PRECLINICAL DERIVATION AND IMAGING OF AUTOLOGOUSLY TRANSPLANTED CANINE INDUCED PLURIPOTENT STEM CELLS

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Derivation of patient-specific induced pluripotent stem cells (iPSCs) opens a new avenue for future applications of regenerative medicine. However, before iPSCs can be used in a clinical setting, it is critical to validate their in vivo fate following autologous transplantation. Thus far, preclinical studies have been limited to small animals, and have yet to be conducted in large animals that are physiologically more similar to humans. In this study, we report the first autologous transplantation of iPSCs in a large animal model through the generation of canine iPSCs (ciPSCs) from the canine adipose stromal cells (cASCs) and canine fibroblasts (c Fibro) of adult Mongrel dogs. We confirmed pluripotency of ciPSCs using the following techniques: 1) immunostaining and quantitative PCR for the presence of pluripotent and germ layer specific markers in differentiated ciPSCs; 2) microarray analysis that demonstrates similar gene expression profiles between ciPSCs and canine embryonic stem cells (cESCs); 3) teratoma formation assays; and 4) karyotyping for genomic stability. Fate of ciPSCs autologously transplanted to the canine heart was tracked in vivo using clinical positron emission tomography (PET), computed tomography (CT), and magnetic resonance (MR) imaging. To demonstrate clinical potential of ciPSCs to treat models of injury, we generated endothelial cells (ciPSC-ECs) and used these cells to treat immunodeficient murine models of myocardial infarction and hindlimb ischemia.

In recent years, human embryonic stem cells (hESCs) have gained popularity as a potentially ideal cell candidate for regenerative medicine (1). Clinical use of hESCs, however, remains challenging due to concerns about the use of human embryos and the risk of transplant rejection (2). One way to circumvent these issues is to generate induced pluripotent stem cells (iPSCs) from adult somatic tissues (3). iPSCs have been successfully generated from a variety of adult cell types through exogenous expression of various transcription factors (3,4). Numerous pre-clinical studies in small animals have demonstrated that adult cells can be successfully reprogrammed into iPSCs, differentiated into therapeutic cell types, and transplanted into damaged tissue for repair (5,6).

Despite promising results in small animal models, further evaluation is needed in large animal models, which are physiologically more similar to humans. Among large animals, the canine model is especially well suited for translational studies of iPSC-based therapies. Unlike the pig, canine embryonic stem cells (cESCs) have already been isolated and characterized and thus provide criteria for pluripotent canine cells (7-9). Although both monkey ESCs and iPSCs have been previously derived, the use of primates remains controversial (7,10). In this study, we generated ciPSCs resembling cESCs from the somatic skin and fat of adult Mongrel dogs. We tested the efficacy of a canine model for pre-clinical
optimization of iPSC delivery to the heart by monitoring stem cell fate using PET reporter gene imaging and iron oxide labeling by MR imaging.

**Experimental Procedures**

**Adipose cell harvest and culture of ciPSCs-**
Adipose tissue and skin were resected from three individual one-year old Mongrel dogs (Marshall Farms, CA) for digestion with collagenase II (Invitrogen, Carlsbad, CA) to a single cell solution for plating and culture in appropriate mediums. Derived ciPSCs were maintained on MEF feeder layers with ciPSC medium.

**Lentivirus production and transduction-**
Lentivirus for reprogramming transgenes or Fluc-RFP-HSVttk was collected from the supernatant of transfected 293FT cells and concentrated as previously described (11,12).

**Immunofluorescence and alkaline phosphatase staining-**
After cells were fixed, permeabilized, and blocked, incubation with primary and secondary antibody was performed. A list of the antibodies used in this study is included in Supplemental Table 1. Alkaline phosphatase (AP) staining was performed using the Quantitative Alkaline Phosphatase ES Characterization Kit (Millipore, Billerica, MA) per manufacturer’s instructions.

**Quantitative PCR-**
Following isolation of total RNA and cDNA, quantitative PCR was performed with Taqman Gene Expression Assays using a StepOnePlus Realtime-PCR System (Applied Biosystems, Foster City, CA). All primers are listed in Supplemental Table 2.

**In vitro differentiation-**
ciPSCs were differentiated into embryoid bodies (EBs) in suspension culture for 8 days. EBs were seeded in adherent culture after which spontaneous differentiation into ectodermal, mesodermal, and endodermal lineages was assessed by quantitative PCR.

**Teratoma formation-**
Approximately 2 million ciPSCs were injected into the dorsal flanks of male SCID mice (n=6). After 8 weeks, tumors were dissected and fixed with 10% formaldehyde in PBS. Paraffin embedded tissue sections were stained with hematoxylin and eosin (H&E).

**Microarray hybridization and data analysis-**
Total RNA samples were hybridized to Affymetrix GeneChip Canine Genome 2.0 Array, and then normalized and annotated by the Affymetrix® Expression Console™ software.

**Canine myocardial delivery of transfected cells-**
Dogs were anesthetized, tracheally intubated, and mechanically ventilated as previously described (12). Cells were delivered by injection through the fourth and fifth intercostal spaces into 3 adjacent sites of the anterior left ventricular myocardium.

**Clinical positron emission tomography-computed tomography (PET/CT) imaging-**
Imaging was performed with a clinical PET-CT scanner (Discovery LightSpeed Plus; GE Medical Systems, Waukesha, Wisconsin) as previously described (12).

**Clinical MR imaging-**
Imaging was performed on a Signa 3.0T Excite HD scanner (GE Healthcare Systems, Milwaukee, Wisconsin) and 8-element cardiac phased array coil. A T2 weighted GRE sequence was used to image ciPSCs incubated overnight with iron particles. Cine images of the left ventricle in short and long axes were acquired with a steady-state free precession sequence as previously described (13,14).

**Generation of canine endothelial cells (ciPSC-ECs) from ciPSCs-**
ciPSCs were differentiated into EBs in ultra-low attachment dishes using differentiation medium. After 16 days in culture, EBs were dissociated into single cells and placed in adherent cell culture conditions. Cells were lifted and FACs sorted for CD31. ciPSC-ECs were cultured using EBM-2 (Lonza, MD).

**Generation of myocardial infarction and intramyocardial delivery of ciPSC-ECs-**
8-10 week old SCID Beige mice (Charles Rivers, MA) were anesthetized by inhaled isoflurane (2% to 3%) and intubated and ventilated. A left thoracotomy was performed followed by ligation of the left anterior descending (LAD) artery for 30 minutes followed by reperfusion as previously described (15). After 30 minutes, 1x10^6 ciPSC-ECs stably expressing Fluc-RFP-HSVttk were injected intramyocardially into 3 sites near the peri-infarct zone at 20 ml of total volume (n=6).
Control animals received PBS injection instead (n=6).  

Optical bioluminescence (BLI) of cell survival and localization- To assess ciPSC-EC survival and engraftment in vivo, BLI was performed on animals receiving 375 mg/kg body weight d-luciferin reporter probe. Living Image 4.0 (Caliper Life Sciences) was used to quantify signal intensity as previously described (15,16).

Echocardiographic measurement of cardiac function- Animals receiving LAD artery ligation were scanned on days 0, 14, 28, and 42 using a Siemens-Acuson Sequoia C512 system equipped with a multifrequency (8–14 MHz) 15L8 transducer. Fractional shortening (FS) was acquired using standard M-mode image acquisitions of left ventricular short axis images as previously described (15,16).

Hindlimb ischemia model- SCID Beige mice (Charles Rivers Laboratories, MA) were anesthetized with isoflurane (2-3%) after which unilateral hindlimb ischemia was induced by ligating the femoral artery as previously described (17). To test the therapeutic potential of ciPSC-ECs, 1x10^6 cells stably expressing mRFP-Fluc were delivered by intra-muscular injection into the region of ischemia (n=5). Control animals received PBS (n=5).

An expanded Methods section is provided in the Supplemental Methods section.

Results

Derivation of ciPSCs from adipose stromal cells and fibroblasts. We successfully reprogrammed canine adipose stromal cells (cASCs) and canine fibroblasts (cFibro) into ciPSCs from three individual dogs. We used lentivirus containing human Oct4, Sox2, Klf4, and c-MYC at a 1:1:1:1 ratio without chemical inhibitors. From days 12-15 post transduction, we observed clearly recognizable, tightly packed colonies with morphologic appearance similar to cESCs under bright field microscopy (Figure 1A). As previous studies characterizing cESCs have used TRA-1-60 as a marker of undifferentiation (7), we used TRA-1-60 in conjunction with typical ESC-like morphology to track the progression of the putative ciPSC colonies. In our reprogramming experiments, we consistently observed the appearance of TRA-1-60 positive colonies as early as day 12, following introduction of virus into cells. From days 15-18, cESC-like colonies grew large enough for mechanical isolation and were transferred onto mouse embryonic fibroblast (MEF) feeder layers. Prior to transfer, we recorded the number of ciPSCs that stained positive for TRA-1-60 and had typical ESC-like morphology to calculate reprogramming efficiency. While the timelines for ciPSC derivation from cASCs and cFibro were nearly identical, cASCs had double the reprogramming efficiency compared to cFibro (1.74% ± 0.09 vs. 0.84% ± 0.07, P <0.05).

Successful reprogramming of ciPSCs was further confirmed by whole genome expression profiling using microarray analysis comparing ciPSCs with cASCs and cESCs. ciPSCs showed a high degree of similarity in their gene expression profile with cESCs and importantly, were distinct from cASCs (Figure 1D, Supplemental Figure 3). In addition, cFibro-derived ciPSCs were found to be similar to cASC-derived iPSCs and cESCs as well (Supplemental Figure 4).

To test pluripotency of ciPSCs derived from cASCs and cFibro, we differentiated ciPSCs in vitro by embryoid body (EB) formation. ciPSCs derived from both cASCs as
well as cFibro gave rise to cells of all three germ layers as evidenced by immunostaining and quantitative PCR (Figure 2A, 2B and Supplemental Figures 5 & 6). Teratoma formation assays confirmed potential of ciPSCs to form tumors composed of derivatives from all three germ layers in vivo (Figure 2C, 2D). ciPSCs were found to have a normal karyotype after extended culture for 30 passages with 78 chromosomes and no translocations, indicating genomic stability of clones over time (Figure 2E).

**In vivo tracking of transplanted canine iPSCs using PET and MR imaging.** To simulate use of ciPSCs for cell therapy, we next prepared c-ASC-iPSCs for autologous delivery into the same animals from which parental cells were isolated. To visualize anatomic localization and survival of cells after in vivo injection, ciPSCs were stably transduced with a lentivirus carrying a triple-fusion (TF) reporter gene consisting of firefly luciferase, red fluorescent protein, and herpes simplex truncated thymidine kinase (LV-Fluc-RFP-HSVttk) driven by the ubiquitin promoter (Figure 3A). Following flow cytometry sorting for RFP positive cells, transfected cells were tested in vitro and in vivo to verify luciferase and HSVttk expression (Figure 3B-D). The minimal number of cells required for detection by PET in vivo was calculated for both murine and canine models by injection of varying cell concentration into the flanks of immunodeficient mice and autologous dogs. The minimum detectable cell concentration in the mouse was 1×10⁶ cell/ml using microPET (Figure 3D). For the dog, the signal could be detected from the subcutaneous regions of the animal at a minimum concentration of 5×10⁶ cells/ml using clinical PET/CT scanner. Specifically, the in vivo reporter gene expression for 5 and 10 million ciPSCs was 0.0004±0.0001 and 0.001±0.0003 percent injected dose per gram of tissue (%ID/g), respectively. As typical human trials for cell-based therapies can utilize billions of cells (18), PET-based reporter gene imaging modalities at a detection level of 5 million cells appears sensitive enough to enable accurate tracking of ciPSC delivery in vivo.

We next tested whether ciPSCs expressing the HSVttk reporter gene could be imaged at an anatomical site where signal attenuation due to tissue thickness is problematic (e.g., myocardium). Due to the therapeutic potential of iPSC therapy for cardiovascular disease and challenge of imaging cell delivery to the heart, we chose the left ventricular myocardial wall as a target. To confirm PET signals in our cardiac model as specific, we labeled ciPSCs with iron oxide particles for co-localization with MR imaging. Following this, 1×10⁶ cells were autologously delivered to the hearts of animals from which ciPSCs were originally derived. Fusion imaging of PET/CT demonstrated focal 9-[4-[(18)F]fluoro-3-(hydroxymethyl)butyl]guanine ([¹⁸F]-FHBG) radiotracer uptake in the anterior myocardial wall (Figure 4A, Supplemental Video 1). The in vivo reporter gene expression after injection of 100 million cells was 0.0025±0.0010 %ID/g of the injected dose per gram of myocardial tissue. MR imaging of these cells co-labeled with iron oxide particles confirmed localization of tracer activity in the anterior wall of the myocardium (Figure 4B). After imaging, canine hearts were explanted immediately after in vivo imaging and cut into 100 mm sections. The location of the cells in the myocardium was further confirmed by ex vivo micro PET imaging (Figure 4C) and histological analysis (Supplemental Figure 7).

**Generation of endothelial cells from ciPSCs and transplantation into murine hindlimb ischemia model.** As undifferentiated iPSCs are not a cell type directly amenable for clinical transplantation, we sought to demonstrate therapeutic applications of these cells through differentiation of ciPSCs into endothelial cells (ciPSC-ECs). Like human and murine ESC-ECs and iPSC-ECs generated in previous studies (15-17,19,20), ciPSC-ECs were positive for CD31, capable of DiI-ac-LDL uptake, and formed tubules when cultured on Matrigel in vitro (Supplemental Figure 8A). To assess in vivo therapeutic potential of ciPSC-ECs, we stably transduced ciPSC-ECs with a TF reporter gene carrying RFP, Fluc, and HSVttk driven by the ubiquitin promoter (Figure 3A), and delivered these cells into a murine model of hindlimb ischemia through intramuscular injection. BLI demonstrated
engraftment of ciPSC-ECs in the ischemic murine hindlimb for 14 days following ligation of the femoral artery (Supplemental Figure 8B). Laser Doppler imaging of blood perfusion in ischemic hindlimbs demonstrated significantly improved revascularization in mice receiving ciPSC-ECs as compared to mice receiving PBS only at day 7 (ischemic leg/control leg ratio; ciPSC-EC: 0.31±0.09 vs PBS: 0.28±0.13; P<0.05) and day 14 (ischemic leg/control leg ratio; ciPSC-EC: 0.35±0.08 vs PBS: 0.30±0.17; P<0.05) (Supplemental Figure 8C-D).

Transplantation of ciPSC-ECs for murine myocardial injury model- Previous studies have demonstrated potential of iPSC- and ESC derived endothelial cells to improve systolic function following myocardial infarction (MI) through revascularization (15,16). Hence we next delivered ciPSC-ECs stably transduced with TF reporter gene into the border zone of infarcted murine hearts. Cardiac engraftment of ciPSC-ECs was monitored noninvasively by BLI for 42 days after cell delivery. Engraftment of ciPSC-ECs was still detectable at 42 days after infarction (Figure 5A). Measurement of systolic function by echocardiography revealed improvement in cardiac contractility for animals receiving ciPSC-ECs as compared to animals receiving PBS at days 14 and 28 post MI. However, as in the murine hindlimb model, BLI demonstrated progressive death of donor cells following transplantation into the ischemic heart. Hence significant differences in systolic function between the two groups of animals was not observed at week 6 (Figure 5B-C). Histologic confirmation of ciPSC-ECs in the heart was performed using RFP immunofluorescence at day 42 following cell delivery (Figure 5D).

Discussion

Previous studies have shown that iPSCs can be derived from small animals such as mice and rats, demonstrating proof of principle for the therapeutic potential of pluripotent stem cell based regenerative therapies (21,22). However, these models have several limitations for extrapolation to human patients due to differences in animal physiology and size. Development of large animal models is likely necessary for the creation of clinical criteria for iPSC-based human trials in the future. Unfortunately, efforts to establish allogeneic or autologous cell transplant models in large animals have been hampered by difficulty in isolation of stable ESC lines from farm animal species and inability to optimize cell culture conditions (23). The reasons for these reported difficulties in derivation of ESCs from large animals are not clear, but likely stem from the inability to isolate cells from the inner cell mass at the appropriate stage of embryonic development (23). Thus, the generation of stable iPSC lines from somatic tissue of large animals is a novel approach by which pluripotent stem cell studies may be established.

Several studies have demonstrated the feasibility of deriving iPSCs from larger animals, including pigs and monkeys (10,24). More recently, the derivation of ciPSCs from canine embryonic fibroblasts and testicular tissue has been described (25,26). However, the ciPSCs derived in these preliminary studies were not shown capable of teratoma formation, nor were compared to cESCs in terms of comprehensive analysis for markers of pluripotency. Derivation of ciPSCs that comprehensively resemble cESCs is thus still required.

Generation and maintenance of ciPSCs had several noticeable differences between that of murine and human iPSCs. First, both murine and human somatic cells have been shown to be capable of induction to a pluripotent state using feeder free systems (11). By comparison, in the generation of ciPSCs, we were unable to propagate undifferentiated cells without a feeder layer of mouse embryonic fibroblasts (MEFs). Withdrawal of MEFs resulted in cell differentiation and loss of colony morphology. Second, both the growth factors LIF and bFGF were necessary for the derivation and maintenance of ciPSCs. This stands in contrast to murine ESCs and iPSCs, which require LIF only (27), and human ESCs and iPSCs, which require bFGF only (23). Without the addition of both LIF and bFGF, ciPSCs lost ESC-like colony morphology as well as expression of Oct-4 and TRA-1-60. Our culture system for ciPSCs may provide additional clues to help establishing other large animal ESC lines like...
pig and sheep for which culture conditions have yet to be determined. Unlike the pig and sheep for which ESC lines are not available, isolation and culture of cESCs has been documented (7-9), thus allowing us to verify pluripotency and self-renewal properties of ciPSCs derived in this study.

In summary, we have successfully generated 12 ciPSC lines from 3 adult dogs. Importantly, we have used a non-invasive multi-modality imaging approach to monitor autologous iPSC transplantation in a large animal model of cardiac delivery. Both the PET reporter gene and the MR iron oxide labeling techniques we employed allow for accurate localization of cells and are directly applicable to human patients (28). Furthermore, we have generated functional endothelial cells from ciPSCs and demonstrated the therapeutic potential of these cells in murine models of hindlimb ischemia and myocardial infarction. Validating pre-clinical iPSC imaging in large animal models can be an arduous process and will be different for every cell type and targeted tissue. Undoubtedly, further endeavors will be needed to further optimize both the imaging protocols and iPSC biology synergistically to allow successful translation of pluripotent stem cell therapies to patients in the future.

References


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Figure Legends

Figure 1. Generation of canine induced pluripotent stem cells (ciPSCs). (A) Schematic diagram of the generation of ciPSC. ciPSC colonies can be picked out around 12-15 days and are alkaline phosphatase positive. (B) Immunofluorescence staining of pluripotent markers. Similar to cESCs, ciPSCs are positive for pluripotent stem cell markers Oct-4 (b, h), Tra-1-60 (c, i), Nanog (e, k), and SSEA-4 (f, l), with nuclear staining by DAPI (a, d, g, j). (C) Q-PCR analysis of expression of pluripotent stem cell markers Oct-4, Nanog, Sox-2, cMyc, and Klf-4. Y-axis value represents fold differences (Log2) in expression of select genes. (D) Pearson correlation analysis for gene expression in cASCs, ciPSCs, and cESCs using transcripts with standard deviation greater than 0.2 among all samples (17,895 probes, P<1.0E-15).

Figure 2. Confirmation of pluripotency of ciPSCs. (A) Immunofluorescence staining of differentiated ciPSCs. Differentiated cells are positive for ectodermal cell maker Tuj1, mesodermal cell marker TNNT2, and endodermal cell marker Sox-17. (B) Q-PCR analysis of genes expressed from the three germ layers. Differentiated ciPSCs express genes from all three germ layers, whereas ciPSCs and cESCs do not express these genes at detectable levels. (C) Longitudinal bioluminescence imaging (BLI) after injection of ciPSCs and cESCs into SCID mice. BLI shows progressive increase in Fluc activity within teratomas. The left side of animals is control cESCs and the right side is ciPSCs. (D) Histological section of a solid, encapsulated tumor removed from the subcutaneous flank of a SCID mouse that had been injected with ciPSCs. The highly differentiated tumor contained a wide range of tissues, composed of endodermal, neuro-ectodermal, and mesodermal lineages (E) Karyotyping of parental cell cASC and ASC-derived ciPSCs at passage 30. All cells had a normal canine cell karyotype of 2n=78 (x,y).

Figure 3. Molecular imaging of ciPSCs in vitro and in vivo. (A) Schematic for the triple-fusion (TF) reporter gene carrying firefly luciferase, red fluorescent protein, and herpes simplex truncated thymidine kinase (LV-Fluc-RFP-HSVttk) driven by the ubiquitin promoter. (B) ciPSCs stably expressing the TF reporter gene were tested in vitro to verify Fluc expression. A linear correlation was observed between cell number and Fluc activity. (C) ciPSCs stably expressing the TF reporter gene were tested in vitro to verify HSVttk expression. A linear correlation was observed between cell number and HSVttk activity. The activities were 0.0008±0.0003 (1x10⁶), 0.0210±0.0010 (2x10⁶), 0.0910±0.0240 (5x10⁶), and 0.1860±0.0670 %ID/g (1x10⁷). (D) 1x10⁶, 2x10⁶, 5x10⁶, and 10x10⁶ ciPSCs stably expressing the TF reporter gene were injected into the subcutaneous flanks of mice. In vivo imaging by microPET was performed 60 minutes later. The activities were 0.0208±0.0033 (1x10⁶), 0.1830±0.0510 (2x10⁶), 0.2910±0.1160 (5x10⁶), and 0.3770±0.0880 %ID/g (10x10⁶). The minimal detectable number of ciPSCs was ~1x10⁶.

Figure 4. Clinical PET-CT and MR imaging of injected cells in autologous canine model. ciPSCs were dual labeled with HSVttk reporter gene and iron oxide particles for PET imaging of cell viability and MR imaging of cell location, respectively. (A) Representative axial non-enhanced PET-CT fusion
image of ciPSCs delivered to the left ventricle acquired 1.5 hours after administration of FHBG. A distinct signal (0.0025% ID/g) can be seen at the site of injection close to the left ventricular apex. (B) T2 weighted GRE images by MRI shows iron labeled cells as hypointense signals (dark areas noted by the single arrow) in the anterior apical wall, corresponding to PET imaging. Images are shown in the two-chamber (lower left) and short-axis views (lower right). Signal voids along two different tissue interfaces are susceptibility artifacts (double arrows). (C) Ex vivo analysis of short-axis sections of myocardium in the same dog confirm distinct signal in the anterior apical wall, which correspond to the \textit{in vivo} PET and MR signals. MicroPET imaging was performed 4 hours after tracer injection and 2 hours after animal sacrifice.

\textbf{Figure 5. Delivery of ciPSC-ECs into murine model of myocardial infarction.} (A) Representative BLI images of an animal receiving 1x10^6 ciPSC-ECs demonstrates strong cell localization to the heart at day 2. Progressive decrease in signal is observed over the next 6 weeks, but persistent cell engraftment is still observed at day 42. Quantitative analysis of BLI signal in animals receiving ciPSC-ECs is expressed in photons/sec/cm^2/sr. (B) Representative M-mode echocardiographic views of infarcted hearts receiving ciPSC-ECs (n=6) or PBS (n=6). (C) Quantification of fractional shortening (F/S) reveals significant improvement in systolic function of animals receiving ciPSC-ECs at days 14 and 28 (P<0.05) post MI, but not at day 42 as compared to animals receiving PBS. (D) Immunofluorescence staining reveals engraftment of RFP cells in the murine heart 42 days after injection.
Figure 1

A: Time line for the process of colony formation and pick up.

B: Immunofluorescence staining of ciPSC and cESC.


D: Heatmap showing fold induction of Sox-2, cMyc, and Klf4 for cASC, ciPSC-1, ciPSC-2, cESC-1, and cESC-2.
Figure 2

A

B

C

D

E

Fold induction

foxA2, TF, AFP, GATA4, MLC, NKX, Gbx

CASC, cESC, ciPSC, cESC-EB, ciPSC-EB

TUJ1, TNNT2, SOX17, DAPI, Merge

Endodermal - gland

Neuro-)Ectodermal - immature

Mesodermal - bone
Figure 3
Figure 4
Figure 5

A

Day 2  Day 7  Day 14  Day 28  Day 42

B

Day 0  Day 14  Day 28  Day 42

PBS

ciPSC-EC

C

Fractional Shortening (%)

Day 0  Day 14  Day 28  Day 42

D

cTNT RFP DAPI
SUPPLEMENTAL METHODS

Isolation of canine adipose stromal cells and skin fibroblasts. The animal protocol was approved by the Stanford Animal Research Committee. Three one-year old Mongrel dogs (Marshall Farm, CA) were anesthetized with 5 mg/kg tiletamine/zolazepam (Webster, Sterling, MA) and were placed prone on the operating table with sterile preparation of the posterior dorsal region. To obtain canine fibroblasts (cFibro), an elliptical skin incision was performed on dorsal surface of these dogs. This incision was carried through the dermis and into the underlying adipose tissue. This ellipse was then sharply resected and underlying hemostasis was obtained. Once the ellipse was removed, the adipose and tissue underlying the dermis was meticulously removed. The ellipse was closed primarily with a layered closure with deep 2-0 vicryl and superficial 2-0 nylon sutures. Subsequently, the dermal and epidermal layers were taken to the laboratory and fibroblast harvest was performed using collagenase II digestion at 37°C overnight. The adipose tissue from dogs was resected sharply and separated from the overlying skin. Adipose specimens were digested with collagenase II at 37°C for 4 hours. The stromal vascular fraction was then pelleted, filtered at 100 micron pore size, and primary cultures of canine adipose stromal cells (cASCs) established at 37°C and 5% CO₂.

Cell culture and maintenance. cASCs were maintained with 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. cFibro were maintained with DMEM containing 10% FBS, L-glutamine (Invitrogen), 4.5 g/L glucose, 100 units/ml penicillin, and 100
ug/ml streptomycin. All cells used for reprogramming were within 5 passages. Cells were passaged upon confluency by trypsinization. Derived ciPSCs were maintained on MEF feeder layers with iPSC medium containing Knockout DMEM (Invitrogen), 20% ES qualified FBS (Invitrogen), 2 mM L-Glutamine (Invitrogen), $1 \times 10^{-4}$ M nonessential amino acids (Invitrogen), $1 \times 10^{-4}$ M 2-mercaptoethanol (Invitrogen), 100 units/ml penicillin, 100 ug/ml streptomycin, 4 ng/ml human bFGF (Invitrogen), and 12.5 ng/ml human LIF (Millipore, Billeria, MA). cESCs were received as a gift from Dr. Beverly Torok Storb (Fred Hutchinson Cancer Research Center, Seattle, Washington) (1). cESCs were cultured under the same conditions as ciPSCs.

**Production of lentiviral reprogramming vectors.** 293FT cells were plated at ~80% confluence per 100 mm dish and transfected with 12 ug of each lentiviral vectors (Oct4, Sox2, Klf4, c-MYC) plus 8 ug of packaging plasmids and 4 ug of VSVG plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer’s instructions. The resulting supernatant was collected 48 hours after transfection, filtered through a 0.45 um pore-size cellulose acetate filter, and mixed with PEG-it Virus Concentration Solution (SystemBio, Mountain View, CA) overnight at 4°C. Viruses were precipitated at 1500g the next day and resuspended with Opti-MEM medium (Invitrogen, Carlsbad, CA).

**Immunofluorescence and alkaline phosphatase staining.** Cells were fixed with 2% formaldehyde in PBS for 2 min, permeabilized with 0.5% tritonX-100 in PBS for 10 min, and blocked with 5% bovine serum albumin in PBS for one hour. Cells were then stained with appropriate primary antibodies and AlexaFluor conjugated secondary antibodies (Invitrogen, Carlsbad, CA). The primary antibodies for Oct3/4 (Santa Cruz, Santa Cruz, CA), Sox2
(Biolegend, San Diego, CA), Klf4 (Abcam, Cambridge, UK), c-MYC (Abcam), SSEA-3 (Millipore, Billerica, MA), SSEA-4 (Millipore), Tra-1-60 (Millipore), Tra-1-81 (Millipore), Nanog (Santa Cruz), Desmin (Sigma, St. Louis, MO), Sox17 (R&D Systems, Minneapolis, MN), and Tuj-1 (Covance, Princeton, NJ) were used in the staining (Supplemental Table 1). Alkaline phosphatase (AP) staining was performed using the Quantitative Alkaline Phosphatase ES Characterization Kit (Millipore Chemicon, Billerica, MA) following the manufacturer’s instructions.

**Quantitative-PCR.** Total RNA and cDNA of each sample were prepared using the RNeasy Mini Plus Kit (Qiagen, Valencia, CA) and the QuantiTect Reverse Transcription Kit (Qiagen), respectively, following the manufacturer’s instructions. Quantitative-PCR to measure mRNA expression levels was done with Taqman Gene Expression Assays (Applied Biosystems, Carlsbad, CA) using a SteponePlus Realtime-PCR System (Applied Biosystems) in the Protein and Nucleic Acid Facility at Stanford University School of Medicine. All primers are listed in Supplemental Table 2.

**In vitro differentiation.** CiPSCs were treated with dispase (Invitrogen, Carlsbad, CA) and transferred to ultra-low attachment plates (Corning Life Sciences, Kennebunk, ME) in suspension culture for 8 days with DMEM/F12 (1:1) (Invitrogen) containing 20% knockout serum (Invitrogen), 4.5 g/L L-glutamine (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), 50 units/ml penicillin (Invitrogen), and 50 ug/ml streptomycin (Invitrogen). Embroid bodies (EBs) were then seeded in 0.25% gelatin-coated tissue culture dishes for another 8 days. Spontaneous differentiation of CiPSCs into cells of
ectoderm, mesoderm, and endoderm lineages was detected with appropriate markers by Q-PCR.

**Teratoma formation.** To form teratomas, ~2 million ciPS cells were harvested from culture dishes and injected subcutaneously to the dorsal flanks of adult SCID mice (n=6). After 8 weeks, tumors were dissected and fixed with 10% formaldehyde in PBS. Paraffin embedded tissue sections were then generated and stained with hematoxylin and eosin (H&E). Histological slides were interpreted by an expert pathologist (AJC).

**Bioluminescence and micro positron emission tomography (microPET) imaging in vitro and in vivo.** ciPSCs and cESCs which were stably transduced with a triple-fusion reporter gene carrying firefly luciferase, red fluorescent protein, and herpes simplex truncated thymidine kinase (LV-Fluc-RFP-HSVttk) driven by the ubiquitin promoter. Tumor growth was monitored noninvasively by BLI. Animals were injected with 250 mg/kg D-luciferin and imaged using a Xenogen IVIS Spectrum (Xenogen, Alameda, CA) as previously described (2). cESCs labeled with Fluc-RFP-HSVttk reporter gene were used as a positive control to monitor teratoma growth. microPET imaging was acquired using a R4 Concorde MicroPET system. Mice were injected with the reporter probe 9-[4-[^18]F]fluoro-3-(hydroxymethyl)butyl]guanine ([^18]F]-FHBG) (0.92±0.19 uCi) and imaged 60 to 75 minutes after injection. Images were reconstructed by filtered back-projection algorithm. For each imaging process, a set of serial microPET images was collected to assess the influx of the tracer for 90 min (18 frames, 5 min each). Regions of interest (ROIs) were drawn over the interested area. 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 to obtain an image ROI-derived[^18]F]-FHBG percentage injected dose per
gram (% ID/g) as described previously (3). For in vitro imaging experiments, 1 ml of cells at the following four different concentrations (1×10^6, 2×10^6, 5×10^6, 10×10^6 cell/ml) were plated on 60 mm dishes. Cells were pre-incubated with 20 µCi/ml [^{18}F]-FHBG for 30 minutes before microPET image acquisition.

**Microarray hybridization and data analysis.** Total RNA samples were hybridized to Affymetrix GeneChip Canine Genome 2.0 Array, and then normalized and annotated by the Affymetrix® Expression Console™ software. A heatmap was generated for 164 genes that showed differences in expression greater than log2 ratio 6. The Pearson Correlation Coefficient was calculated for each pair of samples using the expression level of transcripts, which show standard deviation greater than 0.2 (17895 probes) among all samples. For hierarchical clustering, we used Pearson correlation for average linkage clustering. Scatter plots were generated for whole gene expression.

**Intramyocardial delivery of autologous ciPSCs in canine models.** Dogs were anesthetized with 5 mg/kg tiletamine/zolazepam (Webster, Sterling, Mass), tracheally intubated, and mechanically ventilated. Anesthesia was maintained with 2% isoflurane in 100% oxygen, and electrocardiogram, respiration rate, and intraventricular pressure were recorded. All dogs underwent a left lateral thoracotomy through the fourth and fifth intercostal spaces. The pericardium was opened and an octopus device was placed on the wall of the left ventricle at each injection site to facilitate injections into the heart by reducing the motion of the myocardium. After injection into the anterior myocardium, the chest wall was closed in layers and a pleural tube was placed into the left pleural cavity to drain the remaining free air.
Clinical PET-CT imaging of autologously transplanted ciPSCs. Imaging was performed with a clinical PET-CT scanner (Discovery LightSpeed Plus; GE Medical Systems, Waukesha, Wisconsin), with the animals in a supine position. After an initial scout view (30 mAs, 120 kV), a monenhanced four-detector CT scan of the heart was obtained with the following settings: section thickness of 5 mm, table feed of 38 mm per rotation, 0.8-second gantry rotation time; x-ray tube voltage of 120 kV, and tube current of 150 mA. Eight hours after intramyocardial injection of $1 \times 10^8$ ciPSCs and 4 hours after intravenous administration of $\left[^{18}F\right]$-FHBG (mean, 536.5 MBq; range, 370–777 MBq), a 1-hour static PET scan was acquired. Transverse section reconstructions of the CT data sets were performed with a nominal section thickness of 5 mm at an interval of 4.25 mm. All PET images were reconstructed by using an iterative algorithm (ordered-subset expectation maximization, two iterative steps, 28 subsets), with CT-based attenuation correction applied. No cardiac gating or respiratory motion correction was performed.

Labeling of ciPSCs with iron particles. We labeled ciPSCs with Feridex IV-protamine sulfate (FE-Pro) as described previously (4). The commercially available superparamagnetic iron oxide (SPIO) suspension (Feridex IV; Berlex Laboratories, Inc, Wayne, NJ; http://berlex.bayerhealthcare.com/index.html) contains particles approximately 80–150 nm in size and has a total iron content of 11.2 mg/ml. We prepared preservative-free protamine sulfate (American Pharmaceuticals Partner Inc., Schaumburg, IL; http://www.appdrugs.com/) with a concentration of 10 mg/ml using a fresh stock solution of 1 mg/ml and distilled water. SPIO at a concentration of 100 µg/ml was placed into a mixing tube containing serum-free culture medium. We added protamine sulfate (12 µg/ml) and mixed the entire suspension for 5–10 minutes. We added the final FE-Pro suspension to the existing medium and incubated...
overnight. The final concentrations of Feridex IV and protamine sulfate were 50 and 6 µg/ml of medium, respectively. After overnight incubation, the ciPSCs were washed twice with PBS and harvested by treatment with collagenase IV and trypsin, respectively.

**MR imaging of autologously transplanted ciPSCs.** Imaging was performed on a Signa 3.0T Excite HD scanner (http://www.gehealthcare.com/euen/mri/index.html) with an 8-element phased array coil (GE Healthcare Systems, Milwaukee, Wisconsin). Dogs were intubated, anesthetized, and placed in the supine position for imaging. An electrocardiogram and respiratory gating system was used to acquire images. Briefly, a three-dimensional-plane localization was performed. Iron labeled cells were imaged in oblique planes using a gradient-recalled echo (GRE) sequence (repetition time 100 ms, echo time 10 ms, flip angle 30°, slice thickness 1 mm, slice gap 0 mm). Multiple contiguous slices were acquired for complete coverage of the dog heart.

**Ex vivo imaging of dog heart by microPET.** Following PET-CT imaging, animals were sacrificed by intravenous administration of saturated potassium chloride. Hearts were explanted and sectioned in a short-axis plane for *ex-vivo* imaging by microPET. A 4 minute static image was acquired for each section after which the hearts were fixed for histology.

**Histology of dog heart.** Animal hearts were excised, rinsed, fixed in 10% neutral buffered formalin overnight, and sectioned in transverse slices. Transverse slices from the level of injection were verified by gross examination, and H&E staining was performed by means of paraffin embedding.
Differentiation of ciPSCs into canine endothelial cells (ciPSC-ECs). Generation of ciPSC-ECs was based on previous endothelial cell differentiation protocols using human ESCs and iPSCs (5). Briefly, ciPSCs were cultured in ultra low attachment dishes using EB differentiation medium consisting of Knock out DMEM (KDMEM; Invitrogen), 20% ES qualified FBS (Invitrogen), 2 mM L-Glutamine, $1\times10^{-4}$ M nonessential amino acids (Invitrogen), $1\times10^{-4}$ M 2-mercaptoethanol, and 1X pen/strep (Invitrogen). After two days suspension culture, EBs were collected and transferred to 0.2% gelatin coated dishes using medium containing KDMEM, 20% FBS, 2 mM L-Glutamine, $1\times10^{-4}$ M nonessential amino acids, $1\times10^{-4}$ M 2-mercaptoethanol, 1x pen/strep, 200 ng/ml VEGF (R&D Systems, Minneapolis, MN), 20 ng/ml IGF (R&D), 50 ug/ml Ascorbic acid (R&D), and 1 ug/ml Hydrocortisone (R&D). Medium was changed every other day. After 16 days culture, cells were passaged using 0.25% trypsin and replated on collagen IV coated dishes using endothelial cell culture medium containing EGM-2 (Lonza, MD). For DiI-ac-LDL uptake, iPSC-EC were incubated with 10ug/ml of DiI-AC-LDL (Molecular Probes, OR) at 37 for 6 hours. After washing with PBS twice, cells were fixed and counterstained with DAPI as described. The formation of endothelial tubes was assessed by seeding cells in 24-well plates coated with Matrigel and incubating them at 37°C for 12 hours as described.

Delivery of ciPSC-ECs in a murine model of hindlimb ischemia. SCID Beige mice (Charles Rivers Laboratories, MA) were anesthetized with isoflurane (2-3%) after which unilateral hindlimb ischemia was induced by ligating the femoral artery. Briefly, skin on the right leg of the mouse was dissected from the knee towards the medial thigh. After separation of the femoral artery and vein, a segment of the femoral artery was tied with a 7-0 suture and
subsequently transected with spring scissors (6,7). To test the therapeutic potential of ciPSC-ECs, 1x10^6 cells stably expressing Fluc-RFP-HSVttk were delivered by intra-muscular injection into the region of ischemia (n=5). Control animals received PBS (n=5).

**Laser Doppler of hindlimb perfusion.** Blood perfusion of ligated and control hindlimbs was assessed using a PeriScan PIM3 laser Doppler system (Perimed AB, Sweden) as previously described (6). Animals were placed on a 37°C heatpad, and hindlimb blood flow was measured on days 0, 7, and 14. Perfusion was quantified as mean pixel value within the entire hindlimb and the relative changes in hindlimb blood flow were expressed as the pixel ratio of the ischemic leg over the nonischemic leg.
SUPPLEMENTAL FIGURE LEGEND

Supplemental Figure 1. Pluripotency of ciPSCs derived from cASC and cFibro. ciPSCs derived from cASCs and cFibro both stained positive for standard markers of pluripotency. (A) DAPI staining of ciPSCs (ASC). (B) Oct-4 (green) and Tra-1-60 (red) co-staining of a ciPSC (ASC) colony. (C) ciPSCs (ASC) colonies stain positive for alkaline phosphastase (AKP). (D) DAPI staining of ciPSCs (Fibro) colony. (E) ciPSCs (Fibro) express Oct-4 and Tra-1-60 in an identical fashion to ciPSCs (ASC). (F) ciPSCs (Fibro) express AKP in an indistinguishable manner from c-ASC-iPSCs.

Supplemental Figure 2. Semi-quantative RT-PCR analysis of ciPSCs and cASCs. ciPSCs (ASC) were positive for expression of pluripotency genes Oct-4, Nanog, Sox2, Klf-4, Nanog, and c-Myc. Parental cASCs and emboid bodies differentiated from ciPSCs (ASC) did not express these pluripotency genes.

Supplemental Figure 3. Whole genome analysis of cASCs, ciPSCs (ASC), and cESCs. Scatter plots of whole genome expression show similarities between cESC and ciPSC transcriptional profiles and marked differences between cESCs/ciPSCs and cASCs. Principal component analysis for whole genome expression further verifies the similarities in cESC and ciPSC gene expression profiles as compared to cASCs.

Supplemental Figure 4. Microarray analysis of cASCs, ciPSCs (ASC), ciPSCs (Fibro), and cESCs. Microarray analysis identified 164 genes which showed significant differences in gene expression between cESCs and cASCs (log2 ratio greater than 6). Expression of these genes was
compared in ciPSCs, cESCs, and cASCs. Specifically, 1 cell line of cASCs, 2 cell lines of ciPSCs derived from cASCs denoted as ciPSCs (ASC-1) and ciPSCs (ASC-2), 2 cell lines of ciPSCs derived from cFibro denoted as ciPSCs (Fibro-1) and ciPSCs (Fibro-2), and 2 cell lines of cESCs were compared. ciPSCs (Fibro), ciPSCs (ASC) and cESCs were found to have similar expression profiles for these genes in contrast to cASCs.

**Supplemental Figure 5. Quantitative PCR of ciPSC (ASC)-derived EBs.** Embroid bodies (EBs) derived from ciPSCs (ASC) were subjugated to Q-PCR analysis for an array of genes associated with differentiation. Specifically, these include FoxA2 (endoderm), TJF (ectoderm), AFP (endoderm), Gata4 (mesoderm), Gata6 (mesoderm), MLC (mesoderm), Nkx (mesoderm), and Gbx (ectoderm). Differentiated cells derived from c-ASC-iPSCs were found to express genes from all three germ layers while ciPSCs (ASC) and cESCs did not express these genes.

**Supplemental Figure 6. Quantitative PCR of ciPSC (Fibro)-derived EBs.** EBs derived from ciPSCs (Fibro) were subjugated to Q-PCR analysis for an array of genes associated with differentiation. Specifically, these include FoxA2 (endoderm), TJF (ectoderm), AFP (endoderm), Gata4 (mesoderm), Gata6 (mesoderm), MLC (mesoderm), Nkx (mesoderm), and Gbx (ectoderm). Differentiated cells derived from c-fibro-iPSCs were found to express genes from all three germ layers while ciPSCs (Fibro) and cESCs did not express these genes.

**Supplemental Figure 7. Histology of dog heart.** Histological sections of dog hearts were stained by hematoxylin and eosin (H&E). (A) Myocardial tissue remote to the site of cell injection is healthy with no cell death (20X). (B) Myocardial tissue at site of injection has
minimal cell death and little evidence of inflammatory infiltrate due to autologous nature of transplantation (20x). (C) Magnified view of remote myocardium (40x). (D) Magnified view of injection site (40x).

Supplemental Figure 8. Generation of ciPSC-ECs and delivery into murine model of hindlimb ischemia. (A) ciPSC-ECs stained positive for CD31, demonstrated DiI-ac-LDL uptake, and formed tubules when cultured on Matrigel in vitro. (B) A representative animal post hindlimb ligation injected with 1x10⁶ ciPSC-ECs demonstrates significant bioluminescence activity at day 2, which decreases progressively over the following 2 weeks. (C) Quantitative analysis of BLI signal in animals transplanted with ciPSC-ECs. Signal activity is expressed as photons/sec/cm²/sr. (D) Laser Doppler of blood perfusion in ischemic legs of mice demonstrates improved revascularization in animals receiving ciPSC-ECs as compared to animals receiving saline. (E) Quantification of blood flow reveals improved reperfusion in hindlimbs of mice receiving ciPSC-ECs compared to PBS control.

SUPPLEMENTAL VIDEO LEGEND

Supplemental Video 1: Three dimensional PET-CT reconstruction of the thorax and upper abdomen in the same dog reveals high $^{18}$F-FHBG uptake in the myocardium corresponding to the location of injected ciPSCs. There is high signal in the liver due to natural route of $^{18}$F-FHBG excretion via biliary and hepatic routes as has been shown in human dendritic cell imaging study (lower left) (8).
**Supplemental Table 1: Primary antibody information**

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## Supplemental Table 2: Primer sequences

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SUPPLEMENTAL REFERENCES


Supplemental Figure 1

Supplemental Figure 1
Supplemental Figure 2
Supplemental Figure 3
Supplemental Figure 5
Supplemental Figure 6
Preclinical derivation and imaging of autologously transplanted canine induced pluripotent stem cells

Andrew S. Lee, Dan Xu, Patricia K. Nguyen, Divya Nag, Jennifer K. Lyons, Leng Han, Shijun Hu, Feng Lan, Mei Huang, Junwei Liu, Kazim N. Narsinh, Charles T. Long, Patricia de Almeida, Benjamin Levi, Nigel Kooreman, Charles Bangs, Cholawat Pacharinsak, Alan C. Yeung, Sanjiv S. Gambhir, Michael T. Longaker and Joseph C. Wu

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