Research article

Mouse ES cell–derived cardiac precursor cells are multipotent and facilitate identification of novel cardiac genes

Nicolas Christoforou,[1] Ronald A. Miller,[1] Christine M. Hill,[1] Chunfa C. Jie,[2]
Andrew S. McCallion,[2,3] and John D. Gearhart[1]

[1]Institute for Cell Engineering, [2]McKusick-Nathans Institute of Genetic Medicine, and [3]Department of Comparative and Molecular Pathobiology,
Johns Hopkins Medical Institutions, Baltimore, Maryland, USA.

Although the differentiation of ES cells to cardiomyocytes has been firmly established, the extent to which corresponding cardiac precursor cells can contribute to other cardiac populations remains unclear. To determine the molecular and cellular characteristics of cardiac-fated populations derived from mouse ES (mES) cells, we isolated cardiac progenitor cells (CPCs) from differentiating mES cell cultures by using a reporter cell line that expresses GFP under the control of a cardiac-specific enhancer element of *Nkx2-5*, a transcription factor expressed early in cardiac development. This ES cell–derived CPC population initially expressed genetic markers of both stem cells and mesoderm, while differentiated CPCs displayed markers of 3 distinct cell lineages (cardiomyocytes, vascular smooth muscle cells, and endothelial cells) — *Flk1* (also known as *Kdr*), *c-Kit*, and *Nkx2-5*, but not *Brachyury* — and subsequently expressed *Isl1*. Clonally derived CPCs also demonstrated this multipotent phenotype. By transcription profiling of CPCs, we found that mES cell–derived CPCs displayed a transcriptional signature that paralleled in vivo cardiac development. Additionally, these studies suggested the involvement of genes that we believe were previously unknown to play a role in cardiac development. Taken together, our data demonstrate that ES cell–derived CPCs comprise a multipotent precursor population capable of populating multiple cardiac lineages and suggest that ES cell differentiation is a valid model for studying development of multiple cardiac-fated tissues.

Introduction

The heart is the first organ to function in the vertebrate embryo. In mice, the cardiac mesoderm forms a structure called the cardiac crescent at approximately 7.5 dpc; at 8 dpc the cells of the crescent migrate ventrally to form a linear heart tube and initiate contractions by 8.25 dpc; this corresponds to roughly 3 weeks of gestation in the human (1). Cardiac progenitor cells (CPCs) are derived from the mesoderm which emerges from the primitive streak (PS) during gastrulation (2). CPC migration occurs in an anterior-lateral direction to positions below the head folds, organizing the cells on either side of the midline and merging to form the cardiac crescent, which subsequently extends anteriorly to form the linear heart tube.

Two populations of cardiac progenitors, which arise from distinct mesodermal cell precursors, interact to develop into the heart and great vessels (3–7). The first, termed the *primary heart field*, originates from the anterior splanchnic mesoderm, initially forming the early heart tube and later contributing to the ventricles, the atrio-ventricular canal, and both atria. The second, or *secondary heart field*, originates from the pharyngeal mesoderm, contributing to the outflow tract and all other heart structures except the left ventricle. Both the primary and secondary heart field lineages segregate from a common progenitor cell population prior to the crescent stage, probably at the onset of gastrulation (8).

Molecular analysis of the early stages of cardiogenesis identified 2 genes, *Mesp1* and *Mesp2*, expressed transiently in the mesoderm at the PS stage. These genes are required for cell migration toward the anterior region of the embryo, with descendants of these cells contributing CPCs to both cardiac fields and colonizing the whole myocardium (9). The anterior endoderm and neural tube act on the adjacent mesoderm through extracellular signaling to form an area of reduced *Wnt3a*/*Wnt8* expression and activity. Reduced Wnt signaling activity initiates *Nkx2-5* and *Gata4* expression in the anterior lateral plate mesoderm, with the cells becoming responsive to BMP, and FGF signaling from the endoderm and lateral mesoderm acting to maintain the expression of the homeodomain transcription factor *Nkx2-5* (10). *Nkx2-5* is one of the earliest factors known to be expressed in developing embryonic cardiac regions and can be used to delineate CPCs (11). *Isl1* and *Fgf10* expression distinguishes progenitors of the secondary heart field from those of the primary heart field (3, 4). Initiation of cardiac differentiation is characterized in both heart fields by *Nkx2-5*, although its expression in the secondary heart field is delayed, possibly as a result of elevated Wnt signaling associated with its proximity to neural tube (12).

Heart morphogenesis progresses through coalescence of the cardiac crescent cells and formation of a primitive heart tube consisting of an interior layer of endocardial cells and an exterior layer of myocardial cells separated by extracellular matrix (13). Although interactions between endocardium and myocardium are predicted to play a role during heart morphogenesis, it is not clear how the various cardiac cell lineages are derived. Recent evidence suggests that a cell population expressing *Flk1* (also known as *Kdr*) forms as it exits the PS, migrates to the cardiac crescent, and contributes to
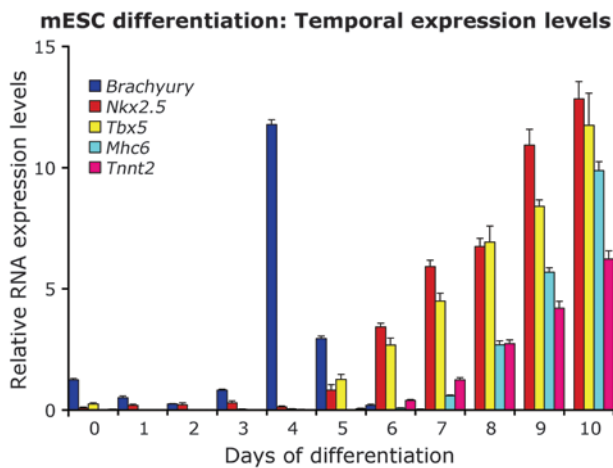
Figure 1
Examination of CPC presence in cultures of differentiating mES cells temporally (qRT-PCR). Relative RNA levels of genes associated with cardiac differentiation in EBs as measured by qRT-PCR. *Brachyury* expression is associated with mesoderm formation. The transcription factors *Nkx2-5* and *Tbx5* are indicators of initiation of cardiac development, and the structural proteins *Myh6* and *Tnnt2* are expressed in functional cardiomyocytes. Error bars indicate SD.

both endocardium and myocardium populations (14, 15), suggesting that CPCs expressing *Flk1* may have the capacity to form both cardiomyocytes and endothelial cells. Vascular smooth muscle comprises a third cell lineage in the heart, and although its origins are unclear, lineage analysis has determined that Nkx2-5^{+} cells in the secondary heart field contribute smooth muscle cells at the base of the aorta and pulmonary artery (16, 17). Moreover, outflow tract smooth muscle cells and yolk sac endothelial cells are derived from progenitor cells (18, 19).

Cardiac induction and heart formation are highly conserved evolutionary developmental processes (20). We posit that cardiogenesis, in vivo through mesoderm induction and heart formation and in vitro through ES cell cardiac differentiation, most likely requires activation of the same signaling pathways. We, and others, have hypothesized that CPCs derived in vitro have the potential for self renewal and the capacity for differentiation into heart cell lineages much like CPCs derived in vivo. In recent reports, CPC populations were isolated and analyzed (21–23), but differences in the approaches used, markers identified, and fate potentials demonstrated have thus far precluded a unifying characterization of such cells.

We isolated mouse ES (mES) cell–derived Nkx2-5[+] CPCs using a cardiac-specific *Nkx2-5* GFP reporter cell line. Isolated CPCs displayed markers consistent with both primary and secondary heart fields and were determined to be multipotent, possessing the capacity to differentiate into cardiomyocytes, vascular smooth muscle cells, and endothelial cells. Clonal cultures of the mES cell–derived CPCs demonstrated an extensive proliferative capacity without any apparent loss of their differentiation potential. Transcript microarray analyses revealed a dynamic expression signature that paralleled in vivo early cardiac induction and development. We strongly believe that we have achieved the derivation of a unique CPC population as related to the markers expressed in the isolated cells as well as their differentiation potential. Moreover, our in-depth temporal transcriptional profile analysis of the isolated

CPCs beginning at the earliest point of cardiac induction provided insights into the molecular events that govern early cardiogenesis.

Results

Differentiation of mES cells into cardiomyocytes. Culture and maintenance of mES cells is described in Methods. mES cells were differentiated through embryoid body (EB) formation using the hanging droplet technique, ensuring uniformity in the microenvironment and number of cells comprising each EB (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI33942DS1). Spontaneous contracting areas, indicative of cardiomyocytes, were observed after 7 days of differentiation in culture (Supplemental Movie 1) and increased in size and number over subsequent days. Cardiomyocytes in the harvested EBs were detected by immunocytochemistry with antibodies against Actn1, Tnni3, and the transcription factor Nkx2-5 (Supplemental Figure 2).

To determine when CPCs were present in the differentiating cultures, we examined the temporal gene expression pattern associated with early cardiogenesis using quantitative RT-PCR (qRT-PCR) to assay the presence and expression levels of precardiac- and cardiac-specific genes. *Brachyury*, a T-box domain–containing transcription factor expressed in all nascent mesoderm, is downregulated following initiation of patterning and specification into derivative tissues (24). *Nkx2-5*, a homeobox transcription factor essential for ventricular cardiogenic differentiation (25, 26), is expressed in CPCs in vivo along with *Tbx5*, a T-box domain–containing transcription factor. Nkx2-5 and Tbx5 interact with Gata factors to regulate gene expression in the developing heart (27), whereas *Mhc6* and *Tnnt2* are expressed in mature functional cardiomyocytes.

Brachyury expression was initiated 4 days after the onset of differentiation, and its subsequent downregulation in concert with the initiation of *Nkx2-5* and *Tbx5* expression on day 5 was consistent with mesoderm induction and specification (Figure 1). The increased *Nkx2-5* and *Tbx5* expression, accompanied by the initiation of *Mhc6* and *Tnnt2* expression on differentiation day 7, coincided with the appearance of spontaneously contracting regions in differentiating EBs. Based on this analysis, we determined that CPCs are most prominent in these cultures after 5–7 days of differentiation; using these time points, we set out to identify the earliest time points at which CPCs could be isolated in culture.

Isolation of mES cell–derived CPCs. To facilitate identification and isolation of CPCs, we established stable transgenic mES cell lines harboring a construct composed of the cardiac-specific enhancer element of the *Nkx2-5* transcription factor regulating the expression of GFP. As *Nkx2-5* is expressed in both the primary and secondary heart field at the earliest stages of heart development in mouse (7.5 dpc) (28), it is an ideal marker for cardiogenic cells, and persists in the adult myocardium (29). The derived mES cell transgenic clones expressed GFP in spontaneously contracting cells during their differentiation to cardiac fates, consistent with CPCs (Supplemental Movies 2 and 3).

We examined the specificity of GFP expression in the differentiating populations (day 6) by assaying for the coexpression of the fluorescent marker and several known cardiac-expressed genes (*Nkx2-5*, *Myocd*, *Gata5*, and *Bmp2/4*; Figure 2, A–D). These data indicated that the *Nkx2-5* enhancer element directed expression of the GFP reporter exclusively in the CPC population during mES cell differentiation. Structural proteins expressed by mature cardiomyocytes (Actn1 and Tnni3) were not detected until day 8
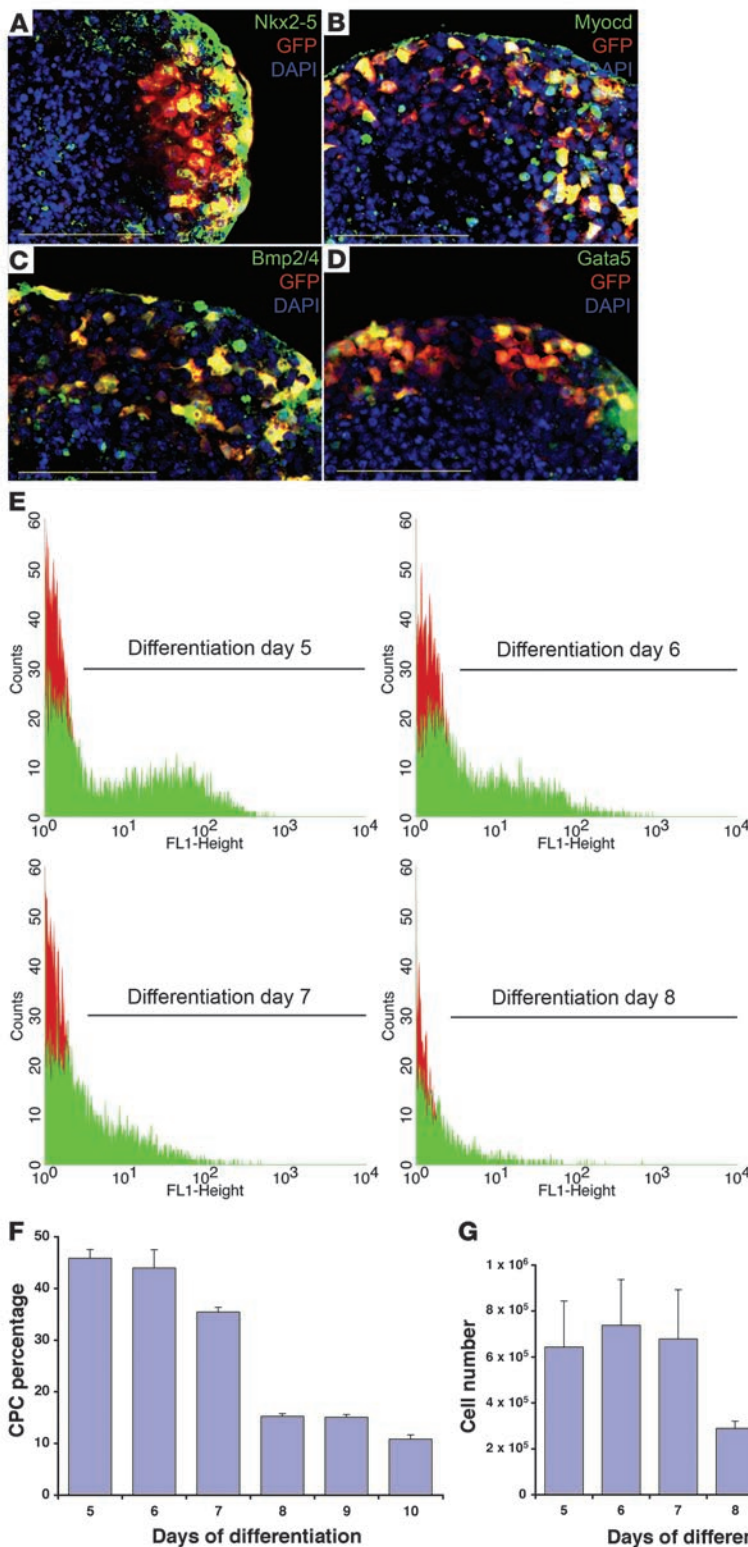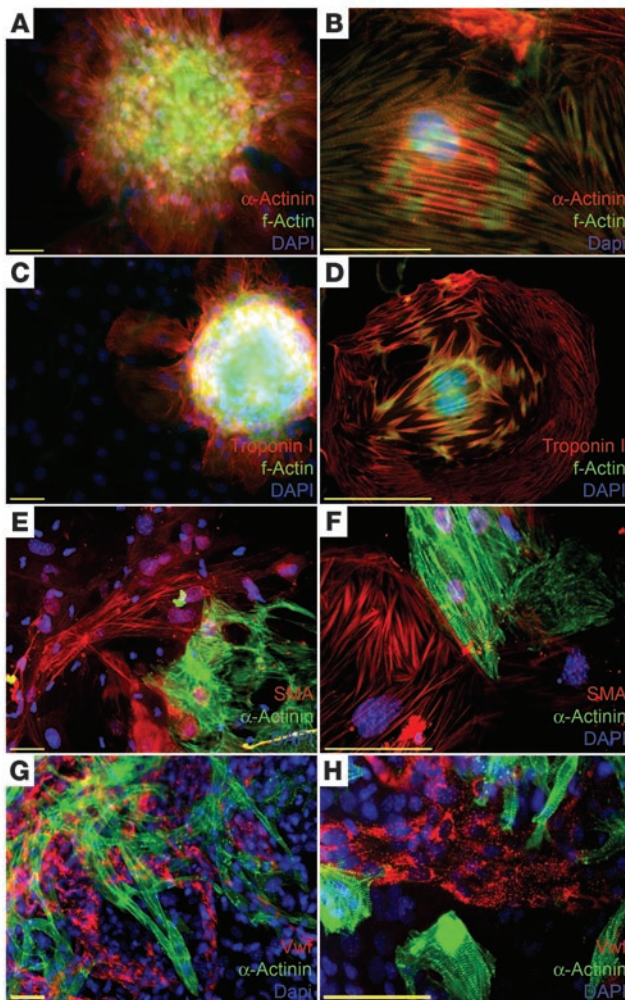
factors, but the absence of both sarcomeres and spontaneous contracting, consistent with the qRT-PCR data (Figure 1).

The efficiency of mES cell differentiation into CPCs was assayed using fluorescence-activated cell sorting (FACS) analysis (Figure 2, E–G) between days 5 and 10 of differentiation. This time interval was selected to coincide with the initial expression of the early cardiac transcription factors *Nkx2-5* and *Tbx5* and the subsequent expression of *Tnnt2* and *Actn1*. On days 5 and 6, a high percentage of the gated cells were GFP+ (45.9% ± 1.7% and 44.0% ± 3.5%, respectively). This number decreased to 35.5% ± 0.9% on day 7 and subsequently dropped substantially on days 8–10 (15.2% ± 0.5%, 15.1% ± 0.5%, and 10.8% ± 0.8%, respectively; Figure 2F). The number of GFP+ cells per 100 EBs varied between 6.4×10^5 and 7.3×10^5 cells with high variance ($\pm 2.0 \times 10^5$ to $\pm 2.1 \times 10^5$) on days 5–7. This number markedly decreased to between 2.1×10^5 and 2.9×10^5 cells per 100 EBs with much lower variance ($\pm 2.7 \times 10^4$ to $\pm 3.1 \times 10^4$) on days 8–10 (Figure 2G). This temporal decrease in the percentage of GFP+ cells is caused by the transcriptional regulation of GFP by the selected enhancer element. Previous studies reported that during in vivo cardiogenesis, this particular enhancer element (–9,435 to –7,353 bps) recapitulated *Nkx2-5* expression only during E7.5–E9.5, with a small remaining amount of expression in the right ventricle by E10.5 (28, 30). Thus the GFP+ cells probably resemble the earliest progenitors of in vivo cardiogenesis. Prior to differentiation day 5, the population of cells expressing GFP was almost undetectable by fluorescent microscopy.

of differentiation in the GFP+ cells (data not shown). Thus, GFP+ cells 5–6 days after the onset of differentiation corresponded to the earliest in vitro stage at which we could identify CPCs. At this time point, the initial transition of nascent mesoderm into CPCs was marked by the expression of the early cardiac transcription

mES cell–derived CPCs are multipotent. Following heart tube formation during in vivo development, CPCs give rise to cell types

Figure 3
Differentiation capacity of mES cell–derived CPCs. mES cell–derived GFP+ CPCs differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells. Cells were dissociated and FACS sorted on differentiation day 6. Following overnight suspension/aggregation cell culture, the CPC aggregates were plated under 3 different conditions and allowed to differentiate for 7 days in culture. Differentiated CPCs stained positive for α-Actinin (Actn1; **A** and **B**), Troponin-I (Tnni3; **C** and **D**), SMA (Acta2; **E** and **F**), and vWf (**G** and **H**; all red). Cells were also stained with phalloidin (f-Actin; green) and DAPI (nuclei; blue). Scale bars: 50μm.

there, stained positive for Acta2; these cells displayed very little staining for the endothelial marker vWf.

To evaluate the presence or absence of a greater number of markers within this cell population, RT-PCR analysis was performed on the differentiated cells. We assayed the expression of cardiomyocyte markers *Nkx2-5*, *Myocd*, *Mef2c*, *Gata4*, *Myl4*, and *Tnnt2*; smooth muscle markers *Acta2* and *Myh11*; endothelial cell marker *Cdh5*; hepatocyte marker *Afp*; skeletal muscle marker *Myod1*; mesodermal markers *Flk1* and *Brachyury*; and stem cell markers *Kit*, *Nanog*, and *Pou5f1* (Figure 4A and data not shown). The CPCs isolated using the *Nkx2-5* reporter did not express *Brachyury* (data not shown) but expressed *Flk1* and *Kit*, and by day 7 also expressed *Isl1* (Figure 4A). The differentiated CPCs displayed strong signals corresponding to the expression for the markers of the 3 cell lineages (cardiomyocytes, vascular smooth muscle cells, and endothelial cells), whereas only weak signal was detectable corresponding to expression of the skeletal muscle marker *Myod1* and no signal was detected for the hepatic marker *Afp*. However, the stem cell marker *Kit* was detected in high serum cultures (Figure 4, conditions I and III), *Pou5f1* — expressed within ES cells — was not detected (data not shown), and signal corresponding to *Nanog* was not detected in condition I and barely reached detectable levels in condition III. In contrast, GFP– cells displayed morphology consistent with undifferentiated mES cell colonies and exhibited high levels of *Nanog* and *Pou5f1* expression (data not shown).

To determine what proportion of this population corresponds to identified lineages, FACS analyses were performed to quantify the capacity of the isolated CPCs to form the 3 cell lineages (Figure 4B). Approximately 55%–65% of the differentiated cells were cardiomyocytes (Actn1+), about 25%–30% were smooth muscle cells (Acta2+), and about 15% were endothelial cells (Cd31+). Differentiation of the GFP– cell population into the 3 cell lineages was greatly reduced compared with that of the CPCs, indicating the presence of other cell types in the culture. Because the GFP– cell population consisted of various differentiated cell lineages, including undifferentiated cells that still retain the capacity to become CPCs, cardiac differentiation — albeit decreased — was also observed in the GFP– population of cells.

CPCs display properties of self renewal and iterative clonal expansion. In order to examine their proliferation potential, GFP+ CPCs were sorted on differentiation day 5 and cultured for 2 weeks as aggregates in the presence of mouse recombinant Igf1, which has previously been demonstrated to promote the survival and proliferation of adult CPCs (31). The aggregates increased in size, with a majority of the cells expressing GFP while exhibiting spontaneously contractile regions, suggestive of a population of cells capable of active division while retaining cardiogenic (*Nkx2-5+*) characteristics. These cells were assayed for the expression of markers of cell cycle

that comprise the adult heart. We set out to determine whether mES cell–derived CPCs also had the potential to differentiate into these cells, particularly cardiomyocytes, vascular smooth muscle cells, and endothelial cells. GFP+ CPCs and GFP– multilineage cells were separated by FACS on differentiation day 6 and allowed to reaggregate in suspension. Within 24 hours of FACS, the GFP+ cell aggregates were spontaneously contracting (Supplemental Movie 4). These cell aggregates were then plated and maintained for an additional 7 days in 3 different conditions: high serum culture medium (condition I), no serum culture medium (condition II), or high serum culture medium with Vegf (condition III). At the end of CPC differentiation, large areas in the cell culture exhibited spontaneous contracting regions characteristic of cardiomyocyte differentiation; however, there were additional cells present in the culture that did not exhibit myocyte morphology. Immunocytochemistry was performed using cardiomyocyte markers Actn2 and Tnni3, smooth muscle cell marker Acta2, and endothelial cell marker vWf (Figure 3). Cells in the aggregates, which were exhibiting contractile regions, stained positive for Actn2 and Tnni3. However, staining for vWf was also detected, indicative of endothelial cell presence, with strong vWf expression in the culture condition supplemented with VEGF. Only adherent cells, whether associated with the aggregates or migrating from
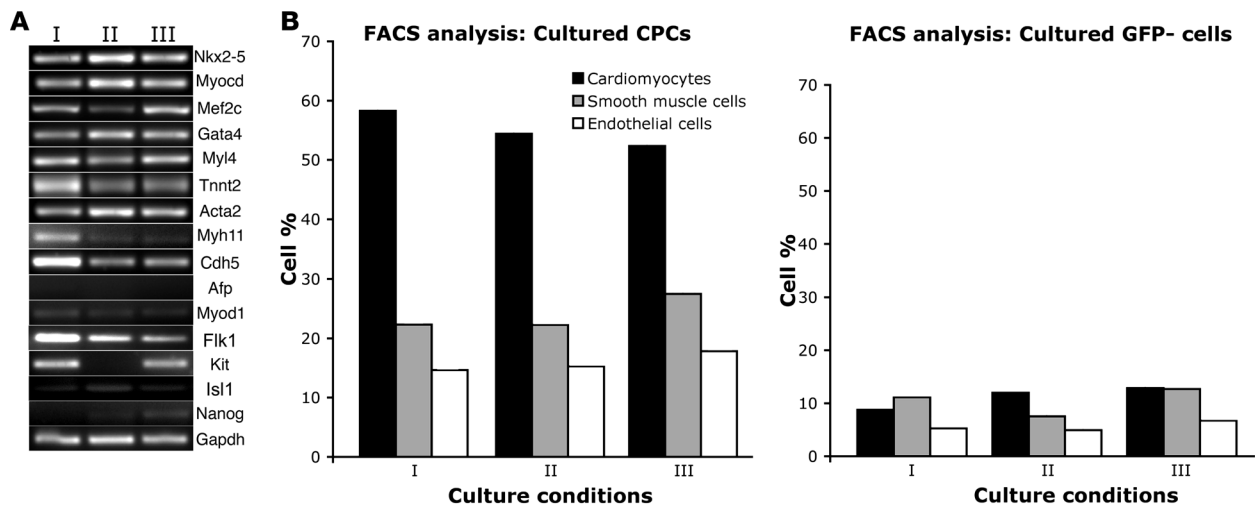
Figure 4
Differentiation capacity and differentiation efficiency of mES cell–derived CPCs. FACS-sorted CPCs were cultured for 7 days in high serum culture medium (condition I), no serum culture medium (condition II), or high serum culture medium plus VEGF (condition III). (**A**) RT-PCR analysis on RNA isolated from FAC sorted and differentiated CPCs. Transcripts analyzed included cardiac markers *Nkx2-5*, *Isl1*, *Flk1*, *Myocd*, *Mef2c*, *Gata4*, *Myl4*, and *Tnnt2*; smooth muscle markers *Acta2* and *Myh11*; endothelial marker *Cdh5*; stem cell markers *Kit* and *Nanog*; skeletal muscle marker *Myod1*; hepatic marker *Afp*; and the positive control marker *Gapdh*. Temporally matched FACS-sorted and differentiated GFP⁻ mES cells expressed high levels of ES cell markers Pou5f1 (not shown) and Nanog. (**B**) Efficiency of GFP⁺ CPC and GFP⁻ cell differentiation into cardiomyocytes (Actn1), smooth muscle cells (Acta2), and endothelial cells (Cd31) as assayed by FACS analysis.

entry and division (Ki-67 and Pcna; Figure 5, A–D). Importantly, these markers were coexpressed with *Nkx2-5* and GFP, consistent with the proliferation of CPCs. This proliferative capacity of the CPC aggregates is probably comparable to that observed in the Nkx2-5⁺ cell population during in vivo development, with the cells proliferating sufficiently to form the entire heart.

Isolated cells were also cultured and passaged for more than 100 population doublings on a layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) in the presence of high serum culture medium. The cultured CPCs differentiated into cardiomyocytes, smooth muscle cells, and endothelial cells upon the removal of serum from the culture medium or in high cell densities (data not shown).

In order to examine their clonal capacity, GFP⁺ CPCs on differentiation day 5 were plated at low plating density (10³ cells/cm²) on mitotically inactivated MEFs (Figure 5, E–H), neonatal rat cardiac fibroblasts (Supplemental Figure 3), or gelatin- or fibronectin-treated plastic. Observation of the cells using fluorescent microscopy immediately following cell plating confirmed single-cell clones on the various substrates. Clonal colonies of GFP⁺ undifferentiated CPCs were evident on the MEFs within 3 days of plating. These colonies increased in size, with the cells exhibiting high levels of GFP expression as before, and could be picked and further passaged or differentiated as above. CPCs plated on heart fibroblasts formed colonies of nonproliferative differentiated cells that eventually downregulated GFP expression. Cardiomyocyte colonies were more prevalent in serum-free medium, with cell division lasting for at least a week. No colonies were formed on gelatin- or fibronectin-treated plastic, with the CPCs primarily differentiating into smooth muscle cells.

Microarray expression analysis of CPCs identifies candidate cardiac genes. Cardiogenesis in vivo is characterized by a unique signature of genes dynamically expressed in concert and activation of cer-

tain molecular pathways (32). We set out to determine the degree to which the in vitro–generated CPC population shared that cardiogenic signature. We hypothesized that the application of a genome-wide approach (transcript profiling) in this context would further our understanding of the molecular pathways active during progenitor determination and facilitate the identification of genes that are enriched in the CPC population in vivo. GFP⁺ CPCs and GFP⁻ cells were sorted by FACS on differentiation days 5, 6, 7, and 8, and the corresponding RNA populations were prepared for array-based evaluation (see Methods). The expression levels of array probe sets were compared between the CPCs and their GFP⁻ counterparts at each analyzed time point. Based on the array analysis, 195 unique genes exhibited at least 1.5-fold upregulation in their expression levels in the CPC population during all 4 time points analyzed compared with the GFP⁻ populations (Supplemental Figure 4). These genes included known cardiac genes *Nkx2-5*, *Myocd*, *Hod*, *Actc1*, *Actn2*, *Flk1*, *Myh6*, *Myh7*, *Myl2*, *Myl3*, *Myl4*, *Myl7*, *Tnnc1*, *Tnnt2*, *Ttn*, *Atp1b1*, *Atp2a2*, *Kcne3*, and *Ryr2*.

Next, canonical pathways that may be active in the CPCs were identified using Ingenuity Pathways Analysis software. Pathways that received a high probability score included the nitric oxide signaling pathway, Vegf signaling, calcium signaling, Igf1 signaling, Notch signaling, and cardiac β-adrenergic signaling. The same analytical process identified 43 genes that were uniquely downregulated in the CPC population during the 4 time points. These included genes *Pou5f1* and *Nanog*, which are expressed in undifferentiated mES cells, as well as the nascent mesoderm-specific transcription factor *Brachyury*.

We confirmed the validity of the microarray data by semi-quantitative RT-PCR analysis of a selection of the identified genes (Supplemental Figure 5) performed on RNAs isolated at the same time points examined in the microarray experiments. The expression levels between the GFP⁺ and GFP⁻ cell populations were
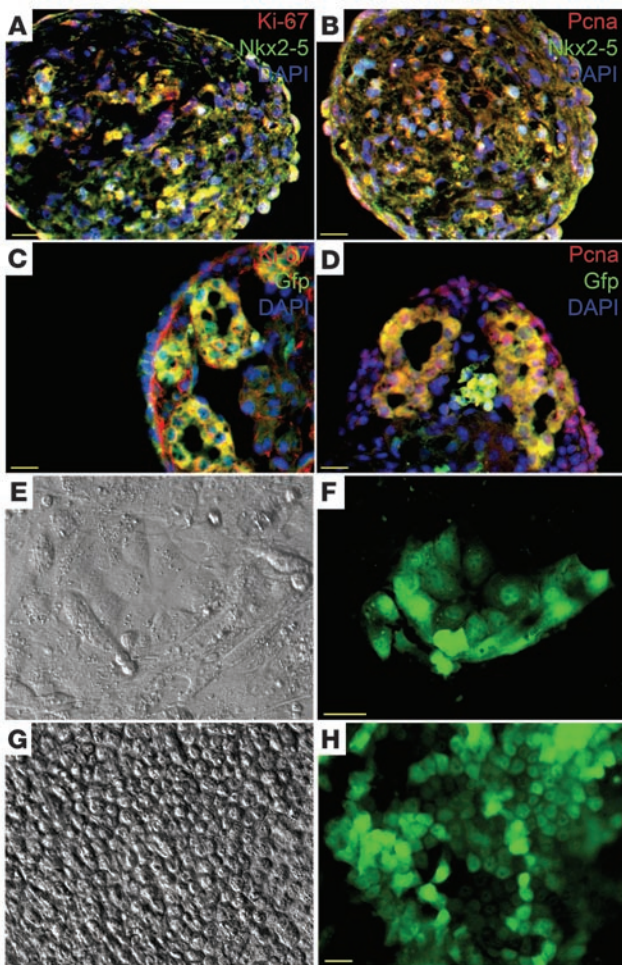
Figure 5
Proliferation and clonal capacity of mES cell–derived CPCs. (**A**–**D**) Immunocytochemical analysis of FACS-sorted GFP+ CPCs isolated on differentiation day 5 and cultured in suspension as aggregates for 10 days in the presence of mouse recombinant Igf1. Nkx2-5 (**A** and **B**) or GFP (**C** and **D**; red) were detected in cells expressing the proliferation markers Ki-67 (**A** and **C**) or Pcna (**B** and **D**; green). Nuclei were stained with DAPI (blue). (**E**–**H**) Clonal capacity of mES cell–derived CPCs. Sorted GFP+ CPCs were plated on mitotically inactivated MEFs at a low plating ratio. Within 4 days after plating, GFP+ colonies were observed (**E** and **F**). The colonies grew in size and were either further passaged or allowed to differentiate (**G** and **H**). Panels **E** and **G** are bright-field images corresponding to **F** and **H**, respectively. Scale bars: 20 μm.

A low expression level of *BC054438* was detected in the cardiac crescent region (E7.5) and the inflow and outflow tracts as well as the branchial arches (E8.5, E9.5). Cardiac expression of *Dpp4* was detected in the common atria, the left and right ventricles, and the edge of the branchial arches (E9.5). *3110004L20Rik* was detected in the inflow and outflow tracts as well as the vasculature (E8.5), with its expression becoming more restricted to the outflow tract and the branchial arches (E9.5).

Discussion
In this study, we used CPCs isolated from differentiating mES cells to recapitulate the early events of in vivo cardiogenesis and, more particularly, cardiac crescent formation and the subsequent formation of the heart tube. Through evaluation of the transcriptional status of known genes at the earliest stages of cardiogenesis and the use of array-based transcription profiling, we clearly demonstrated that in vitro cardiogenesis tightly paralleled in vivo observations. Importantly, this was true for genes associated with both the primary and the secondary heart fields. Finally, we provide evidence of the utility of this approach in identifying novel cardiac gene candidates.

A main undertaking of this work was the determination of the molecular and cellular characteristics of cardiac fated populations derived from mES cells. In order to achieve this, it was crucial to fashion a method that would allow the identification and specific isolation of the cardiac progenitors through the use of a selectable marker whose expression would be controlled by a cardiac-specific gene. It was necessary to set specific criteria allowing us to pick the ideal gene marker expressed in the progenitor population in order to study the earliest steps of cardiac morphogenesis: (a) the mES cell–derived CPC should resemble the earliest identified progenitors present during in vivo cardiogenesis; and (b) the gene marker used for the identification and isolation of the CPCs should have a specific temporal expression pattern only in the embryonic cardiac tissues.

Thus we selected Nkx2-5 as the gene marker on which to isolate the mES cell–derived CPCs, making specific use of the previously characterized cardiac-specific enhancer element of this gene and allowing us to meet the above criteria and derive a CPC population that we believe to be novel compared with the previously described mES cell–derived CPCs. Our clonally derived CPCs initially expressed *Flk1*, *Kit*, and *Nkx2-5*, but not *Brachyury*, and subsequently gave rise to a population expressing *Isl1*. This would suggest that the CPC population identified here is capable of contributing to cells of both heart fields. Consistent with this postulate, our CPCs gave rise to cardiomyocytes, endothelial cells, and vascular smooth muscle.

determined for a subset of genes and compared at each time point. Selected genes included both upregulated and downregulated genes in the CPC population (*Nkx2-5*, *Myocd*, *Sox18*, *Myl4*, *Myl7*, *Tnnc1*, *Tnnt2*, *Cd34*, *Tfpi*, *Flk1*, *Tek*, *Esam*, *Brachyury*, and *Pou5f1*). Importantly, all selected genes exhibited a pattern of relative expression consistent with the microarray analysis.

Establishing the biological relevance of gene candidates by in situ hybridization. Whole-mount in situ hybridization was performed at critical time points in mouse cardiogenesis (E7.5, E8.5, and E9.5) to evaluate the biological relevance of selected genes determined to be upregulated in the CPC. The analysis was done for genes with previously unknown function or developmental expression pattern: *Cap2*, *Zfp69*, *3632451O06Rik*, *Pcdh12*, and *4631423F02Rik* (Figure 6) as well as *BC054438*, *Dpp4*, and *3110004L20Rik* (Supplemental Figure 6). *Cap2* expression was detected in the cardiac crescent (E7.5), in the heart and inflow and outflow tracts (E8.5), and in both ventricles and the common atria (E9.5). *Zfp69* expression was detected in both primary and secondary heart fields (E7.5), the inflow tract and branchial arches (E8.5), and in the branchial arches, outflow tract, and the anterior portion of the ventricle (E9.5). *3632451O06Rik* expression was detected in the vasculature and the outflow tract (E8.5 and E9.5). *4631423F02Rik* was detected in the branchial arches, the inflow tract, and a portion of the common atria (E9.5). *Pcdh12* was expressed in the inflow and outflow tract, the branchial arches, and the vasculature (E8.5, E9.5).
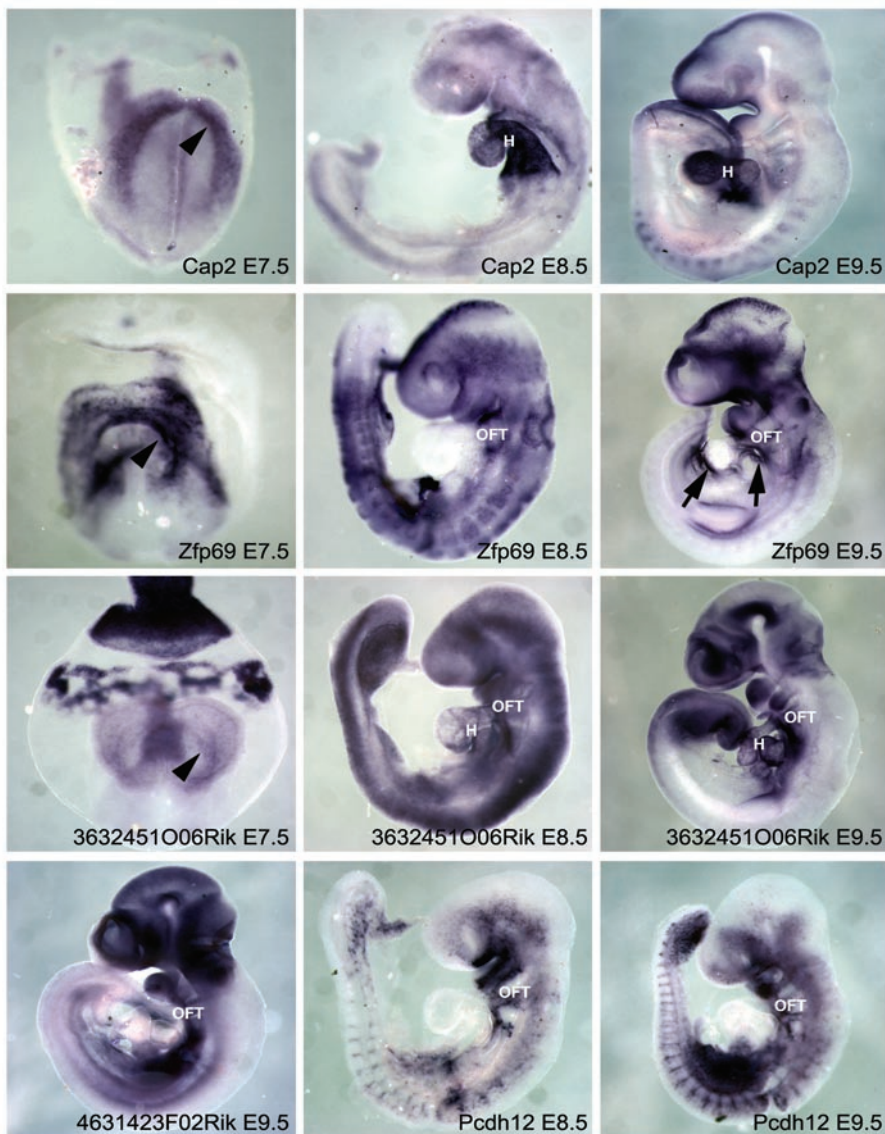
the *Brachyury$^+$Flk1$^+$* progenitors primarily expressed markers of the primary heart field, with very few colonies expressing *Isl1* or *Fgf10*. Finally, even though Wu et al. (22) used the same cardiac-specific *Nkx2-5* enhancer element to isolate mES cell–derived CPCs as the one we used in the present study, their progenitor population is negative for *Flk1* expression and only capable of differentiating into cardiac muscle and smooth muscle, with no reported endothelial differentiation.

One of the main objectives of our study was to examine the earliest molecular events that occur during cardiac progenitor induction, proliferation, and differentiation. In order to achieve this we elected to use the cardiogenic differentiation of mES cells as our model system as it is a well-characterized system allowing us to derive large numbers of CPCs consistently and reproducibly. There were 2 crucial points for this study. First, the earliest induced mES cell–derived CPCs specifically isolated resembled the in vivo cardiac progenitors at the point of cardiac crescent induction. Second, the temporal isolation of the mES cell–derived CPCs allowed us to examine how transcriptional profile of the CPCs varies over time.

We believe the novelty of our study lies precisely in this analysis. In the recent reports describing the isolation of mES cell–derived CPCs, no in-depth characterization of the isolated CPCs prior to their differentiation was reported (21–23). The authors in all 3 studies reported the in vivo and in vitro isolation of the desired progenitor populations based on the described marker genes; however, the in-depth analysis they performed focused on the differentiation characteristics of the progenitor populations, unlike our study, in which we performed in-depth analysis on the CPC population prior to any induction of differentiation. Further evidence of the novelty of our study lies in the discovery of what we believe to be novel genes specifically expressed in the developing heart during in vivo cardiogenesis as confirmed by in situ hybridization analysis. To our knowledge, the identified genes had previously unknown developmental expression pattern or function.

It is apparent that both our study and the 3 recent reports describing the mES cell–derived CPCs may be addressing 4 cell populations

Moretti et al. reported recently the mES cell derivation of an *Isl1$^+$Nkx2-5$^+$Flk1$^+$* CPC (23). We believe that this progenitor is distinct from the one we describe herein, as it is sorted primarily on the basis of *Isl1* expression, which is specifically expressed only in the secondary heart field. Moreover, apart from the secondary heart field, *Isl1* is also expressed in the developing pancreas (33) and motor neuron progenitors (34) at about the same developmental stage. As the authors inserted the β-galactosidase transgene in the genomic locus of *Isl1* it is not clear how they can distinguish among cardiac, pancreatic, and motor neuron progenitors, especially because they did not sort the progenitors prior to plating on the cardiac mesenchyme–supporting cell layer.

The mES cell–derived *Brachyury$^+$Flk1$^+$* progenitors previously described by Kattman et al. also consist of a substantially different progenitor population (21). Based on a fate mapping analysis performed on developing mouse embryos, *Flk1$^+$* progenitors contribute only to a subset of the cardiac muscle cells (14). Moreover, *Flk1* is neither a cardiac-specific marker, nor is it expressed throughout the cardiac crescent during cardiac induction. Once differentiated,

that hold a high level of similarity. The use of a transcriptional profile analysis of the various CPCs at the earliest point of their induction would be an ideal method of comparing these cell populations. Further transcriptional analysis on the 3 described CPC populations (*Brachyury/Flk1*, *Isl1/Nkx2-5/Flk1*, and *Nkx2-5/cKit*) at the time of their induction may allow us to determine their lineage status and help us identify even more genes active during early cardiac development.

Specific transcriptional elements and molecular pathways are active during — and control — the early steps of both in vivo and in vitro cardiogenesis. To further understand these elements, we performed array-based expression analysis on isolated GFP+ CPCs and their corresponding GFP- populations. We identified 195 genes that were upregulated and 43 genes that were downregulated in the CPC population during all 4 time points analyzed. The identified upregulated of genes included candidates with known cardiac expression: *Actc1*, *Actn2*, *Atp2a2*, *Hod*, *Kcne3*, *Myh6*, *Myh7*, *Myl2*, *Myl3*, *Myl4*, *Myl7*, *Myocd*, *Nkx2-5*, *Ryr2*, *Tnnc1*, and *Tnnt2*. A recent study identified 28 transcripts that were enriched in CPCs present during early mouse in vivo development (7.5–9.5 dpc; ref. 30), 17 of which were also present in our analysis. These include *Ramp2*, *Tfpi*, *Esam*, *Flk1*, *Tek*, *Gyg1*, *Myl4*, *Myl7*, *Tnnc1*, *Tnni1*, *Tnnt2*, *Ets1*, *Etv2*, *Fli1*, *Hod*, *Myocd*, *Sox18*, *Bmp2*, *Ggta1*, *Gata4*, and *Sox17*. Another study using mES cells differentiating in EBs identified 100 cardiogenesis-associated genes, 27 of which were also present in our analysis (35).

The CPC expression analysis also identified several genes that to our knowledge have not been previously shown to play a role during cardiac development: *Cap2*, *Zfp69*, *Pcdh12*, *3632451O06Rik*, *4631423F02Rik*, *BC054438*, *Dpp4*, and *3110004L20Rik*. While both *Cap2* and *3632451O06Rik* have recently been described as being expressed in the cardiac region of the looping heart (E9.5; ref. 35), our study clearly demonstrated expression of these genes in the CPCs during cardiac crescent formation in the developing embryo. Whole-mount in situ hybridization of mouse embryos at the earliest stages of cardiac development (E7.5–E9.5) detected expression of the identified genes in cardiac regions of both the primary and the secondary heart fields. These data validate the mES cell–derived CPCs as a cell population that closely recapitulates the molecular events active during the early stages of in vivo cardiogenesis.

Further assessment of uniquely upregulated genes revealed certain molecular pathways that have previously been reported to be active during in vivo cardiac development and may also play a role during in vitro mES cell differentiation: the VEGF signaling pathway (*Flt1*, *Flk1*, *Tie1*, *Tek*, *Cdh5*, *Ets1*, *Ets2*, *Erg*, and *Etv*; refs. 14, 21, 36–38); the nitric oxide signaling pathway (*Flt1*, *Nos2*, *Nos3*, *Cav1*, *Cav2*, *Ryr2*, and *Pde2a*; ref. 39); the IGF1 signaling pathway (*Igf1* and *Igfbp2*; refs. 40, 41); and F-type SOX transcription factors (*Sox7*, *Sox17*, and *Sox18*; refs. 42–44). Genes with a known function during cardiac development were also upregulated in the CPC population between days 6 and 8 of differentiation (*Kit*, *Bmp2*, *Bmp6*, *Tal1/Scl*, *Gata4*, and *Isl1*).

In summary, our data strongly suggest that the molecular characteristics of cardiogenesis within in vitro cultures of mES cell–derived CPCs serve as a valid surrogate for cardiac development in vivo. The CPC-derived cardiogenic populations displayed an expression signature concordant with previously reported in vivo analyses and additionally facilitated the discovery of genes with no a priori (suggesting their potential role in cardiogenesis), yet whose biological relevance was established here through in situ hybridization during mouse embryogenesis.

Methods

Cell culture and cell differentiation. mES cells (ES D3) were cultured and maintained on a feeder layer of mitotically inactivated MEFs in DMEM with 10% FBS (catalog no. S11550, lot no. A0036; Atlanta Biologicals) and ESGRO leukemia inhibitory factor. Differentiation through EB formation was initiated following mES cell dissociation and suspension in basic culture medium (without the leukemia inhibitory factor) at a final concentration of 5×10^4 cells/ml. The hanging droplet technique was used for EB formation. Hanging droplets were plated at a volume of 20 μl/droplet. Two days after initiation of differentiation, the EBs were transferred in suspension on poly-HEMA–treated tissue culture dishes. Basic culture medium was supplemented with ascorbic acid (0.1 mg/ml).

ES cell transgenesis. The mouse Nkx2-5 enhancer fragment 8 (28) and the *Hsp68* minimal promoter was excised from the provided vector (XhoI/NcoI) and inserted at the 5′ end of the enhanced GFP gene in the Bluescript vector. A vector containing the hygromycin phosphotransferase gene under the control of the mouse *polII* promoter was also used in order to select for the transfected mES cells. Prior to transfection, the 2 vectors were linearized. Undifferentiated mES cells were grown to confluency on a layer of primary MEFs and dissociated with trypsin. The cells were combined with the linearized DNA, electroporated, and replated on a fresh layer of MEFs. Hygromycin B was used for 7 days after transfection for selection. Fifty resistant colonies were picked and grown on layers of MEFs in the presence of antibiotic. Once sufficient cells were present, the clones were differentiated, identifying 5 clones with specific GFP expression in spontaneously contracting cells. A selected clone was further amplified and used for characterization of the CPC population.

FACS analysis and sorting. To determine the CPC yield in the differentiating cells, EBs were harvested at the indicated time points (days 6–10 of differentiation). Each sample contained approximately 100 hanging droplets on day 0 of differentiation. The EBs were washed in PBS and resuspended in 0.05% trypsin at 4°C (30 minutes) and 37°C (10 minutes). The percentage of GFP+ cells was determined in a FACSCalibur system (BD). Age-matched EBs from untransfected mES cells were used as negative controls.

The BD Biosciences FACSAria Cell sorting system was used to sort the GFP+ and GFP- cells between days 5 and 10 of differentiation. EBs were harvested and dissociated as described above. The cells were suspended in sorting medium (DMEM plus 1% FBS) at 4°C. RNA was isolated from the sorted cells for the purpose of microarray expression analysis or RT-PCR analysis.

CPC differentiation and proliferation analysis. To assay their differentiation potential, sorted GFP+ CPCs (differentiation day 6) were allowed to aggregate in suspension for 24 hours and then plated in 3 different culture conditions for another 7 days: fibronectin-treated surface in 10% FBS containing medium (condition I); fibronectin-treated surface in DMEM/F12 with B27 and N2 (condition II); and collagen IV–treated surface in 10% FBS containing medium plus VEGF (50 ng/ml; condition III). After the seventh day of culture in these conditions, RNA was isolated for RT-PCR, and cells were fixed with 4% PFA for immunocytochemical analysis or were dissociated and used for FACS analysis using mouse anti-Actn1 for cardiomyocytes, rabbit anti-Acta2 for smooth muscle cells, and mouse anti-Cd31 for endothelial cells.

To assay their proliferation capacity, GFP+ CPCs were sorted by FACS on day 5 of differentiation. Cells were reaggregated in suspension (5×10^5 cells/ml) in the presence of mouse recombinant Igf1 (100 ng/ml) for 14 days. Cell aggregates were fixed and stained for the colocalization of Nkx2-5 with Ki67 or Pcna. The same immunocytochemistry reagents were used as described above.

To assay their clonal capacity, GFP+ CPCs were sorted by FACS on day 5 of differentiation and plated at a low plating ratio (10^3 cells/cm²) on either gelatin-treated plastic, mitotically inactivated MEFs, or mitotically inacti-

vated neonatal rat cardiac fibroblasts in serum-free (DMEM/F12, B2, N27) or serum-containing medium (DMEM plus 10% FBS).

GeneChip microarray hybridization. Total RNA for the microarray expression analysis was isolated from FACS-sorted cells (GFP+ and GFP−) on days 5, 6, 7, and 8 of differentiation as described above. Two separate differentiation batches were prepared for the sake of data reproducibility. Total RNA was also isolated from terminally differentiated mES cell–derived genetically selected cardiomyocytes 3 weeks after initiation of differentiation. The GeneChip 2-cycle target labeling kit was used to convert 100 ng of total RNA to biotinylated labeled cRNA. This was then hybridized to the Affymetrix murine MOE430 2.0 genome GeneChip array. Fluorescence was detected using the Affymetrix GS3000 GeneArray scanner, and image analysis for each chip was done with GeneChip operating system software (GCOS1.3; Affymetrix) using the standard default settings. The raw data were submitted to the gene expression omnibus (GEO) of the National Center for Biotechnology Information (accession no. GSE5671).

Microarray data analysis. To estimate the gene expression signals, data analysis was conducted on the chips' .CEL file probe signal values at the Affymetrix probe pair (perfect match [PM] and mismatch [MM] probe) level, using the robust multiarray analysis statistical technique (45) with the bioconductor package *Affy.* The data normalization procedure used the quantile normalization method (46) to reduce the obscuring variation between microarrays, which might be introduced during the processes of sample preparation, manufacture, fluorescence labeling, hybridization, and/or scanning.

The Spotfire Decision Site software package was used for the identification of uniquely upregulated or downregulated (at least 1.5-fold increase or decrease in expression value) probe sets in the CPC population compared with the rest of the cells in the differentiating EBs. Probe sets that were considered unique for the CPC population were found to be commonly upregulated or downregulated during all 4 days of analysis. Probe sets of the CPC population that exhibited upregulation or downregulation by at least 1.5-fold compared with the mES cell–derived cardiomyocytes were also reported. The final analysis included probe sets that exhibited a different temporal pattern of expression in the CPC population compared with the rest of the cells along the 4 days of differentiation. Specifically, in order to identify these probe sets, gene expression curve over time was modeled flexibly on a natural cubic spline basis. A q value for each gene was computed to estimate the false discovery rate incurred when calling the gene signifi-cant. Differentially expressed genes were obtained by setting a cutoff to the calculated q values (47). The probe level data analysis was implemented with the bioconductor package *Affy* under R environment, and the time course analysis was performed with the software package EDGE. Finally, only probe sets with gene ontology information associated with them were reported. The Ingenuity Pathways Analysis software package was used to identify canonical pathways that are active in the CPC population.

Nucleic acid in situ hybridization. A portion of the probes for candidate genes were generated from E15 whole mouse RNA. An approximately 600-bp region was PCR amplified from cDNA and cloned into the Invitrogen PCRII vector (*Rspo3*, AGGTTTGGAGTCCAGCATTG and GGTTTTGAGGGA-CACTGCAT; *Cap2*, CCCTTTATCAGCGTGGACAT and CCACGTG-CAGAGAAGAGACA; *3110004L20Rik*, GGGGGCAACATTTTTATTTC and TGGAGGACAGGGCTGATAAC). The remaining candidates were obtained through Invitrogen Clone Ranger. Digoxygenin-labeled riboprobes were synthesized using Sp6, T3, and T7 RNA Polymerase. Embryos were removed on days E7.75, E8.5, and E9.5, and in situ hybridization was performed as previously described (48).

Statistics. Data were analyzed using 2-tailed unpaired t tests with Microsoft Excel software. A P value less than 0.05 was considered significant.

Address correspondence to: John D. Gearhart, Institute for Cell Engineering, Johns Hopkins Medical Institutions, BRB 772, 733 North Broadway, Baltimore, Maryland 21205, USA. Phone: (410) 614-3444; Fax: (410) 955-7427; E-mail: gearhart@jhmi.edu.

Nicolas Christoforou and Ronald A. Miller contributed equally to this work.

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