Induction of Vascular Progenitor Cells From Endothelial Cells Stimulates Coronary Collateral Growth

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Rationale: A well-developed coronary collateral circulation improves the morbidity and mortality of patients following an acute coronary occlusion. Although regenerative medicine has great potential in stimulating vascular growth in the heart, to date there have been mixed results, and the ideal cell type for this therapy has not been resolved.

Objective: To generate induced vascular progenitor cells (iVPCs) from endothelial cells, which can differentiate into vascular smooth muscle cells (VSMCs) or endothelial cells (ECs), and test their capability to stimulate coronary collateral growth.

Methods and Results: We reprogrammed rat ECs with the transcription factors Oct4, Klf4, Sox2, and c-Myc. A population of reprogrammed cells was derived that expressed pluripotent markers Oct4, SSEA-1, Rex1, and AP and hemangioblast markers CD133, Flk1, and c-kit. These cells were designated iVPCs because they remained committed to vascular lineage and could differentiate into vascular ECs and VSMCs in vitro. The iVPCs demonstrated better in vitro angiogenic potential (tube network on 2-dimensional culture, tube formation in growth factor reduced Matrigel) than native ECs. The risk of teratoma formation in iVPCs is also reduced in comparison with fully reprogrammed induced pluripotent stem cells (iPSCs). When iVPCs were implanted into myocardium, they engrafted into blood vessels and increased coronary collateral flow (microspheres) and improved cardiac function (echocardiography) better than iPSCs, mesenchymal stem cells, native ECs, and sham treatments.

Conclusions: We conclude that iVPCs, generated by partially reprogramming ECs, are an ideal cell type for cell-based therapy designed to stimulate coronary collateral growth. (Circ Res. 2012;110:00-00-00.)

Key Words: vascular progenitor cells • coronary collateral growth • coronary circulation • induced pluripotent cells

Coronary heart disease (CHD) is the leading cause of mortality and morbidity in the United States. Although there have been numerous advances in the treatment of CHD over the last many years (drug eluting stents, statins), the realization of therapeutic angiogenesis for stimulation of coronary collateral growth remains an elusive goal. Cell-based therapies for cardiovascular diseases offer a new paradigm for treatment of CHD. The outcomes of cell-based therapies in the improvement of left ventricular function and reduction of myocardial ischemia are controversial.1-5 The challenge of growing new myocardium is enormous, involving essentials such as cardiomyocytes, conductive tissue, and a complete circulation. However, the challenge of stimulating coronary collateral growth is far less involved, and is a strategy more likely to produce an immediate benefit in the treatment of ischemic heart disease.6-7 “Ideal” stem/iPS/progenitor cell population for optimal coronary collateral growth (also termed arteriogenesis and collaterogenesis) in ischemic myocardium has not been identified.6-8 Many cell types—such as endothelial progenitor cells from blood or bone marrow, cardiac progenitor cells from the heart, mesenchymal stem cells from bone marrow, and others—are currently being examined as cell sources for cardiovascular regenerative cell therapy. Unfortunately the benefits are modest.9,10 The goal of this study is to generate induced vascular progenitor cells (iVPCs) that are capable of becoming both smooth muscle and endothelium, and stimulating the growth of coronary collateral vessels.

Induced pluripotent stem cells (iPSCs) are somatic cells reprogrammed to pluripotency by introducing a combination of 4 transcription factors out of Oct4, Klf4, Sox2, c-Myc, Nanog, and Lin28.11,12 So far, iPSCs are the strongest example of the plasticity of cells in response to a disruption in the stoichiometry of their transcriptional regulators.13 The
iPSCs potentially can avoid the ethical and legal controversy and practical difficulty associated with using human embryos. Importantly, the autologous source of iPSCs also avoids issues with immuno-incompatibility. The iPSCs are becoming one of the more promising candidates for regenerative medicine, but one drawback of iPSCs is the risk of tumor formation.14–17 Somatic stem cells and mesenchymal stem cells have multipotency, but do not form teratomas. Accordingly, the goal of our study was to reprogram somatic cells, not to full pluripotency, but do not form teratomas. Accordingly, the goal of our study was to reprogram somatic cells, not to full pluripotency, but rather to a progenitor-type cell that remained committed to a specific lineage, which would greatly reduce the risk of tumor formation. Our goal was to partially reprogram endothelial cell into a putative iVPC that hopefully could differentiate into endothelial and vascular smooth muscle cells, but not other cell types.

Since we have an established rat model for coronary collateral growth,17 we reprogrammed rat cells. The reason why we elected to reprogram vascular ECs instead of other cells such as fibroblasts is based on recent studies, which suggest that an “epigenetic memory” of their origins of somatic tissue in early passage of iPSCs favors a commitment to a cell lineage related to the donor cell while restricting alternative cell fate.18,19 Our hypothesis is that implantation of iVPCs would more likely result in more robust vascular growth in the heart than iPSCs, because the former cell type would remain committed to a vascular lineage, which serves as “building blocks” for blood vessels, whereas the latter cell type could differentiate into multiple cell types not necessarily to be involved in vascular growth. Our results show that iVPCs can be generated by reprogramming rat vascular ECs, can demonstrate distinct DNA methylation profiles of the promoters of Nanog and Oct4 and eNOS in comparison with native ECs and iPSCs, have low risk of teratoma formation in comparison with iPSCs, and can better stimulate coronary collateral growth and improve myocardial function than iPSCs, mesenchymal stem cells (MSCs), or native ECs in a rat model of repetitive ischemia.

### Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

#### Viral Transduction of ECs and Doxycycline-Induced Reprogramming

Lentiviral vectors expressing mouse transcription factors Oct4 (O), Klf4 (K), Sox2 (S), and c-Myc (M) were used to reprogram rat ECs. Doxycycline (2 μg/mL) was added at day 3 for induction of the reprogramming and withdrawn at day 15. Colonies were picked at days 21 to 23.

#### Immunocytochemical and Immunohistochemical Analyses

Samples were fixed with 4% (weight by volume) paraformaldehyde, incubated with the primary antibody overnight at 4°C, and then were incubated with a fluorescence-conjugated secondary antibody for 1 hour at room temperature. Gold antifade with DAPI was added for imaging.

#### iVPC Differentiation, Matrigel Assay, and Responses to Shear Stress

For differentiation, iVPCs were cultured on collagen IV coated plates either in EC culture medium with VEGF, or VSMC culture medium with PDGF. For the Matrigel assay, 5 × 10⁴ cells were added on Matrigel in 24 well plate and incubated at 37°C for 16 to 24 hours. For the shear stress experiment, differentiated iVPCs were exposed to a unidirectional laminar shear stress.

#### Teratoma Formation

Cells were injected into kidney or testis capsule of SCID mice for teratoma formation analysis.

#### Fluorescence-Activated Cell Sorting (FACS) Analysis

We used 10⁴ cells for staining. Cells were incubated with 5% BSA for 30 minutes, then stained with the primary antibody for 1 hour at 4°C, washed, stained with the fluorescence-conjugated secondary antibody 30 minutes, and then fixed in 1% PFA for FACS.

#### Quantitative RT Polymerase Chain Reaction (PCR)

RNA was isolated from the cells and real time RT-PCR was performed as described previously.20

#### Bisulfite Genomic Sequencing

Genomic DNA was extracted from cells, and bisulfite treatment was performed. Methylated DNA was purified for PCR and PCR products were cloned in TA vector. At least 8 clones were sent for sequencing, and sequences were analyzed by the software BiQ analyzer.
Karyotyping

Cells were spun down, and hypotonic KCl solution was added. Cells were fixed and spread on glass slides. Glass slides were stained with Giemsa’s stain, and chromosomes were counted.

**tdTomato Labeling of iVPCs**

The tdTomato lentivirus was generated from ptdTomato-N1 Vector and transduced into iVPCs as described above.

**Isolectin-B4 Infusion**

Rats were injected intravenously via the femoral vein with 250 μg isolectin GS-IB4. The heart was fixed, and tissue samples were taken from the collateral dependent and normal zones of the left ventricle.

**Rat Model of Collateral Growth/Repetitive Ischemia (RI) and Cell Transplantation**

Sprague–Dawley rats were used for chronic implantation of a pneumatic occluder over the left anterior descending coronary artery (LAD), as described by Toyota et al.17 Briefly, the rats were anesthetized and intubated, and the left chest was opened between the 3rd and 5th intercostal spaces. An occluder was attached to the left ventricle over the LAD, and 1 million cells were injected into 2 to 3 sites in the myocardium around the balloon. The chest was closed, and rats were allowed to rest for 2 days before undergoing the RI protocol for 10 days.

**Microsphere Measurements of Collateral Flow**

Coronary collateral growth was evaluated from blood flow to the collateral-dependent region by using neutron-activated microspheres injected into the left ventricle (LV) lumen. Collateral flow was calculated as a ratio between activity (dpm/g) of the tissues from the collateral-dependent and normal zones of the left ventricle.

**Echocardiographic Analysis of Cardiac Function**

In vivo heart function was evaluated by echocardiography. All measurements were performed before and after inflation of occluder at day 0 and day 10. Percentage changes of LV EF%, FS%, and LVESV were calculated as the difference between the values obtained before and after the occluder inflation at day 0 and day 10.

**Statistics**

Unpaired 2-tailed t tests with Welch’s correction were used for comparison of 2 or more groups.

**Results**

**Generation of iVPCs**

The protocol for reprogramming ECs with transcription factors Oct4, Klf4, Sox2, and c-Myc is shown in the top panel of Figure 2. Before transduction of ECs, confluent cultures showed typical cobblestone morphology (Figure 2A). At 3 to 5 days after transduction, the cells showed typical ES-like morphology (eg, compact colonies, high nucleus-to-cytoplasm ratio, and prominent nuclei; Figure 2B). These reprogrammed ECs maintained ES-like morphology until days 12 to 16 (Figure 2C). Then their morphology changed to intense ball round colonies at days 20 to 21 (Figure 2D). After expansion, most of the colonies retained the same morphology (Figure 2E). Only 1 colony (#9) out of 36 colonies had some ES-like cells appearing in the round cell population during late passages (Figure 3A). The transduced ECs expressed ES cell marker specific stage embryonic antigen-1 (SSEA-1) (Figure 2F) and Oct4 (Figure 2G) and were positive for alkaline phosphatase (AP) staining (Figure 2H). However, these reprogrammed ECs did not express (or expressed very little) native EC marker PECAM (0.39%), as shown by FACS analysis (Figure 2I). During the passage, these cells kept a normal number of chromosomes. Figure 2J shows iVPCs at passage 16 with 42 chromosomes. The morphology changes in transduced ECs indicated that they went through the early events of reprogramming, but they did not commit to faithful reprogramming to become typical iPSCs at the end. Immunostaining and FACS analysis suggested that a somatic lineage specific marker (eg, PECAM) was down-regulated and some pluripotency markers (eg, SSEA-1, Oct4, and AP) were up-regulated in the transduced.

Figure 1. The iVPCs colocalized with Isolectin B4 after implantation into rat ischemic myocardium. A diagram for surgical procedure was shown in **A**. The iVPCs were labeled with Td-tomato by lentiviral vector before injection (**B**). Fluorescent images show rat myocardium in control group and iVPC-implanted group after 10 days of the RI protocol (**C**). iVPCs were labeled with tdTomato, the native endothelium was labeled with FITC-isoelectin B4, and DAPI reveals nuclei. Note the absence of tdTomato in the control images, but the presence of this fluorochrome and its overlap with FITC in the iVPCs implantation images. Scale bar equals 100 μm in **B** and 10 μm in **C**.

![Figure 1](http://circres.ahajournals.org/)}
ECs. Consistent with morphology changes, these data suggested that the transduced ECs were only partially reprogrammed.

**Gene Expression Profile of iVPCs**

To further characterize the iVPCs, 4 colonies were randomly picked and RT-PCR was performed to compare their endogenous and exogenous gene expression with mouse ES cells and fully reprogrammed rat iPSCs derived from fibroblasts and bone marrow cells in the Xiao laboratory.21 The iPSCs endogenously expressed c-Myc and pluripotent genes such as Sox2, Nanog, Klf4, Oct4, and Rex1 (also known as Zfp42) (Figure 4A). Similarly, colonies of iVPCs expressed c-myc, Klf4, Rex1, and Oct4, but not all iVPCs expressed Sox2 and none expressed Nanog. Figure 4B shows that exogenous expression of transgenes Oct4, Klf4, Sox2, and c-myc in iVPCs were not fully inactivated. These results were consistent with reports that in partially reprogrammed iPSCs, exogenous transgenes were not fully silenced.22,23

Because E-cadherin is crucial for embryonic stem cell pluripotency and fully reprogrammed cells cannot be obtained in the absence of E-cadherin,24 we performed RT-PCR to examine the expression of E-cadherin in iVPCs. Figure 4C showed that mouse ES cells and iPSCs had greater expression of E-cadherin than did iVPCs.

To elucidate the cell lineage of iVPCs, we performed qRT-PCR to detect the expression of 3 germ layers’ lineage markers. In comparison with iPSCs, there were significant differences in the expression level of 3 transcription factors RUNX2 (Figure 4D), GATA6 (Figure 4E), and GATA4 (Figure 4F) between iVPCs and iPSCs. In undifferentiated ES cells and iPSCs, these genes had lower levels of expression than in iVPCs.

FACS analysis was used to further characterize the iVPCs. As Figure 4G illustrates, the majority of iVPCs expressed pluripotent cell markers: 81.8% for Oct4 and 73.5% for SSEA-1. Only a minority of iVPCs expressed hemangioblast markers: 8.37% for CD133, 15.6% for Flk1, and 1.97% for c-kit. Interestingly, iPSCs did not express hemangioblast markers: CD133, Flk1, and c-kit. The majority of iPSCs also expressed Oct4 (71.2%) and SSEA-1 (72.8%). On the basis of a comparison of these lineage markers, iVPCs appeared to be at a more differentiated state than pluripotency, close to mesoderm progenitor cells or hemangioblast progenitor cells.
Epigenetic Status of iVPCs

To address the mechanism of reprogramming to full pluripotency versus that to a progenitor cell type, we investigated the DNA methylation status of the Nanog and Oct4 promoter region of ECs, iPSCs, and iVPCs by bisulfite genomic sequencing analysis. In the promoter region of the rat Nanog gene (−346 to −222 bp upstream of the transcription start site), 67.5% of CpG was methylated in ECs; whereas in iPSCs, CpG dinucleotides in the same region were only 2.5% methylated (Figure 5A). CpG dinucleotides from 4 clones of iVPCs were 5% and 17.5% methylated (Figure 5A). Similarly, 69.6% of CpG in the promoter region of the rat Oct4 gene (−2142 to −1550 bp upstream of transcription start site) was methylated in ECs; whereas in iPSCs, CpG dinucleotides in the same region were 14.3% methylated (Figure 5B). CpG dinucleotides from the 4 clones of iVPCs were 66.1%, 50%, 67.9%, and 60.1% methylated (Figure 5B). These data suggested that the DNA methylation status of the Nanog and Oct4 promoter regions in iVPCs, iPSCs, and ECs were different. The Nanog and Oct4 promoter region in iVPCs were more methylated than those of fully reprogrammed iPSCs; Nanog and Oct4 promoter regions in some iPVC clones are less methylated than those of native ECs. Because 8 rows of lollipop shapes in Figure 5 represent 8 sequences, the statistical analysis showed that in Figure 5C, DNA methylation of the Nanog promoter in ECs was significantly different from iPSCs and iVPCs; iVPC 1 and iVPC 4 were significantly different from iPSCs. Similarly, DNA methylation of the Oct4 promoter in iPSCs was significantly different from ECs and iVPCs; iVPC 2 was significantly different from ECs.

Angiogenic Potential and Differentiation of iVPCs

When iVPCs were selected and expanded on feeder cells, some of these cells spontaneously formed a “tube-like” network (Figure 6A), suggesting a greater angiogenic potential than native endothelial cells that form a confluent monolayer in 2 dimensions. Accordingly, we performed in vitro angiogenesis assays on Matrigel to compare native ECs to iVPCs. In growth factor reduced (GFR) Matrigel, ECs (Figure 6B) and iVPCs (Figure 6C) formed tube-like networks in medium with serum and VEGF. In the absence of serum and VEGF, ECs did not form tubes in the GFR Matrigel (Figure 6D). In contrast, iVPCs formed an extensive tube network in the GFR Matrigel without serum and VEGF (Figure 6E).

When exposed to shear stress for 72 hours, iVPCs responded by aligning parallel to flow (Figure 6F), which is similar to the response of native ECs (Figure 6F). Treatment of iVPCs with VEGF (50 ng/mL) induced differentiation into ECs. RT-PCR results show that differentiated iVPCs expressed EC marker PECAM, Tie2, and VE-cadherin (Figure 6H) that was confirmed by immunostaining of Von Willebrand Factor (VWF) (Figure 6J). The iVPCs could also differentiate into VSMCs after treatment with PDGF (20 Figure 3. Less risk of teratoma formation from iVPCs than from iPSCs. A, Morphology of iPSC, iVPC, and clone #9 (named as iVPC/iPSC because ES-cell-like fully reprogrammed cells appeared in the iVPCs). B, When seeded on low-attachment dishes with differentiation medium, iPSC grew in suspension, aggregated, and formed embryo bodies. In contrast, iVPCs attached to the plates and spread, and did not form embryo bodies. C, Kidneys and testis harvested after injection with iPSCs, iVPCs, and iVPCs/iPSCs. The iVPCs did not form teratomas, whereas iPSCs and iVPCs formed teratomas. D, The probabilities of teratoma formation from iVPCs, iPSCs, and iVPC/iPSC; 1, the teratoma formed; 0, no teratoma formed. The N refers to injection times. So for iPSCs, the probability of teratoma formation was 100% (4 out of 4). For iVPC, the probability of teratoma formation is 0% (0 out of 15). For iVPC/iPSC, the probability of teratoma formation is 100% (3 out of 3). Even if including iVPC/iPSC (clone #9), the probability of teratoma formation of iVPCs is 0.46% (3 out of 18 injections in the context of 1 clone out of 36 clones became iVPC/iPSC). Scale bar equals 100 μm.

DNA methylation of the eNOS promoter in iVPCs was different from that in iPSCs and ECs as shown in Online Figure I (online figures are available in the Data Supplement at http://circres.ahajournals.org). This is consistent with our other data suggesting that iVPCs are neither ECs nor iPSCs after being reprogrammed.
ng/mL). RT-PCR results showed that differentiated iVPCs expressed smooth muscle marker smooth muscle heavy chain (SMHC), smooth muscle 22α, and smoothelin (Figure 6I). This was confirmed by immunostaining of α smooth muscle actin (α-SMA) (Figure 6K). These data show iVPCs had angiogenic potential and could differentiate into ECs and VSMCs in vitro. In contrast, the iVPCs did not differentiate into cardiomyocytes when treated either with BMP4 (10 ng/mL), activin A (10 ng/mL), and bFGF (10 ng/mL) or with 3 μmol/L 5-azacytidine, shown by negative staining of the cardiomyocyte marker α-sarcomeric actin (Figure 6L).

Embryo Body and Teratoma Formation
Rat iPSCs formed embryo bodies in vitro and teratomas in vivo, consistent with previous results.21 In contrast, partially reprogrammed iVPCs did not form embryo bodies or teratomas. We should mention one exception (clone #9), which produced teratomas. This clone, out of 36 clones of iVPCs, appeared to transit from partial to full reprogramming during the passaging, and thus we termed it iVPC/iPSC because of their distinct morphology versus iVPCs (Figure 3A). When iVPCs and iPSCs were seeded on noncoating low-attachment petri dishes in the differentiated medium, iPSCs grew in suspension and aggregated to form embryo bodies. In contrast, iVPCs attached to the plates and spread, but did not form embryo bodies (Figure 3B). In vivo, iPSCs formed teratomas 100% of the time (Figure 3D). In contrast, iVPCs produced no teratomas (Figure 3D). The iVPCs that underwent further dedifferentiation (iVPC/iPSC) also produced teratomas. Online Figure IIB and IIC shows that colony #9 and iPSCs formed teratomas and could differentiate into 3 germ layers. It is interesting to note that the teratoma formed from colony #9 was smaller and contained more blood vessels than typical teratomas from iPSCs. Because embryo body formation is the first step of ES cell differentiation into the 3 germ layers, this is likely a reason why the iPSCs produced teratomas, whereas the iVPCs did not.

iVPCs Engraftment into Blood Vessels After Implantation into Ischemic Rat Myocardium
To test whether iVPCs could augment collateral growth, we implanted the cells in the rat left ventricle in a model of coronary collateral growth as shown in Figure 1A.

First, we studied cell survival and migration after injection. The iVPCs were labeled with tdTomato before implantation and visualized by red fluorescence, as shown in Figure 1B.
This allowed us to assess outcome of the injected cells after the protocol. We mixed FITC labeled microspheres with tdTomato iVPCs and injected this mixture in the rat myocardium. At day 2 and day 10, myocardium was harvested for imaging. In Online Figure II, both the labeled iVPCs and the microspheres were found in the collateral dependent zone 1 (near injection site), but only iVPCs were visualized in the collateral dependent zone 2 (remote from injection site), suggesting that the cells migrated within the collateral dependent zone. Importantly, iVPCs were observed at both day 2 and day 10, which supports the idea that iVPCs survived after transplantation.

Second, we studied the colocalization of iVPCs with cell specific markers. Again, iVPCs were labeled with tdTomato (Figure 1B). FITC-isolectin B4 (endothelial marker) was administered intravenously to visualize ECs. Figure 1C shows tdTomato labeled iVPCs colocalized with isolectin-B4 in the rat myocardium after 10 days of the RI protocol. Note that there were no tdTomato cells present in the shams, suggesting that the fluorescence was specific. We also found integration of iVPCs in blood vessel using multiphoton microscopy (Online Video I, available in the Data Supplement at http://circres.ahajournals.org). Collagen in the arterial wall was shown in green by second-harmonic generation.

Third, we compared the capability of iVPCs and iPSCs to engraft into blood vessels in the heart. For these experiments, iVPCs were not labeled with tdTomato, but rather were visualized (with iPSCs) by immunostaining for ES cell marker SSEA-1 (Figure 7). ECs and VSMCs were visualized by immunostaining for VWF and α-SMA, respectively. The iVPCs colocalized with vascular ECs expressing VWF (red) (Figure 7A and Online Figure III). In the sham control group (without cell injection), there was no obvious SSEA-1 expression. We also observed colocalization of SSEA-1 with α-SMA (red, Figure 7B), suggesting that iVPCs also expressed smooth muscle actin. These data suggested that the implanted iVPCs became components of blood vessels. Interestingly, there were some injected iVPCs that appeared to stimulate the formation of multiple lumens within a vessel, suggesting intussusceptive angiogenesis (Figure 7A, bottom row). The iPSCs expressed SSEA-1, which also colocalized with VWF, but there was no colocalization with α-SMA. To examine whether iVPCs differentiated into nonvascular cell types, we determined whether SSEA-1 would colocalize with the cardiomyocyte marker α-sarcomeric actin. In the iVPC implantation group, no colocalization with α-sarcomeric actin was observed; however, iPSCs were observed to colocalize with cardiomyocytes (Figure 7C).
iVPCs Improved Coronary Collateral Flow and Cardiac Function

To compare the capabilities of different cell types to improve coronary collateral flow, we injected iVPCs, iPSCs, ECs, and MSCs into rat myocardium. After 10 days RI protocol, coronary blood flow was measure by microsphere. Figure 8A shows that iVPCs enhanced coronary collateral growth (increase in CZ/NZ flow ratio) better than any of the other groups. Moreover, this increase in flow translated to an improvement in cardiac function, in terms of an improvement in ejection fraction (Figure 8B), fractional shortening (Figure 8C), and systolic volume (Figure 8D). The improvement in function was greatest and most apparent in the rats treated with iVPCs. These data suggested that iVPCs augment coronary collateral growth in rat myocardium better than either of the other cell types, including iPSCs, MSCs, and ECs, or just repetitive episodes of ischemia.

Discussion

In this study, we generated a vascular progenitor–type cell that we have termed an induced vascular progenitor cell (iVPC) by reprogramming rat vascular ECs with the transcription factors Oct4, Klf4, Sox2, and c-Myc and selecting for markers consistent with hemangioblasts. The iVPCs expressed ES cell markers SSEA-1, Oct4, Rex1, and alkaline phosphatase (AP), but not the EC marker PECAM. The iVPCs showed the ability to stimulate coronary collateral growth and the capability to differentiate into either smooth muscle or endothelial cells. These cells appeared to commit to a vascular lineage, because they did not differentiate into cardiomyocytes. Although the reprogramming strategy we used with the OKSM genes has led to the production of induced pluripotent cells (iPSCs) in mouse and rat fibroblasts, it is not surprising that we did not obtain iPSCs from rat ECs, because a growing concept in reprogramming somatic cells into iPSC is that various cell types are converted to a pluripotency with varying efficiencies.23,25 This suggests that reprogramming is “context”-dependent and the cell type affects the capability to become an iPSC. Also, reprogramming efficiency varies depending on the origin of cell type because more differentiated cells are harder to reprogram than precursor or progenitor cells.23,26,27 Additionally, adult somatic cells are more difficult to reprogram than embryonic somatic cells. Even using the same protocol, some cell types could be reprogrammed but others could not. Studies have shown that successful reprogramming of rat somatic cells is also dependent on the original cell type and that the reprogramming conditions might need to be optimized for each different cell type, eg, for rat liver progenitor cells using retrovirus expressing Oct4, Klf4, and Sox2, plus “cocktail” 3i (containing inhibitors of MEK, GSK3β/H9252, and ALK5)28; for rat bone marrow cells and fibroblasts with lentivirus expressing Oct4, Klf4, Sox2, c-Myc21; and for rat neural precursor and embryonic fibroblasts with retrovirus expressing Oct4, Klf4, and Sox2, plus rat MEF feeder cells and 2i (containing inhibitors of MEK and GSK3β).29
A critical issue is to understand the differences between iVPCs and iPSCs. Our data provide insights into this distinction. First, we have shown that there was no endogenous expression of Nanog in all iVPCs colonies, and not all iVPCs colonies endogenously expressed Sox2. Nanog and Sox2 are both important to maintaining the pluripotent status of ES cells and iPSCs. Expression of Nanog is essential for reprogramming, especially during late steps. It has been reported that missing expression of Nanog protein resulted in partially reprogrammed cells. Our data suggest that it might be the case with iVPCs, too. Expression of Sox2 at some "optimal" level is critical in that lower or higher expression will cause ES cells to differentiate into different cell lineages. Moreover, regulating the amount of Sox2 expression induced alternative cell fate during reprogramming. Second, exogenous expression of transcription factors OKSM were not fully activated. In fully reprogrammed iPSCs, exogenous expression of transgenic transcription factors was fully silenced, and the endogenous expression of pluripotent genes was activated. Third, E-cadherin expression levels were lower in iVPCs than in iPSCs and ES cells, which may explain why iVPCs were only partially reprogrammed. Within this context, E-cadherin seems to be critical for a pluripotent phenotype. Fourth, Nanog and Oct4 promoter regions were more methylated in iVPCs than were fully reprogrammed iPSCs. Epigenetic status is important for the reprogramming and successful generation of the iPSCs. In mammals, 70% to 80% of CpG islands of the genome are methylated. For ES cells and iPSCs, Nanog and Oct4 promoters are typically robustly demethylated. In the iVPCs, DNA methylation status in the Nanog and Oct4 promoter region is different from that in parental ECs and fully reprogrammed iPSCs. From this observation we suggest that high degree of Oct4 promoter methylation may underscore the reason why ECs were not fully reprogrammed to iPSCs. This may also suggest that the epigenetic status of ECs may make them more difficult to fully reprogram into iPSCs. It is worth noting that DNA methylation analysis of Nanog and Oct4 promoters did not completely corroborate the RT-PCR results, in which exogenous Oct4 expression was activated and Nanog expression not activated. Regulation of gene expression by transcription factors can be affected by other events, including DNA methylation as well as histone modification, which we believe may be the cause of the apparent discrepancy between the RT-PCR data and the methylation analysis.

Teratoma formation is one critical standard for pluripotency. When randomly picked colonies from the generated iVPCs were injected into nude mice, teratomas did not form. A teratoma only formed from colony #9 (we named this clone iVPC/iPSC), which had ES-like cells appearing in iVPCs during late passage, implying transition from partial reprogramming to full reprogramming. It is interesting to note that the teratoma formed from #9 was smaller and contained more blood vessels than a typical teratoma from iPSCs. This is consistent with observations that partially reprogrammed iPSCs can lose their epigenetic memory during passage and
revert to an iPSC. In one of our experiments, iVPCs were implanted into rat myocardium, and no tumor or hemangioma was observed for 3 months. Importantly, these data suggest that iVPCs have a lower probability (0.46%) to form tumors than fully reprogrammed iPSCs, which form teratomas 100% of the time. This is in line with our goal to generate multipotent vascular progenitor cells instead of iPSCs to stimulate coronary collateral growth without formation of tumors.

Another observation suggesting that the iPSCs are very different from iVPCs pertained to expression of specific lineage markers such as RUNX2, GATA4, and GATA6. RUNX2 is a mesoderm lineage marker and is essential for osteoblastic differentiation, but it has also been suggested to be involved in angiogenesis. GATA4 and GATA6 are endoderm and mesoderm lineage markers and are important for embryo development including the heart and arterial systems. These data suggested that iVPCs had been trapped in a differentiated state. Expression of these markers in iVPCs was significantly higher than iPSCs. Interestingly, a majority of the iVPC expressed pluripotent markers SSEA1 and Oct4, but some of the iVPC expressed the hemangioblast markers Flk1+, CD133, and c-kit. These data are consistent with epigenetic data, which suggest that ECs have strong epigenetic memory and thus reprogramming them resulted in the development of induced vascular progenitor cells rather than iPSCs.

An important characterization of these iVPCs is their angiogenic potential. In vitro studies showed they could spontaneously form a capillary tube-like network on feeder cells in the absence of VEGF. Typically, endothelial cells do not form tubes on a 2D culture—only when cultured on Matrigel, ECs form tubes. Moreover, iVPCs could form a tube network in GFR Matrigel without serum and VEGF whereas ECs could not form tubes under these conditions. This suggests greater angiogenic potential of these iVPCs compared to native ECs. When implanted in myocardium, iVPCs engrafted into blood vessels, increased coronary collateral blood flow and improved heart function during coronary occlusion—again suggesting that iVPCs enhanced vascular growth in the heart. Consistent with these data, iVPCs could differentiate into ECs and SMCs, which are two important components of blood vessels. Traditionally, ECs and SMCs are regarded to come from different progenitor cells because during embryogenesis, endothelial cells come from hemangioblasts and smooth muscle cells arise from mesenchyme.
and the neural crest. However, Yamashita et al reported that embryonic vascular progenitor cells (Fkl1 +) could differentiate into both ECs and SMCs.41–43 But postnatally, the residence of such common vascular progenitor cells has been controversial.41–43 Our result is the first report of generating iPVCs and applying them to induce coronary collateral growth. However, iPVCs may be applied more broadly for all needs requiring vascular growth, eg, peripheral vascular disease, wound healing, diabetic neuropathy, etc.

Compared to fully reprogrammed iPSCs, MSCs and ECs, iPVCs augmented coronary collateral blood flow and significantly improved heart function. This result is consistent with our hypothesis that iPVCs may be more specific for vessel growth than other cell types. Another highlight of this study is choosing vascular cells as the starting cell type for reprogramming. Recent reports suggest that iPSCs might retain an “epigenetic memory” of their cell type from which they originate and thereby favor differentiation along that lineage. Thus, the cell origin might be important for the fate of the iPSCs as a way of committing to a fate of differentiation into vascular cells. The benefit of iPVCs in this study demonstrated that reprogrammed ECs remain committed to a vascular lineage because of their epigenetic memory and as such, become perfect building blocks for new and/or growing blood vessels in the heart.

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Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**

- Somatic cells (e.g., rat fibroblasts) can be reprogrammed into induced pluripotent stem cells (iPSCs).
- Stem cells (e.g., iPSCs, mesenchymal stem cells [MSC], cardiac stem cells [CSC]) are being used for many types of regenerative therapies.
- Induced pluripotent stem cells have tumorigenic potential and can form teratomas.
- Repetitive ischemia (RI) can induce coronary collateral growth.

**What New Information Does This Article Contribute?**

- Because of epigenetic memory, reprogramming of vascular endothelial cells (ECs) can produce induced vascular progenitor cells (IVPCs).
- Induced vascular progenitor cells remain committed to a vascular differentiation program, becoming smooth muscle or endothelium, but not cardiomyocytes.
- Partially reprogrammed IVPCs have a much lower risk of tumorigenesis and teratoma formation.
- Induced vascular progenitor cells better augment coronary collateral growth than do native ECs, iPSCs, or MSCs in a rat RI model.

Traditionally, cell-based therapies in ischemic heart disease focus on regenerating the myocardium. Other approaches aim to stimulate angiogenesis in the peri-infarct region. Our goal is to use cell-based therapy to stimulate coronary collateral growth to prevent myocardial infarction. Accordingly, we partially reprogrammed vascular ECs into induced vascular progenitor cells (IVPCs), instead of fully reprogramming cells into induced pluripotent stem cells (iPSCs). The risk of tumorigenesis is much lower with IVPCs than with iPSCs because they remain committed to a vascular differentiation program. The vascular commitment of IVPCs is related to the epigenetic memory of ECs, which engenders them as cellular components of growing blood vessels in the heart. When IVPCs were implanted into myocardium, they engrafted in blood vessels and increased coronary collateral flow better than iPSCs, MSCs, or native ECs. We conclude that IVPCs are a useful cell type to stimulate coronary collateral growth as a regenerative therapy. We also believe that partial reprogramming is an effective strategy to generate specific lineage progenitor cells, while avoiding tumorigenesis seen with other types of stem cells. However, the critical, but unresolved issue, pertains to regulating reprogramming to arrive at the partially programmed state.
Induction of Vascular Progenitor Cells From Endothelial Cells Stimulates Coronary Collateral Growth

Liya Yin, Vahagn Ohanyan, Yuh Fen Pung, Angelo DeLucia, Erin Bailey, Molly Enrick, Kelly Stevanov, Christopher L. Kolz, Giacinta Guarini and William M. Chilian

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Expanded Material and Methods

**Cell Culture.** Rat aortic ECs from Sprague Dawley rats were purchased from Cell Application Inc and cultured in the supplied medium. 293T cells were purchased from Clontech and cultured in DMEM with 10% tetracycline-free serum.

**Viral Transduction of ECs and Doxycycline-Induced Reprogramming.** Liao *et al*\(^1\) succeeded in reprogramming rat fibroblasts using lentiviral vectors expressing human transcription factors Oct4 (O), Klf2 (K), Sox2 (S) and c-Myc (M). Accordingly, rat ECs were reprogrammed using a similar approach. In this study, mouse OKSM were used instead of human OKSM because sequence homology between the mouse and rat transcription factors is higher (above 95%). Doxycycline-inducible lentivirus expressing mouse transcription factor of OKSM were used to transduce the ECs. Lentivirus expressing the transcription factors were either purchased from Stemgent or generated in our lab. The plasmids (FUW-M2rtTA, TetO-FUW-cMYC, TetO-FUW-Klf4, TetO-FUW-Oct4, TetO-FUW-Sox2) were purchased from Addgene and plasmid DNA was prepared with an Endo-Free DNA maxi prep kit (Qiagen). The lentiviruses were packed in 293T cells with a Lenti-X HT Packing System (Clontech) and the viruses were concentrated by LentiUp and titered by q-PCR with lentiviral titer kit (both from MellGen Laboratories Inc). Rat vascular EC were transduced with concentrated lentivirus with polybrene (4ug/mL) and incubated for 24 hours. The transduction was repeated on the second day. Two days later, the transduced cells were trypsinized, dispersed and seeded into five 100 mm dishes at a density of 5x10^5 per dish with feeder cells (Mitomycin C-treated MEF) for colony isolation in mouse ES medium (450 mL Knockout DMEM (Invitrogen) supplemented with 50ml ES cell-qualified calf serum (Hyclone), 5 mL penicillin/streptomycin (100x, Invitrogen), 1% non essential amino acid, 5 mL 200 mM L-glutamine, 0.1 mM β-Mercaptoethanol (Sigma) and 56 μl Leukemia inhibitor factor (10^7 unit/mL, Millipore)). Doxycycline (2 μg/mL) was added to induce the expression of the tet-on genes for induction of the reprogramming. The medium was changed every day and the doxycycline was withdrawn at day 15. Colonies were picked at day 21 to 23. The reprogrammed cells were expanded on the feeder cells.

**Immunocytochemical and Immunohistochemical Analyses.** Cells were washed once with PBS, then fixed with 4% (w/v) paraformaldehyde in PBS for 15 minutes, and washed twice in PBS. For intracellular proteins, cells were permeabilized with 0.2% Tween®-20 in PBS for 10 minutes. Non-specific binding was reduced with 5% (w/v) BSA. Cells were incubated with the primary antibody overnight at 4°C, and then after two washing steps with PBS, the cells were incubated with a fluorescence-conjugated secondary antibody for 1 hour at room temperature. For the negative controls, the secondary antibodies were used. After two more washing steps with PBS, Gold anti-fade with DAPI (Invitrogen) was added to identify nuclei and images were obtained using an inverted fluorescent microscope. Tissue was fixed in 4% paraformaldehyde, cryo-protected in 30% sucrose and frozen in OCT and stored at -80°C until sectioning. Seven to ten micron sections were cut and fixed in cold acetone for 20 minutes, washed with PBST, blocked with 5% BSA for 30 minutes, washed twice with PBST, stained with primary antibody overnight at 4°C, and washed and then stained with fluorescence-conjugated secondary antibody for 1 hour. For the negative controls, the secondary antibodies were used. Tissue sections were washed and mounted with Prolong anti-
fade mounting medium with DAPI (Invitrogen). Anti-SSEA-1 (1:200), anti-Oct4 (1:200)(Stemgent), anti-Von Willebrand Factor (1:500)(Abcam), α-Sarcomeric Actin (1:500)(Abcam), and anti-actin alpha smooth muscle-cy3 (1:200) (Sigma) were used as primary antibodies. Alexa Fluor488 goat anti-mouse IgG (1:300), Alexa F594 goat anti-rabbit IgG (1:300), Alexa Fluor 488 goat anti-rabbit IgG(1:300 ) and Alexa Fluor 594 goat anti-mouse IgG(1:300 ) (Invitrogen) were used as secondary antibodies. For the negative controls, the secondary antibodies were used. Alkaline Phosphatase Staining kit (Stemgent) was used for AP staining.

**iVPC Differentiation, Matrigel Assay and Responses to Shear Stress.** For differentiation, iVPCs were first cultured on the feeder free plates in 20% FBS without LIF. Then the iVPCs cultured on 25ug/mL collagen IV coated plates either with rat EC culture medium with VEGF (50ng/mL) or rat SMC culture medium with PDGF (50ng/mL). For the differentiation for cardiomyocytes, iVPCs were treated either with either with BMP4(10ng/ml), Activin A (10ng/ml) and bFGF (10ng/ml) and or with 3 µM 5-azacytidine. For Matrigel assay, 200uL Growth Factor Reduced BD Matrigel™ Matrix was added in each well of 24-well plate and incubated at 37°C for 30 minutes for gelling. Then 5x10^4 cells were added in each well and incubated at 37°C for 16 to 24 hours. Pictures were taken under the microscope. For shear stress experiment, differentiated iVPCs in EC culture medium were plated on 100mm dishes and exposed to a unidirectional laminar shear stress (15 dynes/cm²) by rotating a Teflon cone (0.5° cone angle) from a Biostir 4 (Wheaton).

**Embryo Body and Teratoma Formation Assay.** For embryo body formation, iPSCs and iVPCs were seeded on non coating, low attachment petri dishes with conditional medium (20% FBS without LIF). To characterize if the reprogrammed cells were pluripotent or were more characteristic of progenitor cells, different colonies of cells were injected into kidney and testis capsule in Fox Chase SCID-beige male mice (Applied StemCell, Inc) for teratoma formation assay. Teratomas were examined at 4 to 6 weeks.

**FACS Analysis.** Cells were detached with trypsin and suspended in medium with 10% serum, then washed in staining buffer (10% NaN₃, 5%BSA, 2mM EDTA in PBS), centrifuged at 1000 rpm for 5 minutes, and resuspended at a concentration of 10⁷ cell/mL. Aliquots of 100 µL were used for staining. To assess cell surface markers by FACS, non-specific binding was blocked with 5% BSA for 30 minutes, then washed twice with staining buffer, treated with the primary antibody for one hour at 4°C, washed, stained with the fluorescence-conjugated secondary antibody 30 minutes, and then fixed in 1% PFA. For intracellular markers, cells were fixed in 4% PFA for 15 minutes, washed in PBS, and permeabilized in 0.1% TritonX-100 for 10 minutes. After a wash, cells were stained as described for the cell surface proteins. (1:500)(Abcam). Besides the antibodies used in immunocytochemical staining, anti-CD31 (PECAM), anti- CD117 (c-kit), rabbit polyclonal to CD133 (Abcam) were used.

**Quantitative RT-PCR.** RNA was isolated from the cells and q-PCR was performed as described previously². The housekeeping gene was GAPDH. Primers used are listed in supplemental table 1. Primers for endogenous expression of Oct4, Rex1, Klf4 were described as published¹,³.

**Bisulfite Genomic Sequencing**
Genomic DNA was extracted from cells by DNeasy blood and tissue kit (Qiagen). Bisulfite treatment was performed using the EpiXplore Methyl Detection Kit (Clontech).
Methylated DNA was purified for PCR. PCR primers for Nanog and Oct4 promoters were used as described 1, 3. PCR products were cloned to TA Vectors (Invitrogen). At least 8 randomly selected clones were selected and sequenced. The sequence was analyzed by software “BiQ Analyzer”.

**Karyotyping.** Cells were detached with trypsin and centrifuged. 8 mL of hypotonic KCl solution were added to the cells. Cells were mixed and incubated at 37°C for 10 minutes. 2 mL of fixative (MeOH: Glacial acetic acid 3:1) were added, mixed, and then an additional 6 mL of fixative were added. After mild centrifugation, this step was repeated. Pellets were resuspended in 1 mL of fixative and spread on glass slides. Glass slides were stained with Giemsa's stain for 15 minutes, rinsed with water and air-dried. Chromosome spreads were photographed and chromosome were counted.

**tdTomato Labeling of iVPCs.** ptdTomato-N1 Vector was purchased from Clontech. The tdTomato virus was generated and transduced in iVPCs as described above.

**Rat Model of Collateral Growth/Repetitive Ischemia (RI).** 3 to 5-month-old, 300 to 350 g, male Sprague Dawley (SD) rats were used for chronic implantation of a pneumatic snare over the left anterior descending coronary artery (LAD), as described by Toyota et al4. Briefly, the rats were under anesthesia and intubated, left chest were opened between 3rd-5th intercostal spaces. A balloon was attached to the left ventricle over LAD and tethering, and one million cells were injected in ~2-3 sites in the myocardium around balloon. The chest was closed and rats were rest for 2 days before put in the RI protocols for 10 days. The cells including iVPCs, iPSCs, MSCs, ECs were then injected into the left ventricular myocardium at 2 to 3 sites in the ischemic region around the occluder (10⁶ cells in 250 µL). Rats under RI protocol were divided into five groups: control (no cell injection, 10 rats), iVPCs injection (8 rats), iPSCs injection (8 rats), MSCs injection (9 rats), ECs injection (6 rats). All animal procedures were approved by the NEOUCOM IACUC and were in accordance with all institutional guidelines. The same animals were used for collateral flow measurement and echocardiography.

**Microsphere Measurements of Collateral Flow.** Measurements of coronary collateral growth were evaluated from blood flow to the collateral-dependent region by using neutron-activated microspheres (BioPAL Sterispheres; 15 um diameter; labeled with Gold or Samarium). Microspheres (5 x 10⁵) were injected into the left ventricle (LV) lumen via a 30-G needle over 20 seconds during LAD occlusion at day 0 (the initial of RI) and at day10 (the conclusion of each experiment). Samples dissected from the collateral-dependent zone (CZ) and normal zone (NZ) are weighed and activity during neutron activation of the spheres was measured (BioPAL, Worcester, MA). Collateral flow was calculated as a ratio between activity (dpm/g) of the tissues from the collateral-dependent zone and normal zones (CZ/NZ).

**Echocardiographic Analysis of Cardiac Function.** *In vivo* heart function was evaluated by echocardiography using VEVO 770 High-Resolution Imaging System (Visual Sonics, Inc., Toronto, Ontario, Canada) with a RMV 716 probe specifically for small animal studies. Power was set for the best imaging. Animals were anesthetized using 1.5-2% sevoflurane via nose cone and the chest was cleaned by shaving. Then animals were placed in the supine position on an adjustable platform equipped with ECG electrodes to monitor heart and respiration rates. Rectal temperature probe was placed to monitor body temperature (37-37.5 °C). All presented measurements were averaged
from at least three consecutive cardiac cycles. Calculation and measurement were carried out offline using the Vevo770/3.0.0 software. LV end-diastolic diameter and end-systolic diameter (LVID:d and LVID:s), volumes (LVEDV, LVESV), diastolic and systolic anterior wall (LVAW:d and LVAW:s), and diastolic and systolic posterior wall thicknesses (LVPW:d and LVPW:s) were measured from the short-axis M-mode images obtained at the mid-papillary level and analyzed according to the modified American Society of Echocardiography standards (posterior wall leading-edge to leading-edge and anterior wall trailing-edge to trailing-edge). These measurements were done before inflation of occluder and 10 to 15 seconds after inflation of occluder. During inflation the balloon interrupted myocardial blood flow in the LAD territory and LV wall contraction decreased. Measurement were done before the start of RI (day 0) and repeated after 10 days of RI. Ejection fraction (EF%) and fractional shortening (FS%) were also calculated based on those parameters. Percentage changes of LV EF%, FS% and LVESV were calculated as the difference between the values obtained before and after the occluder inflation at day 0 and day 10.

**Isolectin-B4 Infusion.** On Day 10 of the RI protocol, after the final echocardiography, the rats were sacrificed and the heart was perfused and fixed. Under isoflurane inhalation (1.5-2% via nose cone), rats were injected intravenously via the femoral vein with 250 µg (0.25 mL volume) of isolectin GS-IB4 (Invitrogen). 20 minutes later, with the aorta clamped shut, the heart was perfused with 20 mL 1PBS, followed by 20 mL 4% PFA. The heart was then removed and tissue samples were taken from the ischemic and normal zones of the left ventricle.
Supplemental Figure I. A. DNA methylation status (bisulfite sequencing) of the eNOS promoter regions of endothelial cells (ECs), iPSCs from fully reprogrammed fibroblasts, and 3 randomly selected clones of induced vascular progenitor cells (iVPC). Open and closed lollipops indicate unmethylated and methylated CpGs, respectively. The top panel shows the promoter region of Nanog and Oct4 relative to the translation start site. The bottom numbers indicate the methylation percentage of CpG in the region. B is the statistical graph for A.
Supplemental Figure II. Hematoxylin and eosin staining of teratoma site formed by iVPC/iPSC (iVPC colony #9) (A) and iPSC (B). Images were from gland, pancreatic acini (endoderm); blood vessel, smooth muscle (mesoderm); stratified squamous epithelium and neuron (ectoderm). Interestingly, more blood vessels formed in the terotoma.
Supplemental Figure III. Images of rat myocardium implanted with tdTomato labeled iVPCs mixed with FITC microsphere at day 2 (A) and day 10 (B) of RI protocol. DAPI reveals nuclei. Both the red cells and green microsphere were found in the collateral dependent zone 1 (near injection site) and only red cells presented in collateral dependent zone 2 (remote from injection site). There were no microsphere or Td-tomato labeled cells in normal zone.
Supplemental Figure IV iVPCs engrafted into blood vessels after implantation into rat ischemic myocardium. Images are from immunostaining of rat myocardium in control group, iPSCs and iVPCs delivered group after 10 days of the RI protocol. DAPI staining reveals cell nuclei. Immunostaining of rat myocardium with ES cell marker SSEA-1 (green) and endothelial cell marker VWF (red). In control group, there was no obvious SSEA-1 expression. In the iVPCs and iPSCs implanted group, which highly expressed SSEA-1 and co-localized with vascular ECs.
Reference


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### Supplemental Table II

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Supplementary Movie Legend

Rat was injected with tdTomato labeled iVPCs and went 10 days’ RI protocol. The rat myocardium was imaging under multiphoton fluorescence microscope. iVPCs was shown in red. The image was created from background subtraction of the myocardium to highlight the image of the vessel in the collateral dependent region. Collagen and elastin from arterial wall are shown in green by second-harmonic generation (SHG) technique. From the movie, the tomato red cells were integrated into the blood vessel.