Induced Pluripotent Stem Cell Transplantation in the Treatment of Porcine Chronic Myocardial Ischemia

Yifu Zhou, MD, **Suna Wang, MD**, **Zuxi Yu, MD, PhD**, **Robert F. Hoyt Jr., DVM**, **Timothy Hunt, BS**, **Bogdan Kindzelski, BS**, **David Shou, BS**, **Wen Xie, MD**, **Yubin Du, PhD**, **Chengyu Liu, PhD**, and **Keith A. Horvath, MD**
Cellular Biology Section, Cardiothoracic Surgery Research Program/NHLBI, NIH, Bethesda, MD, USA

Abstract

Background—This study was designed to test the effects of induced pluripotent stem cell (iPSC) in the treatment of chronic myocardial ischemia.

Methods—The reprograming of passage 3 myocardial fibroblasts was performed by using the lentiviral vector containing 4 human factors: OCT4, SOX2, KLF4, and c-MYC. The iPSC Colonies at P12–17 were allogeneically transplanted into ischemic myocardium of ten swine by direct injection. Cohorts of two animals were sacrificed at 2, 4, 6, 8, and 12 weeks after injection.

Results—No signs of graft versus host disease were evident at any time points. At 2 weeks, clusters of SSEA-4-positive iPSCs were detected in the injected area. At 4 to 8 weeks, these cells started to proliferate into small spheres surrounded by thin capsules. At 12 weeks, the cell clusters still existed, but decreased in size and numbers. The cells inside these masses were homogeneous with no sign of differentiation into any specific lineage. Increased smooth muscle actin or vWF positive cells were found inside and around the iPSC clusters, compared with non-injected areas. By RT-PCR, the levels of VEGF, FGF, and ANRT expression were significantly higher in the iPSC treated myocardium compared to untreated areas. These results suggest that iPSCs contributed to angiogenesis.

Conclusions—Allogeneically transplanted pig iPSCs proliferated despite an ischemic environment in first two months and survived for at least three months in immunocompetent hosts. Transplanted iPSCs were also pro-angiogenic and thus might have beneficial effects on the ischemic heart diseases.

Address correspondence to: Yifu Zhou, MD, Cellular Biology Section, Cardiothoracic Surgery Research Program, National Heart, Lung and Blood Institute, National Institutes of Health, 10 Center Drive, MSC 1550, Building 10, Room B1-D47, Bethesda, MD 20892-1550, zhouyifu@mail.nih.gov.

Keywords

Cell Transplantation; Stem cells; Myocardial ischemia; Angiogenesis; Animal model

Introduction

Marrow derived stem or progenitor cells have been used for patients with ischemic myocardial diseases since 2003 (1–8). Functional improvement has been reported by most clinical trials, however, no evidence has shown that marrow derived cells can differentiate into beating myocytes in vitro, even though several studies claimed myocyte-like characteristics or markers after treating these cells with certain chemicals or factors (9–11). Thus, most investigators believe that the beneficial improvement is mainly due to paracrine effects of implanted cells (12–16). Induced pluripotent stem cells (iPSCs) have been recently generated in murine, human, and several other species since 2006 (17–19). These iPSCs have become exciting tools for understanding the mechanisms of diseases and for potentially treating diseases through cell replacement therapy. In order to apply this new technology for future clinical use, it needs to be tested in a large animal model. Recently, pig iPSCs have been derived from fetal fibroblasts by ectopic expression of four human transcriptional factors, OCT4, SOX2, KLF4, and c-MYC using lentiviral vectors (20). These pig iPSCs showed pluripotency via in vitro differentiation assays and teratoma formation assays. However, these cells have failed to differentiate into beating myocytes in vitro. Recent studies have suggested that iPSCs seem to retain epigenetic memory because they have a higher tendency to differentiate into the lineages which they were reprogrammed from (21–23). Therefore, we sought to derive iPSCs from porcine myocardial fibroblasts and hope these iPSCs can facilitate our efforts to generate cardiomyocytes. In this study, we performed allogeneic transplantation of the cardiac fibroblast-derived pig iPSCs into chronic ischemic myocardium, and compared their effects with fetal fibroblast-derived iPSCs (20). The results were compared with those using bone marrow derived mesenchymal stem cell (MSC) previously reported by our laboratory (24,25).

Material and Methods

Pig myocardium-derived fibroblasts preparation and culture

A tissue explant method was used for the myocardium-derived fibroblast culture. Briefly, a full length of left ventricular tissue (1×1 cm) was obtained from a healthy adult donor (Yorkshire, three months old). After washing three times in serum-free 2× penicillin-streptomycin solution containing Dulbecco's Modification of Eagle's Medium (DMEM), the tissue was cut into small pieces (2 mm cube) and then washed twice. The small tissue clumps were transferred into 10 cm-culture dish, and cultured with DMEM supplemented with 10% fetal bovine serum, 1× penicillin-streptomycin solution. Fibroblasts began to migrate out of the tissue fragments and into the surrounding area after 3–7 days in culture. These fibroblasts were harvest by trypsinization and passaged in DMEM with 10% fetal bovine serum (FBS) (Figure 1a–d).

Pig iPSC derivation and culture

Passage 3 myocardium-derived fibroblasts were seeded in six-well culture plates at a density of approximately 1×10^5 per well. In the next day, they were transduced with StemCCA lentiviral vectors (Millipore, Billerica, MA) containing the four human factors: OCT4, SOX2, KLF4, and c-MYC, following the manufacturer provided protocol. After viral transduction, the fibroblasts were cultured in DMEM medium with 10% FBS for five more days, and then trypsinized and plated on 10 cm tissue culture dishes with irradiated mouse embryonic fibroblasts (MEF). The next morning, the culture medium was switched to standard human ES cell medium and changed daily afterwards. The iPSC-like colonies began to appear 5 days after switching to ES medium (Figure 1e and f). These colonies were manually picked about a week later into 24-well plates, and subsequently passed and expanded following standard human ESC/iPSC cultural protocol. Passage 12–17 pig iPSCs were used for the allogeneic transplantation study.

Animals

Yorkshire domestic pigs, initially weighing 15–20 kg were used as allogeneic iPSC recipients for this study. The experimental protocol was approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute, and all procedures conformed to the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, 1996, Washington, D.C.).

Ameroid placement and iPSC transplantation

To create a chronic ischemic model, all animals underwent ameroid constrictor placement around the proximal left circumflex coronary artery (26). Four weeks later, a second left thoracotomy was performed on each animal, the circumflex territory (ischemic zone) was exposed and injected with an average of 1.6×10^7 iPSCs (from four 10-cm culture dishes with confluent iPSC colonies, suspended in 2.5 ml of normal saline, with 25 injection sites). Cohorts of two animals were sacrificed at 2, 4, 6, 8 weeks and 3 months after injection and the hearts were harvested and sectioned to study the differentiation of the injected cells.

To test if different stem cell lines behave differently in terms of their differentiation and proliferation capabilities, iPSCs kindly provided by Dr. Robert's laboratory were used for allogeneic transplantation using the same methods. Before injection, these cells were expanded using the methods suggested by the provider (20).

Histological and immunohistochemistry analysis

Myocardial tissues with the iPSCs transplantation were cut into 5×5 mm-thick pieces, either collected in cassettes and fixed with 10% buffered formalin for paraffin embedding or in O.C.T. for frozen sections with no fixation. Lung, liver, kidney and spleen samples were also collected for possible cell tracking. Paraffin embedded sections were stained with hematoxylin and eosin (H&E) for morphological analysis. The immunofluorescent staining was performed using rabbit polyclonal antibody against human OCT4 and SSEA4 to detect pluripotent marker genes. Antibody against vWF was used to detect vascular endothelial cells, and mouse monoclonal antibody against human smooth muscle actin to detect vascular

smooth muscle cells. Mouse monoclonal antibody against human desmin was used to detect myocytes. All above mentioned antibodies were purchased from Dako North America (Carpinteria, CA). The incubations of primary antibodies were followed by detections of fluorescein isothiocyanate conjugated anti-mouse IgG or Rhodamine conjugated anti-rabbit IgG and the nuclei were labeled with 4',6-diamidino-2-phenylindole..

RNA preparation and real-time reverse-transcription polymerase chain reaction

To test the different gene expression profiles of the tissues with or without iPSC injection, total RNAs from these samples were isolated using RNeasy Kit (Qiagen, Valencia, CA) for real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis. The first-strand cDNAs were synthesized from 500 ng of total RNA using SuperScript™ III kit (Invitrogen Life Technologies, Carlsbad, CA) and random hexamers. The cDNA templates were then mixed with PCR reaction solution containing 100 nM of both forward and reverse primers, Universal PCR master mix with SYBR Green 1 (QuantiTect™ SYBR Green RT-PCR Kit, QIAGEN Inc, Valencia, CA). The PCR amplification was performed on a multicolor qRT-PCR detection system (IQ™5, Bio-Rad Lab. Inc. Hercules, CA) as follows: 10 min of initial denaturation at 95°Cfollowed by 45 cycles of 15 sec. of denaturation at 95°C and 1 min of annealing/extension at 60°C. Each sample was analyzed in duplicates. Three qRT-PCR reactions were performed for the examination of each signal gene. For normalization, the average of the expression level of two housekeeping genes, swine GAPDH and β2-microglobulin were used. Analysis of relative quantification was performed as previously described (27). The sequences of PCR primers are listed as following:

Genes	Primer sequences
FGF F	GGA GAA GAG CGA CCC TCA CA
FGF R	CGG TTT GCA CAC ACT CCT TTG
VEGF F	CGC CAT GCA GAT TAT GCG GAT C
VEGF R	ACT CAA GCT GCC TCG CCT TGC A
ARNT (HIF1B) F	AGC CAT TGT TCA GAG GGC TA
ARNT (HIF1B) R	CCG CCG TTC AAT TTC ACT AT
OCT 3/4 F	AGT GAG AGG CAA CCT GGA GA
OCT 3/4 R	TCG TTG CGA ATA GTC ACT GC

Statistical analysis

Data is presented as means ± standard deviation (SD). Standard *student T-test* was used for gene expression comparison and a *p* value of less than 0.05 was considered significant.

Results

Pluripotency tests of cardiac fibroblast-derived iPSCs

After 5 passages in cultures on MEF feeder layers, the colonies became uniform in appearance with morphology resembling mature human iPSC colonies (Figure 1g and h). They were positive for live staining with Tra-1–60 and Tra-1–81 antibodies. They were also positive for alkaline phosphatase staining, as well as SSEA-4, OCT4, Nanog, and Sox2 antibody staining (Figure 2a–g). After culturing selected large colonies for 4 weeks in ultra-

low attachment plates, embryoid bodies, containing the typical three differentiated germ layers, were found. After injecting into immunocompromised mice, these cells formed teratomas. These cells can also differentiate into neuronal lineages as evidenced by strong staining of Tuj-1 (Figure 2h and i). After culturing selected large colonies for 7 days in ultra-low attachment plates and reseeding on MEF feeder layers, positive GATA-4 staining were found in some of the reseeded cells.

Animal outcome

After transplantation, there were no signs of adverse side effects or graft versus host disease. Three unexpected animal deaths were caused by severe surgical wound infection or pulmonary edema following ameroid placement determined by autopsy examination and not as a result of cell injections.

Histology analysis

Two weeks after cell injection, clusters of SSEA-4 positive cells were detectable in the frozen sections of the injected area using immunofluorescent staining. However, these cells could hardly be seen in H&E stained paraffin sections. This could be resulted from the small number (one tenth) of injected iPSCs as compared to previous MSC studies. Quite to our surprise, at 4 to 8 weeks post transplantation, these clusters of cells started to proliferate into spherical shaped small tumors surrounded by thin layered capsules within the injected area (Figure 3). The cells inside these tumors showed a homogeneous phenotype with no signs of differentiation into any lineages. Most of these tiny tumors were spherical shaped (figure 3b, c and r) and some of them were irregular or elongated (Figure 3e, n and o), but all had thin layers of capsules. There were multiple spherical shaped tumors within an elongated cell cluster (Figure 3o) suggesting variable growth speed within a single cell cluster. All these small tumors stained positive for Oct4 and SSEA4 suggesting these cells were injected iPSCs (Figure 3g–i). Interestingly, three months after injection, the small tumors showed no sign of further growth. Instead, these small tumors became smaller and irregular compared with those seen in the 4–8 week time points. Similar strong SSEA4 positive cell clusters were found in the injected area but in smaller numbers. These results suggested that myocardium-derived iPSCs survived inside the chronic ischemic environment after allogeneic transplantation and started to proliferate after about two weeks. Growth was limited in the immunocompetent host, evidenced by the fact that the small tumors found in the 4–8 week time points were no longer becoming larger at the 12 week time point. In MSC injected animals, cell clusters were also clearly found in the injected area. However, there were more cells in the early time points and the number of MSC decreased gradually over the 3 month period post-injection. Additionally, there was no sign of cell proliferation or evidence of capsules around the MSC clusters.

Angiogenesis related tests

At the 4 and 8 week time points, immunofluorescent staining showed increased smooth muscle actin and vWF positive cells inside and around the iPSC clusters (Figure 4 a, c) compared with those in non-injected areas (Figure 4 d). This finding may suggest that injected iPSCs are associated with angiogenesis. No desmin positive cells were found within or around these cell clusters. The expression of VEGF, FGF, and ANRT, as assessed by

qRT-PCR, were significantly higher in the injected myocardium compared with non-injected myocardium both in the 4 week and 3 month time points (Figure 5). These results suggested that injected iPSCs might have contributed to the formation of new blood vessels to a comparable level as those injected with MSCs. This angiogenic effect may have occurred as a result of paracrine like secretion of pro-angiogenesis factors. This finding suggests a beneficial effect of the iPSC transplantation even though injected iPSCs were not sufficient enough to regenerate the damaged heart.

Allogeneic transplantation of another iPSC cell line

Similar results were found from the animals that received another strain of transplanted iPSCs. Spherical and irregular shaped small tumors were found in 4 to 8 week time points and the sizes decreased over 3 months. The small tumors stained positive for Oct4 and SSEA4 suggesting that injected iPSCs may keep their pluripotency markers, but not differentiate in other cell linages. Figure 6 shows the histology images with a larger amount of small tumors in the 8 week time point, which dramatically decrease in the 3 month time point.

Comment

We report the first in vivo iPSC allogeneic transplantation into the ischemic myocardial environment of a large animal model that focuses on the fate and differentiation abilities of the transplanted cells. We expected myocardium-derived iPSCs developed in our laboratory would preferentially differentiate into the myocyte lineage compared to those derived from skin, blood or other non-muscle lineage cells. The myocardium-derived iPSCs carry myocardial epigenetic memory, which most investigators believe will influence their differentiation capabilities (21–23). Unfortunately, our data did not support this expectation. Rather, the iPSCs derived from pig myocardium only showed limited myocyte differentiation as we only found a few GATA4-positive cells in dissociated embryoid bodies.. No beating myocytes were found when various cultural protocols were performed. The iPSC line provided by Dr. Roberts' group, which was derived from porcine fetal fibroblasts, also lacked the ability to differentiate into beating myocytes in our hands. The underlining reason for this phenomenon is unclear, but one possibility is that the lentiviral vectors with the four reprogramming genes, which are integrated into the genome, are not completely silenced. The residual levels of these transcription factors may hinder the efficient differentiation into cardiac lineages of the injected iPSCs.

Even though both lines of iPSCs failed to show any signs of differentiation when transplanted into ischemic myocardium by direct injection, this study did provide us with several important findings: 1) when allogeneically transplanted, the iPSCs proliferated in the early stage, reached a peak level at 8 weeks post transplantation, then gradually declined in cell number; 2) once transplanted, iPSCs showed pro-angiogenic effects potentially as a result of paracrine-like secretion of factors which enhanced the pro-angiogenic signal pathways of VEGF, FGF and ANRT; 3) compared with our previous study on MSCs transplantation in the same porcine model, they both showed beneficial effects on angiogenesis, but iPSCs seemed to possess a greater aptitude towards survival and

proliferation; 4) The recipient pigs were generally healthy and there were no signs of deleterious tumor formation. However, we did find tumor-like cell clumps in the lung and spleen of one animal at the 6 week time point, suggesting that the injected iPSCs can survive and proliferate in organs other than the heart. This is most likely caused by accidental cell leaking into the blood stream at the time of injection, rather than metastasis. Therefore, extra caution must be exercised to avoid leakage when performing direct injection in future clinical cell-based therapy. The consequences for the leaked iPSCs are not yet clear. More detailed long term studies are needed to investigate whether they will interfere with the function of other organs and whether they will form teratomas, although most of these leaked cells appeared to be short lived. The current study terminates at three months post transplantation. It is very useful for quickly assessing the survival and differentiation potential of iPSCs in vivo, but long term observation and analysis are needed for unequivocally demonstrate whether this method is safe enough for clinical uses.

In summary, in an ischemic environment, pig iPSCs can continue to proliferate in vivo for two months after injection. However, the proliferation ability of the iPSCs was limited within the immunoconpetent hosts. The iPSCs we developed from pig myocardial fibroblasts showed limited ability to differentiate into beating myocytes. For future cell-based therapy, iPSCs with a higher propensity to differentiate into caydiomycytes will be ideal for assessing their potential for treating myocardial infarction. We are planning to use more efficient but non-integrative vectors for reprogramming cardiac fibroblasts into iPSCs.

Acknowledgments

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Abbreviations and Acronyms

|	
ANRT	aryl hydrocarbon receptor nuclear translocator
bFGF	basic fibroblast growth factor
DMEM	Dulbecco's Modified Eagle Medium
ESC	embryonic stem cell
FBS	fetal bovine serum
iPSC	induced pluripotent stem cell
MEF	mouse embryonic fibroblasts
MSC	mesenchymal stromal cell
NHLBI	National Heart, Lung, and Blood Institute
RT-PCR	real-time polymerase chain reaction
VGEF	Vascular endothelial growth factor
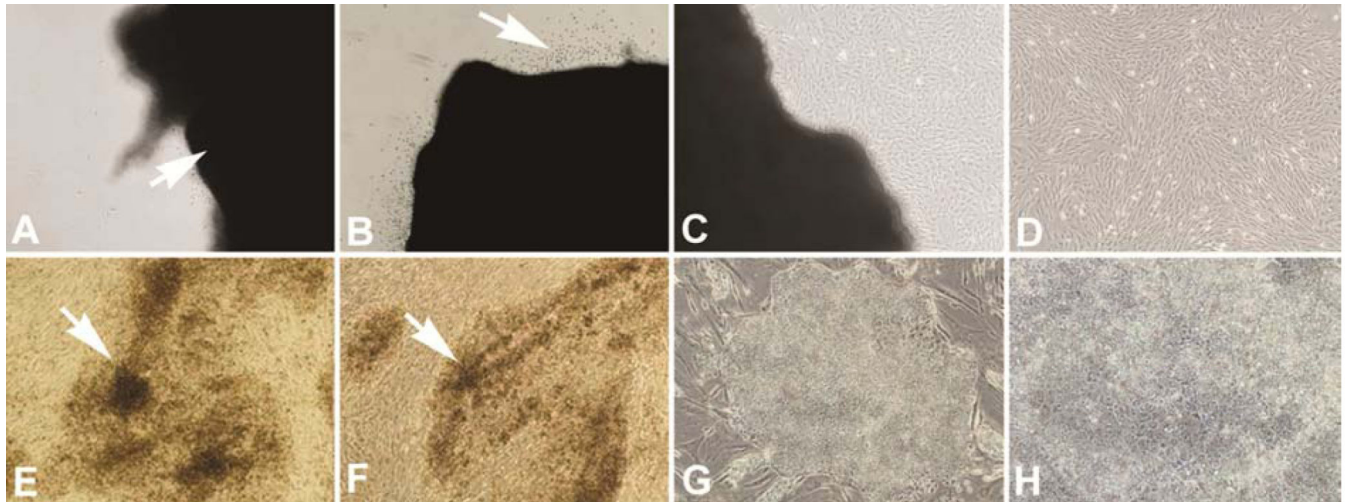
Figure 1.
Top panel shows tissue explant method to obtain myocardial fibroblasts: A. Arrow indicating a tissue clump attached to the culture plate; B. Arrow indicating cells migrating out from tissue clump after three days in culture; C. Migrated cells starting to proliferate to localized confluence; D. Cells derived from the myocardial tissue clump showing homogeneous fibroblast morphology after splitting and subculturing. Bottom panel-Reprograming of myocardial fibroblasts: E and F. Arrows indicating preliminary colonies five days after transfection; G. a mature iPSC colony at passage 5 at day 44; H. Higher magnification of G. Magnifications: A–G = 100×; H = 200×.
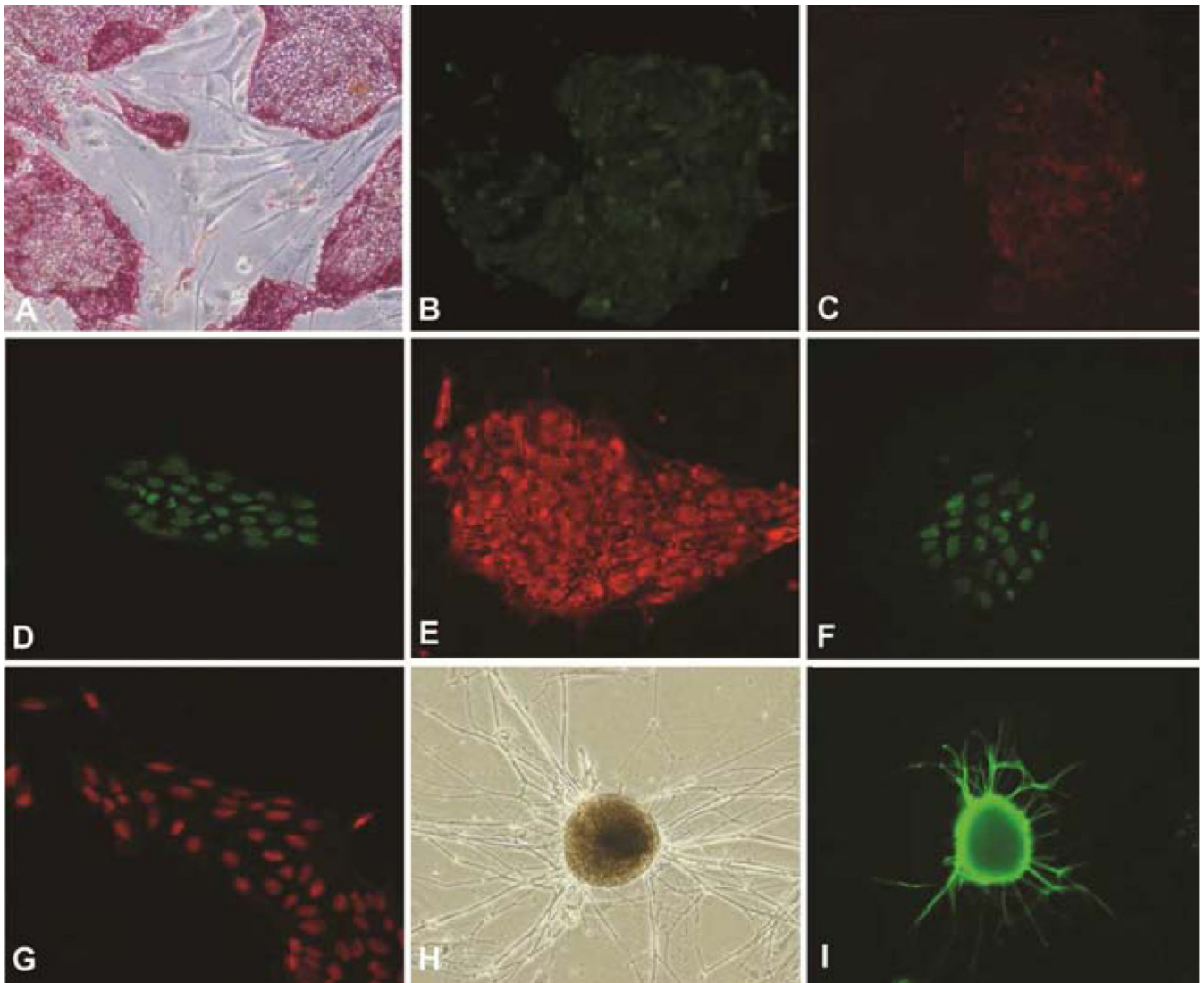
Figure 2.
Pluripotency tests for myocardial fibroblast-derived iPSCs. A. Positive AP staining; B. Tra-1–60; C. Tra-1–80; D. Nanog; E. SSEA4; F. Sox2; G. Oct4; H. Phase contrast image shows neural differentiation; I. Positive Tuj-1 staining. Magnifications: A–C = 100×; D–G = 200×; H and I = 100×.
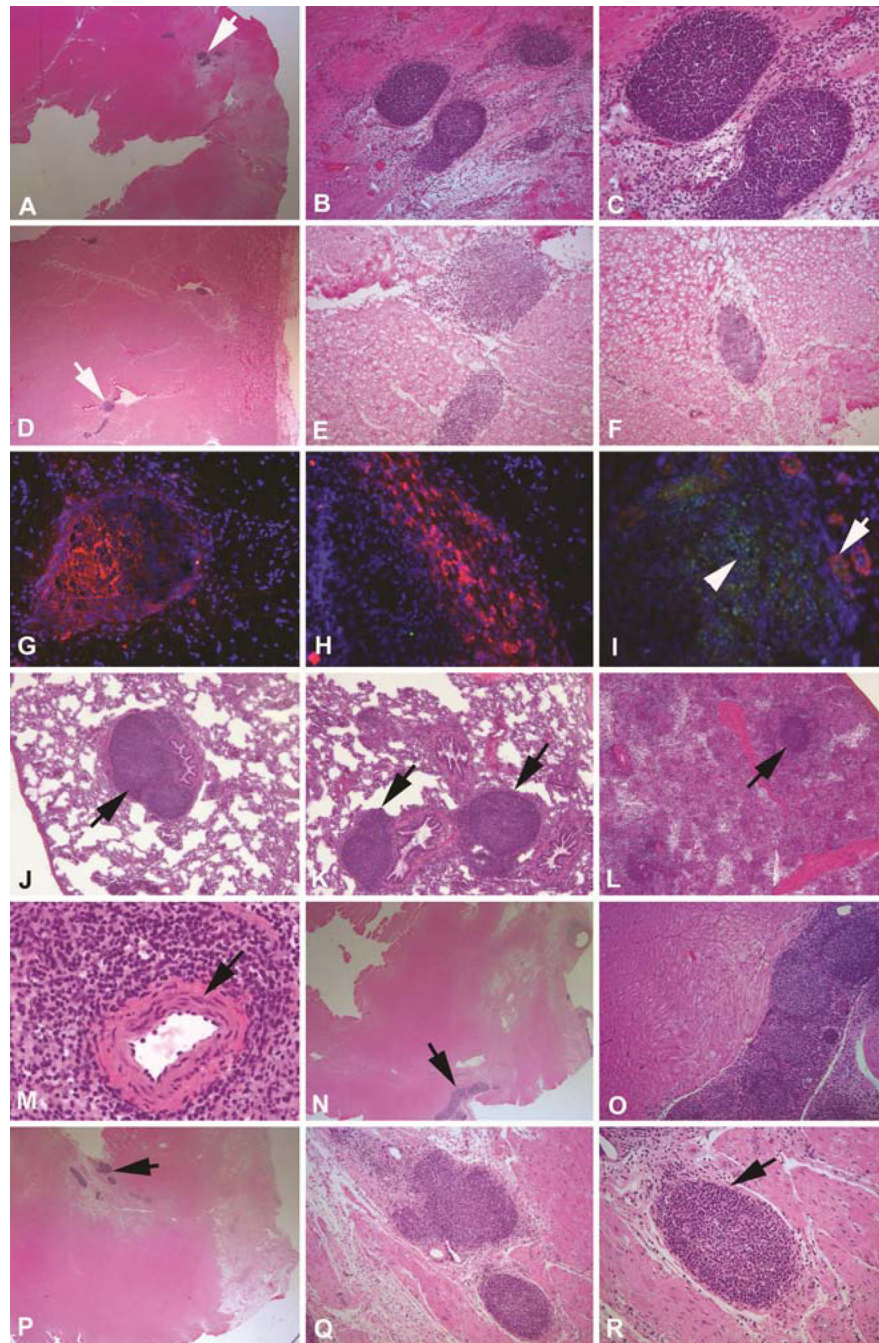
Figure 3.
Allogeneic transplantation of myocardial fibroblast-derived iPSCs. A. Arrow indicating an area with three spherical shaped tumors at 4 weeks after injection (12.5×); B and C. Higher magnification of A (100× and 200×); D. Arrow indicating an area with three irregular shaped tumors at 6 weeks after injection (12.5×); E&F. Higher magnification of D (100×); G&H. Positive SSEA4 staining (red) of the tumor-like cell clumps at 6 weeks (400×); I. Demonstrates positive Oct 4 (green) and smooth muscle actin staining (red) (400×); J–L. Arrows indicating cell clumps were also found in lung (J&K) and spleen (L) at 6 weeks

similar to those found in ischemic heart area (100×); M. A normal splenic nodule with a central artery in the center which was not found in iPSCs formed tumors (400×); N–R. Two sets of photos showing similar spherical shaped small tumors from two different animals at 8 weeks (n and p = 12.5×; o, q and r = 100×).
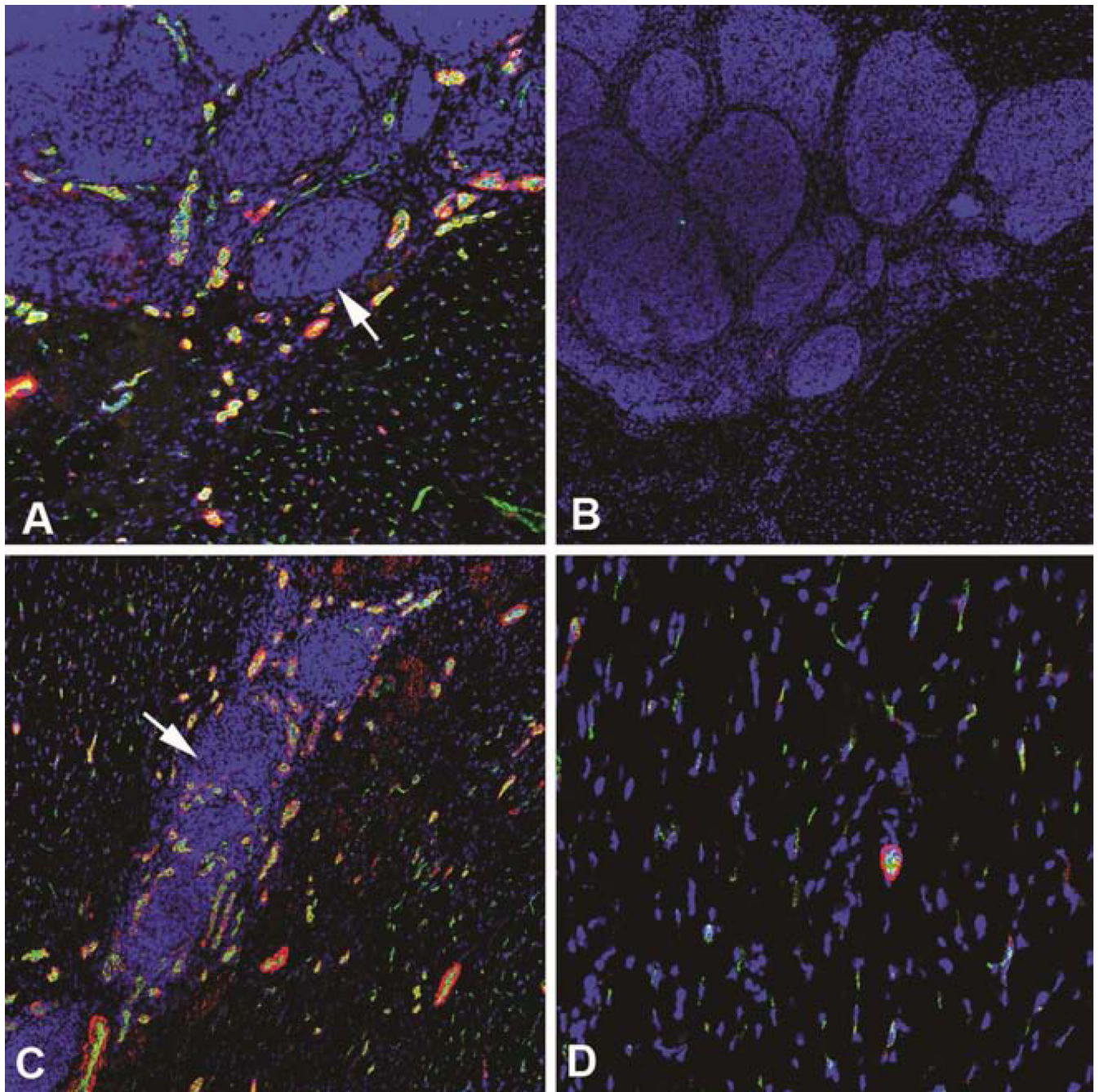
Figure 4.
A. Smooth muscle actin (red) and vWF (green) staining at 8 weeks. Arrow indicating a cell clump. B. Negative control of A. C. Staining of A at 4 weeks; D. Staining of A at non-injected area at 4 weeks after injection. Magnifications a–d = 100×.
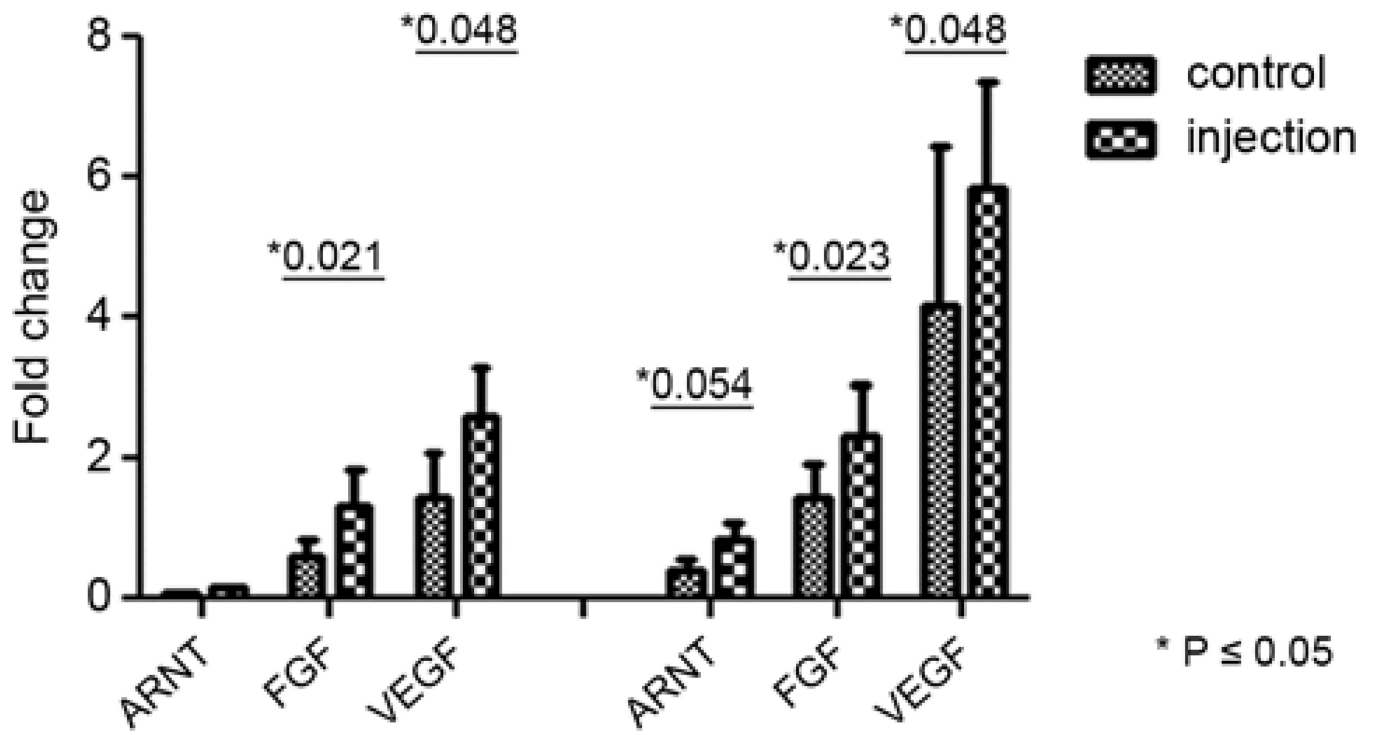
Figure 5.
Expression of VEGF, FGF, ANRT by qRT-PCR were significantly higher in the injected myocardium compared with none injected both in 4 week samples (left panel) and 3 month samples after myocardial-derived iPSC transplantation.
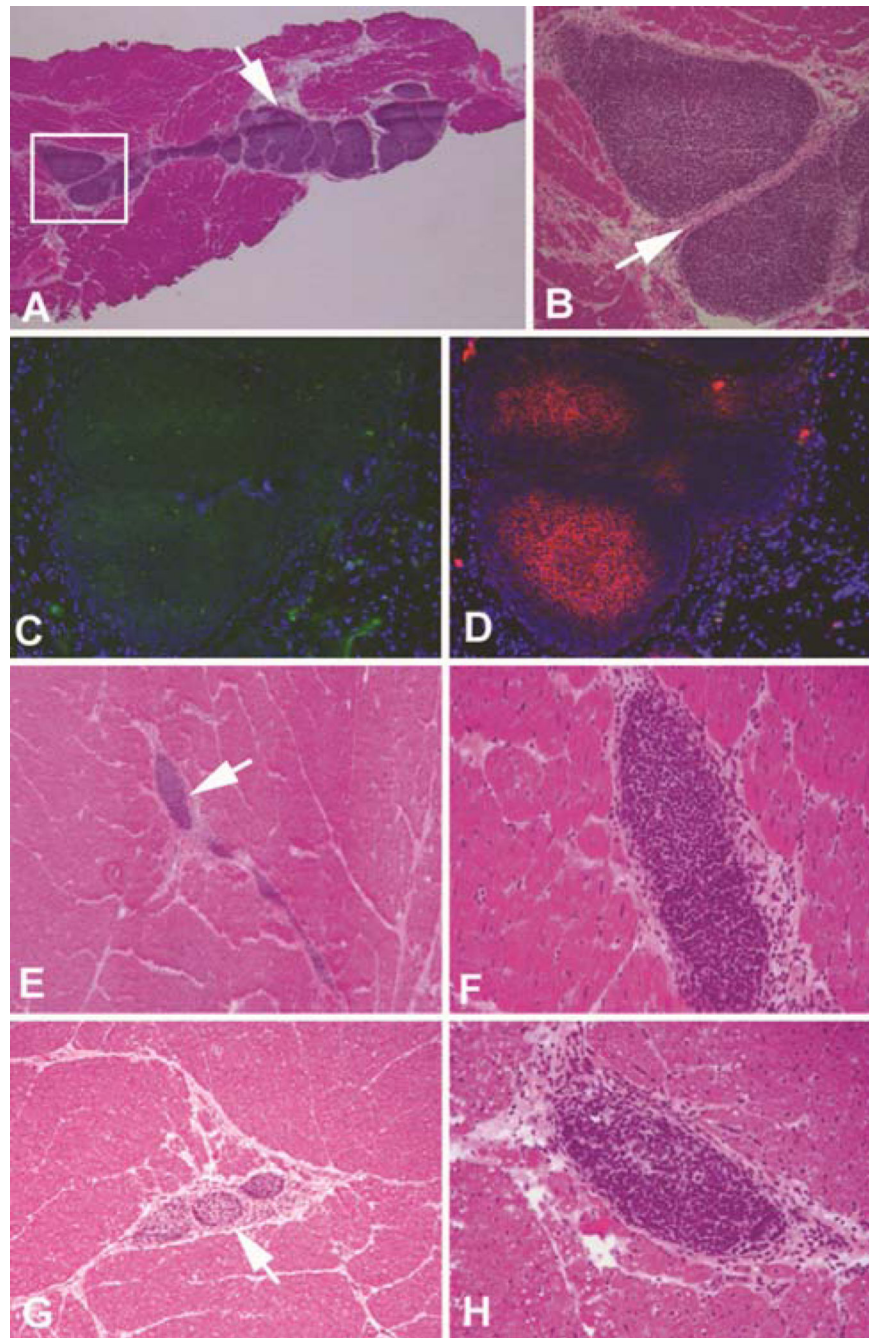
Figure 6.
Histologic analysis of the animals that received cell transplantation with the iPSCs strain provided by Michael Robert's lab. A. Arrow indicating a group of irregular small tumors at 8 weeks (12.5×); B. Higher magnification of the boxed area in A (100×); C. Oct4 staining (400×); D. SSEA4 staining (400×); E. Arrow indicating cell clumps at three months following injection (50×); F. Higher magnification of E (200×); G and H showing images from two separate animals at three months (50× and 200×).