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이학석사 학위논문

Direct Conversion of Adult Skin Fibroblasts to Endothelial Cells by Defined Factors

성체 피부섬유아세포에서
혈관내피세포로 선별된 인자들에 의한
형질전환에 관한 연구

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Abstract

Direct Conversion of Adult Skin Fibroblasts to Endothelial Cells by Defined Factors

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Background—Cell based therapies to augment endothelial cells (ECs) hold great therapeutic promise. Herein, we report the novel approach to generate functional ECs directly from adult fibroblasts.

Methods and Results—11 candidate genes, key regulators of endothelial development, were selected. GFP^- skin fibroblasts (SFBs) were prepared from Tie2-GFP mice, and infected with lentiviruses allowing for simultaneous overexpression of all 11 factors. $Tie2\text{-}GFP^+$ cells (0.9%), representing Tie2 gene activation, were detected by flowcytometry. Serial stepwise screening revealed 5 key factors (5F: Foxo1, Er71, Klf2, Tal1 and Lmo2) which were required for efficient reprogramming of SFBs into $Tie2\text{-}GFP^+$ cells (4%). 5F were able to convert tail-tip FBs into $Tie2\text{-}GFP^+$ cells, suggesting their universal

effects. This reprogramming strategy did not involve pluripotency induction, as neither Oct4 nor Nanog was expressed after 5F transduction. Tie2-GFP^{+} cells were isolated using FACS, and designated as induced endothelial cells (iECs). iECs exhibited endothelial-like cobblestone morphology and expressed molecular markers of EC. iECs possessed endothelial functions such as BS1 lectin binding, acLDL uptake and capillary formation on Matrigel. VE-cadherin- and Tie2-promoters were demethylated in iECs, demonstrating their similar epigenetic profile to authentic ECs. mRNA profiling showed clustering of iECs with authentic ECs, and highly enriched endothelial genes in iECs. In a mouse hindlimb ischemia model, iEC implantation increased capillary density, and enhanced limb perfusion, demonstrating *in vivo* viability and functionality of iECs.

Conclusions－We demonstrated the first direct conversion of adult fibroblasts to functional ECs. These results suggest a novel therapeutic modality for cell therapy in ischemic vascular disease.

Keywords : induced endothelial cells, direct conversion, transdifferentiation

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Introduction

The formation of new vessels by endothelial cells (ECs) has great clinical potential for the treatment of ischemic vascular disease. However, there is no standardized protocol to derive ECs from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Technical obstacles also exist, such as the necessity for complex manipulation of the embryoid body, a low differentiation efficiency and the risk of contamination by feeder cells.[1-4] Recently, several studies demonstrated that these kinds of hurdles could be overcome by directly converting differentiated cells into other lineages such as pancreatic β-cells,[5] neurons,[6, 7] cardiomyocytes,[8] and hepatocytes[9, 10] by defined factors. Here, we report on a novel strategy to provide ECs from differentiated fibroblasts. We screened 11 candidate factors, and showed that the combination of Foxo1, Er71, Klf2, Tal1 and Lmo2 can directly convert mouse adult fibroblasts into ECs. These induced endothelial cells (iECs) exhibit endothelial morphology, endothelial functions, and similar epigenetic and genetic profiles to authentic functional ECs. In addition, transplantation of iECs rescues murine ischemic hindlimbs *in vivo* by inducing new vessel formation. Our findings identify the molecular background of endothelial differentiation and transdifferentiation, and support the notion

that cell fate determination is not eternal, but plastic by the formation of new transcriptional network.

Materials and methods

Animal

Tie2 promoter-driven green fluorescent (GFP) mice (FVB/N background; Jackson laboratory), Nanog promoter-driven GFP mice (129S4/Sv, C57BL/6, DBA/2 mixed background; RIKEN), Oct4 promoter-driven enhanced GFP mice (C57BL/6 × CBA background; Jackson Laboratory), and athymic nude mice (Balb/c background; Orient Bio) were used in this study. Animal care and experiments were performed in accordance with institutional regulations.

Cell Culture

Skin fibroblasts (SFBs), Tail-tip fibroblasts (TTFs) and primary ECs from lungs were isolated from 8 week-old male Tie2-GFP mice. For SFB isolation, trunk skin was peeled off as a single piece using surgical scissors. After fat removal, the dermis was separated from the epidermis using sterile forceps. The dermis tissue was washed three times with phosphate-buffered saline (PBS) and sliced into 4 - 5 pieces. The pieces were incubated in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 0.1% collagenase (Gibco) on 35-mm petri-dishes (SPL) for 1.5 hour at 37°C with 5% CO2. Tissue fragments were then rinsed three times with PBS, and treated

with 0.25% trypsin(Gibco) for 20 minutes. Cells were collected by centrifugation (1300rpm for 7 minutes at 4ºC), resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco), penicillin/streptomycin (Gibco), l-glutamine (Gibco) and sodium pyruvate (Gibco), and seeded onto 0.1% gelatin-coated 6 well plates (Nunc). After 5 days of culture, GFP-cells were sorted using fluorescence-activated cell sorting (FACS). For TTF isolation, tails were collected, the superficial dermis was peeled off by hand, and there maining tail was cut into 1cm pieces. The pieces were placed on 0.1% gelatin-coated 6-well plates, and cultured in DMEM supplemented with 20% FBS, penicillin/streptomycin, l-glutamine and sodium pyruvate. After attachment, fibroblasts migrated out of the tail explants. After culture for 5 days, the explants were removed and discarded. When outgrown TTFs became confluent, GFP-cells were sorted using FACS to obtain pure fibroblasts. For lung EC isolation, lungs were excised after systemic perfusion with PBS to remove blood. Lung tissue was thoroughly minced with a sterile razor, and incubated with pre-warmed digestion buffer containing DMEM, 1 mg/mL collagenaseI (Worthington), 1 mg/mL collagenaseII (Gibco), 10 mg/mL bovine serum albumin (BSA)(Amresco), and 1U/mL dispase (Gibco) at 37ºC with 5% CO2, and stirred every 10minutes. After 30minutes, 1/10 volume of FBS was added to the buffer. Cells were filtered through a 70-μm cell strainer, and incubated on 10-cm culture dishes at

37ºC with 5% CO2. 1hour after incubation, non-attached cells were collected, and cells that were double positive for Tie2-GFP and CD31 were isolated using FACS. Finally, isolated primary ECs were cultured in EBM-2 (Lonza) with 10% FBS and penicillin/streptomycin on 1.5% gelatin-coated plates. MS1 (Mile Sven 1) is a SV40 transformed pancreatic islet endothelial cell line (ATCC CRL-2279). For bone marrow mononuclear cell (BMMNC) isolation, femurs and tibias were harvested using forceps and scissors. BM cells were obtained by flushing the BM cavity with PBS, and filtered through a 100-μm cell strainer. MNCs were isolated by density gradient centrifugation (2700 rpm for 25 minutes at 4ºC) using Histopaque-1083 (Sigma Aldrich). Isolated MNCs were resuspended using the EGM-2MV BulletKit system (Lonza) supplemented with 5% FBS, and seeded onto 1.5% gelatin-coated 6 well plates (Nunc). 7 days after culturing, GFP-cells were sorted using FACS.

Construction of Lentiviral Vectors for the 11 Candidate Factors

The coding sequence of each gene was amplified by reverse transcription-polymerase chain reaction (RT-PCR), and sub-cloned into pENTR1A entry vectors (Invitrogen) to create entry clones. Mouse Er71 cDNA was provided by S. Sumanas, mouse Fli1 and Elf1 cDNAs by B. Göttgens, and mouse Gata2 and Erg cDNAs by P. Oettgen. LR recombination reactions were

performed to insert cDNAs into the pLenti6.3/V5-DEST destination vector (Invitrogen), and pLenti6.3/V5-GW/lacZ (Invitrogen) was used as a mock vector. To produce lentiviruses, the destination vector plasmids, pLP1, pLP2, and pLP/VSVG (Invitrogen) (10 μg for each plasmid) were mixed with 1 mg/mL polyethylenimine (Polysciences), and added to 293T cells. After 1 day of incubation at 37ºC with 5% CO2, media were replaced with fresh DMEM containing 10% FBS. Culture supernatant was then collected 20 hours after the media change. After filtration through a 0.45-μm strainer, viral supernatant was concentrated by centrifugation (25000 rpm for 90 minutes at 4ºC), and the viral pellets were resuspended in DMEM. For viral infection, GFP-target cells were incubated in DMEM containing viral concentrate and 10 mg/mL polybrene (Sigma-Aldrich) after overnight attachment. After 24 hours, viral media were replaced with fresh DMEM containing 10% FBS and penicillin/streptomycin. Media were then changed to EBM-2 containing 10% FBS and penicillin/streptomycin after 24 hours.

FACS Analyses and Sorting

For flow cytometric analyses, cells were harvested 3, 7, and 12 days after viral transduction, and stained with PE-conjugated antibody against VE-cadherin (1:400; eBioscience), CD31 (1:400; BD Biosciences), or Flk-1 (1:400; eBioscience), or primary antibody against von Willebrand factor (vWF) (1:400; Abcam) for

30 minutes on ice. PE-conjugated anti-sheep secondary antibody (1:400; Abcam) was used to detect vWF. Samples were assayed using a FACSCalibur cell analyzer (BD Biosciences). Data were analyzed using BD CellQuest Pro software (version 5.2.1). For Tie2-GFP+ cell sorting, cells were harvested 12 days after viral transduction, and assayed by FACS AriaII cell sorter (BD Biosciences). Data were analyzed using the FACS Diva software (version 6.1.3). Tie2-GFP+ iECs were sorted, and cultured in EBM-2 containing 10% FBS and penicillin/streptomycin on 1.5% gelatin-coated plates.

Western Blot Analyses

Primary antibodies against α-tubulin (1:1000, Santa Cruz Biotechnology), Oct4 (1:1000, Santa Cruz Biotechnology), Nanog (1:1000, Novus Biologicals) or Tie2 (1:1000, Santa Cruz Biotechnology) were used. Horseradish peroxidase (HRP)-conjugated anti-mouse IgM (1:2500, Santa Cruz Biotechnology, for α-tubulin) or anti-rabbit IgG (1:2500, Santa Cruz Biotechnology for Oct4, Nanog and Tie2) antibodies were used as secondary antibodies. Amersham ECL Western blotting detection reagents (GE Healthcare Life Sciences) were used for detection.

Quantitative Real-time PCR

Total RNA was isolated using an RNeasy Mini Kit (Qiagen) and cDNA was synthesized using amfiRivert cDNA Synthesis Premix (GenDEPOT). Quantitative real-time PCR was performed using Power SYBR Green (Applied Biosystems) on a 7500 Real-Time PCR System (Applied Biosystems). Samples were normalized to 18S rRNA. The primer sequences are provided in Table I.

In vitro Fluorescence Staining

Cells were fixed with 1% paraformaldehyde for 10minutes at room temperature. After washing with Tris-buffered saline containing 0.05% Tween-20 (TBS-T) (Amresco) and blocking with PBS containing 1% BSA, cells were incubated with primary antibodies against goat VE-cadherin (1:100, Santa Cruz Biotechnology), goat CD31 (1:100, Santa Cruz Biotechnology) or TRITC-conjugated Bandeiraea simplicifolia (BS)1-lectin (1:20,000, Sigma-Aldrich). After overnight incubation at 4ºC, culture plates were washed with TBS-T and incubated with Alexa Fluor 555-conjugated donkey anti-goat IgG secondary antibody (1:100, Invitrogen, for VE-cadherin and CD31) for 1 hour at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured using a LSM 710 fluorescence microscope (Zeiss).

1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI)-acetylated-low dsity lipoprotein (Ac-LDL) Uptake

After incubation with 10 μg/mL DiI-Ac-LDL (Invitrogen) for 12 hours at 37ºC with 5% CO2, cells were stained with DAPI. To detect DiI-Ac-LDL uptake, cells were imaged by LSM 710 fluorescence microscopy.

Matrigel Tube Formation

Matrigel Matrix (BD Biosciences) was coated onto culture plates, incubated for 30 minutes at 37ºC, and 4×10^4 cells per well were seeded onto the plates. After incubation for 9 hours at 37ºC with 5% CO2, tube formation was analyzed using an IX71 microscope (Olympus).

Nitric oxide (NO) Production

3×10^4 cells were seeded onto each well of 1.5% gelatin-coated 12 well plates, and grown in EBM-2 supplemented with 10% FBS and penicillin/streptomycin. 2 ng/mL vascular endothelial growth factor (VEGF) (R&D Systems), 10μM Acetylcholine (Sigma Aldrich), 1μM Calcium Ionophore A23187 (Sigma Aldrich), or 100μM L-N^G-Nitroarginine methyl ester (L-NAME) (Sigma Aldrich) was added according to the experimental design. After overnight incubation at 37ºC with 5% CO2, culture supernatants

were harvested, and NO was assayed using a NO Detection Kit (Intron Biotechnology). The absorbance at 540 nm was detected using a VersaMax Microplate Reader (Molecular Devices) after 10 minutes of incubation at room temperature.

Apoptosis Assay

1×10^5 cells were seeded onto 1.5% gelatin-coated 60-mm culture dishes (Falcon). After overnight incubation in EBM-2 with 10% FBS, media were replaced with serum free EBM-2. After 24 hours, cells were harvested and stained using a PE AnnexinV Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's protocol. Samples were assayed by FACS Calibur (BD Biosciences). Data were analyzed using BD CellQuest Pro software (version 5.2.1).

Bisulfite Sequencing

Total DNA was isolated using a G-spin Total DNA Extraction Kit (Intron Biotechnology). An EpiTect Bisulfite Kit (Qiagen) was used for bisulfate treatment. RT-PCR was performed using the primers shown in Table I in the online-only Data Supplement. PCR products were cloned into the pCR™2.1 vector (Invitrogen), and randomly selected clones were sequenced using M13 forward and reverse primers.

Microarrays

Mouse genome-wide gene expression analyses were performed using an Affymetrix GeneChip Mouse Gene 1.0 ST Array. Total RNA was extracted using an RNeasy Mini Kit (Qiagen). Microarray analyses were performed in biological duplicate or triplicate, in accordance with the standard Affymetrix GeneChip protocol. Data were analyzed using GeneSpring GX 7.3 (Agilent). Differential gene expression was determined using a combination of statistics ($P<0.05$) and an expression threshold (2-fold). Genes that were differentially expressed between SFBs and primary ECs from Tie2-GFP mouse lungs, or between SFBs and MS1 cells, were selected for analyses. Unsupervised hierarchical clustering was performed using Pearson's correlation. Microarray data have been uploaded to the Gene Expression Omnibus database under the accession number GSE50071.

Murine Model of Hindlimb Ischemia

Target cells were transduced with lentiviral-GFP. 2 days later, GFP-labeled cells were harvested, and 5×10^5 cells were resuspended in 50μL of PBS. 8 week-old male athymic nude mice were anesthetized, and their left femoral arteries were surgically ligated. PBS or cells of interest were injected into 2 sites on the left thighs. Each mouse was then analyzed by serial recording of hindlimb surface blood flow using LDPI (Moor Instruments) on

post-operative days 0, 3, 7, and 14. On day 14, TRITC-conjugated BS1 lectin was injected into the left ventricle. 30 minutes later, mice were euthanized, and perfusion fixation was performed with 4% formalin. After overnight fixing by immersion in 10% formalin, thigh muscles were harvested and embedded in paraffin wax, and multiple 10-μm thick slices were prepared. For analysis of capillary density, tissue sections were stained with DAPI. For detection of implanted cells, tissue sections were stained with Alexa Fluor 488-conjugated rabbit anti-GFP (1:200; Invitrogen) antibody and DAPI. Stained slides were examined using a LSM 710 fluorescence microscope (Zeiss). For the whole cell lysis assays, thigh muscles were harvested and minced with scissors. Samples were incubated with pre-warmed digestion buffer consisting of DMEM, 0.5% collagenaseII (Gibco) at 37ºC with 5% CO2for 30 minutes. Next, DMEM containing 10% FBS was added to the buffer. After filtration through a 40-μm cell strainer, cells were washed twice with PBS, and used for flow cytometric analyses.

Statistics

All data are presented as mean±standard error. Mann-Whitney's U test or Kruskal Wallis test was performed for intergroup comparisons. Dunn's post hoc method was used for the adjustment for multiple comparisons. For the samples which are normally distributed, Student's t-test or one-way analysis of

variance (ANOVA) with post hoc analysis was performed for intergroup comparisons. Repeated-measures ANOVA was used to analyze the serial follow-up data from the murine model of hindlimb ischemia. SPSS version 19.0 (IBM) was used for analysis, and $P < 0.05$ was considered to be statistically significant.

Results

Identification of 5 Key Factors for the Direct Endothelial Reprogramming of Adult Fibroblasts.

We selected 11 genes that are thought to be key regulators of endothelial development (Table Ⅱ).[11] Those were genes that are involved in hemangioblast development from the mesoderm (Gata2, Fli1, Tal1),[12-14] Ets family members as central regulators of endothelial development (Elf1, Erg, Fli1, Ets1, Er71),[15, 16] essential co-workers of endothelial transcription (Foxc2, Foxo1, Lmo2)[17-19] and a gene that regulates endothelial functions (Klf2).[20, 21] We made lentiviruses expressing each gene individually. We prepared SFBs from adult Tie2 promoter-driven GFP (Tie2-GFP) expressing transgenic mice (Figure 1A). We infected all 11 factors into GFP^- SFBs (Figure 1B), and observed the development of a small percentage of GFP^+ cells (0.9%) after 12 days, demonstrating the feasibility of endothelial-enriched Tie2 gene activation by defined factors in adult fibroblasts (Figure 1C). To determine which factors are essential for endothelial gene activation, we performed a series of single factor transduction experiments. Interestingly, only Foxo1 or Er71 transduction could generate Tie2-GFP^+ cells (Figure 1D). The combination of Foxo1 and Er71 increased the percentage of GFP^+ cells to 0.6%. When additional individual

factors were Tie2 gene activation (Figure 1E). Following rounds of single factor addition experiments were performed, revealing that Tal1 and Lmo2 as the fourth and fifth factors, respectively, further improved the efficiency (Figure 1E). The addition of any other single factor to these 5 factors (5F: Foxo1, Er71, Klf2, Tal1 and Lmo2) decreased the percentage of GFP^+ cells (Figure 1E). In addition, the elimination of any single factor from 5F reduced the percentage (Figure 1E). These data suggest that 5F are effective and necessary for the activation of the endothelial program in SFBs. Overexpression of these 5F combination induced Tie2 promoter activation in 4.0% of SFBs (Figure 2A), and derived the cells with an endothelial-like cobblestone appearance from adult SFBs (Figure 2B).

Universal and Unique Effects of 5 Key Factors for Direct Endothelial Reprogramming

Next, we sought to determine if the direct reprogramming of adult skin fibroblasts into ECs by 5F overexpression could be translated to other system. We therefore transduced 5F to TTFs isolated from Tie2-GFP mice (Figure 3A), and successfully generated endothelial-like cells with a cobblestone appearance (Figure 3B). Flow cytometry showed that 5.4% of TTFs exhibited active Tie2 promoters, demonstrating that two different

sources of fibroblasts could be reprogrammed into ECs by 5F (Figure 3C).

While we were performing our study, Ginsberg and his colleagues reported that human amniotic cells from midgestation fetuses could be converted into ECs by the overexpression of 3 ETS members - ER71 (also known as ETV2), ERG and FLI1 - and the suppression of TGFβ[22]. The authors focused only on ETS family, and selected the 3 members out of more than 20 ETS family paralogs,[16] which were abundantly expressed during endothelial differentiation of human ES cells. Nevertheless, they tried to reprogram various adult and fetal non-vascular cells into ECs, but found that only amniotic cells could be successfully converted. Since many factors in addition to the ETS family are involved in endothelial development,[11] this is not surprising. In our study, we tested whether the combination of Er71, Erg and Fli1 could activate the endothelial program in differentiated mouse adult fibroblasts. However, we did not observe any clear morphologic changes to a cobblestone appearance after the transduction of Er71, Erg and Fli into SFBs from Tie2-GFP mice (Figure 3D). Only 0.3% of infected SFBs were transformed into Tie2-GFP^{+} cells (Figure 3E), which was no improvement over Er71 single transduction (Figure 1D). This suggests that those 3 ETS factors are not able to convert adult differentiated fibroblasts into ECs. Moreover, Erg or Fli1

significantly decreased the conversion rate when they were included in the infection combinations used in our study (Figure 1E).

To test whether pluripotency was induced during endothelial repgrogramming of adult fibroblasts, we transduced 5F into SFBs from Oct4 or Nanog promoter-driven GFP- expressing transgenic mice. Although Tie2 expression was successfully induced, Oct4- or Nanog- GFP was not detected, demonstrating that the conversion into ECs was accomplished not through the induction of pluripotency, but through the direct reprogramming (Figure 4A-4D). Western blot analysis confirmed this finding (Figure 4E).

In Vitro Endothelial Characterization of iECs

Tie2-GFP^{+} cells were isolated after 5F transduction using FACS. We designated these cells as iECs (Figure 5A), and characterized their endothelial-like features. The mRNA expression of endothelial-specific genes such as CD31, VE-cadherin, ICAM2 and Tie2 were significantly upregulated in iECs (Figure 5B). Primary ECs isolated from the lungs of Tie2-GFP mice and MS1 EC line were used as positive controls. Although iECs expressed ICAM2 at much lower levels than MS1, the expression was comparable to that detected in

primary ECs from the mouse lung. Immunofluorescence staining revealed that iECs also expressed VE-cadherin and CD31 in protein level (Figure 5C). In addition, iECs displayed endothelial characteristics, such as BS1 lectin binding, and uptake of Ac-LDL (Figure 5C and 5D). iECs, primary ECs and MS1 all formed capillary tubes on Matrigel (Figure 5E). Nitric oxide (NO), which is an important regulator of vascular functions, was produced by iECs at levels comparable with those in primary ECs or MS1 (Figure 5F). The response of iECs to NO inducers such as VEGF, acetylcholine, and A23187, and to NO inhibitor such as L-NAME, was similar to that of primary ECs and MS1 cells.

To examine whether iECs acquired endothelial-like epigenetic reprogramming, their DNA methylation status was analyzed. Bisulfite genomic sequencing demonstrated that cytosine guanine dinucleotides (CpG) in the promoter regions of VE-cadherin and Tie2 were demethylated in iECs, consistent with primary ECs or MS1, whereas they were hypermethylated in mock-infected SFBs (Figure 6A). When the global gene expression profiles were compared, the patterns of iECs were clustered with primary ECs from the mouse lung and MS1, but were distinct from original SFBs or mock-infected SFBs (Figure 6B). In particular, endothelial specific genes were highly enriched in iECs, primary ECs and MS1 (Figure 6C). Further, the gene

ontology analyses showed that iEC mimicked the gene expression patterns of primary ECs or MS1 regarding a set of genes for endothelium development (GO003158), although some differences were also observed (Figure 6D).

In Vivo Viability and Functionality of iECs in a Murine Model of Hindlimb Ischemia

The *in vivo* potential of iECs was then examined using a hindlimb ischemia model. Mock-infected SFBs, iECs, primary ECs from mouse lungs and MS1 cells were infected with GFP-lentivirus in advance for tracing purposes. After left femoral arteries were surgically ligated in athymic nude mice, the cells of interest were intramuscularly injected to the ischemic limbs. Like primary ECs or MS1, implantation of iECs enhanced the recovery of blood flow to ischemic limbs, saving the limbs (Figure 7A and 7B). Histology showed a higher capillary density in the ischemic limbs after transplantation of iECs compared with mock-SFBs (Figure 7C and 7D). The transplanted iECs that were double positive for GFP and BS1 lectin were found at the injection site, demonstrating *in vivo* viability and functionality of iECs (Figure 8).

Discussion

This is the first study demonstrating that adult fibroblasts can be directly converted to ECs by defined factors. Our study provides further evidence that differentiation during embryonic development is not an irreversible process, and that cell fate can not only go back to the primitive and pluripotent stage,[23, 24] but can also cross to other lineages by building new transcriptional networks.[6-10] We identified iEC 5 factors consisting of Foxo1, Er71, Klf2, Tal1 and Lmo2. Foxo1 plays a crucial role in vascular development, and its disruption causes embryonic lethality.[18] Er71 is an ETS family member that regulates early EC development at the top of the transcriptional network.[11] Consequently, knockout of Er71 impairs vasculogenesis and results in embryonic lethality, in contrast to other ETS genes, highlighting its central role in endothelial specification.[11, 25] Remarkably, Foxo1 and Er71 were the only 2 factors that could individually activate the Tie2 promoter in SFBs (Figure 1D). Klf2 is expressed in response to shear stress, and functions as a key "molecular switch" that regulates endothelial functions after initial specification during endothelial development.[11, 20, 21] Tal1 is implicated in the early development of endothelial and blood cells, and is indispensible for normal vascular maturation.[26, 27] Of note, Lmo2 is a transcriptional co-factor that is co-expressed

and associated with Tal1, which constitutes a multi-factorial complex[11] that induces the expression of endothelial markers.[19] Interestingly, the combination of Tal1 and Lmo2 induces hemangioblast formation, and determines its fate to blood- or endothelial-lineage, depending on the presence of blood-inducing factors like Gata1.[19] In the absence of Gata1, Tal1 and Lmo2 induce hemangioblasts to subsequently differentiate into ECs.[19] Taken together, these data suggest that our 5 factors are purposive for endothelial reprogramming.

The direct conversion of adult cells into ECs can provide new therapeutic modalities to overcome the hurdles associated with the clinical application of ESC- or iPSC-derived ECs. In this regard, the conversion of amniotic cells to ECs by Ginsberg and his colleagues[28] has limited clinical relevance, since amniotic cells are not readily available, and because this approach still has critical limitations of ESC-derived ECs such as immunogenicity and possible allograft rejection. Moreover, amniotic cells isolated from amniotic fluid of midgestation human fetuses should be distinct from fully differentiated mature cells, although c-Kit^{+} amniotic cells were excluded for their experiments. Accordingly, the direct conversion of terminally differentiated cells into ECs was not demonstrated by their study.

Recently, some researchers reported that the transduction of a whole or some of Yamanaka's 4 pluripotency factors (OCT4, SOX2, KLF4 and c-MYC)[23, 24] could convert human fibroblasts into ECs without inducing pluripotent status.[29-31] However, because the conversion by these techniques passed through the generation of intermediate progenitor cells or partial iPSCs, these are essentially different from our direct conversion technology. Moreover, because these studies utilized the pluripotent factors, concerns still remain over tumorigenic potential.

Further studies are warranted to develop iEC technology for clinical applications. For increased safety, non-integrative tools for 5F overexpression, such as adenoviruses and plasmid vectors, should be developed. Direct *in vivo* reprogramming using iEC 5F possesses great potential, because fibroblasts comprise a large proportion (40-60%) of the cellular population of the heart which is the major therapeutic target organ of angiogenesis.[32] The validation of this technique in humans will provide substantial benefits. The mechanism for inducing direct conversion into ECs remains a key question to be addressed in future. Answering this question will facilitate understanding the underlying molecular biology, and may optimize the technique, enhancing the conversion efficiency.

The ability to directly convert adult fibroblasts to ECs, which was first demonstrated by our study, therefore sheds new insights on endothelial differentiation and reprogramming, and makes significant progress towards future clinical application.

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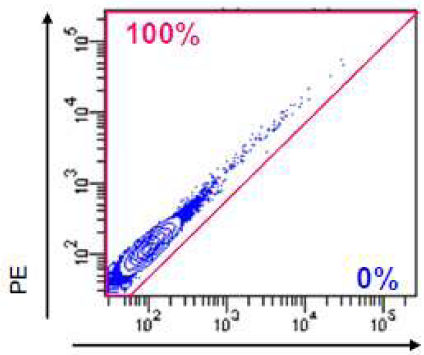
Figures

A.



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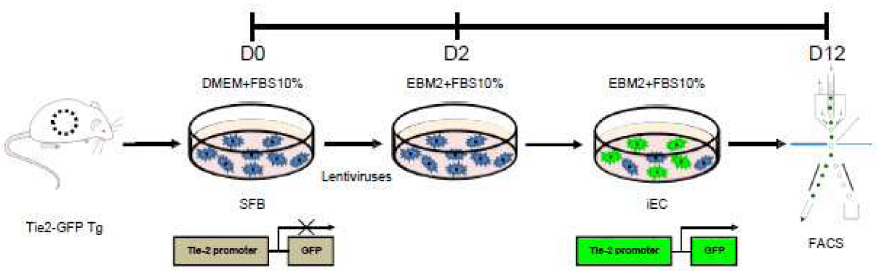
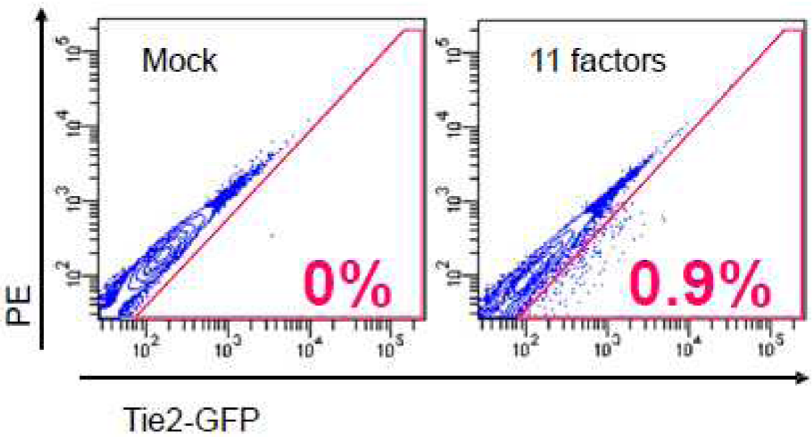


Figure 1. Identification of 5 key factors for the endothelial reprogramming of adult skin fibroblasts (SFBs). (A) SFBs were prepared from Tie2-GFP mice (8 weeks old). Before lentiviral transduction, SFBs were analyzed using flow cytometry. Nearly 100% of cells did not express GFP, showing that endothelial cells (ECs) were not contaminated. To exclude negligible possibility of EC contamination, GFP- cells were isolated by fluorescence-activated cell sorting (FACS), and used for subsequent experiments. (B) Experimental design to determine endothelial reprogramming factors.

C.


Mock
11 factors
0%
0.9%
PE
Tie2-GFP

D.

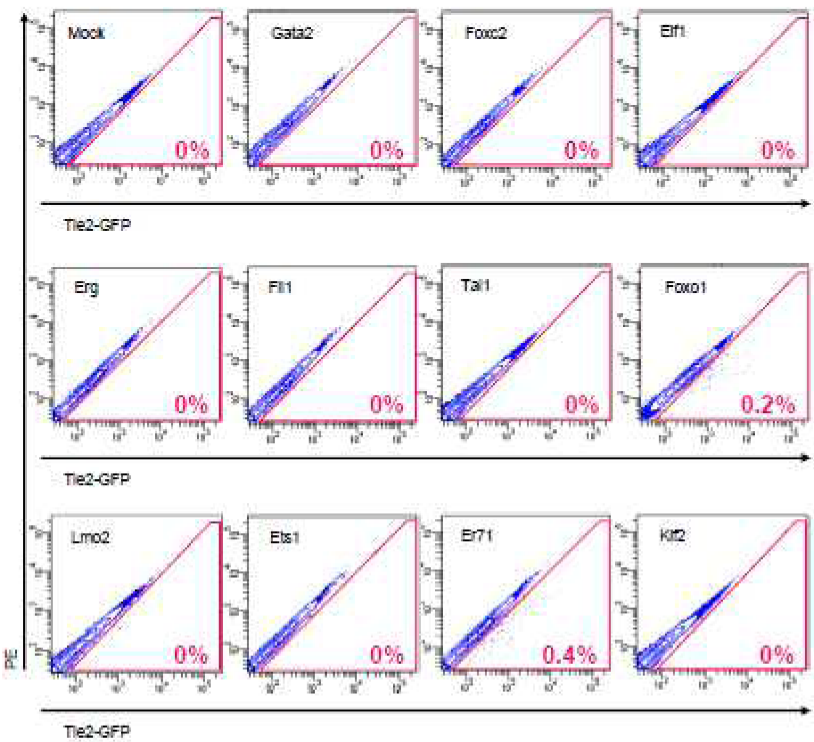

Mock
Gata2
Foxc2
Elf1
Erg
Fli1
Tal1
Foxo1
Lmo2
Ets1
Er71
Klf2
0%
0.2%
0.4%
PE
Tie2-GFP

Figure 1. Identification of 5 key factors for the endothelial reprogramming of adult skin fibroblasts (SFBs). (continued) (C) The transduction of 11 factors together induced Tie2-GFP+ cells. (D) Single factor transduction test to determine essential factors. Only Foxo1 or Er71 generated the significant numbers of Tie2-GFP+ cells after infection of the individual factors. n=3-10

E.

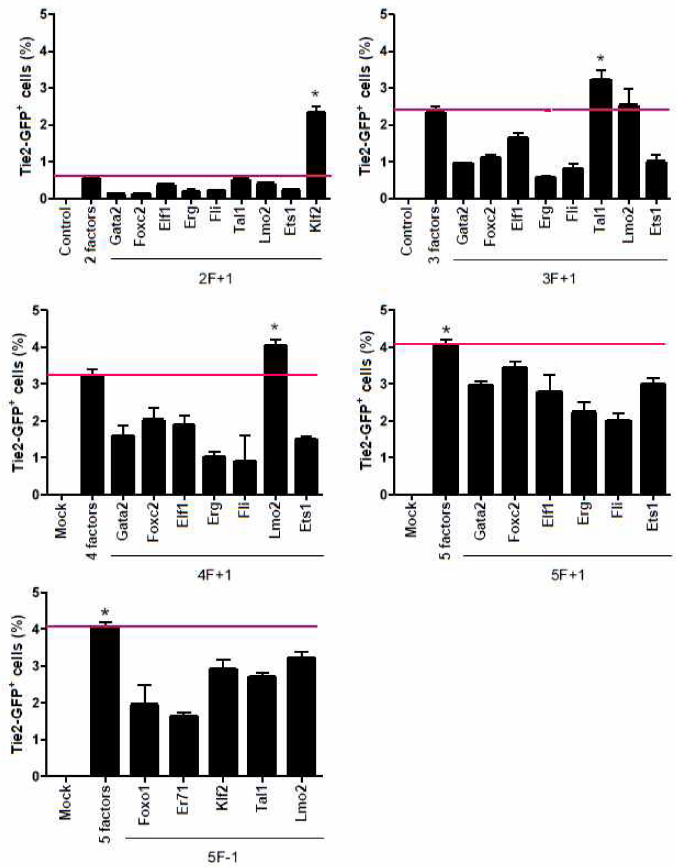
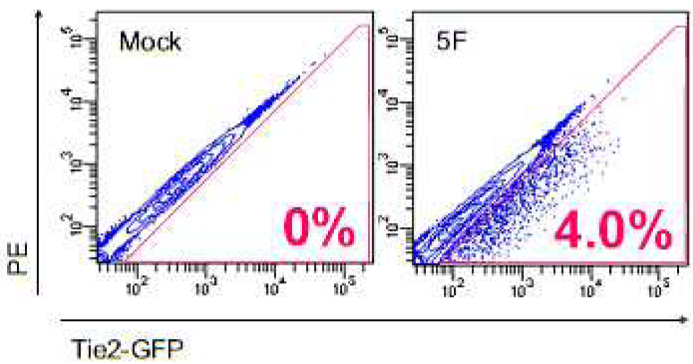


Figure 1. Identification of 5 key factors for the endothelial reprogramming of adult skin fibroblasts (SFBs). (continued) (E) Selection of key factors by sequential stepwise addition of individual factors. Factors that enhanced the efficiency of Tie2-GFP activation were included in the next transduction combination. Transduction tests to confirm 5 key factors. Any single factor addition or elimination decreased the efficiency of

GFP+ cell conversion by iEC 5F, demonstrating that 5F were the ideal combination for endothelial reprogramming.

A.



B.

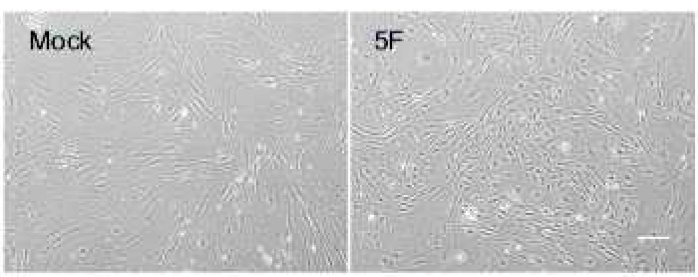
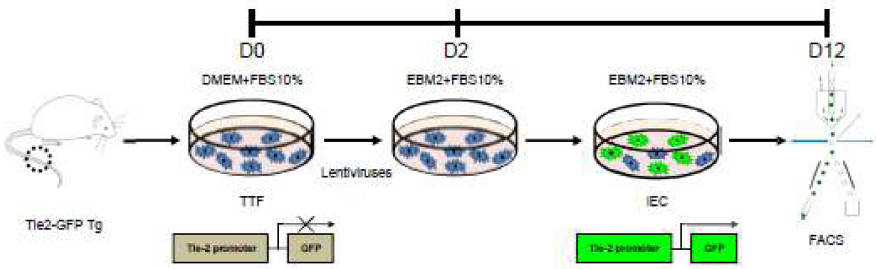
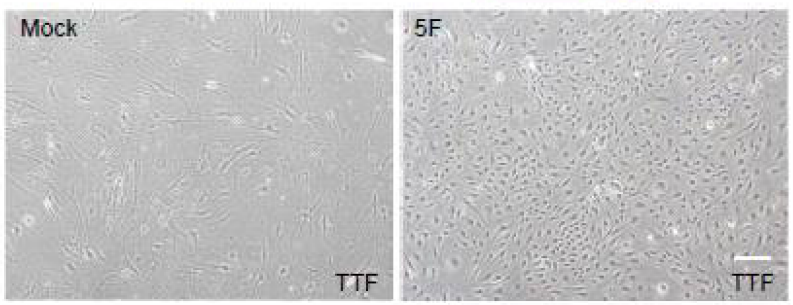


Figure 2. Conversion of adult skin fibroblasts into endothelial cells by 5 factors. (A) The final 5 factors (5F : Foxo1, Er71, Klf2, Tal1 and Lmo2) converted 4.0% of SFBs into Tie2-GFP+ cells. n=11. (B) Transduction with 5F induced endothelial-like cells with a cobblestone appearance. Scale bar: 200μm.

A.



B..



C.

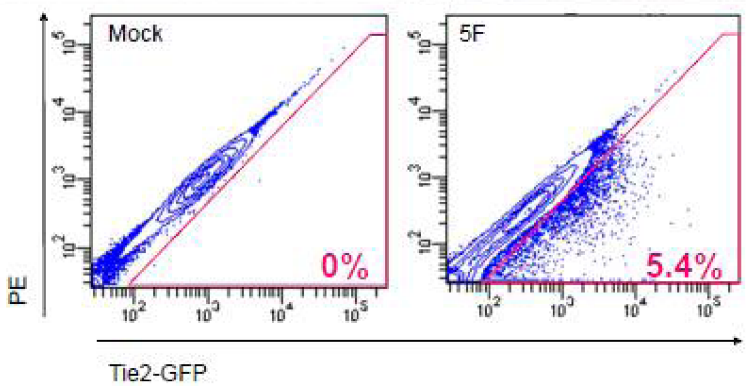
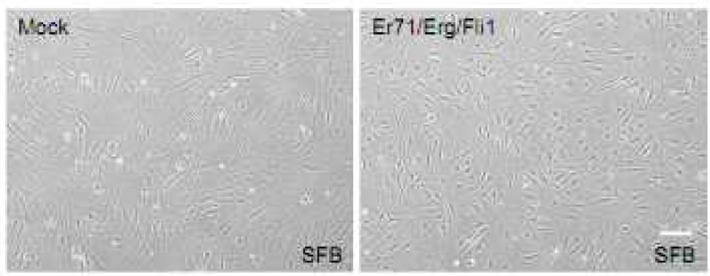


Figure 3. Universal and unique effects of 5 factors for the endothelial reprogramming of adult fibroblasts. (A) Endothelial reprogramming of tail-tip fibroblasts (TTFs). (B) 5F-transduced TTFs showed morphologic changes to an endothelial-like cobblestone appearance. Scale bar: 200μm. (C) 5F transduction resulted in the successful activation of endothelial-enriched Tie2 promoter in TTFs.

D.



E.

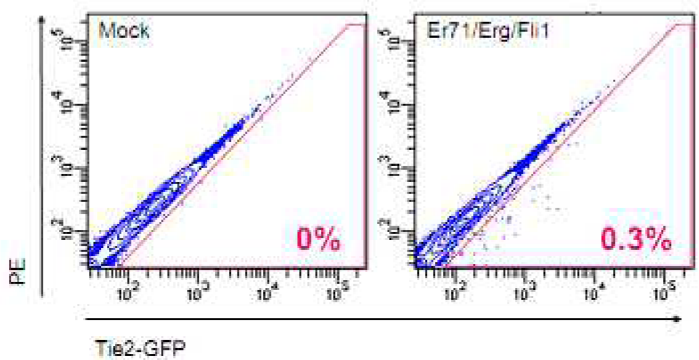
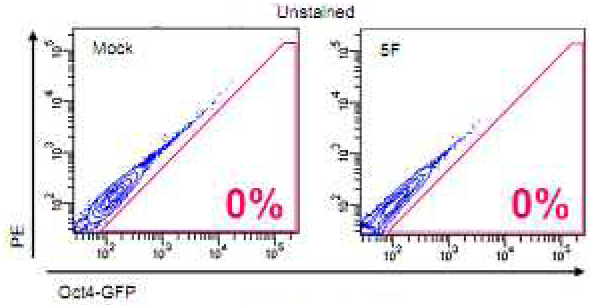
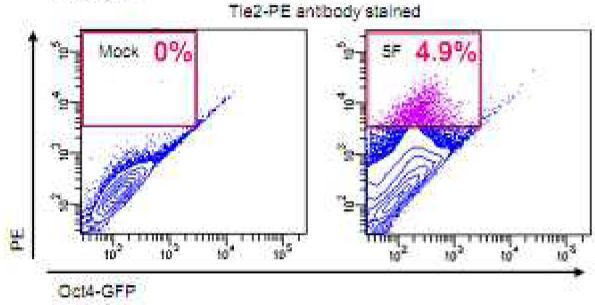


Figure 3. Universal and unique effects of 5 factors for the endothelial reprogramming of adult fibroblasts. (continued) (D) Overexpression of Er71, Erg, and Fli1 did not induce morphologic change to a cobblestone appearance in SFBs. Scale bar: 200μm. (E) Tie2-GFP+ cells were not efficiently induced after those 3 factors transduction.

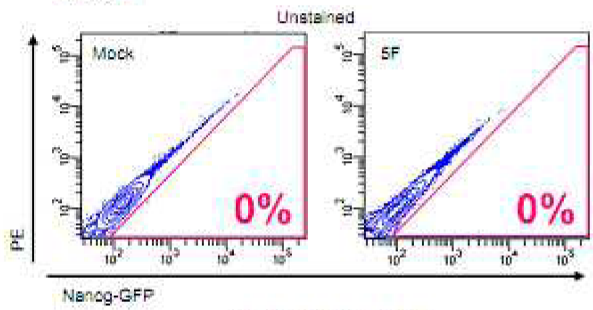
A.


Unstained
Mock
0%
5F
0%
PE
Oct4-GFP

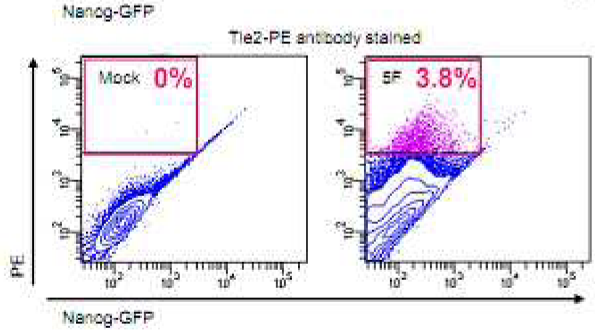
B.


Tie2-PE antibody stained
Mock
0%
5F
4.9%
PE
Oct4-GFP

C.


Unstained
Mock
0%
5F
0%
PE
Nanog-GFP

D.


Tie2-PE antibody stained
Mock
0%
5F
3.8%
PE
Nanog-GFP

E.

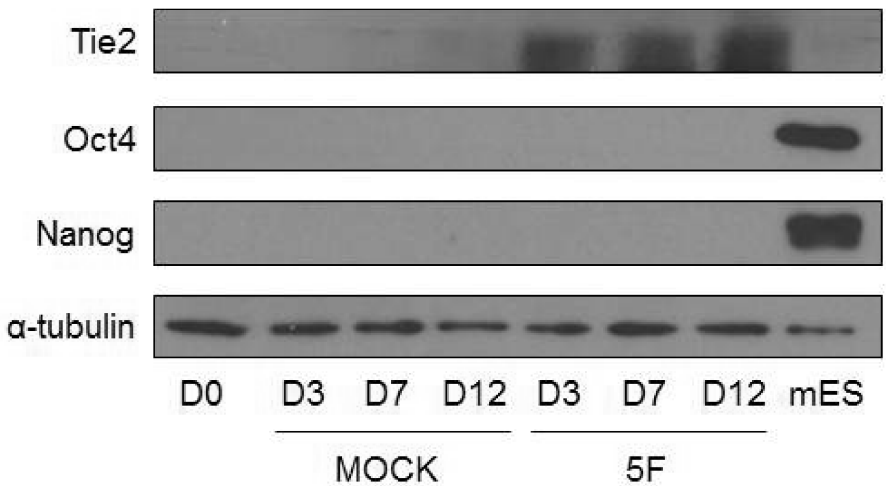
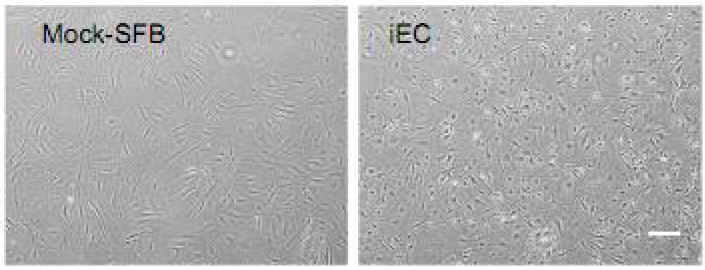


Figure 4. Direct conversion of adult fibroblasts into endothelial cells not involving pluripotency induction. (A to B) 5F transduction did not induce Oct4-GFP+ cells, but successfully activated Tie2 promoter in SFBs from Oct4-GFP mice. (C to D) 5F transduction did not induce Nanog-GFP+ cells as well, but successfully activated Tie2 promoter in SFBs from Nanog-GFP mice. (E) Western blot analysis showed no activation of either Oct4 or Nanog at the protein level after 5F transduction. Mouse embryonic stem cells (mES) were used as positive controls for Oct4, and Nanog.

A.



B.

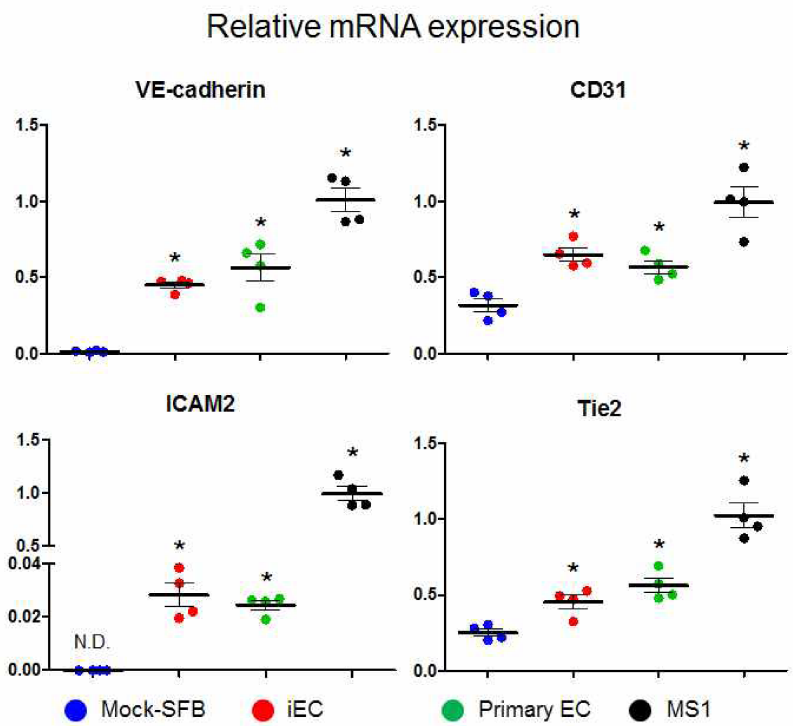
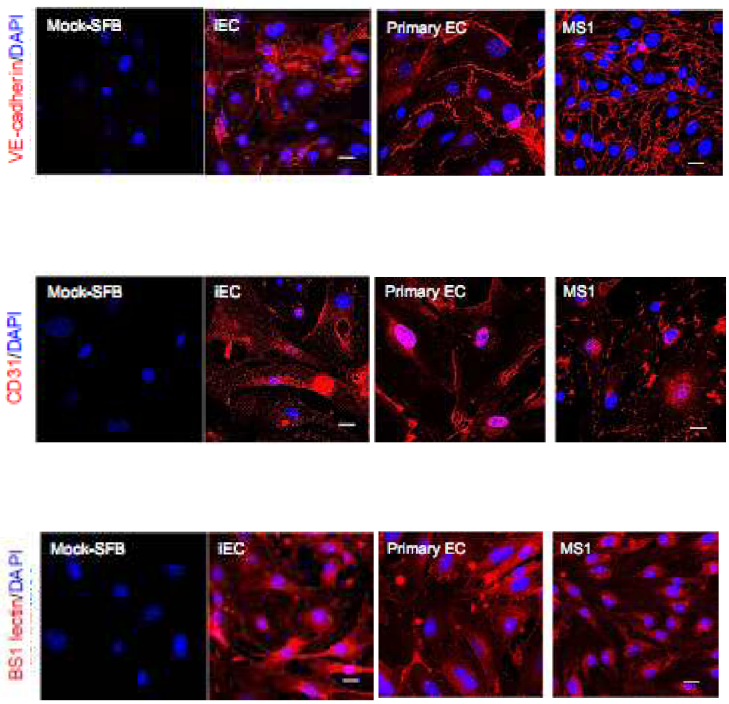


Figure 5. Characterization of iECs. (A) After sorting by FACS, cultured iECs showed a cobblestone appearance, in contrast to mock-infected SFBs (Mock-SFB). Scale bar: 200μm. (B) mRNA expression of endothelial markers was analyzed by real-time PCR.

C.



D.

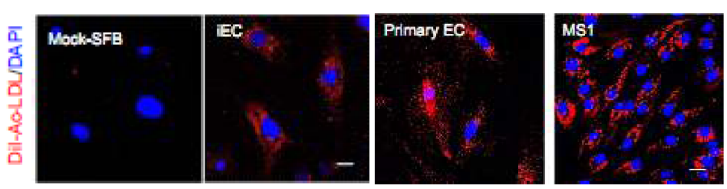


Figure 5. Characterization of iECs. (continued) (C) Fluorescence staining of VE-cadherin, CD31 and BS1 lectin confirmed the endothelial features of iECs. (D) DiI-Ac-LDL uptake by iECs. Scale bars: 20μm.

E.

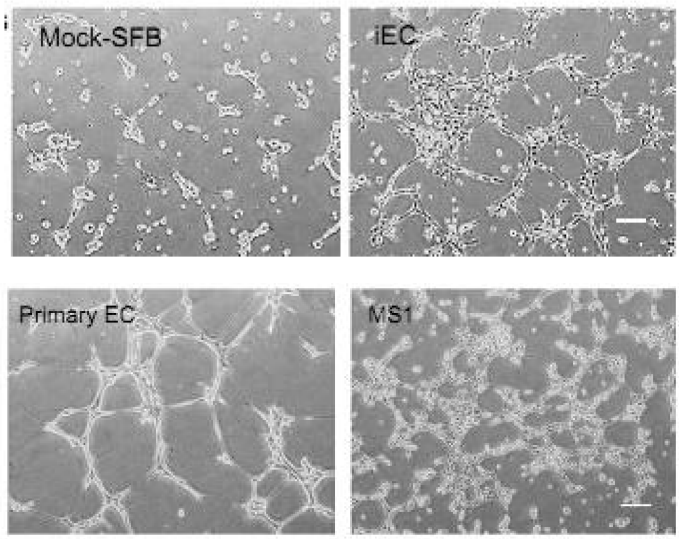


Figure 5. Characterization of iECs. (continued) (E) Capillary tube formation on Matrigel by iECs. Scale bar: 200μm.

F.

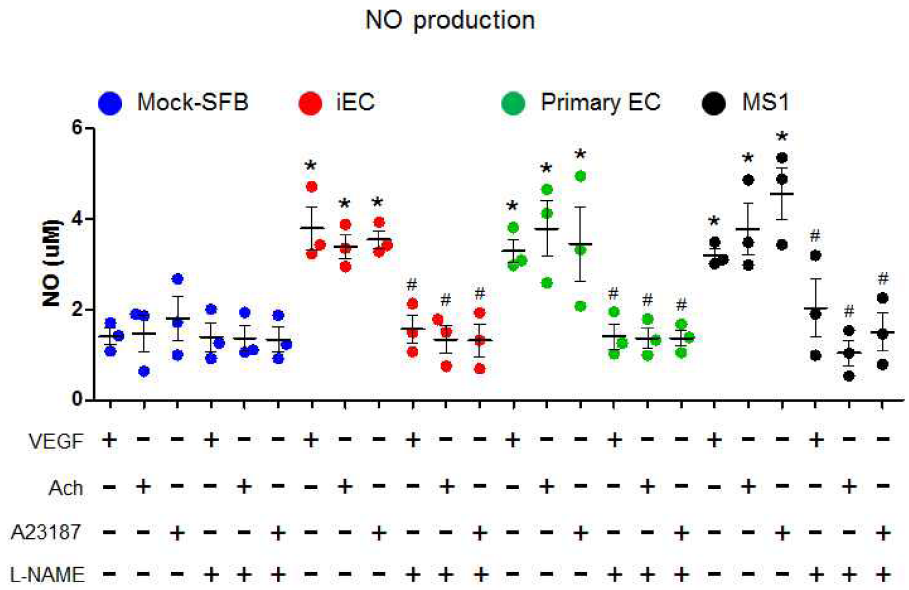


Figure 5. Characterization of iECs. (continued) (F) Significantly higher NO production by iECs compared with mock-SFB control. iECs responded like regular ECs to NO inducers and an inhibitor. Ach denotes acetylcholine.

A.

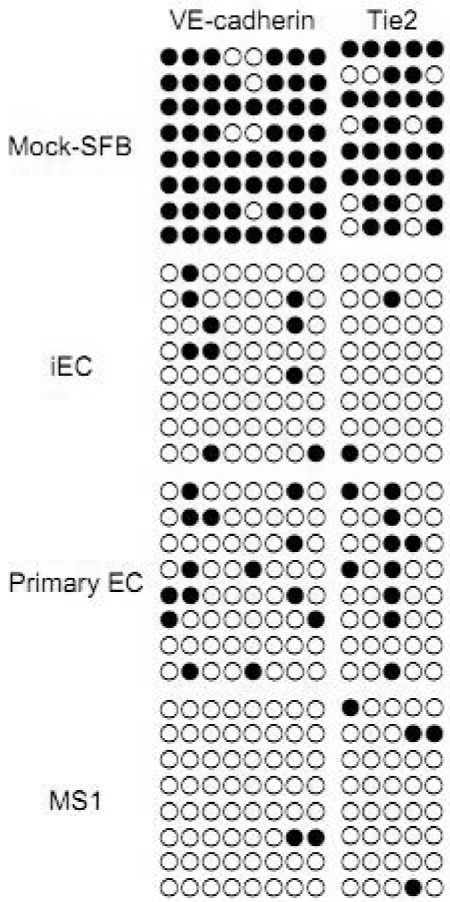


Figure 6. Epigenetic and genetic profiles of iECs. (A) DNA methylation status of the promoter regions was assessed with bisulfite genomic sequencing for VE-cadherin and Tie2 in mock-SFBs, iECs, primary ECs from mouse lungs, and MS1 cells. Open circles represent unmethylated CpG dinucleotides, and closed circles are methylated CpGs.

B.

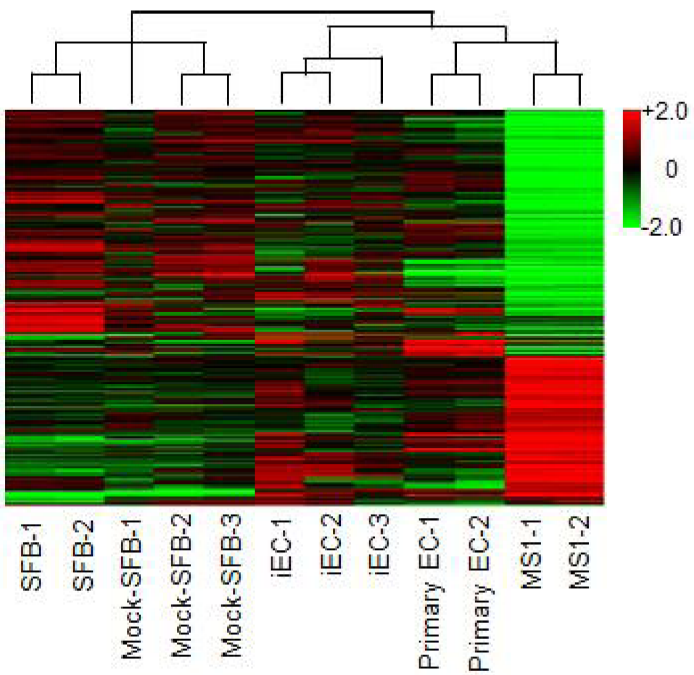


Figure 6. Epigenetic and genetic profiles of iECs. (continued) (B) Global gene expression was analyzed using cDNA microarrays.

C. D.

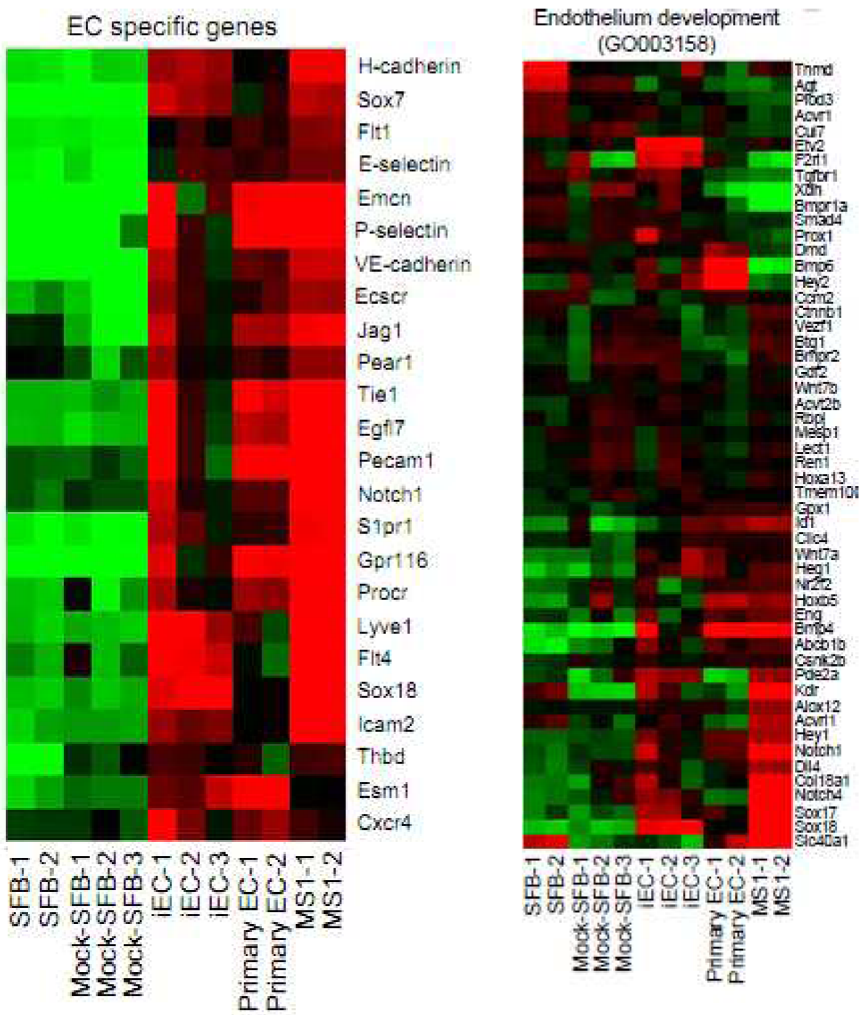


Figure 6. Epigenetic and genetic profiles of iECs. (continued) (C) Endothelial genes were highly enriched in iECs, primary ECs from mouse lungs and MS1 cells but not in original SFBs or mock-SFBs. (D) Gene ontology analysis for endothelium development showed similar expression profiles for iECs, primary ECs from mouse lungs and MS1 cells.

A.

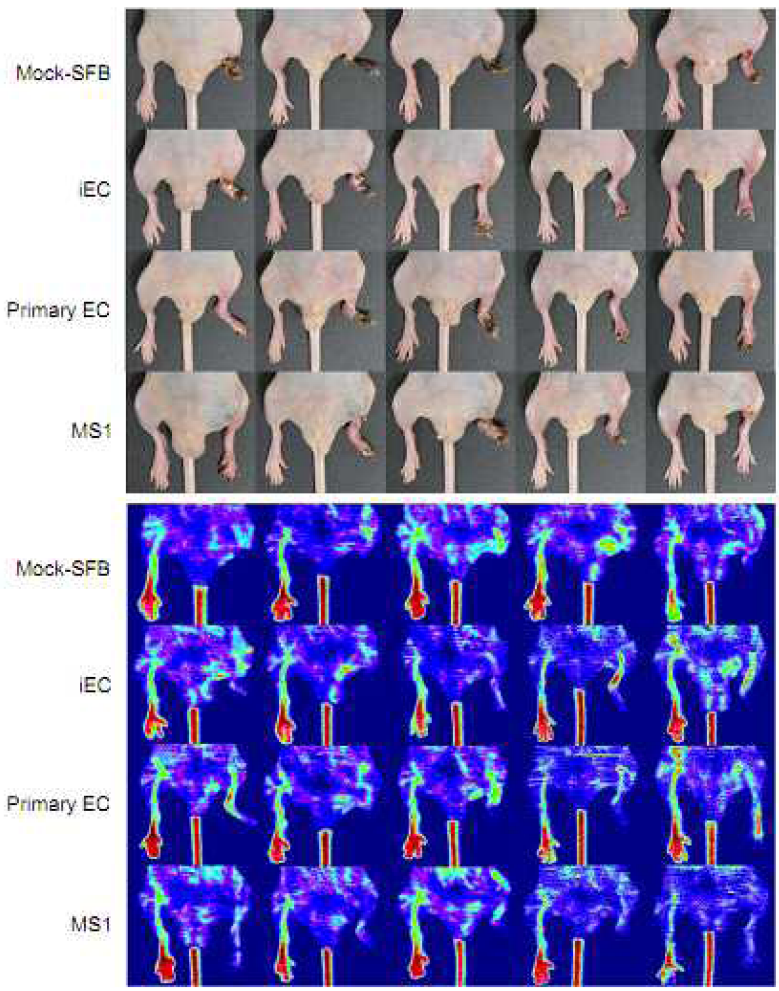
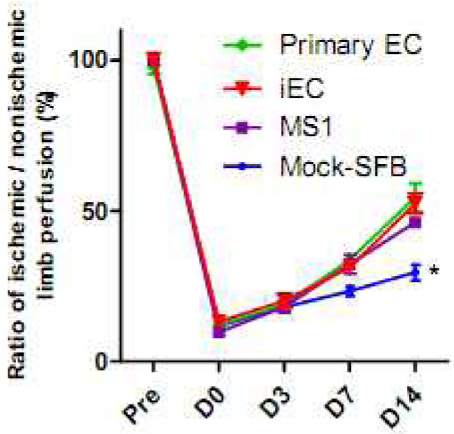


Figure 7. Enhanced limb perfusion and salvage by implanted iECs in a mouse hindlimb ischemia model. (A) Photographs demonstrating limb salvage by intramuscular injection of iECs. Laser Doppler perfusion imaging (LDPI) showing enhanced perfusion recovery after implantation of iECs.

B.



C.

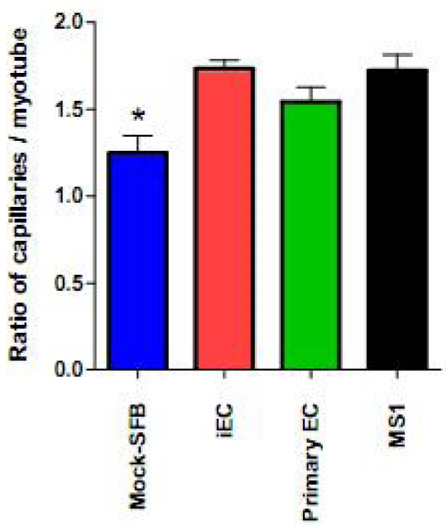


Figure 7. Enhanced limb perfusion and salvage by implanted iECs in a mouse hindlimb ischemia model. (continued) (B) Quantification of LDPI. (C) Quantification of capillary density.

D.

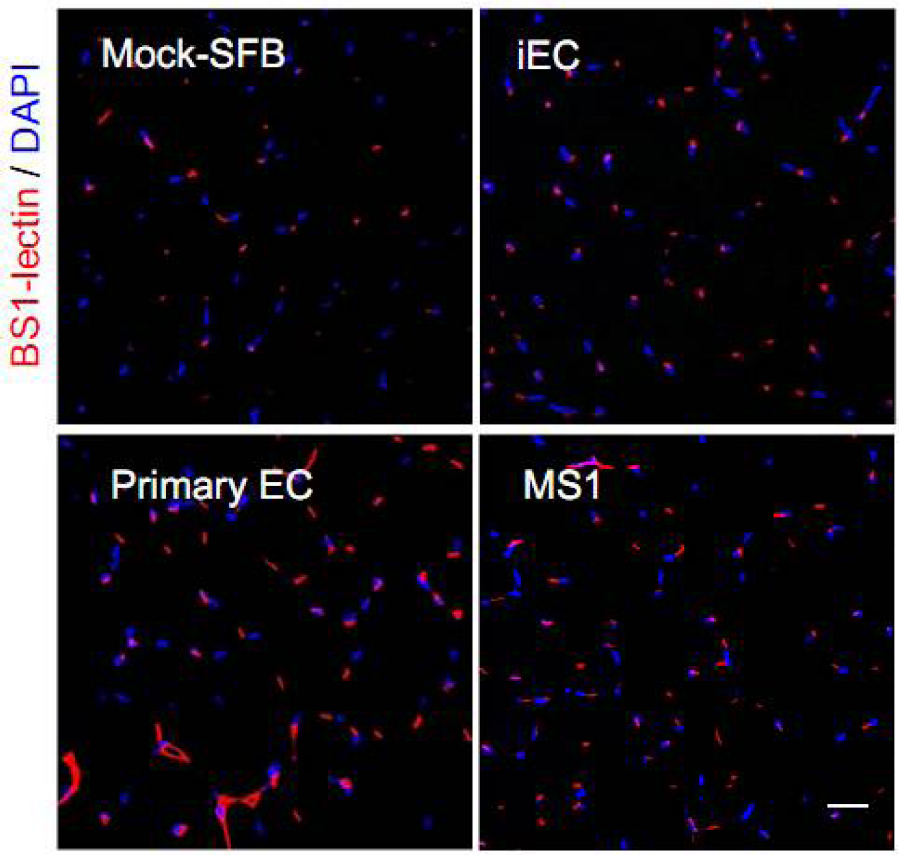


Figure 7. Enhanced limb perfusion and salvage by implanted iECs in a mouse hindlimb ischemia model. (continued) (D) Histological examination of ischemic limbs harvested 14 days after surgery showed a higher capillary density in iEC-implanted mice. Scale bar: 20μm.

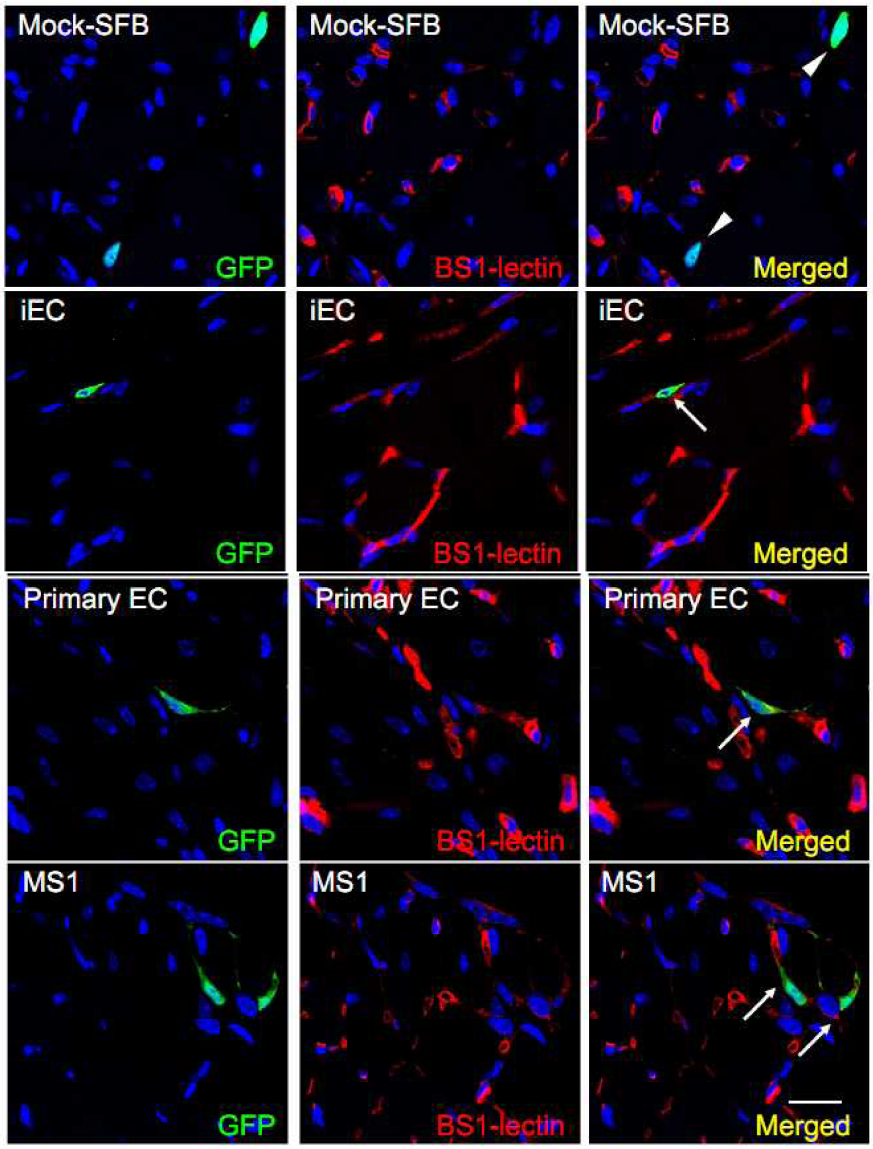


Figure 8. *In vivo* viability and functionality of iECs. In the ischemic hindlimbs of mice implanted with GFP-labeled mock-SFBs, GFP+ cells were not co-localized with BS1

lectin (arrowheads). However, in those implanted with GFP- labeled iECs, GFP+ iECs with BS1 lectin staining (arrow) were incorporated into the vessels, demonstrating *in vivo* viability and functionality of iECs. GFP-labeled primary ECs or MS1 cells were also co-localized with BS1 lectin (arrow). n=5 in each group. Scale bar: 20μm.

Tables

Table Ⅰ. Primers used for quantitative real-time PCR and methylation analysis.

Gene symbol	Accession No.	Primer Sequences	sense/antisense	usage
CD31	NM_008452	gcccaatcacgtttcagttt	sense	real-time
		aaaacgcttgggtgtcattc	antisense	real-time
VE-cadherin	NM_009868	atcgccaaaagagagactgg	sense	real-time
		gcaaactctccttggagcac	antisense	real-time
ICAM2	NM_010494	catcctcaagggaagtggaa	sense	real-time
		acttgagctggaggctggta	antisense	real-time
Tie2	NM_013690	cctcctcaaccagaaaaca	sense	real-time
		ttgccctgaaccttataccg	antisense	real-time
18S rRNA	NR_003278	gtaaccgttgaaccccatt	sense	real-time
		ccatccaatcggtagtagcg	antisense	real-time
VE-cadherin	NM_009868	ttgaggtatgagttgaatatttta	sense	methylation
		ttatatccttacacactaaaaaatata	antisense	methylation
Tie2	NM_013690	ttgtaaaggaaataggaaaaaggaat	sense	methylation
		tccacaaataaacataaatccctaaa	antisense	methylation

Table Ⅱ. Candidate factors screened for direct endothelial conversion of SFBs.

Gene symbol	Accession Number
Gata2	NM_008090
Foxc2	NM_013519
Elf1	NM_007920
Erg	NM_133659
Fli1	NM_008026
Tal1	NM_011527
Foxo1	NM_019739
Lmo2	BC057880
Ets1	BC010588
Er71	NM_007959
Klf2	NM_008452

국문초록

배경- 혈관내피세포를 증가시키기 위한 세포 기반의 치료들은 좋은 치료 방법으로 자리잡아가고 있다. 여기서, 우리는 성체 피부섬유아세포에서 직접적으로 기능성 혈관내피세포를 생성하는 새로운 접근 방식을 연구하였다.

방법과 결과- 혈관내피 발달의 핵심이 되는 11개의 후보 유전자들이 선정되었다. GFP^- 피부섬유아세포는 Tie2-GFP 생쥐에서 얻어졌고, 여기에 렌티바이러스를 이용하여 모든 11가지 인자를 동시에 과발현시켰다. Tie2의 유전자 활성을 나타내는 $Tie2\text{-}GFP^+$ 세포가 유세포 분석에 의해 0.9%로 나타났다. 일련의 단계별 검사는 5가지 주요 인자들 (Foxo1, Er71, Klf2, Tal1 및 LMO2) 을 밝혀내었고, 이것은 피부섬유아세포가 $Tie2\text{-}GFP^+$ 세포로 4%까지 효율적으로 reprogramming 되는 것을 보여주었다. 5가지 인자들은 꼬리 끝 피부섬유아세포를 가지고도 $Tie2\text{-}GFP^+$ 세포로 변환 시킬 수 있었고, 이것은 그들의 보편적인 효과를 보여주었다. 5가지 인자 형질도입 후, 이 reprogamming 전략이 만능 줄기세포 유도를 포함하지 않는다는 것을 증명하기위해 Oct4 또는 Nanog가 발현되지 않는다는 것을 보여주었다. $Tie2\text{-}GFP^+$ 세포는 FACS를 이용하여 분리하였고, 유도된 혈관내피세포 (iECs) 로 명명되었다. iECs는 혈관내피세포와 같은 조약돌 형태를 나타내었고, 혈관내피세포의 분자 마커를 보여주었다. iECs는 BS1 lectin 결합, acLDL 흡수와 Matrigel에 모세 혈관 형성 등의 혈관내피 기능을 보유하였다. VE-cadherin과 Tie2 promotor들이 iECs에서 탈메틸화 되어있었고, 진정한 혈관내피세포와 유사한 후생 유전학적 프로필을 보여주었다.. mRNA의 프로파일

링은 진정한 혈관내피세포와 iECs의 군집화를 보였으며, iECs에서 혈관내피세포 유전자의 높은 발현이 있었다. 생쥐의 뒷다리 허혈 모델에서 iECs의 주입은 모세 혈관 밀도를 증가시키고, 사지 관류를 강화시켰다. 이것은 iECs의 생체 내 생존 및 기능을 보여주었다.

결론- 우리는 성체 피부섬유아세포에서 기능적인 혈관내피세포로의 첫 번째 형질전환을 보여주었다. 이러한 결과는 허혈성 혈관 질환의 세포 치료에 대한 새로운 치료 양상을 제안해준다.

주요어 : induced endothelial cells, direct conversion, transdifferentiation

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