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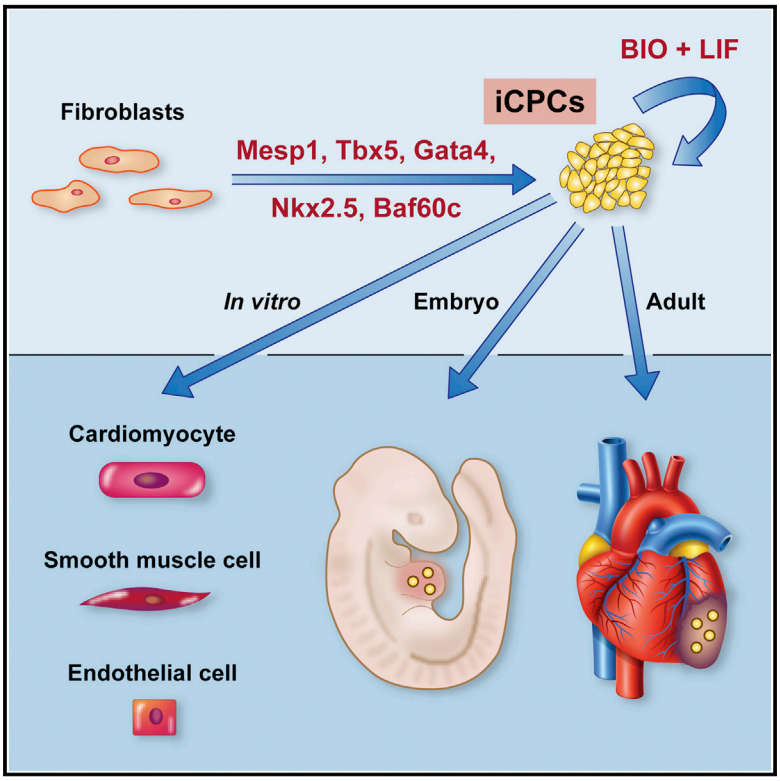
Lineage Reprogramming of Fibroblasts into Proliferative Induced Cardiac Progenitor Cells by Defined Factors

Graphical Abstract



Authors

Pratik A. Lalit, Max R. Salick, Daryl O. Nelson, ..., Karen M. Downs, Gary E. Lyons, Timothy J. Kamp

Correspondence

tjk@medicine.wisc.edu

In Brief

Lalit et al. report that a combination of cardiac factors and signaling molecules reprogram adult mouse fibroblasts into expandable induced cardiac progenitor cells (iCPCs). iCPCs are multipotent and can differentiate into cardiomyocytes, smooth muscle, and endothelial cells. Moreover, iCPCs generate myocardium when injected into the embryonic and adult post-MI mouse heart.

Highlights

- Cardiac factors reprogram fibroblasts into induced cardiac progenitors cells (iCPCs)
- Wnt and JAK/STAT signaling enables robust expansion of iCPCs
- iCPCs differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells
- Injected iCPCs generate myocardium in the embryonic and adult post-MI mouse heart

Accession Numbers

GSE61486

Lalit et al., 2016, Cell Stem Cell *18*, 354–367
March 3, 2016
http://dx.doi.org/10.1016/j.stem.2015.12.001

CellPress

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Article

Lineage Reprogramming of Fibroblasts into Proliferative Induced Cardiac Progenitor Cells by Defined Factors

Pratik A. Lalit,[1,2,3] Max R. Salick,[6,7,8] Daryl O. Nelson,[3,4] Jayne M. Squirrell,[3,5] Christina M. Shafer,[9] Neel G. Patel,[1] Imaan Saeed,[1] Eric G. Schmuck,[1,3] Yogananda S. Markandeya,[1] Rachel Wong,[1] Martin R. Lea,[1] Kevin W. Eliceiri,[3,5] Timothy A. Hacker,[1,3] Wendy C. Crone,[3,6,7,8] Michael Kyba,[10,11,12] Daniel J. Garry,[11,12] Ron Stewart,[9] James A. Thomson,[3,4,9] Karen M. Downs,[3,4] Gary E. Lyons,[3,4] and Timothy J. Kamp[1,2,3,4,*]

[1]Department of Medicine
[2]Molecular and Cellular Pharmacology Program
[3]Stem Cell and Regenerative Medicine Center
[4]Department of Cell and Regenerative Biology
[5]Laboratory for Optical and Computational Instrumentation
[6]Department of Engineering Physics
[7]Wisconsin Institutes for Discovery
[8]Material Science Program
[9]Morgridge Institute for Research
University of Wisconsin-Madison, Madison, WI 53705, USA
[10]Department of Pediatrics
[11]Department of Medicine
[12]Lillehei Heart Institute
University of Minnesota, Minneapolis, MN 55455, USA
*Correspondence: tjk@medicine.wisc.edu
http://dx.doi.org/10.1016/j.stem.2015.12.001

SUMMARY

Several studies have reported reprogramming of fibroblasts into induced cardiomyocytes; however, reprogramming into proliferative induced cardiac progenitor cells (iCPCs) remains to be accomplished. Here we report that a combination of 11 or 5 cardiac factors along with canonical Wnt and JAK/STAT signaling reprogrammed adult mouse cardiac, lung, and tail tip fibroblasts into iCPCs. The iCPCs were cardiac mesoderm-restricted progenitors that could be expanded extensively while maintaining multipotency to differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells in vitro. Moreover, iCPCs injected into the cardiac crescent of mouse embryos differentiated into cardiomyocytes. iCPCs transplanted into the post-myocardial infarction mouse heart improved survival and differentiated into cardiomyocytes, smooth muscle cells, and endothelial cells. Lineage reprogramming of adult somatic cells into iCPCs provides a scalable cell source for drug discovery, disease modeling, and cardiac regenerative therapy.

INTRODUCTION

The advent of induced pluripotent stem cells (iPSCs) has revived interest in earlier research showing that stable transdifferentiation of somatic cells is possible by forced expression of defined factors (Davis et al., 1987). Previous studies have reported lineage reprogramming into a diverse range of differentiated cells types, including neurons (Vierbuchen et al., 2010), hepatocytes (Sekiya and Suzuki, 2011), and cardiomyocytes (CMs) (Ieda et al., 2010; Song et al., 2012). More recently, lineage reprogramming into tissue-specific progenitors has been achieved, including neural (Han et al., 2012) and hepatic progenitor cells (Yu et al., 2013). Using transdifferentiation to produce progenitor cells rather than terminally differentiated cell types provides potential advantages for both drug discovery and regenerative medicine applications. Reprogrammed progenitors are proliferative and, therefore, more scalable. Lineage-restricted induced progenitor cells may be superior for therapeutic applications because of their ability to proliferate and differentiate into the needed complement of cell types required to fully reconstitute the diseased or damaged tissue. Induced progenitor cells may also provide a more efficient and reproducible platform to obtain tissue-specific terminally differentiated cell types compared with pluripotent stem cells (PSCs).

Cardiac progenitor cells (CPCs) have been identified using various markers in the developing and adult heart. During embryogenesis, CPCs of both first and second heart fields reside in the cardiac crescent. Several studies have isolated CPCs from embryos and embryonic stem cells (ESCs) using transcription factor (TF)-based reporters like Mesp1, Isl1, and Nkx2.5, but a master regulator of the CPC state has not yet been identified (Bondue et al., 2011; Masino et al., 2004; Moretti et al., 2006). Cell surface markers, including Cxcr4, Pdgfr-α, Flk1/KDR, and SIRPA, have been used to identify PSC-derived CPCs. (Dubois et al., 2011; Kattman et al., 2011). CPCs have

also been identified in the adult mammalian heart using markers, including Sca1 and cKit, that, in small animal studies, have demonstrated multi-lineage potency following transplantation to the post-myocardial infarction (MI) myocardium (Ellison et al., 2013; Oh et al., 2003). However, in vitro multi-lineage differentiation of adult CPCs has been difficult to demonstrate, especially with regard to differentiation into contracting cardiomyocytes (Noseda et al., 2015), and the regenerative capacity of adult c-kit$^+$ CPCs after cardiac injury has been questioned (van Berlo et al., 2014).

Reprogramming to a stem or progenitor cell state requires knowledge of a specific combination of master regulatory factors as well as appropriate culture conditions that can maintain self-renewal and multipotency. Typically, the culture conditions for reprogramming mimic those optimized for the in vitro culture of native stem cells based on both empiric optimization and knowledge of developmental signaling pathways. For example, in the case of iPSCs, the distinct culture conditions optimized for mouse and human ESC culture were utilized to generate mouse and human iPSCs, respectively (Takahashi and Yamanaka, 2006; Yu et al., 2007). Likewise, reprogramming to induced neural stem cells employed standard adult neural stem cell medium (Han et al., 2012). In contrast to commonly used neural stem cell medium, variable culture conditions have been used for adult heart-derived CPCs (Ellison et al., 2013; Oh et al., 2003). It has also proven difficult to generate culture conditions and appropriate signaling to maintain and expand embryonic or PSC-derived CPCs. Recently, mesodermal SSEA1 progenitors with robust cardiac differentiation potential have been maintained (Cao et al., 2013), but to generate and maintain human PSC-derived, cardiac-restricted progenitors has required transgenic forced expression of an oncogene, c-Myc (Birket et al., 2015). Therefore, the lack of clearly defined culture conditions for the maintenance and expansion of both adult and PSC-derived CPCs has increased the challenge in transdifferentiating cells into CPCs and likely contributes to the limited success to date in converting fibroblasts into proliferative and multipotent CPCs (Islas et al., 2012).

Here we show that a defined set of cardiac factors complemented by appropriate culture conditions can reprogram adult mouse fibroblasts from three different tissues into iCPCs. iCPCs were stably reprogrammed, cardiac mesoderm-restricted, clonal progenitors that could be passaged extensively and showed multipotency toward cardiovascular lineages (CMs, smooth muscle cells [SMs], and endothelial cells [ECs]) in vitro and following transplantation to the embryonic cardiac crescent or the adult post-myocardial infarction heart. Cardiac progenitor reprogramming technology holds promise as a scalable cell source for drug discovery, disease modeling, and cardiac regenerative therapy.

RESULTS

Screening for Cardiac Progenitor Cell-Inducing Factors

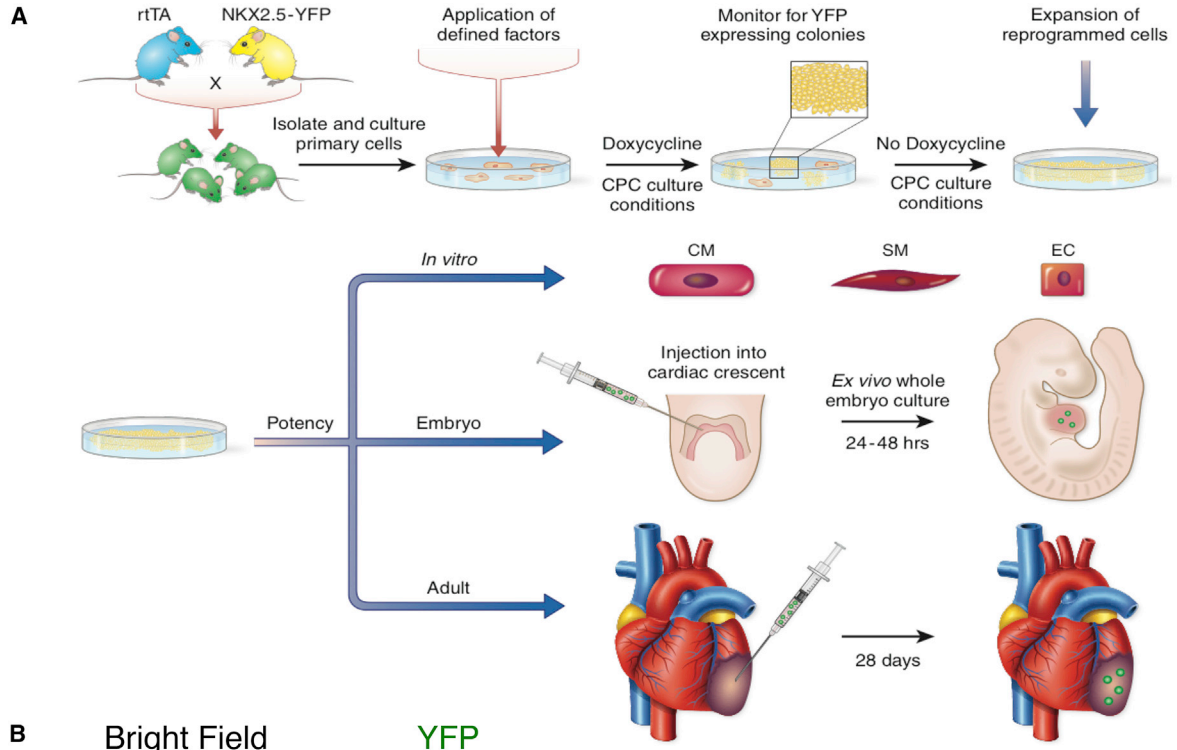
Based on the known expression pattern of genes that are critical during embryonic cardiovascular development, we selected 18 candidate genes to test their reprogramming ability. These included cardiac transcription factors and cardiac chromatin remodeling factors. We also included iPSC factors, reasoning that they may facilitate reprogramming. These 22 genes were cloned individually into a doxycycline (dox)-inducible lentivirus vector, generating an expression library of defined factors (Figure S1A). Dox-induced expression of the factors was confirmed at the mRNA and protein levels in transduced fibroblasts (Figures S1B–S1D; Supplemental Experimental Procedures).

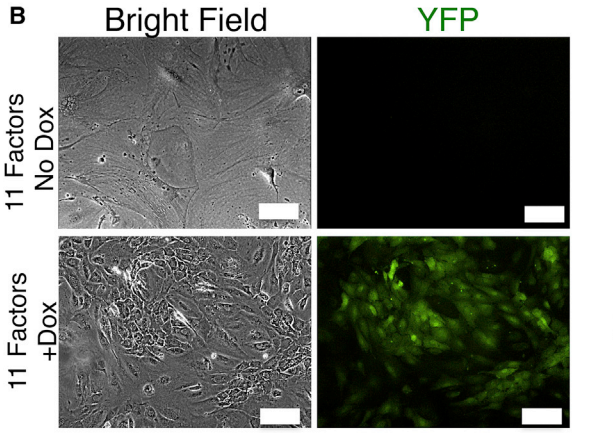
We utilized an Nkx2.5 cardiac reporter mouse model expressing enhanced yellow fluorescent protein (EYFP) in the developing heart during the initial stages of cardiac development (embryonic day [E] 7.75–E 10.5) (Masino et al., 2004). The developmentally restricted Nkx2.5-EYFP reporter identifies early CPCs, but it is inactive during later stages of cardiac development (E11 onward) and in the adult heart. The Nkx2.5-EYFP mouse was crossed with a transgenic mouse expressing a reverse tetracycline transactivator (rtTA) to enable dox-inducible transgene expression. Cardiac fibroblasts were isolated from adult double-transgenic (Nkx2.5-EYFP/rtTA) mice by explant culture (Figure 1A; Figure S1E). Immunostaining of the isolated fibroblasts with markers of CPCs (Irx4, Nkx2.5, and Gata4) as well as cardiac lineage cells such as CMs (α-actinin and α-myosin heavy chain [α-MHC]), SMs (SM-MHC), and ECs (CD31) revealed no staining (Figures S1F–S1J).

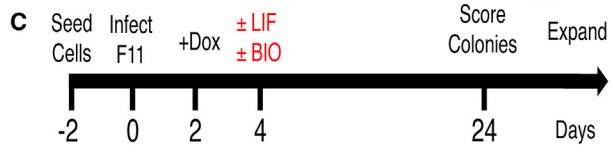
Uninfected adult cardiac fibroblasts (AC Fibs) did not express EYFP and senesced after three to four passages (psgs) (Figure S2A). As a first test of the dox-inducible library for reprogramming, AC Fibs were infected with iPSC factors. Dox treatment produced proliferative cells that formed EYFP$^-$ iPSC colonies (Figure S2B). Next, we tested infection of AC Fibs with induced CM (iCM) factors (Gata4, Mef2c, Tbx5 [GMT]), but even after extended dox induction (6 weeks), we did not observe contracting cells or EYFP$^+$ cells. However, neonatal cardiac fibroblasts infected with iCM factors reprogrammed into spontaneously contracting, EYFP$^-$ iCMs after 4 weeks of dox treatment (Figure S2C; Movie S1). These results demonstrate dox-inducible reprogramming with the described vector system. Furthermore, the Nkx2.5-EYFP reporter is not activated during iPSC or iCM reprogramming.

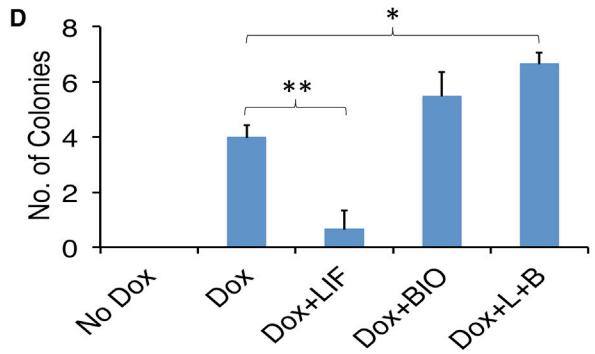
Infection of AC Fibs with a mixture of lentiviruses containing all 22 factors resulted in a small number of EYFP$^+$ proliferative colonies only after dox treatment (Figure S2D). Next, we subtracted iPSC factors from the 22-factor library and infected fibroblasts with 18 cardiac factors. Proliferative EYFP$^+$ cells were again observed 3 weeks after dox treatment (Figure S2D). Reasoning that factors expressed early in cardiac development might have the highest potential to reprogram fibroblasts into iCPCs, we chose 11 early cardiac factors (*Mesp1*, *Mesp2*, *Gata4*, *Gata6*, *Baf60c*, *SRF*, *Isl1*, *Nkx2.5*, *Irx4*, *Tbx5*, and *Tbx20*) to infect AC Fibs. Infection with 11 factors gave rise to proliferative EYFP$^+$ cells only after dox treatment (Figure 1B).

We analyzed the time course of appearance of EYFP$^+$ cells upon infection with the 11 factors. Single EYFP$^+$ cells were detected as early as day 4 after dox treatment. By 3 weeks, these EYFP$^+$ cells developed into two-dimensional, highly proliferative colonies of EYFP$^+$ cells that lost their parental fibroblast morphology and exhibited a high nuclear-cytoplasmic ratio (Figure S2E). Infection with 11 factors reproducibly gave rise to EYFP$^+$ proliferative colonies (4 colonies/50,000 cells; efficiency, 0.008%) (Figures 1C and 1D). We manually isolated these EYFP$^+$ colonies and tried to expand them by splitting. However,



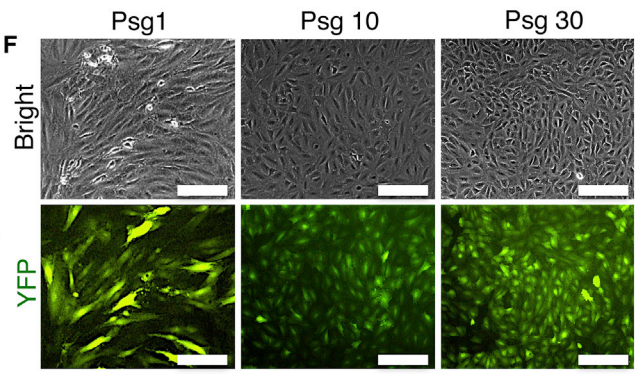


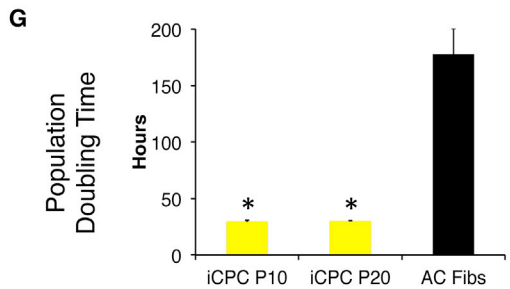




E

Culture Condition	F11 - 5 Passages	
	YFP	Proliferation
Dox only	-	-
Dox+LIF	-	-
Dox+BIO	+	+
Dox+L+B	++	++





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the cells lost EYFP expression and senesced after three to five psgs under the "dox only" culture condition (Figure 1E; Figure S2F).

Wnt and JAK/STAT Signaling Promotes Proliferative Reprogrammed Cells

The overexpression of cardiac factors alone, even though sufficient to produce EYFP$^+$ colonies, was insufficient for maintaining EYFP$^+$ cells in a proliferative, reprogrammed state, indicating that additional signaling cues might be necessary. Canonical Wnt signaling is critical for proliferation of CPCs (Cao et al., 2013; Qyang et al., 2007), and JAK/STAT signaling is important for normal cardiogenesis (Foshay et al., 2005). Therefore, we tested the effect of supplementing the reprogramming medium with 6-bromoindirubin-3′-oxime (BIO, a canonical Wnt activator) and/or LIF (a JAK/STAT activator) on reprogramming efficiency as well as the ability of EYFP$^+$ cells to maintain a proliferative state (Figure 1C). Surprisingly, addition of LIF alone inhibited the generation of EYFP$^+$ cells and colony formation. Addition of BIO alone resulted in a similar reprogramming efficiency as dox only. However, the EYFP$^+$ cells became spindle-like upon passaging and were not highly proliferative. The LIF+BIO combination produced the brightest EYFP$^+$ cells, and the EYFP$^+$ cells were robustly expandable (Figures 1C–1E; Figures S2F–S2H). Therefore, both LIF and BIO were included in our reprogramming medium, "iCPC induction medium." Eleven-factor infection followed by culture in iCPC induction medium produced six to nine EYFP$^+$ colonies (per 50,000 starting cells; 0.013% reprogramming efficiency) that could be expanded continuously on splitting (F11 iCPCs). To determine whether LIF+BIO was necessary for initial reprogramming, we tested whether EYFP$^+$ colonies generated by the dox only condition could be expanded by addition of LIF+BIO later during passaging. We observed that addition of LIF+BIO starting at psg 1 allowed for robust expansion of dox only EYFP$^+$ cells, indicating that the effect of LIF+BIO was more on the maintenance of the reprogrammed state than on initiation of reprogramming.

To determine whether continued forced expression of cardiac factors was required to maintain the iCPC state, we withdrew dox from the iCPC induction medium (iCPC maintenance medium) after two psgs and assessed whether EYFP$^+$ cells remained proliferative. Cells maintained EYFP expression as well as their proliferative ability for over 30 psgs (Figure 1F). The EYFP$^+$ cells continued to reduce in size during the initial psgs until psg 2–3, when they reached a steady state, after which their morphology remained unchanged during further passaging. Next, we determined the population doubling time of iCPCs that had been passaged in iCPC maintenance medium for 10 and 20 psgs. Both psg 10 and 20 iCPCs had a similar population doubling time of about 30 hr, which was significantly less than AC Fibs (Figure 1G). These results suggest that iCPCs were stably reprogrammed and maintained their proliferative state in the presence of LIF+BIO and without exogenous induction of cardiac factors by dox.

Five Factors Are Sufficient to Reprogram Adult Cardiac Fibroblasts into iCPCs

We wanted to determine whether iCPCs could be reprogrammed using a subset of the 11 factors. Initially, we tested a core combination of three factors (within the 11-factor combination) that are expressed earliest in cardiac development: *Mesp1*, *Tbx5*, and *Gata4* (MTG). We infected AC Fibs with MTG and cultured them in iCPC induction medium, but we did not observe emergence of any EYFP$^+$ colonies for up to 4 weeks. Therefore, we tested whether addition of remaining factors within the 11-factor pool to MTG can induce colony formation. Addition of *Baf60c*, *Isl1*, and *Nkx2.5* to MTG reproducibly generated EYFP$^+$ colonies after 3 weeks (Figure 2A; Figure S2I). Although the four-factor combinations produced EYFP$^+$ cells, their proliferative ability as well as EYFP expression declined progressively with subsequent passaging in iCPC maintenance medium. This suggested that four-factor combinations induced partial reprogramming and were insufficient to epigenetically stabilize cells in the iCPC state. Because MTG+*Nkx2.5* (MTGN) produced the most colonies, we tested whether addition of the other factors that produced colonies in the four-factor combination, *Baf60c* or *Isl1*, to MTGN could facilitate stable reprogramming into iCPCs (Figure 2A; Figure S2J). Addition of both *Baf60c* and *Isl1* to MTGN produced proliferative colonies, but *Baf60c*+MTGN (MTGNB) produced the most expandable colonies post-dox withdrawal and, therefore, was selected for further characterization. Infection with MTGNB (factor 5 [F5]) reproducibly gave rise to expandable F5 iCPCs (~7.25 colonies/50,000 cells) (Figure 2B) that could be expanded stably in iCPC maintenance medium (without dox) for at least 20 psgs (Figure S3A). Three F5 iCPC lines underwent karyotype analysis, which demonstrated that two of three lines exhibited normal karyotypes, whereas the third line had numerical and structural abnormalities (Figure S3B). These results suggest that five factors stably reprogrammed AC Fibs into proliferative iCPCs.

Cardiac Factors Stably Reprogram Adult Cardiac Fibroblasts into Cardiac Mesoderm-Restricted iCPCs

To analyze iCPC gene expression, we performed qPCR analysis on psg 1 iCPCs that revealed upregulation of key CPC transcription factors, including *Nkx2.5*, *Tbx5*, *Mef2c*, *Mesp1*, *Tbx20*, and

Figure 1. Screening for iCPC-Inducing Factors and Optimal Culture Conditions

(A) Schematic of the experimental design depicting direct reprogramming of adult fibroblasts into iCPCs by defined factors and culture conditions, expansion of iCPCs, and in vitro as well as in vivo differentiation of iCPCs into cardiac lineage cells.

(B) Infection with a combination of 11 cardiac factors induced Nkx2.5-EYFP expression in AC Fibs only after dox induction.

(C) Strategy to test the effect of culture conditions on F11 reprogramming efficiency as well as the ability of EYFP$^+$ cells to maintain a proliferative state.

(D) Number of EYFP$^+$ colonies formed (per 50,000 starting cells) under the respective culture conditions (**$p < 0.01$, *$p < 0.05$).

(E) Effect of culture conditions on EYFP$^+$ colonies expanded up to five psgs scoring for EYFP$^+$ expression and proliferative ability (Dox only, n = 8; Dox+LIF, n = 3; Dox+BIO, n = 4; Dox+LIF+BIO, n = 9). L, LIF; B, BIO.

(F) F11 iCPCs maintained EYFP expression and proliferative ability for at least 30 psgs after dox withdrawal.

(G) Population doubling time for psg 10 (P10) and psg 20 (P20) F11 iCPCs compared with uninfected AC Fibs (n = 3). *$p < 0.01$.

Data are presented as mean. Error bars indicate SEM. Scale bars, 100 μm (B) and 200 μm (F). See also Figures S1 and S2 and Movie S1.

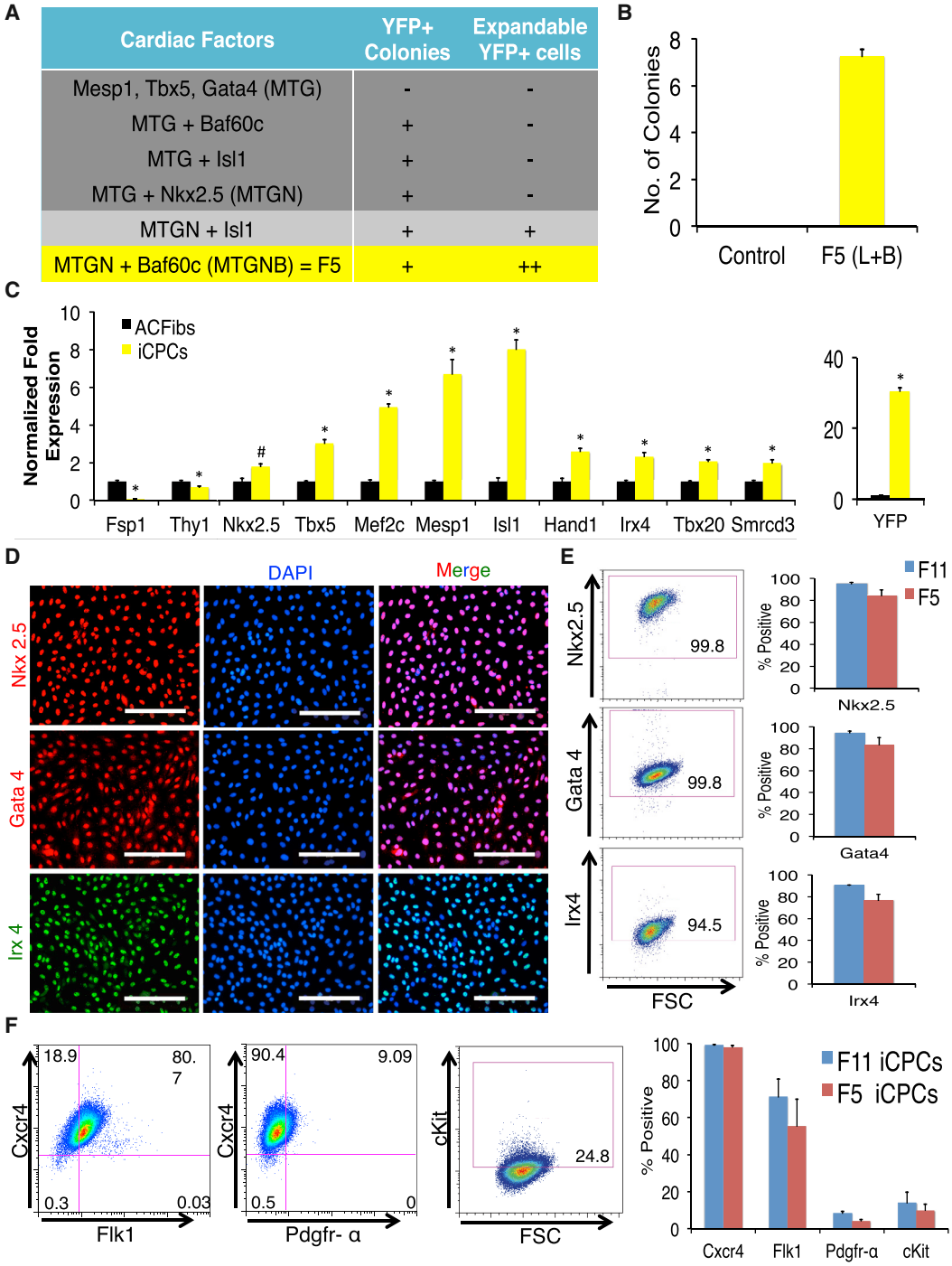


Figure 2. Cardiac Factors Stably Reprogram Adult Cardiac Fibroblasts into Proliferative iCPCs

(A) Factor combinations tested for the ability to produce Nkx2.5-EYFP$^+$ colonies and to expand them for at least five psgs without dox (n = 3–5).

(B) Number of EYFP$^+$ colonies produced after infection with five factors (MTGNB) and cultured in iCPC induction medium for 3 weeks (per 50,000 seeded cells, n = 8). Uninfected fibroblasts cultured in iCPC induction medium were used as control.

(C) qPCR analysis of F11 iCPCs showed upregulation of CPC markers and downregulation of fibroblast markers. Data represent normalized fold expression relative to uninfected AC Fibs (*$p < 0.01$, #$p < 0.05$).

(D) Immunofluorescence labeling of F11 iCPCs showed nuclear localization of the cardiac TFs Nkx2.5, Gata4, and Irx4.

(E) Flow cytometry analysis revealed that the majority of F11 iCPCs expressed cardiac TFs. F11 and F5 iCPCs showed comparable expression of cardiac TFs.

(F) Flow cytometry analysis showed that F11 iCPCs expressed cell surface markers such as Cxcr4, Flk1, Pdgfr-α, and cKit that are associated with CPCs (n = 3). F11 and F5 iCPCs showed comparable expression of CPC-associated cell surface markers.

Error bars indicate SEM. Scale bars, 200 μm. See also Figures S1, S2, and S3 (for F5 iCPCs).

Irx4, accompanied by downregulation of the fibroblast-specific gene *Fsp1* (Figure 2C). These results indicate that iCPCs initiated the cardiac program at the expense of the fibroblast program, a hallmark of lineage reprogramming. Next, we performed immunostaining for CPC-related TFs. In contrast to AC Fibs, which did not immunolabel for Nkx2.5, Gata4, or Irx4 (Figure S1F), iCPCs exhibited nuclear localization of these TFs that remained constant across psgs 5–25 (Figures 2D, S3C, and S4A–C). Flow cytometry demonstrated that greater than 80% of the iCPC expressed Nkx2.5, Gata4, and Irx4 (Figure 2E). Furthermore, we assessed whether iCPCs expressed cell surface markers associated with CPCs (Kattman et al., 2011; Nelson et al., 2008). Flow cytometry analysis revealed that iCPCs homogenously expressed Cxcr4. However, only a fraction of iCPCs expressed Flk1, Pdgfr-α, or cKit (Figure 2F; Figure S3D). We found no protein expression for pluripotency (Oct4) or cardiac lineage differentiation markers (α-actinin, SM-MHC, and CD31), even after extensive passaging (Figure S4F). Comparable results were observed for these experiments whether F11 or F5 iCPCs were used.

To characterize the transcriptome of iCPCs, we performed RNA sequencing (RNA-seq) analysis on early-psg (1–3) as well as late-psg (8–10) iCPCs. As a positive control, we utilized gene expression values for a mouse ESC (mESC)-derived CPC population described previously by Wamstad et al. (2012), and the gene expression was compared with uninfected AC Fibs. We found that genes involved in cardiovascular development, including TFs (*Tbx3*, *Hes1*, *Prrx1*, *Foxa2*, *Gata4/6*, *Meis1*, and *Gli2*), signaling molecules (*LIF*, *Vegfc*, *Grem1*, and *Fgf2*), cell surface markers (*cKit*, *Pdgfr-α*, *Notch1*, and *Gpc3*), and chromatin remodeling genes (*Smarcd3, Hdac 2/5/7/10*, and *Jarid2*) were increasingly upregulated as iCPCs were passaged (Figure 3A). In contrast, fibroblast-specific genes (*Postn*, *Twist2*, and *Thy1*) were increasingly downregulated with passaging (Figure 3B). Furthermore, CM differentiation markers (*Actc1*, *Myh6*, *Myl2*, and *Myl7*) were not expressed in iCPCs. Interestingly, genes associated with SM (*Cnn1* and *Myh11*) and EC (*Pecam1*) were upregulated in one early-psg replicate. However, these genes were downregulated in late-psg iCPCs. Primitive streak genes (*Gsc*, *Mixl1*, and *T*) were not detected (Figure S4D). Likewise, progenitor genes for endoderm, ectoderm, and non-cardiac mesoderm were not expressed by iCPCs (Figure S4E). mESC-CPCs used by Wamstad et al. (2012) were derived from the whole population of cells after 5 days of differentiation. This population was highly enriched for CPCs but potentially contained a minority of cells from non-cardiac lineages. Therefore, mESC-CPC samples had higher expression for some endoderm, ectoderm, and non-cardiac mesoderm markers. Additionally, Bmp (4/6/7) genes that induce cardiac differentiation were also downregulated. Importantly, iCPCs did not express markers of pluripotent stem cells (*Pou5f1*, *Esrrb*, *Dppa2/3*, *Lin28a*, and *Sox2*) (Figure S4D). However, we did observe upregulation of *Nanog* (see Discussion). A pairwise Pearson's correlation analysis of all expressed genes revealed that transcription profiles of both low- and high-psg iCPCs have higher correlation with mESC-CPCs compared with mESC-CMs (Wamstad et al., 2012) (Figure 3C). Gene ontology (GO) terms associated with upregulated genes in iCPCs include categories such as "positive regulation of cell proliferation," "negative regulation of cell differentiation," and "cardiovascular system development," whereas terms associated with the downregulated genes include categories such as "cell adhesion," "cell differentiation," and "apoptosis" (Figure 3D). In aggregate, these data suggest that iCPCs are cardiac mesoderm-restricted precursors.

iCPCs Differentiate into Cardiomyocytes, Smooth Muscle Cells, and Endothelial Cells

To determine whether iCPCs were capable of differentiation into cardiovascular lineages, iCPCs were aggregated in cardiac differentiation medium. iCPCs maintained EYFP expression in aggregates. However, 20 days after plating, cells lost EYFP expression, suggesting that the iCPCs exited the progenitor state and differentiated (Figures 3E and 3F). Immunocytochemistry revealed differentiated cells expressing CM (cardiac actin, α-actinin, MLC-2a, MLC-2v, and α/β MHC), SM (SM-MHC), or EC (CD31) markers (Figure 3G; Figure S3E). Among iCPC-differentiated cells, a majority stained positively for CM markers (80%–90%), and only a fraction stained for SM (5%–10%) and EC markers (1%–5%) (Figure 3G; Figure S3F). These results suggest that iCPCs were multipotent, capable of differentiating into three types of cardiovascular lineage cells. We evaluated the differentiation potential of psg 5 and 30 iCPCs and observed that multipotency was comparable across psgs (Figure S5A). The finding of comparable potency across passages argues against contaminating cell types in the starting AC Fibs, confounding the results. Most MLC-2v-positive CMs also labeled for MLC-2a, indicating that they were relatively immature. However, we did observe cells that exclusively stained for MLC-2v or MLC-2a (<5%), suggesting that iCPCs can differentiate into both atrium-like and ventricle-like CMs (Figures S5B and S5C).

Even after attaining highly organized sarcomeres following extended culture periods under low-serum conditions, the iCPC-derived CMs did not exhibit spontaneous contractions. We reasoned that co-culturing iCPC-CMs with mESC-derived CMs may provide additional mechanical, electrical, and paracrine stimulation to induce further maturation and contraction. Co-culturing with rat CMs has been shown previously to induce contraction in iCMs (Wada et al., 2013). Therefore, we infected iCPC-CMs with a constitutive GFP-expressing lentivirus to identify reprogrammed cells and co-cultured them with mESC-derived CMs expressing td-tomato. We did not detect cells that co-expressed both GFP and td-tomato (Figure 4A), suggesting that cell fusion between iCPC-CMs and mESC-CMs was unlikely. We immunostained the co-cultured cells for CM markers as well as GFP and noticed that GFP^+ iCPC-CMs and GFP^- mESC-CMs both stained positively for CM markers and grew side by side as monolayers (Figure S5D). Moreover, immunostaining for Cx43 revealed that iCPC-CMs developed abundant gap junctions with both mESC-CMs as well as other iCPC-CMs (Figure 4B). After 10–14 days of co-culturing, 5%–10% of iCPC-CMs started contracting synchronously with mESC-CMs (Movie S2). The contracting iCPC-CMs also showed spontaneous calcium transients that were similar to those in mESC-CMs in frequency and amplitude (Figures 4C and 4D; Figures S5E and S5F; Movie S3).

We wanted to determine the cardiovascular potency of single-cell iCPCs. Therefore, iCPCs were seeded at low density and cultured to obtain single cell-derived colonies that were picked,

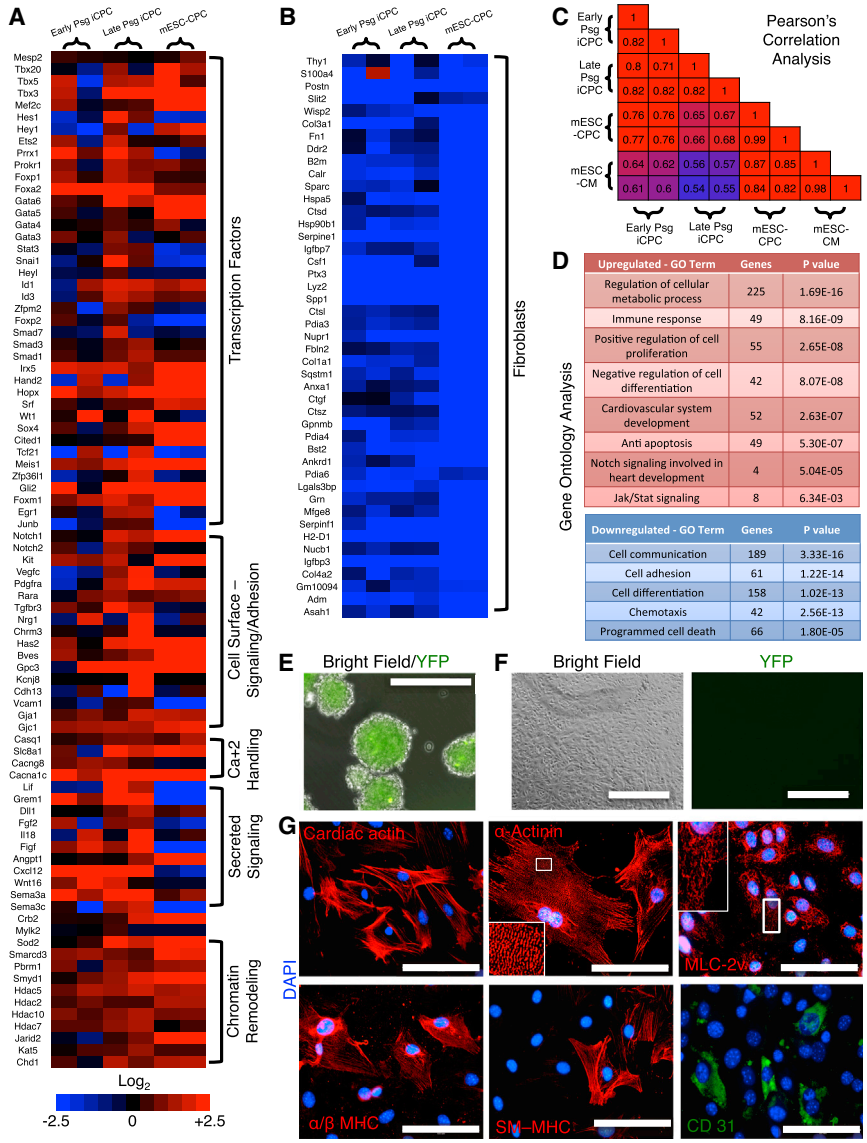


Figure 3. iCPCs Exhibit Cardiac-Mesoderm-Restricted Gene Expression, Are Multipotent, and Differentiate into Cardiomyocytes, Smooth Muscle Cells, and Endothelial Cells In Vitro

(A and B) Heatmaps of RNA-seq data illustrating differentially expressed genes in early-psg (1–3) and late-psg (8–10) iCPCs and mESC-CPCs (Wamstad et al., 2012) compared with AC Fibs. (n = 2 biological replicates in each group). Genes involved in embryonic cardiovascular development were increasingly upregulated in iCPCs with psgs (A). Fibroblast genes were strongly downregulated (B).

(C) Pearson's correlation analysis of all expressed genes among low-psg iCPCs, high-psg iCPCs, mESC-CPCs, and mESC-CMs (Wamstad et al., 2012). Numbers indicate Pearson's *R* values.

(D) Gene ontology analysis performed for upregulated and downregulated genes in late-psg iCPCs compared with AC Fibs.

(E) F11 iCPCs aggregated in cardiac differentiation medium were EYFP$^+$ on day 2.

(F) F11 iCPC aggregates were plated and cultured under low-serum conditions and lost Nkx2.5-EYFP expression by day 20.

(G) Immunocytochemistry on plated cells revealed expression of CM markers such as cardiac actin, α-actinin (note highly organized sarcomere staining), MLC-2v, α/β MHC, the SM marker SM-MHC, and the EC marker CD31.

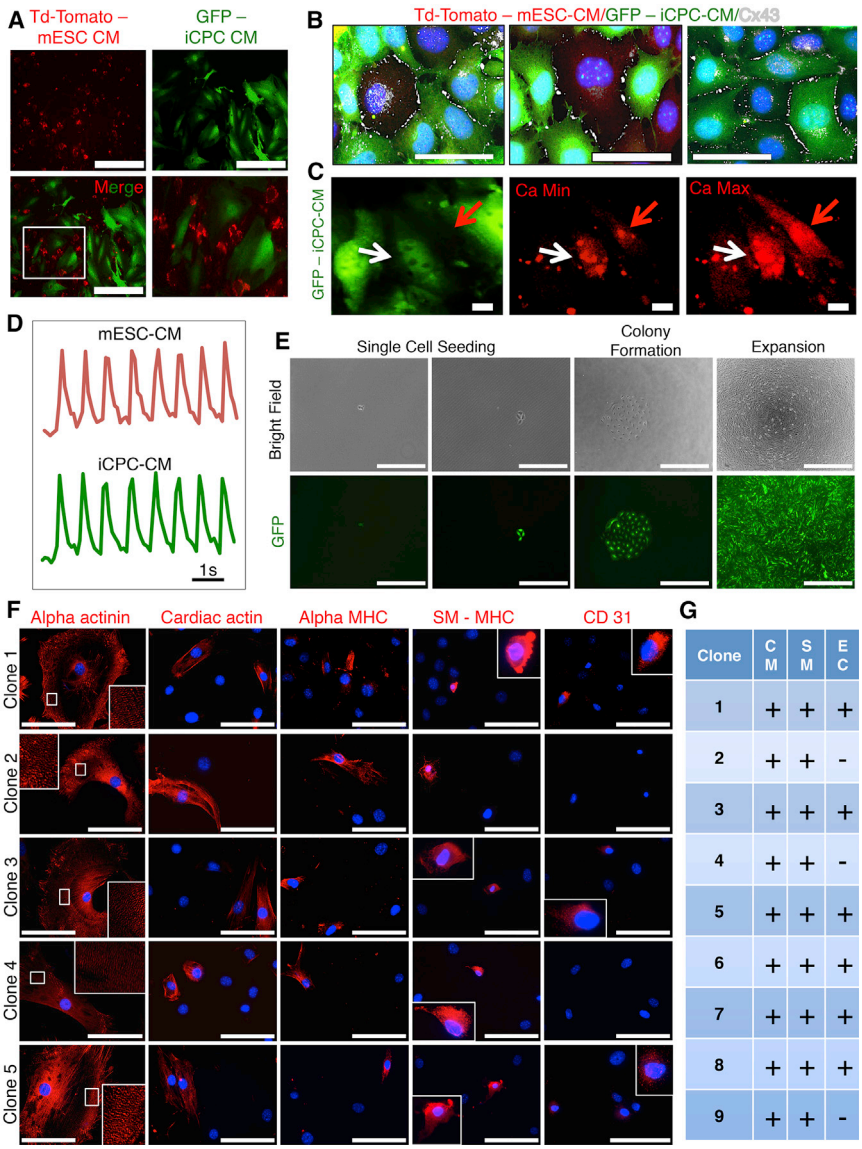
Scale bars, 400 μm (E and F) and 100 μm (G). See also Figures S3 and S5.

expanded, and, subsequently, differentiated (Figure 4E). We found that six of nine clones exhibited tripotency (differentiated into CMs, SMs, and ECs), whereas three of nine clones were bipotent (differentiated into CMs and SMs) (Figures 4F and 4G). This indicates that there is heterogeneity among iCPC clones regarding cardiac lineage potency.

Cardiac Factors Stably Reprogram Adult Lung and Tail Tip Fibroblasts into iCPCs

To determine whether iCPCs could be reprogrammed from non-cardiac sources of fibroblasts, we isolated adult lung fibroblasts (AL Fibs) and adult tail tip fibroblasts (AT Fibs) from Nkx2.5-EYFP/rtTA transgenic mice. Both AL Fibs and AT Fibs stained negatively for CPC TFs as well as cardiac lineage differentiation markers and had no EYFP expression. We infected AL Fibs and AT Fibs with either 11 or 5 factors and cultured them in iCPC induction medium. AL Fibs infected with 11 factors or 5 factors produced 9 or 10.5 EYFP$^+$ colonies, respectively (per 50,000 cells). AT Fibs infected with 11 factors or 5 factors produced 5 or 4 EYFP$^+$ colonies (per 50,000 cells), respectively (Figure 5A). Lung-derived iCPCs could be stably expanded in iCPC maintenance medium for at least 10 psgs, stained positively for CPC markers, and differentiated into CMs (50%–60%), SMs (10%–15%), and ECs (5%–10%) (Figures 5B–5H). Three lung-derived iCPC lines underwent karyotype analysis, which demonstrated that two of three lines exhibited normal karyotypes, whereas the third line was nearly tetraploid (Figure 5G). Similarly, AT Fib-derived iCPCs could be stably expanded in iCPC maintenance medium for at least five psgs, stained positively for CPC markers, and differentiated into CMs (30%–40%), SMs (5%–10%), and ECs (1%–5%) (Figures 5B–5I).

We generated over 40 iCPC lines from three different fibroblast sources (AC Fibs, F11 [10 lines] and F5 [20 lines]; AL Fibs, F11 [4 lines] and F5 [4 lines]; and AT Fibs, F11 [2 lines] and F5 [2 lines]). All iCPC lines had comparable morphologies, EYFP expression, and proliferation rates. Many of these lines were characterized extensively for expression of CPC markers (TFs and cell surface proteins) and cardiac lineage potency at various passages (ranging from psgs 5–30). It is interesting to note that various iCPC lines showed a reproducible expression pattern for CPC markers and possessed cardiac lineage potency regardless of



Clone	CM	SM	EC
1	+	+	+
2	+	+	-
3	+	+	+
4	+	+	-
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	-

Figure 4. iCPC-CMs Show Contraction and Calcium Transients upon Co-culture with mESC-CMs, and Single Cell-Derived iCPC Clones Exhibit Cardiovascular Potency

(A) iCPC-CMs infected with a GFP-expressing lentivirus co-cultured with mESC-CMs that expressed td-tomato. No cell fusion was detected.

(B) Cx43 immunolabeling showed that iCPC-CMs developed gap junctions with mESC-CMs and other iCPC-CMs.

(C) iCPC-CMs showed synchronous calcium transients with mESC-CMs 3 weeks after co-culture. White arrow, iCPC-CM; red arrow, mESC-CM.

(D) Time course of calcium transients.

(E) Images showing clonal expansion of single-cell F5 iCPCs. F5 iCPCs derived from AC Fibs were seeded in low-density cultures to obtain isolated single cells. Single cell-derived colonies were then picked and expanded.

(F) Differentiation of iCPC clones followed by immunostaining for cardiac lineage markers revealed that iCPC clones were either tripotent (differentiated into CMs, SMs, and ECs) or bipotent (differentiated into CMs and SMs).

(G) Table showing the cardiac lineage potency of various iCPC clones determined by lineage-specific immunolabeling.

Scale bars, 200 μm (A), 50 μm (B), 10 μm (C), 1 s (D), 400 μm (E, single-cell seeding), 1,000 μm (E, colony formation and expansion), and 100 μm (F). See also Figures S3 and S5 and Movies S2 and S3.

factor combination (F11 or F5) and tissue of origin. These results indicate that adult fibroblasts from diverse tissues of origin can be reprogrammed reproducibly and stably into proliferative and multipotent iCPCs.

iCPCs Differentiate into Cardiomyocytes when Injected into the Cardiac Crescent of Mouse Embryos

After demonstrating iCPC reprogramming from various tissues of origin and defining their cardiovascular potency in vitro, we wanted to assess iCPC potency in the developing heart at a stage when the Nkx2.5-EYFP reporter used to isolate iCPCs would be expressed. We reasoned that if the iCPCs truly mimic native CPCs, then they would likewise respond to the rich cardiogenic signaling environment in the native cardiac crescent (CC) (Abu-Issa and Kirby, 2007) and differentiate rapidly into cardiomyocytes. iCPCs were first infected with a constitutive GFP-expressing lentivirus to track their progeny and then injected into the CC of mouse conceptus (E7.75, headfold-6 somite pairs stage). We cultured operated and un-operated control conceptus in a whole-embryo ex vivo culture system for 24 or 48 hr. During this culture period, the CC undergoes a morphogenic shift to develop into a beating linear heart tube. Therefore, the endogenous CPCs contained in the CC (E7.75) differentiate into contracting CMs within 24 hr (E8.5).

We injected 200–500 iCPCs per embryo in a total of 20 embryos in two separate experiments and performed live imaging on injected embryos to determine the location of the GFP^+ cells at the end of 24 and 48 hr of whole-embryo culture. In 3 of 20 embryos, no GFP^+ cells were detected, possibly because of leakage of cells out of the injection site during the injection. In 15 of 17 of the remaining embryos (88%), GFP^+ cells localized exclusively to the developing heart and appeared to contract along with the endogenous CMs (Figures 6A and 6B; Figure S6E; Movies S4 and S5). In contrast, AC Fibs injected into the CC were excluded completely from the developing heart tube. The majority of injected AC Fibs were also excluded from the embryo proper and localized to the ectoplacental cone (extra-embryonic region) (Figure 6C; Figures S6A and S6B). The presence of iCPCs in the heart tube suggests that they were able to respond to cardiac-morphogenetic signaling in the developing embryo and localize/differentiate along with host CPCs to the beating heart tube. To assess whether the iCPC-derived cells could integrate

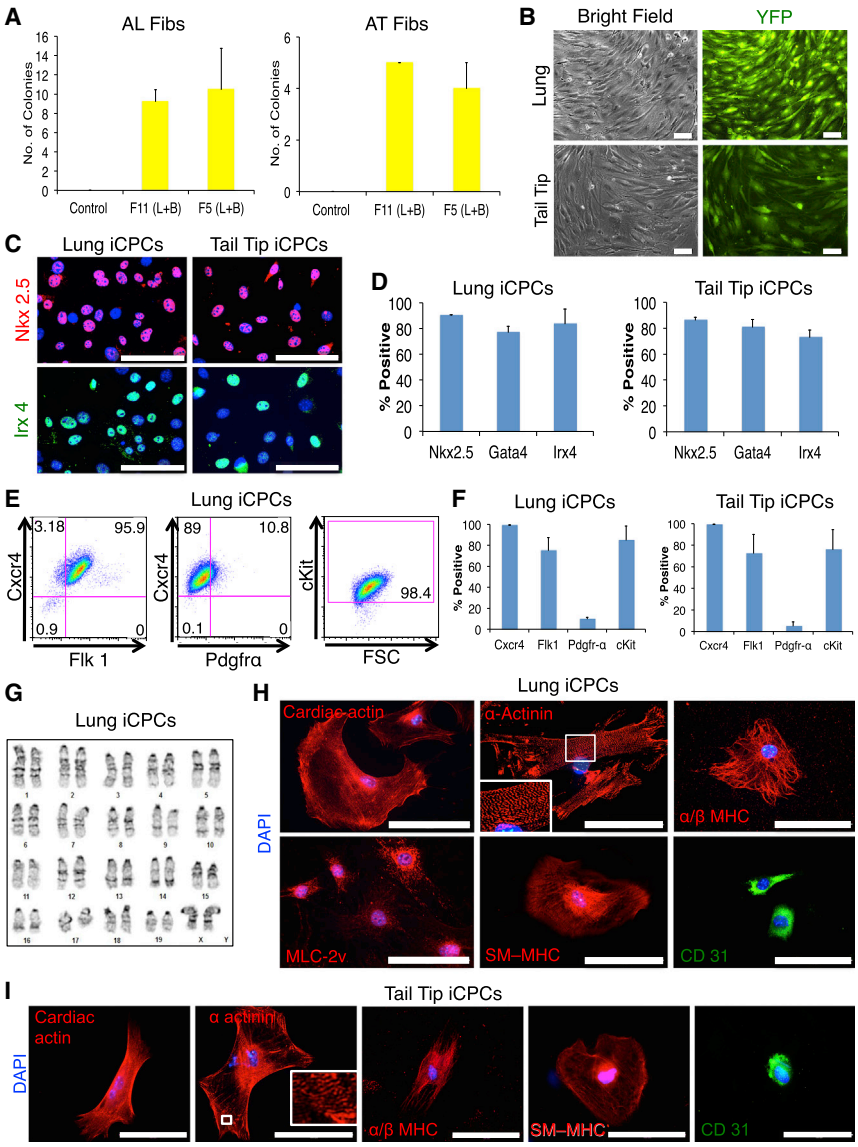


Figure 5. Cardiac Factors Stably Reprogram Adult Lung and Adult Tail Tip Fibroblasts into Proliferative and Multipotent iCPCs

(A) Number of Nkx2.5-EYFP+ colonies produced (per 50,000 seeded cells) after infection of adult lung and adult tail tip fibroblasts with 11 or 5 factors and culture in iCPC induction medium for 3 weeks (n = 4).

(B) EYFP$^+$ cells reprogrammed using five factors could be stably expanded without doxycycline for at least ten psgs (lung) and five psgs (tail).

(C) Immunolabeling revealed that F5 lung iCPCs and tail tip-iCPCs had nuclear localization of CPC TFs (merged images are depicted; red, Nkx2.5; green, Irx4; blue, DAPI).

(D) Quantification of (C) from three experiments with four or five fields of view containing 20–25 cells.

(E and F) Flow cytometry analysis revealed that F5 lung iCPCs (E and F) and tail tip iCPCs (F) expressed cell surface markers associated with CPCs (n = 3).

(G) F5 lung iCPCs showed a normal diploid karyotype.

(H) Lung iCPCs were multipotent and differentiated into CMs (cardiac actin, α-actinin, MLC-2v, and α/β MHC), SMs (SM-MHC), and ECs (CD31). Note the highly organized sarcomere staining for α-actinin.

(I) Tail tip iCPCs were multipotent and differentiated into cardiomyocytes (cardiac actin, α-actinin, and α/β MHC), smooth muscle cells (SM-MHC), and endothelial cells (CD31). Note the organized sarcomere staining for α-actinin.

Data are presented as mean. Error bars indicate SEM. Scale bars, 100 μm. See also Figures S2 and S3.

with host cells, some of the injected embryos were sectioned and immunostained with a GFP antibody. We observed that iCPC-derived cells integrated with host cells within the heart tube (Figure 6D).

To determine whether injected iCPCs differentiated into CMs in vivo, whole-mount embryos were co-immunostained for GFP and CM markers. Specimens were imaged as optical sections (1 μm) using multi-photon excitation microscopy, and three-dimensional reconstructions of the z stack images were taken. In the 24-hr cultured embryos, we detected multiple GFP$^+$ cells in the heart tube that co-stained for CM markers such as MLC-2v and cardiac actin. In 24-hr cultured embryo samples, the iCPC-derived CMs had an elongated appearance and looked morphologically distinct from the native CMs (Figure 6E; Movie S6). However, iCPC-derived CMs in the 48-hr cultured embryos had a rounder morphology and appeared similar in shape and size to the native CMs (Figure 6F; Figure S6F; Movie S7). iCPC-CMs were observed in developing atria and ventricles as well as the outflow track, showing no spatial preference within the heart tube. In contrast, injected AC Fibs failed to differentiate into CMs, as indicated by the absence of staining for CM markers (Figure S6C and S6D).

Although we observed endothelial differentiation from iCPCs in vitro, we were unable to convincingly detect iCPC-derived CD31$^+$ cells in vivo. Because of the limited endothelial potency of iCPCs (only 1%–5% detected during in vitro differentiation), we may have missed rare CD31$^+$ cells within embryos. The whole-embryo culture technique used here cannot be extended beyond E9.75 because the embryo becomes increasingly dependent upon formation of a chorio-allantoic placenta and interaction with its mother (Lawson et al., 1991). Therefore, we were unable to assay embryos for smooth muscle because the onset of smooth muscle differentiation is after E10.5 (Miano et al., 1994), which exceeded our whole-embryo culture duration (E7.75–E9.75).

iCPCs Injected into Adult Heart after Myocardial Infarction Engraft within the Scar Tissue and Differentiate into Cardiac Lineage Cells In Vivo

To examine the applicability of the iCPC technology for cardiac regenerative medicine, we tested the potency of iCPCs in an

A

Ex vivo culture	Total Injected	Abnormal after culture	GFP negative	GFP in heart	GFP outside heart
24 hrs	16	0/16	3/16	11/13 (85%)	2/13 (15%)
48 hrs	4	0/4	0/4	4/4 (100%)	0/4
Total	20	0/20	3/20	15/17 (88%)	2/17 (12%)

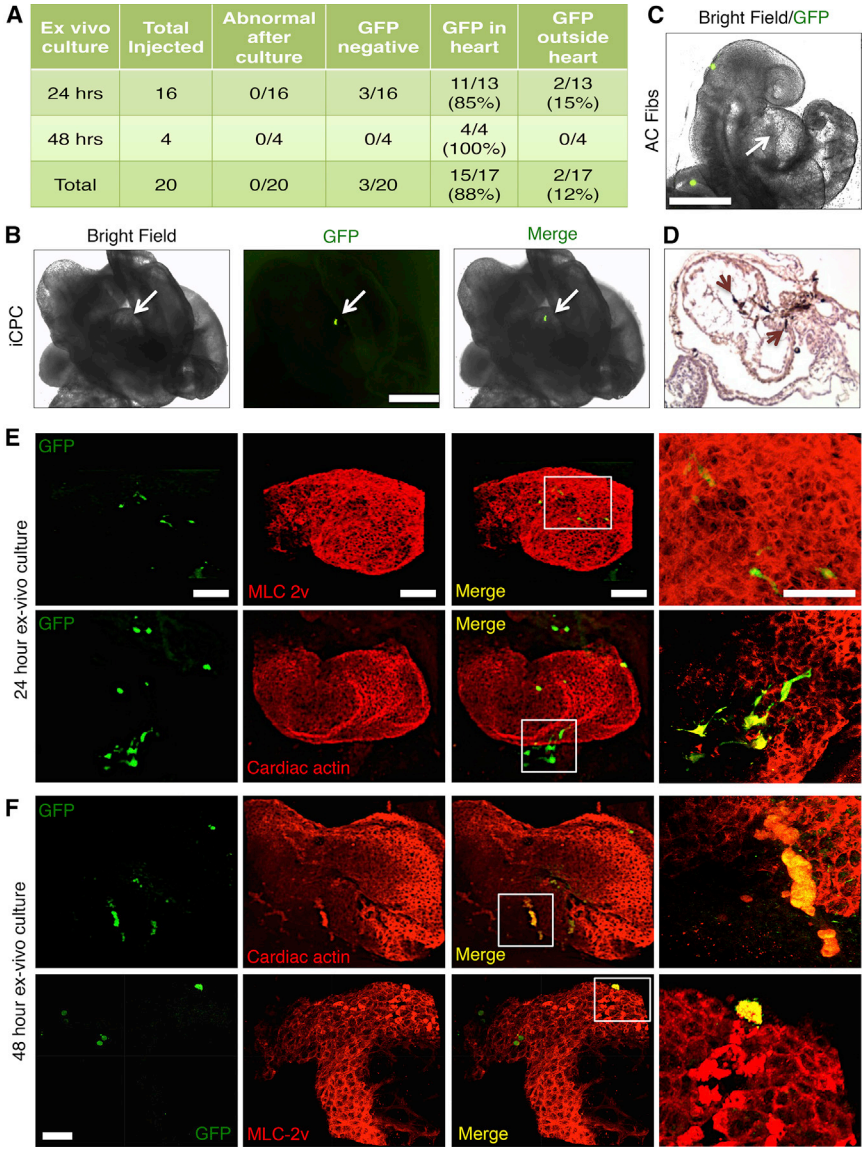


Figure 6. iCPCs Localize to the Developing Heart Tube and Differentiate into Cardiomyocytes upon Injection into the Cardiac Crescent of Mouse Embryos

(A) The number of embryos injected with F11 iCPCs and the location of iCPC-derived cells 24 or 48 hr after whole-embryo culture.

(B) F11 iCPCs (labeled with GFP-expressing lentivirus) injected into the cardiac crescent of mouse embryos colonized the developing heart tube, as assessed after 24 hr of whole-embryo culture. See also Movie S4 for 24-hr cultured embryos and Movie S5 for 48-hr cultured embryos.

(C) AC Fibs (labeled with GFP-expressing lentivirus) injected into the cardiac crescent were excluded from the developing heart tube (arrow), as assessed after 24 hr of whole-embryo culture.

(D) Histological sections of iCPC-injected embryos were stained for GFP antibody (dark brown). iCPC-derived cells (arrows) integrated with the host cells in the developing heart tube.

(E) 24-hr cultured, iCPC-injected embryos were immunostained in whole-mount preparations for CM markers and GFP. Three-dimensional reconstruction images show iCPCs differentiated into CMs, as indicated by co-expression of CM markers and GFP.

(F) iCPC-CMs attained a shape/size similar to native CMs after 48-hr culture.

Scale bars 500 μm (B and C) and 100 μm (E and F). See also Figure S6 and Movies S4, S5, S6, and S7.

adult heart injury model. To induce myocardial infarction, we permanently ligated the left coronary artery in 8-week-old C57BL/6J male mice. Two days post-MI, we injected 1–1.5 million GFP-labeled iCPCs (F5 iCPCs reprogrammed from AC Fibs) or PBS into the border zone of the infarct and monitored the animals for 4 weeks. Kaplan-Meier survival analysis revealed that 75% of the animals that received an iCPC injection survived to 4 weeks, which was significantly higher compared with 11% of mice that survived after PBS injection (Figure 7A). To determine whether injected iCPCs could differentiate into cardiac lineage cells, we harvested the hearts of injected animals and performed immunohistochemistry on tissue sections with cardiac lineage differentiation markers and GFP. We first analyzed the hearts of animals 4 days after iCPC injection. A majority of surviving iCPCs were localized at the border of the infarct zone and showed faint expression of cardiac actin (Figure 7B). This suggested that some iCPCs were differentiating into CMs. When we analyzed the hearts of animals 28 days after injection, we noticed that some iCPC-derived cells migrated and engrafted more than 1 mm deep within the scar tissue (Figure 7C; Figure S7A). Moreover, the iCPC-derived cells exhibited strong expression of cardiac actin, indicating their differentiation into CMs. Even though iCPC-derived CMs did not completely resemble host CMs in morphology, they showed organized cardiac actin staining (Figure 7C; Figure S7A). Some iCPCs-CMs attained a rod-shaped morphology and appeared to be highly aligned, a characteristic of adult CMs (Figure 7D; Figure S7A). The intensity of cardiac actin staining in iCPC-derived CMs (yellow arrow) is comparable with that in host CMs (red arrow), suggesting that iCPCs had differentiated into functional CMs when assessed 28 days after injection. Some of the injected iCPCs differentiated into smooth muscle cells (Figure 7E; Figure S7B) and endothelial cells (Figure 7F; Figure S7C), as indicated by expression of smooth muscle actin and CD31, respectively. During the 4-week time course of the study, we did not observe any tumor formation. These data indicate that iCPCs can survive, engraft, and differentiate into cardiac lineage cells in the post-MI heart and improve survival in treated mice.

DISCUSSION

Here we demonstrate that a combination of 11 (*Mesp1*, *Mesp2*, *Gata4*, *Gata6*, *Baf60c*, *SRF*, *Isl1*, *Nkx2.5*, *Irx4*, *Tbx5*, and *Tbx20*)

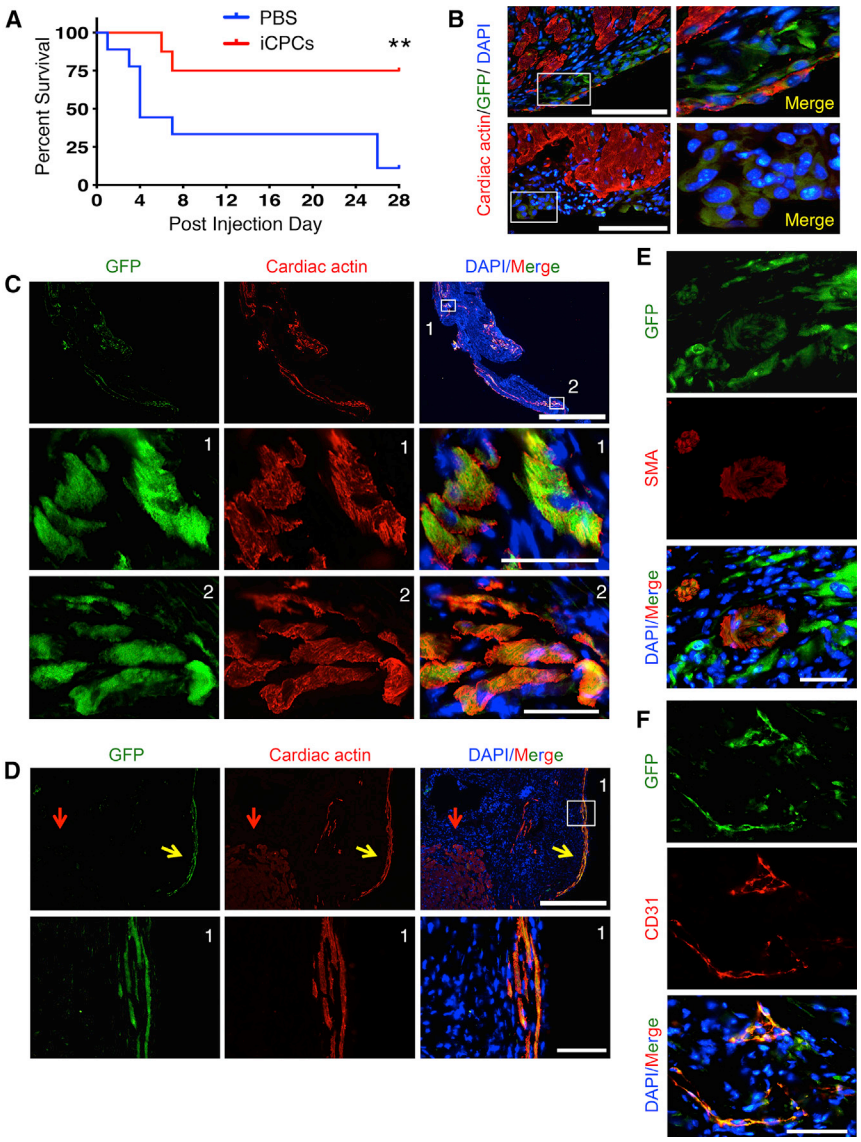


Figure 7. iCPCs Differentiate into Cardiac Lineage Cells In Vivo and Improve Survival in Mice after Myocardial Infarction

(A) Kaplan-Meier survival analysis revealed that iCPC injection significantly improved survival in treated animals compared with controls (n = 8 for iCPCs, n = 9 for PBS). **p < 0.01, Mantel-Cox test.

(B) AC Fib-derived F5 iCPCs (labeled with GFP-expressing lentivirus) were injected into the MI border zone, and hearts were analyzed 4 days after injection. Immunolabeling of tissue sections revealed that a majority of surviving cells were localized on the edge of the scar tissue and showed faint expression of cardiac actin. Merged images are depicted. Scale bars, 100 μm.

(C) Injected iCPCs differentiated into cardiomyocytes based on organized cardiac actin immunolabeling (28 days after injection). The lower two rows of images are from insets in the top row. The tissue section represents the apex region of the left ventricular wall. Scale bars, 1,000 μm and 50 μm in the insets.

(D) Immunolabeling for GFP and cardiac actin revealed that some iCPC-derived cardiomyocytes aligned and attained a rod-shaped morphology 28 days after injection. The lower row of images shows insets from the top row. Red arrow, host CMs; yellow arrow, iCPC-derived CMs. Scale bars, 400 μm and 50 μm in the insets.

(E) iCPCs differentiated into smooth muscle cells within scar tissue based on co-expression of GFP and smooth muscle actin (SMA), as assessed 28 days after injection. Scale bar, 50 μm.

(F) iCPCs differentiated into endothelial cells within scar tissue based on co-expression of GFP and CD31, as assessed 28 days after injection. All images are from Mouse 1. Scale bar, 50 μm.

See also Figure S7 (mice 2 and 3).

or 5 (*Mesp1*, *Tbx5*, *Gata4*, *Nkx2.5*, and *Baf60c*) cardiac factors, in conjunction with Wnt and JAK/STAT signaling, can stably reprogram adult fibroblasts from three different tissue origins into proliferative and multipotent iCPCs. We screened defined factors and culture conditions to induce reprogramming based on two criteria: the capability to activate the Nkx2.5-EYFP reporter and the ability of the resulting EYFP$^+$ cells to maintain a proliferative state on extended passaging without forced expression of reprogramming factors. This stringent approach allowed screening for factors/culture conditions that induced stably reprogrammed iCPCs. The iCPCs differentiate into the major cell lineages found in the heart, including SMs, ECs, and CMs.

Most lineage reprogramming studies to date have utilized embryonic or neonatal fibroblasts as a starting cell source for reprogramming. These cells, although easier to reprogram, are clinically less relevant because comparable cell types for humans are not available. Therefore, we opted to use adult fibroblasts in our study. It is well documented that adult cells are more challenging to reprogram compared with embryonic or neonatal cells (Takahashi and Yamanaka, 2006; Yu et al., 2007). As cells mature, their epigenetic state becomes progressively more rigid and, therefore, less amenable to reprogramming. Therefore, the efficiency of iCPC reprogramming was low (0.01%–0.02%) but comparable with efficiencies reported for iPSC reprogramming (Takahashi and Yamanaka, 2006). We reprogrammed fibroblasts from both male and female mice that ranged from 1–3 months in age, and we did not detect differences in reprogramming efficiency. Although reported in vitro iCM reprogramming efficiencies range from 5%–20%, it should be noted that these values were based on the percentage of cells showing reporter activity 1–2 weeks after infection with iCM factors. Completely reprogrammed iCMs represent a small fraction of the cells that initially showed reporter activity. In spite of low reprogramming efficiencies, we were able to generate millions of stably reprogrammed iCPCs because of their proliferative capacity.

Based on current knowledge, transdifferentiation is thought to be a long and stochastic process in which cells transition through intermediate states before achieving a stable reprogrammed state (Jaenisch and Young, 2008). The first EYFP$^+$ cells

were detected 3–5 days after dox induction and, over the course of 3 weeks, underwent a dramatic morphological change to develop into proliferative colonies. The cells continued to undergo morphological change for the first two to three psgs. Gene expression analysis of psg 1 cells revealed that a relatively small, 2- to 8-fold increase in cardiac marker expression was sufficient to initiate reprogramming toward an iCPC state, whereas late-psg cells demonstrated a further increase in the expression of cardiovascular genes and a decrease in the expression of fibroblasts genes. This indicates that even though the Nkx2.5-EYFP reporter is activated very early (3–5 days), iCPC reprogramming progressively continues over the initial two to three psgs (35–40 days), after which the cells reach a stable reprogrammed state. This is in congruence with reprogramming processes for iCMs (Qian et al., 2013) as well as iPSCs (Brambrink et al., 2008).

RNA-seq analysis revealed that iCPCs have cardiac mesoderm-restricted gene expression. Interestingly, *Nanog* was the only pluripotency-associated gene upregulated in iCPCs. *Nanog* is also expressed in the epiblast and prevents Bmp-induced differentiation (Shin et al., 2011). Correspondingly, Bmp genes (4/6/7) were downregulated in iCPCs, and negative regulators of Bmp signaling, such as *smad6/7* and *Grem1*, were upregulated. Recently, it has been shown that inhibition of Bmp signaling along with Wnt activation can maintain CPCs derived from human PSCs in a stem cell state (Cao et al., 2013).

Ieda et al. (2010) have shown that iCMs are directly reprogrammed from fibroblasts without transitioning through a progenitor state. During iCM reprogramming, cell proliferation is staunchly suppressed along with the activation of CM differentiation-specific genes SM genes are also expressed during iCM reprogramming (Qian et al., 2013; Wada et al., 2013). Conversely, during iCPC reprogramming, cell proliferation is activated, cardiac mesoderm-associated genes are turned on, and cardiac lineage differentiation genes are not expressed. This indicates that the reprogramming route fibroblasts follow during conversion to iCPCs is distinct from the route for iCM reprogramming.

The iCPC-CMs progressively mature with extended low-serum culture based on myofilament immunolabeling and organization. However, unlike ES- or iPSC-derived CMs, iCPC-CMs did not exhibit spontaneous contractions in culture. Adult heart-derived CPCs have also been differentiated into CM-like cells in vitro, which failed to contract spontaneously. However, we were able to induce contractions in iCPC-CMs by co-culturing with mESC-CMs. iCPC-CMs developed gap junctions with mESC-CMs and showed synchronous calcium transients. This result demonstrates the ability of iCPCs to differentiate into fully functional CMs and couple with other CMs. When injected into the cardiac crescent of mouse embryos, iCPCs not only colonized the developing heart tube but also integrated and differentiated into CMs. We noticed that iCPC-CMs appeared to mature in the cultured embryos and attain a shape and size similar to native CMs. It is interesting to note that iCPCs could differentiate into CMs 24–48 hr after in vivo injection. This suggests that iCPCs, in the appropriate microenvironment, can rapidly differentiate into functional CMs.

Lineage tracing studies have shown that the mammalian heart is derived from progenitors of the first and second heart fields as well as the epicardium and neural crest (Abu-Issa and Kirby, 2007). Some of these progenitor populations have been identified in vivo and during in vitro differentiation of PSCs. iCPCs share common properties with other defined CPC populations, such as the ability to self-renew and the potency to differentiate into cardiac lineage cells (CMs, SMs, and ECs). The iCPCs exhibit some heterogeneity, as detected by cell surface markers and gene expression, so it is possible that iCPCs contain subsets of both first heart field and second heart field CPCs, among others. Further characterization is necessary to determine whether iCPCs resemble one or more of these native CPC populations. It is possible that the appropriate combination of CPCs can generate cardiac organ buds that can be used for both drug discovery and regenerative medicine applications (Takebe et al., 2015). Also, the adult heart consists of distinct specialized CMs, including atrial, ventricular, and conduction system cells. Whether iCPCs differentiate into a particular type of CM or a mixture of all three requires further experimentation. However, current data examining MLC2v expression suggest a majority of iCPC-CMs to be ventricle-like.

iCPC reprogramming provides a promising cell source for cardiac regenerative therapy. Direct reprogramming to iCPCs avoids transitioning through an iPSC state and, therefore, theoretically reduces the tumorigenic risk associated with pluripotent cells. Also, iCPCs can be readily expanded (9.14×10^{15} million to 1×10^{16} million cells in 20 psgs), and, therefore, cardiac cell therapy applications that require millions to billions of cells (progenitors or differentiated progeny) are possible (Lalit et al., 2014). iCPCs injected into the adult mouse heart post-MI significantly improved survival in treated mice. Some of the injected iCPCs survived and differentiated into CMs, SMs, and ECs, repopulating the scar tissue with relevant cardiac lineage cells. iCPC-derived CMs showed a mature-like, rod-shaped morphology and alignment as well as organized cardiac actin staining. Importantly, iCPC-derived cells contributed to the vasculature within the scar area by differentiating into SMs and ECs. These results provide promise for future studies that can examine the effects of iCPC therapy on cardiac functionality as well as optimize cell delivery strategies, including tissue-engineered scaffolds. In vivo reprogramming post-MI of native cardiac fibroblasts to iCMs has shown promise in mouse models (Qian et al., 2012; Song et al., 2012), and it is possible that in vivo reprogramming into iCPCs may show benefits. The scalability of iCPCs also enables in vitro applications such as disease modeling, drug discovery, and basic cardiovascular research. Future studies of the reprogramming of human cells into iCPCs will be an important future step to advance these applications.

EXPERIMENTAL PROCEDURES

Isolation of Primary Fibroblasts

For isolation of adult fibroblasts, the respective organs were derived from 1- 3-month-old double-transgenic mice (Nkx2.5-EYFP/rtTA). The organs were washed with PBS and minced in fibroblast medium (DMEM, 10% fetal bovine serum [FBS], 1% non-essential amino acids [NEAAs]/L-glutamine/penicillin [pen]/streptamycin [strep]) to obtain tissue pieces around 1 mm^3 in size. These were briefly trypsinized (0.25% trypsin-EDTA) for 10 min. Explants were then plated on 0.1% gelatin-coated dishes in fibroblast medium for 10–12 days. Fibroblasts migrated from explants were harvested, filtered through a 40-μm cell strainer (BD Biosciences) to avoid contamination of the heart tissue, passaged one to two times, and frozen or used for experiments.

Infection of Fibroblasts with Lentiviruses

Lentivirus particles were produced as detailed in the Supplemental Experimental Procedures. Primary fibroblasts were seeded in a gelatinized 12-well plate at density of 50,000 cells/well 2 days prior to infection. Cells were then fed with lentivirus infection medium (lentivirus supernatant + 8 μg/ml Polybrene, Sigma), and infection was continued for 48 hr.

iCPC Culture/Differentiation Conditions

The lentivirus infection medium was replaced with iCPC induction medium (DMEM, 10% FBS, 1% NEAA, 1% L-glutamine, 1% Pen/strep, 4 μg/ml dox [Sigma], 2.5 μM BIO [Cayman Chemical], and 10^3 units/ml LIF [Millipore]). After reprogramming was achieved, iCPCs were maintained in iCPC maintenance medium (iCPC induction medium without dox). The iCPCs were split 1:6 on a 10-cm dish, and a confluent dish yields 2.5–3.0 million cells. To differentiate, iCPC were aggregated in 24-well low-attachment plates (Corning Life Sciences) for 4–6 days in cardiac differentiation medium (fibroblast medium, 5 μM IWP4 [Stemgent], 50 ng/ml Bmp4 [RD Systems], 10 ng/ml Vegf [RD Systems], and 30 ng/ml bFgf [RD Systems]). Aggregates were then plated on gelatin-coated dishes and cultured in fibroblast medium containing 1% serum for 10–50 days.

Immunocytochemistry and Flow Cytometry

Cells were plated, fixed, and stained with various antibodies via a standard protocol detailed in the Supplemental Experimental Procedures.

qRT-PCR

Total RNA was isolated using the RNAqueous kit (Invitrogen). Reverse transcription was performed using iScriptTM Reserve Transcription Supermix (Bio-Rad). qRT-PCR was performed on a CFX96TM real-time system (Bio-Rad) using SsoFastTM EvaGreen Supermix (Bio-Rad). Minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines were followed when designing qPCR experiments. mRNA levels were normalized by comparison with β-actin (Δ CT), and data are presented as fold change with respect to expression in AC Fibs (ΔΔ CT).

RNA-Seq and Bioinformatics Analysis

RNA was extracted as above from AC Fib-derived iCPCs either at low psg (1–3) or high psg (8–10). Gene expression data for mESC-CPCs and mESC-CMs were obtained from Wamstad et al. (2012) and used as a positive control. For heatmap analysis, gene expression was compared with uninfected AC Fibs. RNA-seq was performed using HiSeq 2500 (Illumina) in duplicates from independent biological samples. Sequencer outputs were processed using CASAVA 1.8.2 (Illumina), and each sample's reads were processed using RSEM version 1.2.3 to obtain expression measures for genes. Differential analysis was done using EBSeq version 1.5.3., and the targeted false discovery rate for each run was 0.05. Transcripts per million values were used for all calculations. The Search Tool for Retrieval of Interacting Genes/Proteins (STRING) database was used for GO analysis (Franceschini et al., 2013). See the Supplemental Experimental Procedures for more details.

Ca^{2+} Imaging

Cells were loaded with Rhod-2, AM (Invitrogen) for 20 min at 37°C in fibroblast medium, washed, and incubated for an additional 30 min at 37°C to allow for de-esterification of the dye. Rhod-2-loaded cells were analyzed with a Nikon epifluorescence microscope with NIS elements software.

Embryo Injections, Immunostaining, and Imaging

Cardiac crescent-stage mouse embryos were obtained by timed matings. iCPCs and AC Fibs were infected with a GFP lentivirus (Addgene, catalog no. 17448) to trace cells in vivo. Approximately 200–500 iCPCs or AC Fibs were introduced into the cardiac crescent of dissected mouse embryos in dissection medium via a mouth-held glass capillary (~20-μm opening). Operated and stage-matched unoperated embryo samples were then placed into whole-embryo culture medium (Downs, 2006) and cultured for 24 or 48 hr. At the end of the culture period, embryos were imaged using a Nikon epifluorescence microscope to determine the location of injected GFP^+ cells. Embryos were then fixed with 4% paraformaldehyde (PFA). Immunofluorescence of whole-mount embryos was done, and imaging was performed using a custom-built multi-photon microscope. Imaris software (Bitplane) was used to make three-dimensional reconstructions. See the Supplemental Experimental Procedures for more details.

Mouse MI Model and iCPC Injections

Animal use and procedures were approved by the University of Wisconsin Animal Care and Use Committee. MIs were induced surgically in 8-week-old male C57BL/6J mice by permanent ligation of the left coronary artery. Two days after MI surgery, in mice with echocardiographically proven large infarctions, a total of 1–1.5 million GFP-labeled iCPCs (reprogrammed using five factors and AC Fibs) or PBS were injected in three injections of approximately 25 μl in the border zone. Animals were monitored for 4 weeks, and the hearts of surviving animals were excised for immunohistochemistry. See the Supplemental Experimental Procedures for more details.

Statistical Analysis

Differences between multiple groups were tested for statistical significance using one-way ANOVA followed by Tukey's post hoc analysis. For comparison of two groups, unpaired Student's t test was used. $p < 0.05$ was regarded as significant.

ACCESSION NUMBERS

The accession number for the RNA-seq data reported in this paper is GEO: GSE61486.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven movies and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2015.12.001.

ACKNOWLEDGMENTS

We would like to thank the UWCCC Experimental Pathology Core (especially Joe Hardin) for help with immunohistochemistry experiments. M.R.S. and W.C.C. would also like to thank the Graduate School at the University of Wisconsin-Madison. The research was supported by NIH Grants U01HL099773 (to T.J.K. and J.A.T.), R01 HL129798 (to T.J.K. and G.E.L.), RO1 HD42706 and RO1 HD079481 (to K.M.D), S10RR025644 (to T.J.K.), and AHA pre-doctoral fellowship 12PRE9520035 (to P.A.L.).

Received: October 15, 2014
Revised: August 14, 2015
Accepted: December 3, 2015
Published: February 11, 2016

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