Differentiation of Adipose Tissue–Derived CD34+/CD31– Cells into Endothelial Cells In Vitro

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Abstract

In this study, CD34$^+$/CD31$^-$ progenitor cells were isolated from the stromal vascular fraction (SVF) of adipose tissue using magnetic activated cell sorting. The endothelial differentiation capability of these cells *in vitro* was evaluated by culturing them in vascular endothelial growth factor (VEGF) induced medium for 14 days. Viability, proliferation, differentiation and tube formation of these cells were evaluated. Cell viability study revealed that both undifferentiated and endothelial differentiated cells remained healthy for 14 days. However, the proliferation rate was higher in undifferentiated cells compared to endothelial differentiated ones. Upregulation of endothelial characteristic genes (Von Willebrand Factor (vWF) and VE Cadherin) was observed in 2D culture. However, PECAM (CD31) was only found to be upregulated after the cells had formed tube-like structures in 3D Matrigel culture. These results indicate that adipose derived CD34$^+$/CD31$^-$ cells when cultured in VEGF induced medium, are capable differentiation into endothelial-like lineages. Tube formation of the cells started 3h after seeding the cells on Matrigel and formed more stable and connected network 24 h post seeding in presence of VEGF.

Keywords

Stromal vascular fraction; CD34+ cells; endothelial differentiation; adipose tissue

1. Introduction

Vascularization is essential to supply cells with nutrients and oxygen in engineered tissue constructs after implantation [1]. Recently it has been suggested that post-natal

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vasculogenesis is influenced by endothelial progenitor cells (EPCs). Although their exact phenotype is unknown they express the CD34 surface marker and can be identified in bone marrow and peripheral blood. As they can home to tissue repair sites and differentiate into mature endothelial cells [2] they have emerged as an attractive source for regenerative medicine applications. Asahara *et al* [3] observed successful *in vitro* endothelial differentiation of isolated CD34+ from peripheral blood using magnetic beads. Others have demonstrated multilineage differentiation potential into endothelial cells and cardiomyocytes in a rat myocardial infarction model [4]. These cells have also been described as pericytes due to their pericyte like properties by co expression of typical pericyte markers including NG2, PDGFRβ, CD105 and CD 90 [5]. Multi-lineage differentiation potentials have also been observed in adipose derived mesenchymal stem cells [6–8]. Additionally, previous studies have reported evidence of pericytes and vascular smooth muscle cells in the stromal vascular fraction of adipose tissue [9]. Sengenes *et al.*, reported that some adipose derived stem/progenitor cells express the CD34+ surface marker and are negative for the endothelial cell marker CD31 [10]. This suggests that adipose tissue may house progenitors for both vasculogenesis and tissue formation. For example, an *in vivo* study recently demonstrated the healing potential of femoral fractures in nude rats after CD34+ cell transplantation [10]. In a very recent study Hertweck and his group tested the impact of CD34+ cells (purified from circulating blood) in collagen scaffolds as monoculture and as co- culture together with human osteoblasts on bone formation in nude mice [11]. They observed better bone healing for monoculture of CD34+ cells as well as co- culture of CD34+/osteoblasts compared to monoculture of human osteoblasts. It is reported that when CD34+ cells, fraction of adipose stromal cells are seeded on a semi solid surface such as Matrigel, vWF is over expressed [12].These studies have shown the importance of CD34+ cells in tissue regeneration and their contribution to endothelial differentiation.

The present study was aimed to investigate if CD34+/CD31− cells isolated from adipose tissue will acquire an endothelial cell phenotype when induced by VEGF *in vitro* and have the ability for tube formation. To characterize endothelial differentiation of CD34+/CD31− cells a study of viability, proliferation and differentiation in VEGF induced medium was conducted over 14 days. Cell viability and proliferation were analyzed using LIVE/DEAD® staining and PicoGreen® DNA quantification assay. Gene expression (CD31, vWF and VE Cadherin) was assessed by qRT-PCR and surface marker expression was analyzed using immunofluorescence staining. Furthermore, tube formation of CD34+/CD31− cells were evaluated by seeding the cell on Matrigel. The results of this study showed successful differentiation of CD34+/CD31− cells, into an endothelial-like lineage after culture in VEGF containing inducing media for up to 14 days *in vitro*.

2. Materials and Methods

2.1. Isolation and culture of CD34+ /CD31− cells:

To obtain human CD34+/CD31− cells, surgically discarded adipose tissues were obtained from patients who underwent an elective adipose tissue removal process (e.g., panniculectomy) at the Pennsylvania State University (Hershey, PA) with patient's consent and approval from the Institutional Review Board (IRB protocol # 00004972). The obtained

tissue samples were minced and rinsed vigorously multiple times with Hanks Balanced Salt Solution (HBSS) until excess blood was removed. Minced adipose tissue was digested with 0.2% of collagenase (Gibco, MD) and incubated at 37 °C on a shaker for 2 h. The collagenase-digested tissue was centrifuged for 10 min at 2,000 rpm and the pellet was obtained. The pellet was then suspended in red blood cell (RBC) lysis buffer and filtered through 100 μm and 40 μm filters to remove large tissue particles and centrifuged. The pellet was re-suspended in HBSS buffer and layered on Ficoll-Paque reagent for density gradient centrifugation for 30 min at 2,000 rpm, and the white from layer (middle) band was collected, denoted as the SVF. We first depleted CD31 positive cells from the SVF using microbeads (Milteyni Biotec, CA). CD31 depleted cells were labelled with CD34 microbeads and sorted using AutoMACs Pro (Milteyni Biotec, CA). Isolated CD34+ cells were tested for purity by flow cytometry [13]. Briefly, the CD34+ cells were centrifuged to obtain a cell pellet and fixed with BD Cytofix (BD Biosciences PharMingen, Franklin Lakes, NJ) for 10–15 minutes, washed and re-suspended in BD perm wash buffer for 10 minutes. CD34 cells were then re-suspended in perm/wash buffer with primary antibody, CD34-FITC, CD31-PE (BD Biosciences, Franklin Lakes, NJ) and incubated at room temperature for 30 minutes and washed. Labeled cells were acquired by a LSR-II flow cytometer (BD Biosciences, Franklin Lakes, NJ) and data analyzed using FlowJo (Ashland, OR) software.

2.2. Endothelial differentiation of CD34+/CD31− cells:

To assess the endothelial differentiation potential of CD34 cells from 5 different patients at passage 1, they were grown to 80% confluency in 24-well plates, and cultured in basal DMEM-F12 medium supplemented with 50 ng/mL VEGF (Sigma) and 2% FBS for 14 days. CD34 cells cultured without VEGF media were used as a negative control. Human Umbilical Vein Endothelial Cells (HUVEC) from Lonza served as a positive control and were maintained in EGM-2 medium (Lonza). The cells were incubated at 37°C in 5% CO_2 and medium was changed every 3 days.

2.3. Cell viability and proliferation:

LIVE/DEAD® staining (Cell viability, Invitrogen – using Olympus fluorescence microscope) was performed at 7 and 14 days to assess cell viability. Total DNA content was used to determine the cell count of each as previously described [14]. Proteinase K of 0.5 mL (Sigma-Aldrich) at a concentration of 0.5 mg/mL was added to each well, and plates were incubated at 56°C overnight for enzymatic lysis of cells and DNA release. Aliquots (50 mL) were mixed with equal volumes of 0.1 g/mL PicoGreen® dye solution (Invitrogen) in 96-well plates. Samples were then excited at 480 nm with an emission wave length of 520 nm using a plate reader (SpectraMax M5). Total DNA concentration was compared to a standard curve generated from serial dilutions of $CD34^+/CD31^-$ cells to calculate the number of the cells in each well. Samples prepared without cells served as blank controls.

2.4. Matrigel tube formation assay:

$CD34^+/CD31^-$ cells cultured for 2 weeks in endothelial differentiation medium and growth medium were plated on top of Matrigel substrate (at density of 25,000 cells/well on 150 μL

Matrigel, Corning® in a 48 well plate) and incubated at 37°C in a 5% CO_2 for up to 24 h. Phase contrast microscopy was used to observe the formation of cord like structures.

2.5. Immunofluorescence staining:

After 14 days in differentiation medium, immunofluorescence staining was performed on induced and un-induced cells to detect the expression of CD31, VE-Cadherin and vWF. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, rinsed with PBS twice, blocked with 1% bovine serum albumin (BSA) for 1 h at 37° C, and incubated with AlexaFluor-488-conjugated Mouse anti-CD31 (Abcam), Rabbit anti-VE Cadherin (Abcam) and Rabbit vWF (Abcam) antibodies at 4° C overnight, followed by washing in PBS three times. AlexaFluor-555-conjugated goat anti-rabbit secondary antibody (ThemoFisher Scientific) was then added to VE-Cadherin and vWF stained samples and incubated for a further 1 h. Cells were then counterstained with DAPI (Sigma) and observed under a confocal microscope.

2.6. Real time quantitative PCR:

Total RNA was extracted from cells as previously described [15]. Total RNA was converted to cDNA using Verso cDNA Synthesis Kit (ThermoFisher Scientific). qRT-PCR was performed using PowerUp™ SYBR® Green Master Mix (ThermoFisher Scientific) and primers for CD31, vWF and VE-Cadherin to quantify endothelial target gene expression of CD34^{+}/CD31^{−} cells cultured in growth medium (GM) and endothelial differentiation medium up to 14 days. Reactions were performed with a QuantStudio 3 PCR system (Thermo Fisher Scientific). The sequences of PCR primers (forward and backward, 5'–3') from Integrated DNA Technologies were as follows: CD31: 5-ACTGGACAAGAAAGAGGCCATCCA-3 and 5-TCCTTCTGGATGGTGAAGTTGGCT −3 [16], VE Cadherin: 5-ATGCACCTGTGTTTGAGAAAAGC-3 and 5-CCTGGATGCGGTATGAGACTT-3[17], vWF: 5-AACGGAAGTCCATGGTTCTG-3 and 5-CCCCATTGAAGGCATACTCC-3[1].

Samples were normalized (Ct) against the house keeping gene 18S rRNA and the - Ct value of the genes in the differentiated cells compared to non-differentiated group was calculated using the - Ct method as described in supporting information.

2.7. Statistical analysis:

Statistical analysis of results was performed using GraphPad Prism. Data was analyzed for statistically significant differences with two-way ANOVA, $p < 0.05$. Fisher's LSD test was used for post comparisons.

3. Results

CD34^{+}/CD31^{−} cells were isolated from SVF based on surface markers utilizing magnetic activated cell sorting. Fig. 1 indicates isolation of these cells by density gradient centrifugation and by flow cytometry. The percentage of CD34+ cells is 50–60% within the SVF isolated from fat tissue.

Viability of CD34$^+$/CD31$^-$ cells was visualized with LIVE/DEAD® staining over 14 days of culture in growth medium and endothelial differentiation medium. Representative images of the cells at day 7 and 14 can be seen with live cells in green and dead cells in red (Fig. 2). More dead cells were observed in growth medium compared to endothelial differentiation medium after 14 days. PicoGreen® total DNA quantification was used to determine the number of the cells at day 1, 7 and 14 as shown in Fig.3. DNA content analysis indicates that proliferation rate is higher in undifferentiated cells compared to endothelial differentiated (ED) ones. A significant difference was observed between the number of the cells in undifferentiated group (3.3 fold increase) and endothelial differentiated group (1.5 fold increase) over 14 days. Images from LIVE/DEAD® staining support the data generated from DNA content analysis that indicate the undifferentiated cells proliferated more than endothelial differentiate cells.

After 14 days of exposure to endothelial differentiation medium, CD34$^+$ cells started to form tube like structure 3 h after seeding on Matrigel and more stable cord like structure was observed after 8 h. Conversely, undifferentiated cells formed aggregates and showed no tube-like after 8 h (Fig. 4).

Expression of vWF, VE Cadherin and CD31 proteins was studied using immunofluorescence staining technique on both differentiated and undifferentiated cells at day 14 in culture. In 2D culture, CD34$^+$/CD31$^-$ cells showed increased expression for vWF and VE Cadherin in endothelial differentiation media compared to the growth media condition (Fig. 5).

None of the 2D culture groups showed positive staining for CD31 surface marker regardless of media condition (Fig. 6 a&b). However, CD31 expression (5 fold upregulation) was observed in endothelial differentiated tubes 24 h after seeding on Matrigel as can be seen in Fig. 6 c&d. Undifferentiated cells however detached and formed aggregation 24 h after seeding on Matrigel (Fig. 6 e).

Gene expression analysis by qRT-PCR demonstrated upregulation of VE Cadherin, CD31 and vWF genes in differentiated cells when normalized to that of undifferentiated group. Fig. 7 shows expression of vWF and VE Cadherin on CD34$^+$/CD31$^-$ cells as well as the positive control (HUVEC cells). No significant difference was observed in expression of VE Cadherin between differentiated cells and HUVEC cells (positive control).

CD31 gene expression was observed after seeding the cells on Matrigel while it did not show any upregulation in 2D (Fig. 8).

4. Discussion

Neovascularization is crucial for cell survival, formation of new tissue and maintaining tissue functionality in tissue engineering [18–20]. Therefore, finding a proper source of progenitor cells capable of endothelial differentiation is of substantial importance in tissue engineering for the vascularization of large volume complex tissues [2]. Mature endothelial cells have shown successful blood vessel development and capillary networks formation within the tissue constructs. However, the source and availability of mature endothelial cells

as well as their proliferative potential is limited [21, 22]. Endothelial progenitor cells are an alternative source for vascularization in damaged tissues. Asahara *et al.*, in 1997 proposed that CD34$^+$ cells can be isolated from peripheral blood and bone marrow as endothelial precursor cells and also can differentiate into endothelial cells *in vitro* [3]. Miranville *et al.*, later demonstrated the presence of CD34$^+$/CD31$^-$ cells within the SVF of human adipose tissue (AT) [12]. They demonstrated that CD34$^+$/CD31$^-$ cells can differentiate into endothelial cells in presence of endothelial cell growth supplement. Moreover, they observed increase in the blood flow and the capillary density in ischemic hindlimb when they injected these cells into the mouse [12].

These cells with the capability of vascularization in damaged tissues can be isolated in abundance unlike other mesenchymal stem cells such as bone marrow stem cells which makes them advantages for vascularization in tissue engineering [23, 24]. Suga et al investigated the differentiation potential of CD34$^+$/CD31$^-$ with and without growth factors in comparison with CD34$^-$/CD31$^-$ cells *in vitro* and they observed capillary network formation of these cell when seeded on Matrigel [25]. VEGF is one of the growth factors that have been used for inducing endothelial differentiation *in vitro*. Oswald *et al.*, first reported that VEGF induction at a concentration of 50 ng/mL for 7 days induces endothelial differentiation of MSCs and they observed major characteristics of mature endothelial-like expression of vWF, VEGF receptors 1 and 2 (FLT-1 and KDR), VE cadherin, and VCAM-1[26].

The fact that acquisition of fat from is a rapid and easy procedure, and CD34$^+$/CD31$^-$ cells exist there prompted us to explore whether these cells show endothelial markers and tube formations in VEGF induced condition as observed in bone marrow stem cells [25] when cultured *in vitro*. Wang *et al.*, observed that endothelial differentiation of mesenchymal stem cells reaches a plateau at day 14 in presence of VEGF [27]. Levenberg *et al.*, also reported expression of CD31 reaching a maximum at 13–15 days. [28]. Therefore, based on literature suggesting two weeks as the optimal time for acquiring endothelial cells, CD34$^+$/CD31$^-$ cells were cultured in VEGF media for up to 14 days.

Cell proliferation and differentiation have an inverse relationship, as cells go through differentiation their rate of proliferation decreases. Precursor cells continue division before acquiring a fully differentiated state and cells usually exit from the division cycle when they are fully differentiated which explains lower proliferation rate in differentiated cells compared to non-differentiated ones. The reason for more dead cells in growth medium is since they proliferated more than differentiated cells over 14 days they passed the confluency point which leads to cell death and that happens late in the log phase of growth. Cell death occurs when cultures become overcrowded which could be caused by lack of nutrients and environmental temperature above or below the tolerance band for the cells [29, 30].

qRT-PCR results revealed 5 fold and 7.5 fold increase in vWF after 7 and 14 days respectively. VE Cadherin also showed 2 and 4 fold upregulation after 7 and 14 days which was close to that of HUVEC (positive control). This *in vitro* observation is consistent with previous work by others that demonstrated expression of endothelial markers in VEGF

induced medium. Zhang *et al.*, reported that human BMSCs after induction with VEGF for 14 days resulted in a twofold increase in vWF [31]. Chen *et al.*, also observed a six fold increase in vWF, VE cadherin, and VEGFR2 expression in bone marrow stem cells with VEGF and EGF treatment [32]. CD31 expression however was not observed in VEGF induced cells on 2D culture and only expressed after seeing on Matrigel. A similar result was observed during endothelial differentiation of mesenchymal stem cells after 10 days in EGM-2 medium by Portalska *et al.* One possible explanation could be that there is a positive feedback loop between the ECM stimulation and CD31 expression in MSC [22]. Other studies also investigated the involvement of PECAM-1 (CD31) in vessel formation of endothelial cells [33]. Matsumura *et al.*, observed a tight correlation between the expression of CD31, tube formation and F actin alignment of HUVECs on Matrigel [34]. This could explain the expression of CD31 on CD34+/CD31− cells after seeding on Matrigel. In their study tube formation were prevented by adhesion-blocking mAb to CD31 both *in vivo* and *in vitro*. Tube formation assay is a simple rapid and reliable assay *in vitro* to screen for various factors that promote endothelial differentiation. Matrigel, a basement membrane extract has been used extensively as a cell culture substratum for three-dimensional cultures to promote endothelial differentiation [35, 36]. Cells form tubes by first attaching to the matrix, migration toward each other and then aligning in a tube-like structure. Synthesis of proteins including collagen and proteases are required in this process [37, 21]. Our tube formation results show that in presence of an angiogenic factor (50 ng/ml VEGF) the cells attach, align and form tubes with the appearance of a network-like lumen area. Formation of tubular-like network is more profound in VEGF induced cells compared to non-differentiated CD34+/CD31− cells. Undifferentiated cells form some alignments but that structure is not stable and the cells detach from the substrate within 24 h. Suga et al observed very similar network formation 6h after seeding their CD34+ cells on in growth factors (−) [38]. In another study Oliver et al observed tube formation after seeding the cells on Matrigel without growth factors, however the cells were all adipose derived stem cells and were not sorted for CD34+/CD31− cells in which the presence of CD31+ cells could have affected capillary network formation despite the lack of growth factors [39].

Immunofluorescence staining and qRT-PCR results show that CD34+/CD31− cells isolated from SVF show endothelial phenotype as indicated by CD31, VE Cadherin and vWF expression when cultured in VEGF induced medium. These results are in agreement with those of hMSCs differentiated into endothelial cells *in vitro*. Oswald *et al.*, observed positive vWF staining on human bone marrow stem cells after 7 days in 50 ng/mL culture medium[26]. Expression of vWF protein was also observed in another study with Miao *et al.*, when differentiating human bone marrow stem cells in presence of VEGF for 7 days [40]. Wang *et al.*, observed expression of CD31 on tube like cells on Matrigel 3 days after induction[27]. However, the ASC whole fraction which contain CD34+/CD31− cells don't always respond to VEGF stimulation *in vitro.* One explanation could be that expression of the CD34 antigen which plays an important role in endothelial differentiation of the cells is not stable in vitro culture and it is lost during expansion or freezing/thawing process[41]. Boquest et al., observed down regulation of adipose derived stem cell surface phenotypes, specifically CD34 marker expression during culture in vitro which can negatively impact endothelial differentiation process of adipose derived cells [42]. Although the ability CD34+/

CD31⁻ cells make them an alternative source for cell therapy applications some factors such as health status [43–45] and age of the donors could impact their differentiation potential [46, 39].

Taken together this study shows differentiation of CD34⁺/CD31⁻ cells into an endothelial cell-like lineage when cultured in VEGF containing media. The ease of isolation from adipose derived SVF and the ability of CD34+/CD31– cells to form EC-like population makes them attractive candidates for the development of vascularized engineered tissues as well as augment vessel growth in ischemic tissue.

Conclusion

In the present study we examined endothelial differentiation of adipose tissue derived CD34⁺/CD31⁻ cells *in vitro*. These cells were isolated from SVF of adipose tissue using magnetic activated cell sorting technique. The results showed that these cells successfully differentiate into endothelial-like cells *in vitro* by acquiring several characteristics of the endothelial cell phenotype when induced with VEGF over 14 days in culture. These features include tube formation in response to Matrigel; upregulation and expression of vWF, VE Cadherin and CD31 genes and proteins after 14 days in VEGF induced medium. Both undifferentiated group and differentiated cells remained healthy and viable over 14 days however differentiated cells proliferated less compared to non-differentiated group. As these cells can be easily harvested from patients in a simple, minimally invasive lipoaspiration procedure and can be expanded ex vivo, they are suitable for autologous cell therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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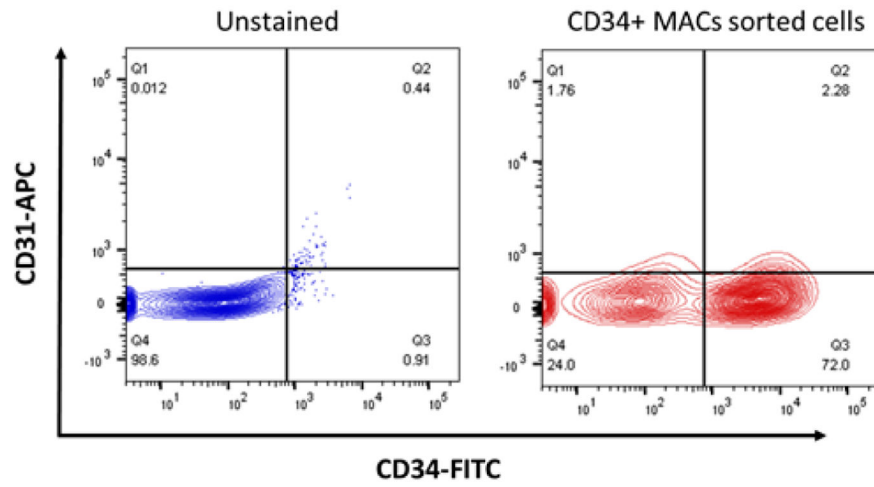
Fig. 1.
CD34+/CD31− cells were isolated from the adipose tissue using MACS microbeads and purity was analyzed by Flow cytometry (CD34-FITC and CD31-APC).
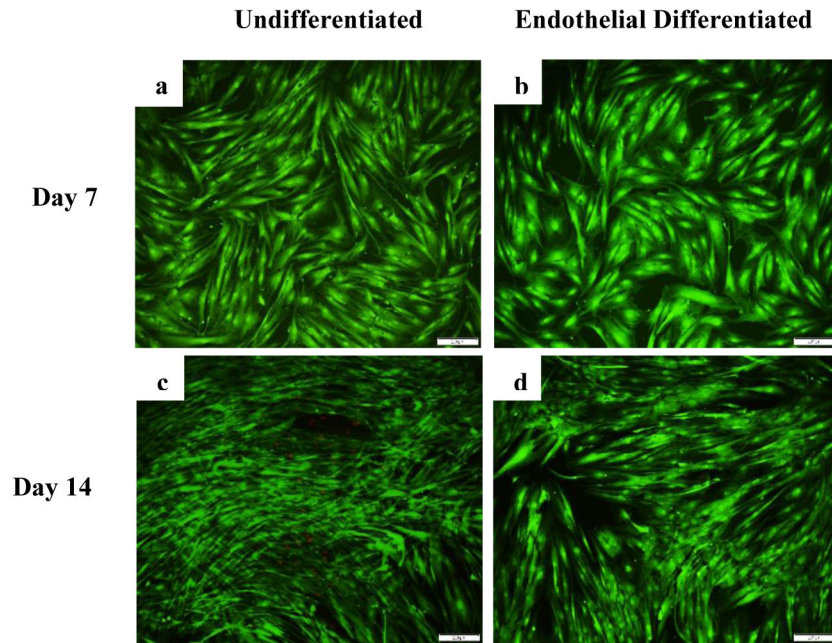
Fig. 2.
LIVE/DEAD® staining assessment over 14 days. LIVE/DEAD® assay stains live cells green and dead cells red. Representative overlay images of cells in growth medium (**a &c**) and endothelial differentiation medium (**b& d**) at days 7 and 14 respectively.
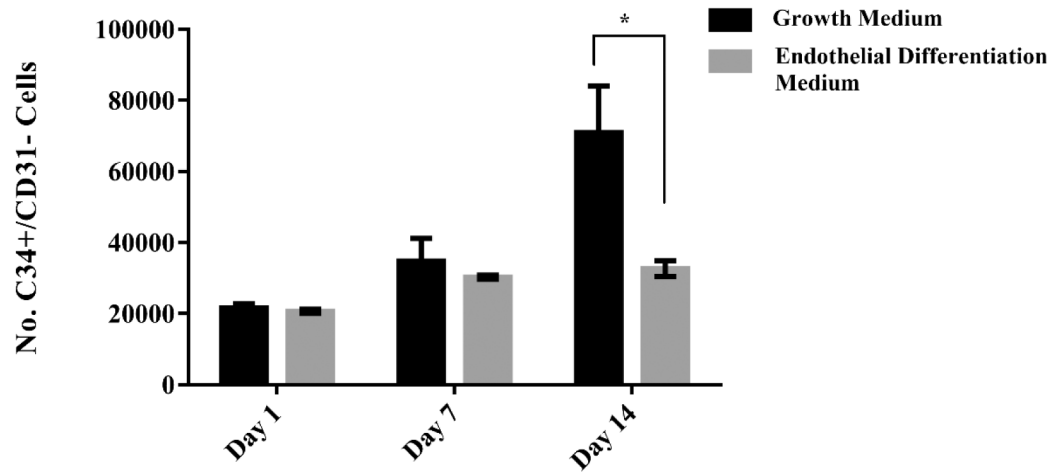
Fig. 3.
Cell proliferation in undifferentiated and differentiated CD34$^+$/CD31$^-$ cells in 14 day culture period. Total number of cells was determined using PicoGreen® DNA quantification assay. Error bars represent the standard error of the mean, for experimental and technical triplicates. * $p < 0.05$
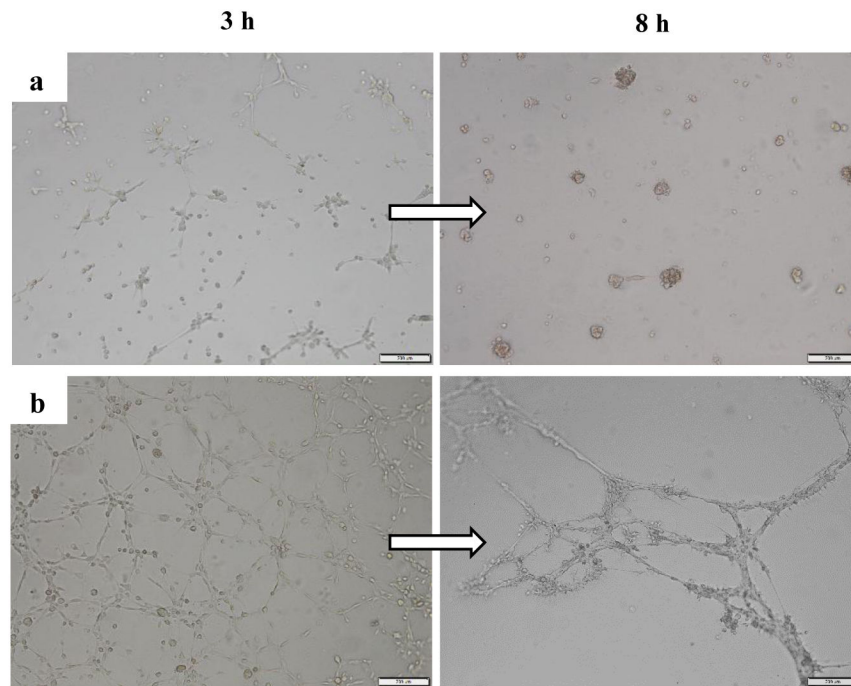
Fig. 4.
Bright field images of tube formation of **a**) non-differentiated and **b**) Endothelial differentiated CD34$^+$/CD31$^-$ cells on Matrigel 3h and 8h after seeding.
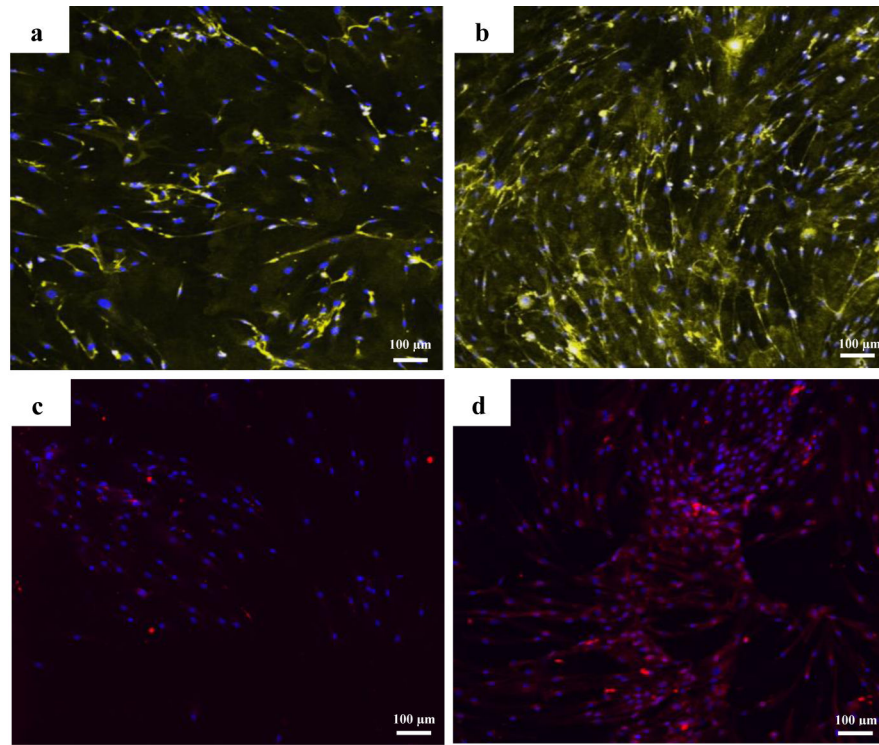
Fig. 5.
Immunofluorescence staining against vWF (yellow) on **a**) non differentiated cells and, **b**) endothelial differentiated cells and against VE Cadherin (red) on **c**) non differentiated cells and, **d**) endothelial differentiated cells after 14 days in 2D culture. The nuclei were counterstained with DAPI.
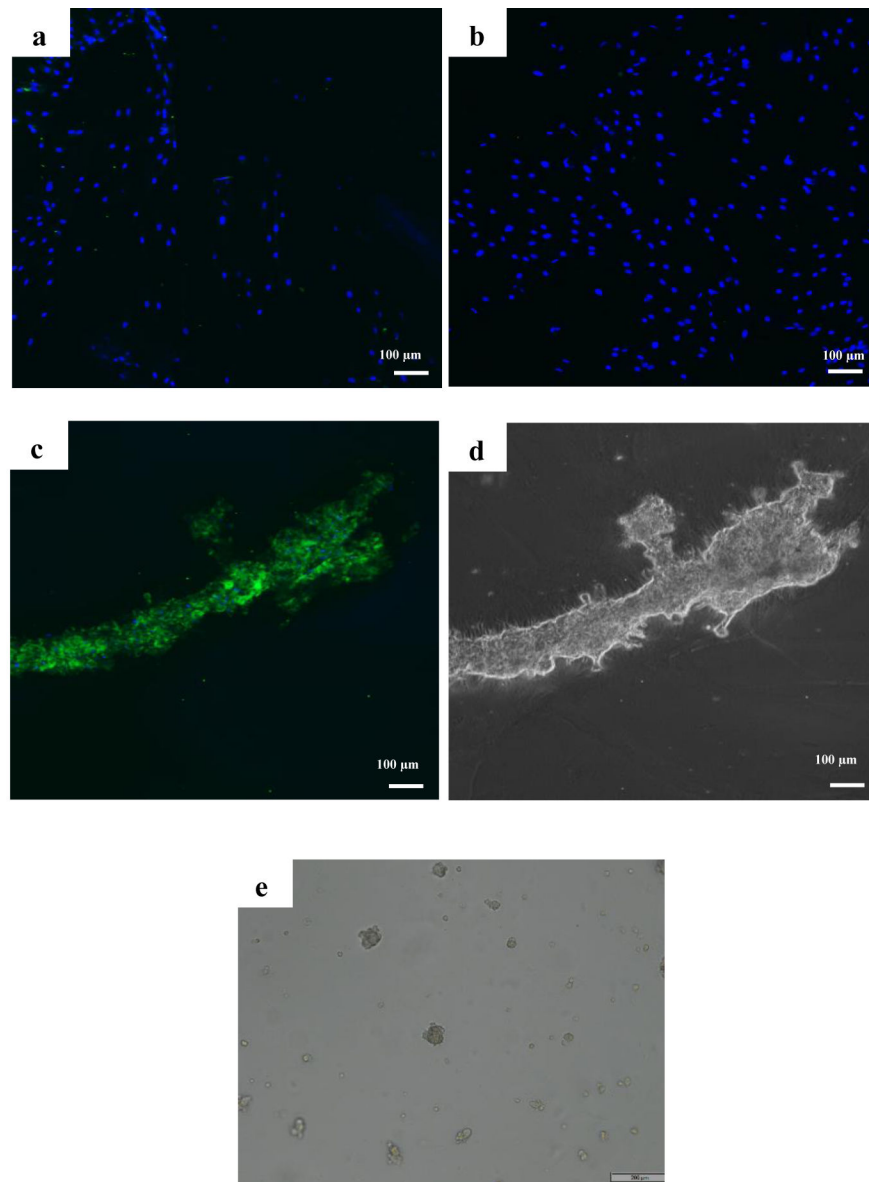
Fig. 6.
Images of immunofluorescence staining against CD31 for **a**) growth medium and **b**) Endothelial differentiation medium cells after 14 days in 2D culture, **c**) on endothelial differentiated cells on Matrigel after 24 h. **d**) bright field image of endothelial differentiated cells forming tube on Matrigel after 24 h. **e**) bright field image of cells in growth medium on Matrigel after 24 h.
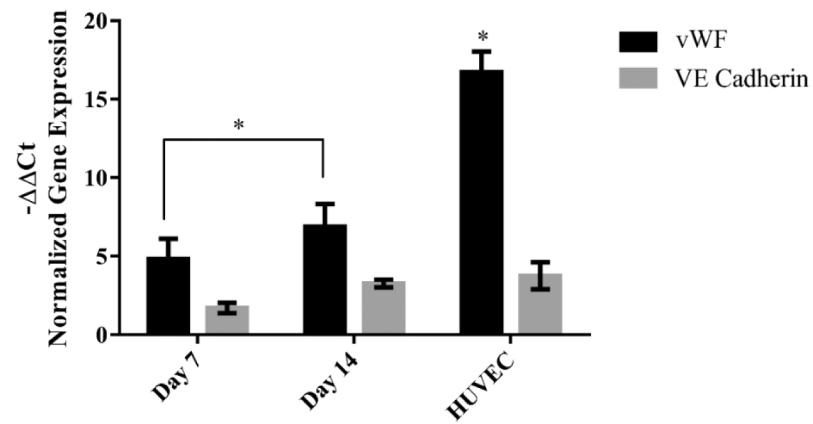
Fig. 7.
Expression of vWF and VE Cadherin genes in endothelial differentiated cells compared to undifferentiated conditions using qRT-PCR. Gene expression was normalized comparing target gene to 18s housekeeping gene. HUVEC cells were used as positive control. Error bars represent the standard error of the mean for experimental and technical duplicates. Significant difference was observed in vWF between HUVEC and Endothelial differentiation groups. * $p<0.05$
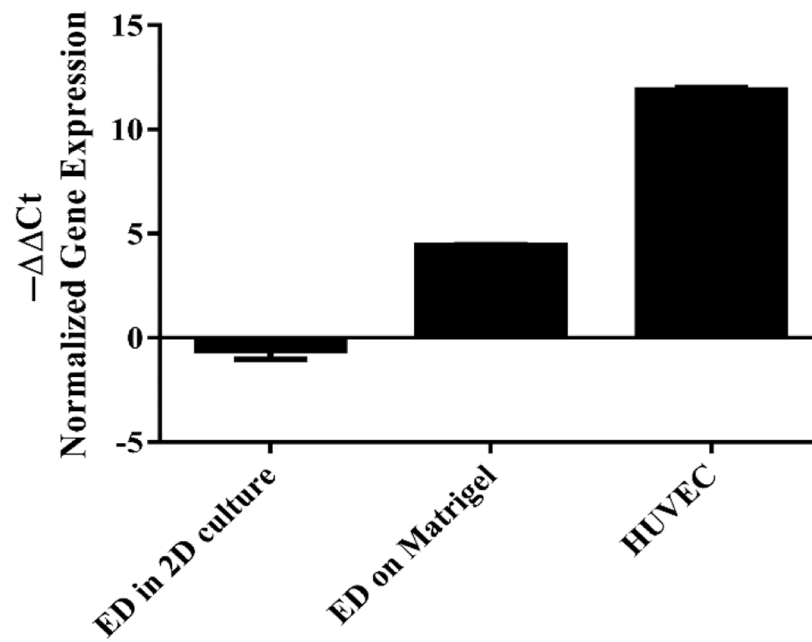
Fig. 8.
Expression of CD31 gene in differentiated cells in 2D and on Matrigel after 24 h compared to undifferentiated conditions using qRT-PCR. Gene expression was normalized comparing target gene to 18s housekeeping gene. HUVEC cells were used as positive control. Error bars represent the standard error of the mean for experimental and technical duplicates. Significant difference was observed between the groups. * $p<0.05$.