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

Running title: Han et al.; Direct Conversion of Adult Fibroblasts into ECs

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Abstract

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

Methods and Results—Eleven candidate genes, which are 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-GFP+ cells (0.9%), representing Tie2 gene activation, were detected by flow cytometry. Serial stepwise screening revealed 5 key factors (5F: Foxo1, Er71, Klf2, Tal1, and Lmo2) which were required for efficient reprogramming of SFBs into Tie2-GFP+ cells (4%). This reprogramming strategy did not involve pluripotency induction, as neither Oct4 nor Nanog was expressed after 5F transduction. Tie2-GFP+ cells were isolated using fluorescence-activated cell sorting, and designated as induced endothelial cells (iECs). iECs exhibited endothelial-like cobblestone morphology and expressed EC molecular markers. iECs possessed endothelial functions such as BS1 lectin binding, acLDL uptake, capillary formation on Matrigel and nitric oxide production. The epigenetic profile of iECs was similar to authentic ECs as the promoters of VE-cadherin and Tie2 genes were demethylated, mRNA profiling showed clustering of iECs with authentic ECs, and highly enriched endothelial genes in iECs. In a murine model of hindlimb ischemia, iEC implantation increased capillary density, and enhanced limb perfusion, demonstrating the 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.

Key words: induced endothelial cell, direct conversion, transdifferentiation
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). Moreover, there are considerable technical obstacles, 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 have demonstrated that these hurdles can be overcome by reprogramming differentiated cells into other lineages such as pancreatic β-cells,5 neurons,6, 7 cardiomyocytes,8 and hepatocytes9, 10 using defined factors. Here, we report a novel strategy to convert differentiated fibroblasts into ECs. 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) exhibited endothelial morphology, endothelial functions, and epigenetic and genetic profiled similar to authentic, functional ECs. In addition, transplantation of iECs rescues murine ischemic hindlimbs in vivo by inducing the formation of new vessels. Our findings clarify the molecular background of endothelial differentiation and transdifferentiation, and support the notion that the determination of cell fate is not fixed, but plastic by the formation of new transcriptional networks.

Methods

An extended methods section is available in the online-only Data Supplement.

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 (1300 rpm for 7 minutes at 4°C), 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 the remaining tail was cut into 1 cm 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 collagenase I.
(Worthington), 1 mg/mL collagenase II (Gibco), 10 mg/mL bovine serum albumin (BSA) (Amresco), and 1U/mL dispase (Gibco) at 37°C with 5% CO₂, and stirred every 10 minutes. After 30 minutes, 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 37°C with 5% CO₂. 1 hour 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 (BM MNC) 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 4°C) 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 polyethyleneimine (Polysciences), and added to 293T cells. After 1 day
of incubation at 37°C 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 4°C), 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 FACSAsria II 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 Supplemental Table 1.

**In vitro Fluorescence Staining**

Cells were fixed with 1% paraformaldehyde for 10 minutes 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 4°C, 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).

**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
(Supplemental Table 2). These candidate genes were (1) genes that are involved in
development of hemangioblasts from mesoderm \( (Gata2, Fli1, Tal1) \), (2) Ets family members,
which are central regulators of endothelial development \( (E1f1, Erg, Fli1, Ets1, Er71) \), (3) essential co-workers of endothelial transcription \( (Foxc2, Foxo1, Lmo2) \) and (4) a gene that
regulates endothelial functions \( (Klf2) \). We constructed lentiviruses that expressed each gene
individually. We prepared SFBs from adult Tie2 promoter-driven GFP \( (\text{Tie2-GFP}) \) expressing
transgenic mice (Supplemental Figure 1). We infected GFP- SFBs with vectors for all 11 factors
(Figure 1A), 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 1B). 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 1C and Supplemental
Figure 2). The combination of Foxo1 and Er71 increased the percentage of GFP\(^+\) cells to 0.6%.
When additional individual factors were added to the combination of Foxo1 and Er71, Klf2 significantly enhanced the efficiency of Tie2 gene activation (Figure 1D). Subsequent rounds of single-factor addition experiments were performed, revealing that Tal1 and Lmo2, the fourth and fifth factors, respectively, further improved activation efficiency (Figure 1E and 1F). 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 1G). In addition, the elimination of any single factor from the 5F reduced the percentage of GFP+ cells (Figure 1H). These data suggest that 5F are effective and necessary for the activation of the endothelial program in SFBs. Overexpression of the 5F combination induced Tie2 promoter activation in 4.0% of SFBs (Figure 2A), and the cells with an endothelial-like cobblestone appearance were derived from adult SFBs (Figure 2B). SFBs transduced with the 5F also strongly expressed endothelial markers such as CD31, VE-cadherin, Flk1, and vWF, demonstrating that they had been successfully reprogrammed as ECs (Figure 3A and 3B). Expression of these markers began as early as 3 days after 5F transduction, and peaked between 7 and 12 days.

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 systems. We therefore transduced the 5F to TTFs isolated from Tie2-GFP mice, and successfully generated endothelial-like cells with a cobblestone appearance (Supplemental Figure 3A and 3B). Flow cytometry showed that 5.4% of TTFs exhibited active Tie2 promoters, demonstrating that fibroblasts from two different sources could be reprogrammed into ECs by 5F transduction (Supplemental Figure 3C).

However, when the 5F were transduced to BM MNCs isolated from Tie2-GFP mice, only 0.1% of cells were converted into Tie2-GFP+ cells, and these cells lacked a cobblestone
appearance (Supplemental Figure 3D and 3E). These findings indicated that endothelial conversion by the iEC 5F was not equally effective in every cell type.

While we were performing our study, Ginsberg and colleagues reported that human amniotic cells from mid-gestation 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 TGFβ²². The authors focused on the ETS family alone, and selected 3 members of more than 20 ETS-family paralogs,¹⁶ which are abundantly expressed during endothelial differentiation of human ES cells. However, when they attempted to reprogram various adult and fetal non-vascular cells into ECs, they found that only amniotic cells could be successfully converted. This is not surprising because many factors, in addition to the ETS family, are involved in endothelial development.¹¹ In this 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 morphological changes to the cobblestone appearance after the transduction of Er71, Erg, and Fli into SFBs from Tie2-GFP mice (Supplemental Figure 4A). Only 0.3% of infected SFBs were transformed into Tie2-GFP⁺ cells (Supplemental Figure 4B), which did not represent an improvement over Er71 single transduction (Figure 1C). This suggests that these 3 ETS factors are not able to convert adult differentiated fibroblasts into ECs. Moreover, inclusion of Erg or Fli1 in the infection combinations in our study significantly decreased the conversion rate. (Figure 1D-1G).

To test whether pluripotency was induced during endothelial reprogramming of adult fibroblasts, we transduced the 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 direct reprogramming (Supplemental Figure 5A-5D). Western blot analysis confirmed this finding (Supplemental Figure 5E).

**In Vitro Endothelial Characterization of iECs**

Tie2-GFP$^+$ cells were isolated after 5F transduction using FACS. We designated these cells as iECs (Figure 4A), and characterized their endothelial-like features. The mRNA expression of endothelial-specific genes such as CD31, VE-cadherin, ICAM2, and Tie2 was significantly upregulated in iECs (Figure 4B). Primary ECs isolated from the lungs of Tie2-GFP mice and the MS1 EC line were used as positive controls. Although iECs expressed ICAM2 at much lower levels than MS1 cells, 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 at the protein level (Figure 4C and 4D, Supplemental Figure 6A and 6B). In addition, iECs displayed endothelial characteristics, such as BS1 lectin binding, and uptake of Ac-LDL (Figure 4E and 4F, Supplemental Figure 6C and 6D). iECs, primary ECs, and MS1 cells all formed capillary tubes on Matrigel (Figure 4G and Supplemental Figure 6E). Nitric oxide (NO), which is an important regulator of vascular functions, was produced by iECs at levels comparable with those produced by primary ECs or MS1 cells (Figure 4H). 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. Proliferation of iECs was similar to that of adult primary ECs isolated from the mouse lung (Supplemental Figure 7A). iECs’ vulnerability to serum starvation of iECs did not differ from that of primary ECs (Supplemental Figure 7B).

To examine whether iECs acquired endothelial-like epigenetic reprogramming, their DNA methylation status was analyzed. Bisulfite genomic sequencing demonstrated that cytosine
guanine dinucleotides (CpGs) in the promoter regions of VE-cadherin and Tie2 were
demethylated in iECs, consistent with primary ECs or MS1 cells, whereas they were
hypermethylated in mock-infected SFBs (Figure 5A). When the global gene expression profiles
were compared, the patterns of iECs were clustered with primary ECs from the mouse lung and
MS1 cells, but were distinct from original SFBs or mock-infected SFBs (Figure 5B). In
particular, endothelial-specific genes were highly enriched in iECs, primary ECs, and MS1
(Figure 5C). Further, the gene ontology analyses showed that iEC mimicked the gene expression
patterns of primary ECs or MS1 cells for the set of genes for endothelium development
(GO003158) and endothelial function (regulation of EC migration) (GO0010594), although
some differences were also observed (Figure 5D and Supplemental Figure 8).

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 the left femoral arteries were surgically ligated
in athymic nude mice, 5×10⁵ cells of interest were intramuscularly injected to the ischemic limbs.
Like primary ECs or MS1 cells, implantation of iECs enhanced the recovery of blood flow to
ischemic limbs, saving the limbs (Figure 6A-6C, Supplemental Figure 9A and 9B). On post-
operative day 14, the ischemic limbs were harvested. Histological analyses showed a higher
capillary density in the ischemic limbs after the implantation of iECs compared with mock-SFBs
or sham controls (Figure 6D and 6E). Implanted iECs that were double positive for GFP and
BS1 lectin were found at the injection site, demonstrating the in vivo viability and functionality
of iECs (Figure 7).

To determine what percentage of capillaries were composed with the engrafted cells, the
ischemic limbs were harvested on day 14, and lysed into single cells (Supplemental Figure 10A). GFP+ implanted cells comprised approximately 0.3% of the BS-1 lectin+ ECs in ischemic limbs in the iEC, primary EC, and MS1 groups (Supplemental Figure 10B). However, no GFP+ cells were detected in the mock-SFB control.

To determine the percentage of implanted cells that were engrafted, 5×10^5 GFP-labeled SFBs were intramuscularly implanted to the ischemic limbs. Just after the injection, the ischemic limbs were harvested and lysed into single cells to assess how many cells were actually implanted in this model (Supplemental Figure 10A). It was found that 2.1×10^4(±0.2×10^4) GFP+ cells were implanted. When this was assumed to be the number of cells that were initially implanted, the percentage of engraftment was calculated as to 9.4±3.2%, 9.8±2.3%, 12.0±3.3% in iEC, primary EC, and MS1 groups, respectively (no significant difference between groups: n=3 for each group).

Conversion Kinetics of iECs

Lastly, we analyzed the time course of EC induction from adult fibroblasts after 5F transduction. Initially, the 12th day after transduction was selected for iEC sorting because previous studies have reported that approximately 2 weeks are required for effective direct conversion of mouse fibroblasts into other cell types.6, 8, 9 However, in our system, the proportion of Tie2-GFP+ cell peaked approximately 1 week after transduction (Figure 8A). Beyond 7 days after transduction, iECs would be outgrown by non-converted fibroblasts. Thus, 7th day post-transduction appeared to be the ideal time point to obtain iECs. Next, we verified time-dependent changes in 5F expression. The expression of the 5F strongly increased after transduction, but returned to the basal level in iECs 7 days after sorting (Figure 8B). This is not surprising given that lentiviral vectors can be subject to gene silencing over time.22 Thus, we hypothesize that the
overexpression of 5F is not necessary to maintain iECs after conversion.

Discussion

This is the first study to demonstrate 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,\textsuperscript{23,24} but can also cross over to other lineages by building new transcriptional networks.\textsuperscript{6-10} We identified 5 iEC factors: Foxo1, Er71, Klf2, Tal1, and Lmo2. Foxo1 plays a crucial role in vascular development, and its disruption causes embryonic lethality.\textsuperscript{18} Er71 is an ETS family member that regulates early EC development at the top of the transcriptional network.\textsuperscript{11} Consequently, knockout of Er71 impairs vasculogenesis and results in embryonic lethality, in contrast to other ETS genes, indicating its central role in endothelial specification.\textsuperscript{11,25} Remarkably, Foxo1 and Er71 were the only 2 factors that could individually activate the Tie2 promoter in SFBs (\textbf{Figure 1C} and \textbf{Supplemental Figure 2}). Klf2 is expressed in response to shear stress, and functions as a key “molecular switch” that regulates endothelial functions after their initial specification during endothelial development.\textsuperscript{11,20,21} Tal1 is implicated in the early development of endothelial and blood cells, and is indispensable for normal vascular maturation.\textsuperscript{26,27} Of note, Lmo2 is a transcriptional co-factor that is co-expressed and associated with Tal1, which constitutes a multi-factorial complex\textsuperscript{11} that induces the expression of endothelial markers.\textsuperscript{19} Interestingly, the combination of Tal1 and Lmo2 induces hemangioblast formation, and determines its fate to the blood- or endothelial-lineages, dependent on the presence of blood-inducing factors like Gata1.\textsuperscript{19} In the absence of Gata1, Tal1 and Lmo2 induce hemangioblasts to subsequently differentiate into ECs.\textsuperscript{19} Taken together, these data
suggest that these 5 factors are purposive for endothelial reprogramming.

In our study, roughly 4% of adult fibroblasts could be converted into ECs by iEC 5 factors. This conversion efficiency is comparable to or higher than those of iPSCs or other directly converted cells. Reprogramming efficiency into iPSCs ranged from 0.01% to 0.1%.24, 28 The efficiency of direct conversion from mouse embryonic fibroblasts to the induced hepatocytes was 0.3%.10 In case of induced neurons, it ranged from 1.8% to 7.7%.6 The efficiency of conversion from fibroblasts into cardiomyocytes was relatively high (20%), which was defined by αMHC-promoter driven GFP expression among the infected fibroblasts.8

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 colleagues29 has limited clinical relevance, since amniotic cells are not readily available, and because this approach still possesses the 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 from their experiments. Accordingly, the direct conversion of terminally differentiated cells into ECs was not demonstrated by their study.

Recently, some researchers have reported that the transduction of all or a subset of Yamanaka’s 4 pluripotency factors (OCT4, SOX2, KLF4 and c-MYC)23, 24 can convert human fibroblasts into ECs without inducing pluripotent status.30-32 However, because conversion by these techniques involves the generation of intermediate progenitor cells or partial iPSCs, these are essentially different from our direct conversion technology. Moreover, because these studies utilized pluripotent factors, there are still concerns over their 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 \textit{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.\textsuperscript{33} 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, first demonstrated here, therefore sheds new light on endothelial differentiation and reprogramming, and constitutes significant progress towards future clinical applications.

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\textbf{Conflict of Interest Disclosures:} None.

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Figure Legends:

Figure 1. Identification of 5 key factors for the endothelial reprogramming of adult skin fibroblasts (SFBs). A, Experimental design to determine endothelial reprogramming factors. B, Transduction of the 11 factors collectively induced Tie2-GFP+ cells. C, Only Foxo1 or Er71 generated Tie2-GFP+ cells in transduction tests using a single factor. D–F, Selection of key factors by sequential stepwise addition of individual factors. Factors that enhanced the efficiency of Tie2-GFP activation were included in the subsequent transduction combination. G–H, Transduction tests to confirm the 5 key factors. Addition (G) or elimination (H) of any single factor decreased the efficiency of GFP+ cell conversion by the iEC 5F, demonstrating that the 5F represent the ideal combination for endothelial reprogramming. Data are presented as mean±s.e.m. n=3 (B), 5 (C), 4-12 (D), 4-15 (E, F), 3-11 (G), 3-15 (H). *P<0.05, compared with each relevant control.
Figure 2. Conversion of adult skin fibroblasts into endothelial cells by 5 the factors. **A**, The final 5 factors (5F : Foxo1, Er71, Klf2, Tal1 and Lmo2) converted 4.0% of SFBs into Tie2-GFP+ cells. n=11 \*P<0.05, compared with the mock control. **B**, Transduction with the 5F induced endothelial-like cells with a cobblestone appearance. Scale bar: 200µm.

Figure 3. Expression of endothelial markers in SFBs after 5 factor transduction. **A**, Flow cytometric analyses were conducted for 12 days following transduction. Endothelial marker expression was detected on day 3 following transduction, and maintained until day 12. **B**, Immunofluorescence staining showed endothelial marker expression localized strongly on the cellular membrane. n=3 (A, B). Scale bar: 20µm (B). \*P<0.05, compared with the mock control.

Figure 4. Characterization of iECs. **A**, After sorting by FACS, cultured iECs showed a cobblestone appearance, in contrast to mock-infected SFBs (Mock-SFB). **B**, mRNA expression of endothelial markers was analyzed using real-time PCR. The y axis represents relative mRNA expression of target genes in cells of interest to those in MS1. **C-E**, Fluorescence staining of VE-cadherin (C), CD31 (D), and BS1 lectin (E) confirmed the endothelial features of iECs. **F**, DiI-Ac-LDL uptake by iECs. **G**, Capillary tube formation on Matrigel by iECs. **H**, Significantly higher NO production by iECs compared with mock-SFB controls. iECs responded like regular ECs to NO inducers and an inhibitor. Ach denotes acetylcholine. Primary ECs from Tie2-GFP mouse lungs and the MS1 EC line were used as positive controls (B, H). Data are presented as mean±s.e.m. n=3-4 (B–H). Scale bars: 200 µm (A, G), 20 µm (C–F). \*P<0.05, compared with corresponding mock-SFB controls. \#P<0.05, compared with each relevant sample without L-NAME treatment.
**Figure 5.** 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 show methylated CpGs. B, Global gene expression was analyzed using cDNA microarrays. 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.

**Figure 6.** Enhanced limb perfusion and salvage by implanted iECs in a murine model of hindlimb ischemia. A, Photographs demonstrating limb salvage by intramuscular injection of iECs. B, Laser Doppler perfusion imaging (LDPI) showing enhanced perfusion recovery after implantation of iECs. C, Quantification of LDPI. D, Histological examination of ischemic limbs harvested 14 days after surgery showed a higher capillary density in iEC-implanted mice. E, Quantification of capillary density. Data are presented as mean±s.e.m. n=3–5 in each group. Scale bars: 20 μm (D). *P<0.05, compared with Mock-SFB or PBS-treated sham controls.

**Figure 7.** 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 the 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.
Figure 8. Conversion kinetics of iECs. A. The proportion of Tie2-GFP+ cells peaked 7 days after 5F transduction. n=10. *P<0.05, compared with D3 cells. D denotes days after transduction. B, The strong induction of 5F expression following transduction was silenced afterwards in iECs. The y axis represents relative mRNA expression of target genes in cells of interest to those in D0 SFBs. n=3-6. *P<0.05, compared with D0 SFBs. D denotes days after transduction. iEC denotes iECs 7 days after Tie2-GFP+ cell sorting.
Figure 1

A

D0

DMEM+FBS10%

DMEM+FBS10%

DMEM+FBS10%

D2

EBM2+FBS10%

EBM2+FBS10%

EBM2+FBS10%

D12

SFB

Lentiviruses

iEC

Tie-2 promoter

GF

Tie2-GFP Tg

Lentiviruses

FACS

Tie2-GFP

Mock

11 factors

0%

0.9%

B

C

Mock

Foxy1

Er71

0%

0.2%

0.4%
Figure 1, cont’d
Figure 2

A

Mock

5F

0%

4.0%

B

Mock

5F

Tie2-GFP
Figure 3

A

D3

0.5% 0.5% 0.5% 0.9%

D7

1.8%* 4.1%* 1.8%* 11.9%*

D12

2.3%* 4.2%* 2.6%* 7.3%

Counts

CD31  VE-Cadherin  Flk1  vWF

mock  5F
Figure 3, cont’d

B

VE-cadherin/DAPI

Mock

5F

MS1

CD31/DAPI

Mock

5F

MS1

Flk1/DAPI

Mock

5F

MS1
Figure 4

A

Mock-SFB  iEC

B

Relative mRNA expression

VE-cadherin  CD31

ICAM2  Tie2

* * * *

N.D.

Mock-SFB  iEC  Primary EC  MS1
Figure 4, cont’d
Figure 4, cont’d

H

NO production

Mock-SFB  iEC  Primary EC  MS1

VEGF

Ach

A23187

L-NAME
Figure 6

A

B

C

Ratio of ischemic/nonischemic limb perfusion (%)

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Figure 6, cont’d

D

BS1-lectin / DAPI

Ratio of capillaries / myotube

PBS  
Mock-SFB  
iEC  
Primary EC  
MS1

* * *
Figure 7
Figure 8, cont’d

B

**Relative mRNA expression**

**Foxo1**

**Er71**

**Klf2**

**Tal1**

**Lmo2**
Direct Conversion of Adult Skin Fibroblasts to Endothelial Cells by Defined Factors
Jung-Kyu Han, Sung-Hwan Chang, Hyun-Ju Cho, Saet-Byeol Choi, Hyo-Suk Ahn, Jaewon Lee, Heewon Jeong, Seock-Won Youn, Ho-Jae Lee, Yoo-Wook Kwon, Hyun-Jai Cho, Byung-Hee Oh, Peter Oettgen, Young-Bae Park and Hyo-Soo Kim

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Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2014/09/03/CIRCULATIONAHA.113.007727.DC1

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Online Supplemental Methods

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

After incubation with 10 µg/mL DiI-Ac-LDL (Invitrogen) for 12 hours at 37°C 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 37°C, and 4×10⁴ cells per well were seeded onto the plates. After incubation for 9 hours at 37°C with 5% CO₂, tube formation was analyzed using an IX71 microscope (Olympus).

Nitric oxide (NO) Production

3×10⁴ 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-N⁵⁴-Nitroarginine methyl ester (L-NAME) (Sigma Aldrich) was added according to the experimental design. After overnight incubation at 37°C 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 Annexin V 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 Supplemental Table 1. 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 $5 \times 10^5$ cells were resuspended in 50 µL 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% collagenase II (Gibco) at 37°C with 5% CO$_2$ for 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.
Online Supplemental Figure Legends

**Supplemental Figure 1.** GFP− cell sorting from Tie2-GFP SFBs.

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 the negligible possibility of EC contamination, GFP− cells were isolated by fluorescence-activated cell sorting (FACS), and used for subsequent experiments.

**Supplemental Figure 2.** Single factor transduction test to determine essential factors.

Only Foxo1 or Er71 generated significant numbers of Tie2-GFP+ cells after infection with the individual factors. n=3-10

**Supplemental Figure 3.** Endothelial reprogramming of other cell types using the 5 factors.

A, Endothelial reprogramming of tail-tip fibroblasts (TTFs).

B, 5F-transduced TTFs showed morphological changes and gained an endothelial-like cobblestone appearance.

C, 5F transduction resulted in the successful activation of the endothelial-enriched Tie2 promoter in TTFs. These findings demonstrated the universal effects of the 5 iEC factors in endothelial conversion of adult fibroblasts.

D, 5F-transduced bone marrow mononuclear cells (BM MNCs) showed no clear morphological changes to an endothelial-like cobblestone appearance.
The Tie2 promoter was activated in only 0.1% of BM MNCs after 5F transduction.

Scale bars: 200\(\mu m\) (B, D). \(n=6\) (C), 4 (E). \(*P<0.05\), compared with mock controls.

**Supplemental Figure 4.** Unique effects of 5 factors for the endothelial reprogramming of adult fibroblasts.

**A,** Overexpression of Er71, Erg, and Fli1 did not induce morphological changes resulting in a cobblestone appearance in SFBs.

**B,** Tie2-GFP+ cells were not effectively induced after transduction with the 3 factors.

Scale bar: 200\(\mu m\). \(n=6\). \(*P<0.05\), compared with mock control.

**Supplemental Figure 5.** Direct conversion of adult fibroblasts into endothelial cells not involving pluripotency induction.

**A,** 5F transduction did not induce Oct4-GFP+ cells,

**B,** but successfully activated the Tie2 promoter in SFBs from Oct4-GFP mice.

**C,** 5F transduction did not induce Nanog-GFP+ cells,

**D,** but successfully activated the 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.

\(n=6\) (A–D). \(*P<0.05\), compared with mock controls. Immunoblot assays were performed in
triplicate, and a representative result is shown.

**Supplemental Figure 6.** *In vitro* endothelial characterization of iECs.

**A–D,** Fluorescence staining of primary ECs and MS1 cells. As positive controls, primary ECs from Tie2-GFP mouse lungs, and MS1 cells were stained for VE-cadherin (**A**), CD31 (**B**) and BS1 lectin (**C**). Primary ECs from mouse lungs and MS1 cells also showed DiI-Ac-LDL uptake (**D**).

**E,** Matrigel tube formation assays showed capillary tube formation by primary ECs from mouse lungs, and MS1 cells, as positive controls.

Scale bar: 20μm (**A–D**), 200μm (**E**).

**Supplemental Figure 7.** Growth kinetics of iECs.

**A,** The cell growth curve of iECs was similar to that of adult primary ECs isolated from the mouse lungs.

**B,** After 24 hours of serum starvation, the proportion of early (Annexin V⁺ 7-AAD⁻) or late (Annexin V⁺ 7-AAD⁺) apoptosis in iECs was not different from that in primary ECs. 7-AAD denotes 7-Aminoactinomycin D.

n=4 (**A**), 3 (**B**).

**Supplemental Figure 8.** Gene ontology analysis for endothelial function.
iEC mimicked the gene expression patterns of primary ECs or MS-1 cells for the set of genes for regulation of EC migration (GO0010594), although some differences were also observed.

**Supplemental Figure 9.** Murine model of hindlimb ischemia.

**A,** Gross pictures of all mice used for experiments.

**B,** LDPI images for all mice used for experiments.

**Supplemental Figure 10.** Replacement of capillaries by the engrafted cells.

**A,** Schematic figure showing the experiments performed to calculate the percentage of capillaries replaced by implanted cells, and the percentage of engraftment.

**B,** Implanted cells accounted for approximately 0.3% of the ECs in the ischemic limbs, indicated by BS-1 lectin binding.

n=3.
**Supplemental Table 1.** Primers used for quantitative real-time PCR and methylation analysis.

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**Supplemental Table 2.** Candidate factors screened for direct endothelial conversion of SFBs.

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Supplemental Figure 1
**[Supplemental Figure 3]**

**A**

- **D0**
  - DMEM+FBS10%
  - Lentiviruses

- **D2**
  - EBM2+FBS10%
  - iEC

- **D12**
  - EBM2+FBS10%

**B**

- **Mock**
  - Tie2-GFP Tg

- **5F**
  - Tie2-GFP Tg

**C**

- **Mock**
  - PE vs Tie2-GFP
  - 0%

- **5F**
  - PE vs Tie2-GFP
  - 5.4%*

---

*Note: *FACS* refers to fluorescence-activated cell sorting.*
[Supplemental Figure 3]
[Supplemental Figure 5]

A

Unstained

Oct4-GFP

Mock 0%

5F 0%

Tie2-PE antibody stained

B

Mock 0%

5F 4.9%

C

Unstained

Oct4-GFP

Mock 0%

5F 0%

D

Nanog-GFP

Mock 0%

5F 3.8%
[Supplemental Figure 5]

E

- Tie2
- Oct4
- Nanog
- α-tubulin

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[Supplemental Figure 6]

A

VE-cadherin/DAPI

Primary EC

MS1

B

CD31/DAPI

Primary EC

MS1

C

BS1 lectin/DAPI

Primary EC

MS1

D

Dil-Ac-LDL/DAPI

Primary EC

MS1
[Supplemental Figure 6]

Primary EC  MS1
Supplemental Figure 7

A

Total cell number (x10^5)

B Serum starvation

Mock-SFB  iEC  Primary EC

7-AAD
Annexin V

3.5 ± 0.3%  3.6 ± 1.0%  4.2 ± 0.2%
2.1 ± 0.9%  1.6 ± 0.3%  2.9 ± 1.8%
Regulation of EC migration
(GO0010594)
[Supplemental Figure 9]
[Supplemental Figure 9]
Supplemental Figure 10

A

Femoral artery ligation → GFP⁺-labeled cells → Ischemic limb harvest → D14

D0 → Ischemic limb harvest → D14

GFP⁺ cell number = “initially implanted cell number”

- % of GFP⁺ cells in capillaries = BS-1 lectin⁺ GFP⁺ cell number / BS1-lectin⁺ cell number x 100
- % of engrafted cells = BS-1 lectin⁺ GFP⁺ cell number / “initially implanted cell number” x 100

BS1-lectin⁺ endothelial cell → Whole cell lysis → BS1-lectin⁺ GFP⁺ engrafted cell
[Supplemental Figure 10]

B

Mock-SFB

iEC

Primary EC

MS1

BS-1 lectin (+)