Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart

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A rodent cardiac side population cell fraction formed clonal spheroids in serum-free medium, which expressed nestin, Musashi-1, and multidrug resistance transporter gene 1, markers of undifferentiated neural precursor cells. These markers were lost following differentiation, and were replaced by the expression of neuron-, glial-, smooth muscle cell–, or cardiomyocyte-specific proteins. Cardiosphere-derived cells transplanted into chick embryos migrated to the truncus arteriosus and cardiac outflow tract and contributed to dorsal root ganglia, spinal nerves, and aortic smooth muscle cells. Lineage studies using double transgenic mice encoding protein 0–Cre/Floxed-EGFP revealed undifferentiated and differentiated neural crest-derived cells in the fetal myocardium. Undifferentiated cells expressed GATA-binding protein 4 and nestin, but not actinin, whereas the differentiated cells were identified as cardiomyocytes. These results suggest that cardiac neural crest-derived cells migrate into the heart, remain there as dormant multipotent stem cells—and under the right conditions—differentiate into cardiomyocytes and typical neural crest-derived cells, including neurons, glia, and smooth muscle.

Introduction

Although the generation of cardiomyocytes in the mammalian heart occurs predominantly during early development, cardiomyocytes in the adult heart are known to proliferate following heart failure or myocardial infarction. Adult cardiac stem cells are self-renewing, clonogenic, and multipotent; they give rise to myocytes, smooth muscle, and endothelial cells to enable the formation of well-differentiated myocardium with blood-carrying new vessels, and myocytes with the characteristics of young cells (Beltrami et al., 2003). Adult heart-derived cardiac progenitor cells expressing stem cell antigen–1 (Sca-1) were shown by Oh et al. (2003) to differentiate into cardiomyocytes in vitro following 5′-azacytidine treatment, and to contribute to the regeneration of damaged myocardium. Although accumulating evidence demonstrates the existence of pluripotent or lineage-committed progenitor cells in the heart, the origin of these cells is unknown.

We previously described a method of isolating murine hematopoietic stem cells by dual-wavelength flow cytometric analysis with the fluorescent DNA binding dye Hoechst 33342 (Goodell et al., 1996, 1997; Matsuzaki et al., 2004). This method relies on the differential ability of stem cells to efflux the Hoechst dye, which—like the activity of P-glycoprotein (Zhou et al., 2001)—defines a small subset of side population (SP) cells. SP cells are observed in various tissue types and generally are considered to be tissue-specific progenitor cells. For example, the SP cells in bone marrow are highly enriched for long-term hematopoietic stem cells, and they were also observed in various tissue types (Kim and Morshead, 2003). It is possible that cardiac SP cells represent a cardiac stem/progenitor cell population that can be enriched by the isolation of the Hoechst 33342 effluxing fraction (Hierlihy et al., 2002).

Neural stem cells can proliferate in vitro as a floating culture and can generate spheres (neurospheres) in serum-free

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Abbreviations used in this paper: α-SMA, α-smooth muscle actin; Ab, antibody; EYFP, enhanced yellow fluorescent protein; GATA4, GATA-binding protein 4; GFAP, glial fibrillary acidic protein; HNK1, human natural killer-1; MAP2, microtubule-associated protein 2; MASH1, neural-specific helix-loop-helix–type transcription factor; MDR-1, multi-drug resistance transporter gene 1; MHC, myosin heavy chain; atrial natriuretic peptide; MP, main population; MSA, migration staging area; P0, protein 0; PNS, peripheral nervous system; Sca-1, stem cell antigen-1; SP, side population; Tα-1, α-tubulin promoter; Tg, transgenic.

The online version of this article contains supplemental material.

The Journal of Cell Biology, Vol. 170, No. 7, September 26, 2005 1135–1146
http://www.jcb.org/cgi/doi/10.1083/jcb.200504061

medium in the presence of EGF, or differentiate into neurons or astrocytes in the absence of EGF (Reynolds and Weiss, 1992). Most cells within neurospheres express nestin and Musashi-1, considered to be markers for undifferentiated neural precursor cells (Lendahl et al., 1990; Sakakibara et al., 1996; Kaneko et al., 2000). In addition to stem cells, Musashi-1 is expressed at least in subsets of astrocytes (Kaneko et al., 2000). The neurosphere culture method is a versatile method for assaying the multipotency of neural stem cells, and for the in vitro expansion of neural stem/progenitor cells (Reynolds and Weiss, 1992). A similar sphere-forming culture method can be applied to tissue-specific stem cells from the retina, pancreas, epidermis, and inner ear, and to neural crest-derived cells (Tropepe et al., 2000; Toma et al., 2001; Zulewski et al., 2001; Li et al., 2003). Common features of sphere formation are the expression of nestin, and the ability of sphere-derived cells to differentiate into other cell types in addition to their own tissue-specific cell type.

Because SP cells are dormant, they cannot be expanded readily in vitro. In the present study, the stem cell fraction of the rodent neonatal heart was enriched and expanded using a combination of the SP cell method and the neurosphere-generation method. We identified multilineage potent progenitor cells in the heart that could generate spheres and differentiate into cardiomyocytes and cells with neural crest characteristics, including peripheral nervous system (PNS)-type neurons, glial cells, and smooth muscle cells. We investigated the neural crest cell–like behavior of isolated sphere-derived cells in vivo by transplanting them into chick embryos. Migration of these cells into the heart region contributed to the neural crest-derived dorsal root ganglia, spinal nerves, and aorta, which subsequently differentiated into neurons, glia, and smooth muscle cells. Analysis of transgenic mice hearts expressing protein 0 (P0)-Cre and Floxed-EGFP confirmed that neural crest-derived cells migrate and lay in a dormant, undifferentiated state in the heart expressing nestin and GATA-binding protein 4 (GATA4), or can differentiate into cardiomyocytes or in vitro–derived cardiospheres.

Results

Isolation and characterization of SP cells in the heart

Because adult tissue stem cells can be enriched as SP cells (Goodell et al., 1996, 1997; Matsuzaki et al., 2004), we initially characterized the SP cells in neonatal and adult mouse hearts. FACS analysis demonstrated the presence of cardiac SP cells that are completely blocked by reserpine (Fig. 1 A). SP cells make up 3.5% of heart tissue cells in 2-d-old mice, which is markedly higher than the range of 0.01–1% that was observed in various other organs, including blood, skeletal muscle, and brain (Goodell et al., 1996). The proportion of heart SP cells decreased rapidly up to postnatal day 7, and made up only 0.02% of heart cells at 6 wk (Fig. 1, B and C); this level was consistent with that found in other organs. Cardiac SP cells were characterized further by immunostaining of cytospin preparations with the anti-sarcomeric myosin antibody (Fig. 1 D). 30% of the main population (MP; non-SP) cell fraction contained myosin-positive cardiomyocytes, whereas the SP fraction contained no myosin-positive cells; this provided evidence that cardiac SP cells are nonmyocytes.

Cardiac SP cells were phenotyped by analysis of cell surface marker expression. Cell suspensions from P2 mouse hearts were treated with Hoechst 33342 and monoclonal antibodies (Abs). Two-dimensional FACS profiles (Fig. 1 E) revealed that cardiac SP cells are negative for all markers of mature hematopoietic cells, including CD11b, 13, and 45 and Ter119, and are positive for the widely expressing antigens CD29 and CD44. Heterogeneous expression was observed for various immature hematopoietic cell or vascular stem cell markers, such as CD34, c-Kit, Flk-1, and Sca-1. These results provide evidence that the cardiac SP cell fraction is phenotypically immature and is not contaminated by mature hematopoietic cells.

Formation of neurosphere-like spheres by cardiac SP cells in serum-free medium

SP cells were expanded in culture for further characterization. An investigation of various culture procedures led to the successful application of a method that was used previously to generate neurospheres from cultured central nervous system stem cells to generate neurosphere-like spheres from cardiac SP cells. Using this approach, culture of isolated cardiac SP cells resulted in cell division followed by detachment from the culture plate to form a sphere of proliferating cells referred to as a cardiosphere. Cardiospheres were similar in appearance to neurospheres that were derived from cultured central nervous system stem cells, and formed after 7–10 d in serum-free medium in the presence of EGF and FGF2 (Fig. 1, F–I). MP cells also proliferated in serum-free medium, although the population of sphere-initiating MP cells was 100-fold lower than that of cardiac SP cells. Proliferation of cardiospheres was not observed in the absence of EGF and FGF2. Cells within the cardiosphere did not express the cardiac myocyte marker myosin (MF20) or differentiation markers of other mature cell types. To compare the phenotype of cardiospheres with neurospheres, the cells from cardiospheres were immunostained for nestin and Musashi-1, markers of undifferentiated neural precursors. Consistent with neurosphere cells, most cardiosphere cells were positive for nestin and Musashi-1 (Fig. 1, J–N). RT-PCR analysis also confirmed *nestin* and *Musashi-1* mRNA expression in the fetal heart, nonmyocyte fraction, and cardiospheres (Fig. 1 O).

Neonatal and adult cardiac nonmyocytes contain cardiosphere-initiating cells

To investigate the origin of cardiosphere-initiating cells, the nonmyocyte and myocyte-enriched fractions were separated on a Percoll density gradient then assessed in the cardiosphere-forming assay (Reynolds and Weiss, 1992). Percoll-purified neonatal rat or mouse cardiac nonmyocytes were plated at a density of 10,000–20,000 cells/cm^2 on uncoated culture dishes (10 cm in diameter). ~50–100 spheres (1–2 spheres/cm^2) were observed after 7–14 d in vitro. After dissociation and subcul-

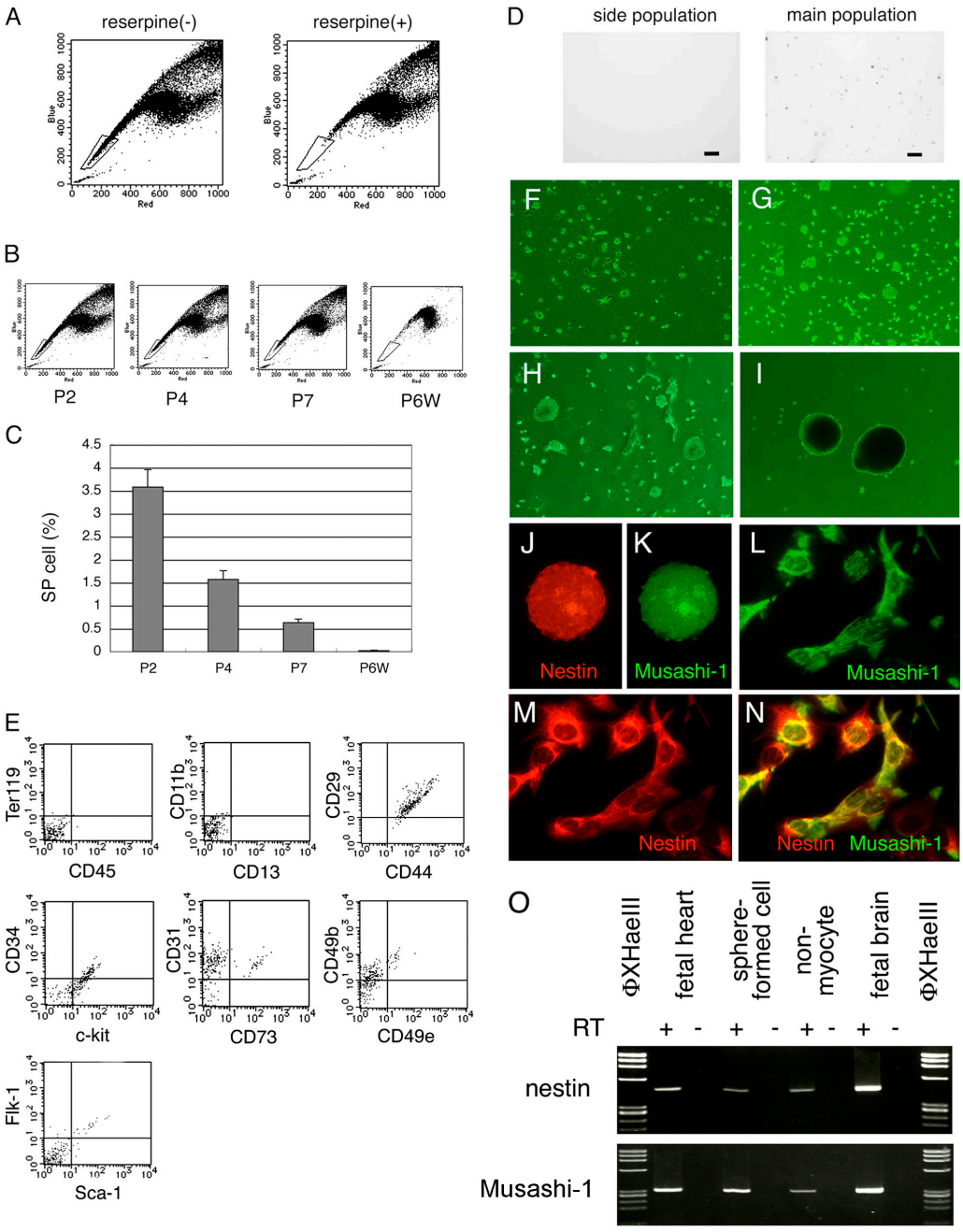

Figure 1. **Isolation, characterization, and cardiosphere formation of SP cells in the neonate and adult heart.** (A) Representative FACS analysis of the 2-d neonatal heart tissues using Hoechst 33342. (B) Postnatal changes in FACS analysis of the SP cell fraction. (C) Percentage of SP cells at P2, P4, P7, and P6 weeks. (D) Cytospin preparations of the SP and MP cells were stained with antisarcomeric myosin monoclonal Ab (MF20). Note that all the cardiomyocytes were classified in the MP cell fraction. (E) FACS analysis of the cell surface marker antigen in SP cells obtained from the neonate heart. (F–I) Phase contrast microscopy of cardiosphere formation. Cardiosphere formation was observed at 0 (F), 3 (G), 7 (H), and 14 (I) d. Cardiospheres stained with antinestin (J) and anti–Musashi-1 (K) Abs. (L–N) Cardiospheres were dissociated with trypsin, and cells were plated onto gelatin-coated glass slides. Double immunostaining with anti–Musashi-1 (L) and antinestin (M) was performed. (N) The merged view. (O) RT-PCR analysis of *nestin* and *Musashi-1*. Fetal brain was used as a positive control.

ture as single cells, ~10% of the primary sphere-derived cells formed secondary spheres. No primary spheres were observed in cardiomyocyte fractions. These results show that cardiosphere-initiating cells are contained within the nonmyocyte cell populations. We also found that cardiosphere formation can be observed in nonmyocytes prepared from adult (10–24-wk-old) murine hearts.

The optimum cell-plating density for formation of cardiospheres was identified as 10,000 cell/ml; most cells that were derived from cardiospheres were positive for nestin and Musashi-1 (unpublished data). This result is consistent with the report by Hulspas et al. (1997) that spheroid colonies displayed clonal growth when murine neural stem cells were cultured at a density of 10,000 cell/ml. We showed that cardiospheres are

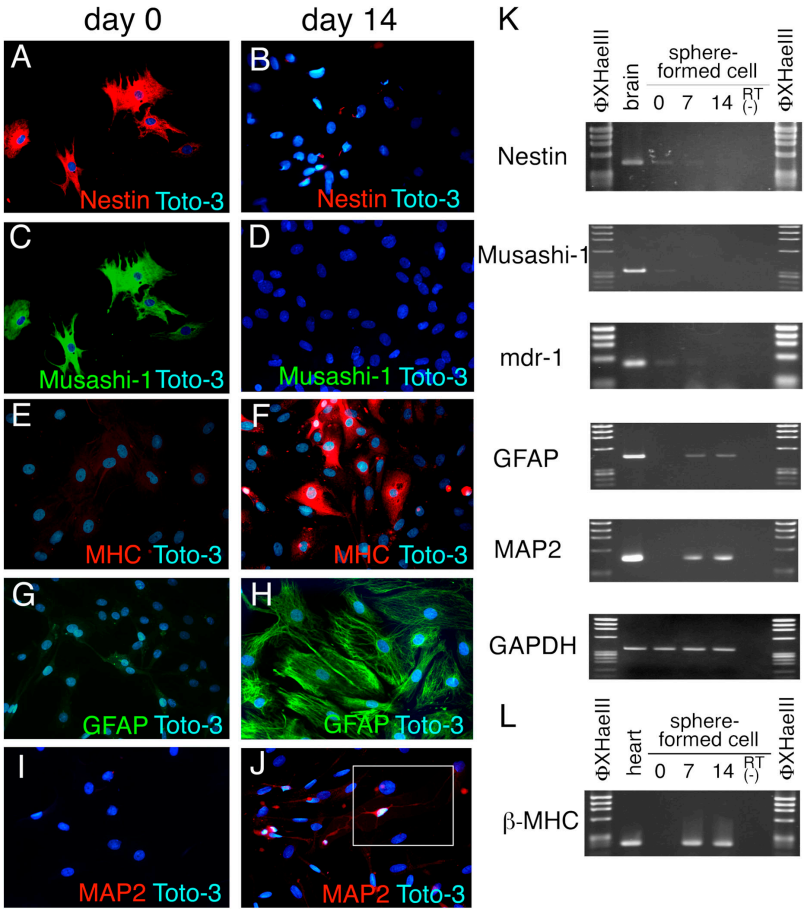

Figure 2. **Differentiation of cardiosphere-derived cells in vitro.** Cardiospheres were obtained from P2 neonates. Dissociated cardiosphere-derived cells were maintained in medium without EGF and FGF-2. At day 0, almost all cells stained with antinestin (A) and anti–Musashi-1 (C), but did not stain with antisarcomeric myosin (MF20) (E and F), anti-GFAP (G), or anti-MAP2 (I) Abs. At day 14, the cells did not stain with antinestin (B), or anti–Musashi-1 (D) Abs. By comparison, some cells stained with antisarcomeric myosin (F), anti-GFAP (H), or anti-MAP2 (J) Abs. The square box in (J) was enlarged in Fig. 3 C. (K) RT-PCR analyses of *nestin*, *Musashi-1*, *mdr-1*, *GFAP*, and *MAP2*. Note that cardiosphere-derived cells at day 0 expressed *nestin*, *Musashi-1*, and *mdr-1*, but not *GFAP* or *MAP2*. By comparison, these stem cell markers disappeared at day 14, and cells began to express GFAP and MAP2. (L) RT-PCR analysis of β-*MHC*. Fetal brain or heart was used as a positive control.

derived from a single cell from primary cultures plated at 10,000 cell/ml. Primary cell cultures derived from the neonatal hearts of wild-type mice and mice ubiquitously expressing GFP (Okabe et al., 1997) were mixed at a ratio of 9:1. Resultant cardiospheres were GFP-negative or GFP-positive; there was no evidence of mixing of the GFP-positive and GFP-negative cells (unpublished data).

Differentiation of cardiosphere-derived cells into multiple cell types in vitro

The multipotent capacity of cardiosphere-derived cells was investigated by dissociating cardiospheres to form a single-cell suspension, and then assessing the ability of these cells to differentiate in the absence of EGF and FGF2. Because cardiospheres are similar to neurospheres, the capacity of cardiosphere-derived

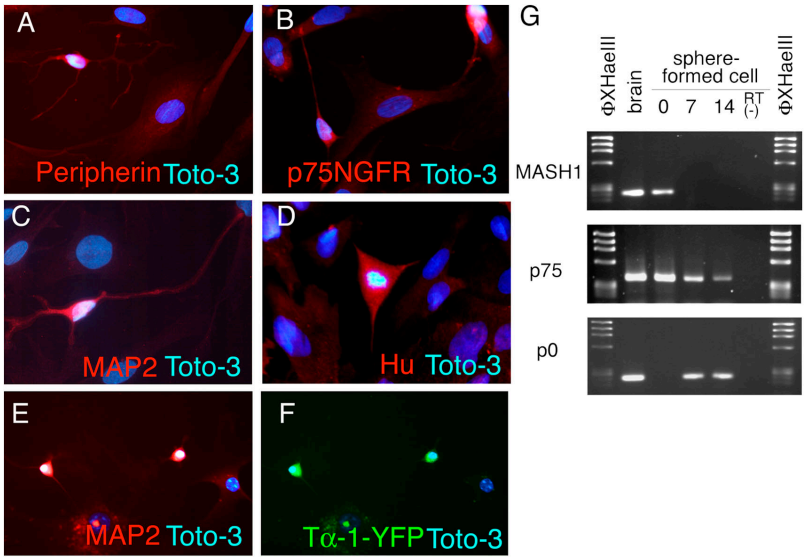

Figure 3. **Analysis of the expression of neuron-specific markers in cardiosphere-derived cells.** Cardiospheres obtained from P2 neonates were induced to differentiate as described in "Materials and methods." (A–D) Immunostaining of the neural markers: (A) peripherin, (B) p*75* NGF receptor, (C) MAP2, (D) Hu. (E and F) Cardiosphere-derived cells were obtained from Tα-1–EYFP transgenic mice, in which all neurons express the yellow fluorescent protein under the control of the neuron-specific α1-tubulin promoter. Some of the cardiosphere-derived cells expressing EYFP (F) were stained with anti-MAP2 Abs. (G) RT-PCR analysis of *MASH-1*, *p75*, and *P0*. *MASH-1*, a proneural basic helix-loop-helix transcription factor, was expressed at day 0, whereas the cells, in turn, expressed *P0*, myelin glycoprotein, at day 14.

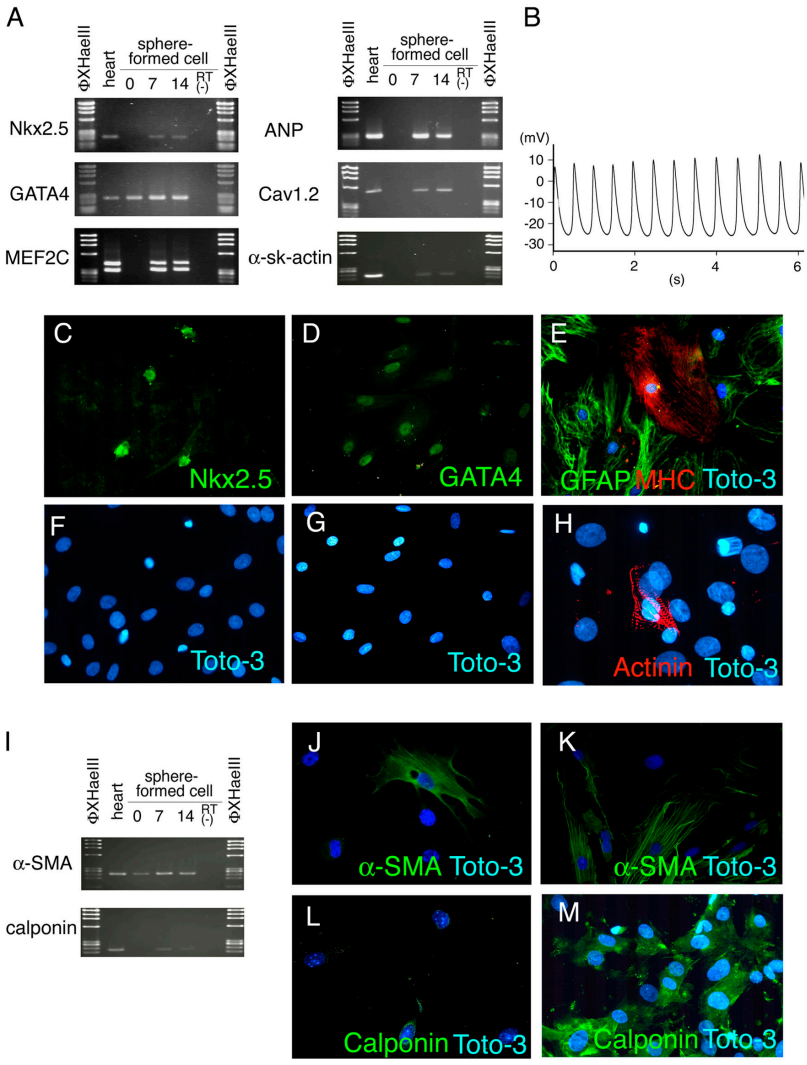

Figure 4. **Characterization of cardiosphere-derived cells as stem cells for cardiomyocytes and smooth muscle cells.** Cardiospheres obtained from P2 neonates were induced to differentiate as described in "Materials and methods." (A) RT-PCR analysis of cardiomyocytes-specific transcription factors and proteins. Cardiospheres were dissociated and replated; at day 0 cells expressed *GATA4*, but not *Nkx2.5,* muscle enhancement factor 2C, atrial natriuretic peptide, a1 subunit of the cardiac L-type Ca^{2+} channel, or α-*skeletal actin*. By comparison, the cells expressed all of these transcription factors and proteins after day 7. (B) A fraction of the cardiosphere-derived cells began spontaneous beating at day 14. This trace is representative of the action potential of these cells at day 14. Double immunostaining of Nkx2.5 (C) and nucleus with DAPI (D). Double immunostaining with GATA4 (E) and DAPI (F). (G) Triple immunostaining of sarcomeric myosin (red), GFAP (green), and DAPI (blue). (H) Double immunostaining of actinin and DAPI. (I) RT-PCR analysis of smooth muscle markers. Double immunostaining of α-smooth muscle actin (green) and nuclei with DAPI at day 0 (J) and day 14 (K). Double immunostaining of the calponin (green) and DAPI at day 0 (L) and 14 (M).

cells to differentiate into neurons, glial cells, and cardiomyocytes was investigated. At day 0, most cardiosphere-derived cells were positive for nestin and Musashi-1 (Fig. 2, A and C). At this stage, most cells were small in size, and did not express microtubule-associated protein 2 (MAP2), glial fibrillary acidic protein (GFAP), or myosin heavy chain (MHC) (Fig. 2, E, G, and I). By day 14, the cells had lost the ability to express nestin and Musashi-1 (Fig. 2, B and D). Differentiation of the cardiosphere-derived cells was associated with the induction of various morphologic changes, including the formation of neuron-like dendrites and the initiation of spontaneous beating within several weeks, a characteristic feature of cardiomyocytes. Cells within the differentiated population stained positively with anti-MHC, anti-GFAP, or anti-MAP2 Abs. The expression of markers for stem/progenitor cells, including *nestin, Musashi-1,* and *mdr-1* (*multi-drug resistance transporter gene 1*) (Zhou et al., 2001), and for differentiated cells, including *MAP2*, *GFAP*, and β-*MHC,* was assessed by RT-PCR (Fig. 2 L). Stem/progenitor cell marker genes were expressed at day 0, but their expression gradually decreased and could not be detected at day 14. By comparison, expression of *GFAP*, *MAP2*, and β-*MHC* were observed from day 7, which provided evidence of the differentiation of cardiosphere-derived cells into neurons, glia, and cardiomyocytes.

Cardiosphere differentiation into peripheral nerve cells

The ability of cardiospheres to differentiate into cells with PNS characteristics also was examined. Differentiated cardiospheres were immunostained with peripherin, a PNS neuronal marker; p75, a common receptor subunit of the nerve growth factor family that is expressed in sensory neurons, neural crest stem cells (Morrison et al., 1999), and Schwann cells (Stemple and Anderson, 1992); and MAP2, a pan-neuronal marker. A population of small cardiosphere-derived cells with long axons stained positive with all antibody markers (Fig. 3, A–C). Immunostaining with anti-Hu (a pan-neuronal marker) showed strong expression of Hu in particular small cells, which confirmed their differentiation into a neuronal cell type (Fig. 3 D).

α-Tubulin promoter (Tα-1)–EYFP transgenic mice, in which all cells with a neuronal lineage express enhanced yellow fluorescent protein (EYFP), were used to evaluate differentiation of cardiosphere-derived cells into neuronal cell types (Sawamoto et al., 2001). Differentiated cardiospheres from

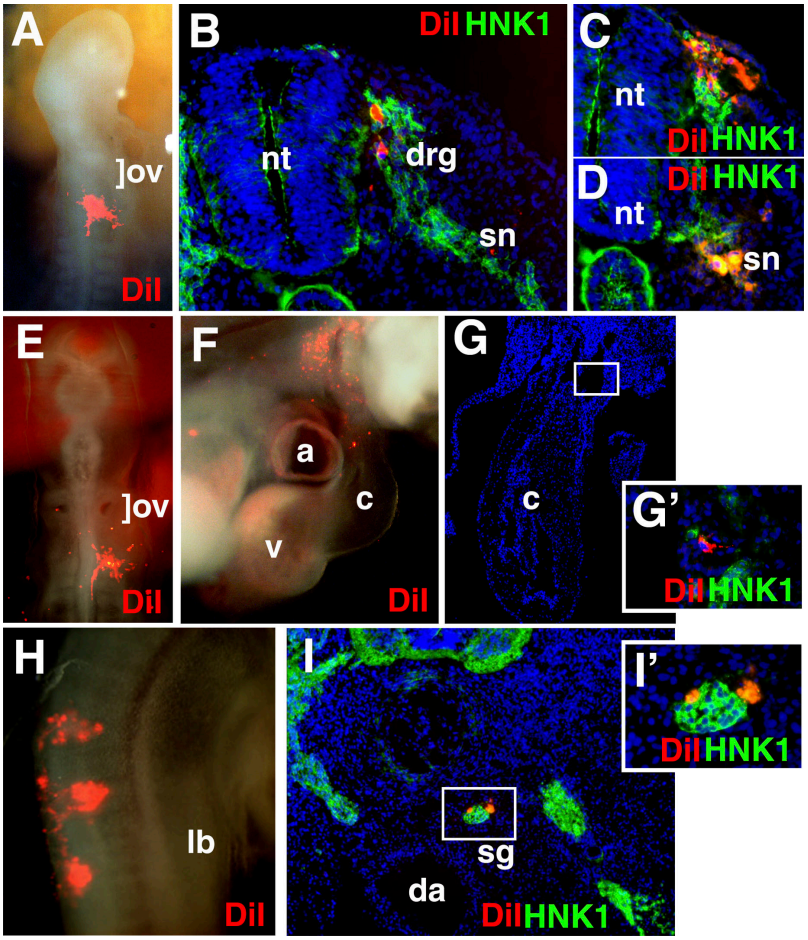

Figure 5. **Cardiosphere cells behave as neural crest in the chicken embryonic environment.** Cardiospheres obtained from P2 neonates were labeled with DiI, and transplanted into the chick neural crest. (A) A dorsal view of a chicken embryo that received cardiosphere cells into the MSA at the second somite level. Cardiosphere-derived cells are well-dispersed among embryonic cells 24 h after transplantation. (B–D) Transverse sections of cardiosphere-transplanted embryos, stained with HNK1 antibody for neural crest-derived cells and with DAPI for nuclei. DiI-labeled cardiosphere-derived cells contribute to the developing dorsal root ganglia (drg, B) and spinal nerve (sn, D). nt, neural tube. Many sphere-derived cells also enter the lateral migration pathway (C). (E) A dorsal view of a chicken embryo that received DiI-labeled cardiosphere cells into the lateral pathway at the second somite level. ov, otic vesicle. (F) A side view of an embryo, 48 h after transplantation into the lateral pathway showing the developing heart. Many cardiosphere-derived cells are entering the outflow tract area. a, atrium; c, conotruncal; v, ventricle. (G) A transverse section of the transplanted embryo showing the outflow tract and conotruncal region. c, conotruncal. (G′) A higher magnification view of the boxed area in (G). A DiI-labeled sphere-derived cell and a HNK1-positive host-derived crest cell are visible. (H) A dorsolateral view of an embryo that received three cardiosphere cells into the MSA at the wing limb bud level, 48 h after transplantation. lb, limb bud. (I) A transverse section of the transplanted embryo, showing developing sympathetic ganglia (sg). da, dorsal aorta. (I′) A high magnification view of the boxed area in (I) stained with HNK1 and DAPI. Cardiosphere-derived cells are integrated into the ganglia.

Tα-1-EYFP transgenic mice were immunostained with the anti-MAP2 antibody, and revealed expression of MAP2 by the $EYFP^+$ cardiosphere-derived cells (Fig. 3, E and F).

RT-PCR of *MASH1*, a proneuronal basic helix-loop-helix protein expressed in immature neuronal cells (Ross et al., 2003); *p75*; and *P0*, a Schwann cell myelin marker (Lemke and Axel, 1985; Lemke et al., 1988), also confirmed the differentiation of cardiosphere-derived cells into neuronal cell types (Fig. 3 G). Differentiation of cardiosphere-derived cells leads to a reduction in *p75* expression, and a reduction—followed by a loss—of *MASH1* expression. By comparison, expression of P0 was induced as a result of cardiosphere cell differentiation. These findings are consistent with the differentiation phenotype of neuronal cells of the PNS lineage.

Cardiosphere differentiation into cardiomyocytes and smooth muscle cells

Cardiospheres and cells dissociated from cardiospheres express *GATA4*, but not *Nkx2.5* or muscle enhancement factor 2C, which indicate that these cells are not cardiomyocytes, but their early progenitors (Fig. 4 A). The cardiac-specific genes, *ANP, Cav1.2*, or α-*skeletal actin*, are activated in cardiosphere-derived cells 7 d after dissociation, whereas spontaneously beating cells are evident at 14 d. Fig. 4 B shows the representative action potentials recorded from the cardiosphere-derived cardiomyocytes at day 14. Compared with mature cardiomyocytes, the resting potential was shallower but the duration of the action potential was similar. Immunofluorescent staining for Nkx2.5, GATA4, and actinin was evident in particular cells at day 14 (Fig. 4, C–H).

In the present study, the capacity of cardiosphere-derived cells to differentiate into smooth muscle was investigated by examining the production of α-smooth muscle actin (α-SMA) and calponin (Fig. 4, J–M). At day 0, $\alpha\text{-SMA}^+$ cells represented 11.8 ± 4.8% of total cells and were negative for calponin. By day 14, the population of $\alpha\text{-SMA}^+$ cells had increased to 42.9 ± 16.2% (Table SI; available at http://www.jcb.org/cgi/content/full/jcb.200504061/DC1); most of these were positive for calponin. These findings show that the cardiosphere-derived cell population consists largely of stem/progenitor cells; a small fraction has the capacity to differentiate, at least in part, into cardiomyocytes and smooth muscle cells.

Cardiosphere cells behave like neural crest cells in vivo

At day 0, 99.8% of the cardiosphere-derived cells were nestin^+ and Musashi-1^+, a characteristic of stem/progenitor cells (Table SI). Following differentiation, the expression of these markers is lost and is replaced by the expression of various markers for differentiation. Many cells were positive for GFAP (68.1 ± 1.8%) and α-SMA (42.9 ± 16.2%), whereas the population of neurons and cardiomyocytes represented 0.45 ± 0.21%

and 0.28 ± 0.17% of the total cell population, respectively. Differentiated neurons and cardiomyocytes did not express nestin. The capacity of cardiosphere-derived cells to generate neurons, glia, and smooth muscle cells in culture suggests that they have neural crest-like characteristics. To investigate these characteristics further, we tested the behavior of cardiosphere-derived cells in vivo. Because neural crest cells that originate from the 1–3 somite level contribute to heart structures in the chick embryo, one to three DiI (a lineage tracing dye)-labeled cardiospheres were transplanted into the migration staging area (MSA) between the dorsal neural tube and somite at the first and/or second somite level of Hamburger and Hamilton (1951) stage 9 chicken embryos. DiI-labeled cardiosphere-derived cells were well-dispersed in the embryonic environment 24 h after transplantation (Fig. 5 A). Many cardiosphere-derived cells seemed to migrate along with host-derived human natrual killer-1 (HNK1)–positive neural crest cells (Fig. 5, B and C). Rodent neural crest cells do not express HNK1. Because DiI is located at the cell surface membrane, the border zone area between the DiI-labeled cardiosphere-derived cells and host-derived HNK1-positive cells appears yellow when many donor cells are concentrated among the recipient cells. Medially migrating cardiosphere-derived cells were found in the developing PNS, such as the dorsal root ganglion (Fig. 5 B) and the ventral spinal nerve (Fig. 5 D). Cardiosphere-derived cells also entered the lateral migration pathway that normally is taken by neural crest-derived melanocyte precursors, ectomesenchymal cells, and those migrating into the cardiac region. Very few cells reached the heart region.

To facilitate the migration of cardiosphere-derived cells into the heart region, DiI-labeled cardiospheres were transplanted directly onto the lateral pathway between the dorsal somite and the overlying epidermal ectoderm. Again, cardiosphere-derived cells were well-dispersed in the embryonic environment 24 h after transplantation (Fig. 5 E). 48 h later, the labeled cells successfully entered the out-flow tract and the conotruncus of the developing heart (Fig. 5 F). On transverse sections of transplanted embryos, cardiosphere-derived cells were found in the heart region along with HNK1-positive host-derived cardiac crest cells (Fig. 5, G and G′). To examine further the migratory capacity of cardiosphere-derived cells in vivo, labeled cardiospheres were transplanted into the MSA at the wing level of stage 12–13 embryos. 48 h after transplantation, cardiosphere-derived cells seemed to migrate along the medial pathway (Fig. 5 H). On transverse sections, many cardiosphere-derived cells were found to contribute to the PNS, such as the sympathetic ganglia (Fig. 5, I and I′), and also to dorsal root ganglia and spinal nerves (not depicted). These results show that cardiosphere-derived cells behave like neural crest cells and migrate in the chick embryonic environment.

To examine the differentiation of transplanted cardiosphere-derived cells in vivo, cardiospheres were prepared from GFP-expressing rat SP cells, because the GFP label allows longer-term identification of individual cells. GFP-labeled cardiospheres were transplanted as described above, and the distribution and differentiation of cardiosphere-derived cells were assessed 2 d later. Many cardiosphere-derived cells formed peripheral ganglia and many also expressed the neuronal marker

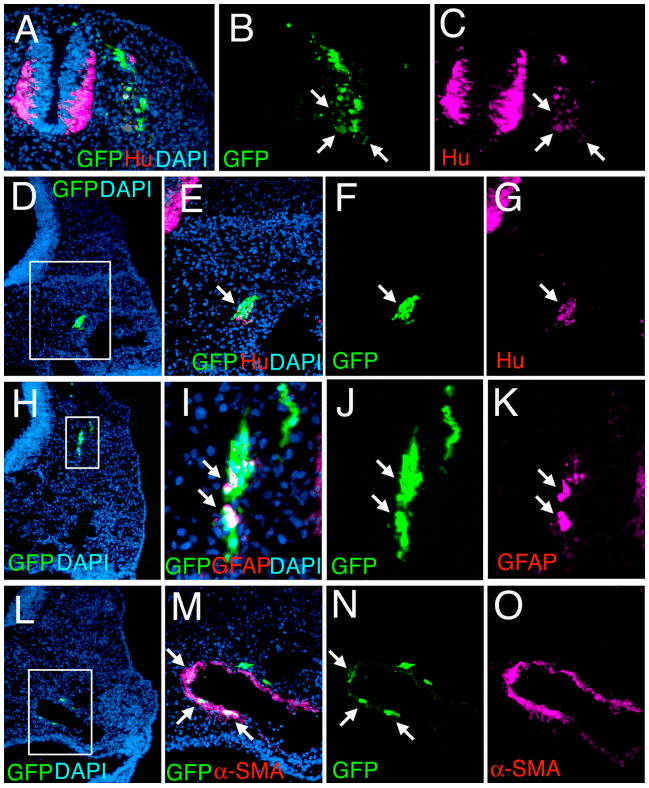

Figure 6. **Differentiation of GFP-labeled cardiosphere-derived cells in the chick embryonic environment.** Cardiospheres obtained from P2 neonates of the GFP-Tg mice were transplanted into chick neural crest. Cardiospheres were transplanted into the MSA at trunk level (A–C), or into the lateral pathway at the first somite level (D–O). The transplanted embryos were allowed to develop for 2 d. Cardiosphere cells contribute to the peripheral ganglia (A–K), and express the neuronal marker Hu (A–C, D–G) or glial marker GFAP (H–K). Arrows indicate double immunofluorescent–positive cells. Some cardiosphere-derived cells also invade the anterior cardinal vein and express smooth muscle actin (M–O, arrows). E–G, I–K, and M–O are higher magnifications of the boxed areas in D, H, and L, respectively.

Hu (Fig. 6, A–G). Expression of the glial marker GFAP at the trunk and cranial levels also was evident (Fig. 6, H–K). Several cardiosphere-derived cells also contributed to the major blood vessels and expressed smooth muscle actin (Fig. 6, L–O). These results indicate that cardiosphere-derived cells have the capacity to contribute to neural crest-derived tissues in the chick embryo.

Distribution and differentiation of neural crest-derived cells in the heart

For the analysis of the neural crest cell lineage, Yamauchi et al. (1999) generated transgenic mice harboring a Cre gene driven by a promoter of P0, and crossed P0-Cre transgenic mice with CAG-CAT-Z indicator transgenic mice, which carry a lacZ gene downstream of a chicken-actin promoter and a “stuffer” fragment flanked by two *loxP* sequences. In three different P0-Cre lines crossed with CAG-CAT-Z transgenic (Tg), embryos carrying both transgenes showed *lacZ* expression in tissues that were derived from neural crest cells, such as spinal dorsal root ganglia, sympathetic nervous system, enteric nervous system, and ventral craniofacial mesenchyme at stages later in development than E9.0.

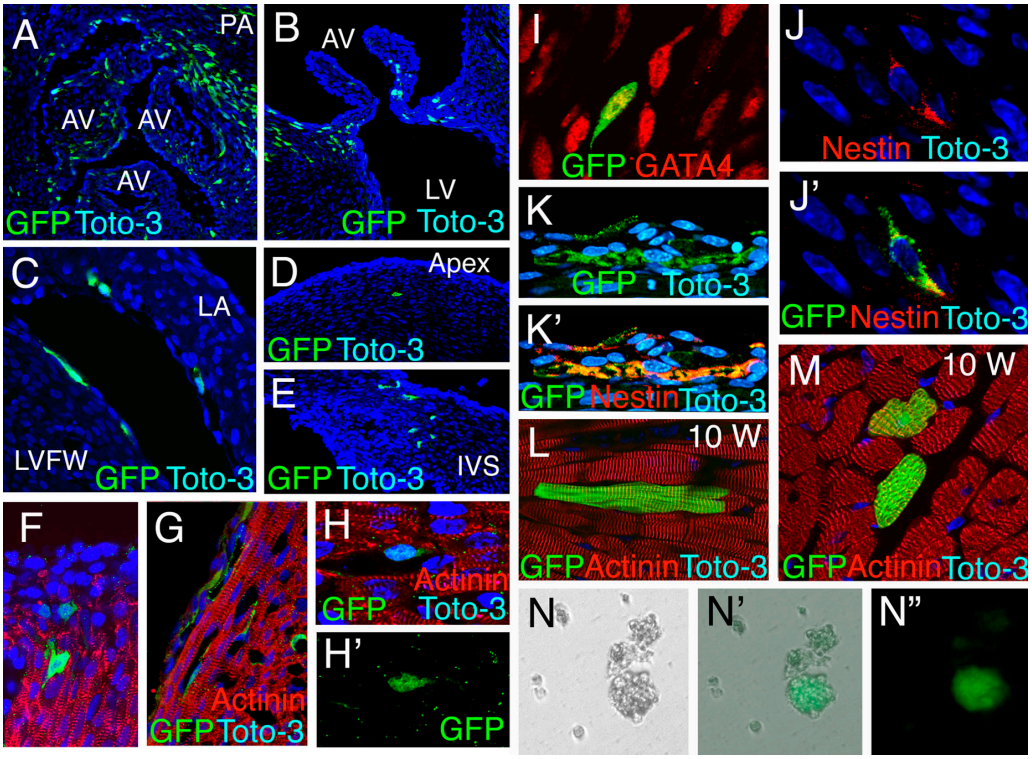

Figure 7. **Distribution and coimmunostaining of neural crest derived cells in the heart.** (A–E) P0 Cre/CAG-CAT-EGFP double Tg mice heart was immunostained with Toto-3 and anti-GFP antibody. Distribution of EGFP$^+$ cells in the heart was demonstrated. EGFP$^+$ cells were concentrated in the outflow tract and aortic valve (A and B) and were observed in the subepicardial layer (C) and intramuscular layer (D and E) of the ventricles. EGFP$^+$ cells were observed at the free wall (C), apex (D), interventricular septum (E), and atrium (C). (F–H) Triple-immunostaining for actinin (red), GFP (green), and Toto-3 in E17.5 P0-Cre/CAG-CAT-EGFP double Tg mice heart. EGFP$^+$ cells did not stain with actinin. (H) and (H′) show the same field. (I) Double immunostaining for GATA4 (red) and GFP (green) in E17.5 double Tg mice heart. Some of the EGFP$^+$ cells stained with anti-GATA4 antibody. (J and K) Triple immunostaining for nestin (red), GFP (green), and Toto-3 (blue) in E17.5 double Tg mice heart. EGFP$^+$ cells were stained with antinestin antibody. (J and J′) and (K and K′) show the same fields. (L and M) Triple-immunostaining for actinin (red), GFP (green), and Toto-3 in 10-wk-old adult P0-Cre/CAG-CAT-EGFP double Tg mouse heart. EGFP$^+$ cells expressed actinin with complete striation indicating cardiomyocytes. (N–N′′) Expression of EGFP in a cardiosphere isolated from a 10-wk-old adult P0-Cre/CAG-CAT-EGFP double Tg mouse indicating its neural crest origin. AV, aortic valve; LA, left atrium; LVFW, left ventricular free wall; IVS, intraventricular septum.

Double Tg mice carrying P0-Cre recombinase and CAG-CAT-EGFP transgenes showed EGFP expression in tissues that were derived from neural crest cells, including spinal dorsal root ganglia, sympathetic nervous system, enteric nervous system, and ventral craniofacial mesenchyme (unpublished data). EGFP$^+$ cells were concentrated at the outflow tract between the aortic and pulmonary arteries and the aortic valves, and at the intramuscular and subepicardial layer of both ventricles, including the intraventricular septum, free wall, and apex (Fig. 7, A–E). EGFP$^+$ cells also were observed at the atrial wall. Triple immunostaining of the heart at E17.5 showed a lack of staining with the anti-actinin antibody (Fig. 7, F–H) and evidence of staining with the anti-GATA4 antibody (Fig. 7 I). Some EGFP$^+$ cells stained with anti-nestin antibody (Fig. 7, J and K). Triple immunostaining of the heart of 10-wk-old mice clearly demonstrated that a portion of the GFP$^+$ cell population were actinin$^+$ cardiomyocytes (Fig. 7, L and M). Consistent with these results, the cardiosphere-derived cells from 10-wk-old double transgenic mice hearts were clearly GFP$^+$ (Fig. 7 N). These findings strongly suggest that neural crest-derived cells remain in the heart as stem cells in adults and have the capacity to differentiate into various cell types, including cardiomyocytes.

Discussion

Cardiosphere-initiating cells are multipotent

The present study used the neurosphere culture method to demonstrate that a certain proportion of SP cells in the heart could proliferate, and that the SP cell–derived cardiosphere-initiating cells were multipotent and could give rise to PNS-type neurons, Schwann cells, smooth muscle cells, and cardiomyocytes. Moreover, cardiosphere-derived cells display characteristics of neural progenitor cells with respect to the expression of nestin, Musashi-1, and p75 (Mujtaba et al., 1998).

Hierlihy et al. (2002) reported that cardiac SP cells were progenitor cells for cardiomyocytes. The present study is the first report to demonstrate that cardiac SP cells are cardiomyocyte progenitor cells and neural crest-like multipotent stem/progenitor cells. Recent studies report that adult tissue-specific stem cells have common characteristics (Reynolds and Weiss, 1992; Tropepe et al., 2000; Kawaguchi et al., 2001). First, all express nestin in their immature and multipotential state. Second, they usually are smaller than the major mature cell type. Third, they proliferate and form floating spheres in serum-free medium in the presence of growth factors, and lose their multi-

potency when cultured in the presence of serum. The present study demonstrated that the characteristics of cardiac SP cell–derived cardiosphere-forming cells are consistent with the common characteristics of tissue-specific stem cells.

The present study showed that a portion of the cardiosphere-derived cell population expressed neuron-specific markers. This result is consistent with previous studies that showed that the conduction system of the heart expresses neuron specific markers, including protein gene product 9.5, neurofilament (H, M, and L), HNK1 (chick), tyrosine hydroxylase, Do170, and RMO270 (Verberne et al., 2000; Mueller et al., 2003). To our knowledge, expression of MAP2, peripherin, and Hu has not been demonstrated in the cardiac conduction system. In the present study, the neuron-like cells that were derived from cardiospheres did not stain with anti-myosin antibody. Taken together, the expression profile of the neuron-like cells that were derived from cardiospheres suggests differentiation into neuronal cells, not conduction cells.

In the present study, we assessed the characteristics and multipotency of cardiospheres that were derived from P2 neonate mice, and demonstrated the formation of cardiospheres from the adult heart with similar characteristics. Cardiospheres that are derived from adult mice hearts are multipotent, but have several characteristics that distinguish them from cardiospheres that are derived from neonatal mice hearts. First, cardiospheres that are derived from adult hearts form with a greatly reduced efficiency than cardiospheres that are derived from those of neonates. Second, neonatal-derived cardiospheres have a greater capacity than adult-derived cardiospheres to form secondary or tertiary cardiospheres following dissociation. Further characterization of adult-derived cardiospheres is required for any future clinical application.

The relationship between cardiosphere-derived cells and neural crest stem cells

Neural crest-derived cells have stem cell characteristics because they have the capacity to proliferate and differentiate into various types of cells, including sensory and sympathetic neurons, glial cells in the PNS, and smooth muscle cells in the blood vessels (Le Douarin and Kalcheim, 1999; Hall, 2000; Gammill and Bronner-Fraser, 2003). Because neural crest-derived cells express nestin, Musashi-1, and p75, and can proliferate and differentiate into multiple lineages in vitro, they are regarded as stem cells (Morrison et al., 1999).

The heart develops in the neck region, directly beneath the pharyngeal arches; the caudal area of the cranial neural crest is sometimes referred to as the cardiac crest (Kirby, 1989; Osumi-Yamashita et al., 1996). Paracrine factors from cardiac neural crest-derived cells play a potentially crucial role in heart maturation and in the formation of the outflow tract and ventricular septum (Waldo et al., 1999). A recent study in zebra fish demonstrated that the cardiac crest cells also can differentiate into cardiomyocytes (Sato and Yost, 2003).

In the present study, transplantation of cardiospheres into chick embryos revealed that these cells migrate by way of dorsolateral pathways to the cardiac outflow tract, and contribute to the smooth muscles of blood vessels and to the PNS, including dorsal root ganglia, sympathetic ganglia, and spinal nerves. The possibility that nonneural crest-derived cells from cardiospheres migrate in the chick embryo is unlikely, because migration is along neural crest-derived cell-specific pathways, and cells are integrated and committed into the neural crest-derived organs. Based on the expression of stem cell markers, multipotency in vitro, and behavior in vivo, it seems that a portion of the cardiosphere-initiating cell population originates from the cardiac crest cells. By lineage analysis using P0-Cre recombinase/Floxed-EGFP double transgenic mice, we also demonstrated that neural crest-derived cells migrated into the myocardium, in addition to the outflow tract and aortic valves; remained in the heart as neural crest-derived cells that could form cardiospheres; and had the capacity to differentiate into various types of cells, including cardiomyocytes.

Along similar lines, recent studies report the presence of neural crest stem cells in the postnatal gut that have the capacity to proliferate and differentiate; this suggests that persistence of neural crest stem cells in the adult tissues may help regeneration after injury or disease (Bixby et al., 2002; Kruger et al., 2002). Consistent with these studies, the present study demonstrated that neural crest-derived cells lay dormant in the neonatal heart, expressing nestin. Some of these cells expressed GATA4, but did not express cardiomyocyte-specific proteins, which indicates that they exist as cardiac stem/progenitor cells. An attractive hypothesis is that neural crest-derived cardiosphere-initiating cells that are present in the neonate will remain as the stem cells in the adult heart. Although the present lineage analysis of the neural crest-derived cells and the formation of cardiospheres by neural crest-derived cells support this hypothesis strongly, further investigation using P0-Cre-EGFP double transgenic mice is required.

The relation between cardiosphere-initiating cells and other cardiac stem cells

Beltrami et al. (2003) reported the existence of stem cells in the adult heart as multipotent $CD45^{-}CD34^{-}c\text{-}kit^{+}Lin^{-}$ cells with the capacity to form spheres. In the present study, cardiac SP cells had some characteristics in common with those described by Beltrami et al. (2003), as well as some distinct characteristics. In this study, clonal cardiospheres could differentiate into cardiomyocytes, smooth muscle cells and PNS-type neurons and glial cells, whereas the $Lin^{-}c\text{-}kit^{+}$ cells that were described by Beltrami et al. (2003) only had the capacity to differentiate into endothelial cells, smooth muscle cells, and cardiomyocytes. This discrepancy suggests that cardiosphere-initiating cells can be derived from other cell lineages, including $c\text{-}kit^{-}$ cells.

Oh et al. (2003) reported the existence of adult heart-derived cardiac progenitor cells expressing Sca-1. According to their analyses, the SP cells in the adult heart were $CD45^{-}c\text{-}kit^{-}CD34^{-}Sca\text{-}1^{+}CD31^{+}CD38^{+}Lin^{-}Flk^{-}Flt^{-}VE\text{-}CAD^{-}vWF^{-}$, which is in partial agreement with the results that were obtained from neonatal heart SP cells in the present study. The cells of the cardiosphere described in the present study and cardiac $Sca\text{-}1^{+}$ cells share characteristics, but also vary. In the present study, expression of Sca-1 by cardiac SP cells was low or negative.

Cardiosphere-derived cells and Sca-1$^+$ cardiomyocyte progenitor cells are distinct cells populations because the cell surface marker, percentage of population, multipotency, and gene expression characteristics are very different.

Cai et al. (2003) reported that hearts of mice lacking isl1, a LIM homeodomain transcription factor, are completely missing the outflow tract, right ventricle, and much of the atria, and that isl1 is a marker for a distinct population of undifferentiated cardiac progenitors. Laugwitz et al. (2005) identified isl1$^+$ cardiac progenitors in postnatal rat, mouse, and human myocardium. Co-culture studies with neonatal myocytes indicated that isl1$^+$ cells represent authentic, endogenous cardiac progenitors that display highly efficient conversion to a mature cardiac phenotype. The finding that isl1$^+$ cells did not express Sca-1 and were relatively concentrated at the outflow tract of the heart is consistent with the findings for SP cell derived-cardiosphere forming cells. isl1$^+$ cells were observed only at the looping heart, whereas we observed EGFP$^+$ cells from P0-Cre/EGFP mice in neural crest-derived tissues. Thus, cardiosphere-forming cells and isl1$^+$ cells have common and distinct characteristics.

Martin et al. (2004) showed that the adult heart contains an Abcg2-positive SP cell population that is capable of proliferation and differentiation, and defined the molecular signature of cardiac SP cells that function as a progenitor cell population for the development, maintenance, and repair of the heart. The multipotency or developmental origin of the cardiac SP cells was not addressed. The present study did address these issues and advanced the characterization of the cardiac SP cell fraction. A portion of the SP cell population could form cardiospheres; differentiate into neurons, glia, smooth muscle cells, and cardiomyocytes; and seemed to originate from the cardiac neural crest.

We have used the cardiosphere formation assay to identify and isolate multipotent stem/progenitor cells in the heart, expand them in vitro, and analyze them in vivo. Further analysis of these cells will contribute to the understanding of the mechanisms that underlie cardiac development. The potential use of these cells for clinical organ repair after injury or disease provides a challenging and exciting prospect.

Materials and methods

Animals

1-d-old neonatal ICR mice and Wistar rats were purchased from Japan CLEA. Tα-1–EYFP Tg mice were generated to express the EYFP under the control of the neuron-specific Tα-1 as described previously (Sawamoto et al., 2001). The P0-Cre recombinase Tg mouse (Yamauchi et al., 1999) was provided by K. Yamamura (Kumamoto University, Kumamoto, Japan). The CAG-CAT-EGFP Tg mouse (Kawamoto et al., 2000) was a gift from J. Miyazaki (Osaka University, Osaka, Japan). All experimental procedures and protocols were approved by the Animal Care and Use Committees of the Keio University and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell preparation

Primary cultures of cardiomyocytes and cardiac nonmyocytes were prepared from the ventricles of 1–7-d-old neonatal and adult rats or mice by enzymatic dissociation. The Percoll gradient method was used to separate cardiomyocytes from nonmyocytes as described previously (Sano et al., 2000).

Isolation of SP cells and FACS analysis

Hoechst 33342 solution (Sigma-Aldrich) was prepared immediately before use. Cells were resuspended at 10^6 cells/ml, and stained with 6.0 μg/ml of Hoechst 33342 in calcium- and magnesium-free HBSS+ (supplemented with 2% FCS, 10 mM Hepes, and 1% penicillin/streptomycin) medium for 90 min at 37°C. As a negative control for SP cells, 10^6 cells were stained in the presence of 100 μM reserpine (Sigma-Aldrich) in parallel experiments.

Antibody staining

For FACS analysis, cells were washed once in cold HBSS+, resuspended at 10^8 cells/ml, and stained with FITC- or phycoerythrin-conjugated Abs against CD11b, 13, 29, 31, 34, 44, 45, 49b, 49e, 73, and 117 (c-kit), Flk-1, Ter119, and Sca-1 (BD Biosciences) at saturating concentrations. After antibody staining, the cells were washed with an excess amount of HBSS+ and resuspended at 10^7 cells/ml in HBSS+ containing 2 μg/ml propidium iodide (Sigma-Aldrich).

Flow cytometry

Cell analysis and sorting was performed on a triple laser FACS Vantage (Becton Dickinson). Hoechst 33342 was excited at 350 nm, and fluorescence emission was detected using a 405/BP30 and 570/BP20 optical filter for Hoechst blue and Hoechst red, and a 550-nm long-pass dichroic mirror (Omega Optical Inc.) to separate the emission wavelengths. Hoechst blue and red emit fluorescence according to a linear scale. Propidium iodide fluorescence was measured through 630BP30 after excitation at 488 nm with an argon laser, and a live cell gate was defined to exclude propidium iodide–positive cells. The SP population was defined as described in previous reports after collecting 10^5 events (Goodell et al., 1996, 1997; Matsuzaki et al., 2004).

Cardiosphere cell culture

Cells were suspended at a density of 1–2 × 10^6 cells/ml in 10-cm uncoated dishes in DME/M199 (1:1) serum-free growth medium containing insulin (25 μg/ml), transferin (100 μg/ml), progesterone (20 nM), sodium selenate (30 nM), putrescine (60 nM) (all from Sigma-Aldrich), recombinant murine EGF (20 ng/ml) (Funakoshi), and recombinant human FGF2 (20 ng/ml) as described previously (Reynolds and Weiss, 1992). Half of the medium was changed every 3 d. Passaging was performed using 0.05% trypsin and 0.53 mM EDTA-4Na every 7–14 d. Cardiospheres were dissociated into a single-cell suspension then reseeded into fresh medium.

For monolayer cultures, cardiospheres were dissociated and seeded onto gelatin-coated glass slides in DME/M199 (1:1) containing 0.5–10% FBS in the presence or absence of EGF and FGF2. Half of this medium, referred to as differentiation medium, was changed every 2–4 d.

Immunofluorescent staining

Cells were cultured on gelatin-coated glass coverslips, fixed with 4% paraformaldehyde/PBS, then incubated with primary Abs against mouse anti-nestin, Rat 401 (Hockfield and McKay, 1985, Hybridoma Bank), anti-MAP2, anti-GFAP, anti-NeuN, anti-sarcomeric myosin MF20, and anti-αSMA (all from Sigma-Aldrich) MoAbs, rat anti–Musashi-1 MoAb, 14H1 (Kaneko et al., 2000), rabbit anti-peripherin (Chemicon) and anti-p75 (Sigma-Aldrich) PoAbs, and human anti-Hu (Okano and Darnell, 1997) MoAb for 12–24 h at 4°C. Cells were incubated with secondary Abs for 2–6 h at room temperature. Secondary Abs were used at the following dilutions: anti–mouse IgG/Texas red (1:500), anti–mouse IgG/FITC (1:100), anti–mouse IgG_1/FITC (1:500) (BD Biosciences), anti–mouse IgG_{2b}/Texas red (1:500), anti–mouse IgG/HRP (DakoCytomation), anti–rabbit IgG-PE, and anti-human IgG-Alexa542. The samples were washed twice with PBS, then treated with 0.5 μg/ml DAPI (Sigma-Aldrich) for 2 min.

RT-PCR analysis

Isolation of RNA and RT-PCR was performed as described previously (Sano et al., 2000). Detection of cardiomyocyte-specific or stem cell marker genes, including nestin, Musashi-1, MDR-1, and p75; neuronal lineage markers, including MAP-2 and MASH1; glia-specific markers, including GFAP and P0; cardiomyocyte markers, including Nkx2.5, GATA4, β-MHC, α-skeletal actin, atrial natriuretic peptide, and α_{1c} subunit of L-type Ca^{2+} channel; and smooth muscle markers, including calponin and αSMA, was performed using 0.01 μg of total RNA. The primers are listed in Table SII (available at http://www.jcb.org/cgi/content/full/jcb.200504061/DC1).

Action potential recording

Action potential of the beating cells was recorded in DME/M199 (1:1) medium containing 1.49 mmol/L $CaCl_2$, 4.23 mmol/L KCl, and 25 mmol/L Hepes (pH 7.4), and was performed as described previously (Makino et al., 1999).

Transplantation of cardiosphere into chick embryos

DiI was made up in 0.5% stock solutions in 100% ethanol, and was diluted in 0.3 M sucrose to 0.5 μg/μl. Cardiospheres were washed in DME/M199, labeled with DiI by for 30 s, and washed three times again in DME/M199.

Fertilized chicken (*Gallus gallus*) eggs were incubated at 38°C. Embryos were staged according to Hamburger and Hamilton (1951). Transplantation procedures essentially were performed as described previously (Wakamatsu et al., 1998). In brief, the medial or lateral pathway of stage 9 or 12–13 host embryos was opened with sharp tungsten needles, and one to three cardiospheres containing ~50–100 cells were inserted with a blunt tungsten needle. Whole-mount images of transplanted embryos were captured with a cooled CCD camera equipped on a fluorescent dissection microscope (MZFLIII, Leica). Fixed embryos were cryosectioned and stained with HNK1 (Tucker et al., 1988), 16A11 anti-Hu (Marusich et al., 1994), anti-GFAP, or anti-SMA Abs. Cell nuclei were counter-stained with DAPI. Immunostaining was performed as described previously (Wakamatsu et al., 1998). Images were captured with cooled CCD camera equipped on a fluorescent microscope (Axio PlanII, Zeiss), and were processed with Adobe Photoshop (v5.0) software.

P0-Cre/EGFP mice

P0-Cre Tg mice were crossed with CAG-CAT-EGFP indicator Tg mice. Hearts from E17.5 mice were dissected, washed in PBS, embedded in OCT Compound (TED PELLA), and quickly frozen in liquid nitrogen. Cryostat sections (8-μm thick) were stained overnight at 4°C using specific Abs. Anti-GFP (MBL), anti-actinin, anti-GATA4, and anti-nestin Abs were used to identify $EGFP^+$ cells, cardiomyocytes, and hematopoietic cells. The sections were incubated with secondary Abs conjugated with Alexa 488 or 594 (Molecular Probes). Nuclei were stained with Toto-3 (Molecular Probes). Slides were observed under a confocal laser scanning microscope (LSM 510 META; Carl Zeiss Microimaging, Inc.).

Online supplemental material

Table SI shows temporal changes of the immunofluorescent staining positive cells of the stem cell or differentiation markers. Table S2 shows the PCR primers used in this study. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200504061/DC1.

Submitted: 12 April 2005
Accepted: 19 August 2005

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