Supporting Information

Tallini *et al.* 10.1073/pnas.0808920106

SI Materials and Methods

BAC Targeting and Founder Genotyping. An EGFP-pA cDNA was inserted into BAC clone RP24-330G11 at the initiation codon of *c-kit* by homologous recombination. The BAC clone contained approximately 77 kb of 5′ and 99 kb of 3′ DNA flanking the *c-kit* locus and included the entire ≈32 kb 1st intron. Five hundred sixty-four- and 541-bp fragments 5′ and 3′, respectively, to the c-kit ATG (exon1) were inserted into the pBS-EGFP-pA-FRT-Neo/Kan-FRT vector and the targeting construct released by XhoI/SacII digestion and electroporated into EL250-RP24–330G11 cells. Chloramphenicol (12.5 μg/mL) and kanamycin (25 μg/mL) resistant clones were genotyped by amplification and sequenced to identify homologous recombinants, followed by removal of the neomycin cassette by L(+)-arabinose (0.1%) recombinase induction, leaving one FRT site. C-kit^{BAC}-EGFP-PA-FRT DNA was cesium chloride purified and injected into the pronuclei of zygotes by standard methods.

Genotyping of c-kit^{BAC}-EGFP mice was with primer pairs befarmI/eGFPR1 (befarmI: GCAGGTGGAGAAACTGAG-CATG; EGFPR1: CCCAGGATGTTGCCGTCCTCCT) yielding a 1,095-bp product and newborns (PN 0–5) were screened for EGFP expression by using a KL2500 cold light source (Schott Fostec) with safety glasses covered with Wratten filter no. 12 (Kodak). The line is registered with the Mouse Genome Informatics as Tg (RP24-330G11-EGFP) 1 Mik, no. 3760303. All procedures were approved by Cornell Institutional Animal Care and Use Committees committee and adhered to the standards published in *Guide for the Care and Use of Laboratory Animals*.

FACS and Culturing Parameters. After dissociation cells were re-suspended in proliferation media (see below) containing 20% FCS and maintained in an incubator at 95% O_2/5% CO_2 and 37 °C until ready for FACS. Cell suspensions from neonatal c-kit^{BAC}-EGFP mice cardiac isolations were analyzed and sorted by flow cytometry on a Becton Dickinson Biosciences FACS Aria. EGFP was excited by a 488-nm laser and emission collected in a FITC channel by using a 502 longpass dichroic mirror and a 530/30 bandpass filter. A parent population of cells that represented only whole cells gated to exclude debris by forward and side scatter was first determined. Autofluorescent cells were then identified as cells with a high PE-585/42 to 530/30 signal ratio and excluded by gating. The total EGFP-positive heart cell population (tEGFP) was identified as those cells with signal exceeding the Gaussian distribution of heart cells obtained from transgene negative littermates. A high EGFP-expressing subgroup (sEGFP) was identified as those cells having a signal >5-fold above control, a population that comprised, on average, the brightest 5% of cells. Cells were sorted at 65 psi through a 70-μm nozzle oscillating at 87 Hz, into proliferating media (see *FACS and Culturing Parameters*). Cell viability was evaluated by propidium iodide exclusion (1) and averaged 92% pre-FACS sort and 78% post-FACS sort.

Cells were plated on Lab-Tek 8 well-chambered cover glass slides (BD Biosciences) pretreated with 0.1% gelatin (Chemicon International), plated at a density of 80,000–160,000 per cm^2 or 8,000–20,000 per cm^2, and cultured for 2–3 days in proliferating media, containing F12K media (Gibco), 5% FCS (Chemicon International), 10 ng/mL basic fibroblast growth factor (bFGF; Molecular Biology Reagents, Sigma-Aldrich), 10 ng/mL leukemia inhibitory factor (LIF; Chemicon International), and 1% penicillin/streptomycin (Gibco). Thereafter, cells were switched to basic media containing DMEM/Ham's F12 (1:1) (Gibco), B27 Supplement (Gibco), 10 ng/mL bFGF, 20 ng/mL epidermal growth factor (EGF; Molecular Biology Reagents, Sigma-Aldrich), 10 ng/mL LIF, and 1% pencillin/streptomycin until the day of the experiment (2–4). To induce smooth muscle differentiation 5 ng/mL tumor growth factor-β1 (TGF β1; R&D) was added to the basic media that in some experiments lacked LIF (5); to promote endothelial cell differentiation the basic media were altered by removal of B27, EGF, and LIF and addition of 10 ng/mL vascular endothelial growth factor (VEGF; Pepro-Tech), 5 units/mL of heparin sodium (Baxter Healthcare), and 5% FCS (6).

For single-cell expansion experiments cells were diluted in proliferating media (see above) to yield one cell per well and plated on 96-well cell culture plates (Corning) that were pretreated with 0.1% gelatin. Three days postplating individual wells with EGFP (GFP emission, 470/20; GFP excitation, 510/20) fluorescent single cells were identified by using a Nikon Eclipse TE300 inverted microscope and maintained in proliferating media for 3 weeks.

Electrophysiology. After 2–3 days in proliferating media, ≈20% of the cells began to spontaneously contract and relax. Glass slides or 35 mm Nunc culture dishes containing sEGFP cells were then placed in a specially designed, sealed chamber and mounted on a Nikon Eclipse TE300 microscope for patch clamping. Current recordings were made by using the Amphotericin B (300 μg/mL) perforated patch-clamp method (7). Patch pipettes were pulled from filamented borosilicate capillary glass (TW 150 F4, World Precision Instruments) to a tip diameter of 1–1.5 μm, and a resistance of 3–5 MΩ, using a P-87 Flaming/Brown micropipette puller (Sutter Instrument Co.). Patch pipettes were tip-filled by briefly dipping them into pipette solution (in mM: 133 KCl, 1 $MgCl_2$, 3 EGTA, 1 $CaCl_2$, 10 Hepes), and then back-filled with the Amphotericin B-containing pipette solution. After gigaseal formation, experiments were initiated once access resistance fell to 10–20 MΩ.

Voltage and current clamp protocols were applied with an Axopatch 200B patch-clamp amplifier (Axon Instruments) interfaced to a computer by a Digidata 1200 series AD/DA converter (Axon Instruments). During experiments, cells were continually superfused with Hanks' solution (in mM: 140 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, 10 glucose) by using a solution heater and controller (SHM-6, TC344 Warner Instruments). The solution-switching dead-space time was ≈5 s. All experiments were performed at 37 °C. Patch-clamp data were analyzed off-line by using Clampex 8.2 (Axon Instruments), and Prism 4 (Graphpad Software).

Cryoablation. Adult, 8- to 12-week-old, male c-kit^{BAC}-EGFP mice underwent cryoablation surgery as described in ref. 8. In brief, mice were anesthetized with 1–2% isoflurane, a thoracotomy was performed to expose the heart, a liquid-nitrogen-cooled 3-mm copper probe was placed against the heart for 10–20 sec 2–3 times and the chest wall, muscle layer, and skin were closed with 6-0 suture. Mice were allowed to recover on a warm surface, given ketoprofen (5 mg/kg) and ceftazidime (25 mg/kg) for 2 days, and euthanized at specific time points.

Immunocytochemistry. Cells cultured on slides or in 96-well plates, along with positive-control tissue slides, were fixed in 4% PFA for 20 min at 25 °C, rinsed 3 times with 0.01 M PBS, and stored at 4 °C until immunocytochemistry was performed. Cells and

control tissues were permeabilized for 5 min (2 min for CD31 and PCNA) with 0.01 M PBS, 0.05% Triton X (0.2% for PCNA nuclear label), and blocked for 1 h with normal mouse IgG (M.O.M. labeling kit, Vector Laboratories), followed by 10% normal donkey serum for 15 min at room temperature (RT). Primary antibodies were combined in TBS/M.O.M. buffer; antibodies and dilutions were mouse anti-α smooth muscle actin (M0851, DAKO, 1:20), mouse anti-PCNA (MO879, DAKO, 1:5), anti-nestin (MAB353, Chemicon, 1:5), anti-troponin T (Ab-1 13-11, Neomarkers, 1:200), Isl1 (Developmental Studies Hybridoma Bank, University of Iowa, 1:50), or rat anti-CD31 PECAM1 (no. 550274, BD PharMingen, 1:5) combined with rabbit anti-GFP (Chemicon, 0.1 mg/mL diluted 1:5). The combined antibodies were applied for 25 min at 37 °C and 40 min at RT. (CD31 colabels were incubated 30 min at 37 °C with CD31 antibody before adding combined antibodies.) To detect GFP, all samples were washed and incubated with FITC donkey anti-rabbit IgG H&L (Jackson ImmunoResearch) diluted 1:80 in TBS at RT for 20 min protected from light. They were then washed and incubated with Texas Red donkey anti-mouse IgG H&L (Jackson), for detection of SMA, Nestin, PCNA, or troponin T, at 1:80 for 15 min in TBS/M.O.M buffer, whereas CD31 colabels were incubated with biotinylated donkey anti-rat 1:50 in TBS (Jackson) followed by Streptavidin Texas Red (Vector) 1:200, for 20 min at RT. Stained cells and sections were mounted with Vectashield DAPI (Vector).

For co-labeling mouse anti-smooth muscle actin or troponin T with rat anti-CD 31, the mouse antibodies were detected with AMCA donkey anti-mouse IgG H&L (Jackson) diluted 1:40 in M.O.M. buffer for 15 min at RT protected from light. CD31 was detected with biotin/Streptavidin Texas Red as in the previous protocol, and DAPI counterstaining was omitted.

Colabeling with rabbit anti-GFP (1:5) and goat anti-CD117 (R&D Systems, 1:30) followed a similar protocol except that cells were incubated for 10 min with TBS 0.2% Triton to permeabilize, M.O.M. blocking was omitted, and cells were blocked with 10% normal donkey serum with 10% nonfat dry milk. Antibodies were diluted in TBS with 0.05% Triton and incubated 1.5 h at 37 °C. CD31 was detected with biotinylated donkey goat diluted 1:100 (Jackson) and Streptavidin Texas Red 1:200.

For colabeling with mouse monoclonal anti-α-SMA (M0851, DAKO) and mouse monoclonal anti-troponin T (Ab-1 13-11, Neomarkers). Cells were washed in 0.01 M PBS, and permeabilized for 5 min with 0.01 M PBS 0.05% Triton X-100. On day 1, cells were blocked with normal mouse IgG MOM labeling kit for 1 h, as per instructions, followed by 10% donkey serum/2× casein for 15 min at RT, and MOM buffer for 5 min. Mouse anti-troponin T primary antibody was diluted 1:150 in PBS/MOM buffer and applied for 25 min at 37 °C and 40 min at RT. Normal mouse IgG at an equivalent micrograms per milliliter was substituted for primary antibody to serve as a negative control. Texas Red conjugated donkey anti-mouse IgG H&L min X secondary antibody (Jackson ImmunoResearch) was diluted 1:80 in MOM buffer and incubated on the slides for 20 min at RT, protected from light. The fluorescent slides were washed and stored overnight in distilled water. On day 2 the cells were blocked 15 min with 10% goat serum, again blocked with normal mouse IgG (MOM. kit), rinsed and incubated with goat mouse fragments (Jackson ImmunoResearch) diluted 1:50 for 30 min at 37 °C, and washed. Mouse anti-α-SMA antibody was applied at 1:20 in MOM buffer as above. After several rinses, slides were incubated with AMCA blue conjugated donkey anti-mouse IgG H&L min X (Jackson ImmunoResearch) diluted 1:40, for 20 min in the dark. Cells were washed several times with PBS and mounted with Vectashield (Vector).

Normal mouse IgG (Santa Cruz Biotechnology), rat IgG, or goat IgG (Vector) combined with normal rabbit IgG (Vector), at final dilutions (μg/mL) equivalent to corresponding primary antibodies served as negative controls. Mouse tissue including embryo and adult stomach and esophagus were fixed and stained along with cultured cells to validate primary antibodies in appropriate cell types.

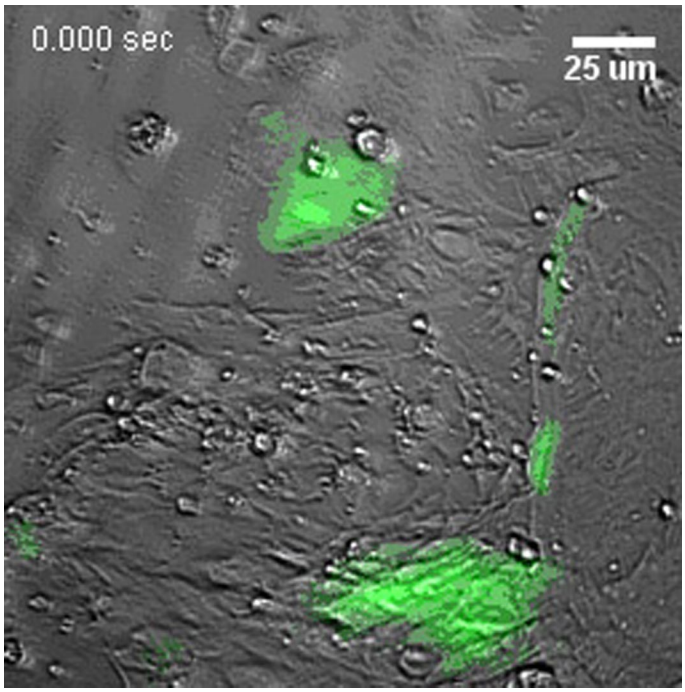
Images were obtained by confocal microscopy (Zeiss 510 Meta Scanning Confocal Microscope), a Kodak DCS 460C camera coupled to an Olympus Provis microscope, or a Leica DMI 6000B microscope coupled to a Retiga 2000R camera.

Whole embryos [14.5 and 18.5 days post coitus (dpc)] and excised PN 2 or 6- to 16-week-old adult hearts were fixed with 4% PFA or Stefanini solution, preserved with 20% sucrose, frozen, and cut into 8- to 10-μm sections. Cryosections were prepared and examined for EGFP expression or immunostained by using primary antibodies directed against α–actinin (Sigma-Aldrich), αSMA (Sigma-Aldrich), CD45 (Lab Vision), GFP, flk-1, or PECAM1 (all from BD PharMingen). All primary antibodies were visualized by appropriate secondary Cy3- or Cy5-conjugated donkey antibodies (Jackson ImmunoResearch). Nuclei were stained with Hoechst 33258 (Sigma). Heart sections were imaged by using a fluorescence stereomicroscope (Leica MZ 16F, LeicaMicrosystems; ProgRes C10+ camera, Jenoptik) or a Zeiss Axiovert 200 microscope (equipped with an ApoTome; AxioCam MRm).

Cell Morphometry. To calculate the total number of cells per neonatal heart, 7–13 sections of each heart ($n = 6$) were imaged with a 20× objective and the total number of Hoechst stained nuclei determined automatically by using AxioVision AutMess Software (Zeiss). The number of EGFP-positive cells was determined by manually counting the nuclei of green cells. Cell numbers were extrapolated to the total amount of heart sections, correcting for overlap based on the average nuclear size.

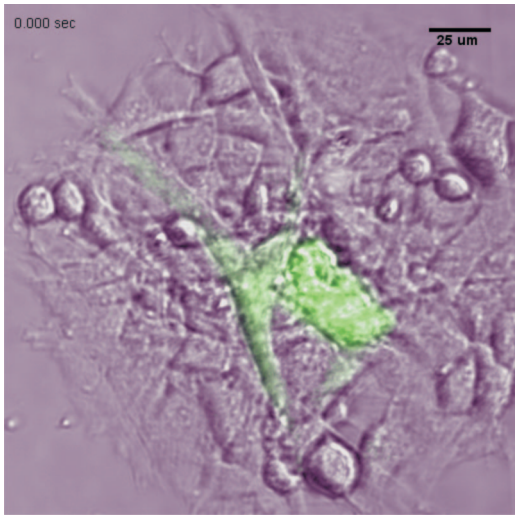
Data Analysis. Data were analyzed with SigmaPlot/SigmaStat/Graphpad Prism 4 software by using one-way analysis of variance with Tukey's post hoc comparisons or a t test. Summary values are mean ± SEM. Differences were accepted as statistically significant with $P < 0.05$.

1. Tumbar T, *et al.* (2004) Defining the epithelial stem cell niche in skin. *Science* 303:359–363.
2. Beltrami AP, *et al.* (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 114:763–776.
3. Tropepe V, *et al.* (1999) Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev Biol* 208:166–188.
4. Laugwitz KL, *et al.* (2005) Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* 433:647–653.
5. Sinha S, Hoofnagle MH, Kingston PA, McCanna ME, Owens GK (2004) Transforming growth factor-beta1 signaling contributes to development of smooth muscle cells from embryonic stem cells. *Am J Physiol* 287:C1560–C1568.
6. Yamamoto H, Kato H, Uruma M, Nitta M, Takamoto S (2007) Identification of two distinct populations of endothelial progenitor cells differing in size and antigen expression from human umbilical cord blood. *Ann Hematol* 87(2):87–95.
7. Rae J, Cooper K, Gates P, Watsky M (1991) Low access resistance perforated patch recordings using amphotericin B. *J Neurosci Methods* 37:15–26.
8. Roell W, *et al.* (2007) Engraftment of connexin 43-expressing cells prevents post-infarct arrhythmia. *Nature* 450:819–824.



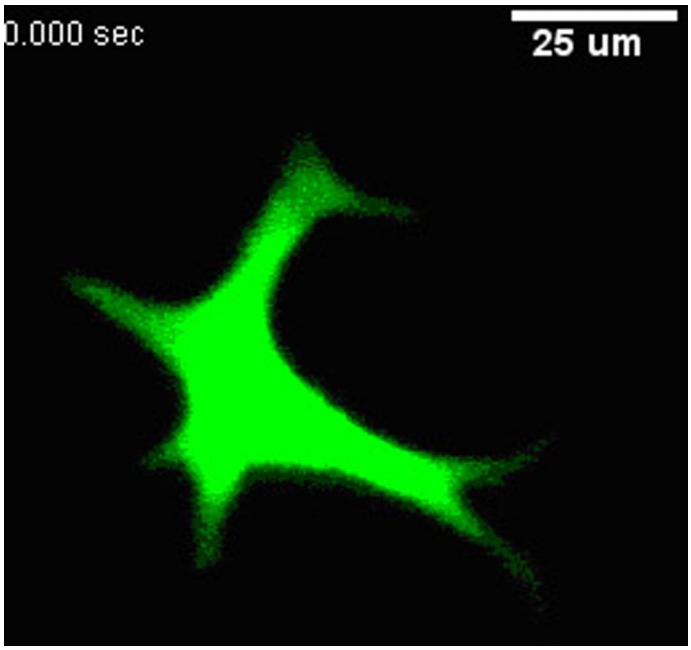
Movie S1. Complex rhythmic contractions in mixed EGFP^{+} and EGFP^{-} population in vitro. tEGFP cells were cultured in vitro and contractions observed in both c-kit-EGFP^{+} and c-kit-EGFP^{-} cell populations. Images acquired and played at 67 Hz.

Movie S1 (MPG)



Movie S2. c-kit-EGFP$^+$ clusters contract in vitro. sEGFP cells were cultured and allowed to expand. Note only the EGFP$^+$ cells are contracting. Image sequence acquired and played at 67 Hz.

Movie S2 (MPG)



Movie S3. Single contracting c-kit-EGFP^{+} cell. Isolated c-kit-EGFP^{+} cell rhythmically contracts at a rate of ≈2 Hz. Images acquired and played at 67 Hz.

Movie S3 (MPG)

Table S1. Action potential characteristics of cultured neonatal cardiac c-kit$^+$-EGFP cells

Parameters	Group 1 (5)	Group 2 (7)	Group 3 (5)
RMP, mV	−46 ± 2.8	−60 ± 0.6	−75 ± 1
dv/dt, V/sec	4.4 ± 1	34.5 ± 6.3	69.5 ± 9.6
APD^{90}, ms	123.6 ± 10.5	77.8 ± 7.7	66.5 ± 13.9
Frequency, min^{-1}	240 ± 34	287 ± 28	205 ± 30

Values are means ± SE for the numberof experiments (in parentheses). RMP, resting membrane potential; APD, action potential duration.