Conversion of Human Fibroblasts to Functional Endothelial Cells by Defined Factors

Jun Li,* Ngan F. Huang,* Jun Zou, Timothy J. Laurent, Jerry C. Lee, Janet Okogbaa, John P. Cooke, Sheng Ding

Objective—Transdifferentiation of fibroblasts to endothelial cells (ECs) may provide a novel therapeutic avenue for diseases, including ischemia and fibrosis. Here, we demonstrate that human fibroblasts can be transdifferentiated into functional ECs by using only 2 factors, Oct4 and Klf4, under inductive signaling conditions.

Approach and Results—To determine whether human fibroblasts could be converted into ECs by transient expression of pluripotency factors, human neonatal fibroblasts were transduced with lentiviruses encoding Oct4 and Klf4 in the presence of soluble factors that promote the induction of an endothelial program. After 28 days, clusters of induced endothelial (iEnd) cells seemed and were isolated for further propagation and subsequent characterization. The iEnd cells resembled primary human ECs in their transcriptional signature by expressing endothelial phenotypic markers, such as CD31, vascular endothelial-cadherin, and von Willebrand Factor. Furthermore, the iEnd cells could incorporate acetylated low-density lipoprotein and form vascular structures in vitro and in vivo. When injected into the ischemic limb of mice, the iEnd cells engrafted, increased capillary density, and enhanced tissue perfusion. During the transdifferentiation process, the endogenous pluripotency network was not activated, suggesting that this process bypassed a pluripotent intermediate step.

Conclusions—Pluripotent factor–induced transdifferentiation can be successfully applied for generating functional autologous ECs for therapeutic applications. (Arterioscler Thromb Vasc Biol. 2013;33:1366-1375.)

Key Words: angiogenesis ■ direct reprogramming ■ endothelium ■ peripheral vascular disease ■ stem cells ■ transdifferentiation

Ischemic vascular disease, where blood flow to tissues is significantly reduced, is a major cause of mortality and morbidity. Ischemic vascular disease–related diseases include peripheral arterial disease, coronary artery disease, and cerebrovascular disease, which in their later stages can result in gangrene, myocardial infarction, or stroke, respectively. Preclinical studies of adult stem cell therapies using bone marrow mononuclear cells, mesenchymal stem cells, adipose stem cells, and endothelial progenitor cells have generated promising results, with evidence for enhanced neovascularization, reduced ischemic injury, and improved organ functions.1,3 Clinical trials using adult stem cells, such as bone marrow mononuclear cells, also showed a modest therapeutic benefit in small clinical studies, although the results need confirmation in larger randomized clinical trials.3,5 However, adult stem cell therapy is limited by the adverse effects of age and disease on the number and quality of adult stem and progenitor cells.6-7 Pluripotent stem cells, such as embryonic stem cells or induced pluripotent stem cells (iPSCs), which can be generated from a patient’s somatic cells, such as skin fibroblasts, provide another potential source of therapeutic endothelial cells (ECs). We previously demonstrated that human pluripotent stem cell–derived ECs can enhance limb perfusion and angiogenesis in murine models of peripheral arterial disease.8,9 However, generating human iPSCs is inefficient, laborious, and time consuming. Furthermore, concerns about the incomplete differentiation and tumorigenicity of these cells are a significant obstacle to their therapeutic application. In searching for an alternative strategy for production of induced endothelial (iEnd) cells, we sought to generate functional ECs from somatic cells directly without transiting through a pluripotent intermediate.

Nuclear reprogramming to pluripotency using current methods is inefficient. Furthermore, our understanding of the determinants of nuclear reprogramming is incomplete. However, it is clear that only a small percentage of induced cells eventually become iPSCs. Furthermore, it is also apparent that pluripotency is achieved only after extended overexpression of iPSC factors and appropriate signaling inputs. We reasoned that, at earlier time points, we could guide the epigenetically
activated cells (induced by the iPSC factors) into lineage-specific cell types under other conditions without traversing pluripotency. We previously found that temporally controlled, transient expression of iPSC factors in mouse fibroblasts, followed by administration of lineage-specific signals, induced generation of nearly homogenous cardiac cells or neural progenitor cells rapidly without activation of pluripotency.\(^\text{10,11}\) Here, we demonstrated that this transient pluripotency-factor-based signaling-directed (TPS) transdifferentiation approach could be further applied to generate functional iEnd cells from human fibroblasts with only 2 factors: Oct4 and Klf4 (OK). The iEnd cells exhibit characteristic EC phenotype in vitro and in vivo and are capable of functionally promoting vascular regeneration and blood perfusion in a murine model of peripheral arterial disease.

In our previous TPS transdifferentiation studies, we showed that 3 or 4 iPSC-factors (Oct4, Klf4, Sox2 [OKS], with or without c-myc) could initially destabilize the epigenetic state of murine fibroblasts, enabling lineage-specific cell fate by soluble factor induction.\(^\text{10,11}\) For clinical applications, developing such strategy in human cells with no or reduced use of genetic manipulation would be highly desirable. Our recent efforts on iPSC generation showed that reprogramming conditions could be enhanced with small molecules to allow generation of iPSCs with fewer exogenously delivered transcription factors.\(^\text{12,13}\) Given that the required ectopic expression of iPSC factors is substantially reduced in the context of TPS transdifferentiation, we hypothesized that it may be feasible to develop a condition with fewer factors for reprogramming human fibroblasts into iEnd cells.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**OK Expression and Inductive Signaling Directs Endothelial Transdifferentiation of Human Fibroblasts**

To determine whether human fibroblasts could be converted into ECs by this TPS transdifferentiation strategy, human neonatal fibroblasts (CRL-2097 and BJ) were transduced with lentiviruses encoding OK, and cultured in the 1:1 mixture of fibroblast medium and chemically defined EC growth medium (Figure 1A). After culturing for 6 to 7 days in this condition, the medium was changed to endothelial induction medium I, supplemented with basic fibroblast growth factor, vascular endothelial growth factor, and bone morphogenetic protein-4, which promote induction of an endothelial program.\(^\text{14}\) Extended bone morphogenetic protein-4 treatment failed to produce any cells positive for the EC marker, CD31. However, when bone morphogenetic protein-4 was withdrawn from the medium at day 14, CD31\(^+\) cells that organized into proliferative clusters became detectable by day 18. The average frequency of CD31\(^+\) cells was relatively low with only \(\approx1\%\) of the total cells expressing this marker at day 28. Previous studies reported that activation of cAMP-dependent protein kinase A enhances endothelial specification.\(^\text{15}\) Therefore, we examined the effect of adding 8-Br-cAMP to the culture medium during days 14 to 28 on EC induction, and found that 8-Br-cAMP could increase endothelial transdifferentiation from human fibroblasts by nearly 4-fold (3.85% efficiency) as measured by the abundance of CD31\(^+\) cells on day 28 based on fluorescence-activated cell sorting (Figure I in the online-only Data Supplement).

At day 28 after induction, cells were immunostained with antibodies for the EC specific markers, CD31 and vascular endothelial-cadherin (VE). At this point, many small clusters of cells with cobblestone morphology had emerged, and nearly all clusters stained positive for these 2 markers (Figure 1B and Figure IIA in the online-only Data Supplement). To enrich for the iEnd cells, these clusters were manually isolated and cultured in EGM2 (Lonza) endothelial expansion medium. To further enhance their expansion, we added to the EGM2 growth media SB431542, a specific transforming growth factor β receptor inhibitor that was reported to promote embryonic stem cell–derived EC growth and sheet formation,\(^\text{16}\) and observed more effective expansion of the iEnd cells.

By manually picking the endothelial-like clusters for expansion, we could enrich the purity of the iEnd cells to 61% after expansion for 3 passages based on the expression of CD31 by fluorescence activated cell sorting (Figure III in the online-only Data Supplement). After fluorescence-activated cell sorting purification, the iEnd cells were 97% pure based on CD31+ expression. The purified iEnd cells showed the typical endothelial cobblestone morphology and were further molecularly and functionally characterized after 5 passages. Based on immunofluorescence staining, the iEnd cells showed colocalized expression of the endothelial markers CD31 and VE (Figure 1C and Figure IIB in the online-only Data Supplement). The iEnd cells also expressed von Willebrand factor, whereas the expression was absent in the parental fibroblasts (Figure 1D and Figure IIC in the online-only Data Supplement).

Functionally, the iEnd cells could efficiently take up fluorescently labeled acetylated low–density lipoprotein, a characteristic of ECs (Figure 1E and Figure IID in the online-only Data Supplement). These iEnd cells also efficiently formed capillary tube-like networks when seeded onto matrigel in vitro, whereas the parental fibroblast remained rounded and failed to organize into networks (Figure 1F and Figure IIE in the online-only Data Supplement).

Furthermore, although various cell types can form networks in matrigel, only ECs can form capillaries with lumens in matrigel.\(^\text{17}\) Therefore, we characterized the endothelial identity of the iEnd cells based on their functional capacity to form capillary lumens when embedded subcutaneously within matrigel plugs in severe combined immunodeficient mice. At 2 weeks postimplantation, histological analysis demonstrated that the iEnd cells within plugs maintained expression of human-specific CD31 and could organize to form capillaries (Figure 1G), whereas the parental fibroblasts neither expressed CD31 nor formed tubular structures in vivo. These results collectively suggested that the iEnd cells exhibited the molecular and functional hallmarks of ECs in vitro and in vivo.
To further characterize the iEnd cells, global gene expression analysis of iEnd-1 (derived from CRL-2097), iEnd-2 (derived from BJ), human fibroblasts, and human umbilical vein ECs (HUVECs) showed that iEnd cells are distinct from fibroblasts and most similar to HUVECs at the transcriptome level (Figure 2A–2C). Many genes known to be involved in EC specification, such as CD31, VE, Tie-1, and von Willebrand factor, were significantly upregulated in the iEnd cells as compared with fibroblasts (Figure 2D). Concomitantly, fibroblast-specific genes, such as Col1A1, Thy1, S100A4, and Twist, were significantly downregulated in the iEnd cells (Figure 2D). Consistent with what has been reported in other reprogramming studies, we also found that a small subset of fibroblast-related transcripts in the iEnd cells were not downregulated to the level in HUVECs (Figure 2E). Although the functional significance of those few genes is unknown, this may indicate an epigenetic memory derived from the parental fibroblasts.

**Figure 1.** Reprogramming of human fibroblasts (CRL2097) to functional endothelial cells by expression of Oct4/Klf4 and sequential induction signals. A, Reprogramming of human fibroblasts (CRL2097) to endothelial cells. The diagram is a schematic overview of the time course and the sequential treatments with different induction media. B, CD31 and vascular endothelial-cadherin (VE) double positive cell clusters were detected on day 28. C, Immunostaining of endothelial cell markers CD31 and VE after fluorescence-activated cell sorting (FACS) purification showing the induced endothelial (iEnd) cells were double positive for CD31 and VE. D, Immunostaining of endothelial cell marker von Willebrand factor (vWF) in iEnd cells. E, Immunostaining of endothelial cell marker von Willebrand factor (vWF) in iEnd cells. F, Formation of a capillary-like network on matrigel by iEnd cells, but not fibroblasts, after 24 hours. G, Formation of capillary structures in matrigel plugs in vivo after subcutaneous transplantation in severe combined immunodeficient mice for 2 weeks. Sections were stained using an antibody specific for human CD31. All the cell samples in (C–G) were characterized after FACS isolation and expansion of iEnd cells in EGM2 medium in the presence of 10 µmol/L SB431542 for 5 passages. Scale bars, 200 µm (B); 20 µm (C); 50 µm (D and E); 100 µm (F); 50 µm (G). bFGF indicates basic fibroblast growth factor; BMP, bone morphogenetic protein; DAPI, 4',6-diamidino-2-phenylindole; FBS, fetal bovine serum; hES, human embryonic stem cells; KSR, knockout serum replacement; and VEGF, vascular endothelial growth factor.
**Figure 2.** Transcriptome profiling of induced endothelial (iEnd) cells and analysis of transgene silencing. 

A, Hierarchical clustering analysis of global gene expression pattern in fibroblast, human umbilical vein endothelial cells (HUVECs), iEnd-1 (CRL-2097–derived iEnd cells) and iEnd-2 (BJ-derived iEnd) cells. The color bar at the right side indicates gene expression in scale. Red and green colors represent higher and lower gene expression levels, respectively. 

B, Homogeneity of gene expression visualized by scatter plot. Scatter plots were drawn with the averaged intensities of gene expression in HUVECs, iEnd-1, and iEnd-2 cells against fibroblasts. 

C, Homogeneity of gene expression between iEnd-1 and iEnd-2 cells with respect to HUVECs. 

D, Heat map of endothelial and fibroblast markers. The expression of the marker genes is shown. 

E, Heat map is shown of a subset of genes having residual expression of fibroblast-related genes in iEnd-1 and iEnd-2 cells. The heat map shows genes that have a comparable expression level in iEnd-1, iEnd-2, and fibroblasts, but a lower expression level in HUVECs. 

F, Genomic polymerase chain reaction (PCR) to verify integration of lentiviral constructs used for direct endothelial reprogramming. β-actin was used as a loading control. Fibroblasts (Fib) (Oct4 and Klf4 [OK]) indicates fibroblasts transduced with OK factors. 

G, A reverse transcriptase-PCR analysis of exogenous (exo) Oct4, exo Klf4, endogenous (endo) Oct4, endo Klf4, CD31, and vascular endothelial-cadherin (VE) in the indicated samples. The GAPDH was used as a loading control. 

H, Teratoma formation assay using 2×10^6 induced pluripotent stem cells (iPS), fibroblast, iEnd-1, iEnd-2, and HUVECs. Cells were injected into severe combined immunodeficient mice. The arrow indicates teratoma formation by iPSCs at 4 weeks after injection. No teratomas could be seen in mice injected with fibroblasts, iEnd-1 cells, iEnd-2 cells, or HUVECs.
Furthermore, reverse transcriptase-polymerase chain reaction analysis revealed that the integrated Oct4-pSin and Klf4-pLove transgenes (Figure 2F) were completely silenced in iEnd cells (Figure 2G). It was further confirmed by reverse transcriptase-polymerase chain reaction that the iEnd cells expressed high level of endogenous CD31 and VE, but no endogenous OK (Figure 2G). Importantly, the iEnd cells did not generate teratomas after injection into severe combined immunodeficient mice (Figure 2H). Taken together, our data demonstrated that the direct reprogramming of fibroblasts into

Figure 3. Direct conversion of fibroblasts into induced endothelial (iEnd) cells is a gradual process that bypasses induced pluripotent stem cell (iPSC) generation. A, Histone modifications during direct reprogramming. Chromatin immunoprecipitation assays examining epigenetic modifications at Oct4, and CD31 and vascular endothelial-cadherin (VE) promoter loci during reprogramming were performed in human umbilical vein endothelial cells (HUVECs) and in the transdifferentiating cells at day 0, day 18, day 28 after transient pluripotency-factor–based signaling–directed (TPS) was initiated. Cell samples at days 18 and 28 were collected by manual selection of clusters having cobblestone-like morphology and assayed without further expansion. Quantitative polymerase chain reaction (PCR) was used to assess changes in histone H3K4 (top) and K27 (bottom) trimethylation levels. Data are represented as percentage of input (*P<0.05; n=3). B, Direct endothelial reprogramming does not involve iPSC generation. Time course analysis is shown for pluripotency and endothelial markers by quantitative reverse transcriptase-PCR. Cell samples at days 18 and 28 were collected by manual selection of clusters based on cobblestone-like morphology. Human embryonic stem cells (hES) and HUVECs were used as positive controls for pluripotency and endothelial markers, respectively. Gene expression levels were normalized to GAPDH and shown as relative fold change (*P<0.05; n=3). C, Immunostaining of Nanog during transdifferentiation on days 18 and 28. Human iPSCs were used as a positive control for Nanog staining. DAPI indicates 4',6-diamidino-2-phenylindole.
ECs by OK factors did not depend on sustained overexpression of the exogenous reprogramming factors.

Direct Conversion of Fibroblasts into iEnd Cells Is a Gradual Process That Bypasses iPSC Generation

To further characterize the epigenetic changes at 3 loci (Oct4, CD31, and VE) during the transdifferentiation, we performed chromatin immunoprecipitation assays on manually dissected iEnd cell clusters on days 18 and 28, in comparison with parental fibroblasts and HUVECs (Figure 3A). Relative levels of histone H3K4me3 and H3K27me3 (markers closely tied to transcriptional activation and repression, respectively) in the promoter regions of these genes were examined. As expected, we observed an increase of H3K4me3 and a concomitant decrease of H3K27me3 at the Oct4 promoter at day 18 after reprogramming induction, followed by a return to the H3K27me3-dominant repressed state at day 28. In contrast, our analysis showed that both CD31 and VE promoter regions underwent a burst of activating H3K4me3 at day 18, compared with the starting fibroblasts at day 0, the level of which activation approached those of control HUVECs by day 28. Concurrently, levels of repressive H3K27me3 at CD31 and VE promoter regions significantly decreased in comparison with those of the starting fibroblasts and approached those of HUVECs at day 28. These results indicated that epigenetic transition to endothelial fate starts as early as day 18 without a parallel commitment to pluripotency.

To confirm whether the iEnd cells were directly generated from human fibroblasts without passing through a pluripotent stage, we examined the expression of pluripotency genes Oct4, Sox2, and Nanog by quantitative polymerase chain reaction. As expected, because of the exogenous expression of Oct4, the total expression of Oct4 at day 18 was higher than at day 0, but returned to the level seen in HUVECs at day 28. Endogenous Nanog and Sox2 transcripts were essentially unchanged compared with intact fibroblasts (day 0) during the entire transdifferentiation process (Figure 3B). We further collected iEnd cell clusters on days 18 and 28 to immunostain for the expression of Nanog. Unsurprisingly, there was no Nanog expression in the CD31+ clusters (Figure 3C). These results indicate that formation of CD31+ clusters does not require activation of the endogenous pluripotency network.

Similarly, we also quantified the gene expression of endothelial progenitors and more mature ECs in the time course samples that had undergone the transdifferentiation. Quantitative polymerase chain reaction analyses showed that the endothelial program was already under way at day 18, as indicated by the upregulated expression of CD31 (Figure 3B). To determine whether the endothelial transdifferentiation program encompassed the formation of mesodermal progenitors, we accessed the temporal expression kinetics of Flk1, a typical marker of mesodermal progenitors. The expression of Flk1 reached a high level at day 18 and then decreased by day 28, indicating that the iEnd cells passed through a progenitor-like state at day 18 before reaching a more mature endothelial fate by day 28. The expression of endothelial markers CD31 and VE at day 28 approached levels observed in HUVECs. These results collectively indicated that our iEnd transdifferentiation bypassed iPSC generation but may still have followed the normal endothelial specification process.

Three Factor (OKS) and 4 Factor (OKSM) Are Also Permissive to Endothelial Transdifferentiation

Our results suggest that 2 reprogramming factors (OK) are sufficient to initiate transdifferentiation of human fibroblasts into ECs with the proper signaling inputs. However, the addition of other reprogramming factors is permissive to initiating such transdifferentiation as well. Induction by ectopic expression of OKS for the first 7 days and cultured under the same conditions for 28 days also resulted in the generation of iEnd cells (Figure IVA–IVE in the online-only Data Supplement) from human fibroblasts. The efficiency of 3-factor–induced
transdifferentiation was 11.8% on day 28 (Figure IVE in the online-only Data Supplement), which was notably higher than the efficiency of transdifferentiation with only 2 factors. In fact, this transdifferentiation approach could also be similarly extended to murine fibroblast cells, where iEnd cells were generated from secondary murine embryonic fibroblasts harboring doxycycline-inducible Oct4, Klf4, Sox2, and cMyc (OKSM; Figure V in the online-only Data Supplement), corroborating previous reports that 4 factors can be used to generate iEnd cells.21 In the doxycycline-inducible murine embryonic fibroblasts system, we could observe formation of iEnd clusters as early as day 12. Taken together, these data suggest that transient expression of the OK reprogramming factors are required for endothelial transdifferentiation, although OKS and OKSM factors are also permissive to transdifferentiation in both human as well as murine fibroblast sources.

**iEnd Cells Show Therapeutic Benefit in a Mouse Model of Peripheral Arterial Disease**

A current limitation of many in vitro transdifferentiated cell types from fibroblasts is the absence or lack of in vivo rescue characterization for disease models.11,22–25 To further functionally characterize our iEnd cells and provide a proof-of-concept demonstration of their potential therapeutic utility, we determined whether the iEnd cells after transplantation could functionally enhance limb perfusion and angiogenesis in a murine hindlimb ischemia model, which is a well-established model of peripheral arterial disease. We genetically labeled the iEnd cells and parental fibroblast with a lentivirus encoding firefly luciferase and green fluorescent protein to enable noninvasive tracking of the cells using bioluminescence imaging and fluorescence-activated cell sorting of transduced cells. After verifying the endothelial identity of the purified iEnd cells by molecular and functional assays, the cells were stably transduced with the double fusion reporter construct with 86% efficiency for double positive expression of green fluorescent protein and CD31 (Figure VI in the online-only Data Supplement). These highly pure iEnd cells were used for intramuscular injection into the ischemic limb at days 0 and 7 after induction of hindlimb ischemia by unilateral ligation of the femoral artery. To verify that transduction of the bioluminescence and fluorescence reporter genes did not affect iEnd cell phenotype, we immunostained for the colocalized expression of EC markers CD31 and VE by immunostaining (Figure 4A).

Using bioluminescence imaging, we noninvasively monitored cell survival and localization of the iEnd cells in the ischemic hindlimb. The cells survived and remained localized to the ischemic limb, although a typical decrease in cell viability after cell transplantation into a site of ischemia was observed (Figure 4B), as was also observed in the parental fibroblasts. Quantification of bioluminescence imaging demonstrated a positive bioluminescence signal (ie, above the background level of 10⁴ p/s per centimeter squared per steradian), confirming the persistence of cells even after 14 days postinjection (Figure 4C). To further verify engraftment of transplanted cells, we performed a histological analysis for the tissues at day 14 after delivery of viable iEnd cells. Immunostaining with the human-specific CD31 antibody demonstrated that the iEnd cells seemed to form both small capillary structures and larger vessels with distinctive lumens (Figure 5). Using a CD31 antibody that was cross-reactive to human and mouse ECs, we observed that human-derived vessels were interspersed within the murine vessels.

To assess the therapeutic benefit of iEnd cell transplantation, we examined the functional recovery of limb perfusion by laser Doppler spectroscopy (Figure 6A and 6B and Figure VII in the online-only Data Supplement). At day 14 after delivery, mice injected with the iEnd cells showed a significant improvement in the mean perfusion ratio (0.64±0.09) when compared with both the control mice injected with saline (0.47±0.07) and fibroblasts (0.47±0.07; P<0.01). We further confirmed the improvement in limb perfusion with immunofluorescence quantification of total vascular density with a CD31 antibody that cross reacts with both human and mouse ECs (Figure 6C).
The results showed that mice transplanted with iEnd cells had a 50% greater total capillary density than those treated with either saline (1437±140 capillaries/mm² iEnd versus 960±114 capillaries/mm² PBS, \( P<0.0025 \)) or fibroblasts (800±156 capillaries/mm² PBS, \( P<0.0025 \)), as shown in Figure 6D. These in vivo results suggested that the iEnd cells are functionally capable of improving limb perfusion by supporting neovascularization of the ischemic hindlimb tissue. These results are consistent with the findings from our previous reports demonstrating the functionality of pluripotent stem cell–derived ECs in the ischemic hindlimb.\(^8,9\)

**Discussion**

In this study, we demonstrated the conversion of fibroblasts to functional ECs in the human system with only 2 factors, OK, by the TPS transdifferentiation strategy. This strategy can also be extended toward generating iEnd cells using 3 (OKS) or 4 (OKSM) factors using human or murine fibroblasts. This approach validates the broad applicability of the TPS transdifferentiation strategy for generating desired lineage-specific cell types from somatic cells. Once the cells have been sufficiently epigenetically activated, they may be directed toward many different lineages and may in part follow natural development.

This method represents an alternative attractive strategy to the derivation of autologous ECs without the generation of iPSCs. Although the transdifferentiation of human fibroblasts requires 28 days, it is still a quicker process than iPSC line establishment followed by endothelial differentiation. In the murine embryonic fibroblasts system, we could observe formation of iEnd clusters as early as day 12. Molecular characterization confirmed cell transition from fibroblasts toward ECs without activating the endogenous pluripotency network. We also demonstrated that iEnd cells resembled primary ECs in phenotype as well as function both in vitro and in vivo. In a disease setting of hindlimb ischemia, the iEnd cells also engrafted and exhibited therapeutic potential. Treatment with these cells increased the capillary density and blood perfusion, and they were able to integrate into the blood vessels and survive. Collectively, those results suggested that these cells could be further developed as a potential therapy for ischemic vascular disease.

Future studies will be necessary to determine whether the iEnd cells represent a novel method to generate patient-specific ECs that traverse through a vascular progenitor state. Although current transdifferentiation paradigm aims to obtain a particular type of cells that are typically isolated from the direct reprogramming culture, characterizing other cell types generated during the reprogramming process would provide new insights and facilitate improving reprogramming conditions. In particular, teratoma formation of OK-transduced fibroblasts at intermediate time points will determine whether transiently overexpressing OK-transduced fibroblasts can avoid generating cells with tumorigenic potential. In addition, further characterization of the genetic profile of iEnd cells at a progenitor-like stage on day 18 will provide new insights into the molecular pathways mediating the transdifferentiation process.

Recently, OKSM factors were shown to induce endothelial transdifferentiation of human fibroblasts.\(^{21,26}\) Our observations using doxycycline-inducible murine embryonic fibroblasts support the finding that transient activation of OSKM together with the appropriate inductive signals (TPS) can generate murine iEnd cells (Figure V in the online-only Data Supplement). Moreover, we demonstrate that only the OK factors are necessary for endothelial transdifferentiation of human fibroblasts, which may be therapeutically more relevant because it obviates the use of oncogene c-myc. This finding is consistent with the reported roles of these 2 factors in maintaining endothelial function or promoting multilineage blood progenitor generation of fibroblasts. Previously, Klf4 was shown to bind to the VE promoter and regulate EC phenotype and function by inducing VE gene expression and endothelial barrier function.\(^{21}\) In addition, ectopic Oct4 expression was shown to activate the adult hematopoiesis program in human fibroblasts to generate multilineage blood progenitors.\(^{23}\) Because
ECs and hematopoietic lineages are thought to originate from a common hemangioblast precursor, it is possible that Oct4 may also exert influence on endothelial specification. Together, these results highlight the role of OK in regulating endothelial-related phenotype and signaling pathways.

It is widely accepted that ECs are heterogeneous in morphology, function, and transcriptome, depending on the local vascular bed. Specification into arterial, venous, and lymphatic subtypes are, in part, regulated by signaling pathways, such as Notch1, and by microenvironmental factors, such as shear stress. It is likely that the iEnd cells represent a heterogeneous mixture of all 3 subtypes, because the nonsubtype-specific endothelial marker, CD31, was used for purification. It remains to be determined whether subtype-specific iEnd cells would be superior to heterogeneous iEnd cells for therapeutic angiogenesis. These experiments are interesting and warranted, but beyond the scope of this study.

In summary, we showed that therapeutically functional ECs can be derived from human fibroblasts with only 2 factors, OK, when combined with the appropriate inductive signals in a process we term TPS transdifferentiation. Because the reprogrammed cells did not go through a pluripotent intermediate state, the risk of tumorigenicity is minimized. Furthermore, this process obviates the use of c-myc, so it may be a safer approach that obviates oncogenesis. TPS transdifferentiation can be successfully applied for transdifferentiation of both human and murine cells. This process might be further optimized, with more small-molecular enhancers, for instance, to generate ECs at a higher efficiency and with faster kinetics. Furthermore, the use of nonintegrating genetic delivery, such as mRNA or episomal vectors, could avoid potential issues associated with viral integration. This approach could be an attractive strategy to derive autologous therapeutic ECs and can serve as a useful model to study lineage specification and conversion.

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Disclosures

None.

References

This study demonstrates that human fibroblasts can be transdifferentiated into functional endothelial cell by using only 2 factors (Oct4 and Klf4) under inductive signaling conditions, while obviating the use of oncogenes, such as c-myc. The induced endothelial cells resemble primary endothelial cells in their phenotype, transcriptome, and functional capacity to enhance vascularization in the setting of hindlimb ischemia. Transient pluripotency-factor–based signaling-directed transdifferentiation can be applied for both human and murine cells, suggesting the universality of this reprogramming approach. We further demonstrate that the transdifferentiation process bypassed a pluripotency intermediate step, because the endogenous pluripotency network was not activated. The epigenetic changes at 3 loci (Oct4, CD31, and vascular endothelial) demonstrated that an epigenetic transition to endothelial fate started as early as day 18. This work highlights the important role of transient nuclear reprogramming in directing cell fate into endothelial lineage and has important implications in the development of safe cell therapies for treatment of vascular diseases.
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SUPPLEMENTAL DATA

Supplemental Tables

Table I. Primers for Genomic PCR

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<th>Genes</th>
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Table II. Primers for RT-PCR

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<td>AGAGGCGTGGTCTCCACAG</td>
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### Table III. Primers for Real Time PCR

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<th>Genes</th>
<th>Forward</th>
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<tr>
<td>CD31</td>
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<td>GAPDH</td>
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<tr>
<td>KDR</td>
<td>CGGCTCTTTGCTTTACTGTT</td>
<td>CCTGTATGGAGGAGGAGGAA</td>
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<tr>
<td>Nanog</td>
<td>GATTTGTGGCCTGAAGAAA</td>
<td>CAGGGCTGTCTGAATAAGC</td>
</tr>
<tr>
<td>Oct4</td>
<td>GAAGGAGAAGCTGGGAGCAA</td>
<td>CTTCTGCTTCAGGAGCTTGG</td>
</tr>
<tr>
<td>Sox2</td>
<td>AACCACCAAGATGCACAACTC</td>
<td>GCTTAGCCTCGTCGATGAAC</td>
</tr>
<tr>
<td>VE</td>
<td>AGAGCTCCACTCAGCCTCAG</td>
<td>CATCTTCCCAGGAGAACAG</td>
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### Table IV. Primers for ChIP assay

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<th>Genes</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>CD31</td>
<td>CGAGACAGAGGGAGGGTCAA</td>
<td>GGCTGATATCTCCTCGAGAA</td>
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<tr>
<td>Oct4</td>
<td>GAAGGAGAAGCTGGGAGCAA</td>
<td>CTTCTGCTTCAGGAGCTTGG</td>
</tr>
<tr>
<td>VE</td>
<td>CACCACCCACACCCACCCCTGC</td>
<td>CTTCCACCACCTCTGACCGATC</td>
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Supplemental Figures

Supplemental Figure I. 8-Br-cAMP enhanced endothelial cell reprogramming from human fibroblasts. Clusters of cells having cobblestone-like morphology were manually expanded for 3 passages before characterization of CD31 expression by flow cytometry. Compared with untreated controls, CD31 positive cells increased in the presence of 8-Br-cAMP. FACS plots are representative of n=3 experiments.
Supplemental Figure II. Reprogramming of human BJ fibroblasts to functional endothelial cells by expression of Oct4/Klf4 and sequential induction signals. (A) CD31 and VE double positive cell clusters were detected on day 28. Immunostaining of iEnd cells after FACS purification for endothelial markers demonstrate colocalization of CD31 and VE (B) and expression of vWF (C). Functionally, the iEnd cells could uptake acetylated LDL (D) and form capillary-like networks on matrigel at 24 hours after cell seeding (E). (F) The iEnd cells formed capillary lumens when subcutaneously implanted within matrigel plugs in SCID mice for 2 weeks. Capillary lumens (arrow) expressed human-specific CD31. Scale bars: 200 µm (A); 50 µm (B-D); 100 µm (E); 50 µm (F).
Supplemental Figure III. CD31 expression of iEnd cells before and after FACS purification. The purity of CD31 expression increased from 61% prior to cell sorting to 97% after purification (n=3).
Supplemental Figure IV. Reprogramming of human fibroblasts to endothelial cells by expression of Oct4/Klf4/Sox2 and sequential induction signals. (A-B) Quantitative gene expression of endothelial markers (CD31 and VE) and pluripotency markers (Oct4 and Nanog) over the time course of 28 days, relative to day 0 (n=3). (C) CD31 and VE double positive colonies were detected on day 28. (D) After manual selection, clusters of iEnd cells functionally resemble endothelial cells in their ability to uptake acetylated LDL. (E) FACS analysis of CD31 positive cells in three-factor (OKS) induced iEnd cells on day 28 of transdifferentiation. Scale bars: 200 µm (C); 50 µm (D). *p<0.05 (n=3).
Supplementary Figure V. Endothelial transdifferentiation of MEFs using 4 factor (OKSM) doxycycline-inducible system. (A-B). Quantitative gene expression of endothelial cell markers (CD31 and VE) and pluripotency markers (Oct4 and Nanog) over the course of 15 days, relative to day 0. (C) CD31 and VE double positive cell clusters were detected on day 12. (D) After manual selection and expansion, the clusters of iEnd cells functionally resemble endothelial cells in their ability to uptake acetylated LDL. Scale bars: 100 µm (C); 20 µm (D). *p<0.05 (n=3).
Supplementary Figure VI. Flow cytometry analysis of GFP and CD31 expression of transduced iEnd cells. The iEnd cells were transduced with a lentiviral construct to enable non-invasive bioluminescence imaging and histological detection. The expression of GFP and CD31 in iEnd cells used for in vivo transplantation was quantified by flow cytometry (n=3).
Supplementary Figure VII. Laser Doppler image showing perfusion time course. Animals were injected at day 0 and day 7 with iEnd cells, fibroblasts, or saline vehicle control. Laser Doppler images of perfusion were measured at days 0, 3, 7, 10 and 14.
Supplementary Figure VIII. Morphological characterization of non-transduced fibroblasts in inductive growth media. Control denotes fibroblasts without OK factors cultured in similar media conditions as OK-transduced cells.
Supplementary Figure IX. Immunofluorescence staining for human-specific CD31 (green) at 2 weeks after subcutaneous implantation of HUVEC embedded within matrigel plugs.
MATERIALS & METHODS

Cell Culture and Generation of iEnd Cells

Primary human dermal fibroblasts (CRL2097 and BJ, ATCC) were cultured in DMEM containing 10% fetal bovine serum (FBS), 1 mM L-glutamine and 1% non-essential amino acids (all from Invitrogen). The cells were then transduced with Oct4 and Klf4 lentivirus, and maintained on gelatin-coated dishes in DMEM containing 10% FBS. Where specified in some cases, Sox2 lentivirus was also added along with OK factors. After overnight culture, the medium was changed to 1:1 ratio of DMEM:Stemline (Sigma) with 7.5% FBS and 7.5% knockout serum replacement (KSR (Invitrogen). Over the course of 7 days this medium was gradually transitioned to a final concentration of 1% FBS and 10% KSR. The cells were then treated with differentiation medium I, containing BMP4 (20 ng/ml, Stemgent), VEGF (50 ng/ml, R&D Systems) and bFGF (20 ng/ml, Stemgent) in Stemline medium for another 7 days, with the medium changed every 2 days. Then the medium was replaced with differentiation medium II containing VEGF (50 ng/ml), bFGF (20 ng/ml) and 8-Br-cAMP (0.1 mM, Tocris) in Stemline for another 14 days, with the medium changed every 2 days. This was followed by cell collection for expansion and molecular analysis. As a negative control, fibroblasts without lentiviral transduction were cultured in the same media conditions as transduced fibroblasts. An overview of the transdifferentiation scheme is depicted in Figure 1A. HUVECs (Lonza) were cultured in EGM2 media and served as positive control cells for endothelial characterization assays. Human iPSCs and ESCs were expanded in culture conditions as previously described.¹
To generate iEnd cells from secondary MEFs carrying doxycycline-inducible reprogramming factors (Oct3/4, Sox2, Klf4, c-myc), MEFs were induced with mouse ES media with the addition of doxycycline (4 µg/ml) for the first 4 days to induce transient expression of the reprogramming factors. On day 4, the cells were switched to differentiation media I, followed by 5 days in differentiation media II.

**Immunocytochemistry**

The iEnd cells were characterized by immunocytochemistry *in vitro* using the following antibodies: CD31 (R&D System; 1:200, mouse), VE-cadherin (R&D System; 1:200, goat), vWF (R&D System; 1:200, mouse), and Nanog (Santa Cruz; 1:200, goat). Details of the staining protocol can be found in Supplementary Methods in the online Data Supplement.

**Endothelial Functional Assays *in Vitro***

Uptake of Ac-LDL was assessed by incubating iEnd cells, normal control fibroblasts, and HMDEC with 5 µg/ml of Alexa Fluor-594–conjugated ac-LDL (Invitrogen) for 4 hours prior to detection by fluorescence microscopy. After incubation, the cells were washed with 1x PBS before being visualized and photographed using a fluorescence microscope. For matrigel tube-like formation assay *in vitro*, 4x10^5 iEnd cells or control fibroblasts were seeded on top of a thin layer of matrigel. After 24 hours, the formation of tube-like structures was imaged under brightfield microscopy.

**Hindlimb Ischemia Animal Studies**

To examine the therapeutic potential of iEnd cells in a mouse model of peripheral arterial disease, we induced unilateral hindlimb ischemia in male SCID mice (17 weeks old) by unilateral excision of the femoral artery. The animals were randomized to receive either...
0.5X10^6 iEnd cells in 30 µl saline (n=5), 0.5X10^6 fibroblasts in 30 µl saline (n=5), or 30 µl of control saline (n=3) injection into the ischemic gastrocnemius by intramuscular injection. After 7 days, an additional 0.5X10^6 cells were injected into the gastrocnemius, in accordance with our previous results suggesting that a second injection of cells could further enhance limb perfusion. For up to 2 weeks, cell survival was monitored by BLI after intraperitoneal injection of D-luciferin. Functional improvement in limb perfusion was assessed by laser Doppler spectroscopy and expressed as the mean perfusion ratio, which is the perfusion of the ischemic limb normalized to that of control limb. After 2 weeks, the animals were euthanized, and the ischemic limbs were explanted for cryosectioning and histological analysis of capillary density based on staining of a CD31 antibody (BD Transduction Labs) that cross-reacts with both human and mouse vessels. In addition, a human-specific CD31 antibody (R&D systems, sheep) was used to distinguish human vs murine cells. All animal experiments were performed with approval by the Administrative Panel on Laboratory Animal Care in Stanford University.

Lentivirus Production

The plasmids used for lentivirus production include pSin-EF2-Puro-hOct4, pLOVE-mKlf4, pSin-EF2-Puro-hSox2, the packaging plasmid psPAX2 and the envelop-coding plasmid pMD2.G. These vectors were used to transfect a 293T packaging cell line. Viral supernatants were harvested 48 hours after transfection. Equal amounts of Oct4 and Klf4 virus were used for fibroblast transduction in the presence of 8 µg/ml polybrene.

Transduction of iEnd Cells With the Double Fusion Reporter Construct

For non-invasive tracking in vivo, the iEnd cells were transduced with a lentiviral vector (LV-pUb-Fluc-GFP) carrying an ubiquitin promoter-driven firefly luciferase (Fluc) and enhanced green fluorescence protein (GFP) as described previously. Cells were harvested and
expression levels of GFP and CD31 were measured by flow cytometry. A linear correlation between bioluminescence intensity and cell density for pluripotent stem cell-derived endothelial cells was demonstrated previously.\textsuperscript{2, 5, 6}

**Immunocytochemical analysis**

Samples were washed once with PBS (Invitrogen; without Ca\textsuperscript{2+} and Mg\textsuperscript{2+}) and were fixed with a 4% formaldehyde solution containing 0.15% picric acid (Sigma-Aldrich) in PBS for 20 min, followed by three washes with PBS. Blocking and permeabilization were done with 10% donkey serum (Jackson ImmunoResearch) and 0.3% Triton X-100 (Sigma-Aldrich) solution in PBS for 1 hour at room temperature. All primary antibodies were diluted in 1% BSA and incubated overnight at 4°C. After 1 hour of washing with 0.1% BSA in PBS, samples were incubated with Alexa-555– or Alexa-488–conjugated secondary antibodies (Invitrogen) for 1 hour at room temperature and nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich). All images were taken using a Nikon Eclipse Ti microscope equipped with a Photometrics CoolSnap HQ2 camera and processed with NIS Elements Basic Research Software (Nikon). The following primary antibodies were used: CD31 (R&D System; 1:200, mouse), VE-cadherin (R&D System; 1:200, goat), vWF (R&D System; 1:200, mouse), and Nanog (Santa Cruz; 1:200, goat).

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Uptake of ac-LDL was assessed by incubating iEnd cells, normal control fibroblasts, and HMDEC with 5 μg/ml of Alexa Fluor-594–conjugated ac-LDL (Invitrogen) for 4 hours prior to detection by fluorescence microscopy. After incubation, the cells were washed with 1x PBS before being visualized and photographed using a fluorescence microscope. For matrigel tube-like formation assay *in vitro*, 4X10\textsuperscript{5} iEnd cells or control fibroblasts were seeded on top of a thin
layer of matrigel. After 24 hours, the formation of tube-like structures was imaged under brightfield microscopy.

Flow Cytometry Analysis and Fluorescence Activated Cell Sorting

Cells were washed with PBS and dissociated with Accutase (Innovative Cell Technologies). After harvesting, the cells were washed twice with ice-cold FACS buffer (HBSS supplemented with 10 mM Hepes, 2% FBS, and 0.1% sodium azide; Sigma-Aldrich). Non-dissociated cells were removed by passing the cell suspension through a cell strainer (BD) twice. Cells were incubated with PE-conjugated anti-human CD31 antibody (eBioscience) for 30 min at 4°C. An isotype-matched antibody was used as a negative control. After incubation, the cells were washed twice with five volumes of FACS buffer, fixed, and suspended in 4% paraformaldehyde solution (Electron Microscopy Sciences) in PBS. More than 20,000 cells were analyzed using FACSCalibur and CellQuest software (BD). Further analysis was performed using FlowJo software (Tree Star). To purify the iEnd cells, single cell suspension was obtained by treatment with Accutase for 20 minutes at 37°C to dissociate cells. The cells were then washed with 1x PBS containing 5% BSA, passed through a 70 μm cell strainer, and incubated with PE-conjugated anti-human CD31 antibody for 30 minutes. An isotype-matched antibody was used as a negative control. Flow cytometry was then performed using a BD Digital Vantage cell sorter (BD). The purified iEnd cells were expanded in EGM2 medium containing 10 μM SB431542.

Microarray Data Analysis

RNA was isolated using the RNeasy-Kit (Qiagen, Hilden, Germany). Target labeling was performed following the protocol of Affymetrix 3’ in vitro transcription (IVT). The mRNA transcription levels were evaluated using the human genome U133 plus 2.0 array which contained more than 54,000 probe sets to detect over 47,000 transcripts. The hybridization data were analyzed using Affymetrix GeneChip Command Console (AGCC) software. The
scanned images were first assessed by visual inspection then analyzed to generate raw data files saved as CEL files using the default setting of AGCC. Background correction was performed using the Affymetrix Robust Multi-array Analysis (RMA) background correction model. Variance stabilization was performed using the log$_2$ scaling and gene expression normalization was calculated using the Quantile method implemented in the Lumi package of R-Bioconductor. All samples were processed in at least triplicates to reduce signals arising from processing artifacts. The post-processing of the data was performed with Matlab (Matrix Laboratory) by the average normalization algorithm. The genes were clustered by using correlation and average linkage method in Cluster 3.0. The microarray data can be accessed online from Gene Expression Omnibus (accession number GSE42672).

**Genome-Wide PCR**

Total DNA was extracted using the Gentra Puregene kit (Qiagen, West Sussex, UK) according to the manufacturer’s instructions. The samples were then treated with RNase solution (Qiagen) for 5 min at 37°C to obtain RNA-free DNA (n=3). Primer sequences are listed in Supplementary Table I.

**RT-PCR and Quantitative RT-PCR**

Total RNA was extracted from samples at the designated time points using the RNeasy Plus mini kit with QiaShredder columns (Qiagen). One microgram of total RNA per sample was reverse-transcribed using the iScript cDNA synthesis kit (Bio- Rad) and the cDNA was diluted with 100 μL of water. A total of 1/50 of the diluted cDNA was used for quantitative PCR with iQ SYBR Green Supermix on the CFX96 system (Bio-Rad). RT-PCR primers sequences are listed in Supplementary Table II. All qPCR reactions were done in triplicate, expression levels were analyzed using CFX manager software (Bio-Rad), with levels normalized to GAPDH. Each set
of reactions was repeated using cDNA from at least three independent experiments. Primer sequences are listed in Supplementary Table III.

**Chromatin immunoprecipitation**

Cells from designated time points and HUVECs were analyzed using a commercially available Magna ChIP G kit (Millipore). Briefly, Histones and DNA were cross-linked with 1% formaldehyde. Chromatin with a DNA fragment length of 200–500 bp was obtained by sonication. Equal amounts of soluble chromatin were incubated with normal rabbit IgG, antitrimethyl-histone H3Lys4 (Millipore), or antitrimethyl-histone H3Lys27 (Millipore) antibodies as well as with magnetic protein G beads. After overnight incubation with the antibodies, DNA-histone cross-linking was reversed and DNA fragments were purified. DNA fragments obtained without antibody were used as the input controls, whereas DNA fragments obtained with normal rabbit IgG were applied as negative controls. A total of 1 μL from each purified DNA solution was subject to real-time PCR analysis for quantification. Primer sequences are listed in Supplementary Table IV.

**In Vivo Matrigel Plug Assay**

To demonstrate formation of functional vessels *in vivo*, 5 x 10^5 iEnd cells were embedded within growth factor-reduced matrigel containing bFGF (500 ng/ml).\(^1\) The plugs were implanted subcutaneously into SCID mice. As a basis for comparison, matrigel plugs composed of the parental fibroblasts were similarly implanted. Matrigel plugs containing HUVECs were used as a positive control. After 14 days, the animals were euthanized and dissected to remove the matrigel plugs. The matrigel plugs were fixed in 4% paraformaldehyde and embedded into paraffin for sectioning. For visualization of vessels derived from iEnd cells, the tissue cross sections were immunofluorescently stained with a human-specific CD31 antibody (R&D Systems, 1:50) that did not cross-react with murine tissues. The tissues were then incubated
with Alexafluor-594-conjugated secondary antibody (Invitrogen, 1:200) and counterstained with Hoechst33342 for total nuclear staining.

Teratoma Formation Assay

For teratoma formation assay, $2 \times 10^6$ cells consisting of human iPSCs, fibroblasts, iEnd-1, iEnd-2 or HUVEC cells were injected into SCID mice. After 4 weeks, the presence of teratomas was assayed.

Hindlimb Ischemia Studies

To examine the therapeutic potential of iEnd cells in a mouse model of peripheral arterial disease, we induced unilateral hindlimb ischemia in male SCID mice (17 weeks old) by unilateral excision of the femoral artery.\textsuperscript{2} The animals were randomized to receive either $0.5 \times 10^6$ iEnd cells in 30 µl saline (n=5), $0.5 \times 10^6$ fibroblasts in 30 µl saline (n=5), or 30 µl of control saline (n=3) injection into the ischemic gastrocnemius by intramuscular injection. After 7 days, an additional $0.5 \times 10^6$ cells were injected into the gastrocnemius, in accordance with our previous results suggesting that a second injection of cells can markedly improve cell survival.\textsuperscript{1} For up to 2 weeks, cell survival was monitored by BLI after intraperitoneal injection of D-luciferin. Since ATP and oxygen are required for Fluc to catalyze the oxidation of D-luciferin and the release of photons, only viable transfected cells and their progeny will generate a bioluminescent signal. Functional improvement in limb perfusion was assessed by laser Doppler spectroscopy and expressed as the mean perfusion ratio, which is the perfusion of the ischemic limb normalized to that of control limb. After 2 weeks, the animals were euthanized, and the ischemic limbs were explanted for cryosectioning and histological analysis. All animal experiments were performed with approval by the Administrative Panel on Laboratory Animal Care in Stanford University.
Histology

To assess total capillary density, cryosections were incubated with a CD31 antibody (BD Transduction Labs) that cross-reacts with both human and mouse vessels. For immunofluorescence quantification of capillary density, the total number of capillaries in 5 high-powered fields in at least 4 cryosections in each sample was counted, as described previously.\(^5\) Capillary density is expressed as #/mm\(^2\). In addition, a human-specific CD31 antibody (R&D systems, sheep) was used to distinguish cells of human from murine vessels. H&E-stained sections of the gastrocnemius were used to verify that the regions used for capillary density analysis were ischemic.

Statistical Analysis

All data are reported as mean ± standard deviation. A statistical analysis between two groups was performed by the Student’s t-test. For comparison of multiple groups, statistical analysis was performed by analysis of variance (ANOVA) with the Tukey post-test. Statistical significance was accepted at p<0.05.
REFERENCES


