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Direct Reprogramming of Human Dermal Fibroblasts Into Endothelial Cells Using ER71/ETV2

Sangho Lee³, Changwon Park^{1,2,4}, Ji Woong Han³, Ju Young Kim^{2,3}, Kyuwon Cho³, Eun Jae Kim¹, Sangsung Kim³, Shin-Jeong Lee⁵, Se Yeong Oh², Yoshiaki Tanaka⁴, In-Hyun Park⁴, Hyo Jae An³, Claire Min Sin³, Shraya Sharma³, and Young-sup Yoon^{3,5}

¹Department of Pharmacology, College of Medicine, University of Illinois at Chicago, Chicago, IL

²Department of Pediatrics, Children's Heart Research and Outcomes Center, Emory University School of Medicine, Atlanta, GA

³Division of Cardiology, Department of Medicine, Emory University School of Medicine, Atlanta, GA

⁴Department of Genetics, Yale Stem Cell Center, Yale School of Medicine, New Haven, CT

⁵Division of Cardiology, Department of Medicine, Severance Biomedical Science Institute, Yonsei University College of Medicine, Seoul, Korea

Abstract

Rationale—Direct conversion or reprogramming of human postnatal cells into endothelial cells (ECs), bypassing stem or progenitor cell status, is crucial for regenerative medicine, cell therapy, and pathophysiological investigation but has remained largely unexplored.

Objective—We sought to directly reprogram human postnatal dermal fibroblasts (HDFs) to ECs with vasculogenic and endothelial transcription factors (TFs) and determine their vascularizing and therapeutic potential.

Methods and Results—We utilized various combinations of seven EC TFs to transduce HDFs and found that ER71/ETV2 alone best induced endothelial features. KDR⁺ cells sorted at day 7 from ER71/ETV2-transduced HDFs showed less mature but enriched endothelial characteristics and thus were referred to as early reprogrammed ECs (rECs), and did not undergo maturation by further culture. After a period of several weeks' transgene-free culture followed by transient re-induction of ER71/ETV2, early rECs matured during three months of culture and showed reduced ETV2 expression, reaching a mature phenotype similar to postnatal human ECs. These were termed late rECs. While early rECs exhibited an immature phenotype, their implantation into ischemic hindlimbs induced enhanced recovery from ischemia. These two rECs showed clear capacity for contributing to new vessel formation through direct vascular incorporation in vivo.

Address correspondence to. Dr. Young-sup Yoon, Division of Cardiology, Department of Medicine, Emory University School of Medicine, 101 Woodruff Dr. WMB 3009, Atlanta, GA, 30322, USA, Tel:404-727-8176, yyoons5@emory.edu, Dr. Changwon Park, Department of Pediatrics, Emory University School of Medicine, 2015 Uppergate Dr. ECC 208B, Atlanta, GA, 30322, USA, Tel: 404-727-7143, cpark23@emory.edu.

S.L. and C.P. contributed equally to this article.

DISCLOSURES

None.

Paracrine or pro-angiogenic effects of implanted early rECs played a significant role in repairing hindlimb ischemia.

Conclusions—This study for the first time demonstrates that ER71/ETV2 alone can directly reprogram human postnatal cells to functional, mature ECs after an intervening transgene free period. These rECs could be valuable for cell therapy, personalized disease investigation, and exploration of the reprogramming process.

Keywords

Direct reprogramming; ER71/ETV2; endothelial cells; ets transcription factors; transdifferentiation; angiogenesis

INTRODUCTION

Endothelial cells (ECs) are a key element of vasculature and are indispensable for repairing injured or ischemic tissues. Over the years, many approaches have been developed to generate ECs for use in cell therapy. Despite early enthusiasm, adult stem or progenitor cells were found to have minimal endothelial transdifferentiation potential^{1–3}. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) emerged as promising alternatives; however, problems such as tumorigenic potential or inefficient cell production have limited their clinical application^{4–6}. Recently, a new approach has been developed for generating target cells through direct lineage conversion or reprogramming with cell-type specific transcription factors (TFs)^{7–12}. This approach has many potential advantages such as procedural simplicity and avoidance of uncontrolled differentiation and tumorigenicity compared to ESCs and iPSCs.

In an attempt to generate ECs directly from somatic cells, one recent study demonstrated direct conversion of lineage committed cells into ECs via lentiviral overexpression of a combination of ETV2 (also known as ER71), ERG, and FLI1 with inhibition of TGF β signaling⁷. This method, however, utilized amniotic fluid-derived c-KIT-negative cells as source cells, and reportedly did not work for postnatal cells. It is also unclear whether the source cells were fully differentiated, since their origin was amniotic tissues¹³ and potential contamination with stem or progenitor cells cannot be entirely excluded. Due to cell heterogeneity, clonal variability of reprogrammed cells was noted as well. Practically, this approach needs invasive amniocentesis and cannot be applied for autologous cell therapy. By employing five TFs, namely FOXO1, ETV2, KLF2, TAL1, and LMO2, which are crucial for vessel development, Han et al directly converted mouse adult skin fibroblasts into ECs¹⁴. Skeletal muscles can also be converted into ECs upon overexpression of *er71* in zebrafish¹⁵. Other approaches employed pluripotency factors, but not vasculogenic/endothelial TFs, to initially induce an intermediate state, and then applied angiogenic factors to produce progenitor-stage endothelial lineage cells^{9, 10}. These results suggest the feasibility of direct reprogramming of non-ECs into ECs, but novel methods for the direct reprogramming need to be designed for potential clinical application.

To date, no studies have clearly shown direct reprogramming of human postnatal cells into mature ECs with vasculogenic/endothelial TF(s). Since the major use for reprogrammed or

induced ECs is for cell therapy or disease investigation, it would be more appropriate to use autologous cells as source cells and lineage-specific TFs for reprogramming agents. This approach would enable autologous cell therapy and personalized disease investigation and avoid or minimize adverse effects. However, no studies have demonstrated such potential. In addition, to reduce the load of external genes in reprogramming, it would be preferable to minimize the number of TFs used. This will also facilitate investigation of yet unknown mechanisms of direct reprogramming.

Accordingly, we sought to directly reprogram human postnatal cells to ECs with TFs critical for EC specification and function. We selected the following seven factors for screening through literature search: ETV2, FOXC2, MEF2C, SOX17/SOX18, SMAD1, HEY1/HEY2, and NANOG^{16–24}. We used various combinations of these factors and found that ETV2 alone was best to reprogram fibroblasts into ECs. Previously, we have demonstrated that ETV2, a member of the ETS TF family, plays an indispensable role in vessel development as evidenced by lack of vasculature in *Etv2* deficient mouse embryos^{21, 25}. ETV2 directly binds promoters of *Flk1*, *VE-cadherin* and *Pecam1*^{21, 25, 26}, and enforced expression of ETV2 in mouse ESCs increased expression of these endothelial genes and generation of FLK1⁺ cells^{21, 27}. Here we report that ETV2 transduction to human postnatal fibroblasts can generate two stages of rECs which are distinguishable by expression levels of ETV2 and more mature EC proteins and duration of culture. We further demonstrate importance of the ETV2 transgene-free period followed by reactivation of ETV2 for maturation of rECs. rECs were also shown to be effective for repairing limb ischemia when transplanted in vivo.

METHODS

Generation of tetracycline-inducible lentiviral vectors expressing transcription factors and production of lentiviral particles

Lentiviral particles of the transcription factors were generated using the FUW-tetO lentiviral system, which was re-engineered from FUW-tetO-hOCT4 (Addgene plasmid 20726)²⁸. Each transcription factor was cloned into the FUW-tetO lentiviral vector. The resulting constructs were co-transfected with pVSVG and pMDL for packaging into 293FT cells (Invitrogen) and the supernatant of the 293FT culture was collected at days 2 and 3 post-transfection, followed by PEG6000-mediated concentration^{29–31}.

Lentiviral transduction

For EC reprogramming, human dermal fibroblasts (HDFs) were incubated with constitutively active lentiviral particles expressing the reverse tetracycline transactivator FUW-M2rtTA (Addgene plasmid 20342), along with doxycycline (DOX)-inducible lentiviral particles containing the EC transcription factors in the presence of polybrene for 18–24 hours, with virus of MOI 4. After washing, the cells were treated with DOX (2 µg/ml, Clontech) containing DMEM supplemented with 10% fetal bovine serum (FBS). The medium, replenished with DOX, was changed every other day. After 7 days, the medium was replaced with EGM-2 medium (Lonza) containing DOX and the cells were further cultured on collagen-coated plates. The medium was changed every 2–3 days for the duration of the culture period.

Induction of hindlimb ischemia and skin wound

All animal experiments were approved by Emory University IACUC. Hindlimb ischemia³² and skin wound³³ were performed on male athymic nude mice as we described previously. For the hindlimb ischemia studies, the estimated minimal number for having a meaningful difference at 4 weeks is 5, so we included 5 to 10 animals in each group (n = 6 for PBS-, 10 for HUVEC-, 5 for rEC-injected groups). Before sacrifice for tissue harvest, the mice were perfused with fluorescein-labeled *Griffonia (Bandeiraea) Simplicifolia* Lectin I (BSL1, Vector Laboratory Inc.) by direct cardiac injection to stain functional endothelial cells in blood vessels. The tissue sections were processed for confocal imaging with a Zeiss LSM 510 Meta confocal laser scanning microscope and LSM 510 Image software (Carl Zeiss).

Details on the materials and methods, including the following items, can be found in the online-only Data Supplement: Flow cytometry³²; Acetylated-LDL uptake and UEA1 lectin staining³²; In vitro tube formation assay³²; Immunohistochemistry and immunocytochemistry³²; Real-time RT-PCR (Table 1 in the online-only Data Supplement)³²; Microarray; Heat map and clustering analysis^{34,35}; RNA-seq analysis; Statistical analysis.

RESULTS

Overexpression of endothelial TFs can convert human postnatal fibroblasts into the EC lineage

First, we generated doxycycline (DOX) inducible lentiviral constructs containing the open reading frame of each gene (Online Figure I). After transduction into human dermal fibroblasts (HDFs), expression of each TF in response to DOX treatment was confirmed by quantitative RT-PCR (qRT-PCR) (Online Figure IB). To determine whether these TFs could induce expression of EC genes in HDFs, we infected HDFs with a mixture of six of the TFs (ETV2, FOXC2, MEF2C, SOX17, SMAD1, HEY1), treated with DOX for 6 or 12 days, and conducted qRT-PCR. mRNA expression of EC genes *CDH5*, *KDR*, *PECAMI*, *CD34*, and *TEK*, but not *VWF*, were markedly increased compared to the uninfected HDFs (Online Figure IC). When *NANOG*, a direct upstream regulator of *KDR* in HUVECs¹⁸, was substituted for *SMAD1*, no difference was observed (data not shown). Next, we modified the protocol: after infecting with the six TFs (with *NANOG* replacing *SMAD1*), the cells were cultured in DMEM for 7 days and then in endothelial cell culture media until D20 with continuous DOX treatment (Figure 1A). From D16, some of the transduced HDFs exhibited a cobblestone morphology, a classic feature of ECs (Figure 1B). mRNA expression of endothelial genes was gradually increased up to D15, but declined at D20 (Figure 1C). We stopped DOX treatment at D20 and cultured these cells up to D39. This change induced rebound of endothelial gene expression. In detail, compared to untransduced HDFs at D0, mRNA expression of *CDH5* at D15 was ~10,000-fold higher, was reduced at D20, but was still ~3,500-fold higher at D39. *KDR* was increased by ~500-fold at D15 and ~1000-fold at D39 compared to the control. Expression of *TEK*, *PECAMI* and *CD34* showed patterns similar to *KDR* but with less elevated levels at D39: *TEK* ~5-fold, *PECAMI* ~40-fold and *CD34* ~20-fold. Flow cytometry confirmed expression of endothelial proteins at D39, showing that approximately 12–15% of the cells expressed *KDR* or *CDH5* (Figure 1D). A

small portion of the cells took up acetylated (Ac)-LDL and formed tube-like structures on Matrigel *in vitro* (Figure 1E). Expression of the six TFs (except *HEY1*) peaked at D7 and declined from D10 (Online Figure II). Taken together, these data indicate that overexpression of these six endothelial TFs was able to induce endothelial characteristics in human postnatal fibroblasts.

Overexpression of ETV2 alone best induces expression of EC markers in HDFs

In order to determine the minimal essential TFs for EC reprogramming, we first started with combinations of 5 factors, omitting one factor from the above six factors. qRT-PCR analyses demonstrated that five-factor combinations minus *ETV2* showed the most significant reduction in major endothelial gene expression such as *CDH5* and *KDR* compared to those which included *ETV2*, suggesting an indispensable role for *ETV2* in endothelial reprogramming (Figure 2A and 2B). We next designed two series of experiments: one with combinations of three or four factors without *ETV2* and the other with combinations of *ETV2* with one or two other factors [*ETV2*, *ETV2*+*FOXC2*²⁶, and *ETV2*+*FOXC2*+*NANOG*¹⁸]. In the former group, no combinations showed higher expression of endothelial genes than the *ETV2* only group (data not shown). In the latter group, we chose those combinations because reports showed potent function of *FOXC2* and *NANOG* in endothelial gene expression (Figure 2C)^{18, 26}. Interestingly, only the *ETV2* single factor-treated condition induced the highest expression levels of *CDH5*, *KDR*, *PECAMI1*, *TEK* and *CD34* (Figure 2C). Inclusion of *FOXC2* into *ETV2* or *ETV2*+*NANOG* did not show any synergistic or additive effects on endothelial gene expression compared to *ETV2* alone. Rather, the expression of endothelial genes decreased upon co-transduction of *FOXC2* with other factors, suggesting that overexpression of *FOXC2* is dispensable for direct reprogramming of HDFs into ECs. Collectively, these data strongly argue that *ETV2* alone may be sufficient to convert human postnatal fibroblasts into ECs.

HDFs transduced with ETV2 and cultured short-term show immature EC characteristics and have the capability for vessel formation

We then optimized infection conditions of *ETV2* and found that multiplicity of infection (MOI) 4 induced the highest infection efficiency and the lowest cell death (Online Figure III and part of data not shown). HDFs infected with only *ETV2* at MOI 4 demonstrated a cobblestone appearance as early as D2 (Figure 3A) and expressed *CDH5* at ~50% and *KDR* at ~39% by flow cytometric analyses at D7 (Figure 3B). Around ~67% of the *KDR*⁺ cells also expressed *CDH5*. During the 7 days culture, 2×10^5 of HDFs used for the infection at D0 gave rise to 1.5×10^6 at D7. Thus, it was calculated that one HDF generates approximately 3.7 *CDH5*⁺ cells, 3 *KDR*⁺ cells, and 2 *CDH5*⁺*KDR*⁺ cells, respectively, upon *ETV2* transduction. Neither *KDR* nor *CDH5* was expressed in control virus-infected HDFs regardless of DOX treatment nor in *ETV2*-infected HDFs without DOX treatment (Online Figure IV). Infection of HDFs with other single factors at MOI 4 did not induce expression of EC markers (data not shown). Compared to HDFs at D0, mRNA expression levels of endothelial genes were consistently higher in *ETV2*-transduced HDFs during the first 7 days, with a peak at day 7 except for *CD34* and *VWF*: *CDH5* (~10,000 fold), *KDR* (~7,000 fold), *TEK* (~50 fold), *PECAMI1* (~50 fold), *CD34* (~5 fold), and *VWF* (~10 fold) (Figure

3C). These results showed higher levels of EC gene expression compared to those of the six factor-infected HDFs at D15 (Figure 1C).

We then FACS sorted the heterogeneous cells using KDR, a comprehensive marker for endothelial-lineage cells, at D7 to enrich endothelial-lineage cells³⁶. The KDR⁺ cells showed substantially higher mRNA expression of endothelial genes compared to the KDR⁻ cells (Figure 3C). Compared to HUVECs, KDR⁺ cells showed similar or higher expression of *CDH5*, *KDR* and *TEK* but lower expression of *PECAM1* and *VWF*, which are known markers of mature ECs. Western blot analysis (Online Figure V) further confirmed expression of all four EC markers, CDH5, KDR, PECAM1, and VWF. Consistent with the qRT-PCR results shown in Figure 3C, PECAM1 and VWF levels were weaker in KDR⁺ cells compared to HUVECs. Thus we referred to these cells as early reprogrammed ECs (rECs). Expression of *S100A4*, a fibroblast marker, was significantly reduced in the KDR⁺ cells compared to the KDR⁻ cells or control HDFs suggesting suppression of fibroblast nature in the reprogrammed cells (Online Figure VIA). Transduction of HDFs with *ETV2* did not induce expression of pluripotency genes *POU5F1* and *NANOG* (Online Figure VIB and VIC). Immunocytochemistry also confirmed that *ETV2*-infected HDFs at D7 expressed CDH5, KDR, VWF and PECAM1 (Figure 3D and Online Figure VII). Approximately 30% of these unsorted cells took up Ac-LDL and stained for UEA1 lectin (Online Figure VID–VIF). KDR⁺ cells sorted at D7 by magnetic bead-based cell sorting (MACS®) readily formed tubular structures, took up Ac-LDL, and stained for UEA1 lectin indicating their functional endothelial capability (Figure 3E).

To further evaluate vessel-forming capability in vivo, *ETV2*-infected HDFs (D7) were injected into a mouse skin wound model after being labeled with a red fluorescent dye, CM-Dil³⁷. Three weeks later, the animals were perfused with BS-1 lectin (BSL1) and the skin tissue was prepared for histologic analysis. Confocal microscopic examination of the skin demonstrated that the injected cells were either incorporated into vessels and stained for BSL1 or localized in close proximity to the vessels, indicating contribution of rECs to vessel formation in vivo (Figure 3F). Together, these results show that overexpression of *ETV2* was able to convert HDFs to functional endothelial cells through direct reprogramming.

Maturation of early rECs to late rECs via booster transduction of *ETV2*

The low expression of *PECAM1* in these early rECs (Figure 3C) indicated a need for further reprogramming into a mature EC stage. Thus we attempted a number of protocols by varying coating substrates (i.e. collagen, gelatin, Matrigel, OP9 cells), serum percentages, culture media, culture duration, and small molecular epigenetic modifiers, and identified efficient working conditions. The 7 day-sorted KDR⁺ cells, i.e. early rECs, were cultured for another 20 days in EGM-2 medium supplemented with 15% FBS and 20 ng/ml of VEGF-A (Figure 4A). However, the endothelial gene expression was significantly reduced at D30 (Figure 4B). We therefore treated these cells with DOX, together with valproic acid (VPA) for another 6 days to reactivate *ETV2*, expecting enhanced reprogramming³⁸ (Figure 4A), and then further cultivated them in EGM-2 medium supplemented with 5% FBS and 20 ng/ml of VEGFA until D105 (Online Figure VIIIA). Immediately after reactivation of *ETV2* (D36), the endothelial mRNA expression increased again to the levels of early rECs. After

termination of DOX treatment, levels were maintained or increased over the course with *CDH5* and *PECAM1* expression reaching the level of HUVECs at D93 (Figure 4B). Immunocytochemistry showed that these cells stained positive for EC markers such as CHD5, KDR, PECAM1/CD31 and VWF (Online Figure VII). Flow cytometry analyses further confirmed gene expression analyses and immunocytochemistry results, showing a gradual increase in both CDH5⁺ cells and PECAM1⁺ cells, reaching ~83% and 60% at D93, respectively (Figure 4C and 4D). During the long-term culture over 86 days through 12~13 passages, 2×10^5 of early rECs (KDR⁺ cells at D7) were culture-expanded to 2×10^9 at D93. Thus, it was estimated that one HDF at D0 generated approximately 24,900 CDH5⁺ cells, and 18,000 PECAM1⁺ cells, respectively, over 93 days. Endothelial morphologies were maintained throughout the process (Online Figure VIII B). They formed tubular structures in a Matrigel assay with LDL uptake and binding of UEA1 lectin (Figure 4E). Some of these cells also stained positive for 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM), a membrane NO-specific fluorescence indicator (Online Figure VIII C), suggesting that these long-term cultured cells are capable of producing nitric oxide. qRT-PCR analysis demonstrated that *ETV2* expression was 16,000-fold higher in early rECs compared to HDFs at D0 (basal level), but in these long-term cultured cells it was similar to basal levels (Online Figure IX and Figure 4B). This low expression of *ETV2* together with high expression of mature EC markers, particularly *PECAM1*, in these long-term cultured cells suggest their postnatal EC nature and they were referred to as late rECs. Our finding is consistent with the previous report⁷, which showed that suppression of *ETV2* expression is critical for EC maturation during the direct reprogramming process. The expression of EC markers was not induced in HDFs transduced with control viral particles with or without DOX, or HDFs transduced with lentiviral *ETV2* without DOX at D93 (Online Figure IV). We again evaluated direct vessel-incorporating capability of late rECs in vivo. CM-DiI-labeled late rECs were injected into mouse hindlimb ischemia (Figure 4F) and skin wound models (Online Figure X) and the animals were perfused with BSL1 and sacrificed for histologic analysis at two weeks or three months, respectively. Confocal microscopic examination demonstrated that the injected cells were heavily localized around vessels and incorporated into vessels as ECs, clearly indicating contribution of late rECs to vessel formation in vivo (Figure 4F and Online Figure X). Collectively, these results suggest that transient re-induction of *ETV2* together with an epigenetic modifier can mature early rECs into late rECs, which are also functionally competent for vessel formation.

To further determine to what extent *ETV2* re-induction or VPA contributed to the re-expression of EC genes at day 36, we performed experiments with the following combinations: 1) none: no re-induction of *ETV2* (no DOX) and no VPA, 2) VPA: VPA without re-induction of *ETV2* (no DOX), 3) DOX: re-induction of *ETV2* (+DOX) without VPA, 4) DOX/VPA: re-induction of *ETV2* (+DOX) with VPA (Figure 5). These treatments were applied between D30 and D36. When analyzing the expression of CDH5, we found that only groups having re-induction of *ETV2* regardless of VPA treatment (DOX and DOX/VPA groups) had significantly increased numbers of CDH5⁺ cells (Figure 5A and 5B). After removing DOX and VPA from D36, both groups maintained the number of CDH5⁺ cells throughout the culture up to D93, with the numbers always being higher in the DOX/VPA group compared to the DOX alone group. During this period, the VPA-only

treated group showed a slight increase in the number of CDH5⁺ cells. Overall, a similar trend was observed for PECAM1⁺ cells which showed a more drastic decrease in its numbers between D51 and D65 and a later catch-up phenomenon in the DOX and DOX/VPA groups (Figure 5C and 5D). The VPA-only treated group showed a marginal increase in PECAM1⁺ cells only at D93. These results suggest that ETV2 is the major factor for the reprogramming and that VPA plays an additive role.

Long-term cultured rECs display a transcriptome profile similar to HUVECs

We then compared transcriptome profiles of early (Online Figure XIA and XIC) and late rECs (Online Figure XIB and XID) with HDFs and HUVECs. Heat map analyses demonstrated that both early- and late rECs showed significantly enriched EC gene expression and that the pattern of EC gene expression was closer to HUVECs than to HDFs, with late rECs being more similar to HUVECs (Online Figure XIA and XIB). In addition, the patterns of fibroblast genes were more analogous between rECs and HUVECs, with late rECs sharing a closer signature with HUVECs and significant turnoff of many fibroblastic genes (Online Figure XIC and XID). To gain more detailed insight into the transcriptome profile during the conversion process, we performed genome-wide RNA sequencing analysis on late rECs together with HDFs, human microvascular endothelial cells (HMVECs), and HUVECs (Figure 6). We selected 174 EC-related genes (Online Table II) and found that late rECs displayed significant induction of EC-related genes, which was more similar to that of HMVECs and HUVECs than HDFs (Figure 6A and 6B, $p < 2.2e-16$). Moreover, Pearson correlation analysis of expression of all genes showed that late rECs possess a global transcriptional pattern similar to that of HMVECs and HUVECs (Figure 6C, Correlation coefficient=0.915 and 0.916, respectively). Through Gene Ontology (GO) analysis with 9,290 differentially-expressed genes (Online Table III), we found that genes specifically expressed in late rECs were related to blood vessel morphogenesis and muscle organ development. Compared to HDFs, late rECs were mainly enriched with GO terms associated with endothelium development, endothelial cell proliferation and epithelial tube morphogenesis (Figure 6D–6F). Comparison of expression profiles (Venn Diagram) of late rECs with those of HMVECs and HUVECs showed that 93% of up-regulated genes in late rECs were also up-regulated in either HMVECs or HUVECs and 75% of down-regulated genes in late rECs were also down-regulated in either HMVECs or HUVECs or (Figure 6E and 6F). Together, these gene expression analyses demonstrated that rECs, particularly late rECs, closely resemble mature ECs.

Early rEC transplantation enhances recovery from limb ischemia and increases neovascularization

Despite immature EC features, early rECs exhibited functional EC characteristics in both *in vitro* and *in vivo* assays as described above. Thus, we investigated the therapeutic effects of early rECs on repair of tissue ischemia. To this end, we intramuscularly injected early rECs into nude mice in a hindlimb ischemia model (Figure 7). Laser Doppler perfusion imaging revealed significantly enhanced blood perfusion in the early rEC-injected limbs compared to the HUVEC-, HDF (transduced with the empty vector + DOX)- or phosphate-buffered saline (PBS)-injected limbs at 1, 2, 3, and 4 weeks (Figure 7A and 7B). Mice receiving the early rECs showed lower limb loss scores at day 28 compared to the HUVEC-, HDF- or PBS-

injected mice, indicating better tissue repair (Figure 7C and Online Figure XIIA). The capillary density in the hindlimb muscle was significantly higher in the early rEC-injected mice than in the HUVEC, HDF-, or PBS-injected mice at day 28 (Figure 7D and Online Figure XIIB). Again, confocal microscopic examination demonstrated incorporation of the rECs labeled with CM-Dil into the vasculature of the ischemic hindlimbs (Figure 7E). Next, to further validate these observations and quantify the incorporated rECs into the vasculature of the hindlimbs, we first transduced HDFs with lentiviral *ETV2* as well as *eGFP* (to avoid any potential problems with CM-Dil), and sorted KDR^+eGFP^+ cells at D7 for injection into the ischemic hindlimbs. Twenty-eight days after the injection, the tissues were harvested and imaged. Three dimensional confocal microscopic analysis revealed that $eGFP^+$ cells were clearly co-localized with BSL-Rhodamine-labeled blood vessels (Figure 7F and Online Figure XIIB and XIIC). Quantitatively, we found that approximately 7% of the injected rECs were incorporated into or anastomosed to the blood vessels in the hindlimb tissue (Online Figure XIIC). Concordantly, detailed gene expression analysis revealed that early rECs exhibited higher expression levels of representative angiogenic factors such as VEGFA, FGF2, ANGPT1, and MMPs compared to HUVECs (Figure 7G). Together these data suggest that early rECs can efficiently enhance recovery of hindlimb ischemia and promote postnatal neovascularization through both direct vascular incorporation and angiogenesis.

DISCUSSION

Recent endothelial reprogramming approaches utilized pluripotency factors, multiple vascular TFs, or embryonic cells, which limit their application in cell therapy or disease investigation⁷⁻¹⁰. In our study, several innovations were made in endothelial reprogramming strategies. First, this study demonstrated that human postnatal cells can be directly converted to ECs, overcoming the major obstacle to using autologous cells for reprogramming. Second, we found that *ETV2* was able to induce reprogramming of HDFs to ECs. This study uncovered a novel role of *ETV2* in fate changes of differentiated cells into ECs. Third, we also identified two different stages of reprogrammed ECs: early and late rECs. Finally, we further demonstrated the therapeutic utility of *ETV2*-induced rECs in repairing tissue ischemia and enhancing neovascularization *in vivo*.

We found that there were two different stages of reprogrammed ECs. Early rECs, which appeared within a week after transduction of *ETV2*, showed characteristics of immature ECs, with mixed signatures of ECs and fibroblasts. The expression of PECAM1 and VWF was low while other endothelial genes or proteins were highly expressed. However, early rECs were capable of taking up Ac-LDL, formed tubular structures, and contributed to vessel formation in animal models, suggesting functional competency as ECs *in vitro* and *in vivo*. These cells were expandable in culture, enabling their use for cell therapy. Although some fibroblast features remained, one could argue that this may be beneficial, as the paracrine pro-angiogenic effects of fibroblasts can augment vessel formation^{39, 40}. The early rECs were more enriched with angiogenic factors such as VEGFA, FGF2, ANGPT1, and MMPs than HUVECs, and after direct injection into ischemic hindlimbs, early rECs demonstrated robust reparative effects for tissue ischemia and promoted vessel formation *in vivo*. Regarding the mechanisms underlying therapeutic and vessel-forming effects of early

rECs in the hindlimb ischemia model, given the effects of rECs on rapid perfusion recovery and vessel formation, the observed effects appear to be attributed more to non-cell autonomous effects such as secretion of angiogenic factors than to direct incorporation of early rECs into the vessels. With future clinical application in mind, for this study, we selected early rECs for testing therapeutic effects on tissue ischemia. The shorter duration of culture and fewer transductions of ETV2 required for early rEC generation are advantages for clinical use.

Late rECs, which appeared after the second round of ETV2 transduction, showed features of more mature stage ECs. We made a number of attempts to increase mature EC genes in early rECs. Our initial approach of continuous overexpression of ETV2 did not work, down-regulating its own expression and most endothelial genes. After a 20-day window without transgene overexpression, early rECs responded to 6-day overexpression of ETV2. After this booster transduction, no evident dip was noted in the expression of CDH5, KDR, CD34 or VWF. We used VPA for enhancing reprogramming as reported for other cell types³⁸. While VPA did not affect reprogramming toward early rECs (data not shown), it played an additive role for induction to late rECs. Late rECs showed dramatically increased PECAM1 expression from ~2% to ~60% by flow cytometric analyses, suggesting a transition to a more mature endothelial phenotype over long-term culture. The mature nature of late rECs was supported by global gene expression analyses that showed repression of fibroblast-specific genes, more robust expression of mature EC genes, minimal expression of ETV2, and production of NO, mimicking the phenotype of HUVECs and HMVECs. Late rECs also had the capability to contribute to vessel formation in vivo, and persisted in vessels as ECs at least 3 months post-injection, indicating long-term durability in vivo. Due to their mature nature, late rECs could be more optimal for the purpose of pathophysiological investigation of vascular disease or drug discovery.

Han *et al.* showed that a combination of five transcription factors (FOXO1, ETV2, KLF2, TAL1, and LMO2) was able to directly convert mouse fibroblasts into ECs¹⁴. Although the four factors without ETV2 resulted in reduced generation of TIE2⁺ cells as a readout of ECs from the fibroblasts, the authors observed that ETV2 was incapable of converting fibroblasts into ECs. However, our study demonstrated that ETV2 alone could reprogram human fibroblasts into ECs. Similar to our finding, Morita *et al.* reported that the single factor ETV2 was able to directly convert HDFs to ECs, referred to as ETVECs⁴¹. However, there are major differences between the two studies. First, the authors claimed that transient induction of ETV2 at the initial stage of the process was sufficient for the conversion into and the maintenance of ETVECs. However, this study demonstrated continued overexpression of ETV2 in their ETVECs. ETVECs are PECAM1^{high} cells selectively sorted at 15 days after ETV2 overexpression and expressed ETV2 at very high levels during the culture period of more than 50 days. It is well known that ETV2 is not or minimally expressed in any mammalian postnatal ECs⁴²⁻⁴⁴. Indeed, sustained expression of ETV2 in vascular endothelial cells during embryogenesis leads to vascular abnormality as evidenced by dilated vessels in the extraembryonic yolk sac⁴⁵, suggesting that a transient expression of ETV2 is critical for ensuring proper development of the vascular system. In agreement with this report, a study showed an inverse relationship between the expression of ETV2 and PECAM1 during the direct conversion of non-ECs to ECs⁷. Further, they found that short-

term, but not continued, expression of ETV2 in the early phase of the reprogramming process is critical for the generation of induced ECs with a mature phenotype. Thus, we argue that ETVECs are not reprogrammed or induced ECs but rather selected cells that show the ectopic expression of EC markers and some characteristics of ECs due to overexpression of ETV2. Since key EC genes including CDH5 and PECAM1 are direct targets of ETV2^{46, 47}, extreme overexpression of ETV2 leads to forced expression of such EC genes in selected cell colonies, as shown in their study. This also explains why ETVECs highly express PECAM1, a direct downstream target of ETV2, from the beginning and throughout, and why no maturation process was shown or observed in the study throughout the duration of culture. In contrast, our study found that PECAM1, which represents a more mature marker, showed low expression at an early stage of reprogramming when ETV2 was highly expressed but PECAM1 expression at both the mRNA and protein levels was progressively and concordantly increased over maturation (Figure 5). Most importantly, our late rECs, had minimal expression of transduced ETV2, which is an obligatory characteristic of genuine postnatal human ECs. Moreover, late rECs, but not ETVECs, produced nitric oxide, which provides additional evidence of mature ECs. Second, our study demonstrated that there is discontinuity in the reprogramming process toward rECs and continuous expression of ETV2 cannot sustain a phenotype of rECs and mature rECs. Only after an ETV2-free period followed by temporary re-induction of ETV2 could the phenotype of early rECs be surpassed, entering into the maturation process. Third, Morita's study reported an important role for FOXC2 in the induction of reprogramming by ETV2. However, we found that overexpression of FOXC2 was dispensable in EC reprogramming. Fourth, there is much critical information missing in their study⁴¹. For example, there is no information regarding the reprogramming efficiency or the role of ETVECs in *in vivo* studies. Also, the vascular incorporation capability is unconvincing.

Elucidating mechanisms of direct endothelial conversion is crucial for understanding the reprogramming process and enhancing the efficiency of generating reprogrammed ECs. It remains to be determined how ETV2 activates EC genes while suppressing fibroblast genes during the reprogramming process. Although ETV2 is a potent vasculogenic TF upregulating endothelial genes in development^{21, 26, 48, 49}, as yet no studies have demonstrated the mechanisms by which ETV2 directly reprograms somatic cells to ECs. We have previously shown that ETV2 can induce *de novo* formation of KDR⁺ cells through direct binding to the promoter of the gene²¹. These results have been supported by other studies^{26, 50-52}. In a subsequent study, using a genome wide ChIP-sequencing approach, we have clearly demonstrated a significant induction of genes critical for endothelial cell generation and function by ETV2⁴⁶. Together with our results showing rapid and significant induction of endothelial genes upon ETV2 transduction into HDFs, it is likely that the reprogramming process can also be achieved in part by direct action of ETV2 on vasculo-angiogenic genes. Moreover, since overexpression of ETV2 in fibroblasts induced global changes in gene expression, ETV2 might have directly modulated epigenetic targets. In fact, ETV2 was shown to interact with Jmjd1a^{53, 54}, a histone demethylase. Thus, it is tempting to speculate that overexpressed ETV2 in HDFs not only activates endothelial genes directly, but also induces epigenetic changes, probably through interactions with histone and/or DNA modifying molecules. As intriguing are the mechanisms underlying maturation or transition

of early rECs to late rECs, because in adult endothelial cells, ETV2 is not, or is very minimally, expressed^{42–44, 55}. Moreover, it remains to be determined why VPA has additive effects in promoting reprogramming into late rECs. Further studies are necessary to address the mechanisms of ETV2-mediated EC reprogramming.

Taken together, the successful demonstration of direct reprogramming of human postnatal somatic cells into ECs via a single TF, ETV2, in this study will facilitate the understanding of direct reprogramming of fibroblasts to ECs and its utilization in biomedical science. Specifically, this novel reprogramming approach and the resultant two stages of rECs will enable autologous cell therapy, individualized drug testing, and personalized disease investigation for various cardiovascular diseases. The next step would be to employ non-integrating episomal vectors or substitute a non-genetic method for lentiviral vectors, as were recently developed for iPSC generation^{56–60}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

rECs	Reprogrammed Endothelial Cells
TFs	Transcription Factors
iPSCs	induced Pluripotent Stem Cells
ESCs	Embryonic Stem Cells
DOX	Doxycycline

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NOVELTY AND SIGNIFICANCE

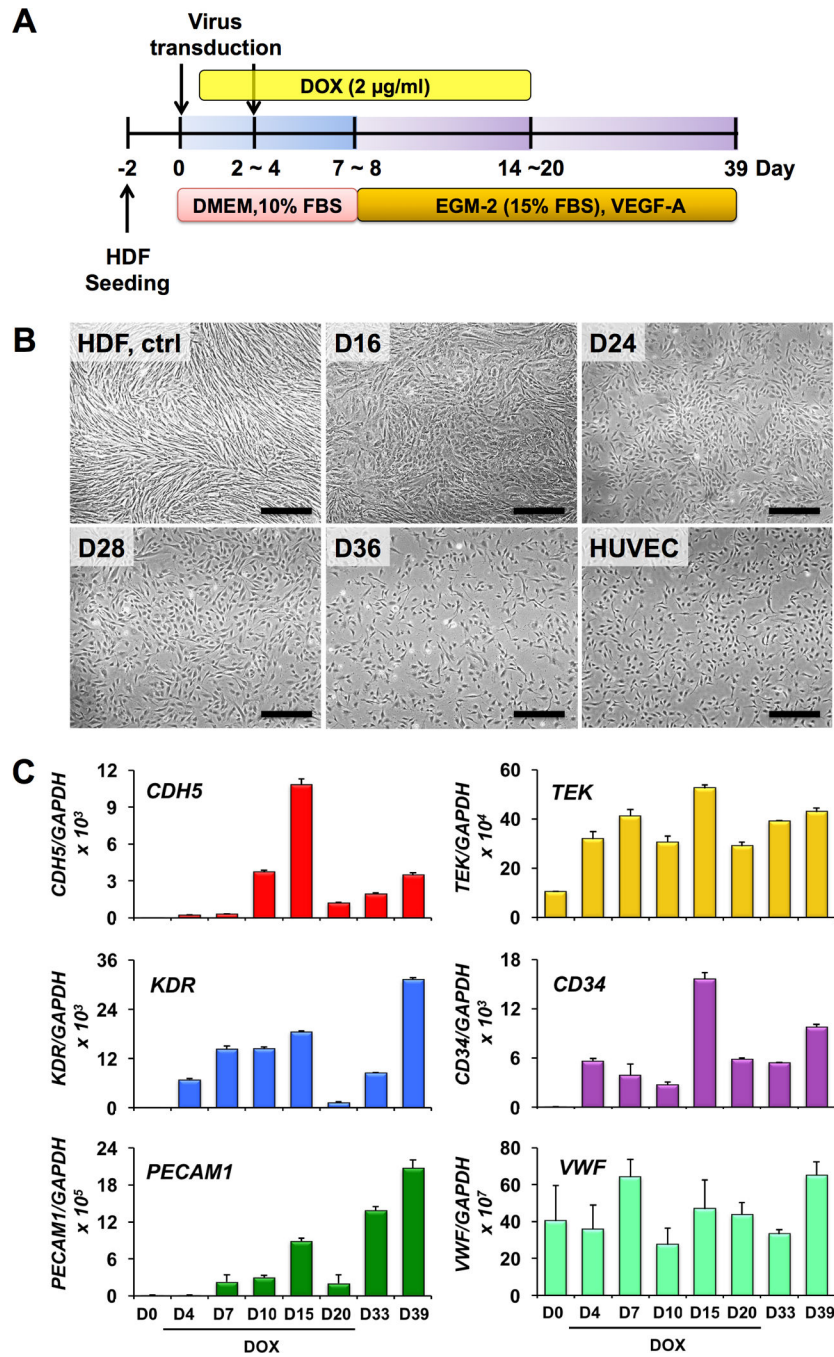
What is Known?

- Vascular Endothelial cells (ECs) plays key roles in vessel formation and ischemic tissue repair.
- ETV2 is an indispensable transcription factor for endothelial lineage specification during development.
- Direct lineage conversion or reprogramming is an emerging technology to generate target cells via overexpression of cell-type-specific transcription factors, bypassing the pluripotent stem cell stage.

What New Information Does This Article Contribute?

- ETV2 alone is sufficient to induce direct conversion of human dermal fibroblasts into ECs, referred to as reprogrammed ECs (rECs).
- Implantation of rECs promote neovascularization and ischemic tissue repair.
- We found at least two different stages of rECs, early and late rECs, which respectively demonstrate immature and mature EC characteristics.

Direct reprogramming with cell-type-specific TFs emerged as a promising strategy to generate target cells. Although studies demonstrated the feasibility of direct conversion of non-ECs into ECs, no studies have shown direct reprogramming of human postnatal cells into mature ECs with endothelial TFs. Among various combinations of seven EC TFs, we found ETV2 alone was sufficient to reprogram human fibroblasts into ECs. We further found that direct reprogramming by ETV2 generated at least two distinct stages of rECs: early and late rECs. Early rECs exhibited characteristics of immature ECs, but had functional and therapeutic potential for repairing ischemic disease and promoting vessel formation. Late rECs, which appeared after further cultivation of early rECs with a second round of ETV2 transduction showed features of more mature ECs and the ability to contribute to vessel formation. These new types of ECs generated by direct reprogramming provide novel opportunities for autologous cell therapy for cardiovascular diseases, drug discovery, disease modeling, and precision medicine. Moreover, this reprogramming protocol as well as the two types of rECs can allow investigation of yet unknown mechanisms of direct endothelial reprogramming from fully differentiated somatic cells.



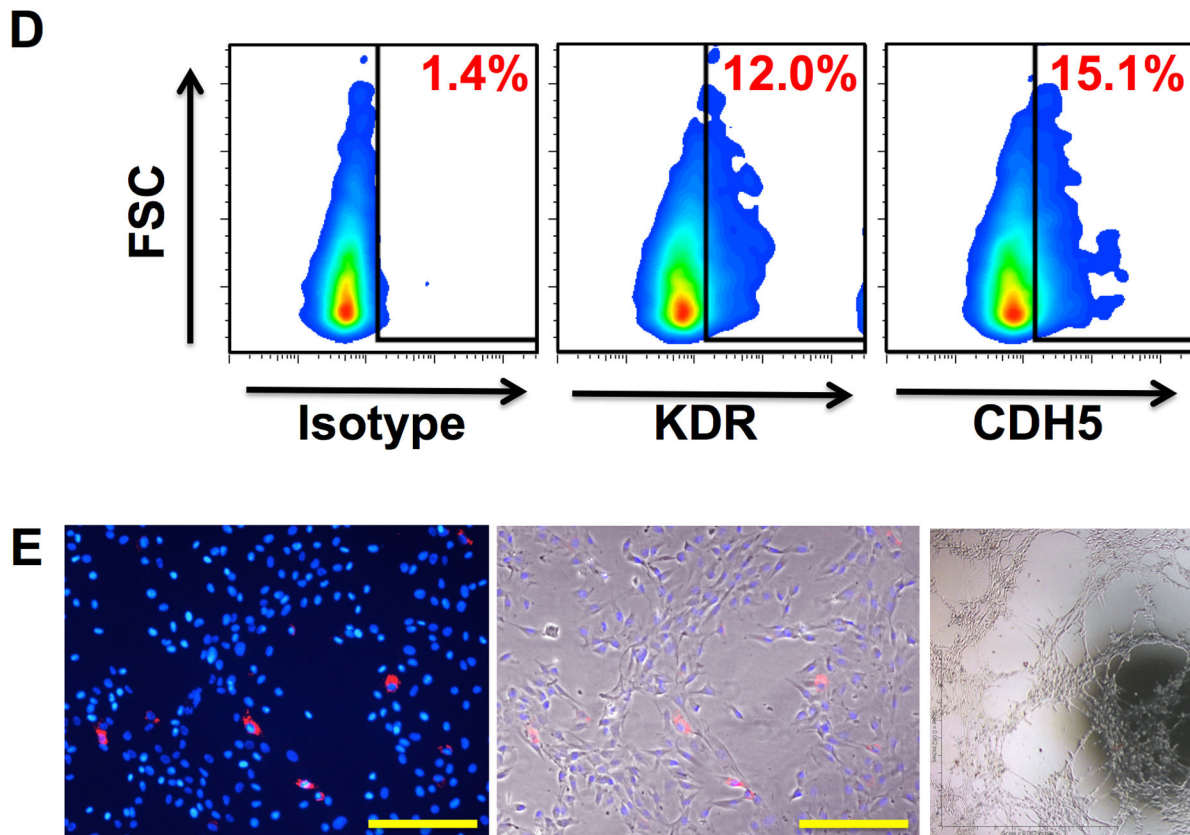


Figure 1. Reprogramming of HDFs to ECs with six EC transcription factors

(A) A schematic of the reprogramming protocol. (B–E) Endothelial characteristics of HDFs infected with lentiviral particles of six TFs (ETV2, FOXC2, MEF2C, SOX17, NANOG, HEY1). The infected HDFs exhibited cobblestone appearance (B), expressed endothelial genes and proteins measured by qRT-PCR (C) and flow cytometry (D), and were able to take up Ac-LDL (red) and form tubular structures (E). Scale bars: (B) 400 μ m, (E) 200 μ m (yellow) and 1 mm (black). All data are presented as mean \pm s.e.m.; three independent experiments, each with technical triplicates.

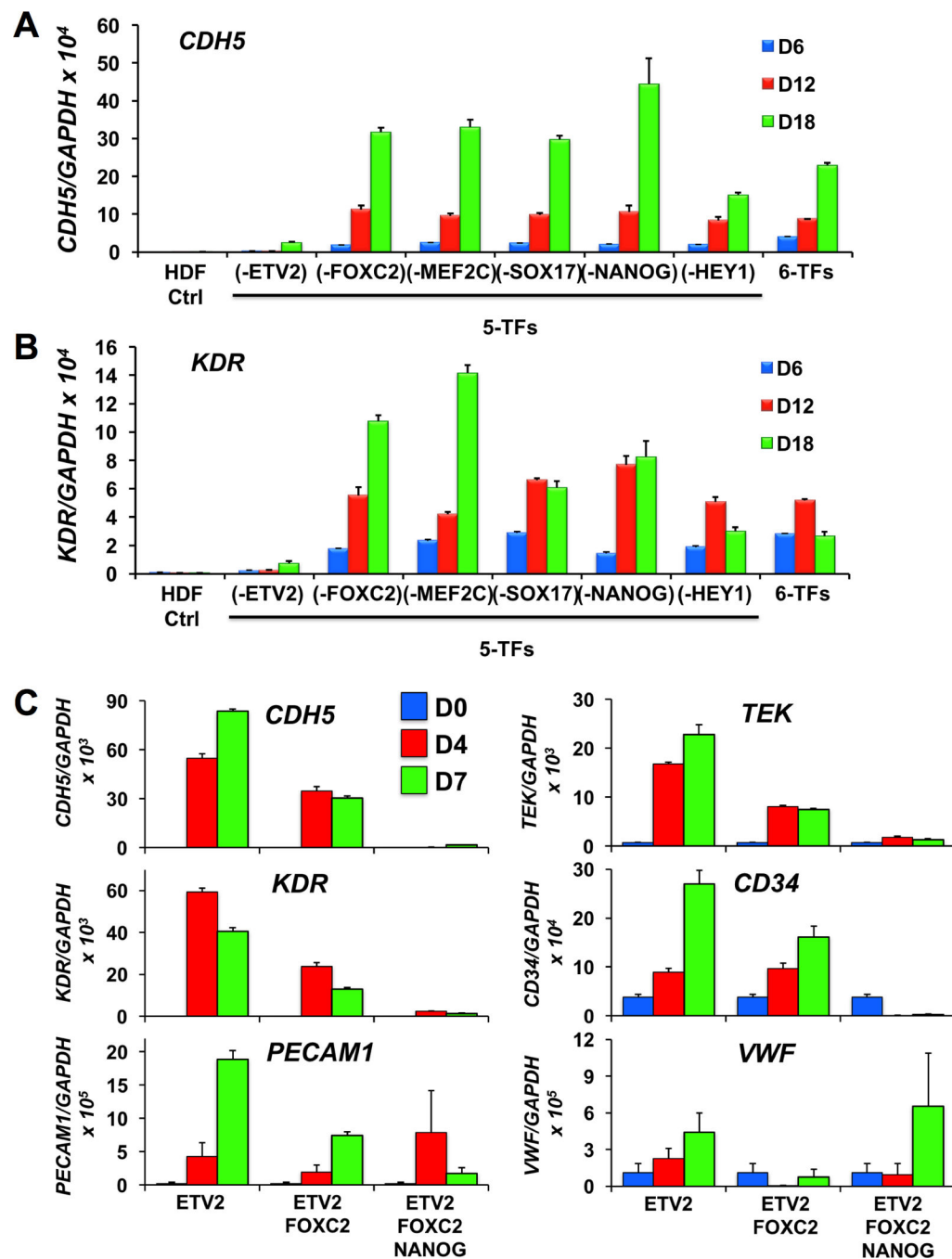
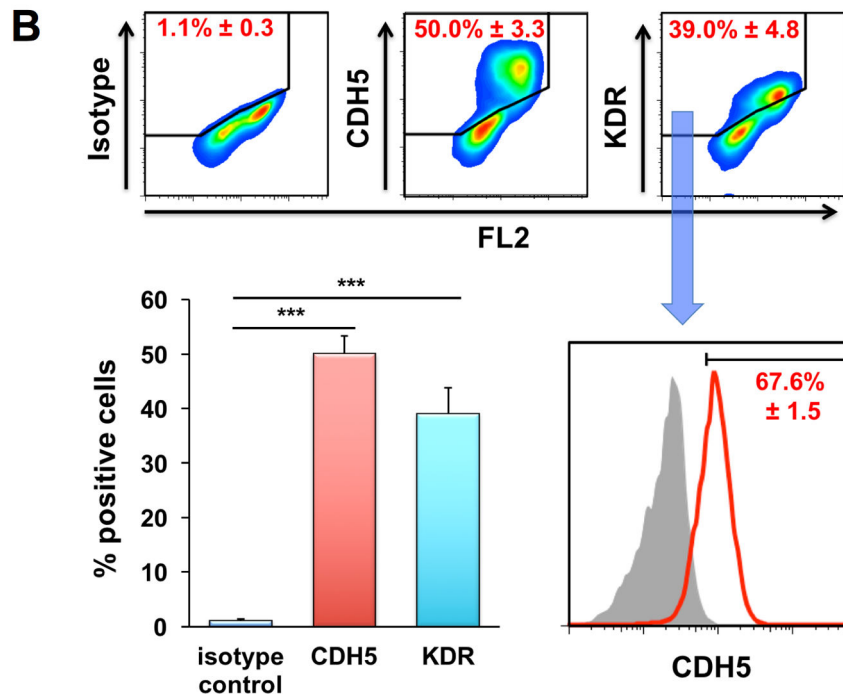
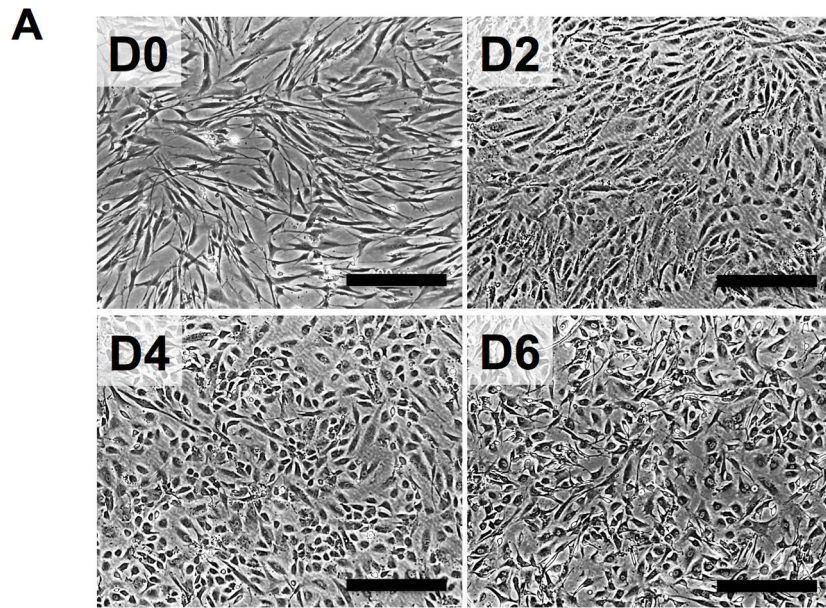
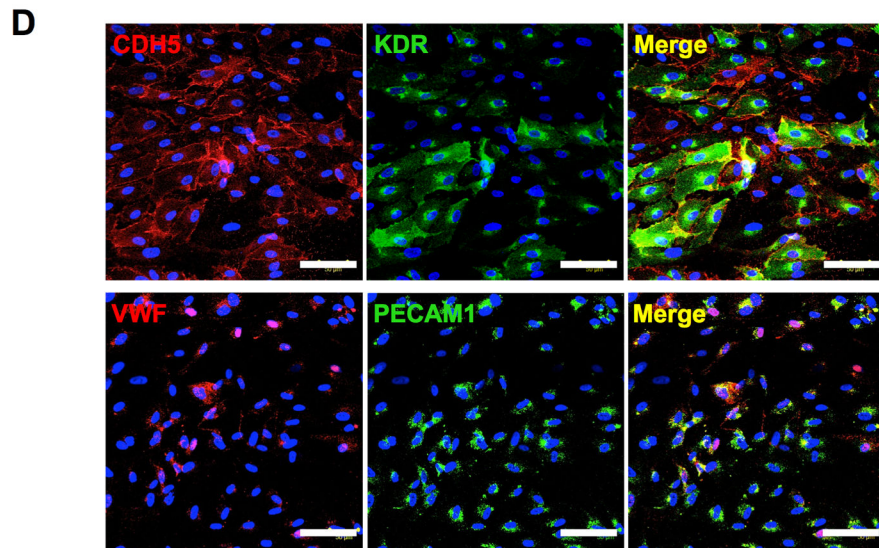
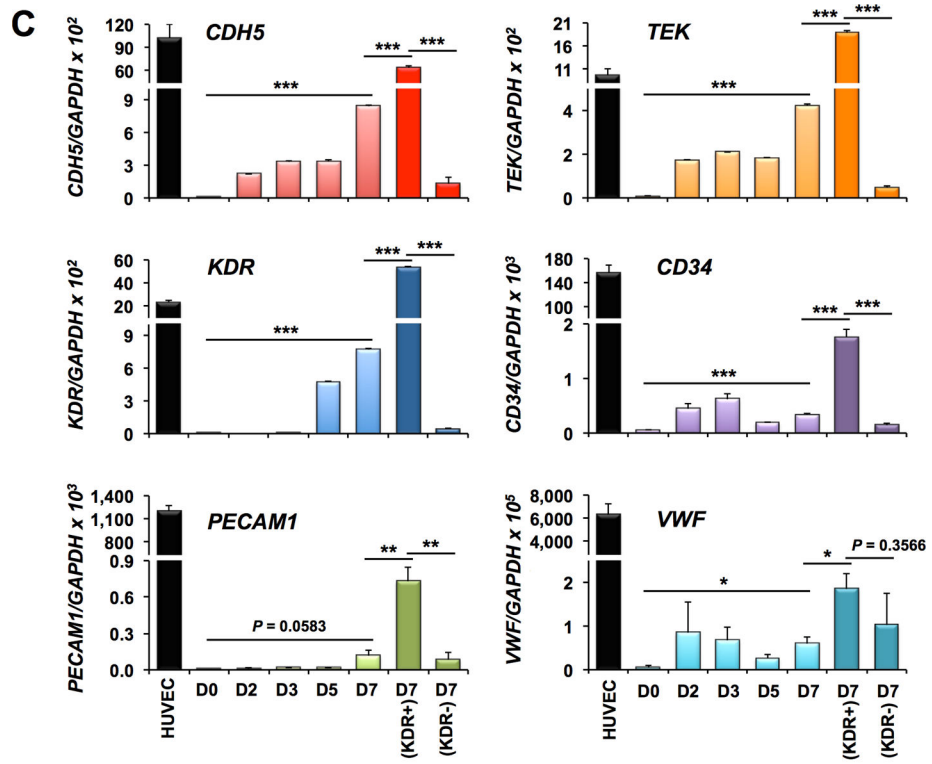


Figure 2. ETV2 is essential for reprogramming of HDFs to endothelial cells

(A and B) qRT-PCR results of HDFs infected with combinations of five TFs for *CDH5* (A) and *KDR* (B) at day (D) 6, 12, and 18. (C) qRT-PCR results of HDFs infected with one to three TFs including *ETV2* for various EC genes at D0, 4, and 7. All data are presented as mean \pm s.e.m.; three independent experiments, each with technical triplicates.





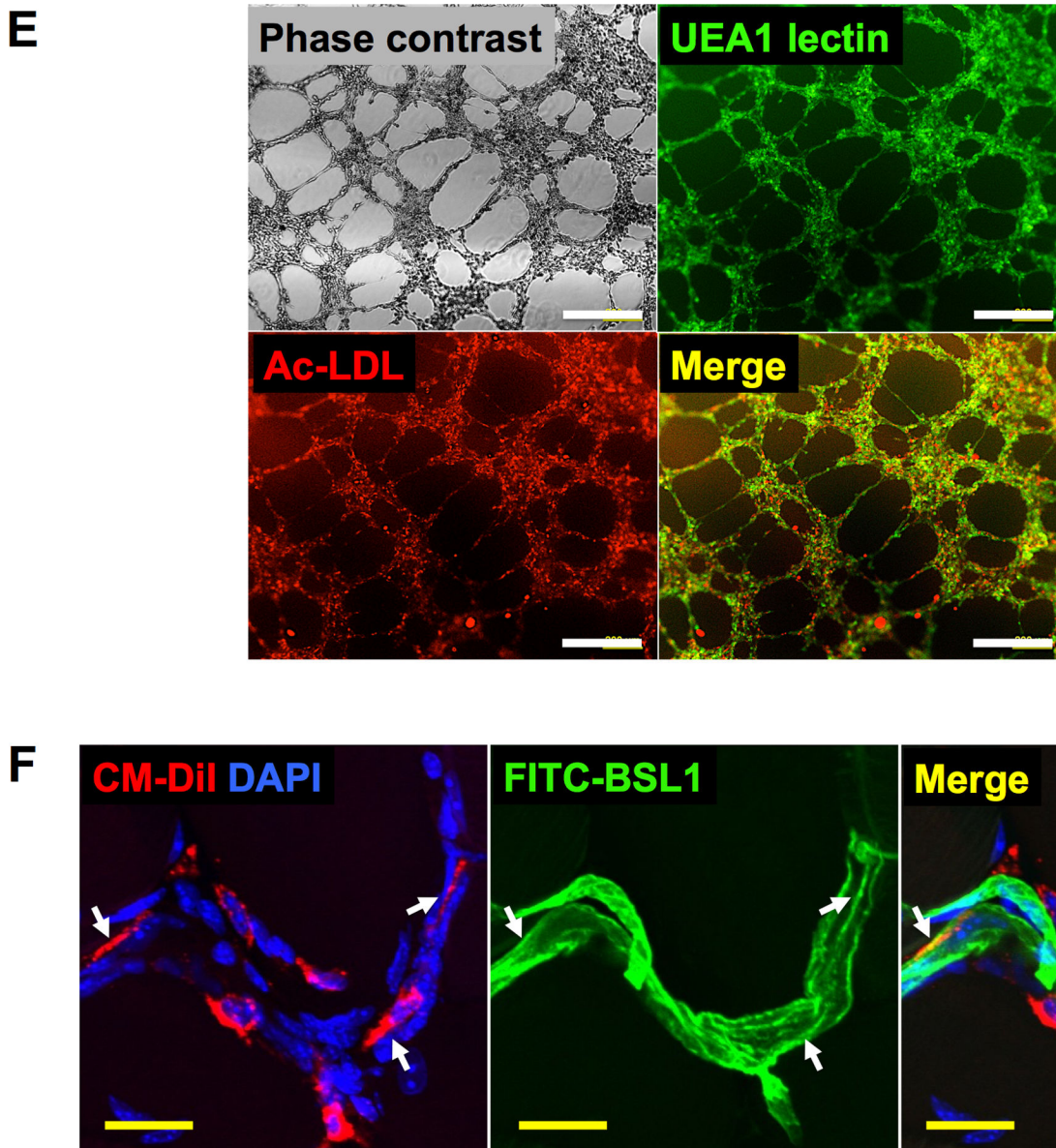
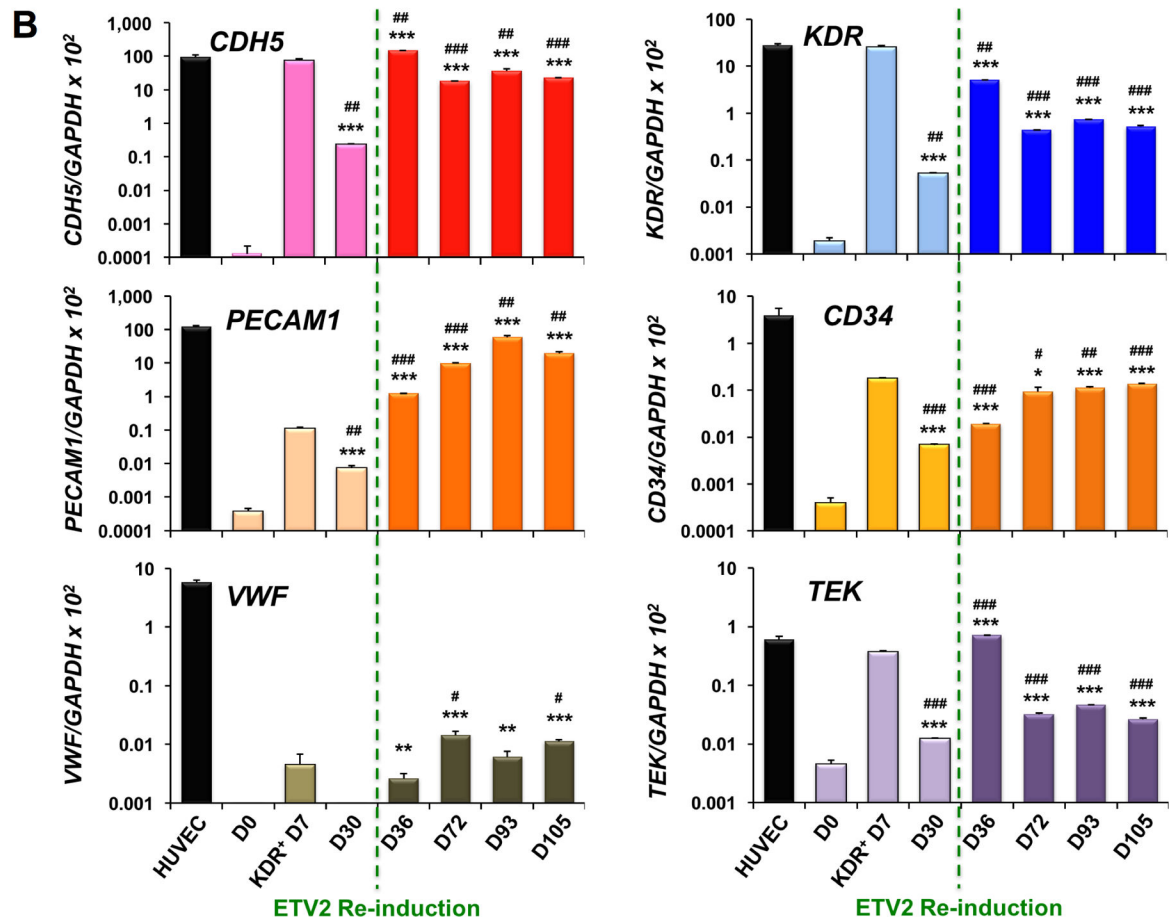
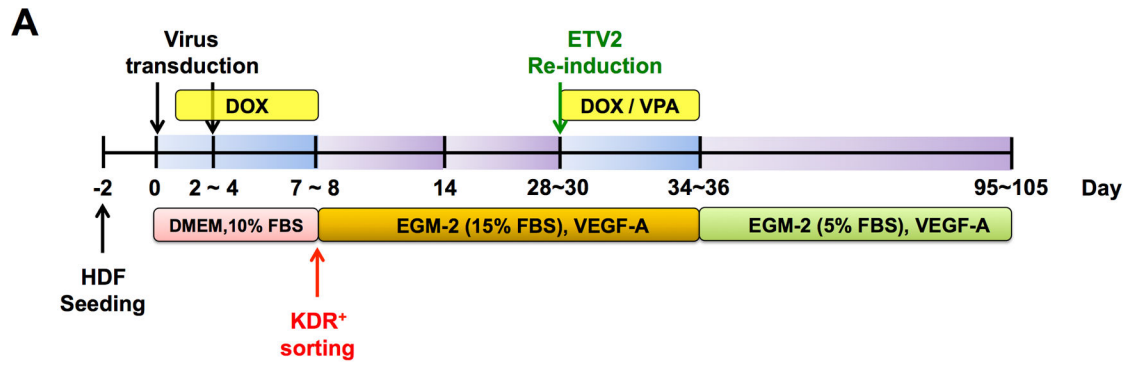


Figure 3. Endothelial characteristics of the single factor ETV2-transduced, short-term cultured HDFs

(A) Morphologic changes showing emergence of cobblestone appearance in HDFs as early as D2 after transduction with *ETV2*. (B) Flow cytometric analyses of transduced HDFs at D7 showing expression of CDH5 and KDR. It also shows that about 67% of KDR⁺ cells expressed CDH5. All data are presented as mean \pm s.e.m.; three independent experiments, each with technical triplicates. *** $P < 0.001$ vs. isotype control, standard unpaired student *t* test. (C) qRT-PCR analyses demonstrated induction of various endothelial genes in ETV2-transduced HDFs. At D7, the results of unsorted cells and the sorted KDR⁺ and KDR⁻ cells are shown. KDR⁺ cells showed significantly higher endothelial gene expression (except *VWF*) compared to unsorted (D7) and KDR⁻ cells. All data are presented as mean \pm s.e.m.; three independent experiments, each with technical triplicates. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, standard unpaired Student's *t* test. (D) Immunohistochemistry showed expression of

CDH5 and KDR (upper panel), and VWF and PECAM1 (lower panel) in transduced HDFs at D7. (E) The sorted KDR⁺ cells at D7 formed tubes on Matrigel, also showing Ac-LDL uptake (red) and UEA1 lectin (green) binding. (F) Contribution of reprogrammed HDFs to vessel formation. The transduced HDFs at D7 were labeled with CM-Dil (red), and injected into the wounded skin of mice. Three weeks later, the mice were perfused with BS1 lectin (FITC-BSL1), and tissues were processed for confocal microscopic imaging. Injected cells (red) were either incorporated into the blood vessels as shown by colocalization with BSL1 (green) (arrows) or localized in close proximity to the vessels, indicating contribution to vessel formation. DAPI (blue). Scale bars: (A, E) 400 μ m, (D) 100 μ m, (G) 25 μ m.



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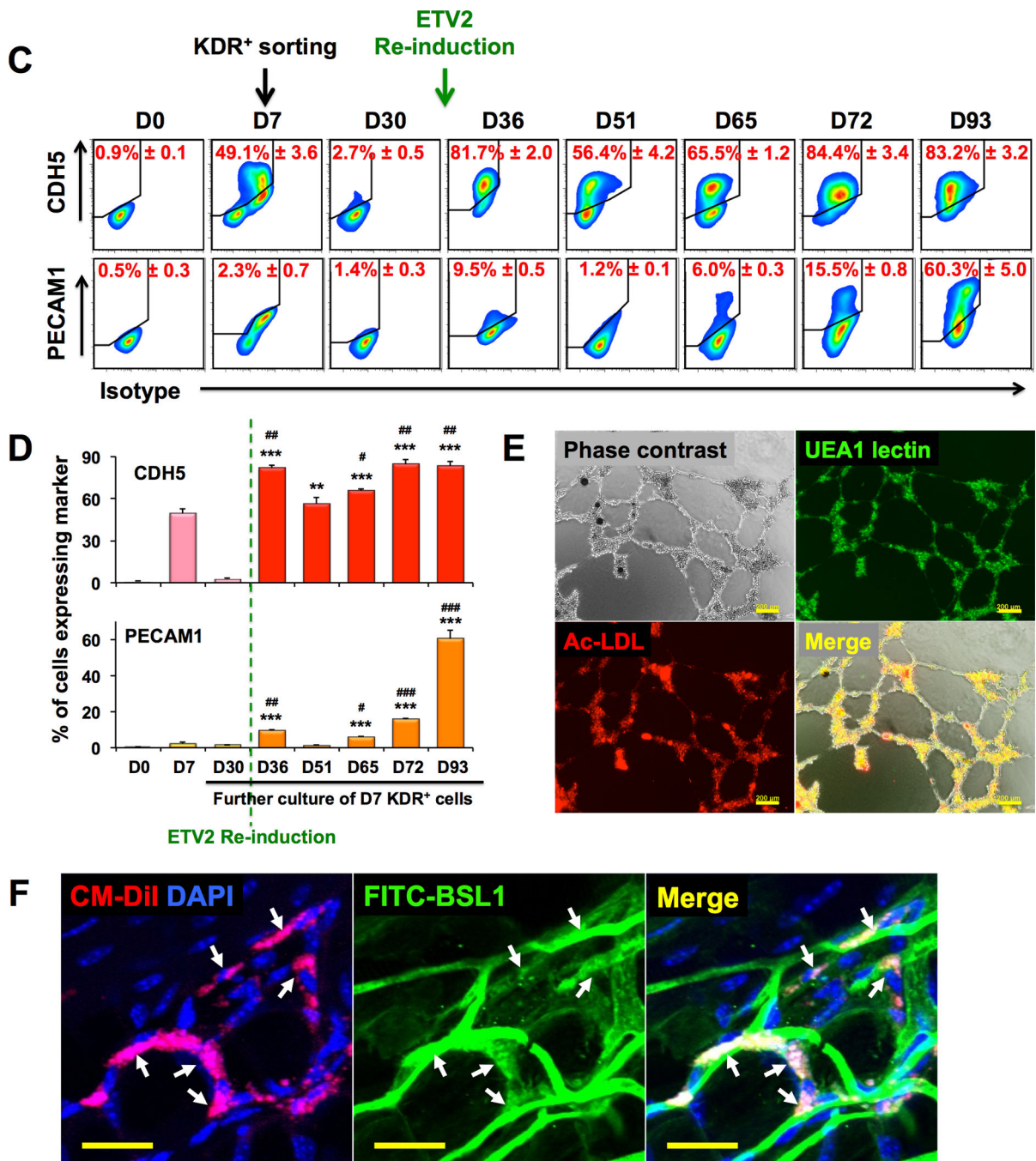
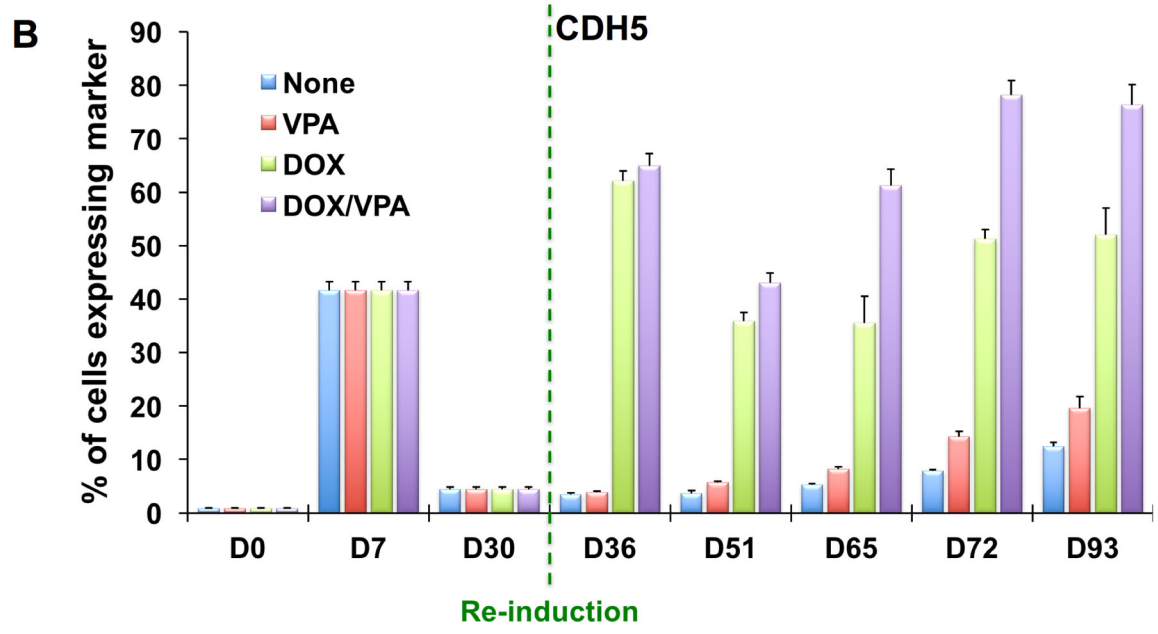
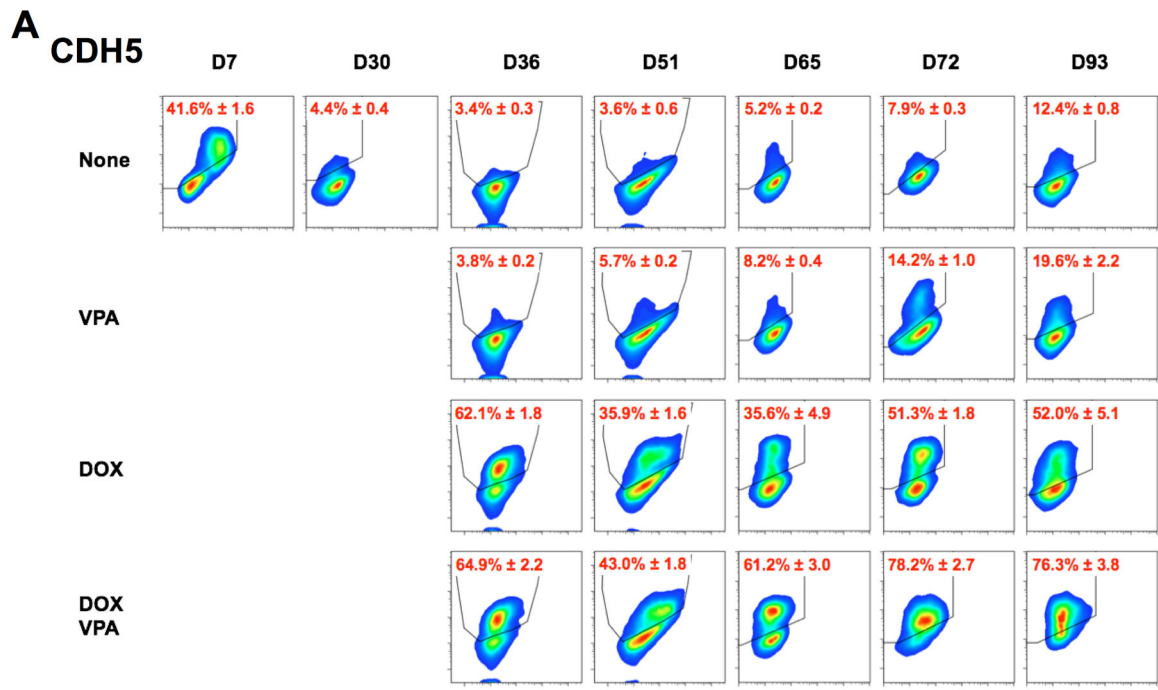


Figure 4. Endothelial characterization of long-term cultured KDR⁺ cells isolated from ETV2-transduced HDFs, late rECs

(A) A schematic of the culture protocol for long-term cultured ETV2-transduced HDFs, late rECs. (B–D) Endothelial characteristics of the long-term cultured KDR⁺ cells *in vitro* which were FACS-sorted from ETV2-transduced HDFs at D7. qRT-PCR analysis (Y axis shown in log scale) (B) and flow cytometric analysis (C, D) demonstrated increased expression and maintenance of EC genes and proteins to D93. The green lines or arrows indicate the time point of reinduction of ETV2 expression via DOX. Note that ~60% of the cells are positive

for PECAM1 at D93 (D). All data are presented as mean \pm s.e.m.; three independent experiments, technical triplicates/experiment for qRT-PCR analysis, single/experiment for flow cytometric analysis. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, vs. D0; ### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$, vs. D7, standard unpaired Student's t test. The cells at D93 were able to form tubular structures and stained positive for Ac-LDL and UEA1-lectin (E). (F) Cells prepared at D93 were labeled with CM-Dil and injected into mice in a hindlimb ischemia model. The mice were perfused with FITC-BSL1 at 3 months, and subjected to immunohistochemistry for confocal microscope imaging. Injected cells (red) were either incorporated into the blood vessels and expressed BSL1 (green) (arrows) or localized close to the vessels, indicating contribution to vessel formation. DAPI (blue). Scale bars: (E) 200 μm , (F) 25 μm .



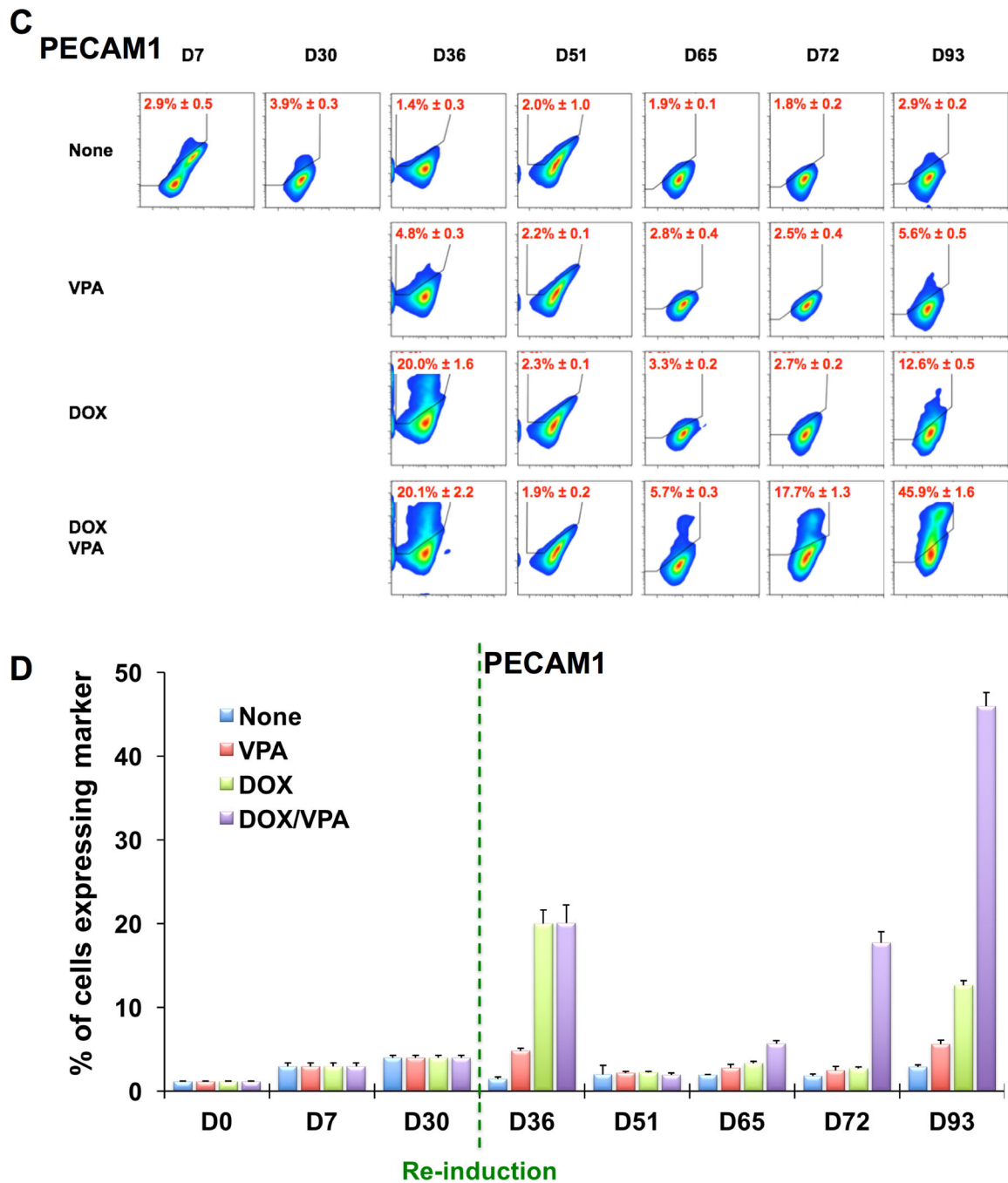


Figure 5. Flow cytometric analysis of rECs after re-induction of ETV2 by DOX and treatment with VPA

Flow cytometric analyses of CDH5 (A and B) and PECAM1 (C and D) in rECs after the following treatments. None (blue): no re-induction of ETV2 (no DOX) and no VPA, VPA (red): VPA without re-induction of ETV2 (no DOX), DOX (green): re-induction of ETV2 (+DOX) without VPA, DOX/VPA (purple): re-induction of ETV2 (+DOX) with VPA.

Shown are the representative plots of flow cytometry (A and C) and their quantification results (B and D). All data are presented as mean ± s.e.m.; three independent experiments.

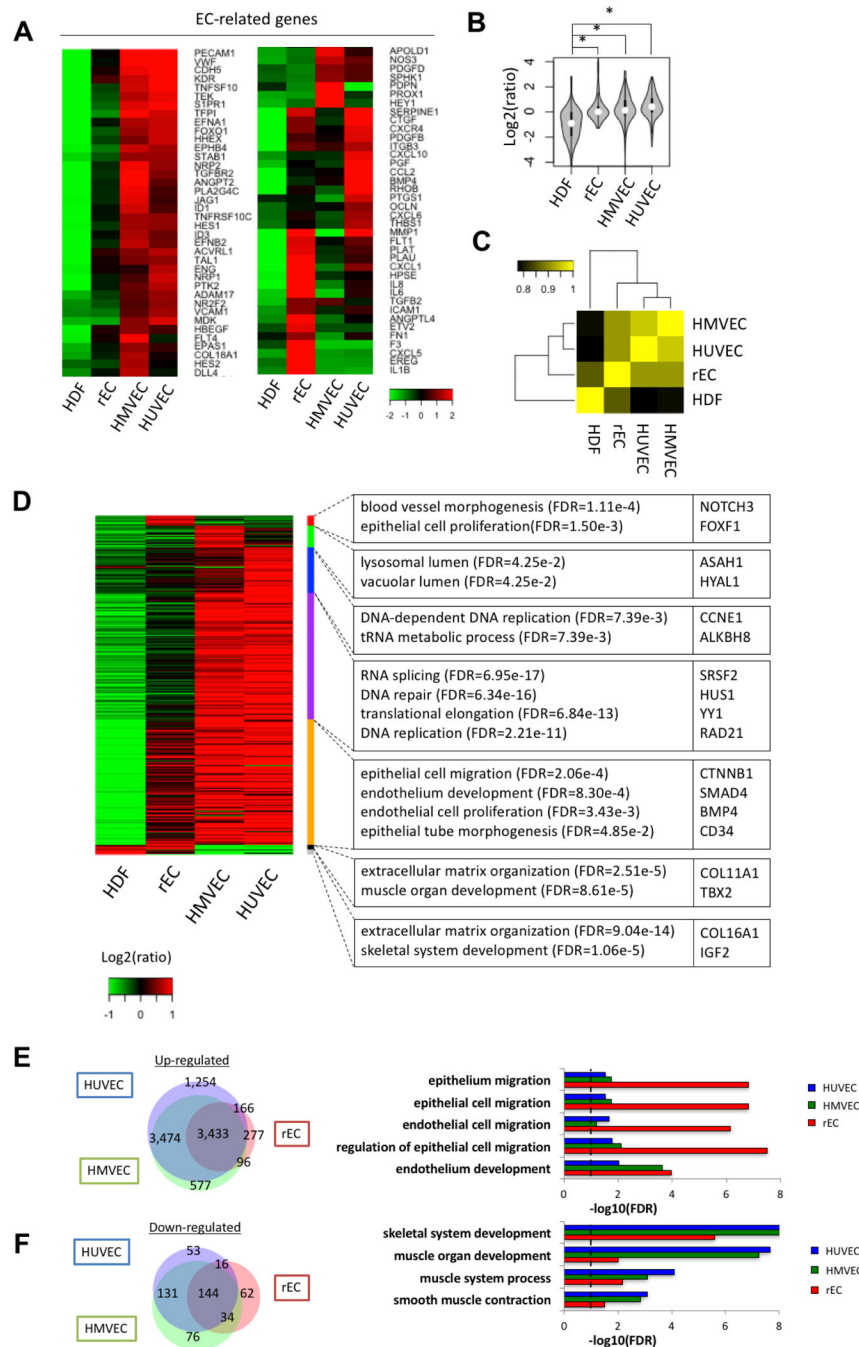
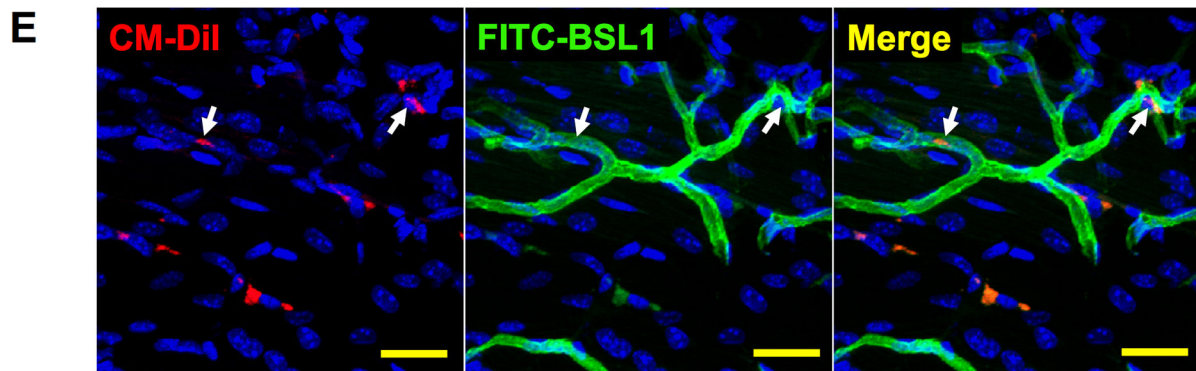
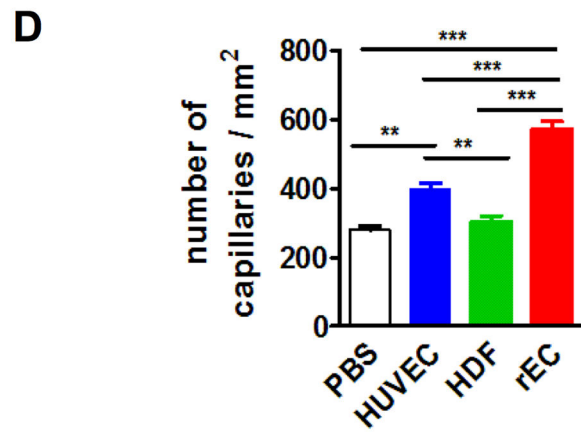
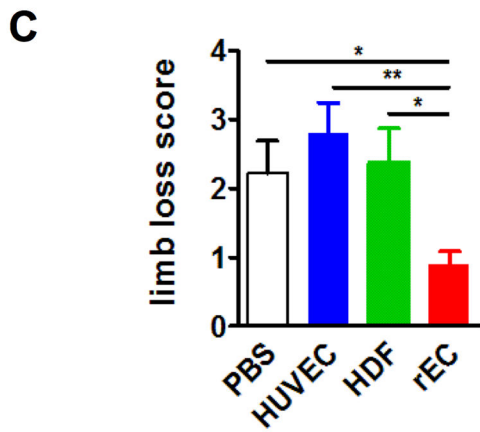
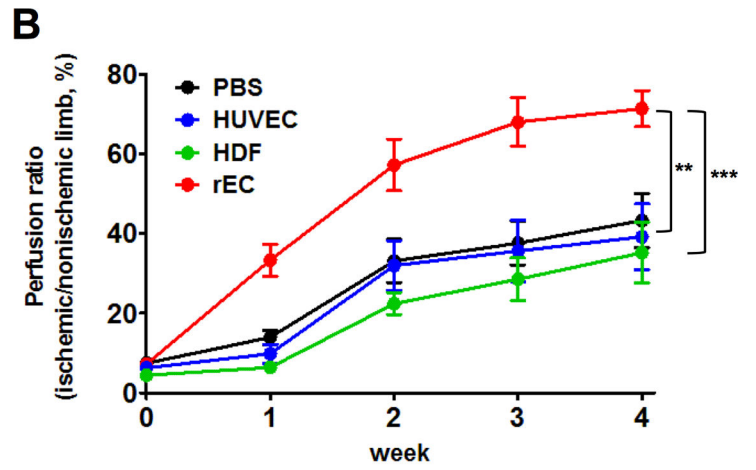
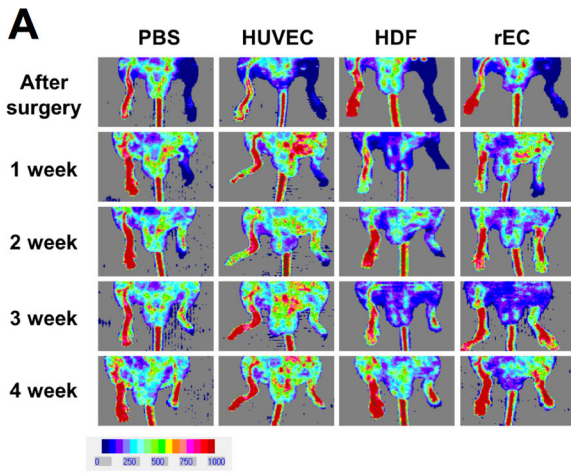


Figure 6. Transcriptome analysis on late rECs via RNA sequencing

(A) Heatmap representing EC-related genes with 2-fold difference. Expression values relative to the average expression values across all samples were represented by colors from green to red (log₂ scale). (B) Comparison of EC-related genes among four samples. **P* < 0.05 by two-sided Student's *t* test. (C) Global gene expression similarity among HDFs, rECs, HMVECs and HUVECs. The color from black to yellow represents Pearson correlation. (D) Heatmap showing differentially-expressed genes in rECs, HMVECs and HUVECs compared to HDFs. Relative expression levels to median value with log₂ scale

were shown by green to red colors. Representative GO terms and gene symbols were also shown in right panel. **(E)** Overlap of highly-expressed genes (> 2 fold) among rECs, HMVECs and HUVECs. The right panel represents significant GO terms of up-regulated genes. **(F)** Overlap of lowly-expressed genes (> 2 fold) among rECs, HMVECs and HUVECs. The right panel represents significant GO terms of down-regulated genes.



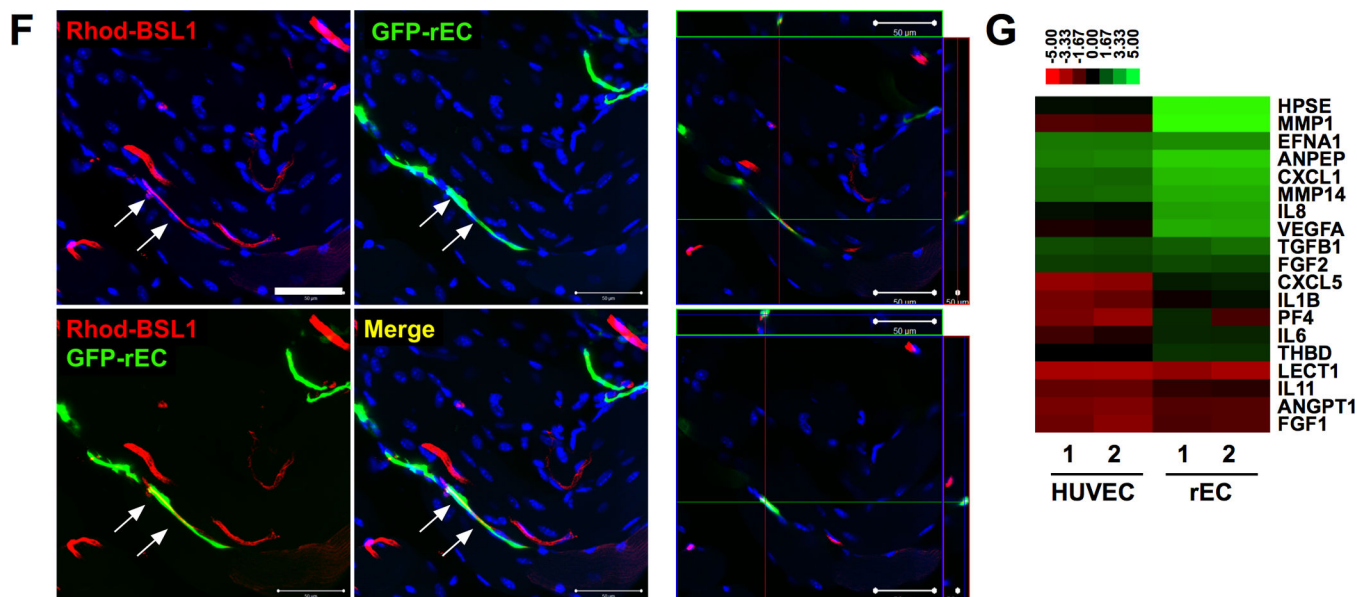


Figure 7. Enhanced blood flow recovery and neovascularization by rECs in ischemic hindlimbs
 Early rECs were intramuscularly injected into ischemic hindlimbs of nude mice. (A, B) Laser Doppler perfusion images (A) and quantitative analysis of blood flow (B) showed improved limb perfusion in the rEC- compared to the HUVEC-, HDF- or PBS-group. All data are presented as mean \pm s.e.m.; n = 5 for rEC-, 10 for HUVEC-, 5 for HDF-, or 6 for PBS-group. *** $P < 0.001$, ** $P < 0.01$, Repeated Measures ANOVA followed by multiple comparisons with Bonferroni's method. (C) The rEC-injected group showed lower limb loss score compared to the HUVEC-, HDF-, or PBS-groups at day 28, suggesting better limb protection. All data are presented mean \pm s.e.m.; n = 5 for rEC-, 10 for HUVEC-, 5 for HDF-, or 6 for PBS-group. ** $P < 0.01$, * $P < 0.05$, standard unpaired Student's t test. (D) The mice were perfused with FITC-BSL1 at 4 weeks and the frozen-sections of the muscle were examined under confocal microscope imaging. Quantitative analysis of vascular density is shown. Note that the vascular density was significantly increased in the rEC-, compared to the HDF-, HUVEC- or PBS-groups. All data are presented as mean \pm s.e.m.; n = 5 for rEC-, 10 for HUVEC-, 5 for HDF-, or 6 for PBS-group. *** $P < 0.001$, ** $P < 0.01$, standard unpaired Student's t test. (E) Injected rECs (red) were incorporated into the blood vessels (green) as indicated by arrows, suggesting contribution of the rECs to vessel formation. DAPI (blue). Scale bars: 25 μ m. (F) Early rECs transduced with lentiviral-*eGFP* (green) were injected into ischemic hindlimbs. The mice were perfused with Rhodamine-BSL1 (red) at 28 days and the frozen-sections of the hindlimb muscle were subjected to confocal microscope imaging. The co-localization of GFP-rEC (green) and BSL1-labeled blood vessel (red) is indicated by arrows and confirmed by orthogonal image. Scale bars: 50 μ m. (G) Increased expression levels of angiogenic genes in early rECs compared to HUVECs. Red and green indicate decreased and increased levels of gene expression, respectively.