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Irx4 marks a multipotent, ventricular-specific progenitor cell

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Abstract

Rationale—While much progress has been made in the resolution of the cellular hierarchy underlying cardiogenesis, our understanding of chamber-specific myocardium differentiation remains incomplete.

Objective—To better understand ventricular myocardium differentiation, we targeted the ventricle-specific gene, *Irx4*, in mouse embryonic stem cells to generate a reporter cell line.

Methods and Results—Using an antibiotic-selection approach, we purified Irx4$^+$ cells in vitro from differentiating embryoid bodies. The isolated Irx4$^+$ cells proved to be highly proliferative and presented Cxcr4, Pdgfr-alpha, Flk1 and Flt1 on the cell surface. Single Irx4$^+$ ventricular progenitor cells (VPC) exhibited cardiovascular potency, generating endothelial cells, smooth muscle cells

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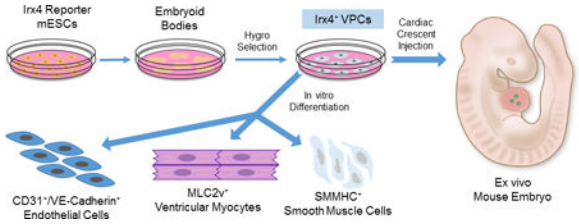
and ventricular myocytes in vitro. The ventricular specificity of the Irx4$^+$ population was further demonstrated in vivo as VPCs injected into the cardiac crescent subsequently produced Mlc2v$^+$ myocytes that exclusively contributed to the nascent ventricle at E9.5. These findings support the existence of a newly identified ventricular myocardial progenitor.

Conclusions—This is the first report of a multipotent cardiac progenitor that contributes progeny specific to the ventricular myocardium.

Graphical Abstract



The *Irx4* locus was targeted in mouse embryonic stem cells (mESCs) allowing antibiotic selection with hygromycin of Irx4$^+$ ventricular progenitor cells (VPCs) during embryoid body differentiation. The Irx4$^+$ VPCs were proliferative and multipotent differentiating *in vivo* to ventricular cardiomyocytes, endothelial cells and smooth muscle cells as well as differentiating when injected into the cardiac crescent of the developing mouse embryo into ventricular cardiomyocytes.

Keywords

Cardiac; Developmental biology; Differentiation; Embryonic stem cells; Stem cell transplantation; Progenitor cells

Introduction

Mammalian cardiogenesis is a complex process encompassing numerous morphogenic steps and cell fate decisions, driven by intricate signaling pathways, all resulting in a functional four-chambered heart[1–4]. The onset of cardiac mesoderm differentiation occurs at embryonic day 6 to 6.5 (E6–6.5), identified by the presence of Mesp1$^+$ cardiac precursors in the mesoderm adjacent to the primitive streak[5–7]. Several groups have demonstrated that the cardiac crescent is composed of cardiac progenitors originating from the first and second heart fields[8–12]. These progenitors are marked by transcription factors such as Nkx2.5, Islet1, and Brachyury, among others[13–15]. To purify these progenitor populations, and evaluate their contribution to cardiovascular differentiation, researchers have targeted these select genetic markers using transgenic methodologies[9]. These cardiac progenitors have proven to be proliferative and exhibit cardiogenic potency, providing evidence that heart development occurs in a hierarchical fashion resembling the process of hematopoiesis[16].

Domian and colleagues used a two-fluorophore approach to identify a bipotent cardiac progenitor that preferentially contributed to the right ventricle, in vivo[17]. This was the first report that heart chamber-specific progenitors exist in the cellular hierarchy underlying cardiovascular differentiation. But because this population exhibited limited proliferation,

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and the potency of a single progenitor cell was not determined, we pursued an alternative strategy to identify proliferative and multipotent progenitors capable of contributing to both ventricular chambers. We employed a recombineering approach to target *Irx4*, establishing a reporter mouse embryonic stem cell line[18,19]. *Irx4* is the earliest known marker of ventricular myocardium differentiation, with transcripts and protein detected in the cardiac crescent at E7.5–8 during mouse embryogenesis[20,21,22]. Irx4 is restricted to the developing ventricular myocardium throughout heart development. It is localized to the ventricular segment of the linear heart tube at E8–8.5 and persists throughout embryonic development and postnatally[20,21,22]. Homozygous knockout of *Irx4* in mice causes aberrant gene expression in ventricles and a maturity onset dilated cardiomyopathy[23]. Thus Irx4 is required for the establishment of some components of the ventricle-specific gene expression program, such as increasing eHand (Hand1) and suppressing ANF and alpha skeletal actin expression[23]. Taken together, the spatiotemporal detection of Irx4 and its role in ventricle myocyte gene expression supports the hypothesis that this homeobox transcription factor is an ideal marker to identify a ventricular myocardium progenitor.

In this study, we report that $Irx4^{+}$ cells purified at day 6 of mouse embryonic stem cell (mESC) differentiation are proliferative, and multipotent, generating cardiomyocytes (CMs), endothelial cells (ECs), and smooth muscle cells (SMCs). The CM progeny exhibited a ventricular phenotype, as evidenced by Mlc2v detection and action potential characteristics. When injected into nascent cardiac mesoderm of mouse gastrulae, the progenitors contribute to the developing ventricular myocardium.

Materials and Methods

Generation of $Irx4^{tdTomato\text{-}hph\text{-}fLuc/wt}$ ES cell line

A recombineering approach was employed to insert tdTomato, hph, and luciferase reporter genes into the 3’ untranslated region of Irx4. A bacterial artificial chromosome (BAC) encompassing the Irx4 gene locus was purchased from the Sanger Institute. The Irx4 targeting construct was introduced into E14 mESCs (passage 29). The Nucleofector A-Max 2 (Lonza, Basel, Switzerland) protocol A24 was used for electroporation. Electroporated cells were cultured in medium supplemented with Geneticin (Gibco, Carlsbad, CA) at 400μg/ml, and Ganciclovir (Roche, Indianapolis, IN), at 1×, for 10 days. After the selection period, sixty clones were picked and fifty clones were successfully expanded on feeder layers comprised of SNL-H1 STO fibroblasts (a gift from Dr. Richard Behringer).

Mouse embryonic stem cells (mESCs) were maintained in cell growth media which was comprised of DMEM basal medium (Gibco, Carlsbad, CA) supplemented with 1% L-Glutamine (Gibco, Carlsbad, CA), 1% Beta-mercaptoethanol (Fisher Scientific, Waltham, MA) diluted in 1× PBS (Fisher Scientific), 1% Penicillin/Streptomycin (Gibco, Carlsbad, CA), 1% Non-essential amino acids (Gibco, Carlsbad, CA), 1% Sodium Pyruvate (Gibco, Carlsbad, CA), 15% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA).), 2μg/ml of leukemia inhibitory factor (EMD Millipore, Billerica, MA).

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Purification of Irx4-tdTomato^{+} progenitors

Irx4$^{tdTomato\text{-}hph\text{-}fLuc/wt}$ mESCs were propagated on STO cell feeder layers, and differentiated in hanging drops for 4.5 days. Mouse embryonic stem cells were differentiated in medium containing the following: DMEM basal media (Gibco) supplemented with 1% L-Glutamine (Gibco), 1% Beta-mercaptoethanol (Fisher Scientific) diluted in 1× PBS, 1% Penicillin/Streptomycin (Gibco), 1% Non-essential amino acids (Gibco), 10% fetal bovine serum (Gibco). Day 4.5 embryoid bodies (EBs) were harvested and suspended in cell growth medium supplemented with Hygromycin B (Invitrogen, Carlsbad, CA) at a concentration of 75μg/ml. EBs remained suspended in the hygromycin B solution for ~36 hours before cell clusters were trypsinized with 0.05% Trypsin-EDTA (Invitrogen, Carlsbad, CA) to obtain a single cell suspension. Day 6 cells were plated onto feeder layers comprised of SNL-H1 STO cells in cell growth media supplemented with 75μg/ml of hygromycin B, to continue selection, and maintain purity of the Irx4^{+} cell population. The expanded VPCs were passaged on SNL-HL STO cells or maintained in feeder-free conditions using mESC media (above) supplemented with 1μl/ml leukemia inhibitory factor (EMD Millipore, Billerica, MA) and 2.5μM BIO (Tocris).

Immunofluorescence analysis of ES cell-derived samples

Single cell samples were obtained by incubating progenitor cell aggregates, or EBs, with 0.05% trypsin (Gibco, Carlsbad, CA) at 37° C. Incubation time was dependent upon the size of cell aggregates. Single cell samples were plated onto glass coverslips, coated with fibronectin (5μg/ml in 1× PBS). When analyzing cells using flow cytometry, the cell samples were maintained in suspension. Cells were incubated for 2 hours at room temperature with blocking solution composed of the following: 2% animal serum (EMD Millipore, Billerica, MA), 0.1% triton X-100 (Fisher Scientific, Waltham, MA), 5% BSA (Fisher Scientific, Waltham, MA) in 1× PBS. Primary antibodies were incubated with cell samples overnight, at 4C. Following three rinses with 1× PBS, the secondary antibodies were incubated with the fixed cells for 2 hours at room temperature. Samples were rinsed three times with 1× PBS and counterstained with Hoechst dye to label nuclei. Primary and secondary antibodies are listed in the supplemental materials and methods. To verify the specificity of each primary antibody for differentiated cell lineages, the antibody was tested on undifferentiated mESCs. Titration of each antibody was performed to optimize the dilution for specific labeling. Finally, secondary antibody alone controls were performed for each flow cytometry experiment.

RNA isolation, cDNA generation, and PCR

RNA was purified using the RNeasy Plus mini-kit (Qiagen, Valencia, CA). One microgram of purified RNA was used for reverse transcription with Superscript III (Invitrogen, Carlsbad, CA) reverse transcriptase. Thirty cycles of PCR were used, 58°C annealing temperature.

For methylcellulose clonal assays, RNA was purified from single colonies using the Ambion RNAqueous RNA extraction kit/protocol. The iScript Reverse Transcription supermix (BioRad) was employed for cDNA synthesis. GoTaq polymerase, and buffer, was used for

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RT-PCR (35 cycles at 55°C). All oligonucleotides used in this study are listed in the supplemental materials and methods.

Detecting bioluminescence during differentiation of Irx4$^{tdTomato\text{-}hph\text{-}fLuc/wt}$ mESCs

Irx4$^{tdTomato\text{-}hph\text{-}fLuc/wt}$ mESCs were differentiated in hanging drops, and embyroid bodies were harvested at 24 hour intervals. Cell samples were lysed with 100μl lysis buffer (Promega), and 20μl of lysate was used for detection of bioluminescence. 50μl of D-luciferin (Promega) was added to lysates to generate bioluminescent signal. Each time point on the graph represents an average of three bioluminescence values, which were obtained from independent samples.

Additional methods – see supplement for methods for Southern blot, doubling time, mouse embryo preparations, methylcellulose assay, and electrophysiology.

Results

Generation of Irx4-reporter mES cell line

The *Irx4* locus (chromosome 13:C1) was targeted in E14 mESCs using a knock-in approach[24] because the Irx4 promoter is unresolved. Three reporter genes (firefly luciferase, hph (hygromycin resistance) and tdTomato) were inserted into the 3' UTR, replacing the endogenous Irx4 stop codon (Figure 1A). Co-expression of *Irx4* and the three reporter genes as separate proteins is mediated using virally-derived 2A peptide sequences(Figure 1A)[25]. Fifty mESC clones were expanded and PCR analysis confirmed that 25 of 50 clones contained the reporter genes in the genome. PCR results for 2 of the 25 positive clones are displayed (Figure 1B). Clones 1 and 14 were selected for Southern blot analysis. Southern blot analysis confirmed that the reporter genes were inserted into the 3' UTR of the *Irx4* locus (Figure 1C). Clone 14 mESCs, and derivatives, were used for subsequent experiments in this study.

To confirm that the reporters were restricted to Irx4^{+} cells, we co-stained differentiated cell samples (day 13) with antibodies to Irx4 and tdTomato or luciferase. Results show that the reporter genes were restricted to Irx4^{+} cells (Figure 1D). To demonstrate the efficacy of the Hph gene, day 7 EBs were suspended in differentiation media, supplemented with hygromycin B and selected over a 7 day period. Following the selection period, we obtained highly enriched Irx4^{+}/Myl2^{+} ventricular myocyte cultures (Figure 1E).

Temporal expression pattern of Irx4 during mESC differentiation

We determined if *Irx4* was expressed prior to myocyte differentiation, as reported in transcript and protein detection assays using mouse embryos[20,22]. We wanted to know how the timing of *Irx4* expression related to other progenitor markers and to determine the earliest time point during mESC differentiation that Irx4^{+} cells could be identified and isolated. RT-PCR analysis was employed using unselected EBs to highlight the temporal expression pattern of *Irx4* during mESC differentiation (Figure 2A). Brachyury expression, which signifies the onset of mesoderm development, first detected at day 3 of differentiation. Mesp1, a marker of cardiac mesoderm specification, is transiently expressed from day 3.5–

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4.25. Irx4 expression ensues at day 4.5 of mESC differentiation, following the upregulation of Nkx2.5. This temporal expression pattern is consistent with in situ hybridization data which shows that Irx4 is downstream of Nkx2.5[20]. Following initial detection at day 4.5, *Irx4* expression persisted throughout the differentiation time course. Cardiomyocyte lineage commitment is not observed until day 7, indicated by the upregulation of cardiac troponin T (cTnT). Expression of the Mlc2v, a myofilment protein expressed primarily in the developing ventricular myocardium and to a lesser extent in the transient outflow tract and AV canal was observed starting on day 8. (Figure 2A). The gene expression data show that Irx4 is present in a population of cells prior to myocyte differentiation.

Flow cytometry data support the RT-PCR time course for Irx4 expression, with Irx4$^+$ cells first detected at day 4.5 of differentiation, indicated by tdTomato expression(Figure 2B). To quantify the extent of *Irx4* translation, we measured the luciferase activity of differentiating cells. Bioluminescence detection indicated that Irx4 was upregulated between day 4.25 and 4.5 (Figure 2C). The level of *Irx4* expression increased until day 10, when the bioluminescent signal plateaus (Figure 2C).

Days 2–6 of mESC differentiation have been established as the period appropriate for identifying, and purifying, cardiac progenitors[13–15,26]. The temporal expression of *Irx4* during mESC differentiation showed that an Irx4$^+$ progenitor population was present during this stage of mESC differentiation.

Purification and expansion of Irx4$^+$ ventricular progenitor cells

Based on these results, we hypothesized that Irx4$^+$ ventricular progenitor cells (VPCs) are present between days 4.5–6 of differentiation. Day 4.5 EBs were harvested from hanging drop cultures and suspended in growth medium containing Hygromycin-B (75μg/ml) (Figure 3A). After 1.5 days of incubation in selection medium, 98% of day 6 cells were positive for Irx4(Figure 3B). In addition, the Irx4 cells were proliferative given expression of Ki67. Interestingly, the VPCs encompass two sub-populations, marked by the first- and second heart field markers, Tbx5 and Islet1, respectively[28, 29] (Figure 3C).

The day 6 purified VPCs were plated onto SNL-H1 STO cell feeder layers to facilitate cell expansion, while continuing antibiotic selection[27]. SNL-H1 STO fibroblasts have been genetically modified to over-express LIF and the Hph gene, which confers resistance to Hygromycin-B. The VPCs were maintained for 10 passages and the cells retained a genetic profile indicative of a cardiac mesoderm progenitor (Figure 3D). RT-PCR data showed that the cells expressed markers of the first (Tbx5) and second (Islet1) heart fields[28,29]. The stem/progenitor cell markers cKit and Sca1 were expressed, while differentiation markers (smMHC, cTnT, and CD31) were not detected (Figure 3D)[30,31]. It is also important to note that the purified population does not express markers of endodermal (FoxA2) or ectodermal (Sox2) germ layers (Figure 3D). The expanded VPCs maintained a progenitor cell genetic profile, without evidence of differentiation on SNL-H1 for more than 22 passages. We determined the population doubling time of VPCs at passages 5,10 and 15. Both passage 5 and 10 VPCs had a similar population doubling time of about 25hrs, but the doubling time increased slightly at passage 15 to 29 hrs. (Figure 3E). These results suggest that VPCs can be stably expanded without undergoing senesence for multiple passages.

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Elucidation of VPCs surface markers

Various cardiac progenitor populations have previously been demonstrated to express select cell surface markers including Flk1, Cxcr4, Pdgfr-alpha, Flt1, and Flt4[15,32–35]. Therefore, we characterized VPCs after 3–5 passages in culture for these cell surface markers using flow cytometry. We found that 82% VPCs expressed the combination of Flk1 and Pdgfr-alpha, which are mesoderm progenitor markers[26](Figure 4A). Nelson and colleagues used a combination of Cxcr4 and Flk1 to purify a cardiopoietic population in differentiating pluripotent cells[36]. The combination of Flk1 and Cxcr4 was detected on 81% of the VPCs (Figure 4B). To enrich for an Islet1^{+}/Nkx2.5^{+} cardiac progenitor in differentiating induced pluripotent stem cells, Nsair et al (2012) used the Flt1/Flt4 antibody combination[32]. While the majority (95%) of the purified VPCs expressed Flt1 on the cell surface, less than 1% expressed Flt4 (Figure 4C). The combination of Pdgfr-alpha and Cxcr4 was found on 80% of the VPCs (Figure 4D). Bondue and colleagues found that Pdgfr-alpha and Cxcr4 marks a subgroup of Mesp1^{+} progenitors between days 3 and 4 of mESC differentiation[26]. These results show that VPCs consistently express cell surface markers associated with cardiac progenitors.

Spontaneous differentiation of VPCs and evaluation of their progeny

To evaluate the capacity of the VPCs to spontaneously differentiate into cardiovascular lineages, expanded VPCs were differentiated in aggregates using the hanging drop method[37]. Following differentiation for 7 days, cell aggregates were dissociated and single-cell suspensions were assayed using immunofluorescence. Because Irx4^{+} cells are present exclusively in the ventricular myocardium in vivo, we hypothesized that myocyte progeny generated by the Irx4^{+} progenitors would be ventricle-specific[22]. Using flow cytometry, we found that 64.4% of the differentiated VPCs were cTnT^{+}/Mlc2v^{+} (Figure 5A). Endothlelial cells (VE-Cadherin^{+}) represent the smallest percentage of the progenitor progeny when differentiated in aggregates, accounting for 12.5% of the differentiated cell sample while SMCs, marked by the SmMHC antibody made up 23% of the progeny (Figure 5A). The 99.9% total indicated that we detected all of the Irx4^{+} progeny. Immunofluorescence examining the co-labeling of Irx4 with markers of differentiated progeny revealed that Mlc2v^{+} cardiomyocytes co-labeled with Irx4, but the VE-Cadherin^{+} endothelial cells and SmMHC^{+} smooth muscle cells did not demonstrate expression of Irx4 (Fig 5B,C). To confirm the VE-cadherin immunolabeling identified endothelial cells, we also immunolabeled samples with CD31, another marker of endothelial cells (Supplemental Figure 1), and detected CD-31 positive cells which likewise did not express Irx4. Overall, these results showed that VPC-derived ventricular myocytes continued to express Irx4 whereas endothelial and smooth muscle cell progeny lost expression of Irx4.

We performed single cell patch clamp analysis to evaluate the characteristics of Irx4^{+} progenitor-derived cardiomyocyte action potentials because prior studies have suggested that cardiomyocytes differentiated from ESCs can be discriminated into different myocyte types (atrial-like, ventricular-like, nodal-like) based on their action potential(AP) morphology[38,39]. We compared the AP profile of progenitor-derived cardiomyocytes to neonatal cardiomyocytes. For this we isolated neonatal ventricular and atrial cells from day 0 mouse pups. Neonatal myocyte APs were measured 24 hrs after isolation. Representative APs are

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as shown in Figure 5D and data are summarized in Table S1. 12/12 progenitor-derived CM showed ventricular-like APs most comparable to neonatal ventricular myocyte APs, characterized by a rapid upstroke, and prolonged plateau phase as reflected by the prolonged action potential durations at 50% of repolarization (APD50) and 90% of repolarization (APD90) (Figure 5E).

To examine the chamber specificity of the differentiating cardiomyocytes from VPCs and mESCs, we performed Q-PCR gene expression analysis on cell progeny derived from feeder-free VPCs and mESCs that were spontaneously differentiated in hanging drops for two days and attaching the resulting aggregates to gelatin-coated 24 well plates for two weeks. We compared the expression of the ventricular expressed Mlc2v and the reportedly more atrial specific CoupTFII and Hey1. Because the VPCs more efficiently generated cardiomyocytes, we normalized the gene expression to cTnT (which is expressed ubiquitously in all CMs). The expression of Mlc2v was significantly greater in VPC derived cardiomyocytes than mESC-derived cardiomyocytes consistent with a preference for generation of ventricular cardiomyocytes (Fig 5F). In contrast there was a trend toward lower expression of the atrial markers CoupTFII and Hey1.

VPCs exhibit clonal expansion and cardiovascular potency

To test the clonality and cardiovascular potency of expanded VPCs, we suspended single cells in methylcellulose containing medium supplemented with cardiogenic growth factors (DKK1, BMP4, VEGF, bFGF) and allowed them to differentiate[15]. Single Irx4$^+$ cells formed colonies of differentiating cells (Figure 6A). We picked 24 independent cell aggregates after 10 days of differentiation for further analysis.

RT-PCR was performed with RNA purified from the clones using primers to lineage-specific genes (Figure 6C). Results showed that 45% of the clones expressed the first heart field marker, Tbx5[29], and 36% expressed the second heart field marker, Islet1[28]. Nkx2.5, a regulator of Irx4, was expressed in 50% of the colonies. The differentiated progenitors also expressed genes specific for CMs (83% of the colonies expressed cTnT; 79% expressed *Myl7* (MLC2A). The progenitors exhibited vascular potency, as indicated by the expression of smooth muscle and endothelial markers. The majority of the clones expressed Calponin (87%) and/or CD31 (95%), which mark SMCs and ECs, respectively.

Interestingly, following the 10 day differentiation in methylcellulose, only 16% of the clones expressed *Myl2* (Mlc2v), a myofilament protein expressed in ventricular myocytes[40]. This was unexpected based upon the ventricle-specific localization of Irx4$^+$ cells during murine cardiogenesis[22]. Hence, we picked a subset of the day 10 differentiated colonies, plated them individually in a 96 well plate and allowed them to differentiate in liquid medium for an additonal 14 days. Following this extended differentiation period (24 days total), all clones expressed *Myl2* (Figure 6C). The delay in *Myl2* expression is most likely due to the conditions of differentiation, as cell samples used for the initial RT-PCR analysis (Figure 6B) were maintained in suspension media (methylcellulose) only. Of note, *Myl7* was expressed in a significant number of clones and this myosin light chain marker has been shown to be expressed in immature ventricular myocytes before being more restricted to atrial CMs as the cells mature[41]. Further, we dissociated 6 individual clones subjected to the

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extended differentiation period and immunolabled for cardiovascular lineage-specific markers. Co-immunofluorescence data showed that all 6 clones differentiated into Mlc-2v+ ventricular CMs, $SmMHC^+$ SMCs, along with clusters of $CD31^+$ ECs (Figure 6D). These results showed that majority of single $Irx4^+$ cells exhibited cardiovascular potency, generating CMs, SMCs, and ECs.

VPCs generate cardiovascular cell types that contribute to the developing ventricle during murine embryogenesis

After demonstrating the cardiovascular potency of the VPCs in vitro, we assessed the potency of the expanded, day 6 VPCs in the developing mouse embryo. VPCs were maintained on SNL-H1 STO cells for one passage, with continued selective pressure. A sample of the expanded VPCs was used for Irx4-antibody staining and assessed by flow cytometry which confirmed that >99% of the cells were $Irx4^+$ (data not shown). To trace the progenitor progeny, we introduced a GFP reporter into the genome of the VPCs using a pSam2-GFP lentiviral construct. tdTomato and luciferase could not be used for lineage tracing as these two reporters are expressed only in $Irx4^+$ cells, and Irx4 is not expressed in smooth muscle and endothelial cell progeny (Figure 5B,C). GFP^+ VPCs were isolated via fluorescence-activated cell sorting (FACS) to ensure a pure labeled population for embryo injections.

VPCs were injected into the cardiac crescent of ~E8.25 mouse embryos (headfold-6 somite pairs stage; Figure 7A) (staging of mouse embryos according to Downs and Davies, 1993)[42]. A total of 25 embryonic hearts were injected in two experiments (Figure 7B). Following injection, the operated and unoperated control embryos were cultured in whole embryo culture, for 15–18 hours, according to Downs (2006)[43]. At the end of the culture period, the specimens were evaluated, and scored according to developmental criteria set forth by Brown and Fabro (1981) prior to sample fixation[44]. After culture, only 1/25 injected embryos was judged abnormal. Of the 24 normal embryos, 21 were immunostained for GFP and cardiovascular-specific proteins to determine the localization of exogenous cells post-injection, and their progeny. As confocal microscopy was used to optically section the embryos, immunofluorescence images represent a single optical section (Figure 7C–E). Because the embryos had developed for 15–18 hours, the cardiac progenitors present in the cardiac crescent had undergone a remarkable morphogenic shift to a contracting heart tube conformation (Figure 7C–E). Seventy-one percent of the immunostained embryos exhibited GFP (15/21).

GFP^+ cells were predominantly found in the ventricular myocardium. The majority of these were ventricular myocytes as the cells were also positive for the ventricular myocyte-specific marker, Mlc2v (Figure 7C). $GFP^+/Mlc2v^+$ cells were found clustered in the ventricular segment of the heart tube, with no spatial preference within the developing ventricle (Figure 7C). A small population of $GFP^+/Mlc2v^-$ cells was detected adjacent to the ventricular segment (Figure 7C, arrowhead).

To illustrate the commitment of GFP^+ cells to the developing ventricular myocardium, E9.5 embryos were co-stained with antibodies to GFP and cTnT (Figure 7D). cTnT stains all myocytes of the developing cardiac tissue, but GFP^+ cells were restricted to the ventricular

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segment of the looping heart tube (Figure 7D). We never observed GFP^+ cells at the atrial pole of the looping heart tube. Majority of the GFP^+ cells were also positive for cTnT (Figure 7D). A small population of $GFP^+/cTnT^-$ cells was observed in the ventricular segment (Figure 7D). This population likely represents endothelial derivatives provided the proximity of the cells to the lumen, but the presence of undifferentiated $Irx4^+$ progenitors could not be ruled out.

In addition to ventricular myocytes, the $Irx4^+$ progenitors gave rise to ECs, marked by CD31 (Figure 7E). $GFP^+/CD31^+$ cell clusters were primarily observed in the developing ventricular myocardium. This clustered spatial arrangement is possibly due to clonal derivation of the endothelial cell population. Of note, all $CD31^+$ cells derived from the $Irx4^+$ progenitors localized to the heart, further demonstrating the cardiac-specificity of the progenitors.

The whole embryo culture technique used here was specifically designed for early gastrulae that culture from ~E6.75 to E9.75 and cannot be extended beyond E9.75 (K. Downs, unpublished data). Hence, we were unable to assay the smooth muscle cell potency of injected VPCs as the onset of smooth muscle differentiation occurs after E12.5[45], as this is beyond our whole embryo culture duration (E7.75–E9.75). As a control experiment for this embryo potency assay, we injected a terminally differentiated cardiac lineage, eGFP labeled adult cardiac fibroblasts, into the cardiac crescent of ~E8.5 day mouse embryos. In contrast to the VPCs, the adult fibroblasts in the 4 different embryos tested did not localize to the heart or demonstrate differentiation to cardiomyocytes (Supplemental Figure 2).

Discussion

In this study we report the elucidation of a novel, ventricular myocardium-specific progenitor population. To identify this ventricular progenitor, we targeted the homeobox transcription factor gene, *Irx4*. $Irx4^+$ ventricular progenitor cells (VPCs) were isolated on day 6 of mESC differentiation using selection with the antibiotic, hygromycin B. The VPCs proved to be highly proliferative, with more than 95% of the purified population positive for the proliferation marker, Ki67. Furthermore, the VPCs presented several cell surface markers associated with cardiac progenitors (Flk1, Flt1, Pdgfr-alpha, and Cxcr4)[15,32,33,35]. The $Irx4^+$ progenitors exhibited multipotency, giving rise to ECs, SMCs and CMs. The cardiomyocyte phenotype is exclusively ventricular, based on chamber specific gene expression and single cell patch clamp analysis. Further, when injected into the developing mouse embryo, the VPCs maintained their cardiogenic potency and chamber-specificity, contributing cardiovascular lineages to the developing ventricular myocardium.

To resolve the contribution of cardiac progenitor cell (CPC) populations to the developing heart, several groups have employed lineage tracing methodologies[10,46,47]. Results of these studies have revealed that the cardiac mesoderm can be traced back as early as E6–6.5, where $Mesp1^+$ CPCs contribute to the early cardiac mesoderm, adjacent to the primitive streak[6]. This temporal expression is supported in vitro by the identification of $Mesp1^+$ cardiac presursors as early as day 2 in differentiating mESCs[26].

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Mesp1$^+$ CPCs migrate to the anterior region of the developing murine embryo and contribute to the cardiac crescent, which is comprised primarily of two populations of cardiac progenitors (first- and second heart fields), distinguished by their spatial & temporal emergence during embryogenesis[11,12,48,49]. Resolution of the gene expression profile of the crescent has revealed potential gene targets, some of which have since been used to facilitate purification of cardiac progenitors[13–15]. By generating transgenes using regulatory elements for Islet1, Flk1, and Nkx2.5, researchers have isolated cardiac progenitors with similar and differing characteristics. These progenitor populations, including Mesp1$^+$ cells, all exhibit the ability to proliferate and differentiate into at least two of the three primary cardiovascular lineages (CMs, SMCs, ECs). While each of these populations generates myocytes, none of these progenitors exhibit chamber-specific potency.

Like other prominent cardiac progenitor markers, Irx4 is also expressed in cells of the cardiac crescent[20,21,22]. But unlike other cardiac factors, Irx4 exhibits a ventricle-specific expression pattern following heart chamber formation[20]. Unlike the previously reported ventricular progenitor population[17], Irx4$^+$ VPCs are multipotent, not only giving rise to ventricular myocytes and SMCs, but also ECs. The previously identified Isl1$^+$ progenitor population contributes to the right ventricle, and exhibits limited proliferative capacity[17]. Irx4$^+$ VPCs presented in this study are highly proliferative, evidenced by culture for more than 22 passages.

It is important to note that Nkx2.5 is a regulator of Irx4 expression[20]. The relationship of the two transcription factors suggests that the Irx4$^+$ VPCs are a subpopulation of the Nkx2.5 progenitor pool. Nkx2.5$^+$/cKit$^+$ progenitors were bipotent, lacking the ability to generate ECs, but Irx4$^+$ VPCs are tripotent, producing the three primary cardiovascular lineages[13]. This difference in potency suggests that the two progenitor populations do not completely overlap.

As we unravel more of the cell hierarchy responsible for cardiovascular development, it is important to update the cardiovascular differentiation paradigm, including the novel VPCs. Following mesoderm differentiation, signified by Brachyury expression, Mesp1$^+$ cells are the earliest detected cardiac precursors in vivo and in vitro[6,26]. Nkx2.5 marks progenitors of both the first and second heart fields, but the transcription factor is not detected until cardiac crescent formation in vivo and day 4–4.25 of mESC differentiation[20,22]. Islet1$^+$/Nkx2.5$^+$/Flk1$^+$ and Nkx2.5$^+$/cKit$^+$ cardiac progenitors are detected within day 4–6 of mESC differentiation[13,14]. Provided that Islet1 marks progenitors of the second heart field and Nkx2.5 labels myocyte progenitors of both heart fields, the Islet1$^+$/Nkx2.5$^+$/Flk1$^+$ progenitors likely represent a combination of first- and second heart field progenitors. The limited potency of Nkx2.5$^+$/cKit$^+$ progenitors suggest that the population is downstream of the Islet1$^+$ precursors, or potentially a subpopulation. During this same differentiation period, Irx4$^+$ ventricular progenitors were detected. The ventricle-specific phenotype of the myocyte progeny distinguish the VPCs from previously identified multipotent populations.

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Summary

In summary, we have elucidated a novel ventricular myocardium-specific progenitor population, marked by the expression of the homeobox transcription factor, Irx4. The purified progenitors are proliferative and, when differentiated, the Irx4$^+$ population gives rise to ECs, SMCs, and ventricular CMs. To our knowledge this is the first report of a tripotent, ventricular-specific progenitor. Identification of the Irx4$^+$ VPCs is a significant step in the resolution of the ventricular myocardium differentiation process.

In addition to identifying an important component of the cardiovascular hierarchy, we believe the Irx4$^+$ VPCs will have significant clinical implications. Because ischemic damage primarily manifests in the ventricular myocardium, the VPCs may be uniquely equipped to engraft, and potentially regenerate the myocardial layer of the ventricles, post-infarction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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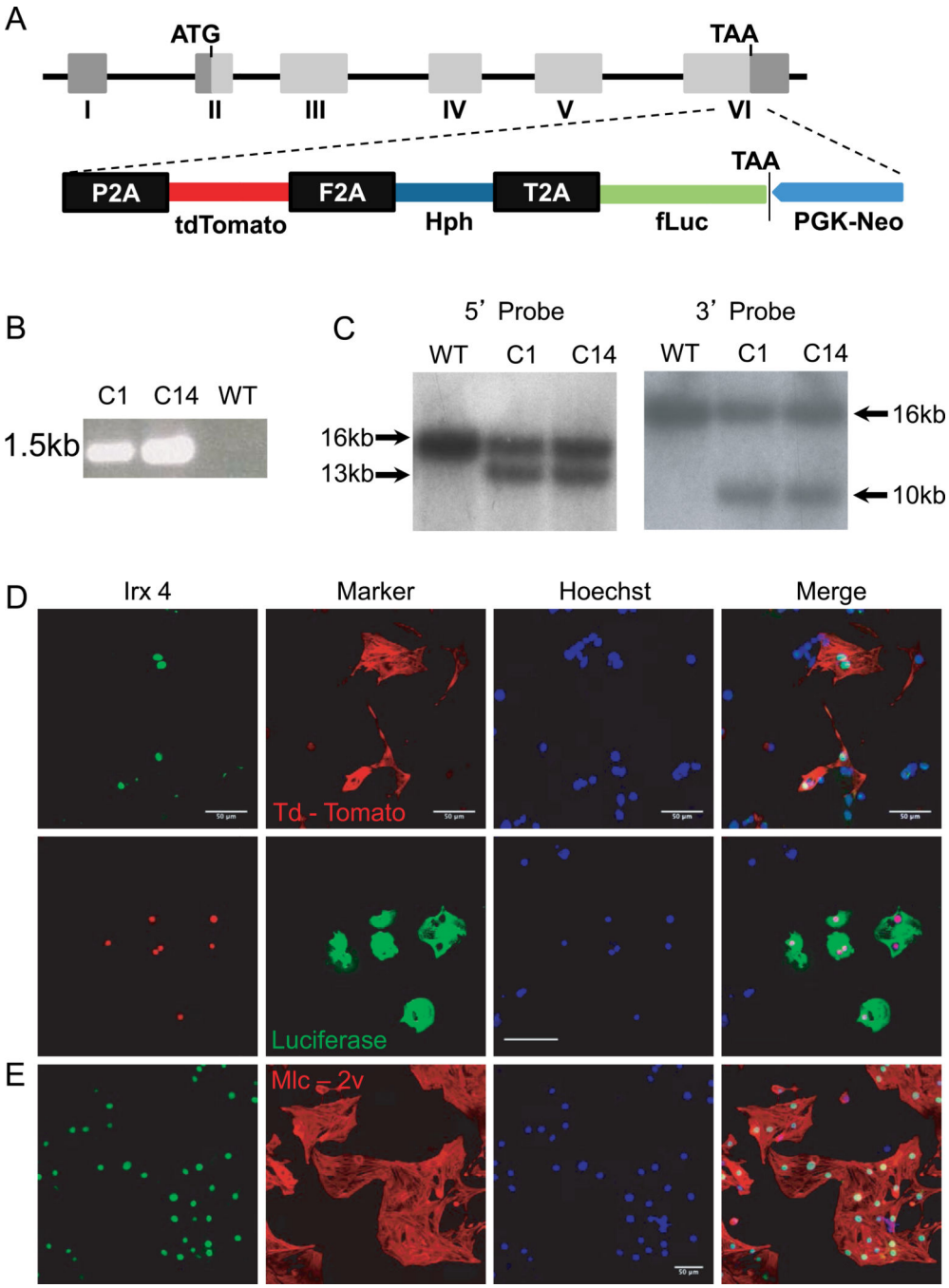

Figure 1. Generation of Irx4 reporter cell line & assessment of reporter function and fidelity

(A) Schematic illustrating the targeting of the *Irx4* gene and the reporters and selection cassette that were knocked in. Boxes in the Irx4 gene map represent exons. Dark gray regions of the Irx4 map are untranslated regions, and light gray regions are translated. (B) PCR screening using genomic DNA purified from 2 of 50 mESC clones. WT= water only (no DNA) control. (C) Southern blot analysis to verify insertion of reporter genes into the Irx4 locus. (D) Unselected, day 13 EB cells co-stained for Irx4 and Td-Tomato or luciferase. Hoechst dye was used to identify nuclei. Images were merged to identify the co-localization

of the labeled cells. (E) Hygromycin B-selected EB day 14 cells co-stained with the ventricle-specific marker, Mlc2v, and Irx4. Hoechst dye identifies nuclei. Scale bars= 50μm.

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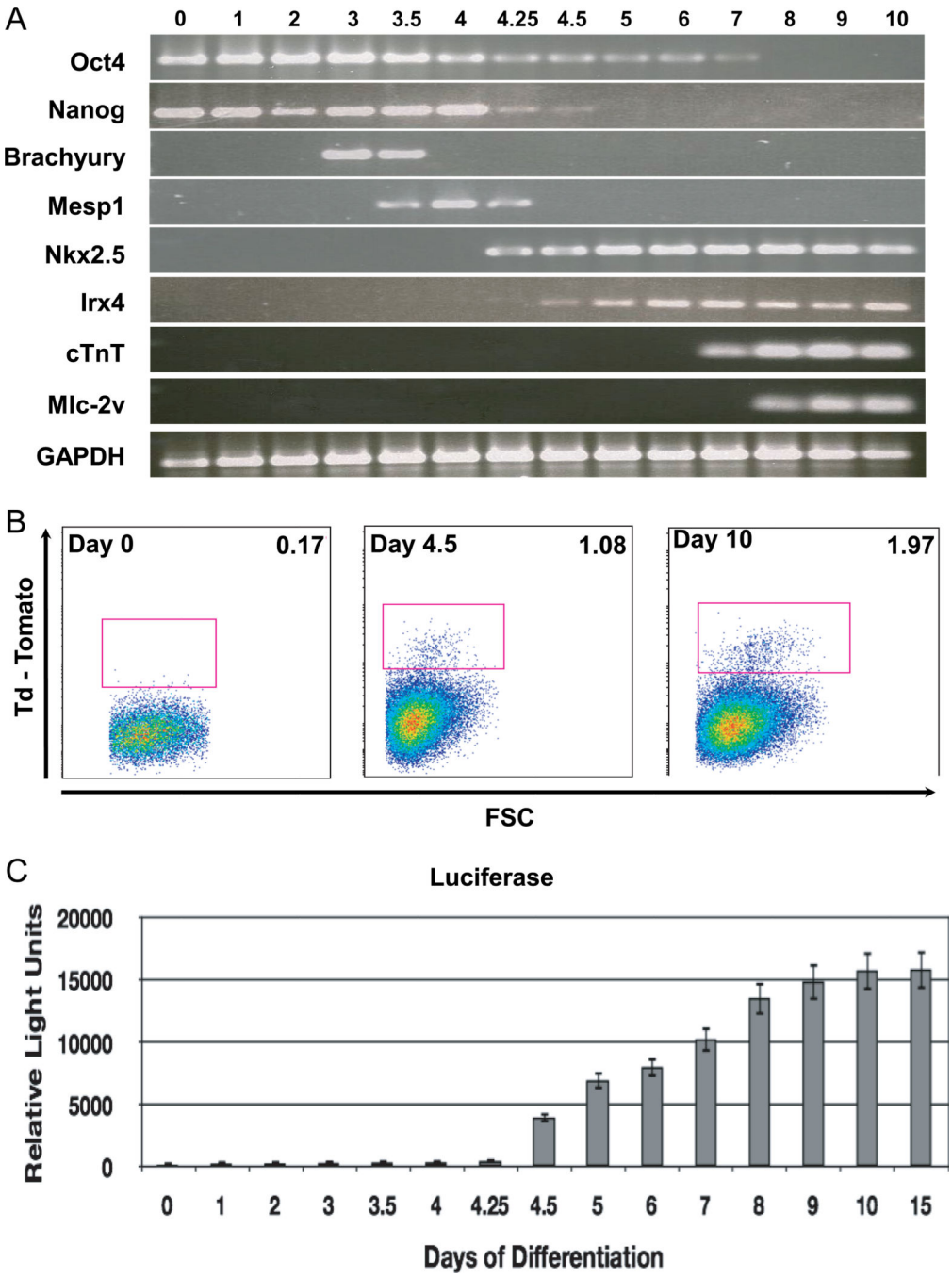

Figure 2. Temporal expression of Irx4 during mESC differentiation

(A) RT-PCR analysis of relevant gene expression using cDNA from cells at days 0–10 of mESC differentiation. (B) mESCs and EB differentiated cell samples were assessed for tdTomato expression using flow cytometry. FSC= forward scatter. Dot plots are representative of the flow cytometry data set (n=3). (C) A bioluminescence time course was established to determine the level of *Irx4* expression during mESC differentiation. Error bars represent standard error of the mean. Each bar represents an average (n=3 cell samples).

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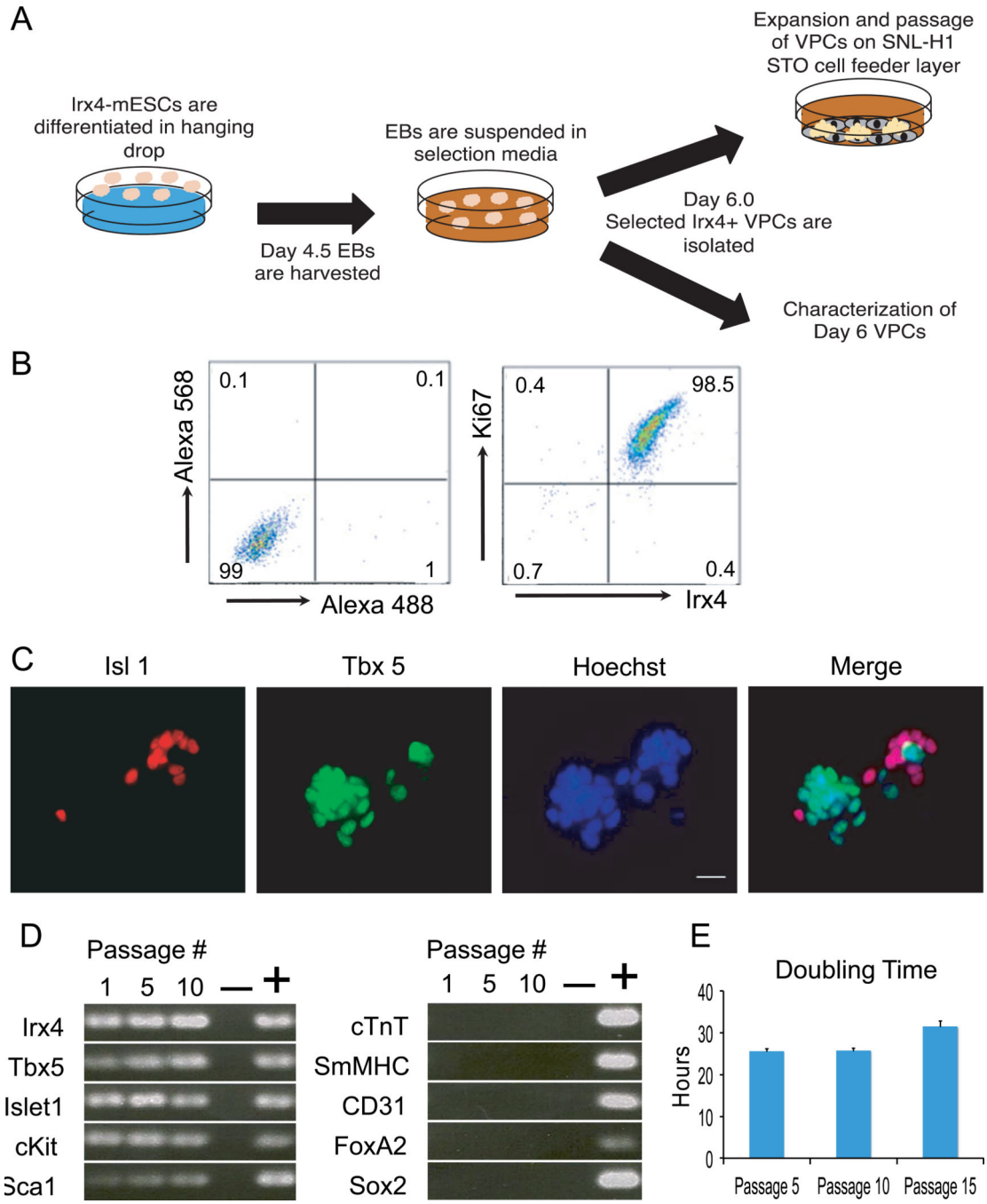

Figure 3. Purification of Irx4^{+} progenitors in vitro

(A) Schematic representation of the process of VPC purification from differentiating mESCs and their expansion using SNL-H1 STO feeder cells. (B) Flow cytometric analysis of day 6, hygromycin B-purified cells labeled with antibodies to Irx4 and Ki67. For comparison, the primary antibodies were omitted and served as a secondary antibody-only control. Dot plots are representative of the flow cytometry data set (n=3). (C) Purified day 6 Irx4^{+} cells were labeled with antibodies to Tbx5 and Islet1. Nuclei were labeled with Hoechst dye. The images were merged to illustrate co-localization of labels. Scale bar= 25μm. (D) RT-PCR

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analysis was used to assess gene expression of purified Irx4^{+} population over several passages. Negative= water, positive= cDNA from differentiated mESCs (unselected) or neuron preparation. E) Population doubling time for passage 5, 10 and 15 VPCs. Data represented as mean. Error bars indicate SEM (n=3).

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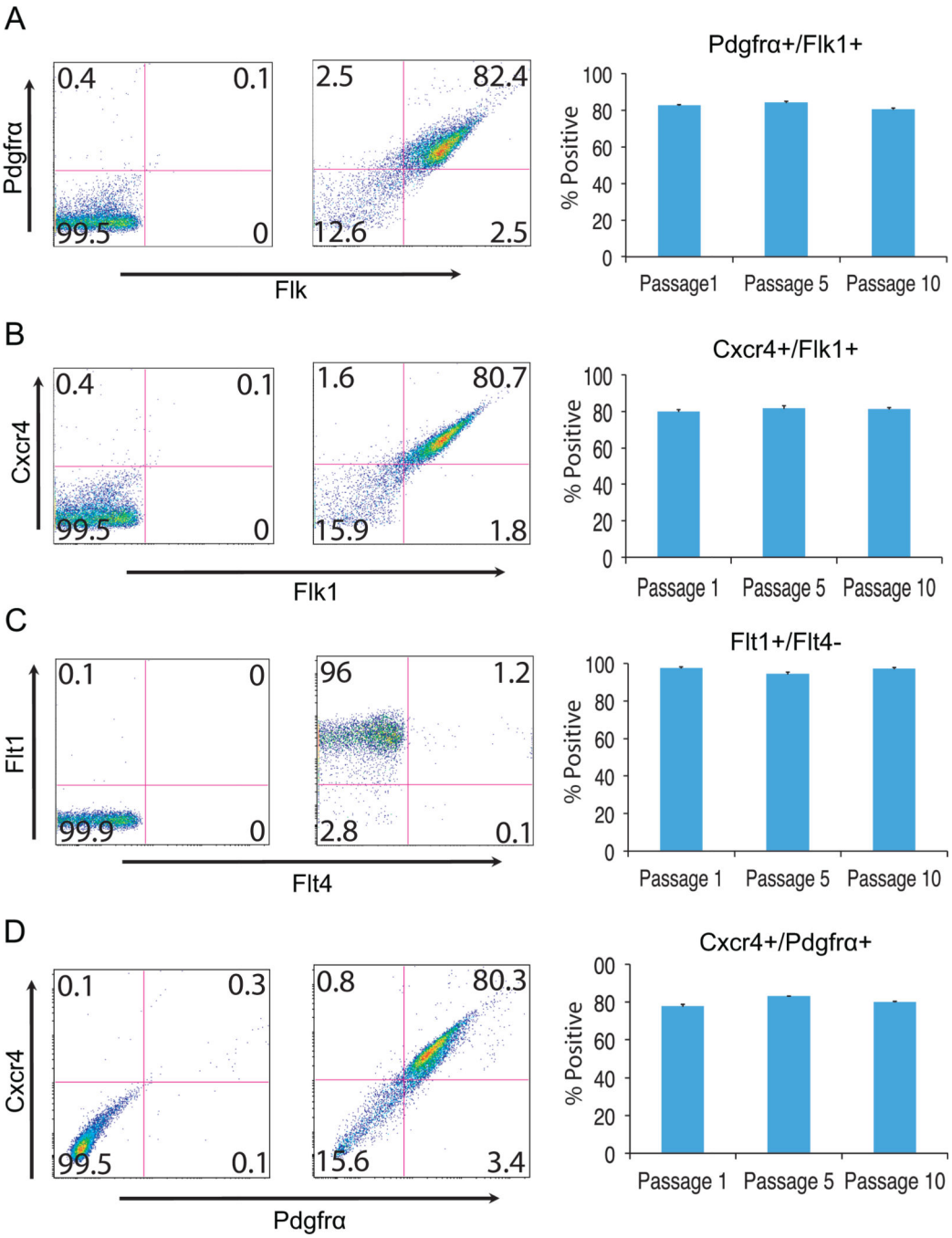

Figure 4. Resolving the cell surface marker profile of Irx4^{+} VPCs

Suspension cultures of purified and expanded progenitors were stained following 1, 5 and 10 passages with Pdgfr-alpha and Flk1 (A), Cxcr4 and Flk1 (B), Flt1 and Flt4 (C), and Cxcr4 and Pdgfr-alpha (D) antibody combinations. (A–D) Left dot plots in each panel represent secondary antibody only controls. Dot plots are representative of the flow cytometry data set (n=3). Bar graphs represent average data from corresponding passages. Error bars indicate SEM (n=3).

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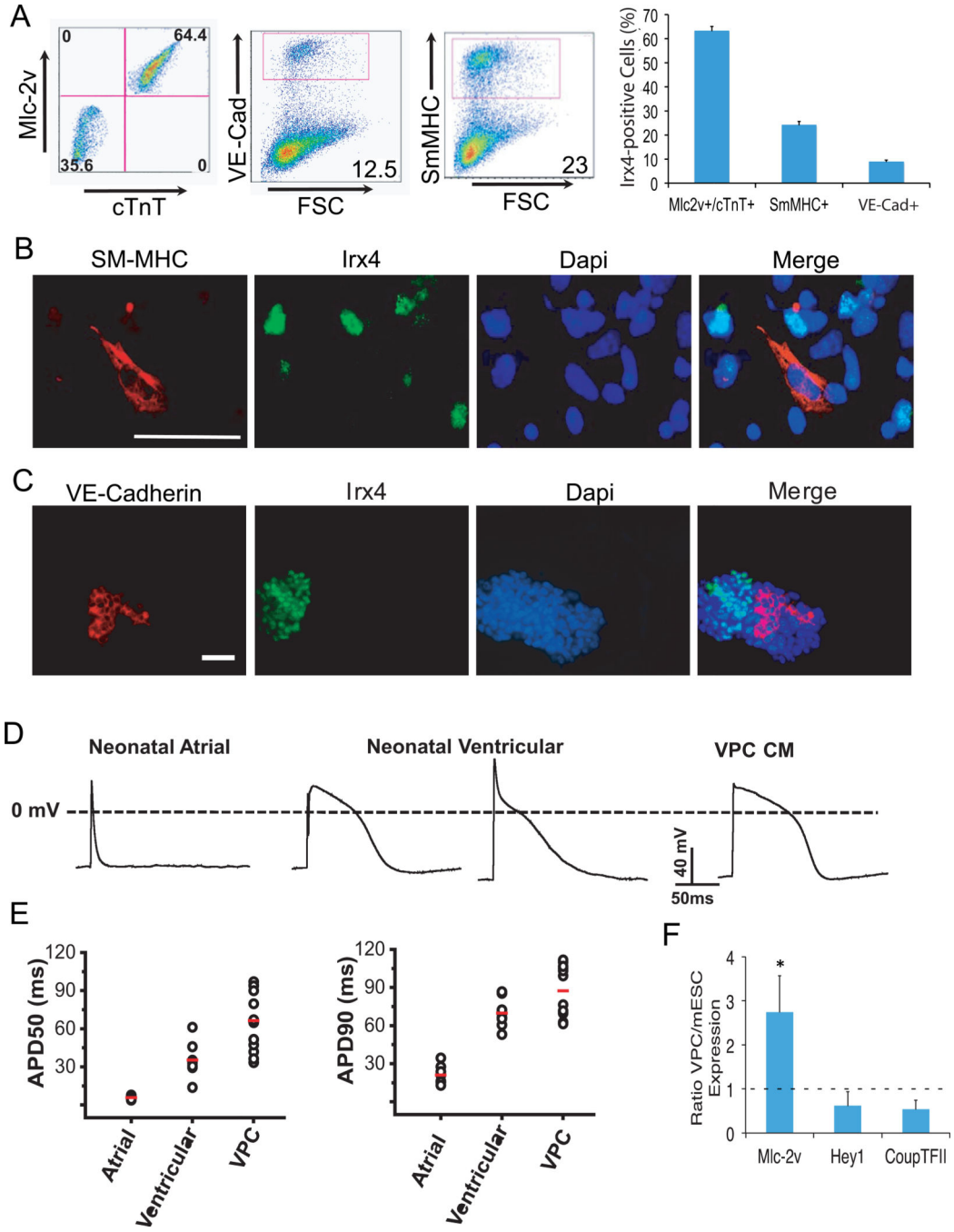

Figure 5. Irx4^{+} VPCs generate ventricular myocytes, endothelial cells and smooth muscle cells

Expanded VPCs were differentiated for 18 days and singularized for evaluation. (A) Flow cytometry of differentiated cells from VPCs co-stained with antibodies against cTnT and Mlc2v to identify ventricular cardiomyocytes, VE-Cadherin to identify endothelial cells, and SmMHC to mark smooth muscle cells. Bar graph presents average flow cytometry results (n=3). (B) Immunofluorescence images of VPC derivatives labeled for SM-MHC, a smooth muscle cell marker, and Irx4. (C) Immunofluorescence images of VPC derivatives labeled with VE-Cadherin, an endothelial cell marker, and Irx4. (D) Representative action potentials

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recorded from a cultured neonatal mouse atrial myocyte, 2 neonatal mouse ventricular myocytes and a CPC-derived myocyte. (E) Scatter plots for action potential duration at 50% repolarization (APD50) and 90% repolarization (APD90) for all cells measured from neonatal atria, neonatal ventricle and VPCs with red bars indicating averages. See also table S1 (AP stimulated at rate of 5Hz at 37°C). (F) Q-PCR analysis of differentiated progeny from VPCs and mESCs for ventricular (Mlc2v) and atrial (CoupTFII and Hey1) gene expression (n=4±SEM, *p<0.05 for VPC vs mESC).

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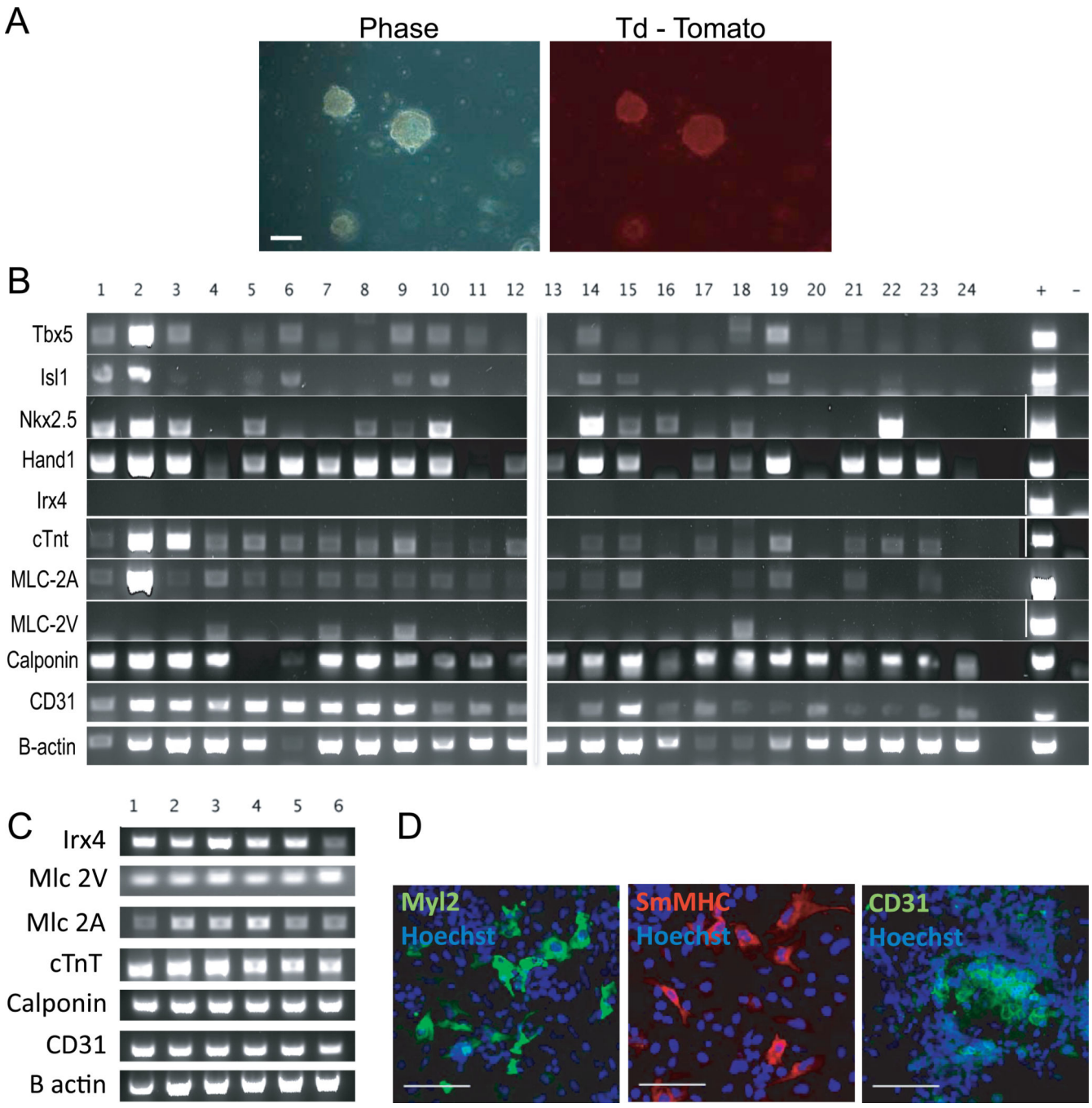

Figure 6. Single cell VPCs exhibit cardiovascular potency in vitro

(A) Phase and fluorescent tdTomato images of day 10 differentiated clones aggregates in methylcellulose. (B) RT-PCR analysis of individual clones after 10 days of differentiation in methylcellulose using cardiac-lineage specific primers revealed that majority of VPC clones were tripotent and differentiated into CMs, SMs and ECs. Negative= water only control, positive= Day 9 differentiated mESCs. (C) RT-PCR analysis of individual clones after 24 days of differentiation (days in methylcellulose +14 days in liquid medium) revealed that all analyzed clone were tripotent and expressed the ventricular-specific marker Mlc-2v. (D)

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Clones differentiated for 24 days were immunostained for Mlc2v, Smooth muscle myosin heavy chain, and CD31. All analyzed clones were tripotent (n=6). Scale bar= 50μm.

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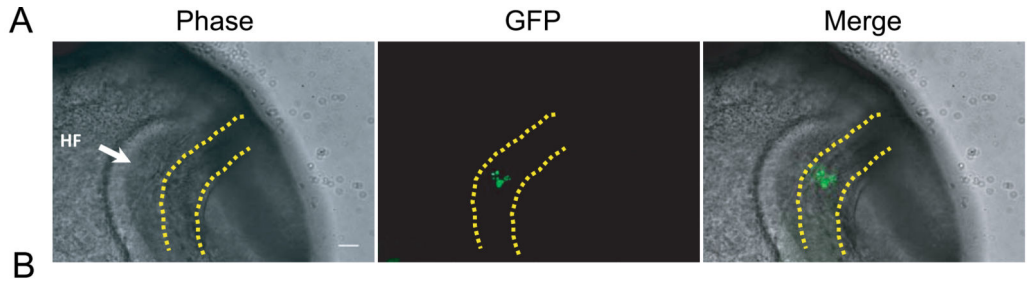

B

Total No. Embryos Injected	Abnormal after Culture	Number Immunostained	No. GFP-negative	GFP+ in Ventricle	GFP+ in Outflow Tracts	GFP outside of heart
25	1/25 (4%)	21/24	6/21 (29%)	15/15 (100%)	10/15 (67%)	0/15

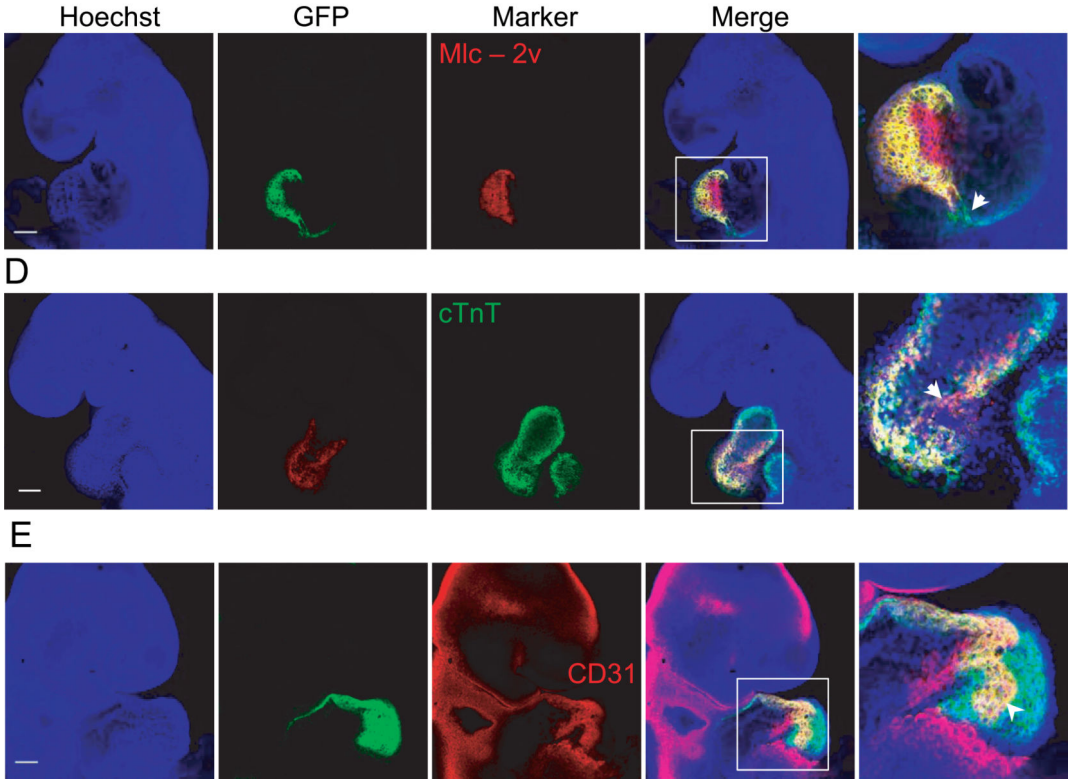

Figure 7. Irx4^{+} progenitors exhibit cardiovascular potency in vivo

(A) Phase and fluorescence images of ~E8.25 embryo to illustrate the injection site. Green region represents injected GFP^{+} cells. Dotted pattern outlines the cardiac crescent. (B) Table shows the number of embryos injected with VPCs and the location of VPC-derived cells 18 hrs after whole embryo culture. (C) Confocal fluorescence images of mouse embryo co-stained with antibodies to GFP and Mlc2v. Arrowhead highlights GFP^{+}/Mlc2v^{-} cells. (D) Confocal images of embryo co-labeled with antibodies to GFP and cTnT. Arrowhead points to GFP^{+}/cTnT^{-} cells. (E) Confocal fluorescence image of embryo stained with antibodies to

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GFP and CD31. Arrowhead highlights $GFP^+/CD31^+$ cells. Scale bars= 100μm. Abbreviations: HF= head fold.