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ORIGINAL RESEARCH

Direct reprogramming of fibroblasts into endothelial progenitor cells by defined factors

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Abstract— Introduction: Endothelial progenitor cells (EPCs) are important progenitor cells in vasculogenesis as well as in tissue engineering. However, few EPCs can be isolated from bone marrow, peripheral blood and umbilical cord blood. Moreover, their *in vitro* proliferation potential is also limited. Therefore, this study aimed to produce EPCs from direct reprogramming of fibroblasts by transduction with certain specific factors. **Methods**: Human fibroblasts were collected from human skin by published protocols. The cells were transduced with 2 viral vectors containing 5 factors, including Oct3/4, Sox2, Klf4, c-Myc (plasmid 1), and VEGFR2 (plasmid 2). Transduced cells were treated with endothelial cell medium for 21 days. The cells were analyzed for expression of Oct3/4, Sox3, Klf4, c-Myc and VEGFR2 at day 5, and for EPC phenotype at day 21. **Results**: The results showed that after 5 days of transduction, fibroblasts acquired partial pluripotency. After 21 days of transduction and culture in endothelial cell medium, the cells exhibited endothelial markers (e.g. CD31 and VEGFR2) and formed blood vessel-like capillaries. **Conclusion**: Our findings suggest another strategy for direct reprogramming of fibroblasts into EPCs.

Keywords: Direct reprogramming, Endothelial progenitor cells, Fibroblasts

INTRODUCTION

Direct reprogramming is a process wherein a differentiated adult cell converts into another differentiated somatic cell. As a result, direct conversion does not pass through an undifferentiated pluripotent stage (Van Pham, 2015). This strategy enables the generation of patient-specific cell types, without formation of pluripotent stem cell-induced tumors prior to differentiation. Therefore, this reprogramming technology has been a potent tool for regenerative medicine.

Endothelial progenitor cells (EPCs) are one of the most important progenitor cells in tissue engineering, especially in vasculogenesis and angiogenesis (Chong et al., 2016). The use of EPCs has provided effective results in treating hindlimb ischemia (Flex et al., 2016; Yu et al., 2009), stroke (Bai et al., 2015; Li et al., 2015), diabetic ulcer (Gallagher et al., 2006), and myocardial infarction (Kawamoto et al., 2003). Although EPCs have many advantages, one major disadvantage is that very low numbers of these cells can be isolated from bone marrow (Hristov et al., 2003), peripheral blood (Donndorf et al., 2015), and umbilical cord blood (Schmidt et al., 2004; Van Phuc et al., 2012). Moreover, their proliferation potential is limited. Therefore, this study investigated the production of EPCs from direct reprogramming of fibroblasts via transduction with defined factors. We show that fibroblasts were, indeed, differentiated into EPCs by endothelial cell medium containing specific endothelial differentiation factors. Thus, the study herein demonstrates the application of a direct reprogramming technology to reprogram fibroblasts into partial pluripotent stem cells.

MATERIALS AND METHODS

Isolation and culture of human fibroblasts and HEK 293T

Human fibroblasts (HFs) were isolated from foreskin, according to a previously published protocol (Van Pham et al., 2016). Cells were cultured in DMEM/F12 complete (DMEM/F12, 10% FBS and 1% antibiotic; all reagents were bought from Life Technologies, Carlsbad, CA) until cells reached 80-90% confluency on surface flask. The HFs were then sub-cultured to the 3rd passage. Cells were cryopreserved in liquid nitrogen until use in experiments. HEK 293T cells were obtained from a commercial source (Life Technologies, Carlsbad, CA). The cells were thawed and cultured at 5×10^6 cells in a $25cm^2$ flask. HEK 293T were also cultured in DMEM/F12 complete.

Viral vector production

There were 2 viral vectors used in this study. Vector 1 contained four factors: Oct3/4, Sox2, Klf4 and c-Myc (OKSIM); Vector 2 contained VEGFR2. HEK 293T were trypsinized to collect single cells. Then, a plasmid containing c-Myc (OKSIM) was co-transfected into HEK293T cells with pCMV-VSV-G-RSV-Rev and pCMV-dR8.2 (Addgene, Cambridge, MA) to produce Vector 1. Similarly, Vector 2 was produced by transfection of HEK293T cells with plasmids expressing VEGFR2, pCMV-VSV-G-RSV-Rev, and pCMV-dR8.2. The mix was transferred into 2 mm electroporation cuvettes and transfected into cells. Immediately after, pre-warmed medium was gently added to the transfection mix and transferred into 6-well plates. The plates was incubated at 37^oC, 5% CO_2 for 24 h. After 36 h, supernatant was collected to extract viral particles by centrifugation.

Transduction into HFs

On the day of transduction, HFs were treated with polyprene at the final concentration of 8 µg/mL in 6 h, then transduced with both viral vector 1 (containing Oct3/4, Sox2, Klf4, and c-Myc) and viral vector 2 (containing VEGFR2 only). Transduction was repeated two independent times, without polyprene treatment the second time. The medium was refreshed after 2 d of transduction by endothelial cell medium and every 3 d until day 21. The endothelial cell medium was prepared by M200 medium supplemented with 2% fetal bovine serum (FBS), 10 ng/mL vascular endothelial growth factor (VEGF), 5 ng/mL epithelial growth factor (EGF), 5 ng/mL basic fibroblast growth factor (bFGF), 1 µg/ml hydrocortisone, and 90 µg/mL heparin (all chemicals and media were bought from Life Technologies, Carlsbad, CA).

Gene expression by reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted with the use of easy-BLUETM Total RNA Extraction Kit (iNtRON, Korea) from cells at day 5. Inducible EPC (iEPC) gene expression was detected using a one-step RT-PCR premix kit (iNtRON, Korea), according to the manufacturer's protocol. The reaction was performed in a thermal realtime PCR cycler (Eppendorf, Germany) with amplification of over 30 cycles at 94°C for 20 s (denaturing), 50-60°C for 10 s (annealing), and 72°C for 30 min (primer extension). The primers (AIT Biotech, Singapore) used in this experiment are listed in **Table 1** (F: forward, R: reverse). RNA for GAPDH was co-amplified to assess the quality of the samples.

Table 1. Primer sequences of iEPC genes in RT-PCR analysis

STT	Gene	Forward primer (5–3')	Reverse primer (5–3')
1	OCT4	AAACCCTGGCACAAACTCC	GACCAGTGTCCTTTCCTCTG
2	SOX2	CACATGTCCCAGCACTACC	CCATGCTGTTTCTTACTCTCCTC
3	NANOG	ACTCTCCAACATCCTGAACCTC	CTTCTGCGTCACACCATTGC
4	REX1	GTGGGAAAGCGTTCGTTGAG	CGCTTTCCGCACCCTTC
5	VEGFR2	CTCGGCTCACGCAGAACTT	GCTGCACAGATAGCGTCCC
6	GAPDH	GGGAGCCAAAAGGGTCATCA	TGATGGCATGGACTGTGGTC

Immunocytochemistry

HFs were seeded in 48-well plates the day before the this experiment. The medium was removed and washed twice with PBS. The cells were then fixed with 4% paraformaldehyde for 30 minutes and washed twice with PBS. Cells were stained with anti-CD31 monoclonal antibody conjugated with PE (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min, then counterstained with Hoechst 33342 for 15 min to visualize the nuclei. Cells were washed twice with PBS and observed under a fluorescent microscope (Carl-Zeiss, Oberkochen, Germany).

Capillary-like structure formation assay

Cells (2×10^4) were seeded on 96-well flat-bottom plates coated with 30 µL Matrigel (Lifetechnologies) and cultured in EGM-2 medium. Eighteen hours after incubation, capillary-like structures were observed under an Axiovert microscope (Carl-Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical analyses of all endpoints were performed using the two-sided Student's *t* test or one-way analysis of variance. All data are presented as mean ± SD. $p < 0.05$ was considered statistically significant. Data were analyzed with Prism 6.0 software.

RESULTS

Culture and proliferation of human fibroblasts and HEK293T cells

Dermal foreskin fragments were cultured for 21 d to isolate dermal fibroblasts. Under microscopy, fibroblasts could be seen migrating from the tissue fragments at day 5. From day 15, cells began to rapidly proliferate (**Fig. 1A**). The cells became homogenous after 3 passages of sub-culturing (**Fig. 1B**). The hemogenicity of the cell population was confirmed by CD90 surface expression; flow cytometry analysis of CD90 expression showed that 100% fibroblasts expressed the gene (**Fig. 1C**).

HEK293T cells were thawed from the commercial kit, and then cultured in standard conditions, according to the manufacturer's guidelines. After thawing, the cells were cultured until they reached confluency (typically after 5 d). All cells were sub-cultured at least 2 times before use in further studies (**Fig. 1D**).

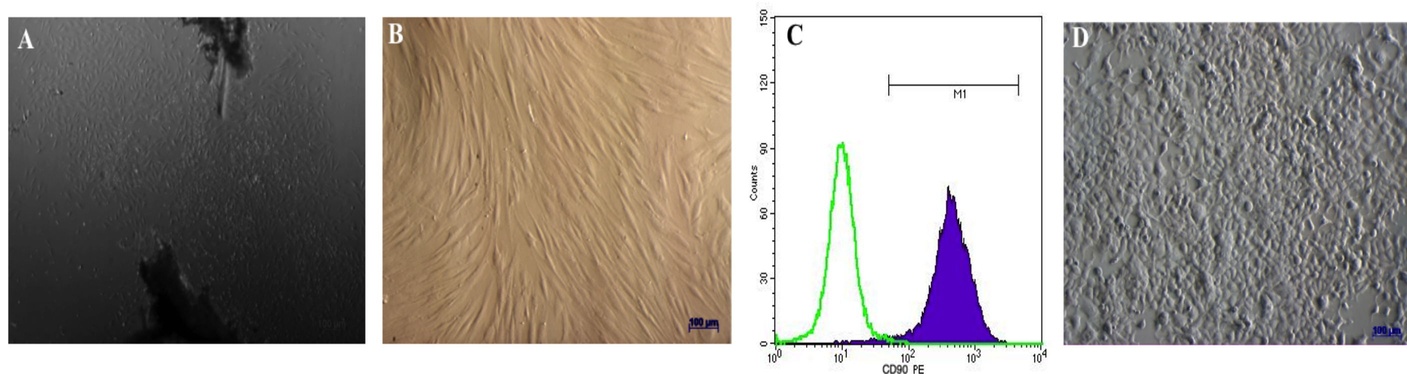

Figure 1. Culture and proliferation of skin fibroblasts and HEK293T cells. Dermal fibroblasts cultured from foreskin shown at day 15 (A); the sub-cultured cells reached a homogenous population after the 3rd passage (B). Fibroblast homogeneity was confirmed by flow cytometry (100% cells expressed CD90) (C). HEK293T cultured from a commercialized cell line (D).

Transduced fibroblasts were enhanced to express pluripotent genes

After transduction with 2 viral vectors for 7 d, transduced fibroblasts were evaluated for expression of Oct4, Sox2, cMyc and Klf4. The expression of these factors were vital for triggering the epigenetic reprogramming. The RT-PCR results showed that after 7 d of transduction, fibroblasts showed increased expression of the epigenetic reprogramming markers, i.e. Oct4, Sox2, cMyc and Klf4 (**Fig. 2**).

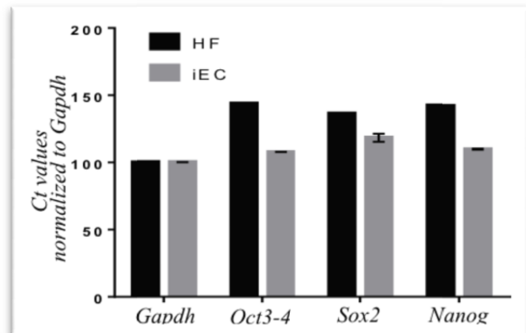

Figure 2. Transduced fibroblasts enhanced to express pluripotent markers. After 7 d of transduction, fibroblasts showed increased expression of reprogramming markers, including Oct4, Sox2, and Nanog. The differences were significant, p <0.05.

Changes in cell shape during mesenchymal to endothelial transition

After transducing with 2 retroviral vectors containing 5 factors, the fibroblasts were cultured in the endothelial cell medium. This medium was derived from endothelial cells cultured with 2% FBS(to inhibit growth of non-transduced fibroblasts) and supplemented with hydrocortisone (to stimulate the growth of endothelial cells). Under this condition, the majority of cells died after 48-72h of treatment (**Fig. 3A**). Some surviving cells formed clusters of cells (**Fig. 3B**). From day 5 (after culture in endothelial cell medium), surviving cells clearly changed their cell shape. Almost all cells converted from a spindle shape to a round shape (similar to that of HUVECs) (**Fig. 3C**). The cells gradually became a homogenous cell population after 21 d of culture (**Fig. 3D**).

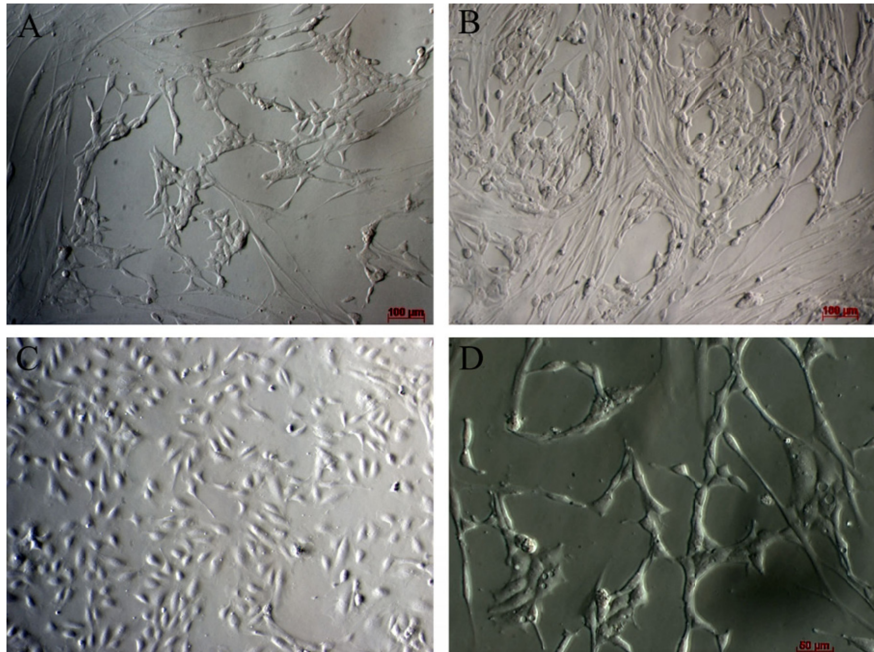

Figure 3. Transduced fibroblasts change their shape from mesenchymal to endothelial cells. After transduction, cells were cultured to enrich for endothelial cells in endothelial cell medium, while the majority of cells died (A); some surviving cells grew to form clusters or colonies (B). After 5 d of enrichment culture, transduced fibroblasts changed their shape from spindle shape to round, similar to HUVECs (C); the cell population became more hemogenous after 21 d of culture (D).

Cell size cell population

Besides the change in shape that was observed under microscopy, the transduced cells also changed in cell size. The size of the cells before and after transduction with viral vectors containing 5 target genes were analyzed by flow cytometry and are presented at Fig. 4. After transduction with viral vectors containing the target genes and cultured in endothelial cell medium, the fibroblast size significantly changed. From flow cytometry data, a small population with higher forward scatter (FSC) and the same side scatter (SSC) became visible (**Fig. 4**). The cell size was larger than that of fibroblasts.

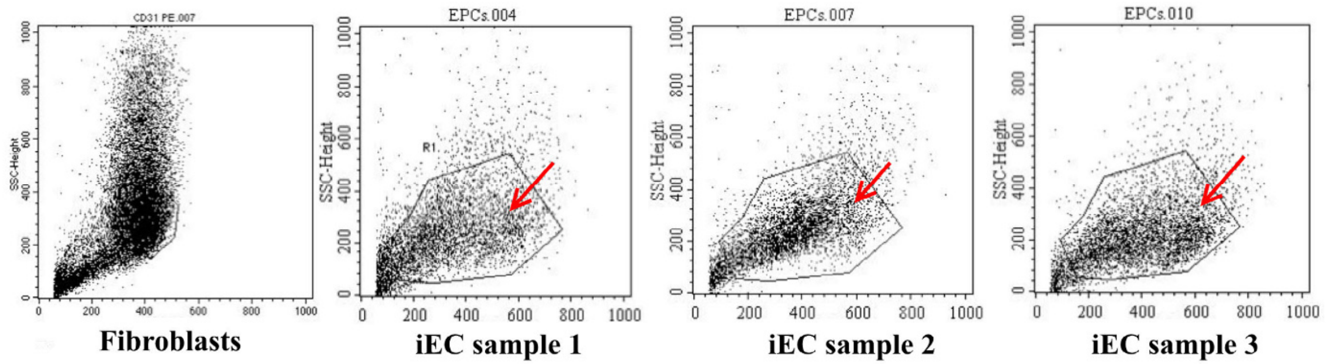

Figure 4. Flow cytometry analysis of cell size. A cell population with higher FSC than fibroblasts formed after transduction with 2 vectors and enrichment in endothelial cell medium.

Upregulation of CD31 and VEGFR2

Expression of CD31 and VEGFR2 were determined by flow cytometry and immunocytochemistry. The percentage of CD31 positive cells was 3.48 ±0.92 % ($p>0.05$) in transduced cells, and 0% in the control. There was a significant increase in the percentage of VEGFR2 positive cells too; the percentage was 91.84 ± 6.41% in transduced cells, and 43.07 ± 3.43% in the control.

The expression of CD31 was also evaluated but via immunocytochemistry with anti-CD31 monoclonal antibody-PE. The results are represented in Figure 5. The results showed that fibroblasts did not express CD31 yet the transduced cells did express the CD31 marker (**Fig. 5**).

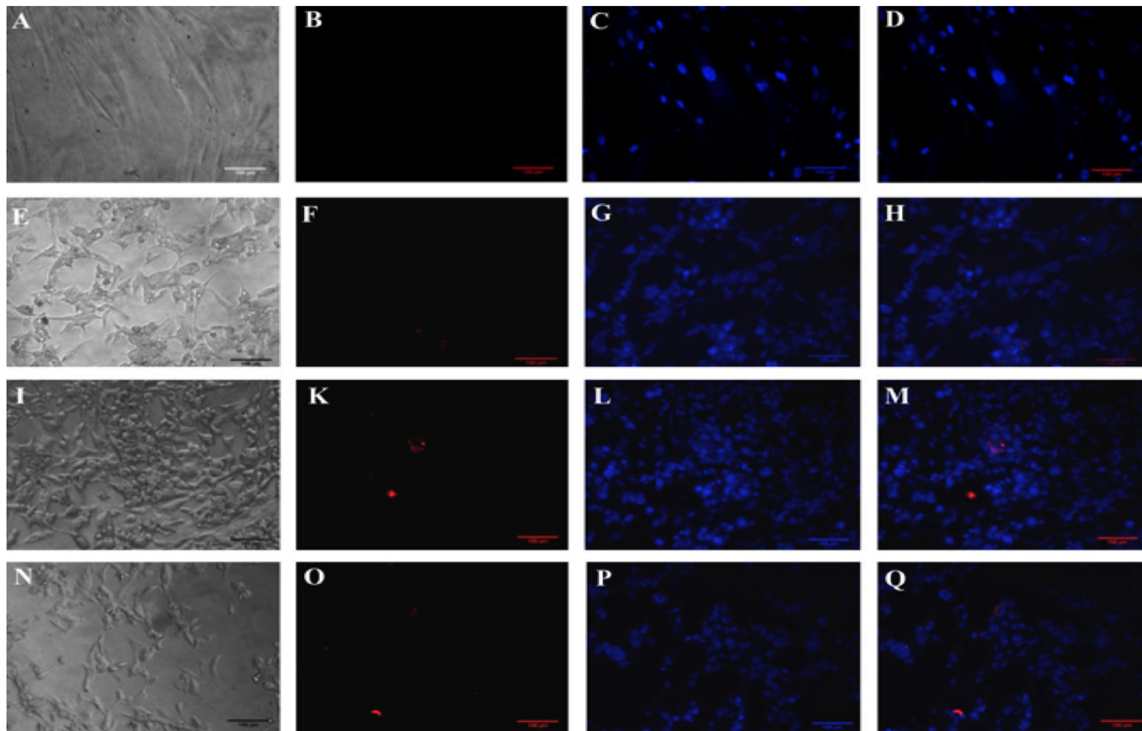

Figure 5. Transduced fibroblasts express CD31. Transduced cells expressed CD31 were confirmed by immunocytochemistry with anti-CD31 monoclonal antibody conjugated with PE for 3 samples (E-Q), while the control samples was negative (A-D). A,E,I,N: cells were captured under white light; B,F,K,O: cells were captured under PE filter; C,G,L,P: cells were captured under Hoeschst 33342 filter; and D,H,M,Q: the merged images.

In vitro formation of blood vessel-like capillaries

Transduced fibroblasts were cultured in culture medium for 20 d. They were used to evaluate the *invitro* formation of blood vessel like capillary in matrigel. The results showed that after 18 h of incubation, transduced cells were capable of forming capillary structure similar to blood vessels (**Fig. 6**).Results are similar to using HUVECs; results suggest that transduced cells cultured in endothelial cell medium can exhibit endothelial progenitor cell bio-activity *in vitro*.

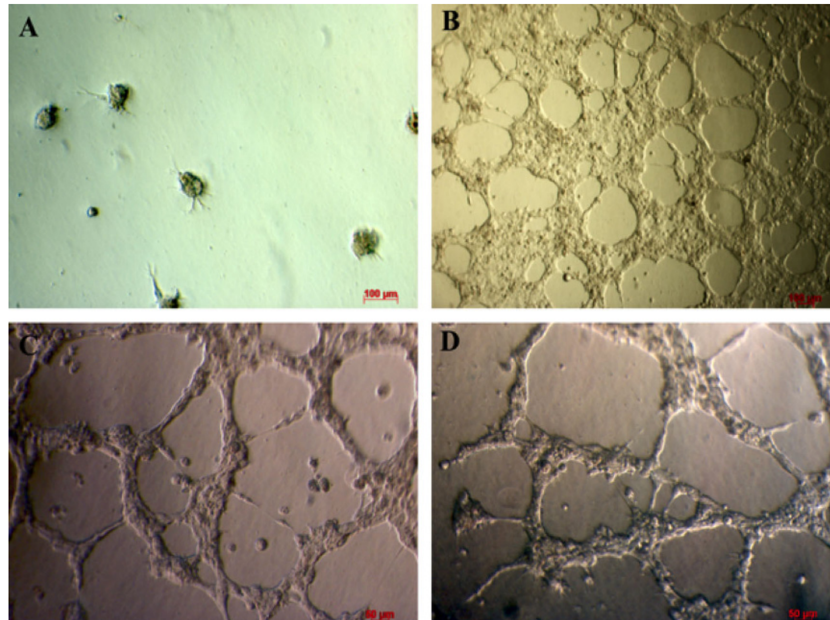

Figure 6. *In vitro* formation of blood vessel like capillary of transduced cells. Transduced fibroblasts after culture in endothelial cell medium can form blood vessel like capillary (B,C,D), but not in fibroblasts (A).

DISCUSSION

Endothelial progenitor cells as well as endothelial cells are important cell sources for regenerative medicine and tissue engineering. Therefore, this study aimed at generating EPCs by direct reprogramming of fibroblasts with 5 factors, including Oct3/4, Sox-2, Klf4, c-Myc and VEGFR2, in combination with culture in endothelial cell medium. Our study showed that the above conditions induced the generation of functional EPCs.

Fibroblasts were successfully isolated from foreskin. This cell population was a homogenous population with 100% CD90-expressing cells. Indeed, CD90 expression is considered a fibroblast marker (Kisselbach et al., 2009). Fibroblasts are common cells in the human body, and are especially easily to isolate and are very proliferative. Therefore, fibroblasts are the predominant cells used in the study as well as in applications of epigenetic reprogramming, including direct reprogramming. Indeed, fibroblasts are used to reprogram and induce pluripotent stem cells (Alawad et al., 2016; Takahashi and Yamanaka, 2006; Yu et al., 2007), myoblasts (Choi et al., 1990; Davis et al., 1987; Lassar et al., 1989), adipocytes (Tontonoz et al., 1994), macrophages (Feng et al., 2008), cardiomyocytes (Efe et al., 2011), neuron (Yoo et al., 2011), myocytes (Bichsel et al., 2013), and endothelial progenitor cells (Li et al., 2013; Van Pham et al., 2016; Wong and Cooke, 2016). In this study, we used fibroblasts to directly induce reprogramming into EPCs.

To date, there have been various approaches used different induction factors to direct reprogramming of fibroblasts into EPCs. For example, in the first study involving direct reprogramming of fibroblasts to EPCs, Margariti *et* al. used 4 factors, including OCT4, SOX2, KLF4, and c-MYC to induce fibroblasts for 4 days. Then, differentiation of particle induced pluripotent stem cells into endothelial cells was done by treatment with defined media and culture

conditions (Margariti et al., 2012). Subsequently, Li *et* al investigated the removal of the Sox-2 gene and two other genes (*Oct4 and Klf4*), in combination with soluble factors (Li et al., 2013). Since the efficacy of these procedures was extremely low, Han *et* al. (2014) tried a combination of *Foxo1, Er71, Klf2, Tal1, and Lmo2*(Han et al., 2014). Recently, another factor for use as a single factor for direct reprogramming of fibroblasts to EPCs was suggested (Lee et al., 2014; Morita et al., 2015); single factor ETV2 was capable of inducing fibroblasts to acquire EPC phenotype. In 2016, the efficacy of this procedure was increased by combining ETV2 transfection with hypoxia (Van Pham et al., 2016).

In this study, we investigated a strategy similar to the one by Margariti *et* al. (Margariti et al., 2012). Initially, fibroblasts are induced to acquire partial pluripotency using 4 factors expressed in the first viral vectors (Oct3/4, Sox-2, Klf4 and c-Myc). Indeed, these factors could successfully reprogram many adult cells toward pluripotent stem cells. To improve on this strategy, we used another vector containing VEGFR2, a receptor for VEGF that has many roles. VEGF is critical signaling protein involved in both vasculogenesis and angiogenesis (Shibuya and Claesson-Welsh, 2006), and it serves as a mitogen for endothelial cells (Hoeben et al., 2004). It can also stimulate the migration of endothelial cells. Upregulation of VEGFR2 on fibroblasts enhances the effects of VEGF on both mitogenesis and migration.

Thus, we investigated if partial pluripotent stem cells could be directly induced into EPCs by endothelial cell medium. The endothelial cell medium contains critical factors to direct induction of partial pluripotent stem cells into EPCs. For example, the medium contains low fetal bovine serum (2% as opposed to 10% used for fibroblasts), VEGF, hydrocortisone, and heparin. The low serum medium is not only suitable for endothelial cells but also can inhibit the proliferation of fibroblasts, particularly non-transduced fibroblasts. Hydrocortisone is an important factor in the endothelial cell medium, with an important role of triggering endothelial cell differentiation, while inhibiting mesenchymal characteristics (Furihata et al., 2015). Heparin also has an essential role in endothelial cell growth; it is considered important in modulating the availability and stability of potent growth-regulating agents for endothelial cells (D'Amore, 1990). Moreover, heparin also protects vascular endothelial cells from injury induced by TNFα and sepsis; the protective mechanisms are related to effects of heparin on the histone methylation of promoter region and the regulation of heparin on the MAPK and NF-κB signal pathways (Ma and Bai, 2015). Heparin also binds to angiogenic growth factors and some pro-angiogenic receptors, as well as to angiogenic inhibitors which regulate angiogenesis (Chiodelli et al., 2015). Taken together, all the above factors can drive partial-pluripotent stem cells toward to EPCs. The process and mechanism of direct reprogramming of fibroblasts into EPCs are proposed in **Figure 7**.

Importantly, results from our study show that the process of reprogramming occurs and that it is feasible to produce functional endothelial progenitor cells. Although the efficacy of this process was low, a small population with markers of endothelial progenitor cells ($CD31^{+}VEGFR2^{+}$) could be produced. Moreover, these cells could perform *in vitro* functions of EPCs, such as blood vessel-like capillary formation. The properties of our EPCs are similar to ones of previously published studies (Lee et al., 2014; Morita et al., 2015; Van Pham et al., 2016).

CONCLUSION

EPCs can participate in both vasculogenesis and angiogenesis. Therefore, they can be used in treatment of ischemia-related diseases or blood vessel regeneration. However, the percentage of EPCs in blood and other tissues is extremely low. Our study demonstrated that EPCs could be feasibly produced by direct reprogramming of fibroblasts. Five factors were necessary in the cell medium: Oct3/4, Sox-2, Klf4, c-Myc and VRGFR2. Additionally, endothelial cell medium (containing low fetal bovine serum, hydrocortisone and heparin) was also critical for the induction. The EPCs expressed CD31 and VEGFR2, and formed blood vessel-like capillaries *in vitro*. These results suggest another strategy to directly reprogram fibroblasts into EPCs for potential use in regenerative medicine.

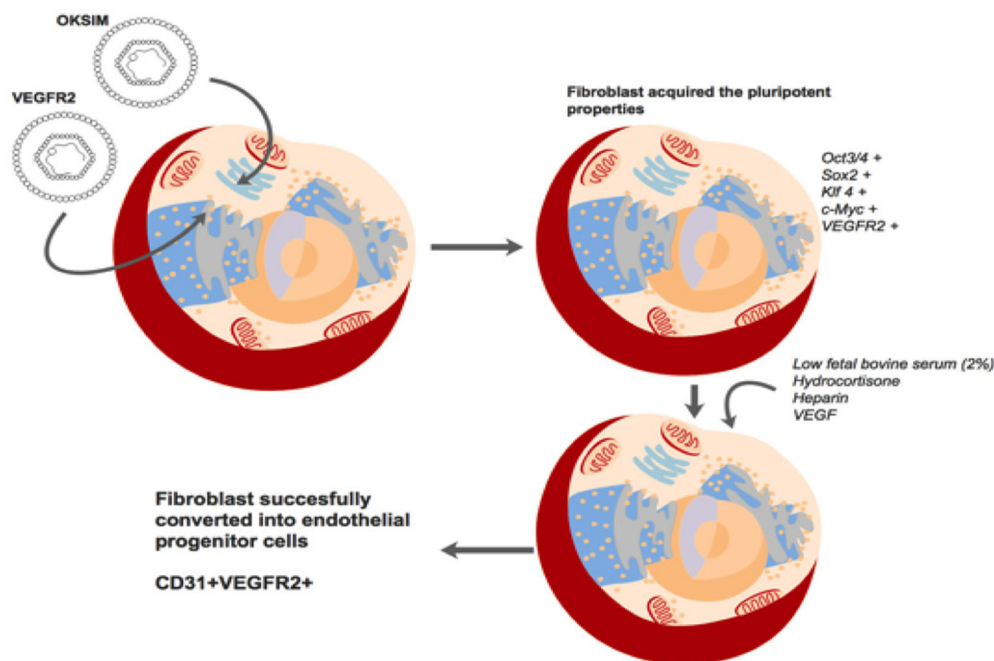

Figure 7. Epigenetic direct reprogramming of fibroblasts into endothelial progenitor cells. Fibroblasts are reprogrammed into partial pluripotent stem cells; these cells are then differentiated into endothelial progenitor cells after culture in endothelial cell medium with low fetal bovine serum, hydrocortisone and heparin.

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Competing interests

The authors declare they have no competing interests.

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