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Direct conversion of human adipose derived stem cells into endothelial progenitor cells

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Abstract

Introduction: Endothelial cells (ECs) or endothelial progenitor cells (EPCs) are essential cells for blood vascular regeneration and tissue engineering. However, the source of EPCs is limited. Indeed, these cells only exist with the low rate in some tissues such as bone marrow, umbilical cord blood, and peripheral blood. This study aimed to produce EPCs from direct epigenetic reprogramming of adipose tissue-derived mesenchymal stem cells (ADSCs) by ETV2 transfection *in vitro*. **Methods**: ADSCs were isolated according to our published work. They were confirmed as mesenchymal stem cells (MSCs) following the minimal criteria suggested by ISCT included positive with CD44, CD73, CD90, but negative with CD14, CD45, and HLA-DR; *in vitro* their differentiation into adipocytes, and osteoblasts. ETV2 mRNA was in vitro produced by the commercial kit. ETV2 mRNA molecules were transfected into ADSCs by Fugenes and Lipofectamine agents. **Results**: These transfected cells were evaluated the expression of EPC properties included expression of CD31, VEGFR2 in the cell surface by flow cytometry, immunocytochemistry, and *in vitro* microvascular blood vessel formation. The results showed that ETV2 could convert the ADSCs from mesenchymal cell phenotype into endothelial cell phenotype with 10% transfected ADSCs expressing the CD31 in their surface, and they also could form *in vitro* microvascular blood vessel. **Conclusion**: Although the low efficacy of direct reprogramming, this study gave the new strategy to produce EPCs from the favorite cell sources as ADSCs for stem cell therapy as well as tissue engineering.

1. Background

Endothelial progenitor cells (EPCs) are the progenitor cells that can become the endothelial cells (ECs) to form the blood vessels. The roles of EPCs in the vascular repair and angiogenesis were determined in the some previously published works [1–4].

Recently, some preclinical and clinical applications of EPCs showed the promising results [5–8]. Fan *et al*. (2010) investigated the effects of EPCs on ischemic brain injury in a mouse model of transient middle cerebral artery occlusion (tMCAO). About 10^6 EPCs were systemic injected into the nude mice after 1 hour of tMCAO. After 24hrs of injection, these EPCs were detected in ischemic brain regions. After three days of transplantation, the ischemic infarct volume significantly reduced compared to control [6]. Similarly, Geng *et al*. (2017) transplanted the EPCs to cure cerebral ischemia. Moreover, the results showed that neurological deficits were attenuated and brain infarct volume was reduced in EPC-transplanted diabetic mice compared to the controls ($p < 0.05$) [5]. Clinically, EPCs also were used to treat some conditions. In 2010, Burt *et al.* reported the effects of EPCs on the critical limb ischemia in human. In this study, EPCs were collected from peripheral blood from the same patients after the injection of GCSF (10mcg/kg/day). The results showed that this treatment could save seven lower extremity limb of the nine treated patients [7]. EPCs also were clinically used to treat the myocardial infarction [8,9]. Bartunek *et al*. (2005) isolated EPCs from the autologous bone marrow; then intracoronary administration in patients with recent myocardial infarction. There were 35 patients treated in this study. The results showed that the transplantation seems to be associated with improved left ventricular performance paralleled with increased myocardial perfusion and viability [8]. These results also were recorded by Li *et al.* (2007) [9].

However, the sources of EPCs are so limited. EPCs usually were collected from bone marrow, peripheral blood and umbilical cord blood with the low efficacy. Notably, different to mesenchymal stem cells (MSCs), it is so difficult to *in vitro* expand. While MSCs, particularly adipose-derived stem cells - ADSCs are favorite cell sources, easy to *in vitro* expand. Therefore, this study aimed to directly covert the ADSCs into EPCs through the transcription EVT2.

2. Methods

(a) **Isolation and proliferation of adipose-derived stem cells**

The adipose-derived stem cells were isolated and proliferated according to the previously published study [10–12]. Briefly, the human adipose tissues were collected at the hospital with the consent form and approved by the local ethical committee. Adipose tissue was minced with the knife into the small fragments, then incubated in the Superdigest solution (collagenase, from Regenmedlab, Ho Chi Minh City, Viet Nam) for 30 min. This treated adipose solution was centrifuged at 3000 rpm for 15 min to get the pellet at the bottom of centrifuge tubes. The pellet was washed twice with PBS before it was suspended in the MSCCult medium; and incubated in the incubator at 37 ^{0}C, 5% CO_2. Cells were continuously sub-cultured to fourth passage and used for further experiments and evaluations.

(b) **ADSC characterization and phenotyping**

ADSCs were characterized as MSCs according to the minimal criteria of MSCs suggested Domini *et al*. (2006) [13] included plastic adherence with fibroblast-like shape, expression of MSC marker profile (positive for CD44, CD73, and CD90; negative for CD14, CD45, and HLA-DR), *in vitro* differentiation into adipocytes, and osteoblasts. The methods for these assays were demonstrated in the previously published study [12].

(c) ETV2 mRNA *in vitro* production

ETV2 mRNA was produced as in the previous publication [14,15].

(d) mRNA transfection into ADSCs

The mRNA transfection into target cells was performed according to the previous study. Briefly, ADSCs were seeded into the 6-well plates in the MSCCult medium (Regenemedlab Ltd, Ho Chi Minh City, Viet Nam) for 24 hr before they were treated with 1 ug mRNA of ETV2 resuspended in 70 ul FuGENE in 37^0 C, 5% CO_2 for three days. Then these cells were changed the medium to EPC medium (Medium 200, supplemented with LVES, Gibco, Waltham, MA, United States) with or without VEGF (Gibco, Waltham, MA, United States) and repeatedly treated with mRNA ETV2 as the first time. The cells were further cultured for three days. Similarly, these cells were transfected with ETV2 4 times.

To evaluate the effects of ETV2 transfection and effects of VEGF on the efficacy of direct reprogramming of ADSCs toward to EPCs, there were five groups were investigated, included:

G1: HUVECs cells were cultured in EPC medium as positive control

G2: ADSCs were cultured in the MSCCult medium as negative control

G3: ADSCs were cultured in EPC medium as placebo

G4: ADSCs were transfected with ETV2 and cultured in EPC medium

G5: ADSCs were transfected with ETV2 and cultured in EPC medium supplemented with 10 ng/mL VEGF

(e) Expression of CD31 and VEGFR2

The expression of CD31 and VEGFR2 were evaluated in 5 groups (G1-G5) by the methods published in the previous works [14,15].

(f) *In vitro* microvascularblood vessel formation

In vitro microvascular blood vessel formation is the essential assay to demonstrate the function of EPCs. In this study, ADSCs/EPCs in 5 groups (G1-G5) by the methods published in the previous works [14,15].

3. Results

(a) Adipose-derived stem cells exhibited the mesenchymal stem cell phenotypes

Adipose-derived stem cells (ADSCs) adhered to the flask surface with fibroblast-like cells **Figure 1A**). These cells rapidly proliferated and got the confluence 70-80 % after 14 days. These cells were negative with markers of hematopoietic cells included CD14, CD34, and CD45; while positive with mesenchymal cells included CD44, CD73, and CD90 (**Figure 1B**). Under inducing conditions, they were successfully differentiated into adipocytes that accumulated the lipid drops in the cytosol (**Figure 1C**); and into osteoblasts that accumulated the calcium phosphate. These characteristics suggested that these obtained cells are mesenchymal stem cells.

(b) Adipose-derived stem cells can be trans-differentiated into endothelial progenitor cells by ETV2 mRNA transfection

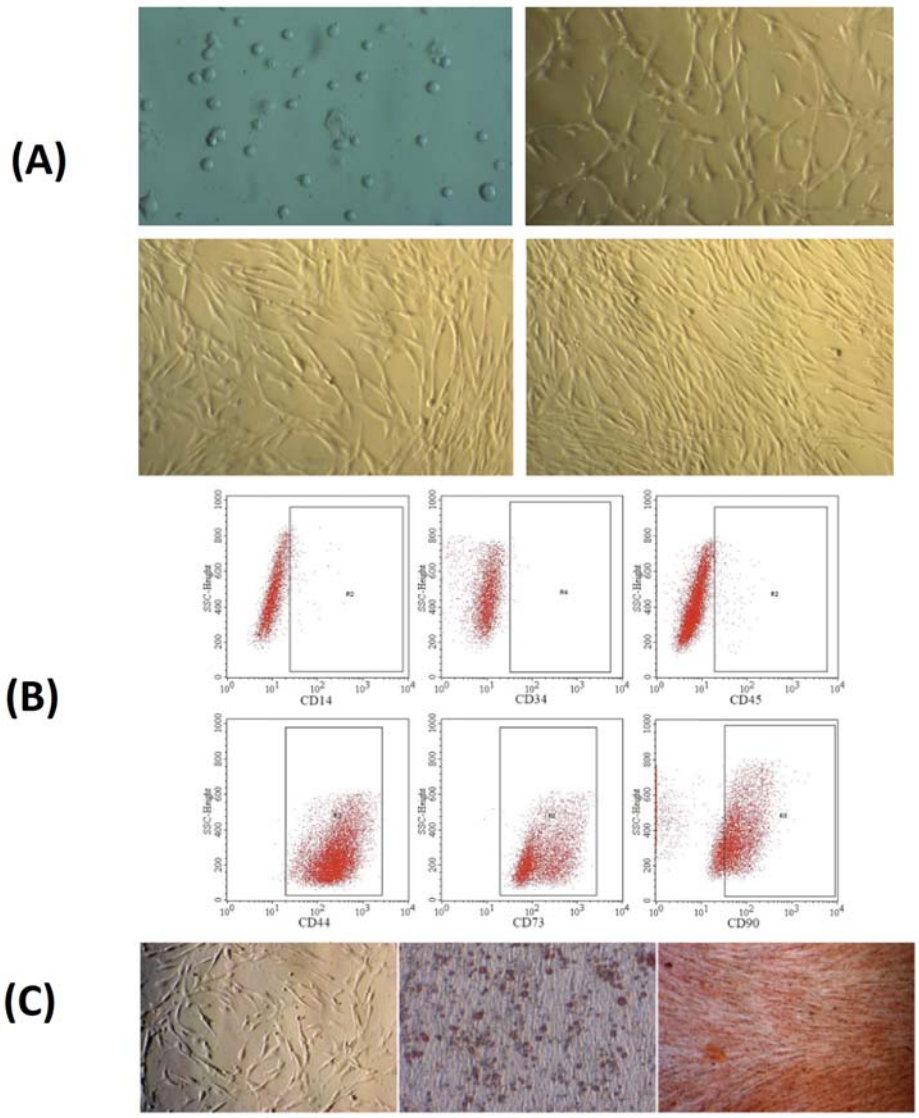

Figure 1. Adipose-derived stem cells (ADSCs) exhibited the mesenchymal stem cell phenotype. (**A**) ADSCs adhered into the surface of the flask and displayed the fibroblast-like shape; (**B**) They expressed the popular marker profile of MSCs included positive with CD44, CD73, and CD90; negative with CD14, CD34 and 45. (**C**) They could be differentiated into adipocytes that positive with Oil Red staining; into osteoblasts that positive with Alizarin Red.

(i) The changes of cell shape after ETV2 mRNA transfection

After 14 days of transfection, ADSCs started to change their shapes. Under the inverted microscope, some cells changed from fibroblast-like cells to cone-like cells. Indeed, ADSCs exhibited the fibroblast-like cells in adherent condition on the flask surface (**Figure 2A**), while the endothelial cells displayed the cone shapes (**Figure 2B**). In the **Figure 2C**, some transfected ADSCs (in group G4) achieved the cone shapes after 14 days of ETV2 mRNA transfection.

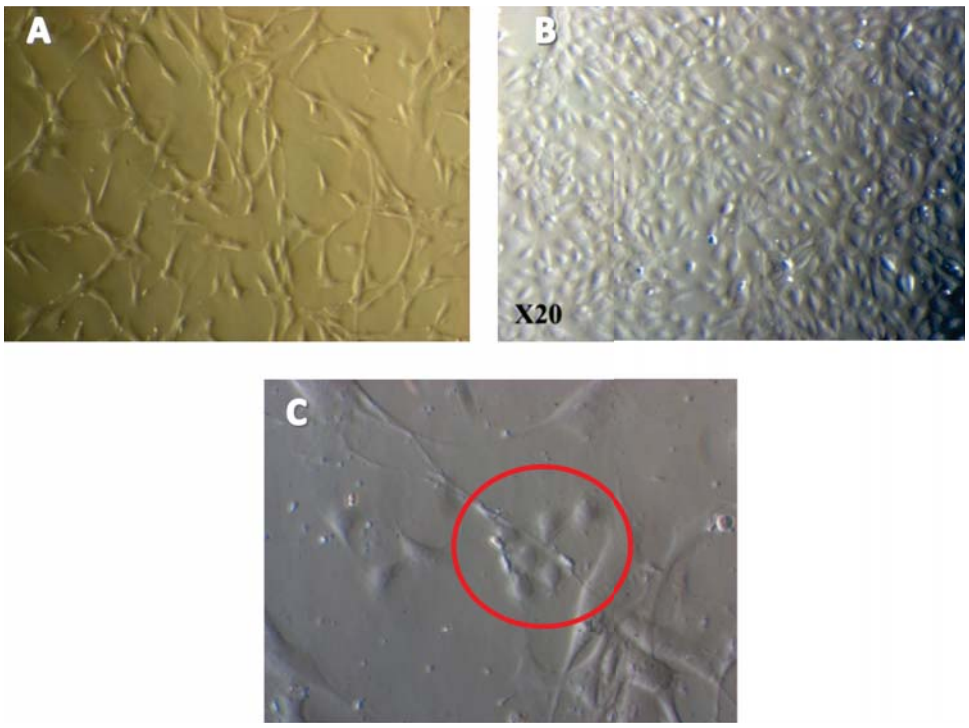

Figure 2. Morphological changes of ADSCs after transfected with ETV2 mRNA. The ADSCs commonly exhibited the fibroblast-like cells (**A**); while the human umbilical vascular cells (HUVECs) displays the cone-like cells (**B**). The ADSCs had changed their shapes from long shape to round shape after 14 days of ETV2 mRNA transfection (**C**).

The expression of CD31 and VEGR2 also were confirmed by flow cytometry (**Figure 3**). In the groups of G4, there was a subpopulation of CD31-expressing ADSCs (2.13 $\pm$ 0.47), while in other groups of ADSCs (G2, G3), there was not this population. Although the in all groups of ADSCs (G2, G3, and G4) and HUVECs (G1), cells expressed the VEGFR2, the expression of VEGFR2 on the HUVECs was higher than other groups of ADSCs. In the group of ADSCs transfected with ETV2 and cultured in EPC medium (G4), there was a sub-population of ADSCs highly expressing of VEGFR2 (2.53 $\pm$ 0.76). We also confirmed the expression of CD31 by immunocytochemistry (**Figure 4**). The results confirmed that there were some transfected cells expressing CD31.

(ii) The microvascular blood vessel formation *in vitro*

The *in vitro* microvascular blood vessel formation is the critical characteristic of endothelial progenitor cells. In this study, cells in all groups were cultured in the Matrigel and monitored the formation of microvascular blood vessels. The results represented in **Figure 5**. Both ADSCs in non-transfected groups (G2, G3) could not form the micro-vessels in the matrigel; while in the

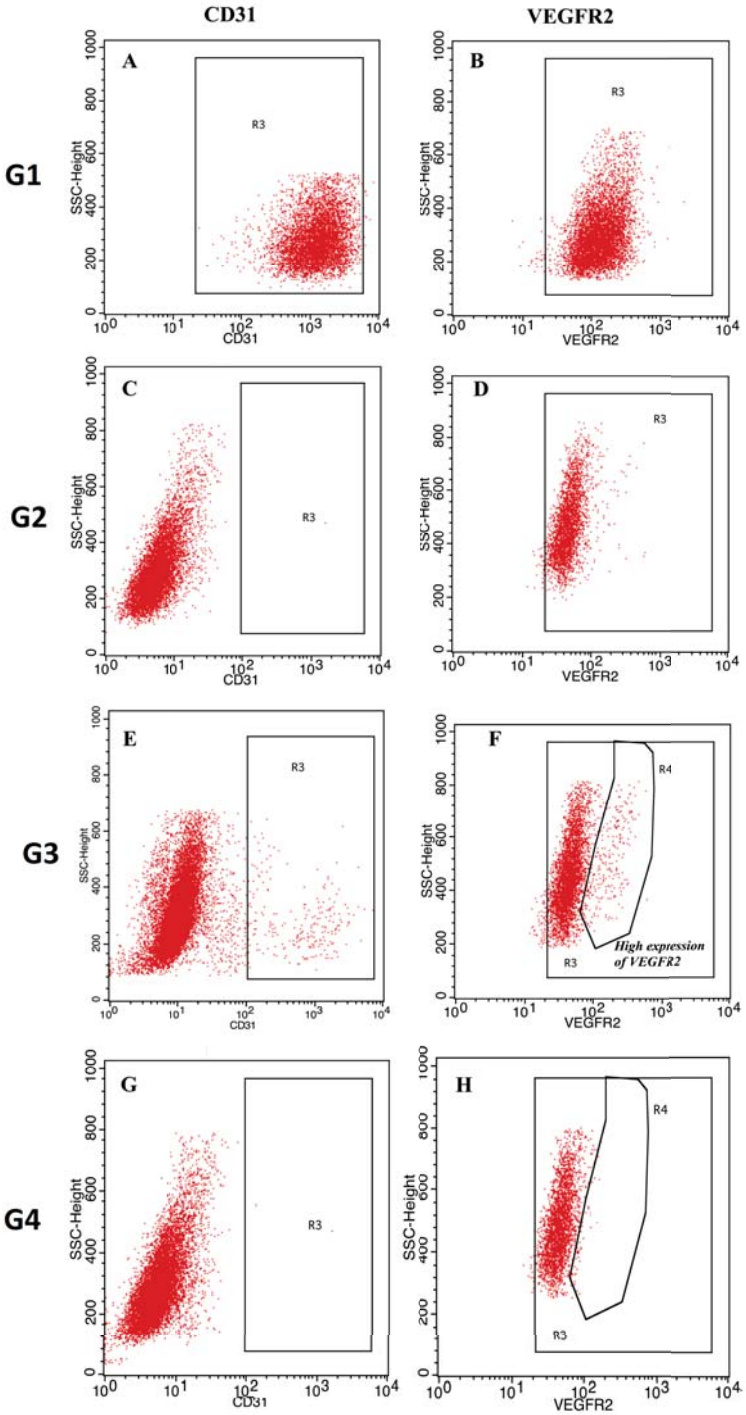

Figure 3. Expression of CD31 and VEGFR2 on the transfected ADSCs confirmed by flow cytometry. HUVECs (G1) highly expressed both CD31 and VEGFR2 (**A, B**), while ADSCs (G2, G3) were negative with CD31 and positive with VEGFR2 (**C, D**). After transfected with ETV-2 mRNA and cultured in the EPC medium (G4), there was a subpopulation of ADSCs expressing CD31, and bright expression of VEGFR2 (**E, F**). This subpopulation did not appear in control (**G, H**).

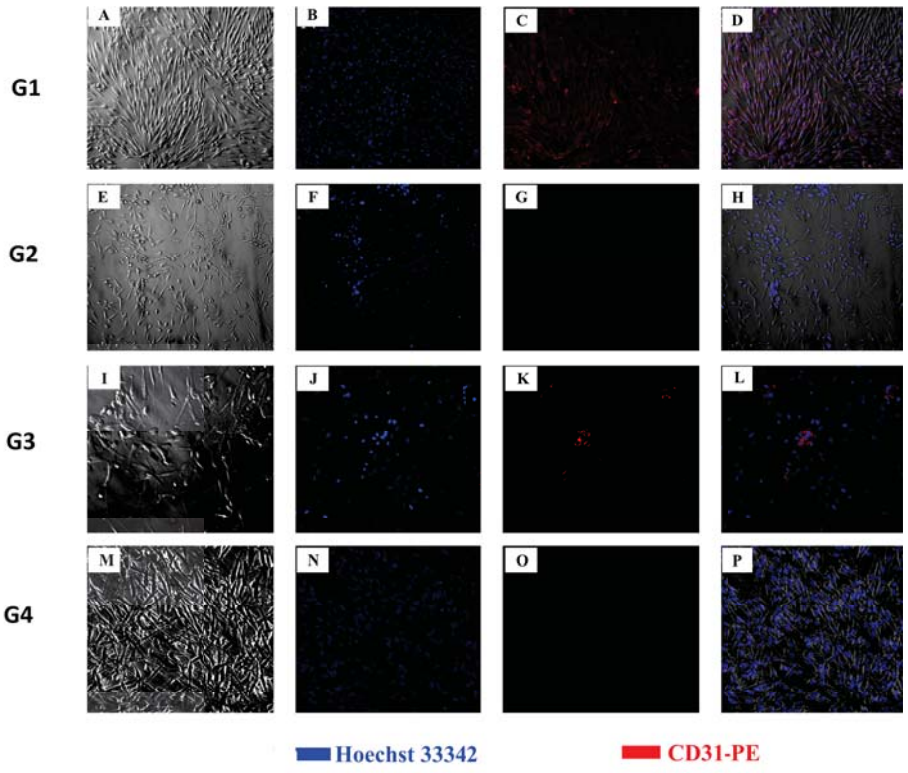

Figure 4. The expression of CD31 on transfected ADSCs evaluated by immunocytochemistry. HUVECs highly expressed both CD31 (**A-B**); while ADSCs were negative with this marker (**E-H**). There are a few ADSCs positive with CD31 (**I-L**), but not in the placebo (**M-P**).

HUVEC cells (G1) and transfected ADSCs (G4) could form the micro-vessels (**Figure 5**). However, we can observe the difference in the microvascular blood vessel network formed by HUVECs (G1) and transfected ADSCs (G4) in **Figure 5**. The microvascular blood vessel networks from HUVEs are so clearer than from transfected ADSCs.

(c) VEGF significantly increased the efficacy of trans-differentiation of adipose-derived stem cells into endothelial progenitor cells by ETV-2 mRNA transfection

To increase the efficacy of trans-differentiation of ADSCs to EPCs by ETV-2 transfection, VEGF was added to the culture medium (G5). At the concentration of 10 ng/mL of culture medium, VEGF could significantly increase the efficacy of trans-differentiation of ADSCs toward to EPCs by ETV2 transfection. As the in **Figure 6**, the percentage of CD31 positive cells dramatically increased in the group G5 ($10.45 \pm 5.67\%$) compared to G4 ($1.45 \pm 0.67\%$).

4. Discussion

EPCs are the critical source of cells used in vascular therapy and engineering. Different to MSCs, *in vitro* EPCs culture and expansion are so difficult while they exist in tissues at low density. This

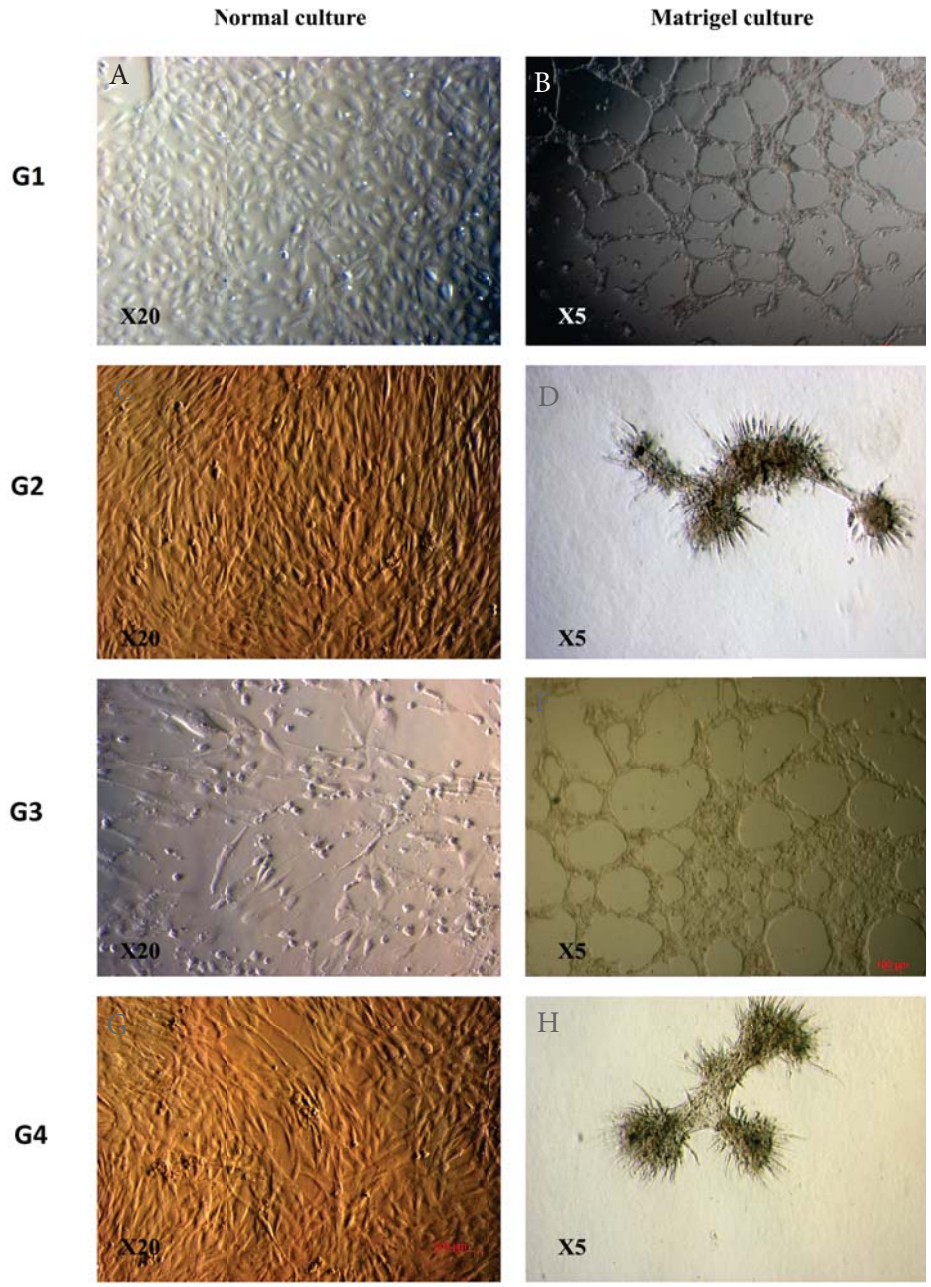

Figure 5. The microvascular blood vessel formation *in vitro* of transfected ADSCs. HUVECs could form the microvessels in vitro in the suitable condition (**A-B**), but ADSCs could not form these vessels (**C-D**). Transfected ADSCs could form these vessels (**E-F**) when cultured in the suitable condition, but the ADSCs in placebo could not do that (**G-H**).

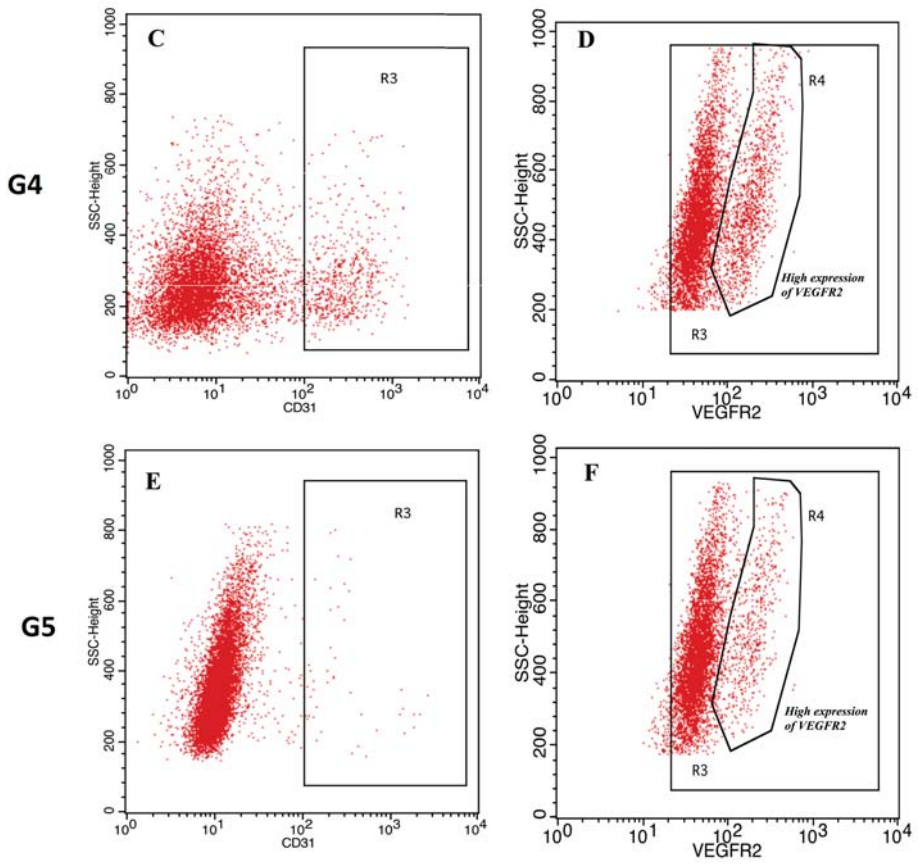

Figure 6. The expression of CD31 and VEGFR2 in groups of ADSCs transfected with ETV2 cultured in the medium with VEGF or without VEGF2.

is the reason why some efforts were performed to expand or produce enough EPCs for clinical applications. This study aimed to develop a new technique to produce EPCs from MSC through the trans-differentiation using the ETV2 transcription factor.

In this first experiment, the ADSCs were successfully isolated from human adipose tissue. These cells meet all minimal criteria of mesenchymal stem cells that suggested by ISCT [13]. Indeed they can adhere to the surface of the T-flasks and exhibit the fibroblast-like cells. They also displayed the particular marker profile of MSCs such as positive with CD44, CD73, and CD90; negative with CD14, CD34, and CD45. They were successfully induced into adipocytes and osteoblasts *in vitro*. These results are similar to all our studies about ADSC isolation and characterization [10–12,16] and similar to other groups [17–20].

In the next experiment, ADSCs were transfected with ETV2 mRNA. To determine the roles of ETV2 in trans-differentiation, some control groups also were performed included positive control (HUVECs), negative control (non-transfected ADSCs) and placebo (non-transfected ADSCs and cultured in inducing medium). The results confirmed that only in the group of ADSCs transfected with ETV2 contained a subpopulation of EPCs like cells with $CD31^{+}VEGFR^{+}$ phenotype. The expression of CD31 and VEGFR2 have confirmed some various methods included RT-PCR, flow cytometry, and immunocytochemistry. CD31 and VEGFR2 are specific markers of endothelial

cells or EPCs [21–23]. In the ADSCs, there were not any cells expressing CD31. This result also was determined in some previous publications [18,24,25]. The expression of CD31 in transfected ADSCs showed that there was a trans-differentiation process of ADSCs – mesenchymal cells – toward EPCs – endothelial cells that induced by ETV2. ETV2 is an important transcription factor in the vascular development [26], injury-mediated vascular regeneration [27]. Using this transcription factor, some authors successfully to trans-differentiate or directly convert human fibroblasts to functional endothelial cells [15,28–33], human amniotic cells into endothelial cells [34,35], *in vitro* trans-differentiation of fast skeletal muscle into functional endothelium [14, 36]…

In this study, we also found that effect of VEGF on the efficacy of direct conversion of ADSCs toward to endothelial cells. The increase of VEGF concentration in the culture medium of transfected ADSCs significantly increased the percentage of EPCs like ADSCs with CD31+VEGFR+. We hypothesized VEGF could trigger the proliferation of induced EPCs like transfected ADSCs proliferation. Indeed, transfected ADSCs highly expressed the VEGFR2 compared original ADSCs. This role of VEGF was proved by Yang et al. (2012) that VEGF increased the proliferation of EPCs through the calcineurin/NFAT signaling pathway [37]. The role of VEGF in the promotion of EPC differentiation was determined in the previous study [38].

5. Conclusions

EPCs play the important roles in vascular repair and angiogenesis. In the stem cell therapy and tissue engineering for vascular diseases, EPCs are essential materials for these applications. This study initially produced the EPCs from the trans-differentiation of ADSCs through the direct epigenetic reprogramming using the ETV-2 transcription factor. This method can produce up to 10% of CD31+VEGFR+ cells in combination with VEGF treatment. The transfected cell population displayed some of the EPC phenotypes, especially the micro blood vessel formation in the Matrigel. Although this study should be improved the efficacy and further evaluations, these initial results gave the promising strategy to produce the EPCs for stem cell transplantation as well as tissue engineering.

6. Open Access

7. List of abbreviations

ADSCs: Adipose derived stem cells, **ECs**: Endothelial cells, **EPCs**: endothelial progenitor cells, **HUVEC:** Human umbilical vein endothelial cells, **VEGF**: Vascular endothelial growth factor; **VEGFR2**: Vascular endothelial growth factor receptor 2

8. Competing interests

The authors declare they have no competing interests.

9. Funding

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10. Authors' contributions

PVP was responsible for suggesting the idea for this study, creating the experiment design, vector preparation, data analysis, writing the Discussion, preparing the figures, a nd revising the manuscript. VHT was responsible for performing the essays of ADSC culture, analysis, flow cytometry analysis, and writing the Methods, Results. HTN was responsible for performing the HUVEC cultures, Matrigel assays, and writing the Introduction. All authors read and approved the manuscript.

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