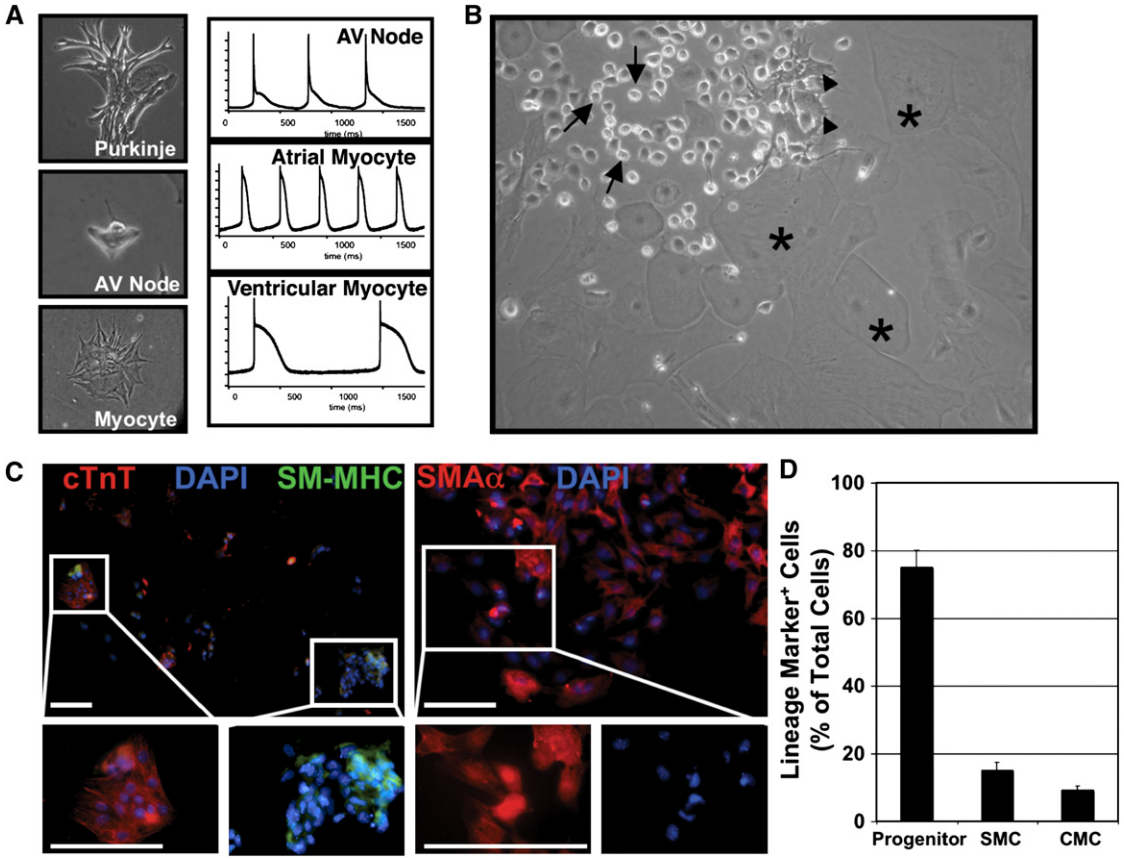


Figure 2. In Vitro Differentiation of Embryonic Nkx2.5-eGFP$^+$ Cells

(A) Myocardial differentiation of FACS purified E9.5 eGFP$^+$ cells. The morphologies of the differentiated progenies were assessed 8 days after sorting (left panel). Spontaneous action potentials are also shown (right panel).

(B) A representative eGFP$^+$ progenitor cell cluster. Progenitor cells, arrows; cardiomyocytes, arrowheads; and smooth muscle cells, asterisks.

(C) Immunofluorescence microscopy of eGFP$^+$ cells. Cardiac troponin T, cTnT; smooth muscle myosin heavy chain, SM-MHC; and smooth muscle actin-α, SMAα. Cell nuclei were counterstained with DAPI.

(D) Myocardial versus smooth muscle cell differentiation of eGFP$^+$ progenitor cells. Cardiomyocytes (CMC) or smooth muscle cell (SMC) differentiation was assessed by immunofluorescence staining for cTnT or SM-MHC, respectively. Progenitor cells were defined by the absence of cTnT or SM-MHC staining. Each bar represents means ± SEM of the percentage of cells that expressed the respective lineage marker from total DAPI$^+$ cells.

postinjection (Figure 4C, D7 arrows). At 35 days postinjection, sprouts of new myofibrils were found within areas of cell injection (Figure 4C, D35 arrows). Immunohistochemical staining revealed the coexpression of eGFP with GATA-4, cardiac MHC, and cTnT in some cells. The expression of SMAα was also detected, but in a nonoverlapping pattern with eGFP. This is more likely due to the downregulation of eGFP expression in transplanted cells that have differentiated towards smooth muscle lineage and not from the detection of endogenous smooth muscle cells.

c-Kit Is a Marker for a Progenitor Cell Subpopulation within Nkx2.5$^+$ Cells

Since the expression of Nkx2.5 marks a mixture of progenitors and immature myocytes, we sought to identify surface markers that predict progenitor status within Nkx2.5$^+$ cells. We examined the expression of >30 surface proteins using commercially available flow cytometry competent antibodies and found a modest level of c-kit and Sca-1 expression, but no expression of endothelial or hematopoietic lineage markers (Figure 5A). The expression levels of CD71 (transferrin receptor) and CD29 (integrin β1) were high, but the expression of these markers failed to correlate with progenitor cell status (data not shown). We fractionated Nkx2.5$^+$ cells based on their c-kit expression and found that the c-kit$^+$ cells appeared less differentiated morphologically than c-kit$^-$ cells (Figure 5B, upper panels). Consistent with this, c-kit$^+$ cells also possessed 40-fold greater capacity for proliferation than c-kit$^-$ cells (Figure 5B, lower left panel). c-Kit$^-$ cells, on the other hand, were more likely to form beating cardiomyocytes (Figure 5B, lower right panel). The expression of Sca-1 did not distinguish proliferating from differentiating cells (data not shown).

Using c-kit as a marker for a progenitor cell subpopulation within Nkx2.5$^+$ cells, we investigated the capacity for

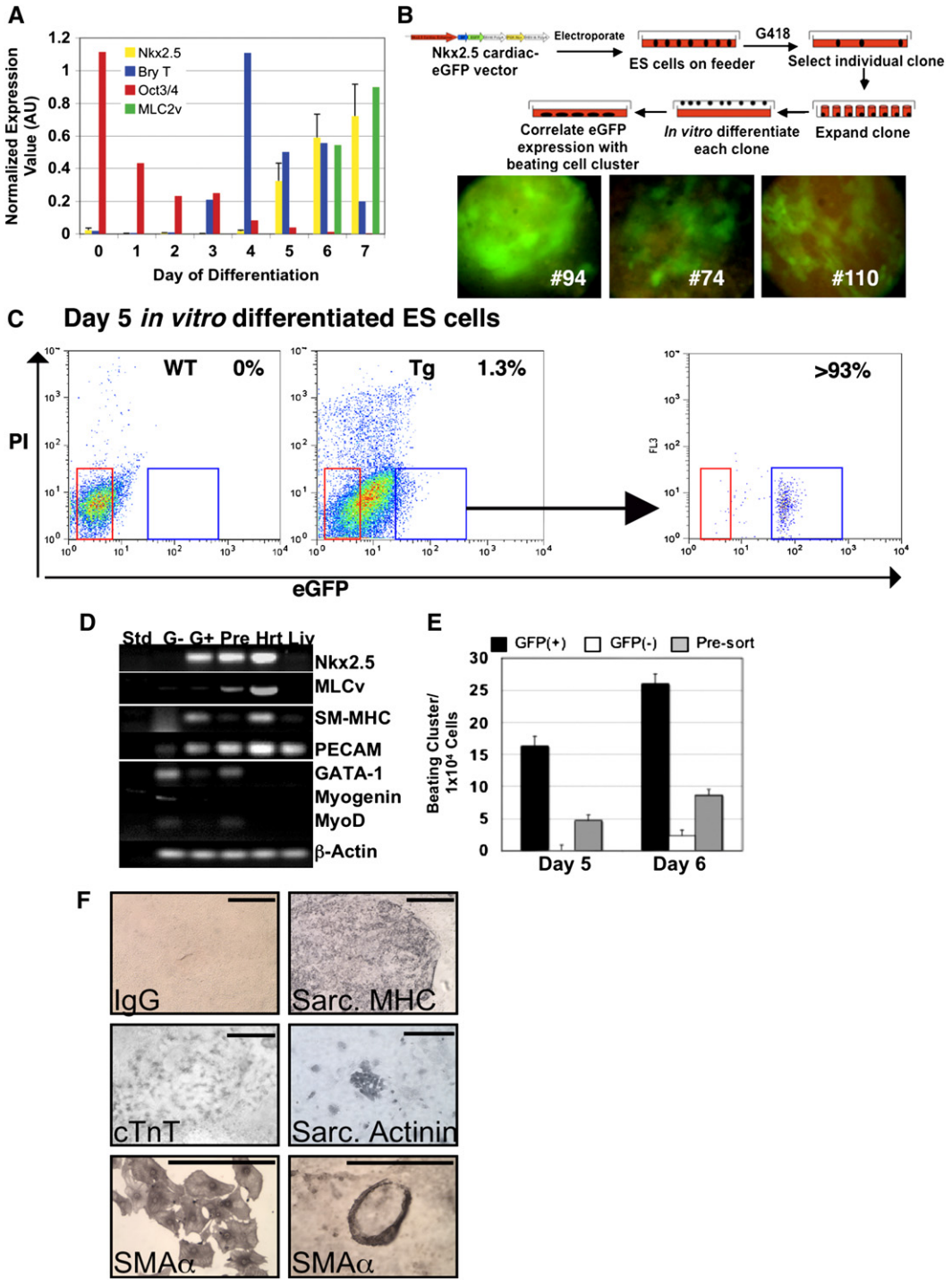


Figure 3. Isolation of Nkx2.5$^+$ Progenitor Cells from In Vitro Differentiated ES Cells

(A) Quantitative PCR analysis of developmental cardiac gene expression in differentiating ES cells. Expression of each gene was normalized to β-actin level.

(B) Schematic diagram for cardiac-specific Nkx2.5-eGFP transgenic ES cell line generation (upper panel). Three clones (numbers 74, 94, and 110) expressed eGFP specifically in beating cell clusters (lower panels).

(C) Isolation of cardiac-specific Nkx2.5-eGFP$^+$ cells by FACS. eGFP$^+$ (blue gate) and eGFP$^-$ (red gate) live cells were purified from in vitro differentiated ES cells at days 5 or 6.

(D) Analysis of gene expression in eGFP$^+$ and eGFP$^-$ cells by RT-PCR. Qualitative PCR for the expression of cardiac (Nkx2.5, MLCv), smooth muscle (SM-MHC), endothelial (PECAM), hematopoietic (GATA-1), or skeletal muscle (Myogenin, MyoD) genes in eGFP$^+$ (G$^+$), eGFP$^-$ (G$^-$), or presort (Pre) cell populations is shown. Hrt, adult heart tissue; Liv, adult liver tissue as negative control; Std, molecular weight standard.

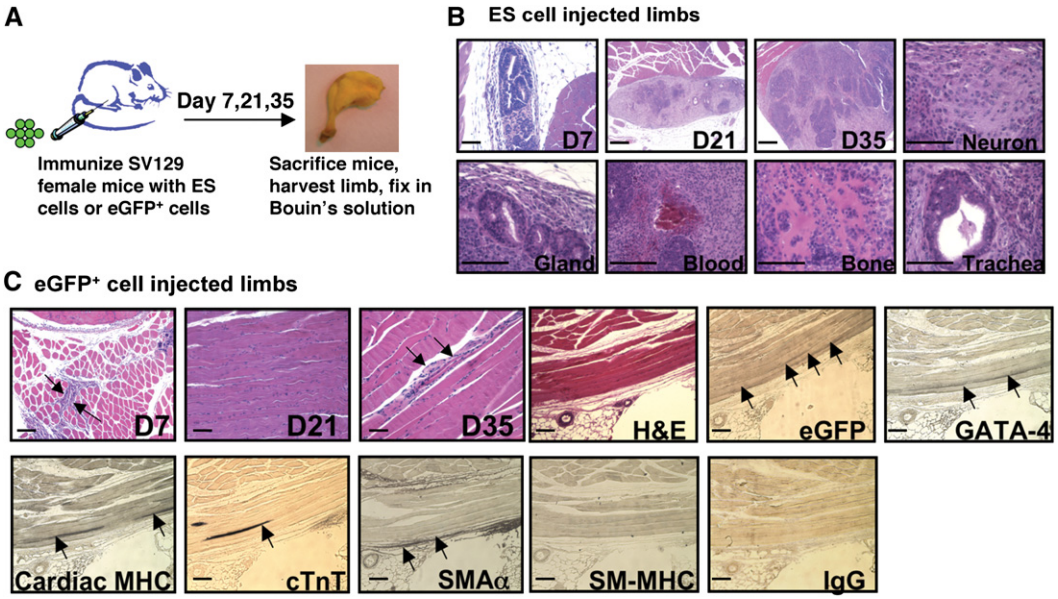


Figure 4. In Vivo Differentiation of ES Cell-Derived Nkx2.5$^+$ Cells

(A) Schematic diagram of transplantation of ES cell or eGFP$^+$ cells into the hind limbs of syngeneic mice.

(B) Formation of teratomas in ES cell-injected limbs. Hind limbs were isolated on days 7 (D7), 21 (D21), and 35 (D35). Black bar, 50 μM.

(C) Formation of cardiac cells in eGFP$^+$ cell-injected limbs. No teratoma (0/15) was found. Injected cells can be seen infiltrating existing skeletal muscle bundles on day 7 (D7, arrows). On day 35 new myofibrils can be observed (D35, arrows). Immunohistochemical staining for eGFP, GATA-4, cardiac MHC, cTnT, SMAα, and SM-MHC are also shown. Black bar, 50 μM.

a single c-kit$^+$Nkx2.5$^+$ cell to differentiate into both myocardial and smooth muscle lineages. c-Kit$^+$Nkx2.5$^+$ single cells were plated into each well of 96-well plates on day 0 and cultured in vitro until day 13 (Figure 5C). Approximately 5% of 672 plated cells survived single cell cloning. Examination of cells within each well on day 13 revealed three distinct patterns of differentiation. Approximately 42% of the single cell clones differentiated into beating cardiomyocytes (clones 1–13) and expressed α-cardiac actin and ventricular myosin light chain (MLCv) (Figure 5D, blue bars). Thirty-two percent differentiated into cells expressing smooth muscle markers, SM22α and SMAα, and failed to beat spontaneously (clones 22–31) (Figure 5D, red bars). The remaining wells (clones 14–20) contained both beating cardiomyocytes and vascular smooth muscle cells, confirming their bipotential differentiation from a single cell (Figures 5C and 5D and Video S3).

Identification of Bipotential c-Kit$^+$Nkx2.5$^+$ Progenitor Cells in the Developing Embryo

The ES-cell studies above suggest that early Nkx2.5$^+$ progenitors expressed c-kit. We investigated whether c-kit$^+$Nkx2.5$^+$ cells are present in the developing heart field in vivo. Flow cytometry analysis of embryonic hearts from Nkx2.5-eGFP transgenic mice revealed that 13% ± 6% of E8.5 eGFP$^+$ cells express c-kit (Figure 6A). This expression decreased with myocardial maturation, such that by day15.5, c-kit expression was nearly undetectable within the Nkx2.5$^+$ population. The expression of Sca-1 within eGFP$^+$ cells appeared later at E13.5 and increased with myocardial differentiation (Figure 6A).

To assess whether c-kit expression confers progenitor cell status within Nkx2.5$^+$ cells, we separated E8.5 eGFP$^+$ cells based on their level of c-kit expression and analyzed their cell morphology and differentiation status by quantitative and qualitative PCR. As shown in Figure 6B, c-kit$^+$ cells appeared to be morphologically less differentiated compared with c-kit$^-$ cells. Gene expression analysis confirmed that c-kit$^-$ cells were more differentiated and preferentially expressed myocardial and smooth muscle lineage markers compared with c-kit$^+$ cells (Figure 6C). These c-kit$^+$ cells are unlikely to originate from hematopoietic sources, as blood-forming cells have not entered the heart tube at this stage of embryonic development. To determine the role of Sca-1 expression, we fractionated E15.5 eGFP$^+$ heart cells based on their Sca-1 status. We found the expression of Sca-1 did not predict progenitor cell status within eGFP$^+$ cells (Figure S3A).

The expression of c-kit in eGFP$^+$ cells was further confirmed by immunofluorescence analysis of frozen sections

(E) Formation of beating cell clusters by FACS-purified eGFP$^+$ cells.

(F) Immunocytochemical analysis of differentiated eGFP$^+$ cells. Sarcomeric MHC, Sarc MHC; cardiac troponin T, cTnT, sarcomeric actinin, Sarc. Actinin; smooth muscle actin-α, SMAα; control IgG, IgG. The SMAα$^+$ cells were found to occasionally self-assemble into tubular structures in vitro (lower right panel).

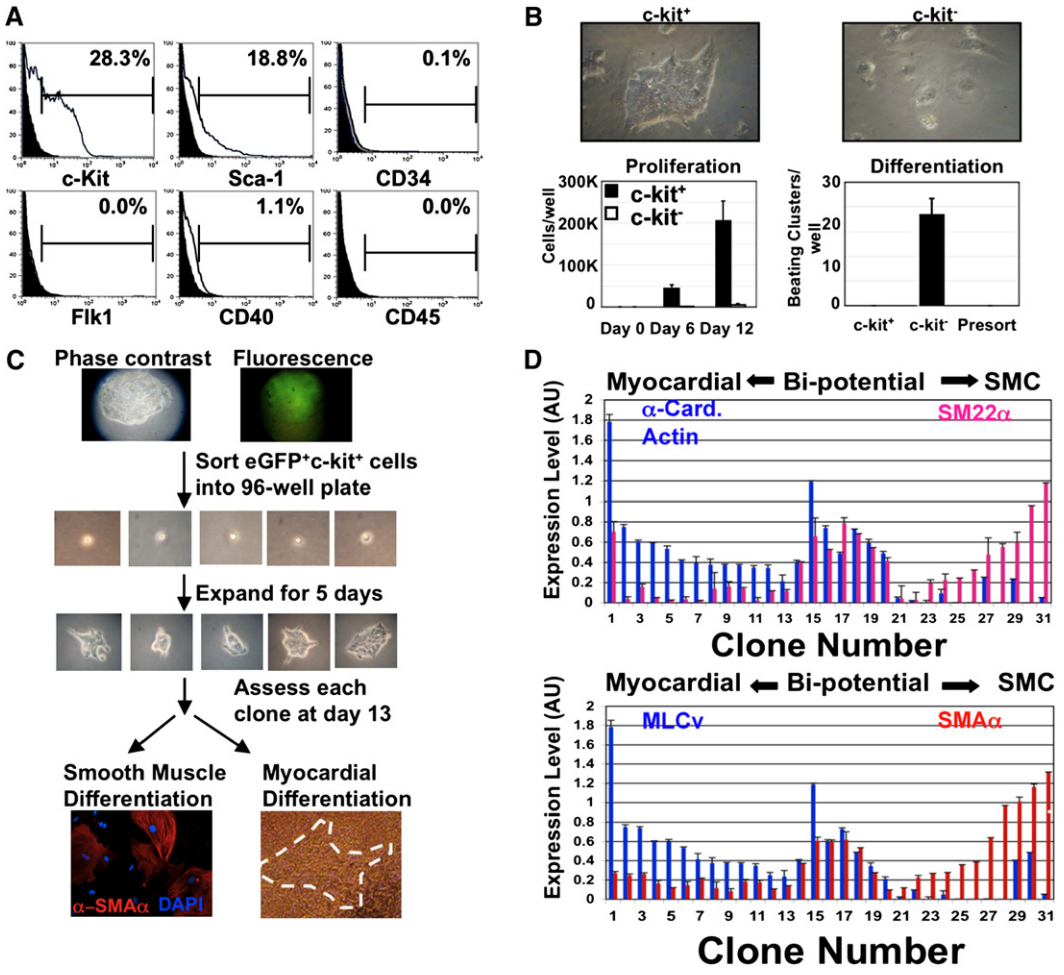


Figure 5. Identification of a c-Kit$^+$ Subpopulation as Progenitor Cells within eGFP$^+$ Cells

(A) Expression of selected surface markers by Nkx2.5-eGFP$^+$ cells.

(B) Differential capacity for proliferation and differentiation of FACS-purified eGFP$^+$c-kit$^+$ versus eGFP$^+$c-kit$^-$ cells from day 5 in vitro differentiated ES cells. The morphologies of c-kit$^+$ and c-kit$^-$ sorted cells are shown (upper panels). The total number of cells in culture at days 6 and 12 after isolation is displayed in lower left panel. The number of beating cell clusters formed was assessed on day 12 (lower right panel).

(C) Clonal differentiation of eGFP$^+$c-kit$^+$ single cells. eGFP$^+$c-kit$^+$ cells from day 5 in vitro differentiated ES cells were deposited as single cells into each well of a 96-well plate. At day 13, the presence of beating cell cluster (demarcated by dashed white line) and the expression of SMAα (by immunofluorescence) were assessed.

(D) Quantitative PCR analysis of differentiated progenies from each c-kit$^+$Nkx2.5$^+$ single-cell clone. The β-actin adjusted expression of α-cardiac actin (blue bar) and SM22α (pink bar) (top panel), MLCv (blue bar), and SMAα (red bar) (bottom panel) in each well is shown.

from E9.5 and E10.5 hearts (Figure S3B). Interestingly, c-kit$^+$Nkx2.5$^+$ cells localized in the outer layer of compact myocardium, suggesting that the niche for these developing progenitor cells is adjacent to the epicardium. This observation is consistent with an essential role for epicardial-secreted factors that stimulate myocardial cell proliferation and expansion of the compact myocardial layer (Lavine et al., 2005).

Bipotential Differentiation of c-Kit$^+$Nkx2.5$^+$ Progenitor Cells

We postulated that smooth muscle cell differentiation from a common progenitor cell results in an alteration of its molecular phenotype from that of differentiating myocardial cells. To test whether the continued expression of eGFP could distinguish myocardial from smooth muscle cells, we isolated eGFP$^+$ cells from E9.5 Nkx2.5-eGFP transgenic mouse hearts and allowed them to undergo spontaneous differentiation in vitro (Figure 6D, a′). Following 3 days of culture, we separated cells that continued to express eGFP from cells that became eGFP$^-$ and analyzed their gene expression. We observed a clear segregation of myocardial lineage markers into the eGFP$^+$ population and smooth muscle markers into the eGFP$^-$ population (Figure 6D, b′). The maintenance of Nkx2.5 expression appeared to characterize myocardial differentiation from a bipotent-progenitor cell.

To establish whether c-kit$^+$Nkx2.5$^+$ cells can undergo bipotential differentiation in vivo, we employed a

well-established chick xenograft transplantation assay and injected freshly isolated c-kit$^+$eGFP$^+$ heart cells (~2500 cells/heart) from E9.5 Nkx2.5-eGFP/ROSA-LacZ double-heterozygous mouse embryos into each of stages 8–13 chick embryos (Figure 6E) (N = 15). One of five surviving embryos demonstrated engraftment of transplanted cells at the aortic arch/ branchial vessel region. Immunohistochemical analysis revealed smooth muscle cell differentiation and integration of LacZ$^+$ cells into an existing blood vessel (Figure 6E, a′–d′). Myocardial differentiation occurred as well (Figure 6E, e′–g ′), but these cells appeared unable to integrate into chick myocardium, presumably due to their ectopic site of engraftment.

DISCUSSION

In this paper, we explored the developmental progression from precardiac mesoderm to myocardial commitment using a genetic tagging approach that allowed for the prospective isolation of cardiac progenitor cells. Importantly, we demonstrated that a c-kit$^+$Nkx2.5$^+$ population differentiates spontaneously into both myocardial and smooth muscle cells, as revealed by in vitro differentiation of ES cells and by embryonic transplantation studies. These bipotent progenitors appear unable to form hematopoietic, neuronal, or skeletal muscle cells; thus, they represent a population that is restricted with respect to other lineages. Taken together, these studies support a new paradigm for cardiovascular development involving the divergence of myocardial and smooth muscle cell lineages from a common precursor in the primordial heart field.

Relationship between Nkx2.5$^+$ Cells and other Embryonic Progenitor Cells

Prior lineage tracing studies in mice have demonstrated diverse developmental origins for vascular smooth muscle cells. In particular, the cardiac neural crest can give rise to vascular smooth muscle cells of the aortic arch, as well as of the coronary vessels (Le Lievre and Le Douarin, 1975; Waldo and Kirby, 1993; Hungerford et al., 1996; Hutson and Kirby, 2003; Stoller and Epstein, 2005). In addition, cells from the secondary heart field have been shown to generate cardiomyocytes of the outflow tract, as well as vascular smooth muscle cells of the aortic ring (Verzi et al., 2005; Waldo et al., 2005). The contribution of these cells to multiple lineages suggests either a broad developmental potential of a common precursor or the existence of multiple lineage-committed progenitors at the same anatomical site.

In this regard, it is of interest that Moretti et al. report the identification of an isl1$^+$ progenitor cell capable of multilineage differentiation in vitro into smooth muscle cells, cardiomyocytes, as well as endothelial cells (Moretti et al., 2006). The relationship between these isl1$^+$ progenitors and the c-kit$^+$Nkx2.5$^+$ cells that we have described herein remains to be clarified. Given that cells of both the primary and secondary heart fields express Nkx2.5, whereas isl1 is expressed exclusively in the secondary heart field, the two progenitor cell populations may either be completely distinct (if c-kit$^+$Nkx2.5$^+$ cells are derived entirely from the primary heart field) or related (if isl1-expressing cells in the secondary heart field are descendents of the mesodermal c-kit$^+$ cells). Of note, our analysis of E10.5 embryos revealed eGFP$^+$ cells in the outflow tract that colocalized with isl1$^+$ cells (Figure 1C, E10.5). These cells are located in the secondary heart field in a region that Waldo et al. had postulated as a site where a bipotential smooth muscle and myocardial progenitor cell might arise (Waldo et al., 2005). Further experiments using cells doubly marked for isl1 and Nkx2.5 will help to rigorously define the relationship between these populations.

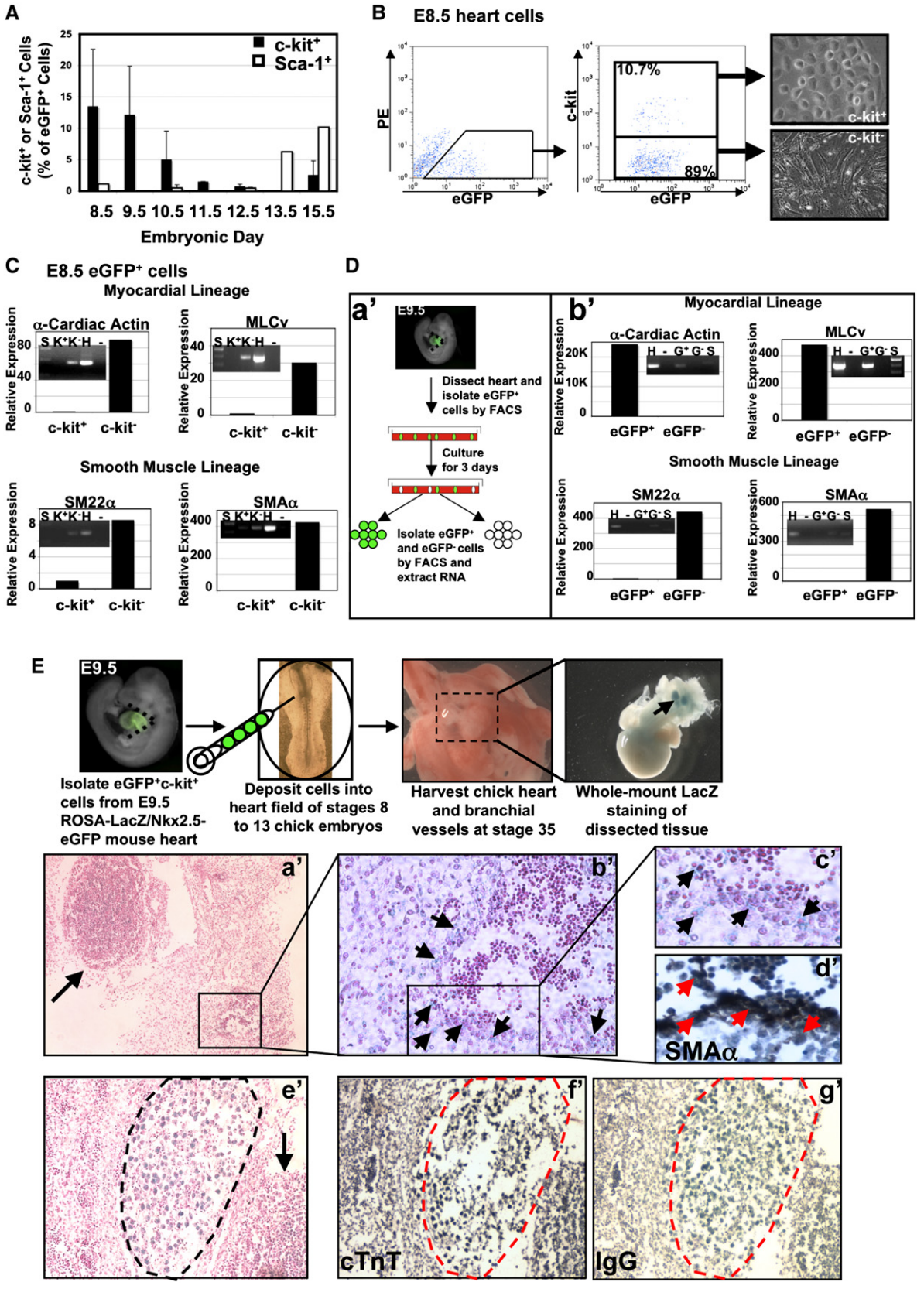
Relationship between c-Kit$^+$Nkx2.5$^+$ Cells and c-Kit$^+$ Cardiac Stem Cells

The adult c-kit$^+$ cardiac stem cell is characterized by the expression of c-kit in the absence of lineage markers such as Nkx2.5 (Beltrami et al., 2003). The developmental potential of these adult c-kit$^+$ cells also appears to be broader. These c-kit$^+$ cells may represent a developmental remnant from a multipotent mesodermal cell population that has persisted in the heart throughout embryonic and postnatal development. Alternatively, these c-kit$^+$ cells may be "itinerant" mesodermal remnants residing in the bone marrow and are mobilized in response to myocardial injury. These cells, although not specifically programmed for myocardial differentiation, have been shown to improve cardiac function in a myocardial injury/reconstitution assay (Fazel et al., 2006). Further studies will be required to determine the ancestral relationship between the c-kit$^+$Nkx2.5$^+$ cells described here and the adult c-kit$^+$ cardiac stem cells.

During development, c-kit is likely dispensable for myocardial or smooth muscle cell proliferation and differentiation. Studies from spontaneous mutation of the *W* locus revealed no essential role for c-kit in cardiovascular development or hematopoietic stem-cell formation despite the established role of this gene as a stem-cell marker (Reith et al., 1990). We believe the expression of c-kit serves as an indicator for developmental potential in different organ systems and, in conjunction with Nkx2.5, defines a bipotent population of cardiovascular progenitor cells.

The Existence of a Bipotential Myogenic Precursor Cell Supports a New Developmental Paradigm for Cardiogenesis

Our data support the existence of a common myocardial and smooth muscle cell precursor in the developing embryo. This is consistent with in vivo studies demonstrating the coexpression of numerous smooth muscle genes in myocardial progenitor cells (Figure S1) (Samaha et al., 1996; Li et al., 1996; Jain et al., 1998). Although it has been proposed that myocardial cells undergo a smooth muscle cell-like transition during early development (Li et al., 1996), we demonstrated that the coexpression of myocardial and vascular smooth muscle cell markers represents evidence for the divergence of these two lineages from a common myogenic precursor. In this regard, recent


A
c-kit+ or Sca-1+ Cells (% of eGFP+ Cells)
c-kit+
Sca-1+
8.5 9.5 10.5 11.5 12.5 13.5 15.5
Embryonic Day
B
E8.5 heart cells
PE
eGFP
c-kit
10.7%
89%
c-kit+
c-kit-
C
E8.5 eGFP+ cells
Myocardial Lineage
α-Cardiac Actin
MLCv
Smooth Muscle Lineage
SM22α
SMAα
Relative Expression
c-kit+ c-kit-
D
a'
E9.5
Dissect heart and isolate eGFP+ cells by FACS
Culture for 3 days
Isolate eGFP+ and eGFP- cells by FACS and extract RNA
b'
Myocardial Lineage
α-Cardiac Actin
MLCv
Smooth Muscle Lineage
SM22α
SMAα
eGFP+ eGFP-
E
E9.5
Isolate eGFP+c-kit+ cells from E9.5 ROSA-LacZ/Nkx2.5-eGFP mouse heart
Deposit cells into heart field of stages 8 to 13 chick embryos
Harvest chick heart and branchial vessels at stage 35
Whole-mount LacZ staining of dissected tissue
a'
b'
c'
d'
SMAα
e'
f'
cTnT
g'
IgG

reports of common progenitor cells for paraxial skeletal muscle cells and aortic smooth muscle cells (Esner et al., 2005), head skeletal muscle and myocardial cells of the outflow tract (Tirosh-Finkel et al., 2006), myocardial and hematopoietic cells (Masino et al., 2004), and endothelial and vascular smooth muscle cells (Ema et al., 2003), all suggest that developmental multipotentiality is more prevalent than that supported by earlier studies. The common myogenic precursor that we prospectively isolated and characterized in this study points to a new paradigm of cardiogenesis where a single multipotent stem/progenitor cell contributes to cells of diverse lineages within the heart.

A Model for the Differentiation of Precardiac Mesoderm into Cells of Divergent Fates within the Heart

A model for the developmental progression from precardiac mesoderm to committed cardiovascular progenitor cell prior to chamber-specific differentiation is depicted in Figure 7. In this model the expression of c-kit precedes the expression of Nkx2.5 at the precardiac mesoderm stage. The initiation of Nkx2.5 expression denotes commitment into a precursor for myocardial and smooth muscle lineages. The expression of c-kit within Nkx2.5$^+$ cells is lost when these cells transition into either smooth muscle or myocardial precursors. With further differentiation, these precursor cells become atrial or ventricular-specific myocytes or cells of the conduction system. Although not specifically investigated in these studies, it has been established that Purkinje fibers are derived from cardiomyocyte precursors (Gourdie et al., 1995). Given that Nkx2.5 mutation is responsible for conduction system disease in both mouse and human (Schott et al., 1998; Pashmforoush et al., 2004), our ability to monitor the differentiation of Nkx2.5$^+$ progenitors into cells of the conduction system in vitro may provide unique insights into the pathogenesis of cardiac arrhythmia.

In summary, we have established the existence of a common myogenic precursor cell that gives rise to both myocardial and smooth muscle lineages. This bipotential progenitor cell makes a lineage choice decision at a single-cell level. These findings reveal a hierarchy for myogenic differentiation in vivo and suggest a new developmental paradigm for cardiogenesis where a single multipotent progenitor cell gives rise to cells of diverse lineages within the heart.

EXPERIMENTAL PROCEDURES

Generation of Nkx2.5-eGFP Transgenic Mice

A 2.1 kb enhancer fragment from the 5′ regulatory region of murine *Nkx2.5* (Lien et al., 1999) (a kind gift from Dr. Eric Olson at UT Southwestern, Dallas, TX) along with a 500 bp *Nkx2.5* base promoter was inserted into a promoterless eGFP expression vector (BD Biosciences-Clontech, Mt. View, CA). The DNA insert, including the eGFP expression sequence, was introduced into the pronucleus from C57Bl/6 mice (Charles River Laboratories, Wilmington, MA). Of the initial founders, one (number 3) was further expanded. All animal experiments described in this paper have been approved by Animal Resources at Children's Hospital (ARCH) of Boston, MA.

Isolation and In Vitro Differentiation of Embryonic Nkx2.5$^+$ Progenitor Cells

Embryos from timed matings of Nkx2.5-eGFP transgenic mice were dissected to release their heart tubes. eGFP$^+$ hearts were immediately digested with collagenase solution (collagenase A [10 mg/mL] and B [10 mg/mL] [Roche Diagnostics, Indianapolis, IN] in 10 mM HEPES buffed saline [HBS] and 20% FCS) for 60 min at 37°C. A single-cell suspension was obtained by gentle triturition followed by passing through a 40 μM cell strainer. eGFP$^+$ and eGFP$^-$ live cells (as defined by the lack of propidium iodine [PI] staining) were isolated by FACS on a MoFlo cell sorter (Cytomation Systems) and cultured in differentiation medium (DM) containing IMDM with high glucose, 20% FCS, 5000 i.u./mL penicillin/ streptomycin, 200 mM L-glutamine , 1-thioglycerol (1.5×10^{-4}M), and ascorbid acid (50 μg/mL). Flow cytometry data was acquired by CellQuest^T v3.3 (BD Biosciences, San Jose, CA) and processed using FlowJo® v4.6.2 (Tree Star, Ashland, OR) software.

Generation of Nkx2.5-eGFP ES Cell Lines

The Nkx2.5 cardiac enhancer-eGFP plasmid was linearized and electroporated into 11th passage CJ7 ES cells and cultured in the presence of G418 to select for neomycin resistant colonies. A total of 168 colonies were expanded and individually in vitro differentiated. Day 9 ES cell aggregates (i.e., embryoid bodies [EBs]) were scored for their expression of eGFP by fluorescence microscopy. Three colonies (numbers 74, 94, and 110) showed complete correlation of eGFP expression with beating cells. One line (number 74) was further studied.

Figure 6. Isolation and Characterization of Nkx2.5$^+$c-kit$^+$ Cells from Developing Embryos

(A) Expression of c-kit (black bar) or Sca-1 (white bar) in eGFP$^+$ cells during embryogenesis. Each bar represents mean ± SEM from four hearts.

(B) The morphologies of E8.5 eGFP$^+$c-kit$^+$ and eGFP$^+$c-kit$^-$ heart cells.

(C) Quantitative and qualitative PCR analysis of lineage marker expression in E8.5 eGFP$^+$c-kit$^+$ versus eGFP$^+$c-kit$^-$ cells. Bar graphs represent β-actin adjusted fold overexpression by c-kit$^-$ cells compared with c-kit$^+$ cells. S, molecular weight standard; K$^+$, c-kit$^+$; K$^-$, c-kit$^-$; H, adult heart cells; and –, H_20 blank.

(D) Maintenance of eGFP expression distinguishes myocardial from smooth muscle cells in culture. eGFP$^+$ cells from E9.5 hearts were cultured for 3 days and resegregated by FACS based on their continued expression of eGFP (panel a′). Quantitative and qualitative expression of myocardial and smooth muscle markers are shown in panel b′. For myocardial lineage markers, bar graphs represent β-actin-adjusted fold overexpression by eGFP$^+$ cells compared with eGFP$^-$ cells. For smooth muscle lineage markers, bar graphs represent fold overexpression by eGFP$^-$ cells compared with eGFP$^+$ cells. H, adult heart cells; –, H_20 blank: G$^+$, eGFP$^+$; G$^-$, eGFP$^-$; S, molecular weight standard.

(E) Transplantation of eGFP$^+$c-kit$^+$ cells from E9.5 ROSA-LacZ/Nkx2.5-eGFP transgenic mouse embryos into chick embryos. Tissues containing LacZ$^+$ cells (arrow in X-gal stained whole heart) were paraffin sectioned and counterstained with nuclear fast red (a′, b′, c′, e′). These same sections were then stained with SMAα (d′), cTnT (f′), or control (IgG) (g′) antibodies, developed with DAB substrate, and counterstained with Hematoxylin Qs. The arrows in (a′) and (e′) denote the major site of LacZ$^+$ cell engraftment. Arrows in (c′) mark individual LacZ$^+$ cells along a nearby blood vessel wall. These same cells (red arrows in [d′]) also express SMAα (black stain). A cluster of LacZ$^+$ cells (within dashed circle in [e′]) also express cTnT (dashed red circle in [f′]).

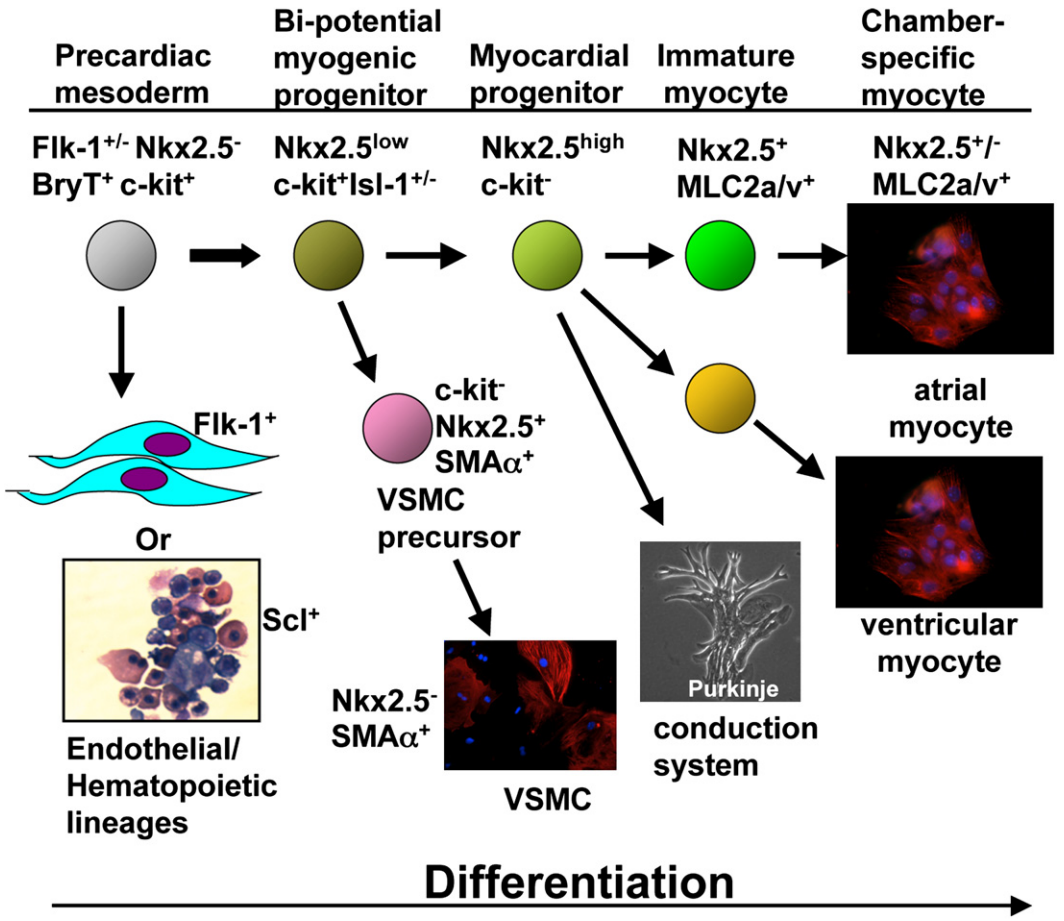


Figure 7. Differentiation of Cardiovascular Lineages from a Common Precursor in the Precardiac Mesoderm

The expression of lineage markers shown is based on our current data as well as other published studies. The divergence of endothelial and hematopoietic lineages from myocardial lineage precedes the expression of Nkx2.5 in the precardiac mesoderm. BryT, brachyury T; VSMC, vascular smooth muscle cell.

In Vitro Differentiation of ES Cell-Derived Nkx2.5$^+$ Cells

ES cells were cultured according to Fehling et al. (Fehling et al., 2003) and adapted to gelatin-coated dishes in the presence of leukemia inhibitory factor for 2 days prior to differentiation. ES cell differentiation was performed according to a previous published protocol (Wobus et al., 1991). On the day of sorting, EBs were digested with either trypsin/EDTA for 5 min or collagenase solution (if EBs were day 6 or later) for 30 min. These cells were then resuspended in HBS with 20% FCS and analyzed on a MoFlo cytometer. This treatment protocol resulted in greater than 90% live single cells.

Immunohistochemical, Immunocytochemical, and Immunofluorescence Analysis of Embryonic and ES Cell-Derived Cells

Nkx2.5-eGFP transgenic mouse embryos or ES cell-injected mouse hind limbs were fixed in 4% paraformaldehyde in phosphate buffered saline and embedded in paraffin prior to 5 μM sectioning or in OCT compound prior to 10 μM cryo-sectioning. Immunohistochemical analysis was performed as previously described (Li et al., 2005).

Molecular Analysis of Gene Expression by Qualitative or Quantitative PCR

Sorted cells from embryonic hearts or in vitro differentiated ES cells were immediately added to Trizol® reagent (Invitrogen, Carlsbad, CA) and stored at −80° C until processed. Total RNA from each sample was purified from cell lysate using the SV Total RNA kit (Promega, Madison, WI) according to manufacturer's suggested protocol. Qualitative and quantitative PCR were performed on cDNA made from reverse transcribed RNA using the I-script® cDNA synthesis kit (BioRad, Hercules, CA) for total cell number >100,000, or Cells-Direct cDNA synthesis system (Invitrogen, Carlsbad, CA) for cell number less than 100,000. Qualitative RT-PCR was performed using Taq® polymerase (Roche Diagnostics, Indianapolis, IN) within the linear range of amplification (25–33 cycles) for each primer. Quantitative PCR was performed using the I-cycler® system with SYBR® Green substrate (BioRad, Hercules, CA) for 40 cycles. Primers sequences used for qualitative and quantitative PCR are available by request.

Mouse Hind-Limb Cell Transplantation Assay

Undifferentiated Nkx2.5-eGFP transgenic ES cells (N = 5) or FACS-isolated eGFP$^+$ cells (N = 15) from day 5 in vitro differentiated Nkx2.5-eGFP transgenic ES cells were injected into the quadriceps of anesthesized SV129 syngeneic mice (1×10^6 cells per injection). At days 7, 21, and 35 (N = 5 each), mice were sacrificed and their quadriceps harvested and fixed immediately in Bouin's solution before paraffin embedding and sectioning.

Electrophysiological Studies of Differentiated Cardiomyocytes

FACS-purified Nkx2.5-eGFP$^+$ cells from embryonic hearts were cultured in DM for 8 days. During experiment, cells were bathed in extracellular solution containing 140 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose at pH 7.4. Patch electrodes were filled with an intracellular solution containing140 mM potassium gluconate, 10 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, 1 mM EGTA, 4 mM Mg-ATP, and 0.3 mM Na-GTP at pH 7.3, giving resistances of ~2–5 MΩ. Spontaneous cardiomyocyte action potentials were recorded at room temperature using the whole-cell patch clamp method in current clamp mode with an Axopatch 200A amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA). Holding currents were sometimes applied to alter the frequency of firing. Recorded data were filtered online at 2 kHz, sampled, and digitized at 10 kHz (Digidata 1322A and pClamp 9.2 software; Axon Instruments/Molecular Devices, Sunnyvale, CA).

Chick Embryo Transplantation Assay

FACS-purified eGFP$^+$c-kit$^+$ cells from E9.5 heart tubes of mice carrying a β-galactosidase expressing transgene at the ROSA locus (*Gt(ROSA26) Sor*, Jackson Lab, Bar Harbor, ME) (ROSA-LacZ) and the Nkx2.5-eGFP transgene were transplanted into the anterolateral plate mesoderm region of stage 8 or dorsomesocardial region of stage 13 chick embryos. At stage 35, the heart and aortic arch region of chick embryos were harvested and fixed in 4% PFA for 15 min followed by X-gal staining overnight. LacZ$^+$ tissues were paraffin sectioned and analyzed by nuclear counterstain and immunohistochemistry.

Supplemental Data

Supplemental data include Supplemental Experimental Procedures, three figures, and three videos and can be found with this article online at http://www.cell.com/cgi/content/full/127/6/1137/DC1/.

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