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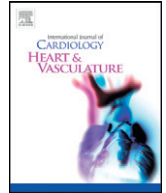
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Soluble CD54 induces human endothelial cells ex vivo expansion useful for cardiovascular regeneration and tissue engineering application



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ABSTRACT

Aim: Consistent expansion of primary human endothelial cells in vitro is critical in the development of engineered tissue. A variety of complex culture media and techniques developed from different basal media have been reported with alternate success. Incongruous results are further confounded by donor-to-donor variability and cellular source of derivation. Our results demonstrate how to overcome these limitations using soluble CD54 (sCD54) as additive to conventional culture medium.

Methods and results: Isolated primary fragment of different vessel types was expanded in Ham's F12 DMEM, enriched with growth factors, Fetal Calf Serum and conditioned medium of Human Umbilical Vein Endothelial Cells (HUVEC) collected at different passages. Cytokine content of culture media was analyzed in order to identify the soluble factors correlating with better proliferation profile. sCD54 was found to induce the in vitro expansion of human endothelial cells (HECs) independently from the vessels source and even in the absence of HUVEC-conditioned medium. The HECs cultivated in the presence of sCD54 (50 ng/ml), resulted positive for the expression of CD146 and negative for CD45, and lower fibroblast contamination. Cells were capable to proliferate with an S phase of 25%, to produce vascular endothelial growth factor, VEGF, (10 ng/ml) and to give origin to vessel-like tubule in vitro.

Conclusion: Our results demonstrate that sCD54 is an essential factor for the in-vitro expansion of HECs without donor and vessel-source variability. Resulting primary cultures can be useful, for tissue engineering in regenerative medicine (e.g. artificial micro tissue generation, coating artificial heart valve etc.) and bio-nanotechnology applications.

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1. Introduction

CD54 is a major functional molecule expressed in Human Endothelial Cells (HECs). Membrane-bound CD54 (mCD54) on the surface of HECs acts as a co-stimulatory/adhesion molecule toward T cell adhesion and activation through binding with lymphocyte function associated antigen 1 (LFA-1) [1,2]. Soluble CD54 (sCD54), derived from membrane-CD54 by shedding, is produced by a variety of different cells including human umbilical vessel endothelial cell (HUVEC),

human saphenous vein endothelial cell, human aortic smooth muscle cell, melanoma cells and hematopoietic cell lines. sCD54 is present in normal human serum at concentrations between 100 and 450 ng/ml [3]. This concentration increases in the sera of patients with lymphoma, leukemia, hypertension, and hepatitis [4–6]. It has also been described that sCD54 can stimulate chemotactic HEC migration, HEC tube formation on Matrigel, sprouting in an aortic ring assay and angiogenesis in chick chorioallantoic membrane assays [7].

In our study, for the first time, we report a protocol that uses sCD54 as additive to promote the in vitro expansion of HECs. Human endothelial cells can be used for various applications including molecular profiling (e.g., transcriptomics [8], proteomics, micro RNA analysis, genomic analysis [9]) as well as cell culture (e.g., generation of cell lines through immortalization [10], in vitro drug testing [11], tissue engineering [12]).

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In particular, cultured endothelial cells are essential, for the elucidation of cellular and molecular mechanisms underlying involvement of endothelial cells in wound healing, atherosclerosis, thrombosis, and abnormal angiogenesis [13–16]. Many publications showed that human differentiated endothelial cells can be isolated from different arterial/venous biopsy. Van Beijnum, JR et al. (2008) [17] isolated HECs by making a single-cell suspension of tissue by mechanical disruption and enzymatic digestion. After tissue disaggregation, HECs are purified by immunomagnetic separation or using high-speed cell sorting. Similarly, Yu SY et al. [18] showed a method to obtain and identify human coronary artery-derived endothelial cells during routine percutaneous coronary interventions by immunomagnetic bead isolation technique. However, incomplete purity of resulting endothelial cells and poor yield due to removal of cell surface epitopes during dissociation are strong limitations of this approaches. The protocol of HEC isolation presented here is standardized and without donor and source variability guarantees a good yield of CD146 positive and CD45 negative HECs.

2. Materials and methods

2.1. Patients enrolled

A cohort of patients was involved, undergoing coronary artery bypass graft surgery (CABG) with pedicle left internal mammary artery, (LIMA), performed on pump in the Cardiovascular Surgery of the Medicine and Surgery Sciences Department of Salvatore Venuta Campus of the University “Magna Graecia” of Catanzaro in Italy. Each patient signed an informed consent, approved by our bioethical commission (Ethics Committee of the “Mater Domini” University Hospital, reference for University Magna Graecia of Catanzaro, approved document number 2012.65). All surgical procedures were performed following standard protocols. The LIMA was harvested semi-skeletonized i.e. with the concomitants veins but without the thoracic fascia. Low voltage (20 mV) electro-cautery was always used for the dissection, and the side branches were always clipped on the arterial side.

2.2. Procedure of isolation

Biopsied vessels were immediately immersed in standard medium with complete F-12K growth medium, 0.1 mg/ml Heparin, 0.03 mg/ml endothelial cell growth supplement (ECGS), fetal bovine serum (FBS) 10%, Gentamicin 3%, and Amphotricin D 3% and rinsed with phosphate buffered saline (PBS). The vessel fragment was placed in a culture dish, the adipose tissue was removed and the vessel was de-endothelialized cutting lengthwise and the remaining layer was cut into 1.5 mm pieces. Each piece was placed in a 96-well culture plate and 50 μ l of fresh F-12K complete growth medium, supplemented with 5 μ l (1:10) of ECGS. Successively cells were incubated at 37 °C and 5% CO₂.

2.3. HUVEC conditioned media

Conditioned culture medium (CCM) was obtained from 70% confluent HUVEC cultured for 48 h in fresh medium. The CCM collection was performed after the first subculture and successively after fourth (p4) and sixth (p6) sub-cultivations respectively. The unconditioned culture medium served as control.

2.4. Characterization of HUVEC CCM

Analysis of human cytokines of CCM was performed with Proteome Profiler RD system, (Catalogue number ARY005). For the experiments involving conditioned media, media from 10- to 14-day-old cultures were collected and filtered with 0.2- μ m filters. The protein concentration of conditioned media was determined with the Bradford protein

assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad), using bovine gamma globulin as the standard. The protein concentration ranged from 50 to 70 μ g/ μ l. Analyses of collected sample were performed as reported in literature [19]. Cytokine array data on developed X-ray film were quantified scanning the film with a transmission-mode scanner and analyzing the array image file using image analysis Shion Corp software. Each array contains internal control spots, two positive and one negative.

2.5. Antibodies and reagents

Monoclonal anti-human ICAM/CD54 (mouse IgG1, clone BBIG-I1) and related isotype control were purchased from, R&D Systems, Inc. When needed, anti-CD54 and isotype control were added at the same concentration (15 ng/ml) to the Ham's F-12 medium (GIBCO) containing 0.25% BSA (Sigma-Aldrich, France). Recombinant human ICAM-1/CD54 (murine myeloma cell line, NSO-derived; Accession #CAA30051), CD146, KDR and CD31 were purchased from R&D Systems, Inc.; CD45 was purchased from AbD Serotec, CD34 and CD14 were purchased from Becton Dickinson Pharmingen, and fibronectin was purchased from Southern Biotech. Anti-alpha smooth muscle Actin antibody was purchased from Abcam.

2.6. Cell cultures

Primary cell cultures were established from endothelial biopsy. Endothelial cells were seeded on culture dishes in standard medium with complete F-12K growth medium (Gibco, Catalogue number 21765-029), 0.1 mg/ml Heparin, 0.03 mg/ml endothelial cell growth supplement (ECGS) (Sigma-Aldrich, France. Catalogue number E0760), FBS 10%, Gentamicin 3%, and Amphotricin D 3% (GIBCO). The cell cultures were maintained at a confluence of 75% before use. Moreover, one thousand cells for each patient, were plated in 96-well flat-bottom plates with sphere growth medium D-MEM/F-12 (0.1 mg/ml Heparin, 25 ng/ml FGF, 50 ng/ml EGF, BSA1%, Penicillin Streptomycin 1% (GIBCO)).

2.7. Immunofluorescence and flowcytometry

2.7.1. Immunofluorescence

Slides fixed in formaldehyde 2% were dipped in working solution (WS) PBS 0.1% (GIBCO), BSA 0.2% (Sigma-Aldrich). Monoclonal antibodies diluted in WS (1:100) were added, and the slides were incubated for 60 min at room temperature (RT). The slides were washed with three changes of PBS for 5 min and then mounted with coverslip using aqueous mounting medium 90% of glycerol in PBS. Cells were observed with an Inverted Confocal Microscopy Nikon TE2000 using 408 nm (blue fluorescence, DAPI excitation), 543 nm (red fluorescence, fluorochrome PE) and 488 nm (green fluorescence, fluorochrome FITC) excitation length. The microscope is located within a clean room (class 1000, T = 25 °C). The magnification used was of 20 \times and 40 \times with numerical aperture of the objective lens of 0.45 and 0.75 respectively. Images were acquired through the camera (QICAM model FAST 1394), using the NIKON EZC1 software, n.3.7 and merged using Adobe Photoshop software (v7.0).

2.7.2. Flow cytometry

The cells were washed in PBS, and incubated in PBS + 10 mmol EDTA at 37 °C for 15 min. Cell suspension was centrifuged. Following, the cells were incubated at 4 °C with primary antibodies diluted 1:100. After the staining, cells were washed and resuspended in PBS and 1% FCS at a final concentration of 1.5×10^5 cells/ml. Acquisition was performed with FACSCanto (Becton Dickinson, San Diego, CA) and analysis was done with Cellquest software. *S-phase analysis*: was performed as reported in literature [20]. Briefly, 1.5×10^5 cells were washed with cold PBS 1X and fixed with 70% ethanol at –20 °C

overnight. The cell pellets were resuspended in 500 μ l PBS containing 2 mg/ml RNase A (Sigma-Aldrich) and kept at 37 °C for 60 min. Pellets were resuspended in staining buffer (500 μ l PBS containing 50 μ g/ml of Propidium Iodide) and incubated at RT for 60 min. DNA content was analyzed by the FACSCanto. The cell-cycle phase distribution was determined with Modfit 3.1 Software (Becton and Dickinson)

2.8. ELISA-based VEGF quantification

Antibodies anti hVEGF (R&D Systems) and a detection antibody conjugated to horseradish peroxidase (HRP) (Southern Biotech) were used to develop ELISA test. The substrate for peroxidase labels procedure was included in the kit Super signal ELISA Pico Chemiluminescent Substrate (Thermo Scientific). The intensity of signals was proportional to the amount of secondary antibody. Relative light units were measured in a luminometer (Perkin Elmer, Victor 3) operating at 425 nm wavelength. VEGF value was determined on the basis of three standard curves media realized with know VEGF quantities.

2.9. Matrigel seeding

Conditioned media were collected, centrifuged, and transferred to fresh tubes from HUVEC cell lines at 80% of confluence growing in RPMI640 (GIBCO). The endothelial cells at 70% of confluence, were trypsinized, washed and seeded in each well with conditioned culture medium on growth factor-containing BD Matrigel (Basement Membrane Matrix, Becton Dickinson) in a 24-well-plate, and incubated in 5% CO₂ at 37 °C at density of 8×10^5 ml. Tube formation was detected with an inverted light optical microscope (Leica). Three random fields per well were examined at 20 \times magnification, and the values were averaged. The pattern/value association criteria for tube formation are:

0 = individual cells, well separated; 1 = cells beginning to migrate and align themselves; 2 = capillary tubes visible without sprouting; 3 = sprouting of new capillary tubes; 4 = closed polygons beginning to form; and 5 = complex mesh like structures developing. Each well was photographed using an inverted optical microscope with a digital camera. The images were taken at 20 and 40 \times magnification and the total lengths of the tubes were measured with Image J (Image Processing Analysis in Java, ver. 1.42; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

2.10. Statistical analysis

All results are reported as mean \pm standard error of the mean (SEM). Data were analyzed using Student's *t* test. A *P* value of less than 0.05 was considered significant and a *P* value of less than 0.01 was considered highly significant. IBM SPSS Statistics 20 software and Microsoft® Office Excel 2010 were used for data analysis and graphing.

3. Results

3.1. Expansion of human endothelial cells isolated from vessel walls in the presence of a specific HUVEC-conditioned medium

Twelve man volunteers from 50 to 83 years old (68 ± 3) were involved, undergoing CABG with pedicle LIMA. Their characteristics are reported in Supplemental Table 1. None of the collected radial arteries and veins (diameter 8.1 ± 0.8 mm), were affected by intima thickening and all artery walls were disease free (atherosclerosis or medial calcification). Freshly collected cells, from internal mammary artery (Fig. 1A) and vein biopsy (Fig. 1B), were negative for CD45 marker

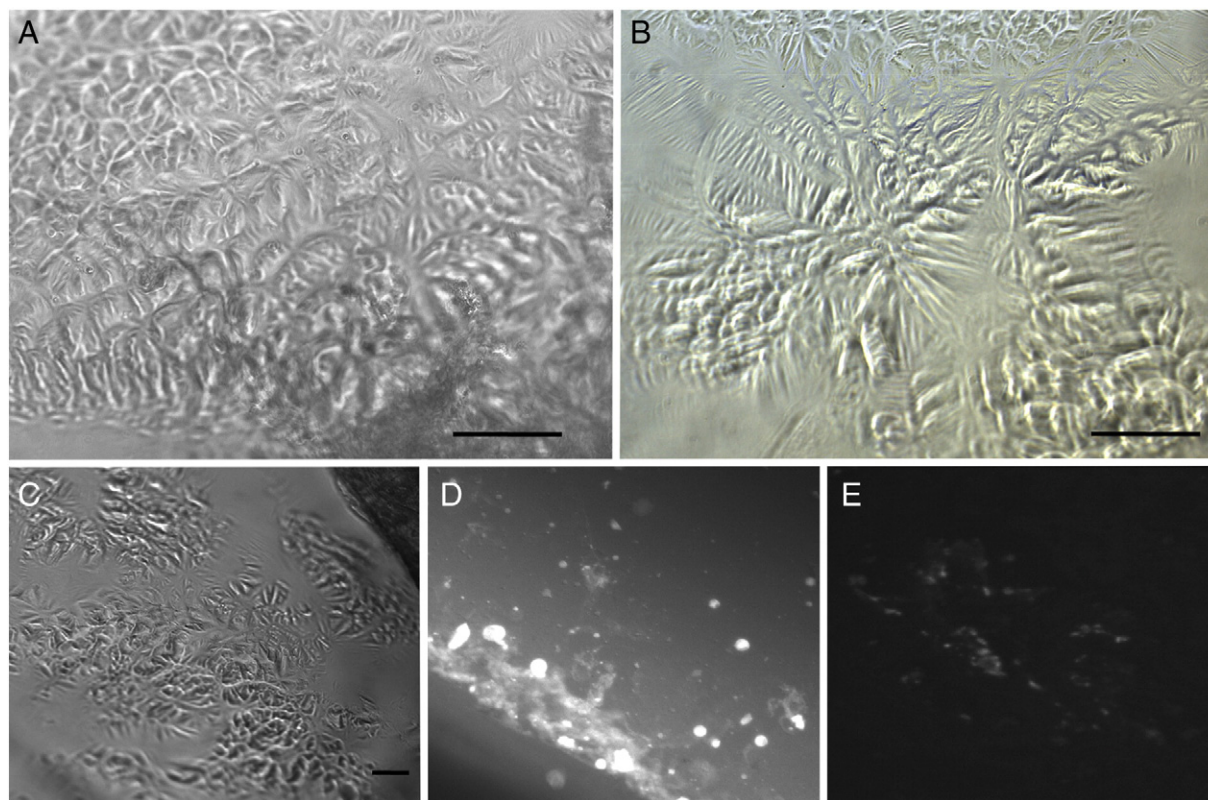


Fig. 1. Cultivated human endothelial cells. Adherent endothelial cells were obtained from artery (A) and vein (B) biopsy. Cells were cultivated in the presence of conditioned medium collected at passage 4 (p4) of HUVEC cultivation. Immunostaining for CD146 (C–E) on adherent cells demonstrates the presence of CD146-positive cells within adherent monolayer obtained from artery biopsy. DNA staining with DAPI (4,6-diamidino-2-phenylindole) was used to evidence nuclei distribution (E). Scale bars: A and B 100 μ m; C, D, and E 20 μ m.

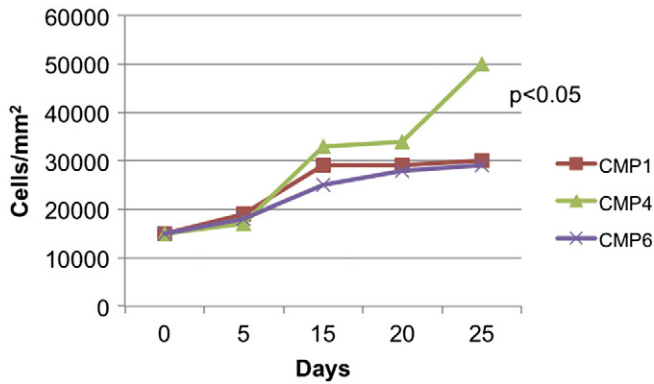


Fig. 2. Long-lived endothelial cultures treated with HUVEC-conditioned medium (CM) collected at different subcultures. Cell density measured as number of cells for mm² (y-axis) increases in relation to the time of cultivation (day) (x-axis), in the presence of conditioned medium collected at passage p1 (CMP1), p4 (CMP4) and p6 (CMP6) of HUVEC cultures. The best increase in cell density was registered in the culture treated with the CM collected at fourth passages of HUVEC sub cultivation (p4).

and positive for CD146 $15 \pm 7\%$, CD31 $19 \pm 3\%$ and KDR $13 \pm 7\%$ markers. Immunostaining on adherent cells of nuclei and CD146 was reported in Fig. 1(C–E). In order to describe growth under the different culture condition, we counted the number of cells. We detected 1.5×10^3 cells/mm² for up to 5 passages without losing the proliferative capacity with the 1:1 addition of the conditioned medium collected at

different passages (p1, p4, p6) of HUVEC cultures to standard medium. The cells are long-lived in culture and have different capacities of expansion. Highest cell numbers were generated with conditioned medium collected at p4 of HUVEC cultivation. The cell density calculated in terms of cell number/mm² is shown in the graphic of Fig. 2. After the second replanting, cells were negative for hematopoietic lineage markers (CD34, CD45, CD133) and monocyte markers (CD14) and positive for endothelial markers such as CD146 $45 \pm 5\%$, CD31 $41 \pm 2\%$, KDR $22 \pm 6\%$. Flow cytometry of cells at the third passages showed a stable phenotype and the fibronectin expression was of $3 \pm 1\%$. Remaining cultivated cells have shown immunofluorescence positivity for α -actin antibody, suggesting a smooth muscle cellular phenotype.

3.2. Characterization of HUVEC-conditioned medium paracrine effects

Conditioned culture medium obtained from 70% confluent HUVEC cultured 48 h in fresh medium, was collected after the first subculture and successively after p4 and p6 subcultivations respectively, and was added to standard medium to cultivate endothelial cells. The results of the cytokine array of the three medium showed a different composition for qualitative and quantitative difference, as shown in Fig. 3(A–C). The paracrine activity of the HUVEC was characterized by the production of CCL1, CD54, IL-6, CXCL8, IL23, IL27, MCP-1, MIF, and Serpin E1. The production of CD54 is restricted to the medium collected after the fourth passage indicating this molecule as a good candidate to explain the efficacy of p4 conditioned medium in sustaining cell proliferation.

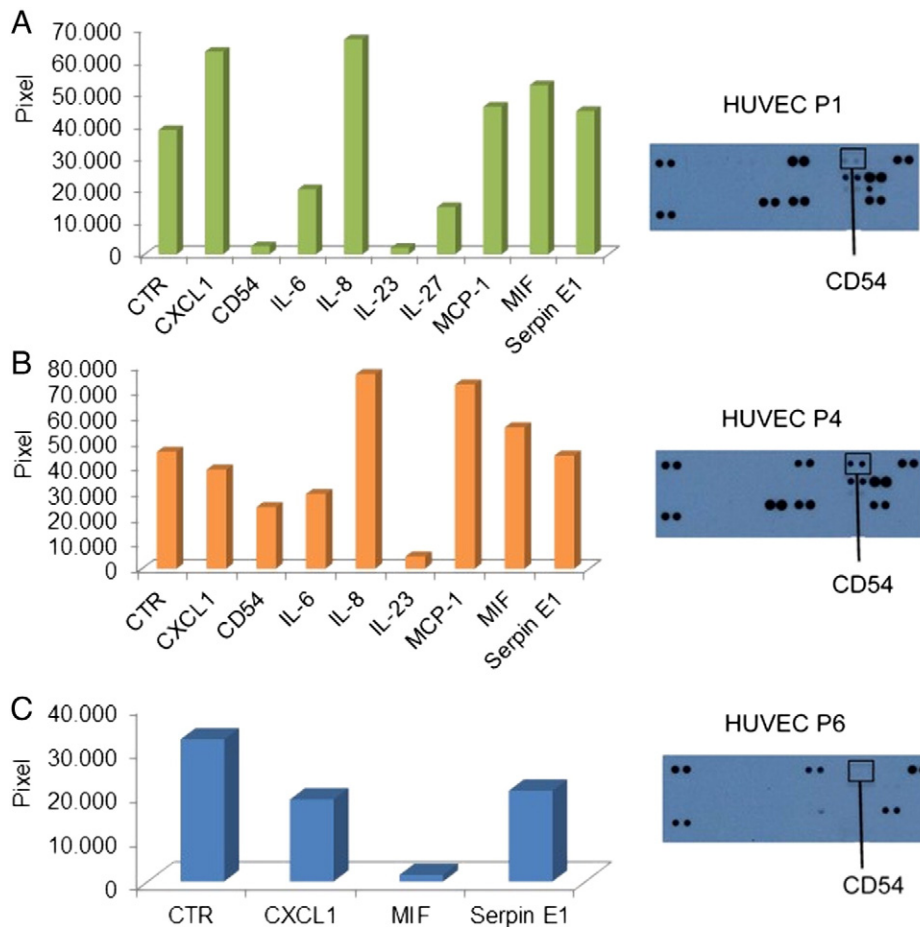


Fig. 3. Cytokine proteomic array of HUVEC-conditioned medium. HUVEC conditioned medium was collected after first passage HUVEC p1 (A), fourth passage HUVEC p4 (B), and sixth passage HUVEC p6 (C) and was analyzed for the presence of soluble factors. Membrane array shows the spot corresponding to the reaction of soluble cytokine and relative antibody printed in duplicated. Internal positive and negative spots control were reported at the top and to the right of each membrane.

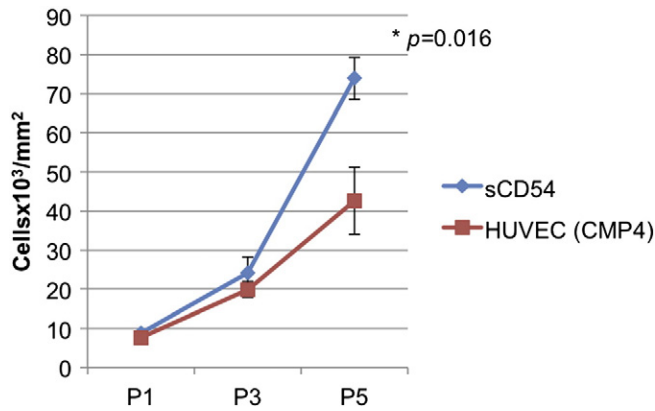


Fig. 4. Cell density of endothelial cells cultivated in the presence of soluble CD54. Endothelial cells cultivated in the presence of soluble CD54 (sCD54) were expanded for five splits. After the first (p1), second (p2) and fifth (p5) passages (as shown in the x-axis) the cellular densities were measured (y-axis) and compared between different growing conditions: in the presence of sCD54 alone, resulting in higher propagation rate, than cell culture treated with HUVEC-conditioned medium collected at p4 (CMP4) alone.

3.3. High harvest yield, high expansion and phenotype stability of CD146 endothelial cells with *in vitro* addition of soluble CD54

Endothelial cells cultivated in the presence of sCD54 added at the concentration of 50 ng/ml to standard medium are long-lived in culture and have a significant capacity of expansion. The dosage of 50 ng/ml was identified examining a dose–response curve; corresponding doses used were 10, 30, 50, 70, and 80 ng/ml and cellular density was checked. On day 3 complete medium exchanges were performed and cells still floating in the supernatant were discarded. Cell proliferation appeared

to be exponential from the third day. The S-phase at fifth passage was of $25 \pm 2\%$ in the presence of sCD54 versus $19 \pm 2\%$ of corresponding culture in the presence of HUVEC-conditioned medium p4. Cell density calculated as number of cells per mm^2 is shown in Fig. 4. The expansion observed after the third passage continued in similar fashion after the fifth passage. In particular the cellular density registered in the presence of sCD54 after the first passage was of $9 \pm 1 \times 10^3$ cells/ mm^2 , after the third of $24 \pm 9 \times 10^3$ cells/ mm^2 , after the fifth of $73 \pm 12 \times 10^3$ cells/ mm^2 . Significant difference was observed comparing the cellular density at the fifth passage of endothelial cells cultivated with sCD54 and Huvec-p4 medium: $p = 0.008$. After the second replanting, cultivated cells were positive for endothelial markers as CD146 $45 \pm 5\%$, CD31 $41 \pm 2\%$, and KDR $22 \pm 6\%$. Flow cytometry of cells at the third passages showed a stable phenotype and the fibronectin expression was of $1.3 \pm 1\%$. Inhibition assay using antibody against CD54 (15 ng/ml) and IgG 1 (15 ng/ml) as isotype control was used and confirms that in the absence of CD54 or in the presence of reciprocal monoclonal antibody, we didn't obtain endothelial colony structure, as shown in histogram of Fig. 5.

3.4. Soluble CD54 preserves long-term *in vitro* differentiation and promote self-organization vessel-model

The ability to form sphere was developed to test the power of cell replacement. Moreover, we tested endothelial functionality through VEGF production and vessel-like structure *in vitro* generation. No difference was registered in terms of ability to form sphere (Fig. 1 Supplementary). Different behavior was observed in terms of VEGF production (Fig. 2 Supplementary). In particular the amount of VEGF secretion was normalized to cell number (cells/ mm^2) and expressed as amount of factor released in extracellular medium per 48 h. The amount of VEGF produced by endothelial cells cultivated with sCD54

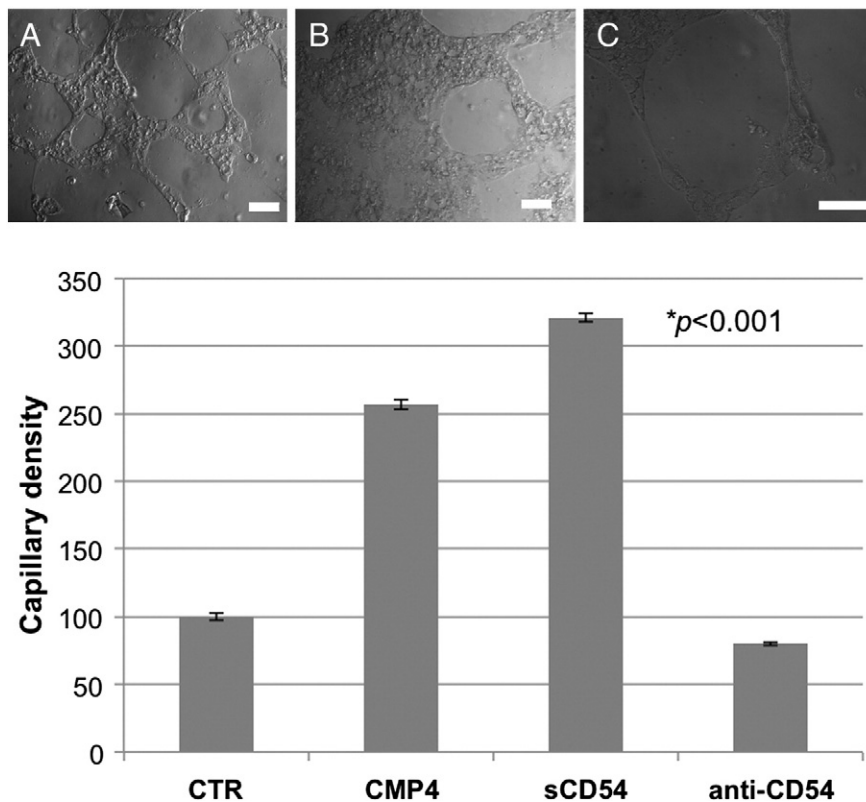


Fig. 5. Capillary density. Tube formation occurred through an ordered sequence of events and was investigated with an inverted optical light microscope; (A) cells beginning to migrate and align themselves to close polygons beginning (B) to form complete tubules (C) of endothelial cells cultivated with the addition of sCD54. The histogram shows the data mean \pm SD of the quantitative analysis of tube formation area; in the x-axis was reported capillary density in the presence of HUVEC-conditioned medium collected at p4 (CMP4), sCD54 and antibody direct against CD54 at the concentration of 15 ng/ml (y-axis). Scale bars: 200 μm .

was of 10 ng/ml. The sequence of tube formation was investigated and results are shown in Fig. 5A–C. Capillary density produced by endothelial cells seeded in matrigel, with the addition of sCD54, increased by 30% with respect to conventional procedure, as shown in histogram of Fig. 5). In the presence of the anti-CD54 no tube formation was observed.

4. Discussion

The development of cell-based therapies to assist/replace diseased tissue is one of the most promising initiatives in regenerative medicine [21]. Yet, a major challenge on our way towards the design of artificial organs is controlling tissue vascularization to provide oxygen, nutrient and to remove catabolites [18,19]. Precise knowledge of how tissue microenvironments impacts on metabolism, proliferation and growth-factor production will be essential for elucidation of microvessel formation [22]. Today, transplantation remains the preferred clinical intervention for the treatment of organ failures; nevertheless, owing to a global shortage in donor organs, alternative strategies providing bioengineered tissue for replacement of damaged, injured or missing tissues are of high clinical priority [23]. An artificial tissue larger than a few cubic millimeters cannot survive by simple diffusion and requires formation of new blood capillaries to supply essential nutrients/oxygen and enable connection to the host vascular system following implantation [23,24]. Likewise, tumors do not grow beyond a few millimeters unless they become vascularized, in most cases by directing an ingrowth of capillaries from adjacent blood vessels [25]. Therefore, precise control of pro- and anti-angiogenic activities is of prime clinical interest in current cancer therapy and tissue engineering initiatives [26,27].

Although most of today's knowledge on vascularization regulatory networks has been derived from in vitro monolayer cultures [28], three-dimensional (3D) culture technologies may reveal further insight. Current 3D models include formation of primitive vascular networks in vitro by co culturing endothelial cells with mural cells or their precursors [29,30]. The interplay between environmental and genetic impact on tumor angiogenesis/proliferation is complex and remains largely elusive [31]. A major challenge is to establish a generic cell culture model mimicking tumor development and angiogenesis of a wide variety of tumorigenic cell types [32,33].

5. Conclusion

In this study, we utilized a modified procedure for the isolation of endothelial cells; to our knowledge, this isolation strategy is a first report that enabled a valid side-by-side assessment in the propagation of isolated endothelial cells, whilst minimizing potential donor sample variability. More importantly, this study is done with a goal towards defining and establishing more robust culture methodologies to initiate further development of suitable tissue engineered endothelial graft alternatives.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ijcha.2015.01.004>.

Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

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