Developmental Cell *11*, 723–732, November, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.devcel.2006.10.002

Multipotent Flk-1$^+$ Cardiovascular Progenitor Cells Give Rise to the Cardiomyocyte, Endothelial, and Vascular Smooth Muscle Lineages

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Summary

Cell-tracing studies in the mouse indicate that the cardiac lineage arises from a population that expresses the vascular endothelial growth factor receptor 2 (VEGFR2, Flk-1), suggesting that it may develop from a progenitor with vascular potential. Using the embryonic stem (ES) cell differentiation model, we have identified a cardiovascular progenitor based on the temporal expression of the primitive streak (PS) marker *brachyury* and Flk-1. Comparable progenitors could also be isolated from head-fold stage embryos. When cultured with cytokines known to function during cardiogenesis, individual cardiovascular progenitors generated colonies that displayed cardiomyocyte, endothelial, and vascular smooth muscle (VSM) potential. Isolation and characterization of this previously unidentified population suggests that the mammalian cardiovascular system develops from multipotential progenitors.

Introduction

The heart primordium is derived from mesodermal cells that migrate through the primitive streak (PS) to the anterior-proximal side of the embryo where they organize as an epithelial layer to initially form a structure known as the cardiac crescent at approximately E7.75 of development (Garcia-Martinez and Schoenwolf, 1993; Tam et al., 1997). Shortly thereafter, the cardiac crescent fuses at the midline and gives rise to the primordial heart tube, which undergoes a process of looping and rapid growth to generate the embryonic heart. It is now well established that the heart develops from two separate progenitor pools or heart fields that arise from distinct mesodermal populations at different times (Cai et al., 2003; Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001; Zaffran et al., 2004). The first population to migrate to the heart region originates from the anterior splanchnic mesoderm and establishes the primary heart field that gives rise to the early heart tube and ultimately contributes to the left ventricle and atria (Dehaan, 1963; Zaffran et al., 2004). The second cardiogenic population of the embryo is derived from pharyngeal mesoderm (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001; Zaffran et al., 2004). These cells constitute the secondary or anterior heart field and contribute initially to the arterial end of the developing heart tube at E9.5 and subsequently to the right ventricle and outflow tract of the mature heart (Kelly et al., 2001; Zaffran et al., 2004). Retrospective clonal analyses have shown that the two heart fields are established as distinct lineages early in development and likely segregate from a common progenitor at the time of gastrulation (Meilhac et al., 2004).

Molecular studies have uncovered a number of different key transcription factors that are expressed in and function at specific stages of myocardial development and heart formation. Two of the earliest markers of the cardiac lineage, Mesp1 and Mesp2, are expressed transiently in the PS populations that contribute to both heart fields (Kitajima et al., 2000; Saga et al., 1999). Both genes are required for proper movement of the cells to the anterior region of the embryo (Kitajima et al., 2000). In the anterior region, cells of the cardiac crescent are exposed to cytokines of the BMP (Schultheiss et al., 1997) and FGF (Reifers et al., 2000) families as well as to inhibitors of the Wnt pathway (Marvin et al., 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001), resulting in the onset of cardiac differentiation marked by expression of two key regulators of the lineage, Nkx2.5 (Lints et al., 1993) and GATA-4 (Arceci et al., 1993; Heikinheimo et al., 1994; Kelley et al., 1993). Myocardial maturation within the cardiac crescent is associated with expression of contractile proteins including myosin light chain-2a, (MLC2a) (Kubalak et al., 1994) and sarcomeric-MHC (Han et al., 1992), expressed in the cardiac crescent and throughout the nascent heart tube. In addition to defining specific stages of cardiac development, gene expression patterns were instrumental in establishing the concept of two heart fields. Expression of the lim domain gene *Isl1* and the growth factor FGF10 distinguish the two populations as they are both detected in progenitors of the secondary but not in those of the primary heart field (Cai et al., 2003; Kelly et al., 2001). Tbx5, a T-box transcription factor, is initially expressed throughout the cardiac crescent and then appears to be restricted to derivatives of the primary heart field (Bruneau et al., 1999, 2001; Buckingham et al., 2005; Zaffran et al., 2004). Genes expressed uniquely in the earliest primary heart field progenitors have not been described.

While the two origins of heart progenitors are now well demonstrated, the specification and relationship of the earliest cardiac lineages, the myocardium and endocardium, have not been elucidated. During heart development, the cooperative interactions of these lineages are critical for many aspects of heart morphogenesis, including establishment of the conduction system (Carraway et al., 1997; Meyer and Birchmeier, 1995; Meyer et al., 1997; Rentschler et al., 2002), trabeculation (Gassmann et al., 1995; Lee et al., 1995), and myocyte growth and survival (Lavine et al., 2005; Zhao et al., 1998). Insights into these early stages of cardiac development have been provided by lineage-tracing studies in the mouse that demonstrate that the endocardium and a population of the myocardium develop from an intermediate population that expresses Flk-1 (Ema et al., 2006; Motoike et al., 2003). This Flk-1$^+$ population appears to develop as the cells exit the PS and begin to migrate to form the cardiac crescent (Ema et al., 2006).

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These observations support the notion that the myocardial and endothelial lineages develop from a common Flk-1$^+$ progenitor. Such a progenitor could represent the cardiac equivalent of the hemangioblast of the hematopoietic system, a Flk-1$^+$ progenitor that is specified at the onset of embryonic blood cell development and displays the potential to generate both hematopoietic and vascular progeny (Huber et al., 2004). Although the concept of a comparable multipotential cardiovascular progenitor is attractive and consistent with findings from lineage tracing experiments, the identification and characterization of such a cell has not been possible due to the small size and inaccessibility of the early mouse embryo.

The outstanding capacity of embryonic stem (ES) cells to generate differentiated progeny in vitro offers a powerful model system for studying mammalian development and lineage commitment as it overcomes the problems of accessibility and cell numbers associated with working with the early embryo (Keller, 2005). The ES-cell model was instrumental in the identification and characterization of the hemangioblast and in providing approaches for the isolation of a comparable progenitor in the early embryo (Choi et al., 1998; Huber et al., 2004). Through the use of an ES-cell line with the green fluorescent protein cDNA targeted to the mesodermal gene *brachyury* (GFP-Bry cells), more recent studies have demonstrated that the sequential allocation of mesodermal cells to hemangioblast and cardiac fates, found during embryonic development (Kinder et al., 1999), also takes place in ES-cell-derived embryoid bodies (EBs) (Fehling et al., 2003; Kouskoff et al., 2005). They showed that within 72 hr of differentiation, the hemangioblast and cardiac potential within the developing embryoid bodies (EBs) segregated to distinct populations, defined as GFP-Bry$^+$/Flk-1$^+$ (D3.25 Flk-1$^+$) and GFP-Bry$^+$/Flk-1$^-$ (D3.25 Flk-1$^-$), respectively (Kouskoff et al., 2005). These findings established the identity of the earliest cardiac mesoderm population, both from the perspective of markers and the temporal pattern of development.

To further define the developmental progression of this cardiac mesoderm, we followed the maturation of the GFP-Bry$^+$/Flk-1$^-$ population and demonstrated that it generates a second Flk-1$^+$ population that displays robust cardiac potential. Clonal assays revealed that the second Flk-1$^+$ population contained progenitors with cardiomyocyte, vascular smooth muscle (VSM), and endothelial potential. Furthermore, these clones displayed molecular profiles consistent with cells of the primary and secondary heart fields. Similar cardiovascular progenitor cells could be isolated from the anterior side of head-fold-stage mouse embryos. Together, these findings document the existence of a Flk-1$^+$ cardiovascular progenitor, which is distinct from the hemangioblast, and in doing so strongly support the concept that the mammalian cardiovascular system develops from multipotential progenitor cells.

Results

Cardiac Potential Is Enriched in the D4.25 GFP-Bry$^+$/Flk-1$^+$ Population during ES-Cell Differentiation

To identify and characterize cardiac progenitor cells within the D3.25 Flk-1$^-$ cell population, we analyzed its developmental progression in more detail. In the protocol established in our earlier study, D3.25 GFP-Bry$^+$/Flk-1$^-$ cells were allowed to reaggregate for 24 hr to enable the continued differentiation of the *brachyury*-expressing mesodermal populations. Following 2–3 days of culture on gelatin-coated wells in serum-free (SF) conditions, cells from these aggregates differentiated into populations of contracting cardiomyocytes (Kouskoff et al., 2005). Analysis of the GFP-Bry$^+$/Flk-1$^-$-derived aggregates prior to replating revealed that a subpopulation of these cells upregulated Flk-1 expression during the 24 hr reaggregation step (Figure 1A). The size of the Flk-1$^+$ population varied from 5% to 10% of the total GFP-Bry population when the cells were allowed to aggregate in SF media in the absence of added cytokines (data not shown). The proportion of Flk-1$^+$ cells increased to greater than 20% of the total population if VEGF was included in the reaggregation culture.

To determine which population contained the cardiomyocyte potential, the Flk-1$^+$ and Flk-1$^-$ populations were isolated from the aggregates (day 4.25 presort) and plated onto gelatinized microtiter wells. Virtually all cardiomyocyte potential segregated to the Flk-1$^+$ population. Within 2–3 days of culture, the Flk-1$^+$ population generated discrete areas of contracting cells. Following an additional 2–3 days, these areas expanded, resulting in the development of a single mass of contracting cells covering the entire well (Supplemental Data and Movie S1 available with this article online). In contrast to the Flk-1$^+$ population, very little cardiomyocyte development was observed in cultures of Flk-1$^-$ cells. To quantify the extent of cardiomyocyte development in each population, the cardiac isoform of TroponinT (cTnT) was evaluated by intracellular flow cytometry. Greater than 50% of the cells in the D4.25 Flk-1$^+$ cultures expressed cTnT as compared to 8% of the cells from the D3.25 Flk-1$^+$ population and 4% from the D3.25 Flk-1$^-$ population (Figure 1B).

The D4.25 Flk-1$^+$ cardiac population is generated from D3.25 Flk-1$^-$ cells following a 24 hr reaggregation step, whereas the D3.25 Flk-1$^+$ population that displayed little cardiac potential was assayed directly without reaggregation. One possible explanation for the difference in cardiac potential between the D3.25 Flk-1$^-$ and D3.25 Flk-1$^+$ populations is that one was reaggregated, while the other was not. To determine if this is the case, D3.25 Flk-1$^+$ cells were reaggregated for 24 hr in the same conditions used for the D3.25 Flk-1$^-$ population and then replated in the monolayer assay. Very few contracting cells were observed following 4 days of culture. Flow cytometric analysis revealed the presence of very low numbers of cTnT-positive cells, consistent with the lack of contracting myocytes (Figure 1C). These findings strongly suggest that the ES-cell cardiomyocyte lineage develops from a Flk-1$^+$ population that emerges later than, and is distinct from, the earliest Flk-1$^+$ population that has hemangioplast potential and displays little cardiac potential (Figure 1D). These populations will now be referred to as D3.25 Flk-1$^+$ and D4.25 Flk-1$^+$ cells.

Emergence of the Flk-1$^+$ Cardiac Progenitor Population Precedes Expression of Early Cardiac Transcription Factors

Contracting cardiomyocytes are first observed following 2 days of culture of the D4.25 Flk-1$^+$ population,

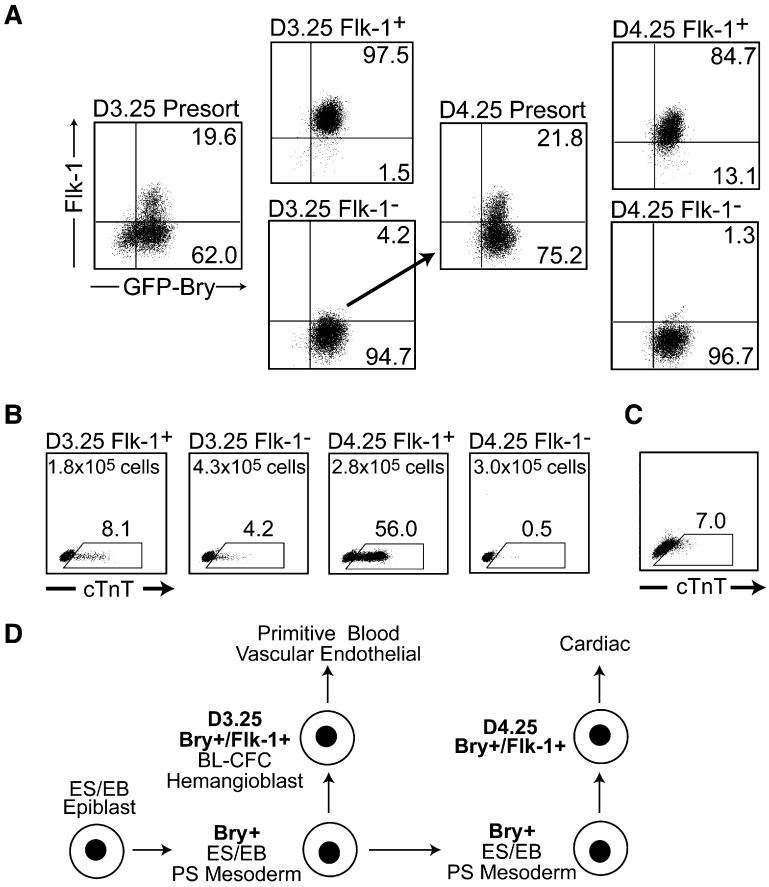

Figure 1. Development of D3.25 and D4.25 Flk-1$^+$ Populations from Serum-Stimulated EBs and Their Commitment to the Cardiac Lineage

(A) GFP-Bry$^+$/Flk-1$^+$ and GFP-Bry$^+$/Flk-1$^-$ populations were isolated from day 3.25 EBs by cell sorting. The GFP-Bry$^+$/Flk-1$^-$ cells were reaggreaged in SF media supplemented with VEGF and bFGF for 24 hr. At this stage (D4.25), the aggregates were dispersed and separated into Flk-1$^+$ and Flk-1$^-$ fractions by cell sorting.

(B) Cells (6×10^4) from the D3.25 Flk-1$^+$, D3.25 Flk-1$^-$, D4.25 Flk-1$^+$, and D4.25 Flk-1$^-$ population were plated directly into microtiter wells in SF conditions. Following 5 days of culture, the cells were harvested, counted, stained for cTnT, and analyzed by flow cytometric analysis.

(C) Cells (6×10^4) from the D3.25 Flk-1$^+$ population were reaggregated for 24 hr in SF conditions followed by trypsinization and replating onto microtiter wells. Following 5 days of culture, cells were harvested and analyzed as in (B).

(D) Schematic of mesoderm, hemangioblast, and cardiac formation during ES/EB differentiation and embryonic development.

indicating that it represents an early stage of lineage development. To further define the developmental status of this D4.25 Flk-1$^+$ population with respect to the other ES-cell populations and to the contracting myocytes, we analyzed the expression patterns of genes known to mark specific stages of cardiac development (Figure 2). The PS marker *Mesp1* was expressed in each of the isolated GFP-Bry populations (Saga et al., 1999). Its expression declined as the D4.25 Flk-1$^+$ population matured to form contracting cells. *Isl1*, a gene expressed in progenitors giving rise to the secondary heart field (Cai et al., 2003) as well as limbs (Yang et al., 2006), neuronal subsets, and pancreatic endoderm (Karlsson et al., 1990; Thor et al., 1991), was also detected in each of the sorted populations, although levels tended to be higher in the day 4 populations. Expression was readily detected in the contracting cultures. *GATA-4*, expressed in visceral and definitive endoderm and cardiac mesoderm of the embryo (Arceci et al., 1993; Heikinheimo et al., 1994; Kelley et al., 1993) was detected in all populations analyzed. The early acting transcription factors *Nkx2.5* and *Mef2c* were first expressed following 1 day of culture of the D4.25 Flk-1$^+$ progenitors. *Tbx5* as well as *Tbx20*, a gene normally expressed in the endocardium and myocardium of the developing heart (Stennard et al., 2003), and *Mlc2v*, a gene that encodes contractile proteins, were all detected with the onset of contraction in the cultures. The endothelial markers *Flk-1* and *CD31* were expressed in all of the isolated fractions as well as in the replated contracting populations. Together, the findings from these analyses demonstrate that the molecular progression of mesoderm to nascent cardiac tissue and contracting myocytes found in the early embryo is recapitulated in the ES-cell model. In addition, they position the D4.25 Flk-1$^+$ cardiac progenitor population

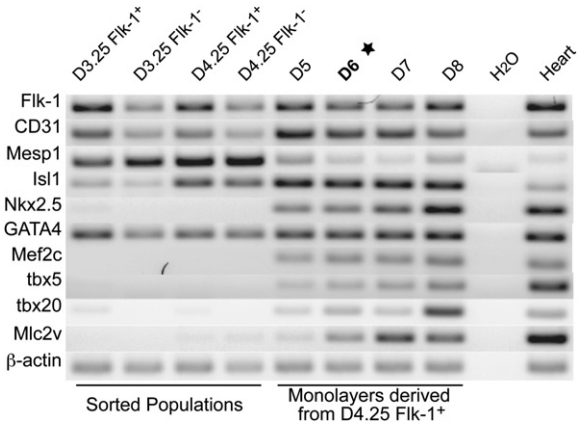

Figure 2. Gene Expression Profiles of Sorted GFP-Bry/Flk-1 Populations and Developing Cardiac Cultures

D3.25 Flk-1$^+$, D3.25 Flk-1$^-$, D4.25 Flk-1$^+$, and D4.25 Flk-1$^-$ sorted populations and developing cardiac cultures derived from the D4.25 Flk-1$^+$ fraction were isolated and analyzed by PCR. The star and bolding at D6 indicate the onset of contraction. Expression profiles representing endothelium (*Flk-1*, *CD31*), mesoderm (*Mesp1*), and cardiac markers (*Isl1*, *Nkx2.5*, *GATA4*, *Mef2c*, *Tbx5*, *Tbx20*, and *Mlc2v*) were analyzed. Controls include H_2O and neonatal mouse heart.

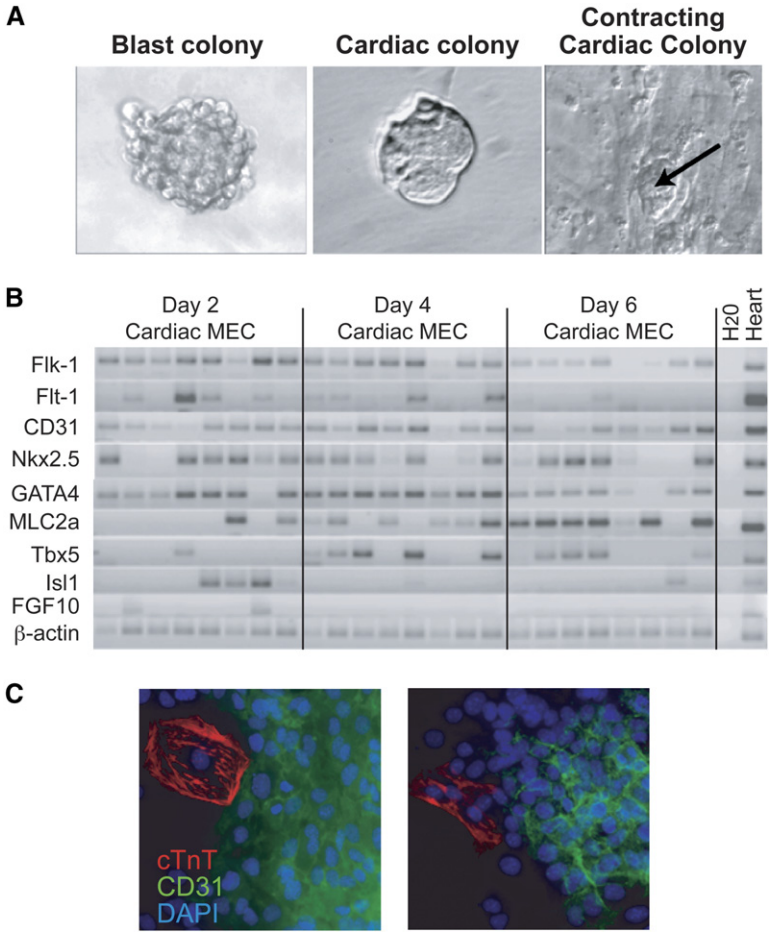

Figure 3. Identification of Cardiac Colonies and Lineage Analysis of Individual Colonies

(A) Images of a blast colony generated from D3.25 Flk-1$^+$ cells, a cardiac colony generated from D4.25 Flk-1$^+$ cells, and a cardiac colony expanded in liquid culture. Arrow indicates the region of contracting cells (see Supplemental Data and Movie S2).

(B) PCR analysis of individual colonies grown for 2, 4, and 6 days in MEC cultures. Colonies were harvested and subject to molecular analysis at the indicated days. Lanes represent individual colonies.

(C) Immunostaining for cTNT and CD31 protein expression of cardiac colonies expanded for 2 days in liquid culture. Two representative colonies are shown.

at a developmental stage preceding the upregulation of expression of the transcription factors *Nkx2.5* and *Mef2c*.

The D4.25 Flk-1$^+$ Population Contains Cardiac Progenitors that Display Cardiomyocyte, Endothelial and VSM Lineage Potential

We have previously shown that the D3.25 Flk-1$^+$ EB-derived population has hemangioblast potential defined by the presence of progenitors known as blast-colony-forming cells (BL-CFCs) that display the ability to generate blast colonies consisting of the hematopoietic, vascular endothelial, and VSM lineages (Choi et al., 1998; Fehling et al., 2003; Kouskoff et al., 2005; Nishikawa et al., 1998) (Figure 3A, blast colony). To determine if the D4.25 Flk-1$^+$ population contained comparable colony-forming cells for the cardiac lineages, we cultured these cells in methylcellulose (MEC) containing VEGF and bFGF as well as cytokines known to function in early heart formation including BMP4 (Schultheiss et al., 1997) and the Wnt inhibitor DKK1 (Marvin et al., 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001) (cardiac MEC). Following 4–6 days of culture in these conditions, the D4.25 Flk-1$^+$ cells generated tightly packed colonies that could easily be distinguished from the D3.25 Flk-1$^+$ BL-CFC-derived blast-cell colonies (Figure 3A, cardiac colony). Between 300 and 1000 cardiac colonies developed from 2×10^4 plated cells. When 6-day-old colonies were picked from the MEC and replated onto a gelatin substrate in SF conditions, 25%–90% of the colonies began spontaneous contraction (Figure 3A, contracting cardiac colony, Supplemental Data, and Movie S2). These findings demonstrate that progenitors within the D4.25 Flk-1$^+$ population respond to cytokines found in the heart field of the mouse embryo and generate colonies that contain cardiomyocte potential.

Molecular analysis of colonies at days 2, 4, and 6 of growth revealed that greater than 60% expressed *Nkx2.5* and *Flk-1*, suggesting most have both cardiac and endothelial potential (Figure 3B). Most colonies from the three different days also expressed *GATA4*. The overall expression patterns did change with colony age as the 2- and 4-day-old colonies expressed readily detectable levels of cardiac and vascular genes whereas those isolated at day 6 of growth preferentially expressed genes indicative of cardiomyocyte maturation. In addition to *Nkx2.5*, some of the day 2 colonies expressed other genes associated with cardiac development, including *MLC2a*, *Tbx5*, and *Isl1*. Expression of *Isl1* in some of these colonies suggests that they may contain progenitors of the anterior heart field. Coexpression of *FGF10* in one of the *Isl1*$^+$ colonies is consistent with this interpretation. The *Isl1*-negative colonies may be representative of the primary heart field as suggested by the expression of *Tbx5* in one of them. By day 4 of growth, a larger portion of the colonies expressed *MLC-2a* and *Tbx5* than was found at day 2, suggesting

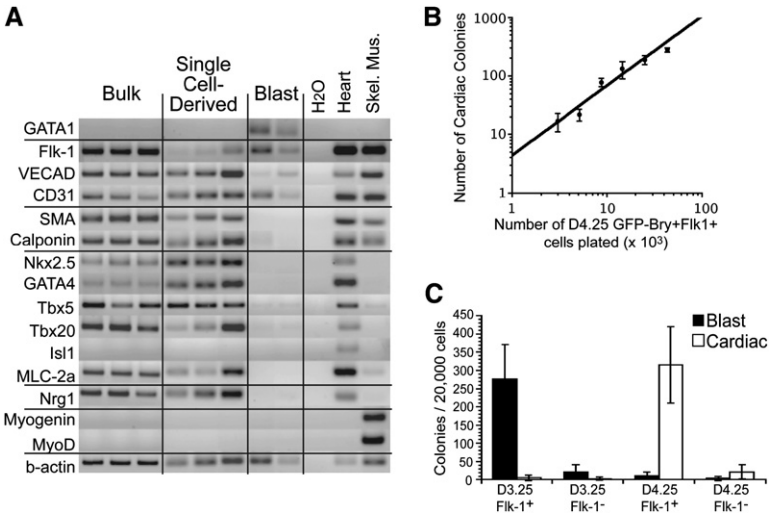

Figure 4. Multilineage Potential of D4.25 Flk-1$^+$-Derived Progenitors

(A) PCR analysis of expanded colonies generated from D4.25 Flk-1$^+$ cells. Four-day-old colonies were picked either from bulk cultures initiated in 35 mm dishes (2×10^4 cells/ml; D4.25 Flk-1$^+$ bulk; three representative colonies are shown) or from microtiter wells seeded with single D4.25 Flk-1$^+$ cells (D4.25 Flk-1$^+$ single cell). Colonies were expanded for 2 days in liquid culture prior to analysis. Comparable aged blast colonies generated from D3.25 Flk-1$^+$ cultured in Blast MEC for 4 days (D3.25 Flk-1$^+$ Blast) were also analyzed. Controls include H_2O, neonatal heart, and skeletal-muscle cDNA.

(B) Relationship between the number of cardiac colonies and the number of D4.25 Flk-1$^+$ cells plated. D4.25 Flk-1$^+$ cells were plated at different densities into cardiac MEC and grown for 6 days. At day 6, the colonies were counted and replated in liquid culture for an additional 3–5 days. The percentage of contracting cardiac colonies was multiplied by the number of colonies from D6 to determine the number of cardiac colonies. Bars represent standard error of the mean.

(C) BL-CFC and CV-CFC potential of the indicated day 3.25 and 4.25 populations. Blast colonies were scored following 4 days of growth, whereas the cardiac colonies were counted at day 6. Forty-eight cardiac colonies were picked in each of six experiments and plated into liquid culture. The proportion of expanded colonies per 2×10^4 cells that generated contracting cells was scored 3–5 days later. The number of cardiac colonies was determined by multiplying the proportion of colonies that generated contracting cells by the number of colonies scored in the MEC culture. Bars represent standard error of the mean number of colonies from six cultures.

maturation to a population consistent with primary heart field myocytes. One *Tbx5*$^+$ colony at this stage faintly coexpressed *Isl1*. All *Nkx2.5*$^+$ 6-day-old colonies expressed *MLC-2a*, and four of six expressed *Tbx5*. Only one colony expressed *Isl1*, and most showed reduced levels of the endothelial genes *Flk-1* and *Flt-1*. Collectively, these expression patterns support the interpretation that the compact colonies contain populations of cardiomyocytes and endothelial cells. The expression patterns of *Isl1* and *Tbx5* suggest that colonies representing both primary and secondary heart field populations develop early in the cultures. The loss of *Isl1*-expressing colonies and the increase in *Tbx5*-expressing colonies may imply that the conditions in the cardiac MEC assay do not support the growth and maturation of those containing secondary heart field cells.

To further evaluate their lineage potential, individual 4-day-old colonies were picked and expanded on fibronectin-coated microtiter wells in SF medium supplemented with BMP4, Dkk1, VEGF, bFGF, D4T, and FCS. After 2–3 days of culture, 30% of colonies gave rise to detectable contracting cells. Immunofluorescence analysis of expanded populations from individual colonies showed the presence of cells that expressed the endothelial marker CD31 in the majority (39 of 46) of colonies that expressed the cardiomyocyte marker cTnT (Figure 3C). Molecular analyses of the expanded contracting cardiac colonies demonstrate the expression of genes indicative of the endothelial (*Flk-1*, *VECAD*, *CD31*), VSM (*smooth muscle actin* [*SMA*] and *Calponin*), and cardiomyocyte (*Nkx2.5*, *GATA-4*, *Tbx5*, *Tbx20*, *MLC-2a*) (Figure 4A, bulk) lineages. Consistent with the later stage methylcellulose colonies, *Isl1* was not expressed in any of the expanded populations. Of 18 expanded colonies analyzed, all expressed *Tbx5*, and none expressed *Isl1*. In addition to these genes, *neuregulin* (*Nrg1*) a gene expressed in endocardial cells (Meyer and Birchmeier, 1995; Meyer et al., 1997), was also detected, suggesting the presence of endocardium. *Gata-1*, a hematopoietic-specific gene, and *Myogenin* and *MyoD* genes associated with skeletal muscle were not expressed in these cultures. BL-CFC-derived blast colonies expressed *Gata-1* and the endothelial genes, but not the genes associated with cardiac development (Figure 4A, blast).

The above replating studies demonstrate that progenitors with cardiomyocyte, endothelial, and VSM lineage potential are contained within individual D4.25 Flk-1$^+$-derived cardiac colonies and support the notion that these populations share a common ancestor, provided that the colonies are clonal. As a first approach to address the issue of clonality, the relationship between the number of D4.25 Flk-1$^+$ plated cells and the number of cardiac colonies formed was determined. As shown in Figure 4B, this relationship is linear with a slope of one, suggesting that the cardiac colonies are derived from a single-colony-forming unit and not a result of aggregation. As a second method to establish the clonality of the cardiac colonies, single D4.25 Flk-1$^+$ cells were deposited into microtiter wells containing cardiac MEC. In seven separate experiments, cardiac colonies developed at a frequency of 30–48 per 480 cells plated. Analysis of the expanded populations from these colonies indicated that they expressed the same spectrum of cardiac, endothelial, and VSM genes found in the cells generated from cardiac colonies from the bulk cultures (Figure 4A, single-cell derived). Colonies generated from single cells also gave rise to contracting cardiomyocytes when cultured in cardiac MEC for 6 days. The results from these analyses indicate that the D4.25 Flk-1$^+$ population contains multipotential progenitors that have the ability to generate colonies containing cells of the cardiac, endothelial, and VSM lineages.

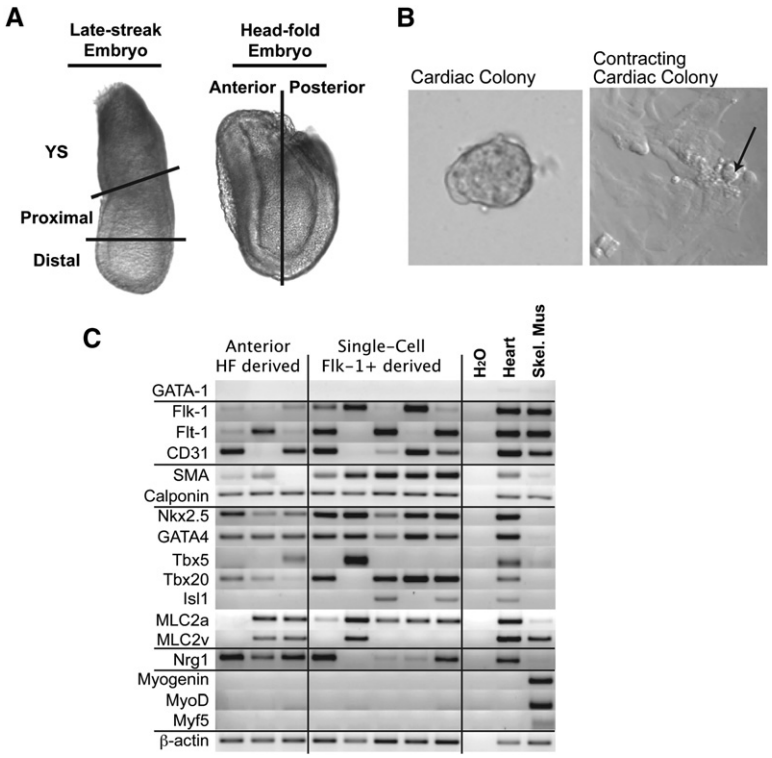

Figure 5. Identification of Multipotential CV-CFCs in the Early Embryo

(A) Early and late PS embryos were dissected into proximal and distal sections. Neural-plate and head-fold embryos were dissected into anterior and posterior sections. The YS was removed from all embryos. Embryo fragments were dispersed into a single-cell suspension and assayed for CV-CFC potential in cardiac MEC.

(B) Six-day-old colonies (cardiac colony) were picked and expanded in liquid culture for 5 days (contracting cardiac colony). The arrow indicates area of contracting cells (see Supplemental Data and Movie S3).

(C) PCR analysis of expanded embryo-derived cardiac colonies picked from bulk cultures of anterior embryo cells (anterior head fold [HF]) or from microtiter wells seeded with single Flk-1$^+$ cells (single-cell Flk-1$^+$) isolated from entire head-fold-stage embryos. Colonies were picked at day 4 of growth in the MEC cultures and expanded for an additional 3 days in liquid culture prior to molecular analysis. Lanes represent individual colonies. Controls are the same as for Figure 4A.

Hereafter, these progenitors will be referred to as cardiovascular-colony-forming cells (CV-CFCs).

Analysis of the different GFP-Bry/Flk-1 fractions for hemangioblast and cardiovascular progenitor potential revealed that the majority of BL-CFC potential was found in the D3.25 Flk-1$^+$ population, whereas the CV-CFCs segregated to the D4.25 Flk-1$^+$ fraction (Figure 4C). The lack of CV-CFC potential in the D3.25 Flk-1$^-$ population from which the D4.25 Flk-1$^+$ population arises suggests that these progenitors represent a specific stage of maturation during cardiac mesoderm development. These findings demonstrate that the BL-CFC and the CV-CFC represent distinct progenitors and that the hemangioblast and cardiac lineages are generated within Flk-1$^+$ populations that develop in a defined temporal pattern of differentiation.

Multipotential Cardiovascular-Colony-Forming Cells Can Be Isolated from the Head-Fold-Stage Embryo

To determine if the early embryo contains progenitors similar to the CV-CFCs, we isolated various regions from different staged embryos and cultured the cells in cardiac MEC. For these analyses, we assayed the proximal/distal regions of the PS from midstreak and late-streak embryos and the anterior/posterior region containing the heart primordium of neural plate and head-fold embryos (Tam et al., 1997) (Figure 5A). The yolk sac (YS) was removed from all embryos prior to dissection. Cardiac colonies, similar to those obtained in the ES-cell system, able to differentiate into contracting cells were obtained only from the anterior region of the neural plate and head-fold-stage embryos (Figure 5B, Supplemental Data, and Movie S3). The anterior neural plate embryos gave rise to 0–1 contracting colonies per embryonic section of 13 embryos analyzed. The later stage head-fold embryos contained 1–8 colonies per equivalent region of 50 embryos analyzed. No colonies were generated from PS embryos (40 total embryos) or from the posterior sections of neural-plate and head-fold-stage embryos.

When 4-day-old embryo-derived colonies were picked and expanded in liquid culture for 3 days, 3 out of 29 generated contracting cells. Molecular analysis of these colonies revealed expression of cardiac-, endothelial-, and VSM-associated genes, in a pattern similar to that observed for the cells derived from the CV-CFC. (Figure 5C, anterior head fold). To determine if the embryo-derived colonies represent clones, single Flk-1$^+$ cells from head-fold-stage embryos were deposited into microtiter wells containing cardiac MEC. In two separate experiments, 33 and 31 cardiac colonies were detected from 830 cells plated. After expansion, a representative analysis of the cells generated from these colonies revealed the expression of endothelium-, VSM-, and cardiac-associated genes (Figure 5C, single-cell Flk-1$^+$). *Isl1* was expressed in two of ten colonies analyzed, whereas *Tbx5* was detected in one, suggesting that both heart fields are represented. Of a total of 21 bulk- and clonal-embryo-derived colonies analyzed, 11 expressed *Tbx5*, while 2 expressed *Isl1*. In all, the findings from these analyses demonstrate the existence of Flk-1$^+$ multipotential cardiovascular progenitors in the region of head-fold-stage embryos that will give rise to the heart.

Discussion

The identification of a Flk-1$^+$ cardiovascular progenitor in this study is consistent with observations from in vivo lineage-tracing studies and expression analysis

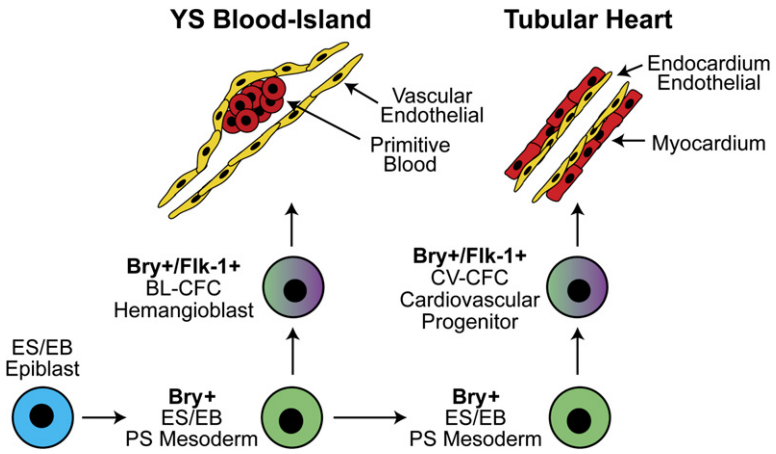

Figure 6. Model of Mesodermal Specification to the Hematopoietic and Vascular Lineages of the YS Blood Islands and to the Developing Heart

In the ES-cell differentiation system (ES/EB) and the developing embryo (epiblast) the Bry$^+$/Flk-1$^+$ BL-CFC develops first to generate the hematopoietic and vascular lineages of the blood islands, and this is followed by the development of the Bry$^+$/Flk-1$^+$ CV-CFC to establish the myocardial and endothelial lineages of the heart.

that demonstrate Flk-1 expression early in cells of the cardiac crescent and later in the endocardium and myocardium (Ema et al., 2006; Motoike et al., 2003). Of significance in the most recent of these studies was the finding that Flk-1 was not expressed in cells of the PS but was upregulated as they exited the streak (Ema et al., 2006). The progression of the D3.25 GFP-Bry$^+$/Flk-1$^-$ cells to the D4.25 Flk-1$^+$ population defined here may be representative of the onset of Flk-1 expression in the cardiogenic population as the cells exit the PS in vivo. The presence of the CV-CFC in the D4.25 but not in the D3.25 population and in the anterior region of the mouse but not in the PS suggests that this progenitor represents a specific stage of cardiovascular development and not an uncommitted mesoderm cell. The fact that it responds to molecules found in the heart field of the embryo to generate distinct colonies in MEC cultures further supports the interpretation that it represent a normal stage of development.

In a previous study, Yamashita et al. demonstrated that cardiomyocytes could be generated from Flk-1$^+$ progenitors isolated from ES-cell differentiation cultures and early embryos (Yamashita et al., 2005). Our current study extends these findings in several important ways. First, based on temporal patterns of development, we have identified the cardiogenic Flk-1$^+$ population as distinct from the Flk-1 population that gives rise to the hemangioblast. Of significance is the finding that the sequential development of the hemangioblast and cardiac Flk-1 populations in the ES-cell model recapitulates the temporal development of the hemangioblast and cardiac lineages in vivo (Kinder et al., 1999). The identification of this cardiogenic Flk-1 population enables the routine generation of populations with an unprecedented high frequency of cardiomyocytes (>50%). Second, our molecular analyses revealed that specification of the cardiogenic Flk-1 population precedes the onset of expression of transcription-factor genes associated with the earliest stage of cardiac induction. Third, we have shown that the cardiogenic Flk-1$^+$ population contains a progenitor that we have named the CV-CFC that will grow in methylcellulose and generate distinct colonies that display both cardiac and vascular potential. The development of the cardiac colonies provides a new quantitative assay for cardiovascular progenitors. Fourth, our study has demonstrated the existence of comparable CV-CFCs that develop in the anterior region of the head-fold-stage embryos. The CV-CFC identified in this study can be considered the cardiovascular equivalent of the hemangioblast of the hematopoietic system and represents one of the earliest stages of cardiac development described to date.

The identification of the CV-CFC in both the ES-cell differentiation model as well as in the early embryo adds strong support to the notion that the cardiac lineages develop from a progenitor with endothelial potential (Lee et al., 1994; Linask and Lash, 1993). The presence of a common progenitor for myocardium and endocardium is supported by studies in the zebrafish that demonstrate that single cells in the ventral marginal zone have the potential to generate both lineages (Lee et al., 1994). The observation that the chick cell line QCE-1 can be induced to both endothelial and myocardial lineages is also consistent with the notion of a common progenitor for both lineages (Eisenberg and Bader, 1995). Analyses of cardiac development in the chick embryo, however, have failed to identify a common progenitor in the early embryo (Cohen-Gould and Mikawa, 1996). This discrepancy may reflect the fact that the investigators analyzed a later stage of development than we have done in this study.

Segregation of two distinct progenitors with endothelial potential, the BL-CFC and the CV-CFC, to different Flk-1 populations that appear in a sequential fashion is not only consistent with the temporal development of the hemangioblast and cardiac lineages in vivo but also raises the intriguing possibility that the types of endothelial cells generated by them may differ. Expression of the endocardial gene *neuregulin* in the cardiac but not in the blast colonies is consistent with this interpretation and suggests that at least some of the vascular potential of the cardiac colonies could represent endocardium (Meyer and Birchmeier, 1995; Meyer et al., 1997). Together, these observations support a model in which the BL-CFC develops first to generate the hematopoietic and vascular components of the blood islands (Huber et al., 2004), and the CV-CFC develops next to establish the myocardial and endocardial lineages of the heart (Figure 6, model). The notion that the myocardial and early endothelial heart lineages develop from a common progenitor could account for their close proximity and cooperative effects during heart development.

By E8.5 of mouse development, neuregulin-1 is expressed in endocardium, while its receptors ErbB2 and ErbB4 are expressed in the underlying myocardium. Paracrine secretion of neuregulin-1 from the endocardium has been found to be sufficient for induction of the cardiac conduction system from myocardium (Rentschler et al., 2002). Similarly, cooperative interactions between these cell types have been demonstrated for trabeculation (Gassmann et al., 1995; Lee et al., 1995) and myocyte growth and survival (Lavine et al., 2005; Zhao et al., 1998). Access to the CV-CFC and cardiac colonies provides a powerful model for further investigating interactions between these different populations at a clonal level.

The existence of two distinct heart fields that develop sequentially and express unique molecular profiles has been described by a number of groups (Cai et al., 2003; Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001; Zaffran et al., 2004). While it is difficult to unequivocally define a lineage based on the expression patterns of several genes, our findings do suggest that both the primary and secondary heart fields are represented in the colony assay. In addition, the expression of vascular genes in ES-cell-derived *Isl1*+*Tbx5*$^{-}$ and *Isl1*-*Tbx5*$^{+}$ colonies (Figure 3B) as well as in expanded populations from those grown from the embryo (Figure 5C) suggests that both heart fields develop from progenitors with vascular potential. To date, our analyses have identified only one colony that may express markers of both primary and secondary lineages. Identification of colonies that express markers of both lineages is expected to be rare, as Meihlac et al. have provided evidence that these lineages segregate at much earlier stages during gastrulation (Meilhac et al., 2004). Our analysis of different aged colonies and of expanded outgrowths from the colonies indicates a loss of *Isl1* with concomitant gain of *Tbx5* expression with time. If these patterns are indicative of the presence of secondary and primary heart-field populations, respectively, these findings would suggest that our culture conditions favor the growth of the primary heart field cells. In the embryo, the secondary heart field develops both anterior and dorsally to the cardiac crescent, positioning it closer to the signals derived from the neural tube. These observations suggest that progenitors of the secondary heart field may develop in a cytokine environment different from those of primary heart field. The combination of cytokines in our cardiac MEC assay may be more representative of the environment of the primary heart field. The availability of a clonogenic assay for cardiac progenitors provides a unique opportunity to investigate the signaling pathways that regulate the development of these distinct cardiogenic lineages.

In summary, the findings reported here have identified and characterized a Flk-1^{+} multipotential cardiovascular progenitor in the ES-cell differentiation culture as well as in the early embryo. The identification of this progenitor provides a novel cell population for studies aimed at uncovering the mechanisms of diversification of these lineages and the establishment of the cardiovascular system. In addition to providing novel cell types for developmental studies, the D4.25 Flk-1^{+} population enriched for CV-CFC is an ideal population for transplantation for the treatment of cardiovascular disease. Given the fact that the CV-CFC represents a progenitor with both cardiac and vascular potential, one might expect significant regeneration of both the vasculature and myocardium following transplantation to the damaged heart.

Experimental Procedures

ES Cell Growth and Differentiation

ES cells were cultured and differentiated as previously described (Fehling et al., 2003). For reaggregation, 5×10^5 sorted cells were reaggregated in 500 ul StemPro-34 SF medium (GIBCO), 2 mM L-glutamine, transferrin (200 ug/ml), 0.5 mM ascorbic acid (Sigma), 4.5×10^{-4} M MTG, human (h) VEGF (5 ng/ml) (R&D Systems), hbFGF (30 ng/ml) (R&D Systems) per well in 24-well low-cluster plates (Costar). For direct plating of populations, 6×10^4 cells were seeded onto individual wells of a 96-well flat bottom plate (Becton Dickenson, Franklin Lakes, NJ) coated with gelatin in StemPro-34 SF medium, 2 mM L-glutamine, hVEGF (5 ng/ml), and hbFGF (30 ng/ml).

Flow Cytometry

EBs were harvested and trypsinized, and single-cell suspensions were analyzed on an LSR flow cytometer (Becton Dickinson) or sorted on either a Moflow cell sorter (Cytomation, Ft. Collins, CO) or Vantage cell sorter (Becton Dickinson). Cells were incubated with mAb in IMDM/10% FCS. Stains included biotinylated Flk-1 mAb, revealed with streptavidin PE-Cy5 (BD Pharmingen) and, with or without, PE-conjugated anti-CD117 (BD Pharmingen), of which the highest 3%–5% was excluded during the D3.25 sorts. Anti-cardiac isoform of TroponinT (cTnT) (NeoMarkers, Fremont, CA) revealed with Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) staining was performed on cells fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS. Staining was done in the presence of 0.5% Saponin (Sigma).

Colony Assays

Blast cell colonies were generated as previously described (Choi et al., 1998). For the generation of cardiovascular cell colonies (CV-CFC assay), EB and embryonic cells were plated in the same conditions of the BL-CFC assay minus mIL-6 and with the addition of hVEGF (5 ng/ml), hbFGF (30 ng/ml), hBMP4 (50 ng/ml) (R&D Systems), hDKK (150 ng/ml) (R&D Systems). For CV-CFC frequency analysis, colonies were counted after 6 days of culture, and a representative number were deposited into a gelatinized 96-well microtiter plate in StemPro 34 media as above. Contracting cardiac colonies were scored after an additional 5 days. For CV-CFC molecular analysis, cardiac colonies were isolated after 4 days and deposited into fibronectin coated 96-well microtiter plates with StemPro 34, 25% D4T endothelial-cell-conditioned medium, 2% FCS, 2 mM L-glutamine, hVEGF (5 ng/ml), hbFGF (30 ng/ml), hBMP4 (50 ng/ml), and hDKK (150 ng/ml).

Molecular Analysis

The preparation of 3′ cDNA was performed as previously described (Brady and Iscove, 1993; Robertson et al., 2000). 3′ cDNA was diluted and underwent gene-specific PCR by using primers designed within the most 3′ 400 bp, including the UTR (Figure S1). PCR conditions were: 94°C, 5 min followed by X cycles (94°C, 30 s; 55°C, 30 s; 72°C, 30 s); 72°C, 10 min.

Immunohistochemical Staining

Colonies were fixed in the 96-well microtiter plate with 4% paraformaldehyde for 30 min, washed, and stained with rat anti-mouse CD31 revealed with Cy2-conjugated donkey anti-rat IgG (Jackson ImmunoResearch). Cells were permeablized with PBS/0.1% Tween-20, washed, and stained in PBS/10% FCS for cTnT revealed with Cy3-conjugated donkey anti-mouse IgG. Bright-field and fluorescent images were acquired on a Leica DMIRB inverted fluorescent microscope with MagnaFire software (Optronics, Goleta, CA).

Dissection and Culture of Embryos

Male mice heterozygous for GFP knockin of the Brachyury locus (GFP-Bry$^{+/-}$) were mated with Swiss Webster female mice to

generate mid- and late-PS embryos, isolated at E7.0, or neural-plate and head-fold embryos, isolated at E7.5. Embryos were removed from decidua and Reicher's membrane and dissected in IMDM containing 15% FCS. Dissections were done under a Leica MZFLIII fluorescence dissecting stereomicroscope with tungsten needles (Fine Science Tools). Embryo fragments were dispersed into a single-cell suspension by incubation in a 2.5% trypsin-EDTA solution (Sigma) for 5 min at 37°C and by vortexing. 20,000–1 × 10^5 dispersed cells from each fragment were added to 1 ml of Cardiac-MEC, incubated in 5% oxygen for 6 days, and then counted. A representative number of colonies from each population were picked and replated onto gelatin-coated microtiter wells containing cardiac expansion medium. After an additional 4–6 days, contracting cardiac colonies were counted. Alternatively, for clonal analysis, head-fold embryos were dispersed into a single-cell suspension stained for Flk-1 and individually deposited into cardiac MEC. After 4 days, colonies were picked and expanded for molecular analysis.

Animal Care and Use

All experiments conducted on animals were performed in accordance with institutional guidelines and regulations. These experiments were approved by Mount Sinai School of Medicine Institutional Animal Care and Use Committee review board.

Supplemental Data

Supplemental Data include a figure and three movies and are available at http://www.developmentalcell.com/cgi/content/full/11/5/723/DC1/.

Acknowledgments

We thank members of the Keller lab for critically reading this manuscript. We also thank the Mount Sinai School of Medicine Flow Cytometry Shared Research Facility for assistance with cell sorting. This work was supported by National Institutes of Health grants R01 HL 48834-09 and R01 HL 71800 to G.K. and National Institutes of Health/National Heart, Lung, and Blood Institute grant F32 HL078112 to S.K. The authors declare that they have no competing financial interests.

Received: March 6, 2006
Revised: September 16, 2006
Accepted: October 5, 2006
Published: November 6, 2006

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