Induced pluripotent stem cells (iPSCs) hold great promise for the development of patient-specific therapies for cardiovascular disease. However, clinical translation will require preclinical optimization and validation of large-animal iPSC models.

**Objective:** To successfully derive endothelial cells from porcine iPSCs and demonstrate their potential utility for the treatment of myocardial ischemia.

**Methods and Results:** Porcine adipose stromal cells were reprogrammed to generate porcine iPSCs (piPSCs). Immunohistochemistry, quantitative PCR, microarray hybridization, and angiogenic assays confirmed that piPSC-derived endothelial cells (piPSC-ECs) shared similar morphological and functional properties as endothelial cells isolated from the autologous pig aorta. To demonstrate their therapeutic potential, piPSC-ECs were transplanted into mice with myocardial infarction. Compared with control, animals transplanted with piPSC-ECs showed significant functional improvement measured by echocardiography (fractional shortening at week 4: 27.2±1.3% versus 22.3±1.1%; P<0.001) and MRI (ejection fraction at week 4: 45.8±1.3% versus 42.3±0.9%; P<0.05). Quantitative protein assays and microfluidic single-cell PCR profiling showed that piPSC-ECs released proangiogenic and antiapoptotic factors in the ischemic microenvironment, which promoted neovascularization and cardiomyocyte survival, respectively. Release of paracrine factors varied significantly among subpopulations of transplanted cells, suggesting that transplantation of specific cell populations may result in greater functional recovery.

**Conclusions:** In summary, this is the first study to successfully differentiate piPSCs-ECs from piPSCs and demonstrate that transplantation of piPSC-ECs improved cardiac function after myocardial infarction via paracrine activation. Further development of these large animal iPSC models will yield significant insights into their therapeutic potential and accelerate the clinical translation of autologous iPSC-based therapy. (Circ Res. 2012;111:882-893.)

**Key Words:** induced pluripotent stem cells ■ large-animal models ■ paracrine activation ■ myocardial infarction ■ molecular imaging ■ ischemic heart disease ■ vascular biology

In recent years, induced pluripotent stem cells (iPSCs) have become a popular alternative to embryonic stem cells (ESCs) for regenerative medicine. Not only do iPSCs provide an unlimited source of pluripotent cells that are capable of self-renewal, but they also circumvent the ethical concerns associated with ESC derivation and can be transplanted autologously, limiting potential immunologic rejection. To date, iPSCs have been generated from a growing number of adult somatic cell types.
Swine are an ideal large-animal model for the clinical translation of experimental medical therapies due to their resemblance to human organ size and physiology and their relatively long lifespan. Consequently, several recent studies have utilized the porcine model as a preclinical tool for the clinical translation of cell-based therapy. These studies, however, have been limited to porcine mesenchymal stem cells. Perhaps the most significant achievement is the development of porcine iPSCs, which can potentially fill the gap between the transplantation of these cells into mice and the initiation of the first clinical trials using iPSCs in humans.

**In This Issue, see p 815**

**Editorial, see p 824**

It has long been appreciated that endothelial cells play an important role in the survival and function of nearby cardiomyocytes by promoting vascular regeneration and angiogenesis. In the normal heart, capillary networks can be found near almost every cardiomyocyte, modulating cardiac performance, contraction, and growth. Thus, it is not surprising that in the ischemic myocardium, a deficit of blood flow leads to progressive cardiomyocyte death and myocardial dysfunction. To exploit the potential therapeutic benefits of endothelial cells, several investigators have differentiated endothelial cells from iPSCs generated from mice, dogs, and humans, demonstrating that paracrine release of proangiogenic factors promote functional recovery in small animal models of ischemia. Prior studies, however, have analyzed entire cell populations. Because cell populations are heterogeneous, especially those generated from exogenous factors such as iPSCs and their derivatives, it is critical to study the regulation of angiogenesis at the single-cell level.

In this study, we lay the foundation for the clinical application of iPSCs for the treatment of ischemic heart disease. After generation of iPSCs from adipose tissue of adult miniswine, we successfully differentiated these cells into endothelial cells that share similar morphological and functional properties to their endogenous counterparts (ie, endothelial cells isolated from the porcine aorta). We then demonstrated that porcine iPSC-derived endothelial cells (piPSC-ECs) could provide therapeutic benefit in a murine model of myocardial infarction (MI). To explore the biological mechanisms for functional recovery, we showed that piPSC-ECs released both proangiogenic and antiapoptotic factors that promote neoangiogenesis and cardiomyocyte survival, respectively, at the single-cell level using microfluidic dynamic arrays. We then demonstrated that the release of paracrine factors varied among specific subpopulations of iPSC-ECs, suggesting that transplantation of specific cell subpopulations may result in greater functional recovery. Finally, cell delivery and engraftment are monitored using positron emission tomography/computed tomography (PET/CT) as well as MRI in a porcine model of MI, laying the groundwork for the development of future imaging protocols to monitor cell delivery and engraftment in humans.

### Methods

**Derivation of Porcine iPSCs**

Porcine adipose tissue was harvested and cultured, as described in detail in the Online Supplemental Methods section. Approximately $1 \times 10^7$ porcine adipose stromal cells (pASCs) were seeded onto a 24-well tissue culture dish and maintained with human ASC growth medium. Cells were then transduced with 5 μg/mL polybrene (Sigma) plus individual lentivirus containing Oct4, Sox2, Klf4, and c-MYC in a 4:2:2:1 ratio. On day 6, cells were dissociated with 0.05% trypsin-EDTA (Gibco) and counted with a hemocytometer. Reprogramming of piPSCs was performed on mouse embryonic fibroblast (MEF) feeder cells. Ten thousand cells were then transfected onto an MEF feeder layer in a gelatin-coated 6-well plate and cultured with human ESC growth medium containing knockout DMEM (Invitrogen), 20% knockout FBS (Invitrogen), 2 mmol/L L-glutamine (Invitrogen), 1X nonessential amino acids (Invitrogen), 1X-2-mercaptoethanol (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 ng/mL human β-fibroblast growth factor (bFGF) (Invitrogen). ESC-like colonies with distinct edges appeared on day 13 to 15. On day 15, positive colonies with ESC-like morphologies were isolated with a glass needle and seeded onto new...
MEFs. These pASC-derived iPSCs were maintained on MEF feeder layers with iPSC medium containing knockout DMEM (Invitrogen), 20% ES-qualified FBS (Invitrogen), 2 mMol/L L-glutamine (Invitrogen), 1% nonessential amino acids (Invitrogen), 1x2-mercaptoethanol (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 ng/mL human bFGF (Invitrogen). Each selected colony was then established as one individual pASC-iPSC line. A total of 10 lines from the 5 miniswine were generated.

**Differentiation of pASC-Derived ECs**

For endothelial cell differentiation, well maintained iPSCs at passage number 25 were treated with type IV collagenase (Invitrogen, Carlsbad, CA) for 15 minutes at 37°C and transferred to ultra-low attachment plates (Corning Life Sciences, Kennebunk, ME) containing differentiation medium for 2 days. The differentiation medium consisted of DMEM (Invitrogen) containing 20% knockout serum (Invitrogen), 4.5 g/L L-glutamine (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mMol/L 2-mercaptoethanol (Invitrogen), 50 U/mL penicillin (Invitrogen), 50 μg/mL streptomycin (Invitrogen), 10 ng/mL Activin A (R&D Systems), and 20 ng/mL bone morphogenetic protein-4 (BMP-4) as described previously. The differentiation medium was supplemented with 25 ng/mL vascular endothelial growth factor (VEGF) (R&D Systems), 8 ng/mL bFGF (R&D Systems), and 10 μg/mL TGF-β inhibitor SB431542 (Sigma). The 2-day embryoid bodies (EBs) were then seeded onto 0.5% gelatin-coated dishes and cultured for another 8 days in differentiation media in absence of BMP-4. On day 10, plated EBs were digested and sorted by fluorescence activated cell sorting (FACS) for CD31-positive cells. The CD31-positive cells were collected and seeded into one well of a 6-well plate with EGM-2 medium (Lonza).

**Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis to Confirm Pluripotent State in iPSCs**

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed after isolation of total RNA and cDNA. Using Taqman Gene Expression Assays, PCR reactions were performed with a StepOnePlus Realtime-PCR 7900 HT System (Applied Biosystems, Foster City, CA). Primers for genes defining pluripotency and confirming germ layer differentiation are listed in Online Table I.

**Microarray Hybridization and Data Analysis of iPSCs and piPSC-ECs**

Total RNA samples were hybridized to Affymetrix GeneChip Porcine Genome Array, and then normalized and annotated by the Affymetrix Expression Console software. For comparison to our generated lines, we obtained piPSC lines (piPSC-ID4, piPSC-ID6, and piPSC-IC1) from R. Michael Roberts and Toshihiko Ezashi (University of Missouri, Columbia, MO) and are grateful for their contribution. We also obtained the expression data for piPSC lines and fibroblast from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo, GSE15472). The Pearson correlation coefficient was calculated for each pair of samples using the expression level of transcripts. For hierarchical clustering, a Pearson correlation for average linkage clustering was used.

**Generation of a Murine Model of MI and Intramyocardial Delivery of Therapeutic Cells**

Ligation of the mid left anterior descending (LAD) artery was performed in 8- to 10-week-old female NOD SCID mice (Jackson Laboratory) under anesthesia (2% to 3% inhaled isoflurane) by a single experienced microsurgeon (Y.G.). Survival rate for each surgery group was ~90%. Myocardial blanching and ECG changes confirmed MI after ligation. Animals were randomized into 4 groups: (1) phosphate-buffered saline (PBS) as control, (2) 1x10⁶ pASCs, (3) 1x10⁶ aorta-ECs, and (4) 1x10⁶ pASCs-ECs (n=20/group). Immediately after LAD artery ligation, animals were injected intramyocardially near the peri-infarct zone at 2 different sites using a 31-gauge Hamilton syringe (25 μL per injection). Study protocols were approved by the Stanford Animal Research Committee. Animal care was provided in accordance with the Stanford University School of Medicine guidelines and policies for the use of laboratory animals.

**Optical Bioluminescence Imaging and Micro Positron Emission Tomography for Tracking Cell Fate**

Please see Online Data Supplement Methods.

**Angiogenesis and Antiapoptosis Cytokine Array After Hypoxia Exposure**

Please see Online Data Supplement Methods.

**Single-Cell Gene Expression Profiling of Transplanted iPSC-ECs and ASCs**

Please see Online Data Supplement Methods.

**Assessment of Paracrine Activation by Specific Subpopulations**

Because cell populations of pASC and piPSC-ECs are heterogeneous, we hypothesized that the release of paracrine factors varied by specific cell subpopulations. To investigate this hypothesis, we used single-cell expression profiling to analyze the release of paracrine factors by subpopulations of pASCs and piPSC-ECs. One week after transplantation of pASCs and piPSC-ECs into mice undergoing LAD artery infarction (n=5), we euthanized the mice and harvested and dissociated their hearts as described above. We then used FACS to isolate subpopulations of cells and sort them into a 96-well 0.2-μL PCR plate, based on their expression of endothelial specific markers, specifically CD31, CD34, and CD144. We then performed single-cell gene expression analysis to determine the relative amounts of paracrine factors released by each subpopulation of pASC and piPSC-ECs: (1) CD31⁺/CD34⁻/CD144⁺, (2) CD31⁺/CD34⁺/CD144⁻, and (3) CD31⁺/CD34⁺/CD144⁻. Very few cells expressed all three markers and this subpopulation was, therefore, not analyzed.

**Tracking Transplanted Cells in Miniswine Using PET and CT Imaging**

Please see Online Data Supplement Methods.

**Statistical Analysis**

For analysis of microarray data, a Pearson correlation coefficient was utilized. A probability value of <0.05 was considered significant. ANOVA with post hoc testing was used to analyze the differences in qRT-PCR, single-cell PCR, angiogenesis, and antiapoptotic cytokine data between groups, percent infarct size by triphenyltetrazolium chloride (TTC), and density of tDd-mediated dUTP nick-end labeling (TUNEL)-positive nuclei. The percent infarct size is calculated by dividing the area of infarct by the total area of the left ventricle. Repeated measures of ANOVA were used to analyze MRI, bioluminescence imaging (BLI), and echocardiographic data. All data are expressed as mean±SD.

**Results**

**Derivation of iPSCs From Adult Miniswine**

Porcine adipose stromal cells (pASCs) were harvested and successfully reprogrammed into iPSCs from 5 young adult Yucatan miniswine pigs. Retroviral overexpression of Oct4, Sox2, Klf4, and c-Myc was used in a 4:2:2:1 ratio to induce pluripotency. Compact colonies of cells staining positive for alkaline phosphatase, a universal pluripotent marker (Online Figure IA), were initially observed under bright-field microscopy 15 days after transduction (Figure 1A). Individual colonies were mechanically dissociated using a pulled Pasteur pipette and transferred onto irradiated MEF feeder layers for clonal expansion into distinct cell lines. A total of 10 iPSC lines were generated, 2 lines from each miniswine. iPSC colonies stained...
positive for Nanog, Oct4, c-Myc, Klf4, and SSEA-1 (Figure 1B) as well as Tra-1 to 60 and Tra-1 to 81 (Online Figure IB). By contrast, adult somatic pASCs from which piPSCs were derived revealed very low or no expression of these genes (Figure 1B and Online Figure IB). piPSCs were also characterized by semiquantitative reverse transcription PCR, which showed upregulation of pluripotency genes (Figure 1C). Microarray analyses were conducted to determine the degree of similarity between different piPSC lines. Hierarchical clustering of the whole genome expression showed that the generated piPSC lines were highly similar to the piPSC lines derived by Ezashi et al27 (piPSC-ID4, piPSC-ID6, and piPSC-ID6) generated from another laboratory and were distinct from pfibroblasts and pASCs. The range of Pearson correlation coefficients is displayed in the color bar (top).

Figure 1. Generation and characterization of piPSCs. A, Representative timeline of piPSC generation. Compact clones were observed on day 15. B, Immunofluorescence staining of pluripotent markers. Unlike the porcine adipose stromal cells (pASCs) from which they were derived, piPSCs stained positive for the traditional markers of pluripotency (eg, Nanog, Oct4, Klf4 and SSEA-1). pASCs have a weak expression of c-Myc, which is consistent with other reports. C, Reverse transcription PCR analysis of pluripotent markers. Expression of Oct4, Klf4, Sox2, and Nanog is present in derived piPSCs but absent from pASC and negative control (blank). D, Pearson correlation analysis for gene expression in piPSCs versus pASCs and porcine fibroblasts (pfibroblasts). Hierarchical clustering of whole genome expression showed that our generated piPSC lines were similar to reference piPSC lines (piPSC-ID1, piPSC-ID4, and piPSC-ID6) and were distinct from their somatic precursors (pASC) and porcine fibroblasts (pfibroblasts). The range of Pearson correlation coefficients is displayed in the color bar (top).

Confirmation of Pluripotency by In Vitro and In Vivo Differentiation
To further confirm the pluripotency of piPSCs derived from pASCs, we differentiated piPSCs in vitro by 3-dimensional EB formation. After 1 week of spontaneous differentiation using the hanging drop assay, piPSCs gave rise to cell types from the 3 different germ layers, as evidenced by immunostaining and quantitative PCR (Online Figure IIA and B). We then applied the in vivo teratoma formation assay as a definitive test to assess pluripotency. We transplanted piPSCs at passage 50 into the kidney capsule of SCID mice. Palpable tumors were seen 4 weeks after transplantation. Tumors were explanted 10 weeks after injection. Histological examination of the teratoma revealed the presence of derivatives of all 3 germ layers, including neural epithelium (ectoderm), chondrogenic stroma (mesoderm), and glandular epithelium (endoderm) (Online Figure IIC).

In Vivo Tracking of Teratoma Growth by BLI
Teratoma development was tracked in vivo to provide further evidence of pluripotency. To track teratoma growth in vivo, we stably transduced piPSCs with a lentiviral triple-fusion reporter gene construct containing firefly luciferase (Fluc), monomeric red fluorescent protein (mRFP), and herpes simplex virus thymidine kinase (HSVtk) genes. After 4 weeks, tumors were harvested and subjected to brightness level imaging (BLI). The brightness levels were consistently higher in the transduced tumors compared to the control tumors, indicating the engraftment of transduced stem cells.
virus truncated thymidine kinase (HSVttk) driven by ubiquitin promoter. Cells expressing the reporter gene were selected by cell sorting for mRFP (Online Figure IIIA). After injection into the kidney capsule of immunodeficient mice, teratoma growth was confirmed by BLI (Online Figure IIIB).

Generation of Functional Porcine iPSC-Derived ECs
We next tested whether we could differentiate piPSCs into a therapeutic cell population. Transplantation of endothelial cells is known to restore function to ischemic tissues through revascularization.11,28,29 Generation of piPSC-ECs was conducted via directed cell differentiation of EBs followed by FACS for the endothelial cell marker CD31 at day 12 after induction of differentiation (Figure 2A). Cells isolated by sorting stained positive for CD31 (green) and DAPI nuclear stain (blue). Similar to pAorta-ECs, piPSC-ECs take up acetylated LDL (red) and form a capillary-like network on Matrigel 24 hours after seeding the cells (far right). C, Microarray analysis confirmed that, similar to pAorta-ECs, piPSC-ECs express genes related to endothelial function.

Figure 2. Derivation and characterization of piPSC-derived endothelial cells. A, Bright-field images of endothelial cell differentiation from piPSCs using embryoid bodies. Differentiated cells were dissociated and sorted by FACS on day 12. B, Morphological and functional similarities between piPSC-ECs and endothelial cells harvested from the autologous porcine aorta (pAorta-ECs). Both piPSC-ECs and pAorta-ECs show positive immunofluorescence staining for the endothelial marker CD31 (green) and DAPI nuclear stain (blue). Similar to pAorta-ECs, piPSC-ECs take up acetylated LDL (red) and form a capillary-like network on Matrigel 24 hours after seeding the cells (far right). C, Microarray analysis confirmed that, similar to pAorta-ECs, piPSC-ECs express genes related to endothelial function.
persistent cell engraftment is still noted at week 4. Additionally, we demonstrated robust cell engraftment at day 2 after injection. Progressive decrease in signal was observed over the next several weeks, but the engraftment was still noted at week 4.

Short-term engraftment into the myocardium was also confirmed by BLI (Figure 3C), which showed focal [18F]FHBG radiotracer uptake in the anterior left ventricle. Interestingly, there was a trend toward greater improvement in fractional shortening in mice injected with piPSC-ECs and pAorta-ECs compared with mice injected with pASCs.

Delivery of piPSC-ECs Results in Improvement of Cardiac Function After MI

To confirm the therapeutic efficacy of the piPSC-ECs in vivo, we injected 1×10^6 piPSC-ECs into a murine MI model and compared the functional recovery among mice treated with PBS (negative control), 1×10^6 pASCs given their reported therapeutic benefit,30,31 and 1×10^6 pAorta-ECs (positive control). Functional recovery was assessed by echocardiography (fractional shortening at week 2, 23.7±0.9% versus 19.3±0.5%, *P<0.01; week 4, 27.2±1.3% versus 22.3±1.1%, **P<0.001). Greater improvement was also seen in animals receiving piPSC-ECs compared with pASCs, although this did not reach statistical significance (week 2, 23.7±0.9% versus 21.5±1.5%, P=NS; week 4, 27.2±1.3% versus 24.2±2.5%, P=NS). Comparable improvement is observed between animals receiving piPSC-ECs and their endogenous counterpart, pAorta-ECs (week 2, 23.7±0.9% versus 23.4±1.3%; week 4, 27.2±1.3% versus 27.1±0.6%; P=NS). Greater improvement was also seen in animals receiving piPSC-ECs compared with pASCs, although this did not reach statistical significance (week 2, 23.7±0.9% versus 21.5±1.5%, P=NS; week 4, 27.2±1.3% versus 24.2±2.5%, P=NS). Comparable improvement is observed between animals receiving piPSC-ECs and their endogenous counterpart, pAorta-ECs (week 2, 23.7±0.9% versus 23.4±1.3%; week 4, 27.2±1.3% versus 27.1±0.6%; P=NS).}

Histological Evaluation of Transplanted piPSC-ECs Into the Peri-Infarct Area

To confirm in vivo imaging data, animals in a subset were euthanized and their hearts were explanted at week 4. On gross histochemical analysis by triphenyltetrazolium chloride (TTC) (Figure 4A and 4B), animals treated with piPSC-ECs showed smaller average infarct area when compared with control (25.2±1.5% versus 32.7±0.8%; P<0.05). Similarly, microscopic analysis by hematoxylin and eosin staining showed thicker left ventricular walls in mice treated with piPSC-ECs compared with the control group (281±12 μm versus 161±6 μm; P<0.05) (Figure 4C and 4D). Immunohistochemistry of the peri-infarct area by mouse CD31 staining showed the number of capillaries per high power field was higher in the group receiving piPSC-ECs than those receiving PBS (395±28 vessels/mm² versus 107±9 vessels/mm²; P<0.001) (Figure 4E and 4F), suggesting that neovascularization may be a potential mechanism for improvement in cardiac function and decreased scar size. To evaluate whether the piPSC-ECs can form mature and functional vessels in the mouse infarction model and contribute to neovascularization, we performed immunofluorescence staining with anti-porcine CD31 antibodies (green) and anti-murine CD31 antibodies (red) in areas of increased capillary formation.
from explanted murine hearts. We found that microvessels were only immunoreactive to murine-specific antibodies (Online Figure VIA and B). However, piPSC-ECs could be found surrounding murine microvessels even though the cells themselves do not form mature vessels in hearts explanted 4 weeks after transplantation (Online Figure VIC and D). The coalescing of piPSC-ECs adjacent to murine microvessels supports our findings that piPSC-ECs release proangiogenic factors that increase native vessel formation. The failure of piPSC-ECs to form discrete microvessels in the mouse infarcted heart may be due to an insufficient number of piPSC-ECs present in the infarcted mouse heart to support vessel formation. As shown by bioluminescence imaging (Figure 3C), the majority of transplanted piPSC-ECs do not survive 4 weeks after transplantation. In contrast, formation of discrete microvessels was demonstrated by the in vivo Matrigel plug assay 7 days after implantation, which is a very different environmental niche (Online Figure IV).

Assessment of Paracrine Factor Release In Vitro by piPSC-ECs in Response to Hypoxia

Given that the majority of cells do not survive 4 weeks after transplantation (Figure 3C), we hypothesize that piPSC-ECs may release paracrine factors that promote angiogenesis in the setting of ischemia, accounting for the longer-term functional benefit that was observed. To explore this hypothesis, we compared the secretion of proangiogenic cytokines in pASCs, pAorta-ECs, and piPSC-ECs after 24 hours of hypoxia (Figure 5). The culture medium served as control. Both pASCs and piPSC-ECs secreted more angiogenic factors than control. In addition, piPSC-ECs secreted more tumor necrosis factor-α (TNF-α), fibroblast growth factor-β (FGF-β), interleukin (IL)-1α, IL-6, IL-8, and leptin than pASCs (P<0.05, P<0.01 versus pASCs). The pattern of cytokine secretion was similar between piPSC-ECs and pAorta-ECs. Taken together, these in vitro data suggest that piPSC-ECs can provide a framework for new vessel growth via secretion of paracrine factors regulating angiogenesis.

Single-Cell Profiling of Transplanted piPSC-ECs Reveals In Vivo Paracrine Activation

Because of the heterogeneous nature of all cell populations, especially those derived from exogenous factors such as piPSCs and their derivatives, it is important to study the expression of proangiogenic genes at the single-cell level. To meet these objectives, we injected the piPSC-ECs into the peri-infarct zone of mice treated with PBS, pASCs, pAorta-ECs, and piPSC-ECs. Quantitative analysis of capillary density (vessels/mm²) showed a significant increase in vessel density (number of vessels per high-power field) in animals treated with piPSC-ECs compared with PBS (395±28 vessels/mm² versus 107±9 vessels/mm², *P<0.001).
cells injected into the noninjured myocardium, including VEGF-A, IL-6, IL-10, TNF, interferon alpha 1, tissue inhibitor of metalloprotease (TIMP)1, TIMP2, GATA4, angiopoietin 1, and colony-stimulating factor 3 (CSF3) (ischemia versus sham; \( P < 0.05, P < 0.001 \)). These results correlated well with our in vitro findings that piPSC-ECs were capable of secreting a series of angiogenic factors under hypoxic conditions, accounting for the functional improvement observed in animals treated with piPSC-ECs. Because populations of pASCs and piPSC-ECs are heterogeneous, we characterized the pattern of paracrine secretion by different cell subpopulations (CD31/CD34/CD144 versus CD31/CD34/CD144) using single-cell qRT-PCR analysis. We found that the release of paracrine factors varied among different subpopulations of pASC and piPSC-ECs (Figure 6C and 6D). We also found that cells expressing CD34 and CD144 released more proangiogenic and antiapoptotic factors than other subpopulations of piPSC-ECs and pASCs. These data suggest that greater functional recovery may be achieved by transplanting a select population of cells rather than a heterogeneous mixture of cells.

Transplanted piPSC-ECs Inhibit Apoptosis by TUNEL Staining
Interestingly, the same angiogenic genes (ie, VEGF-A, IL6, IL10, TIMP1, GATA4, angiopoietin 1, and CSF3) that are upregulated in the peri-infarct zone after piPSC-EC transplantation also play an important role in regulating apoptosis and promoting cell survival. To explore whether the transplantation of piPSC-ECs inhibited cardiomyocyte and endothelial cell apoptosis, we measured the amount of DNA fragmentation in explanted hearts of treated and control animals (n=3 animals per group) using the TUNEL assay. The density of TUNEL-positive nuclei, shown as the percentage of total nuclei, was significantly lower in mice transplanted with piPSC-ECs compared with PBS control (6.1±0.6% versus 18.9±0.9%, \( P < 0.002 \)). These findings suggest that inhibition of apoptosis by piPSC-ECs may also contribute to functional recovery (Online Figure VII).

Imaging piPSC Fate in a Porcine Model of MI Using PET/CT and MRI
Before the clinical application of iPSCs in humans, successful delivery and monitoring of cells in a large animal model must first be established. Swine are an ideal large animal model for the clinical translation of iPSC-based therapy due to their resemblance to human organ size and physiology and their relatively long lifespan.16 To demonstrate feasibility of tracking piPSCs in swine using PET/CT and MRI, we injected piPSCs into Yucatan miniswine that previously underwent LAD artery balloon catheter occlusion to induce a myocardial infarction (Online Video I). Before intramyocardial cell transplantation, piPSCs were directly labeled with \[^{18}F\]-FDG for PET imaging, iron particles for co-localization with MRI, and a fluorescent...
carbocyanine dye for histological identification. We chose direct labeling of cells with [18F]-FDG rather than reporter gene imaging due to the robustness of this technique and its application in current clinical trials.37–39 After intramyocardial delivery of 2 x 10^6 piPSCs into the peri-infarct area under direct visualization, PET/CT and MRI demonstrated that transplanted cells localized to the peri-infarct area of the apical lateral wall of the left ventricle (Online Figure VIIIA and B). After imaging, hearts were explanted, and immunofluorescence staining demonstrated the presence of cells expressing the carbocyanine dye (red) as well as the pluripotent marker Oct-4 (green) in the peri-infarct area (Online Figure VIIIC). Histology revealed piPSCs with a large nucleus and abundant cytoplasm in the peri-infarct area (Online Figure VIIID).

**Discussion**

Because swine have a close resemblance to humans in terms of anatomy and physiology, the results of this study have several important implications for the clinical translation of iPSC therapy. First, we have shown that piPSCs can be successfully differentiated into a therapeutic cell population that mimics their endogenous counterparts, paving the way for the development of additional therapeutic subtypes. Second, we have demonstrated that the therapeutic effectiveness of piPSC-ECs in repairing the damaged myocardium is mediated by the release of proangiogenic and antiapoptotic modulating factors, using a novel microfluidic single-cell analysis. Third, we have also shown that paracrine release varied among different subpopulations of piPSC and piPSC-ECs. One week after injection into infarcted hearts, injected cells were harvested, subpopulations of pASCs and piPSC-ECs were isolated using FACS, and single-cell qRT-PCR was performed. D. Comparison of fold-change obtained by single-cell qRT-PCR showed patterns of paracrine release varied among 3 populations of piPSC-ECs and ASCs and cells: (1) CD34+/CD31+ /CD144+, (2) CD34+/CD31+/CD144−, and (3) CD34+/CD144−/CD31−.
Large-animal iPSCs have been recently derived from a number of species including monkeys, dogs, humans, and pigs. Successful differentiation of these cells into therapeutic cell populations, however, is necessary to harness the clinical potential of iPSC technology. Surprisingly, except for human iPSCs, reports on large-animal iPSC differentiation into therapeutic cell types for the treatment of cardiovascular disease have been limited. Using a modified protocol designed for human iPSC-EC differentiation, we generated piPSC-ECs that were morphologically and functionally similar to endothelial cells harvested from the autologous porcine aorta, based on standard in vitro and in vivo assays. Interestingly, these cells secrete paracrine factors at comparable levels and improve cardiac function to a similar extent as their endogenous counterparts. Although cell transplantation with native endothelial cells has been shown to promote angiogenesis and improve cardiac function, we believe that iPSC derivatives are a superior alternative because they have a potentially unlimited source unlike native endogenous endothelial cells, which are challenging to harvest and have a restricted supply. However, further optimization and modification of established human differentiation protocols are still needed for efficiently generating large quantities of iPSC derivatives such as endothelial cells or cardiomyocytes.

Although the debate continues over the optimal stem cell type for the clinical application of cardiovascular regenerative therapy, iPSCs appear promising because of their unlimited supply, immune privilege via autologous transplantation, and lack of ethical or regulatory hurdles impeding their clinical application. These cells may also be more effective than other cell types, such as adult somatic stem cells, which have been most often applied in preclinical and clinical trials to date. The efficacy of ASCs, for example, has been demonstrated in several studies with reported benefits due to the secretion of paracrine factors promoting angiogenesis and cell survival. Our head-to-head comparison revealed that piPSC-ECs secrete significantly more proangiogenic and anti-apoptotic modulating cytokines than pASCs in vitro, a finding that is analogous to a recent report showing that iPSC-derived mesenchymal cells release more paracrine factors than adult bone marrow stem cells. Findings from our in vivo study confirmed that transplantation of piPSC-ECs resulted in more neovascularization in the peri-infarct area than transplantation of pASCs. Additional studies directly comparing the efficacy of different stem cell types, however, are needed to determine the best option for clinical translation.

The release of cytokines and growth factors that promote neovascularization and cytoprotection has emerged as a leading mechanism to explain the observed functional improvement after stem cell therapy, in light of results from numerous studies demonstrating poor long-term survival and engraftment of transplanted cells and a paucity of newly generated cardiomyocytes. Improvement in cardiac function has been associated with stem cell induced release of VEGF, IL-1α, IL6, TNF-α, FGF-β, TIMP1, TIMP2, and angiopoietin 1, all of which were significantly increased in our study. Importantly, in a porcine model of myocardial infarction, Doyle et al found that pigs treated with conditioned media containing paracrine factors produced equivalent improvement in regional myocardial function and mass compared with those treated with transplanted cells; recovery was attenuated after administration of cytokine specific antibodies. Interestingly, Cho et al found that transplanted cells not only directly release cytokines but also induced the release of these factors from host tissues that is sustained for greater than 2 weeks, much longer than donor derived factors.

Previous findings, however, have been performed in whole cell populations, which are heterogeneous, and may reflect the average gene expression profile, not necessarily the expression profile of the cells of interest. Because whole-cell populations of iPSCs and their derivatives are even more heterogeneous than other stem cell types, perhaps as a result of limitations in reprogramming, variations in differentiation, and/or persistent donor cell memory, we used a novel microfluidic platform to examine gene expression at a single-cell level. We confirmed that transplanted piPSC-ECs release proangiogenic and antiapoptotic modulators that lead to new vessel formation, decreased apoptosis, and significant functional recovery of injured myocardium. Despite these encouraging results, further research is needed to define methods to improve survival and engraftment of transplanted cells, which will not only enhance paracrine release of these beneficial factors, but also promote cardiac regeneration.

Because of the heterogeneous nature of iPSC derivatives, we also compared the pattern of paracrine release from different subpopulations, specifically cells expressing the endothelial specific markers CD34 and CD144 (VE-cadherin). Single-cell expression analysis revealed that piPSC-ECs expressing either CD34 or CD144 released more proangiogenic and anti-apoptotic factors than other subpopulations of piPSC-ECs and pASCs. This is not surprising, given the function of these proteins in vasculogenesis. CD34 is a transmembrane protein that is expressed on early hematopoietic precursors and vascular associated structures. CD144 (VE-cadherin) is an endothelial specific adhesion molecule that maintains and controls endothelial cell-to-cell adhesion and, thus, can regulate vascular permeability and leukocyte extravasation. In addition, it regulates cell proliferation, apoptosis, and the function of vascular endothelial growth factor, making it is essential for embryonic angiogenesis. Furthermore, previous studies have shown that endothelial progenitor cells expressing CD34 and CD144 augment angiogenesis by release of paracrine factors. Given our findings that cells expressing these markers release more proangiogenic and antiapoptotic factors than other subpopulations, future studies should consider transplantation of specific iPSC-derivative cell subpopulations to further enhance functional recovery.

Finally, the development of a robust platform for noninvasive in vivo imaging in large animals is critical to ensure that therapeutic cells have been safely delivered near the target area of injury and have integrated into the target tissue. In the present study, we directly labeled cells with [18F]-FDG and iron particles for in vivo monitoring by PET/CT and MRI, respectively. Previous studies have reported successful monitoring of cell engraftment in large animals using these approaches, albeit with other stem cell subtypes. Similarly, we demonstrate the feasibility of this approach for monitoring the piPSC delivery and engraftment into the peri-infarct area. A major limitation of our study is that we
did not evaluate the functional efficacy of piPSC-ECs in a porcine model. This is primarily due to the large number of animals that would be needed to demonstrate robust statistical differences, which would need to be carefully evaluated in follow-up studies. Future studies could also determine whether a dual approach using direct labeling and reporter gene expression could exploit the advantages of both techniques to monitor stem cell delivery in large animals.

In summary, we successfully derived piPSCs and differentiated them into piPSC-ECs, which share similar morphological and functional properties to their endogenous counterparts. We found that piPSC-EC therapy promotes proangiogenic and antiapoptotic cytokine release in vitro and in vivo, leading to improved cardiac function in ischemic injury models. We also used multimodality noninvasive imaging to monitor cell engraftment into the peri-infarct area in small- and large-animal models of myocardial infarction. Taken together, this study provides preclinical validation and optimization of piPSCs-ECs as an effective therapeutic cell population, laying the groundwork for the eventual application of autologous iPSC-based therapy in the future.

Acknowledgments

We would like to thank C. Dana Bangs for performing the cytogenetic analysis for this study. We are grateful to the Stanford Functional Genomics Facility for their assistance with the microarray experiment.

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Disclosures

None.

References

What Is Known?

- Induced pluripotent stem cells (iPSCs) have been created from adult somatic cells of many small and large animals.
- More recently, iPSCs have been derived from swine, an animal that shares a cardiovascular anatomy and physiology similar to humans.

What New Information Does This Article Contribute?

- We successfully created iPSCs from porcine adipose tissue.
- For the first time, we also injected the iPSCs into the myocardium in a porcine model of myocardial infarction and tracked their localization to peri-infarct area by multimodality MRI and PET/CT imaging.
- In addition, we successfully differentiated porcine iPSCs into endothelial cells (iPSC-ECs) in vitro and demonstrated that intramyocardial injection of iPSC-ECs improved cardiac function in a murine model of myocardial infarction.

Although several groups have created iPSCs from porcine adult tissue, the generation of a therapeutic cell type from these large animals has remained challenging. Using a modified protocol for the derivation of human endothelial cells from iPSCs, we successfully generated endothelial cells from porcine iPSCs that shared similar morphological and functional properties as endothelial cells from the aorta. Transplantation of these iPSC-ECs into the murine myocardium after myocardial infarction resulted in significant improvement in ejection fraction 4 weeks after transplantation. Using a novel microfluidic PCR technique to determine gene expression at the single-cell level, we discovered that iPSC-ECs are capable of releasing proangiogenic and anti-apoptotic factors in the ischemic environment. These paracrine factors promoted the formation of new blood vessels in the peri-infarct area. We also demonstrated that the pattern of paracrine release varied among different cell subpopulations. In summary, further development of these large-animal iPSC models may provide additional insight to facilitate the development of autologous iPSC-based therapy in humans.
Microfluidic Single-Cell Analysis Shows That Porcine Induced Pluripotent Stem Cell–Derived Endothelial Cells Improve Myocardial Function by Paracrine Activation
Mingxia Gu, Patricia K. Nguyen, Andrew S. Lee, Dan Xu, Shijun Hu, Jordan R. Plews, Leng Han, Bruno C. Huber, Won Hee Lee, Yongquan Gong, Patricia E. de Almeida, Jennifer Lyons, Fumi Ikeno, Cholawat Pacharinsak, Andrew J. Connolly, Sanjiv S. Gambhir, Robert C. Robbins, Michael T. Longaker and Joseph C. Wu

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SUPPLEMENTAL METHODS

Porcine adipose stem cell harvest and culture. Adipose tissue and skin were resected from five juvenile, female Yucatan pigs (Pork Power Farms, Turlock CA). Adipose tissue was separated from the overlying skin and digested with 0.2% collagenase II at 37°C on a shaker for 1 to 2 hours, followed by vigorous manually shaking for 5-10 seconds every 20 min. After digestion, the stromal vascular fraction was separated from lipid-filled adipocytes by centrifugation at room temperature at 300×g for 10 min. Each stromal vascular fraction pellet was then resuspended thoroughly in 20 ml of PBS and filtered through a 100-micron pore size filter. All adherent cells acquired a fibroblast-like morphology within 5-7 days. Primary porcine adipose stromal cells (pASCs) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA), containing 10% fetal bovine serum (FBS) (Invitrogen), Glutamax-I (Invitrogen), 4.5 g/L glucose (Sigma, St. Louis, MO), 110 mg/L sodium pyruvate (Invitrogen), 50 units/ml penicillin, and 50 µg/ml streptomycin (Invitrogen) at 37°C, 95% air, and 5% CO₂ in a humidified incubator. All pASCs used for reprogramming were within passage 15.

Lentivirus production and transduction. Lentivirus carrying the reprogramming transgenes (Oct4, Sox2, Klf4, and cMyc) or reporter genes (Fluc-mRFP-HSVttk) was generated as previously described. Briefly, 293FT cells (Invitrogen) were transfected with lentiviral vectors carrying the appropriate gene (12 µg lentivirus/gene), packaging plasmids (8 µg), and VSVG plasmids (4 µg) using Lipofectamine 2000 (Invitrogen). Two days after transfection, the supernatant was collected, filtered, and mixed with PEG-it Virus Concentration Solution (System Biosciences) overnight at 4°C. The following day, viruses carrying the appropriate genes were precipitated at 1,500×g and resuspended with Opti-MEM medium (Invitrogen).

Immunofluorescence, alkaline phosphatase staining, and triphenyltetrazolium chloride (TTC) staining. After fixation with 4% paraformaldehyde for 10 minutes, cells were blocked with 5% bovine serum albumin in PBS for 1 hour at room temperature. For histological sections requiring nuclear staining, cells were treated with 0.3% tritonX-100 in PBS for 10 min for membrane permeabilization prior to adding antibodies. The following primary antibodies were used: 1) markers of pluripotency: Oct3/4 (Santa Cruz, Santa Cruz, CA), Sox2 (Biolegend, San Diego, CA), Klf4 (Abcam, Cambridge, UK), c-MYC (Abcam), SSEA-1 (Millipore, Billerica, MA), Tra-1-60 (Millipore), Tra-1-81 (Millipore), and Nanog (Santa Cruz); 2) markers of germ layer differentiation: AFP (Santa Cruz), Sox17 (R&D Systems, Minneapolis, MN), SMA (Santa Cruz), Desmin (Sigma, St. Louis, MO), and Nestin (Millipore). AlexaFluor conjugated secondary antibodies (Invitrogen, Carlsbad, CA) were used after primary antibody incubation for detection by fluorescent microscopy. For alkaline phosphatase (AP) staining, the quantitative alkaline phosphatase ES characterization kit (Millipore Chemicon, Billerica, MA) was used according to manufacturer’s instructions. For histological analysis by TTC, H&E, and TUNEL staining, animals were sacrificed and their hearts explanted at week 4. For TTC staining (n=5 per group), tissue sections were incubated in TTC for 15-20 minutes. After living tissue sections turned a deep red and infarcted areas turned white, sections were fixed in 10% formalin for 20 min. NIH Image J software was used to contour the infarcted area. The percent infarct size was calculated as the area of the infarct divided by the total area of the left ventricle. For the TUNEL assay (Roche Applied Science) (n=3 per group), the in situ cell death detection kit was used according to manufacturer’s instructions. After staining, murine sections were examined by
confocal microscopy. Three heart tissue slides were examined per mouse and five different fields were examined per murine section. For capillary density analysis, 3 sections from each mouse heart were stained using a CD31 antibody (BD Pharmingen), followed by a secondary antibody (Alexafluor-594). Capillary density was assessed by counting the number of capillaries in 6 random high-powered fields in each of 3 tissue sections. Images were then analyzed using ImageJ analysis software (NIH) and the data were expressed as capillaries/mm².

**Karyotype analysis.** Karyotype analyses of pASC-derived piPSCs were performed at the Cytogenetics Laboratory in the Department of Pathology at Stanford Hospital and Clinics. Cells were treated with 0.1 mg/mL colcemid for induction of mitotic arrest and were subsequently harvested by trypsin dispersal, hypotonic shock, and fixation with 3:1 methanol:acetic acid. For each cell line, 20 metaphases were analyzed by the standard G-banding method.

**Angiogenesis Matrigel plug assay.** Using appropriate anesthesia and under sterile conditions, 0.5 ml of Matrigel mixed with 1x10⁶ piPSCs-ECs (n=2 mice) or PBS (n=2 mice) was injected subcutaneously near the dorsal midline of SCID mice, per manufacturer's instructions. The Matrigel plug formed within seconds subcutaneously as Matrigel solidifies at body temperature. Seven days after implantation, the Matrigel plugs were explanted and prepared for histological and immunohistochemical analysis.

**Echocardiographic evaluation of murine cardiac function.** Echocardiography was performed at 0 (day 2 post MI), 1, 2, and 4 weeks post LAD artery ligation, using a Siemens-Acuson Sequoia C512 system equipped with a multi-frequency (8-14 MHz) 15L8 transducer (n=20 per group). Two-dimensional and M-mode echocardiography of the left ventricular short-axis were used to estimate fractional shortening (FS), as previously described.³ ⁴

**MRI evaluation of murine cardiac function.** A subset of mice was randomly selected for MRI evaluation of cardiac function (n=5 per PBS and iPSC-EC group) at 2 and 4 weeks post MI. Using a Signa 3.0T Excite HD scanner (GE, Milwaukee, Wisconsin) equipped with a Mayo Clinic T/R MRI coil (Mayo Clinic Medical Devices, Rochester, Minnesota), mice were placed in the prone position after anesthesia (1.5% isoflurane with 1 ml/min of oxygen). Heart rate, respiratory rate, and body temperature were monitored (Small Animal Instruments, Stony Brook, New York). After cardiac localization using gradient recalled echo (GRE), fast spoiled GRE (FSPGR) was used to acquire 20 short axis cine frames of the heart over 1 cardiac cycle. The following imaging parameters were applied: TR = 10 ms, TE = 4.6 ms, number of excitations (NEX) = 10, field of view (FOV) = 40 x 49 mm, matrix = 256 x 256, flip angle (FA) = 45°, slice thickness 1.5 mm, spacing = 0 mm. Ejection fraction was calculated using a commercial analysis program (Osirix Version 3.81) that was used to trace the endocardial border of the left ventricle (LV) at end diastole and end systole.³

**Optical bioluminescence imaging (BLI) and micro positron emission tomography (microPET) for tracking cell fate.** To enable in vivo tracking, piPSCs were stably transduced with a triple fusion reporter gene consisting of firefly luciferase, monomeric red fluorescent protein, and herpes simplex virus truncated thymidine kinase (Fluc-mRFP-HSVttk). BLI of the reporter gene FLuc was used to monitor cell survival and engraftment (n=5). Briefly, following intravenous tail injection with 250 µg/kg of D-luciferin, animals were imaged using a Xenogen IVIS Spectrum (Xenogen, Alameda, CA). Image analysis was performed using Living Image 4.0 (Caliper Life Sciences), as previously described.³ ⁴ MicroPET imaging of the reporter gene HSVttk was performed using a R4 Concorde MicroPET system (n=5). The PET reporter probe (9-[[18F]fluoro-3-[(hydroxymethyl)]butyl]guanine ([18F]-FHBG) (150uCi/animal) was injected into mice. Sixty to seventy five minutes after injection, images were taken and then reconstructed
using a filtered back-projection algorithm. A set of serial microPET images was obtained for 90 minutes (18 frames, 5 min each) to assess tracer influx. Regions of interest (ROIs) were defined for each area of interest. To obtain an image ROI-derived $^{18}$F-FHBG percentage injected dose per gram (% ID/g), the ROI counts per milliliter per minute were converted to counts per gram per minute (assuming a tissue density of 1 g/mL) and divided by the injected dose, as described previously.\(^2\)

**Angiogenesis cytokine array following hypoxia exposure.** To determine whether differentiated piPSC-ECs are capable of producing paracrine factors under hypoxic exposure, angiogenesis cytokine array (Panomics) experiments were performed (2 biological replicates). One million pASCs, pAorta-ECs (positive control), and piPSC-ECs were seeded onto a 6-well plate and exposed to 5% O\(_2\) hypoxia for 24 hours. Culture medium was used as the negative control. Supernatants were collected for the angiogenesis protein array. The arrays were hybridized and developed using a Kodak X-OMAT 2000 camera. The total pixel intensity for each spot was combined to calculate the expression intensities. Inter-array data was normalized by using positive control spots (eight per array) on each array. Protein expression levels were also normalized to PBS controls.

**Single cell gene expression profiling of transplanted piPSC-ECs and ASCs:** To determine the gene expression of different pro-angiogenic and anti-apoptotic cytokines in vivo at the single cell level, we harvested the transplanted piPSC-ECs and performed single cell gene expression profiling of piPSC-ECs injected into mice receiving LAD ligation and sham surgeries (n=5 per group). One week after transplantation, animals were sacrificed and hearts were explanted and dissociated using Liberase TH Research Grade high Thermolysin and TM Research Grade medium Thermolysin (Roche Applied Science) per manufacturer’s instructions. For single cell expression profiling, piPSC-ECs were first sorted by FACS into a 96 well 0.2-ml PCR plate for reverse transcription and specific target amplification using two different antibodies to prevent murine cell contamination. For negative selection, we used mouse Anti-H2kb monoclonal antibody, PE conjugated (BD 553570, BD Sciences). For selecting piPSCs, we used a mouse anti-porcine SLA Class I JM1E3 antibody (AM05880FC-N, Acris Antibodies GmbH). Each well contained a reverse transcription-specific target amplification master mix that included the following reagents: 1) 2x CellDirect Reaction Mix (CellsDirect One Step qRT-PCR kit, Invitrogen), 2) SUPERase-In (Applied Biosystems) for prevention of RNA degradation by RNases, 3) Taqman primers defining angiogenic factors (Applied Biosystems) for real time qPCR using the dynamic array, 4) SuperScript II Reverse Transcriptase for specific cDNA synthesis, and 5) Platinum Taq DNA polymerase (Invitrogen) for inhibiting polymerase activity and stopping the reaction at room temperature. Reverse transcription and specific target amplification was conducted on a thermocycler (Applied Biosystems Veriti) using the following parameters: 1) RNA release and reverse transcription at 50°C for 15 minutes, 2) reverse transcription reaction inhibition and activation of Platinum Taq DNA at 70°C for 2 minutes, and 3) specific target amplification for 18 cycles with each cycle consisting of denaturation (95°C for 15 minutes) followed by annealing and extension (60°C for 4 minutes). Quantitative real-time PCR was then conducted on a Fluidigm 48.48 Dynamic Array chip by placing the samples into a 48 microfluidic chamber with angiogenic genes and one housekeeping gene to be analyzed in the opposite chamber. Samples and genes were loaded into the reaction chamber using the Nanoflex 4-IFC controller. Single cell qPCR is performed on a BioMark HD reader in nanoliter reaction volumes, and analyzed using the Fluidigm Real-time PCR analysis software. Results are expressed as threshold cycles (C\(_T\)), which measures the amount of transcript present in the sample. A total of thirteen genes were analyzed in parallel with three replicates per chip. Two chips were analyzed, Genes were selected based their role in the regulation of angiogenesis
and apoptosis. Genes included positive (VEGF, TNF-α, FGF-β, IFN-γ, IL-1α, IL6, IL8, GATA4, Leptin, PIGF, ANGPT1, FGFR2, BCL2, and CSF3) and negative modulators of angiogenesis (IL-10, IL12, Ip10, IFNA1, TIMP1, TIMP2, and ANGPT2), which are both required for normal vessel development. A subset of these genes also has apoptotic (i.e., TNF, IFN-γ, Ip10, TIMP2, and ANGPT2), and anti-apoptotic effects (i.e., VEGF, IL6, IL10, TIMP1, Leptin, GATA4, ANGPT1, BCL2, and CSF3).

**Generation of a porcine model of myocardial infarction and intramyocardial delivery of piPSC.** After induction of anesthesia (5 mg/kg tiletamine/zolazepam with 4-5% inhaled isoflurane) and intubation, Yucatan miniswine underwent carotid balloon catheter occlusion of the proximal to mid left anterior descending artery under fluoroscopic guidance, as previously described. At 12 weeks post myocardial infarction, pigs underwent intramyocardial injection of piPSC (2x10^8 cells) via lateral thoracotomy. Animals were injected into the peri-infarct zone of the left ventricle at 8 different sites using a 25-gauge syringe (500 µl per injection). Study protocols were approved by the Stanford Animal Research Committee. Animal care was provided in accordance with the Stanford University School of Medicine guidelines and policies for the use of laboratory animals.

**Tracking transplanted piPSCs in miniswine using positron emission tomography / computed tomography (PET/CT) imaging and MRI.** For in vivo PET/CT imaging, piPSCs were directly radiolabeled with [18F]-Fluorodeoxyglucose (([18F]-FDG). One hour prior to injection, piPSCs were incubated with 5 mCi of [18F]-FDG in a 10 ml test tube containing 10 U/mL heparin (Roche), 0.1 U/ml recombinant human insulin (Novo Nordisk), and serum-free PBS (pH 7.2) at room temperature. After a 30-minute incubation period, centrifugation was performed at 7x g, 27x g, and then 60x g for 120 seconds each, followed by washing in heparinized PBS. A dose calibrator (CRC 55-T, Capintec Inc, Ramsey, NJ) was used to measure the radioactivity in the pellet and supernatant. Prior to injection, labeled cells were resuspended into 2 ml of heparinized PBS (10 U/mL). PET/CT imaging was performed with a clinical PET/CT scanner (Discovery Lightspeed Plus; GE Medical Systems, Waukesha, Wisconsin), with the animals in a supine position, as previously described. Briefly, an initial three-plane scout was obtained for cardiac localization (30 mAs, 120 kV), followed by a non-enhanced 4-detector CT scan using the following parameters: 5 mm thickness, 38 mm per rotation, 0.8-second gantry rotation time, 120 kV x-ray tube voltage, and 150 mA tube current. A 1-hour static non-gated PET scan was acquired two hours after intramyocardial injection of 2x10^8 piPSCs. CT data sets were then reconstructed in the transverse plane with 5 mm section thickness at a 4.25 mm interval. An iterative algorithm (ordered-subset expectation maximization, 2 iterative steps, 28 subsets) with CT-based attenuation correction was used to reconstruct the images. For cellular localization by MRI, piPSCs were magnetically labeled with iron particles (Resovist, Schering AG, Germany) at a concentration of 250 µg Fe/ml and protamine sulfate (5 µg/ml) for 24 h, as previously described. MR images were acquired by using a Signa 3.0 Tesla Excite HD Scanner (GE Medical Systems, Milwaukee, Wisconsin) and an 8-element cardiac phased array coil. For imaging cells labeled with iron, a T2 weighted GRE sequence was performed in two orthogonal views. Evaluation of left ventricular function and infarct size was also performed using steady state free precession and gadolinium delayed enhancement sequences, respectively. Finally, cells were identified by histology by labeling with a fluorescent carbocyanine dye (Vybrant CM-Dil, Invitrogen) that binds to phospholipid cell membranes. Labeling was performed prior to cell transplantation per manufacturer’s instructions.
ONLINE SUPPLEMENTAL FIGURE LEGEND

Online Figure I: Successful derivation of piPSCs from pASCs. A) piPSC colonies stain positive for alkaline phosphatase (AKP), a universal marker of pluripotency for all types of pluripotent cells. B) Immunofluorescence staining shows expression of additional pluripotent markers (e.g., Tra1-60 and Tra1-81 shown in green) in piPSCs. DAPI nuclear staining shown in blue. C) Karyotyping of piPSCs at passage 20 shows normal porcine karyotype: 2n=38, XX.

Online Figure II: piPSCs are capable of in vitro and in vivo differentiation into the three germ layers. A) Immunofluorescence staining of differentiated piPSCs. Differentiated cells are positive for endodermal (AFP and Sox-17), mesodermal (SMA and Desmin), and ectodermal (Nestin) markers. B) Quantitative-PCR analysis confirms that differentiated piPSCs express genes from all three germ layers. Spontaneously differentiated piPSCs (e.g., iPS1-EB, iPS2-EB, iPS3-EB) express genes from the endoderm (Foxa2, AFP), mesoderm (GATA4, GATA6, MLC, Nkx), and ectoderm (TJF, Gbx). These genes, however, are not expressed at detectable levels in piPSCs and pASCs. Foxa2: forkhead box protein A2; AFP: alpha-fetoprotein; GATA4: GATA-binding protein 4; GATA6: GATA-binding protein 6; MLC: myosin light chain; Gbx: gastrulation-binding-homeobox. C) Hematoxylin and eosin (H&E) staining of a teratoma derived from porcine iPSCs reveal the presence of tissues from all three germ layers, shown by the asterisk: 1) neural epithelium (ectoderm), chondrogenic stroma (mesoderm), and 3) glandular epithelium (endoderm).

Online Figure III: Transplantation of cells expressing the triple fusion reporter gene for in vivo cell tracking by BLI and microPET. A) Diagram for the triple fusion reporter gene expressing firefly luciferase (Fluc), monomeric red fluorescent protein (mRFP), and herpes simplex virus truncated thymidine kinase (HSVttk) driven by the ubiquitin promoter (top). Expression of Fluc and HSVttk enables in vivo cell tracking by BLI and PET, respectively. Expression of mRFP ex vivo confirms stable integration of the construct in cells and identifies cell engraftment for post-mortem histology (bottom). B) Representative BLI of teratoma development in the kidney capsule of a SCID mouse after injection of 1x10^6 piPSCs. Continuous growth of the tumor is noted over 60 days as demonstrated by BLI. C) Representative microPET images of a SCID mouse after intramyocardial injection of 1x10^6 piPSC-ECs. Transverse and coronal images of [18F]-FHBG uptake in the anterior wall of the left ventricle.

Online Figure IV: In vivo angiogenesis within Matrigel plugs mixed with piPSC-ECs. Ten days after implantation into the dorsum of SCID mice (n=2 per group), plugs were explanted. A) Representative images of Matrigel plugs mixed with piPSC-ECs showing formation of vascular-like structures. B) Similar structures are not observed in Matrigel plugs mixed with PBS. C) Representative immunofluorescence staining of Matrigel plugs mixed with piPSC-ECs. Vascular-like structures in the Matrigel plugs show strong expression of the endothelial marker CD31. D) Representative hematoxylin and eosin (H&E) sections from the Matrigel plugs mixed with piPSC-ECs confirm the presence of capillary formations.

Online Figure V: Improvement of left ventricular function after delivery of piPSC-ECs confirmed by MRI. A) Representative MRI short-axis views taken at the level of the papillary muscles at week 2 and 4 post-MI in mice treated with piPSC-ECs and PBS control. Increased
wall thickening and contractility is noted at end systole in mice receiving piPSC-ECs compared to PBS control. **B)** Quantification of left ventricular ejection fraction (LVEF) reveals significant improvement at week 2 and 4 post MI (\(P<0.05\))

**Online Figure VI:** CD31 staining of the microvasculature using anti-porcine and anti-murine antibodies revealed that porcine iPSC-ECs do not form discrete vasculature, but can be found adjacent to murine vessels. **A)** Immunofluorescence staining with anti-porcine CD31 antibodies (green) and **B)** anti-murine CD31 antibodies (red) in areas of increased capillary formation from explanted hearts show that microvessels are only immunoreactive to murine-specific antibodies. Overlay images at low (C) and high (D) magnification demonstrate that piPSC-ECs can be found surrounding murine microvessels, supporting our findings that piPSC-ECs release pro-angiogenic factors that increase native vessel formation. Scale bars=200 \(\mu\)m at low magnification and \(\mu\)m at high magnification.

**Online Figure VII:** Decreased apoptosis in murine hearts injected with iPSC-ECs compared to those injected with PBS. Four weeks after treatment, murine hearts were explanted and mouse frozen sections were examined for DNA fragmentation using TUNEL assay. **A)** Immunofluorescence staining with TUNEL demonstrates fewer cardiomyocytes with DNA fragmentation in the transplanted vs. control group (shown in green). DAPI nuclear staining shown in blue. Cardiac troponin T shown in red. Overlay of TUNEL, DAPI and cardiac troponin T (far right). **B)** Quantification of the density of TUNEL positive nuclei. Murine hearts transplanted with piPSC-ECs have a lower average density of apoptotic cells (6.1±0.6% compared to 18.9±0.9%, \(P<0.005\)).

**Online Figure VIII:** In vivo tracking of injected piPSCs by PET/CT and MRI in a porcine model of myocardial infarction. piPSCs were directly labeled with \(^{18}\)F-FDG, iron oxide particles, and carbocyanine dye (i.e., Vybrant CM-Dil) for localization by PET/CT, MRI, and histological analysis, respectively. **A)** Representative axial non-enhanced PET-CT fusion image of piPSCs (left) with coronal reconstruction of PET data set (right) one hour after delivery of cells into the peri-infarct area. A strong signal (0.05±0.2 %ID/g) can be seen at the sites of the injection in the distal left ventricular wall (noted by single arrow). **B)** Representative T2 weighted GRE images by MRI shows iron-labeled cells as hypointense signals (dark areas noted by the single arrow) in the apical lateral wall (left), which corresponds to areas of strong signal noted on PET imaging. Cells were injected near the region of infarct, noted by areas of hyperenhancement on gadolinium enhanced MRI (bright white areas shown on right). **C)** Immunostaining reveals the expression of Vybrant CM-Dil fluorescent dye (red), Oct4 (green), and DAPI (blue) cells injected into the porcine myocardium. Overlay of Vybrant CM-Dil fluorescent dye, Oct4, and DAPI (far right). **D)** Histological sections of porcine hearts confirm the absence of cells in the adjacent, non-injected myocardium (left) and successful delivery of the piPSCs in the myocardium (center), piPSCs have a large nucleus (12-14 \(\mu\)m) with significant cytoplasmic volume (single white arrow on 100x high power field).

**Online Video I:** Cine of MRI of left ventricular function in a porcine model of myocardial infarction. Representative short-axis steady-state free precession cine MRI of left ventricle in a pig that underwent balloon occlusion of the left anterior descending artery. Ejection fraction is moderately to severely depressed post-MI. Thinning and poor contractility of the mid to distal anterior wall and apex are noted.

**Online Table I:** Primer sequences for evaluating pluripotency by RT-PCR. A list of primer sequences used to perform RT-PCR to confirm that generated porcine induced pluripotent stem
cells express pluripotency genes and can successful differentiate into the three embryonic germ layers.
Supplemental References


Online Figure I
Online Figure III
Online Figure IV
Online Figure V
Online Figure VI
Online Figure VII
Online Figure VIII
Supplemental Table I: Primer sequences for evaluating pluripotency by RT-PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’-3’)</th>
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| Oct-4   | F: GCTGACAACAAACGAGAATCTGC  
  R: ACGCGGACCACATCCTTCTCTAG |
| Nanog   | F: CCGAAGCATCCATTTCCAGCG  
  R: GGTATTCTGTACTGGCTGAGCC |
| Sox-2   | F: AATGCGCAGACACGGCGGCT  
  R: GCCCATGGAACCAGCGGT |
| Klf-4   | F: CCATGGGCCAAACTACCCAC  
  R: TGGGGTCAACACCATTTCCGT |
| cMYC    | F: GCCAAAAGGTCGGAATCGGGG  
  R: CGCAGCAGTCTTTTTCTGACAC |
| GATA6   | F: CATTGGAGGAAACCGTGAA  
  R: CCAGCAAATGCAGATTCCCTT |
| Rex1    | F: GAGAAAGCATCTCTCCGATCCA  
  R: CCAGCAAATGCAGATTCCCTT |
| Gbx     | F: TGCAAGCCGTCGCTCGTAG  
  R: TCCGAGCTGTAGTCCAGATCA |
| APF     | F: CGCGTTTCTGGTTGCCATACAC  
  R: ACTTCTTGCTCTGGCCTTG |
| MLC     | F: CCACTCTGTTAGAGAGGCTA  
  R: GGGCTGCGTTAGGATTCTC |
| Nkx     | F: CCAAGGACCCCTCGAGCTGA  
  R: CGACAGATACCGGTGCTGCT |
| GAPDH   | F: CTCAACGGGAAGCTCACTGG  
  R: CATTGTGTCGAGAAATGAGC |