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Research article

The effects of human platelet lysate versus commercial endothelial growth medium on the endothelial differentiation potential of human amniotic fluid mesenchymal stem cells

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ABSTRACT

To differentiate stem cells into endothelial cells, vascular endothelia growth factors (VEGF) serve as the major signal for stimulating the cells. However, there are other cytokines or growth factors associated with endothelial cell development and differentiation. Human platelet lysate (hPL) has been a promising reagent in cell-based therapy since it is considered as a source of bioactive molecules and growth factors. The aim of this study was to investigate the in vitro differentiation of human amniotic fluid mesenchymal stem cells (hAF-MSCs) into endothelial-like cells under hPL together with VEGF or endothelial cell growth medium 2 (EGM-2), a commercially induced medium. In this study, hAF-MSCs were isolated from human amniotic fluid cells (hAFCs) using the direct adherence method. The cells expressed CD44, CD73, CD90, and HLA-ABC at high levels and expressed Oct-4 (octamer-binding transcription factor 4) at low levels. The cells were negative for CD31, CD34, CD45, CD105 and HLA-DR. This study found that hAF-MSCs induced with hPL and VEGF had the ability to differentiate into endothelial-like cells by presenting endothelial specific markers (vWF, VEGFR2 and eNOS), forming a network-like structure on Matrigel, and producing nitric oxide (NO). This outcome was similar to those of experiments involving EGM-2 induced cells. The present findings indicate that hPL + VEGF can induce hAF-MSCs to express endothelial cell characteristics. Our findings represent an important step forward in the development of a clinically compliant process for the production of endothelial cell-derived hAF-MSCs, and their subsequent testing in future clinical trials.

1. Introduction

Cardiovascular disease (CVD) is a major cause of death and disabilities worldwide. The risk factors associated with the pathology of this disease are obesity and insulin resistance, diabetes, smoking, hypertension, poor diet, and an increasing ageing population. CVD includes coronary artery disease (CAD), peripheral vascular disease (PVD), congestive heart failure, atrial fibrillation (AF), and stroke [1, 2, 3]. Generally, endothelium plays an important role in cardiovascular biology and in the responses to these risk factors. An injury to endothelium leads to a loss of appropriate endothelial physiology and/or dysfunction [4]. Previous studies have shown that endothelial cells have the ability to repair damaged endothelium and assist in the formation of new blood vessels [5]. However, the regenerative capacity of mature endothelial cells is limited. This is due to an insufficiency of the expanded cells and a decrease in function that is associated with an increase in age [6, 7]. Therefore, it is critical to identify the alternative cellular sources that are necessary to facilitate successful autologous or allogeneic transplantation.

Recently, MSCs have been acknowledged as a promising agent for treatment of vascular-related diseases through functional revascularization [8, 9]. Since MSCs have the ability to regenerate and differentiate into specific cells such as adipocytes, chondrocytes, osteocytes, hepatocytes, cardiomyocytes and endothelial cells under certain physiological or experimental conditions, they have been considered for using in cell-based therapies to treat or restore damaged tissue [10, 11, 12, 13]. When exposed to endothelial growth factors, they exhibited endothelial

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specific markers and were able to effectively differentiate into endothelial-like cells [7, 13, 14, 15].

To successfully differentiate stem cells into endothelial cells, preclinical studies usually differentiate stem cells into endothelial cells using endothelial cell growth medium 2 (EGM-2), the commercial medium developed for the culture system. It is loaded with serum supplement, fetal bovine serum (FBS), and the necessary cytokines for endothelial proliferation such as hydrocortisone, human fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), ascorbic acid, human epidermal growth factor (hEGF) and heparin [16, 17, 18, 19]. One possible alternative to this commercial medium is the use of human platelet lysate (hPL). It is well known that there are many growth factors, hormones, and other bioactive molecules that are contained within hPL. The growth factors present in hPL include platelet-derived growth factor (PDGF), EGF, VEGF, FGF, IGF-1, hepatocyte growth factor (HGF), and transforming growth factor β1 (TGF-β1), all of which have been shown to promote vasculogenesis, improve wound healing and promote the vessel growth of endothelial cells [20, 21, 22, 23].

A number of other advantages of hPL include its human blood derivatives, the absence of any risk from xenogeneic proteins, viruses or prion transmissions and the ease with which it can be prepared [24]. The ability of hPL has already been shown for different cells type such as fibroblasts, chondrocytes, endothelial cells and stem cells obtained from various sources [25, 26, 27]. However, there have been only a few research studies on the differentiation of human amniotic fluid MSCs (hAF-MSCs) with hPL. Therefore, we evaluated the endothelial differentiation potential of human amniotic fluid mesenchymal stem cells after they were induced in the different conditions of VEGF, hPL or EGM-2. We have hypothesized that a supplement of hPL and VEGF was able to increase in endothelial differentiation potential of hAF-MSCs and used in the clinical trials alternative to EGM-2.

2. Materials and methods

2.1. Cell source and preparation

Back-up flasks of human amniotic fluid cell (hAFCs) samples (n = 5) were obtained during weeks 16–22 of gestation from the Human Genetics Laboratory of Anatomy Department, Faculty of Medicine, Chiang Mai University. After being analyzed, normal karyotype cells were then used in this study. This study was approved by the Research Ethics Committee of the Faculty of Medicine, Chiang Mai University on March 13th, 2018 (no. ANA-2561-05343).

To isolate MSCs, the direct adherent method was performed as it has been previously explained [28]. The hAFCs were washed with sterile phosphate buffer saline (PBS) (Sigma-Aldrich, USA) and trypsinized with 0.25% trypsin-EDTA (Gibco, USA). After observed, detached cells were then diluted with basal medium consisting of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) – high glucose with 10% fetal bovine serum (FBS) (Gibco, South America), gentamycin (T.P. Drug Laboratories, Thailand) and Pen Strep (penicillin and streptomycin) (Gibco, USA). The hAFCs were plated in non-pyrogenic polyprolene 25 cm^2 (Corning®, NY, USA) at 37 °C and 5 % CO_2 at 95% humidity. Upon reaching 80% confluence, the cells were sub-cultured to remove non-adherent cells. After observing the presence of a fibroblast-like morphology, the cells were then used in the experiments.

2.2. Flow cytometry analysis

Cell surface antigens were used to characterize the MSC properties by flow cytometry. The cells were detached with 0.25% trypsin-EDTA and centrifuged at 3,700 rpm for 6 min at room temperature to obtain the cell pellets. Non-specific binding was then blocked with 10% human AB serum at 4 °C for 30 min. For the detection of Oct-4, the cells were permeablised with 1% Triton X-100 (amresco®, Ohio, USA) for 1 min before incubated with antibody. Detroit 551 (fibroblast) (Sigma-Aldrich, USA) was used a negative control for the detection of Oct-4. Subsequently, cells pellets were incubated with the following monoclonal antibodies: Mouse anti-human CD31-PE (Immuno Tools GmbH, Germany), CD34-FITC (Immuno Tools GmbH, Germany), CD44-PE (Pierce Biotechnology, USA), CD45-PE (Immuno Tools GmbH, Germany), CD73-PE (Immuno Tools GmbH, Germany), CD90-FITC (Biolegend, USA), CD105-PE (Pierce Biotechnology, USA), HLA-ABC-FITC (Immuno Tools GmbH, Germany), HLA-DR-PE (Immuno Tools GmbH, Germany), and Oct-4-Alexa Fluor® 488 conjugate (Sigma-Aldrich, USA). Isotype antibodies served as a control to exclude nonspecific binding. Quantitative analysis was performed using FACScan (BD Biosciences) and the results were analyzed using CellQuest™ Pro 9.0 software (BD Biosciences).

2.3. Alamar blue cell proliferation assay

Alamar blue cell proliferation assay was used to evaluate the proliferation of hAF-MSCs. The cells were tested with alamarBlue® reagent (Sigma-Aldrich, USA) which functions as an indicator by measuring cell viability based on the detection of metabolic activity. Blue-colored resazurin was ultimately converted to red-colored resorufin. The change in color was indicative of the metabolic activity of the living cells. Resazurin fluorescence was measured as the level of absorbance with an excitation wavelength at 540–630 nm using a spectrophotometer plate reader (Thermoscientific, UK). Briefly, hAF-MSCs were washed, trypsinized and centrifuged at 3,700 rpm for 6 min. Next, the cell pellets were re-suspended in DMEM and plated in a 24 well-plate (Corning Incorporated, USA) at a density of 2 x 10^3 cells/well (5 wells/sample). The cells were then cultured in basal medium at 37 °C, 5% CO_2, and 95% humidity. After 24 h, the medium was removed and 100 μl of 10% (v/v) alamar blue solution in DMEM was added to these wells, as well as to the wells without cells so as to serve as a negative control. The samples were returned to an incubator and were incubated for 4 h in the same environment. After which, the alamar blue solution in each well was removed and added to the 96 well-plate (Corning®,USA), and the fluorescence was measured. Each group was continuously cultured under the same conditions. Alamar blue cell proliferation assay was performed every other day for 21 days.

2.4. Preparation of human platelet lysate

Platelet concentrates (PCs) were obtained from the blood bank of Maharaj Nakorn Chiang Mai Hospital using the apheresis method. PCs were then prepared from pooled PC (n = 15) as has been previously described [29, 30]. Briefly, the pooled PL was frozen at -80 °C, re-thawed 3 times at 37 °C and centrifuged at 4,000 rpm for 20 min to remove cellular fragments. After that, the supernatant was passed through a 0.2 μm filter (Corning®, USA) to remove cellular debris and 4U/ml heparin (LEO Pharma A/S) was added to avoid gel formation. Finally, the PL was stored at -20 °C.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Commercially available enzyme-linked immunosorbent assay (ELISA) kits (Sigma-Aldrich, USA) were used according to the manufacturer's instructions to quantify the concentrations of the relevant growth factors including b-FGF, IGF-1, TGF-β1, and VEGF. All sample measurements were performed in duplicate.

2.6. Differentiation of hAF-MSCs into endothelial cells

To differentiate the hAF-MSCs into endothelial cells, the cells were treated under different conditions, including the medium supplemented with 10% hPL only, 50 ng/ml VEGF only, 10% hPL +50 ng/ml VEGF, 20% hPL +50 ng/ml VEGF, and EGM-2. The positive control was comprised of human umbilical vein endothelial cells (HUVECs). The cells

were cultured at 37 °C, 5% CO_2, and 95% humidity. The medium was changed every 3 days.

2.7. Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

After induction, total RNA of cells from all conditions was extracted using an Illutra RNAspin Mini RNA Isolation kit (GE Healthcare, UK). Next, the first complementary strand of DNA (cDNA) was synthesized from total RNA using Tetro cDNA synthesis kit (Bioline, USA) according to the manufacturer's instructions. Then, cDNA was amplified by 7500 FAST Real-time PCR System (Applied Biosystems, USA) in 20 μl of reaction mixture containing cDNA, SensiFAST™ SYBR® No-ROX Kit (Bioline, USA), gene specific primers including *von Willebrand Factor (vWF), VEGF receptor 2 (VEGFR2),* and *endothelial nitric oxide synthase (eNOS)* [31] (Table 1), and nuclease free water. The housekeeping gene of *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was amplified to serve as an internal control. The expression levels of the endothelial specific genes were plotted using the $2^{-\Delta Ct}$ method.

2.8. Immunofluorescence analysis

Cells were detected for the expression of endothelial specific markers including vWF, VEGFR2, and eNOS. Briefly, the cells were washed twice with PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) at room temperature for 15 min and permeabilized with 0.2% Triton X-100 (amresco®, Ohio, USA) in PBS at room temperature for 10 min. Blocking for non-specific binding was then conducted with 10% human AB serum in 1% BSA-PBS for 30 min at room temperature, followed by incubation with mouse monoclonal antibody against human vWF (Pierce; Thermo Fisher Scientific, USA), rabbit monoclonal antibody against human VEGFR2 (Pierce; Thermo Fisher Scientific, USA) or mouse monoclonal antibody against eNOS (Pierce; Thermo Fisher Scientific, USA) at 37 °C for 2 h. After washed with PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG antibody (ImmunoTools, Germany) or PE-conjugated goat anti-rabbit IgG antibody (Thermo Fisher Scientific, USA) at 37 °C for 1 h. For nuclear staining, the cells were incubated with antifade blended with 4′-6-diamidino-2-phenylindole (DAPI) (Prolong® Gold, Thermo Fisher Scientific, USA) for 10 min at room temperature. The fluorescent signal of the endothelial specific marker was observed by fluorescent microscope and photos were taken with a DP manager and DP controller. Statistical analysis of the fluorescent signal was conducted using ImageJ 1.50i software and presented as the corrected total cell fluorescence (CTCF).

2.9. Network formation

The capillary formation potential of the cells was performed by incubating the cells in Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning®, Netherlands) according to the manufacturer's instructions. Briefly, the Matrigel was thawed at 4 °C overnight and added to a 96 well-plate at a concentration of 50 μl/cm^2 with pre-cooled pipette tips. Next, the plate was incubated at 37 °C for 30 min. The cells were then prepared by being trypsinized and re-suspended in the medium. After that, cells were counted and adjusted to a concentration of 2 x 10^5 cells per 100 μl. Finally, the cells were gently added at the selected density to the gel-coated well and incubated at 37 °C, 5% CO_2 and 95% humidity overnight. The capillary formation was observed under a light microscope. The ImageJ angiogenesis analyzer was used to analyze statistics of mesh area and number of mesh.

2.10. Nitric oxide production by griess assay

Nitric oxide (NO) production was measured within the supernatant of the cultured cells. The metabolic product of NO or nitrite ($NO2^-$) was detected by using Griess reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. In brief, the supernatant and the standard solution were added into the 96 well-plate at 50 μl in triplicate. Next, 50 μl of the Griess reagent was added to all samples and they were incubated for 10 min at room temperature and protected from the light. The color of the supernatant was used to measure the absorbance within 30 min in a microplate reader at a wavelength of 540 nm.

2.11. Cell proliferation assay by MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (Sigma-Aldrich, USA) was used to evaluate the proliferation of the cells that were treated by 10% hPL + VEGF, 20% hPL + VEGF and EGM-2. The cells were seeded in a 96 well-plate at a density of 5 × 10^3 cells in triplicate and incubated at 37 °C with 5% CO_2 and 95% humidity for 15 days. At the indicated time points (day 1,3,5,7,9,11,13, and 15), the medium was removed and replaced with MTT solution (0.5 mg/ml in DMEM). After a further 4 h of incubation under the same conditions as for culture, MTT solution was removed and 100 μl DMSO was added to dissolve the formazan crystals. The absorbance was determined at 540 nm with a spectrophotometer.

2.12. Statistical analysis

Statistical analysis was performed for descriptive analysis with SPSS statistic 22. All results were presented as mean ± SEM values. Comparisons between multiple groups were performed using one-way ANOVA with post hoc test by employing SPSS version 22.0 software. Significance was considered when the p value was less than 0.05 ($p < 0.05$).

3. Results

3.1. Morphology of hAF-MSCs

Notably, hAF-MSCs were isolated from hAFCs using the direct adherence method. Cell adhesion occurred 3–5 days after the initiation of the culture. At first passage, cells expanded in the monolayer and revealed differing relevant characteristics including a spindle-shaped, polygonal and epithelioid morphological feature. When sub-cultured as

Table 1. Rt-qPCR primer sequences.

Primer		Real-time PCR primer sequence
vWF	Forward 5′-3′	CAAGGAAGAAAATAACACAGGTGAA
	Reverse 5′-3′	TCATTGACCTTGCAGAAGTGAGTAT
VEGFR2	Forward 5′-3′	GACTTCCTGACCTTGGAGCATCT
	Reverse 5′-3′	GATTTTAACCACGTTCTTCTCCGA
eNOS	Forward 5′-3′	TCCCCCAGAACTCTTCCTT
	Reverse 5′-3′	CTCATTCTCCAGGTGCTTCA
GAPDH	Forward 5′-3′	ATGGGGAAGGTGAAGGTCG
	Reverse 5′-3′	TAAAAGCAGCCCTGGTGACC

second passage, the polygonal and epithelioid cells gradually disappeared. After 5–7 days, cells reached a 80% level of the confluence and revealed a homogenous spindle-shaped morphology (Figure 1A).

3.2. Immunophenotypic characteristics of hAF-MSCs

Flow cytometry analysis revealed that the cells at second passage were found to be positive for the markers of CD44, CD73, CD90 and HLA-ABC at 96.31 ± 0.53%, 98.45 ± 0.47%, 89.10 ± 0.82% and 85.21 ± 1.32%, respectively. The cells expressed Oct-4 at low levels (35.21 ± 0.98%) and a negative control cells showed a negative expression of this marker (1.38 ± 0.09%). The cells were negative for CD31, CD34, CD45, CD105 and HLA-DR at 0.21 ± 0.08%, 0.18 ± 0.05%, 0.19 ± 0.02%, 0.30 ± 0.02 and 0.12 ± 0.02%, respectively (Figure 1B).

3.3. Proliferation of hAF-MSCs

Cell proliferation was evaluated throughout alamar blue assay to represent the percentage of reduction by referencing the number of living cells. The growth characteristic of hAF-MSCs indicated that the percentage of reduction of cells gradually increased in the log phase within 9 days. Cells then entered into the stationary phase and this phase was maintained through day 21 (Figure 1C).

3.4. Growth factor concentrations

ELISA kits were used to measure the concentrations of b-FGF, IGF-1, TGF-β1, and VEGF in hPL obtained from a batch of 15 samples of platelet units. Growth factor concentrations in lysate preparations are listed in Table 2. The concentration values of b-FGF, IGF-1, TGF-β1, and VEGF were 40.60 ± 0.58 pg/ml, 4.26 ± 0.14 ng/ml, 103 ± 0.96 pg/ml, and 46.52 ± 1.74 pg/ml respectively. The data are shown as mean ± SEM values.

3.5. Morphology of treated cells

Subsequently, hAF-MSCs were induced to be differentiated into endothelial cells under different conditions involving the media supplemented 10% hPL, VEGF, 10% hPL + VEGF, 20% hPL + VEGF or EGM-2. HUVECs were used as a positive control. After 14 days, the cells that were exposed to 10%hPL or VEGF only maintained a spindle-shaped morphology. Some of the cells in 10%hPL + VEGF exhibited a thin process of the cytoplasm. Furthermore, the cells tended to realign and form the lumen-like structure. While, the cells in 20% hPL + VEGF showed a short and thin cytoplasm with a network-like structure. The EGM-2 induced cells displayed a polygonal shape. HUVECs displayed an oval shape of morphology (Figure 2A).

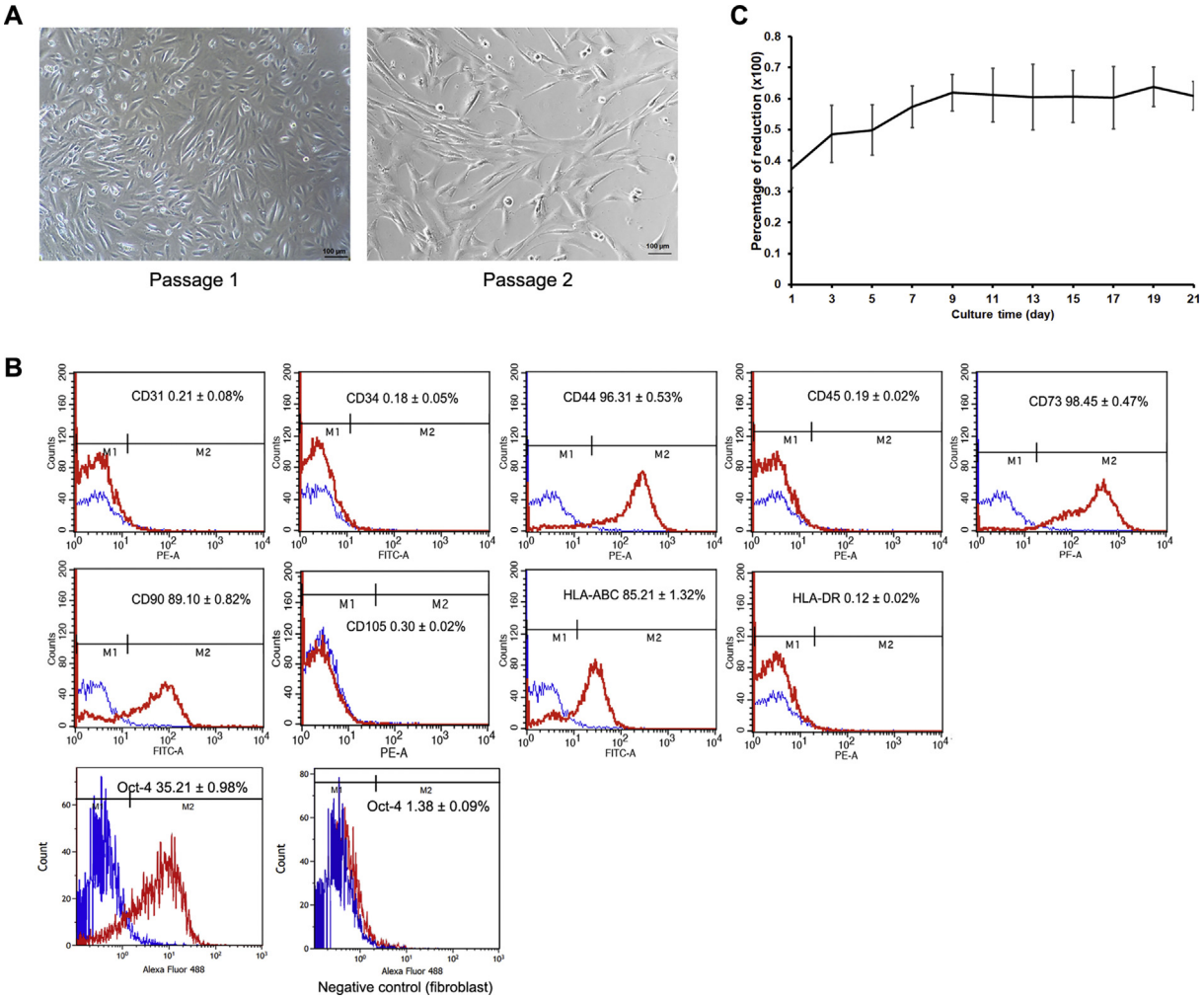

Figure 1. Characteristics of hAF-MSCs. Morphology of cells obtained from human amniotic fluid at passage 1 and 2 (magnification, x10; scale bar, 100 μm) (A). Detection of mesenchymal stem cell specific marker expression by flow cytometry (B). A blue line is representative of an antibody isotype control and a red line is indicative of a signal from the cell surface marker antibodies. Data is presented as mean ± SEM values. Growth curve by alamar blue assay in hAF-MSCs throughout 21 days. Data are presented as mean ± SEM values (C).

Table 2. Growth factor concentrations of hPL.

Growth factors	Concentration
b-FGF	40.60 ± 0.58 pg/ml
IGF-I	4.26 ± 0.14 ng/ml
TGF-β1	103 ± 0.96 pg/ml
VEGF	46.52 ± 1.74 pg/ml

3.6. Detection of endothelial-related gene expression

The hAF-MSCs were used to detect the expression of endothelial-related genes including *vWF*, *VEGFR2*, and *eNOS* and then normalized with *GAPDH* using RT-qPCR. HUVECs were used as a positive control. The results at Figure 2B showed a significant increase in the level of *vWF*, *VEGFR2*, and *eNOS* in VEGF only, 10% hPL + VEGF, 20% hPL + VEGF, EGM-2, comparable to that of *vWF*, *VEGFR2*, and *eNOS* in 10% hPL only. By statistical analysis, cells were found to be significant in *vWF* level when compared between 10% hPL + VEGF and EGM-2. While, there were no significant differences in VEGFR2 and eNOS levels among 10% hPL + VEGF, 20% hPL + VEGF and EGM-2.

3.7. Detection of endothelial-specific marker expression

The levels of expression of vWF, VEGFR2 and eNOS were investigated by immunofluorescent analysis. The results are presented in Figure 3A demonstrating the fluorescent signals of vWf (green), VEGFR2 (red), and eNOS (green) located in cells treated in VEGF, 10% hPL + VEGF, 20% hPL + VEGF and EGM-2. This outcome was similar to the fluorescent signals of of HUVECs. Conversely, no signal was detected for these proteins under 10% hPL condition. Analysis using ImageJ 1.50i software was used to calculate the CTCF. There was an significant increase in CTCF levels of vWF Figure 3B, VEGFR2 Figure 3C and eNOS Figure 3D in VEGF,

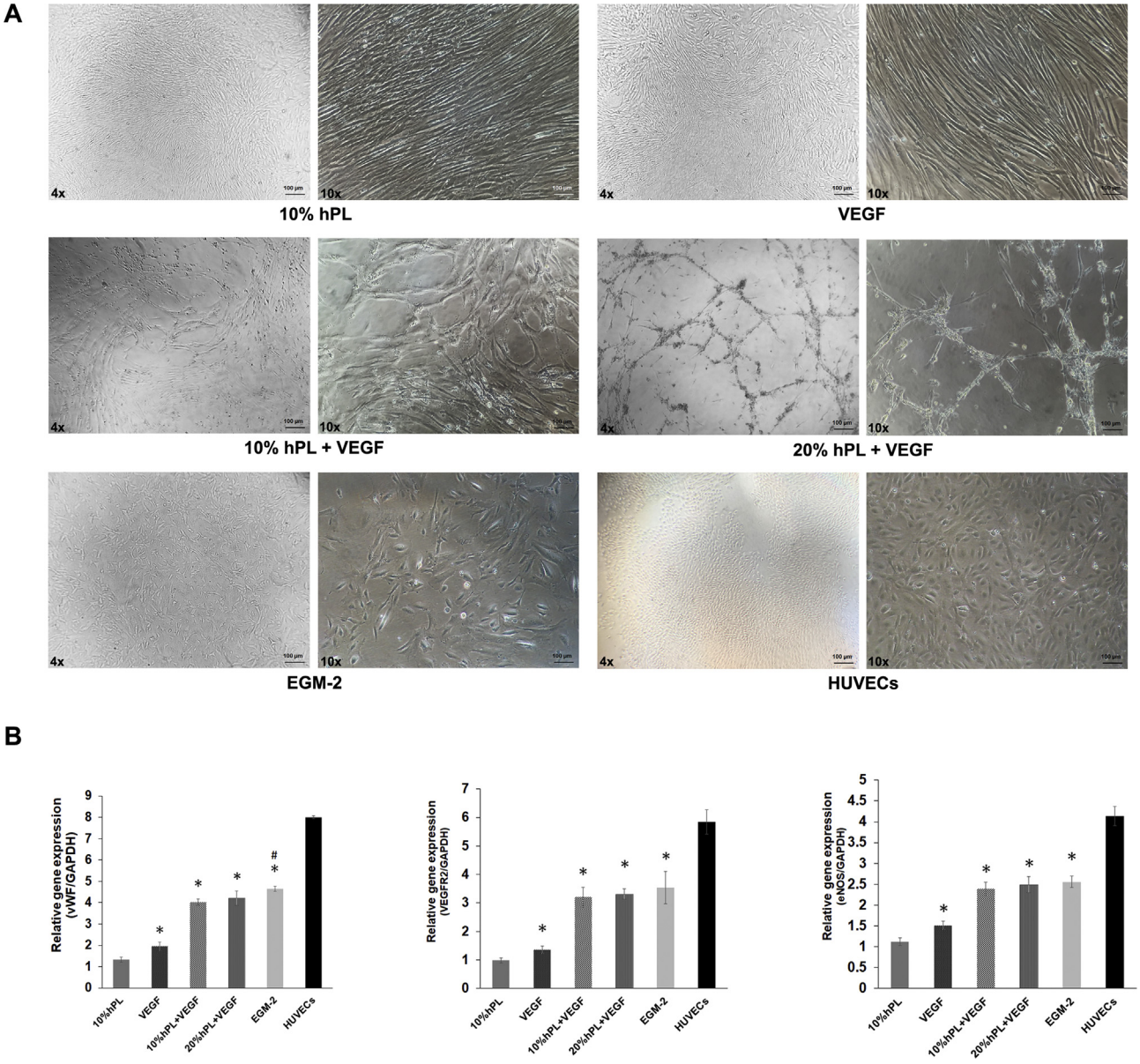

Figure 2. Morphology of treated cells and HUVECs (A). Representative phase contrast of cells treated by 10% hPL, VEGF, 10%hPL + VEGF, 20% hPL + VEGF, EGM-2 and HUVECs. Magnifications are represented in 4X (left column) and 10X (right column) (scale bar, 100 μm). Detection of endothelial-related gene expression by RT-qPCR (B). The treated cells and HUVECs were analyzed to determine endothelial-related gene expression levels. The relative expression values of vWF, VEGFR2 and Enos were determined. Gene expression levels were normalized to GAPDH. Data is presented as the mean ± SEM. *P < 0.05 indicates a statistical difference, comparable to 10% hPL. #P < 0.05 indicates a statistical difference, comparable to 10% hPL + VEGF.

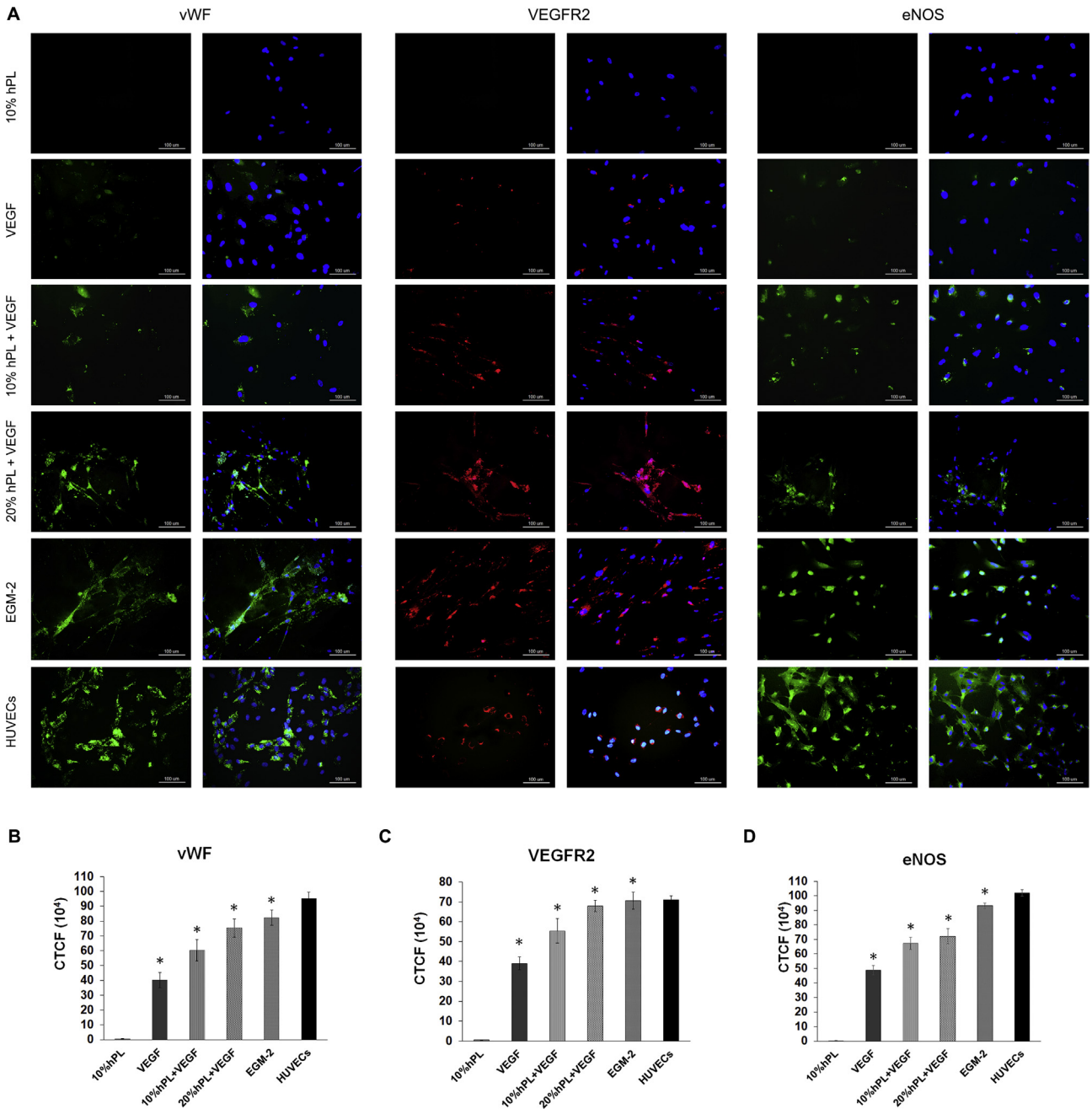

Figure 3. Immunofluorescence staining for endothelial associated markers. The treated cells and HUVECs were stained with antibodies against vWF, VEGFR2 or eNOS. Cell nuclei were stained with DAPI (magnification x20; scale bar 100 μm) (A). Quantification of fluorescent signals was represented by using CTCF of vWF (B), VEGFR2 (C) and eNOS (D). Data is presented as the mean ± SEM. *P < 0.05 indicates a statistical difference, comparable to 10% hPL.

10% hPL + VEGF, 20% hPL + VEGF, EGM-2 when compared with those of vWF, VEGFR2 and eNOS in 10% hPL only.

3.8. Ability to form networks

The ability to form a network in Matrigel was tested. After the cells were incubated in different conditions for 14 days, they were then harvested with trypsin and plated in a Martigel-coated plate for 24 h Figure 4A revealed the network-like characteristic of cells. Cells that were cultured in 10% hPL only did not present a network-like structure. In contrast, cells in VEGF only presented some connection to the cell processes. The cells in 10% hPL + VEGF, 20% hPL + VEGF and EGM-2 displayed a network-like structure that was similar to HUVECs. The quantitative data of the network-like structure was analyzed by angiogenesis analyzer, ImageJ 1.50i software. The data was presented in terms of the total mesh area (Figure 4B) and number of mesh (Figure 4C). The cells in VEGF only, 10% hPL + VEGF, 20% hPL + VEGF, EGM-2 and HUVECs showed a higher level of both parameters. However, the data showed no significant difference in the term of total mesh area and number of mesh when compared between 10% hPL + VEGF and 20% hPL + VEGF.

3.9. Nitric oxide production

Total byproducts of nitric oxide were measured in the form of nitrites after the conversion of nitrites via nitrate reductase. Total nitrite levels of the treated cells and HUVECs were calculated based on a nitrite calibration curve. The results Figure 5A revealed that the levels of nitrite in

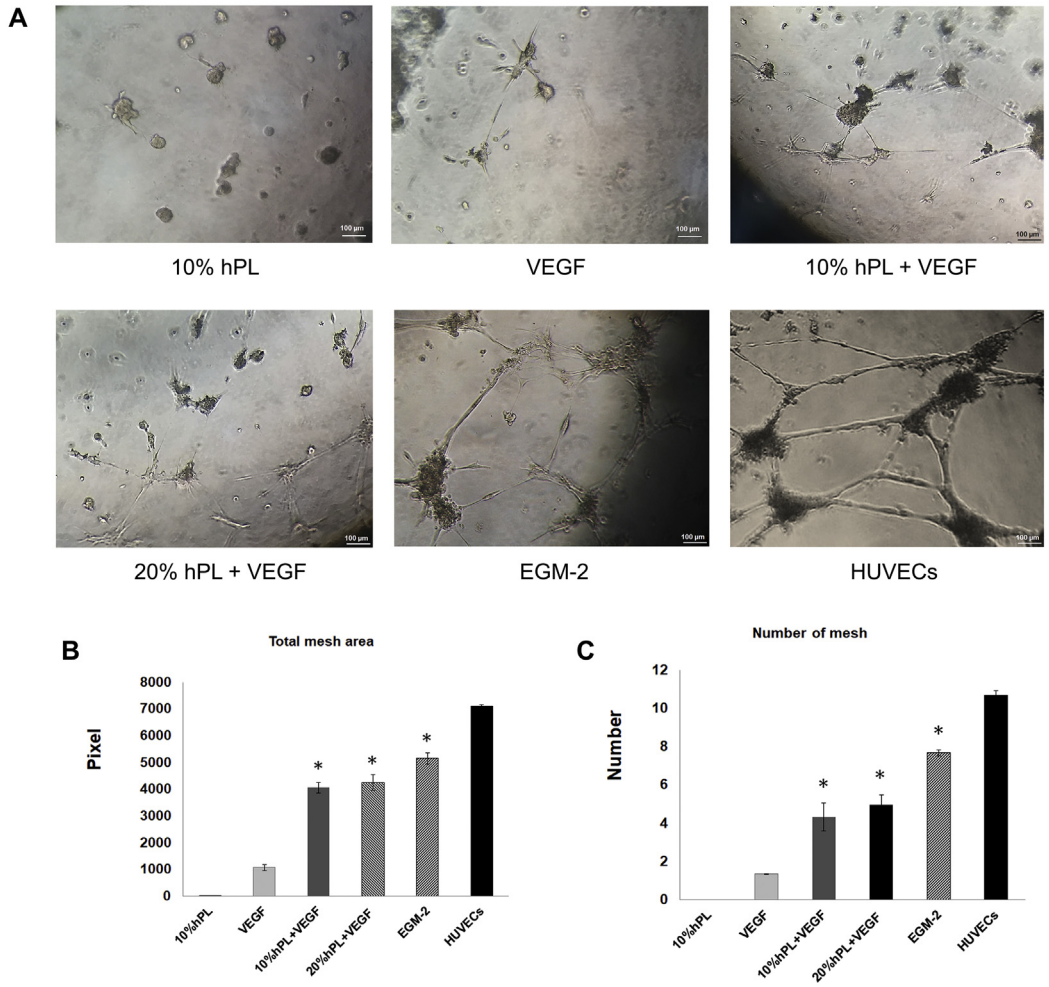

Figure 4. Network formation in Matrigel. Ability of cells in 10% hPL, VEGF, 10% hPL + VEGF, 20% hPL + VEGF, EGM-2 and HUVECs to form a network-like structure (magnification x10; scale bar 100 μm) (A). The quantitative data of the network formation was analyzed by angiogenesis analyzer, ImageJ 1.50i software. The data is presented in terms of the total mesh area (B) and number of mesh (C). *P < 0.05 indicates a statistical difference, comparable to 10% hPL or VEGF only.

VEGF, 10% hPL + VEGF, 20% hPL + VEGF, and EGM-2 significantly increased when compared with 10% hPL only. Among 10% hPL + VEGF, 20% hPL + VEGF and EGM-2, the levels of nitrites showed an insignificant difference ($p > 0.05$).

3.10. Proliferation of treated cells

The proliferation of cells exposed to 10% hPL + VEGF, 20% hPL + VEGF and EGM-2 was determined by MTT assay. The data showed no significant difference in the proliferation of cells from three conditions. Until day 13, the cells treated by 20% hPL + VEGF showed a decrease in cell proliferation and the data was found to be significant when compared with EGM-2 at day 15 Figure 5B.

4. Discussion

MSCs have received attention as a promising autologous source for cell-based therapy in restoring endothelial functions and promoting vascularization [8, 32]. As a source of MSCs, human amniotic fluid presents many advantages. The hAF-MSCs can be easily obtained by amniocentesis, which is a minimally invasive procedure and has yielded approximately 0.9–1.5% of the cellular population [28, 33].

In this study, we investigated the endothelial differentiation potential of hAF-MSCs with regard to the induction of hPL, VEGF and EGM-2. MSCs were isolated from human amniotic fluid using the direct adherence method [28]. During the early period of the culturing process, the adherent cells obtained from human amniotic fluid displayed both epithelial-like and fibroblast-like morphological characteristics. After being sub-cultured to passage 2, epithelial-like cells gradually disappeared and fibroblast-like cells expanded rapidly. This result was supported by the findings of a previous study in which epithelial-like cells appeared during the early period and significantly decreased thereafter. The fibroblast-like cells exhibited phenotype characteristics and differentiation potential that were similar to MSCs [34, 35].

Flow cytometry analysis revealed that hAF-MSCs were positive for CD44, CD73, CD90 and HLA-ABC and negative for CD31, CD34, CD45, and HLA-DR. The surface antigen markers used for immunophenotyping were the same as those used in previous studies [25, 26, 27, 36]. The negative expression values of CD31, CD34, and CD45 confirmed that the cells were clearly discriminated from endothelial cells (CD31) and hematopoietic stem cells (CD34 and CD45) [25]. Additionally, the hAF-MSCs were stained positively for HLA-ABC and were found to be negative for HLA-DR. This outcome suggested that the cells could reduce the need for immunosuppression following allogeneic transplantation [37]. However, CD105 showed the negative expression. According to the criteria for defending the MSCs, the cells must express CD73, CD90 and CD105 [10]. Tsai et al. (2004) [38] found that MSCs from second-trimester amniotic fluid was low positive for CD90 and CD105. In contrast, Kern et al., (2006) [39] showed that MSCs derived from bone marrow, umbilical cord blood, and adipose tissue expressed CD105 in

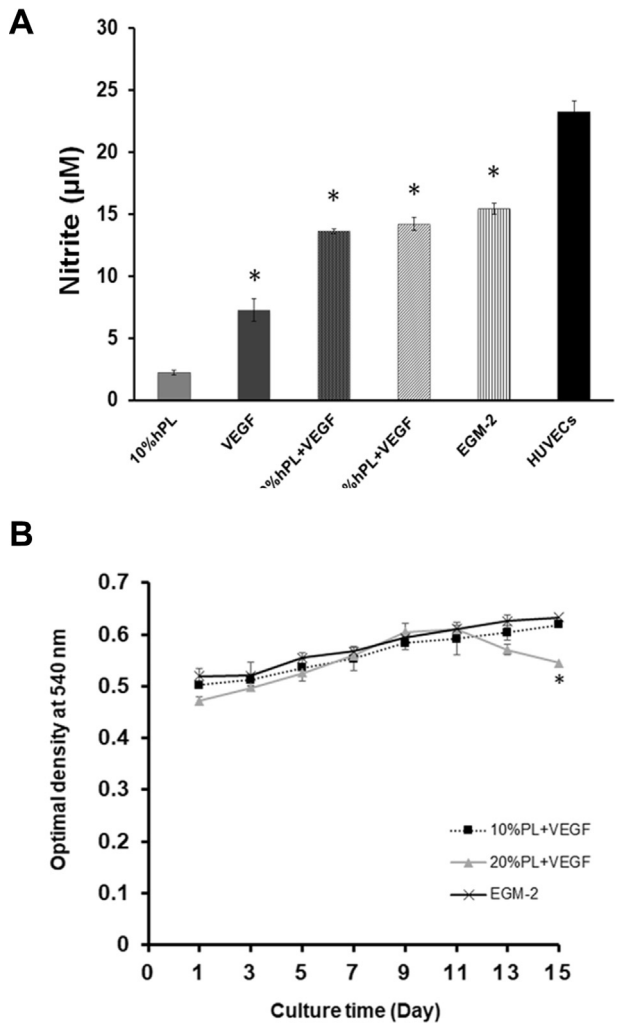

Figure 5. Nitric oxide production via the detection of nitrites (A). Nitrite levels produced by treated cells and HUVECs. Data is presented as the mean ± SEM. *P < 0.05 indicates a statistical difference, comparable to 10% hPL. Determination of cell proliferation (B). The cells were treated by 10% hPL + VEGF, 20% hPL + VEGF or EGM-2 using the MTT assay throughout day 3, 5, 7, 11, 13 and 15. The data is represented as the mean of optimal density ±SEM. *P < 0.05 indicates a statistical difference, comparable to EGM-2.

high level. The result from present study found that, the cells also showed the expression of CD90 less than 95%, similarly to previous finding [40]. The results from flow cytometry suggested that sources, the gestational age, the cell passage and the donor's heterogeneity are associated with the phenotype of MSCs [31, 41]. Interestingly, the cells expressed Oct-4 at low levels. This result was consistent with those investigated by Zhang et al. (2009) [16] and Phermthai et al. (2010) [42]. Oct-4 is a key regulator of stem cell pluripotency and differentiation, known to be expressed in embryonic stem cells (ESCs) [43]. The data indicated that hAF-MSCs represented an intermediate stage between embryonic and adult stem cells and had the potential for multipotent differentiation. The potency of hAF-MSCs was confirmed by recent studies that isolated MSCs from the same source. The results showed the differentiation potential of MSCs into chondrocyte, cardiomyocyte and osteoblast [44, 45, 46].

The growth characteristics of hAF-MSCs were further investigated using alamar blue assay. The cells showed an increase in the proliferation within 9 days and maintained their viability through day 21. Previous studies indicated that hAF-MSCs possess a high expansion potential and grow easily with a stable morphology [47]. In addition, the lifespan of AF-MSCs is superior to that of bone marrow derived MSCs (BM-MSCs) because of a decrease in proliferation and differentiation potential that occur along with an increase in age [47, 48].

Several studies have demonstrated that MSCs isolated from adult tissues under specific conditions were capable of differentiation into multilineage cell types including endothelial cells [13, 17, 49, 50, 51]. A number of studies have shown that mesenchymal stem cells obtained from various sources can differentiate into endothelial cells in the presence of VEGF [7, 15, 49, 52]. In addition, angiogenic cytokines and growth factor supplements, such as b-FGF and IGF-1 were added into the induced medium [49, 53]. As an alternative to commercially prepared induced medium, hPL combined with VEGF was considered to be the stimulator of endothelial differentiation of hAF-MSCs. The present study evaluated the endothelial differentiation potential of hAF-MSCs when they were induced under hPL, VEGF or EGM-2.

The hPL was obtained from platelet concentrates via a standardized platelet apheresis technique that provided a high concentration of platelets and a low level of leukocyte contamination [36]. To lysis the platelet concentrates, repeated freeze/thaw cycles were selected in the present study. This was due to the establishment of a systematic study wherein this procedure was found to ensure the maximum release of growth factors and cytokines from platelets [54].

ELISA was preformed to determine the concentration of growth factors in hPL. Growth factors including b-FGF, IGF-1, TGF-β1, and VEGF were detected as they were frequently reported in recent studies [24, 29, 55, 56]. The results of previous analyses are not comparable with our results because of the different preparation methods employed and variations in the components of the hPL values between batches. According to previously presented evidence, PL was added to the medium at concentrations that ranged from 5-20%. Concentrations were then often used at values of 10% [26, 30, 36, 57, 58]. To induce the cells, 10% hPL was used in the condition according to the method described in a previous study. This was used because this concentration did not produce a negative effect on cell viability [31].

Following 14 days of stimulation, the cells induced by 10% hPL + VEGF, 20% hPL + VEFG and EGM-2 adopted the arrangement and morphology. To assess the degree of endothelial differentiation potential of hAF-MSCs, we investigated the expression of some endothelial related-markers including *vWF*, *VEGFR2*, and *eNOS*. The result from RT-qPCR revealed that the cells treated by hPL only showed the lowest expression of endothelial specific genes. Moreover, there were no specific proteins locating in the cells. When investigated the functional abilities, the cells didn't exhibit the network as well as the nitric oxide production. In contrast, the cells in VEGF only showed an increase in endothelial differentiation potential by expressing endothelial specific markers in both gene and protein levels. Furthermore, the cells displayed the ability to form network and produce nitric oxide partially. The results indicated that VEGF is the main signal that induced hAF-MSCs to differentiate into endothelial cells. VEGF is well known as a major vasculogenic and angiogenic molecule during development as well as adult tissues [59]. Cells that respond to VEGF must first express its receptors [60]. Although, several studies evidenced that VEGF is a key signal of endothelial differentiation [59, 60, 61, 62], some study reported that VEGF itself cannot be the most proximal signal, and it is still not clear precisely when VEGF first participates in vascular specification [63].

A number of studies have shown that mesenchymal stem cells from various sources can differentiate into endothelial cells in the presence of 50 ng/ml VEGF [7, 15, 49, 52]. The evidence from previous study showed that this concentration of VEGF resulted in maximal expressions of endothelial specific markers [19]. In addition, angiogenic cytokines and growth factor supplements such as basic fibroblast growth factor (bFGF) and insulin-like growth factor-I (IGF-I) were added in the induced medium to enhance the differentiation potential [49, 53]. Pre-clinical studies often differentiate the stem cells into endothelial cells by using EGM-2, a commercial medium for the culture system. It contains the necessary cytokines for endothelial proliferation such as VEGF, b-FGF, EGF, and IGF-1 [14,16-18].

As an alternative to EGM-2, hPL has become an alternative source of growth factors in this study. Previous studies have been reported that hPL contains various cytokines and growth factors that are involved in cellular migration, proliferation, differentiation, extracellular matrix organization and remodeling, and cell survival [25, 30, 36, 58, 64]. Growth factors in hPL, especially, VEGF, stimulate Akt to promote cell survival and ensure adequate vascular development in cardiovascular function [65, 66].

When hPL and VEGF were combined in the induced media, the treated cells from both 10% and 20% hPL with VEGF presented a higher ability of endothelial differentiation. Both induced media can enhance the expression of vWF, VEGFR2 and eNOS in the cells. The results indicated that growth factors in hPL may involve in the endothelial differentiation. The data were also supported by Barsotti1 et al. (2013) [22]. The study showed that the PL treatment of endothelial cells induced viability and proliferation and was able to stimulate wound healing via both ERK1/2 and NFκB pathways. Additionally, previous study reported that growth factors in hPL may enhance cell survival of circulating endothelial colony forming cells by activating Akt1 and enhancing the expression of pro-survival/anti-apoptotic molecules against the apoptotic signal [21].

Our study showed that hAF-MSCs were capable of differentiation into endothelial cells *in vitro* by expressing levels of endothelial specific markers including vWF, VEGFR2 and eNOS similarly to HUVECs. The expression of these markers was determined at mRNA levels, which supported the results of protein accumulation in the cells as shown by immunofluorescence. The results suggested that the cells responded to the signal of VEGF by expressing its receptor and showed an improvement in endothelial differentiation potential after they were supplemented by hPL. Although the mechanism of endothelial differentiation is still unclear, the studies have reported that the binding of VEGF to its receptor has been shown to activate the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signalling pathway [63, 67]. The activation of this pathway to histone deacetylases (HDACs) leads to endothelial marker gene and protein expressions [68]. In current study, the expression of vWF, VEGFR2 and eNOS in differentiated hAF-MSCs appeared within 14 days. The results were supported by the previous reports [15, 69]. vWF gene first appeared in 1 week and completely decreased at the third week [69]. vWF, considered as one of the endothelial specific markers, is synthesized exclusively in endothelial cells [70]. While VEGFR2 and eNOS genes were documented that they exhibited at the early stage of differentiation [69, 71]. VEGFR2 is primary responder to VEGF signal [72]. It is the major mediator of endothelial cell mitogenesis, survival, and microvascular permeability [60, 63]. eNOS is the enzyme playing an essential role downstream of VEGF signaling pathway. *In vivo*, it stimulates migration, proliferation, and vasodilation through nitric oxide production [19]. Therefore, the expression of endothelial specific markers on differentiated hAF-MSCs implied that the cells could have the potential in further clinical applications and may be an alternative source for repairing vascular pathologies.

VEGF binding to VEGFR2 also activates downstream signaling pathways which is essential for angiogenesis and several cellular responses [73]. To prove these data, the cells induced by VEGF with 10% hPL or 20% hPL were investigated their endothelial function. They displayed a network-like structure in Matrigel as well as produced nitric oxide which were similar to the cells induced by EGM-2. Interestingly, the cells that were cultured in 20% hPL and VEGF exhibited the connection not only within Matrigel but also within the culture flask. This finding was in agreement with the results of previous studies wherein PL had the potential to induce vasculogenic and angiogenic responses that were mediated by growth factors including VEGF, b-FGF and PDGF. All of which could lead to the formation of the capillary network [21, 23]. Several studies used Matrigel as model for in vitro study of endothelial cell activity [15, 16, 18, 50]. The induced cells performed the network by interacting with laminin, collagen IV, and enactin, [74]. A portion of laminin A chain mediated cells attachment to laminin resulting in the alterations in cell morphology and cell migration. This process mimics the endothelial cell alignment, cell-cell adhesion, lumen formation, and basal secretion of basement membrane that occurs during formation of new microvessels [75]. The ability to form new capillary-like structures upon plating on Matrigel suggested that differentiated hAF-MSCs had the potential to participate in angiogenesis.

In the view of vascular activity, the combination of hPL, both 10% and 20%, and VEGF can enhance the ability of nitric oxide production in the cells. This finding is in accordance with the expression levels of eNOS in the cells that were evaluated by RT-qPCR and immunofluorescent analysis. This result indicated that the eNOS expression of differentiated hAF-MSCs can catalyze the reaction to produce NO. This activity provides the evidence for the ability of differentiated hAF-MSCs to commit to an endothelial cells lineage. The data were supported by previous reports in which eNOS was activated through direct phosphorylation by Akt. This was found to link the signal transduction from VEGF to the release of NO in endothelial cells [76]. It was previously explained that the exposure of endothelial progenitor cells or endothelial cells to growth factors, such as IGF-1 and TGF-β1 resulted in an increase in eNOS expression associated with an increase in production of NO [77, 78, 79].

The results from this study indicated that hAF-MSCs can differentiate into endothetlial cells. The supplementation of 10% and 20% hPL together with VEGF can promote the endothelial differentiation potential. The ability of the cells was similar to the cells induced by EGM-2. Recently, hPL has been originally suggested as an alternative to the xenogenic serum [55]. The advantages of PL have been documented that it is applicable for a wide range of different cell types, enriched in growth factors, no risk of xenogeneic immune reactions or transmission of bovine pathogens and no critical side effects were reported [24].

Although hPL at concentration 20% has a positive effect on endothelial differentiation of hAF-MSCs, the cells from this condition showed a lower proliferation than the cells induced by 10% hPL and VEGF. The previous report documented that the cell growth in stationary phase is supposed to be limited by one of three factors, including exhaustion of available nutrients, the accumulation of inhibitory metabolites or end products, and a lack of biological space [80]. Therefore, the supplementation with 10% hPL and VEGF seem to be the suggested condition to use in clinical trials.

In conclusion, the present study found that hAF-MSCs had potential to differentiate into endothelial cells. The signal of VEGF and the supplementation with hPL can enhance the endothelial differentiation potential of hAF-MSCs. This suggested that the supplement of hPL and VEGF in media can be used as an alternative to commercial endothelial-induced media such as EGM-2 to differentiate stem cells into endothelial cells. The study demonstrates another process of endothelial differentiation to save costs and reduce the risk of transmission of known and unknown pathogens by using the human derivative for the production of the cells in pre-clinical study or therapeutic application.

Declarations

Author contribution statement

W. Tancharoen: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

S. Aungsuchawan: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

R. Markmee: Performed the experiments.

S. Narakornsak: Analyzed and interpreted the data.

P. Pothacharoen: Conceived and designed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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