STEM CELLS®

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

# Hypoxia Promotes Efficient Differentiation of Human Embryonic Stem Cells to Functional Endothelium

SONIA PRADO-LOPEZ,[a] ANA CONESA,[b] ANA ARMIÑÁN,[c] MAGDALENA MARTÍNEZ-LOSA,[e] CARMEN ESCOBEDO-LUCEA,[a] CAROLINA GANDIA,[c] SONIA TARAZONA,[b] DARIO MELGUIZO,[a] DAVID BLESA,[f] DAVID MONTANER,[b] SILVIA SANZ-GONZÁLEZ,[d] PILAR SEPÚLVEDA,[c] STEFAN GÖTZ,[b] JOSÉ ENRIQUE O'CONNOR,[e] RUBÉN MORENO,[a] JOAQUÍN DOPAZO,[b] DEBORAH J. BURKS,[d] MIODRAG STOJKOVIC[a]

[a]Cellular Reprogramming Laboratory, [b]Bioinformatic Department, [c]Cardio-regeneration Unit, [d]CIBERDEM and Molecular Endocrinology Laboratory, [e]Cytomics Laboratory, and [f]Microarray Analysis Service, Príncipe Felipe Research Center (CIPF), Avenida del Autopista del Saler 16, 46013 Valencia, Spain

**Key Words.** Angiogenesis • Vasculature • Hypoxia • Embryonic stem cells • Development

ABSTRACT

**Early development of mammalian embryos occurs in an environment of relative hypoxia. Nevertheless, human embryonic stem cells (hESC), which are derived from the inner cell mass of blastocyst, are routinely cultured under the same atmospheric conditions (21% $O_2$) as somatic cells. We hypothesized that $O_2$ levels modulate gene expression and differentiation potential of hESC, and thus, we performed gene profiling of hESC maintained under normoxic or hypoxic (1% or 5% $O_2$) conditions. Our analysis revealed that hypoxia downregulates expression of pluripotency markers in hESC but increases significantly the expression of genes associated with angio- and vasculogenesis including vascular endothelial growth factor and angiopoitein-like proteins. Consequently, we were able to efficiently differentiate hESC to functional endothelial cells (EC) by varying $O_2$ levels; after 24 hours at 5% $O_2$, more than 50% of cells were CD34+. Transplantation of resulting endothelial-like cells improved both systolic function and fractional shortening in a rodent model of myocardial infarction. Moreover, analysis of the infarcted zone revealed that transplanted EC reduced the area of fibrous scar tissue by 50%. Thus, use of hypoxic conditions to specify the endothelial lineage suggests a novel strategy for cellular therapies aimed at repair of damaged vasculature in pathologies such as cerebral ischemia and myocardial infarction.** STEM CELLS *2010;28:407–418*

Disclosure of potential conflicts of interest is found at the end of this article.

## INTRODUCTION

The first stages of human embryogenesis occur in a hypoxic or even anoxic environment [1–3] given that the outer layer of trophoblasts prevent entry of maternal blood and that oxygen ($O_2$) levels are low within the oviducts and uterus. Various studies [1–3] have suggested that the developing blastocyst migrates from the Fallopian tubes to the uterus by pursuing an $O_2$ gradient. However, not until around 11 weeks of human gestation does maternal circulation fully inhabit the placental space, causing a rise in $O_2$ tension and initiating a phase of branching angiogenesis [2]. The few studies [1–3] that have addressed the effects of low $O_2$ on embryo development suggest that this condition benefits the embryo, whereas culture under atmospheric $O_2$ has a detrimental effect on embryonic development. For instance, culture of mouse embryos in 5% $O_2$ significantly enhanced development to the morula and blastocyst stages than culture in the presence of 20% $O_2$ [3]. Thus, it is likely that fluctuations of $O_2$ tension play an important role throughout embryo development and may influence different cellular lineages by activating and suppressing gene expression. It is well established that hypoxia inducible factors (HIFs) are activated by reduced oxygen levels and, therefore, exert critical roles during embryonic and postnatal development through transcriptional regulation of genes related with angiogenesis, endothelial cell development, and glycolysis [4]. Indeed, deletion of HIF1-$\alpha$ in mice is lethal because it causes vascular malformations and developmental arrest [5, 6].

Specification of the circulatory system is one of the first events during embryo development, since a sufficient supply of $O_2$ and nutrients is essential for growth and survival of developing tissues. The formation of new blood vessels

Author contributions: S.L.P. and M.S.: Designed the experiments, performed all studies of hESC in hypoxia, and interpreted data; A.C., S.T., D. Montaner, S.G., and J.D.: Analyzed the results of the microarrays; A.A., C.G., and P.S.: Performed experiments and collected data related with transplantation of EPC/EC; M.M.L. and J.E.O.: Performed flow cytometry analysis; C.E.L., D. Melguizo, and R.M.: Prepared the ECM for differentiation of hESC; D.B.: Performed CGH-Microarray analysis; S.S.G.: Performed Western analysis; D.J.B.: Performed Western analysis, wrote the manuscript; S.P.L.: Wrote the manuscript; M.S.: Wrote the manuscript, approved the final manuscript.

Correspondence: Miodrag Stojkovic, PhD, Cellular Reprogramming Laboratory, Príncipe Felipe Research Center (CIPF), Avenida del Autopista del Saler 16, 46013 Valencia, Spain. Telephone: +34-96-328-96-80; Fax: +34-96-328-97-01; e-mail: mstojkovic@cipf.es Received August 14, 2009; accepted for publication December 21, 2009; first published online in STEM CELLS *EXPRESS* January 4, 2010. © AlphaMed Press 1066-5099/2009/$30.00/0 doi: 10.1002/stem.295

involves both vasculogenesis, the de novo emergence of a vascular network from endothelial progenitor cells (EPC), and angiogenesis, the generation of vessels by sprouting of preexisting vessels [7]. EPC share many markers with the hematopoietic stem cells (HSC) [8, 9], as both cell types originated from a common progenitor, the hemangioblast. Thus, EPC are defined as a group of cells that present different states of differentiation between the hemangioblast and the mature endothelial cells (EC) [9]. Since the first identification of circulating cells with properties of EPC by Asahara et al. [10] in 1997, various sources of adult EC-like multipotent progenitors have been reported, including bone marrow, myeloid cells, adult stem cells from fat, and cardiac tissue [11–19]. These studies have suggested the possibility to generate EC in vitro for therapeutic applications such as cell transplantation to repair damaged vessels as well to improve our basic understanding of the role of angiogenesis in hemangiomas, rheumatoid arthritis, and cancer.

Human embryonic stem cells (hESC) provide a unique model to study mechanisms of human development including early lineage commitment, differentiation, and maturation [20]. These cells are self-renewing and can give rise to many different types of cells of the human body. The effects of low oxygen levels on the biology of hESC remain controversial. Some reports have suggested that low $O_2$ promotes the undifferentiated state of hESC [21], whereas more recent publications demonstrate that reduced $O_2$ tension facilitates the expression of early cardiac genes in embryoid bodies (EB) [22] and enhances the chondrogenic potential of hESC [23]. However, many of these published studies employed EB cultured under low $O_2$ and the subsequent analysis of morphologic changes and expression of pluripotency markers. Here we present a study of the effects of hypoxia on stem cell biology, which is based on transcriptome profiling of hESC cultured at different $O_2$ levels. Given that early embryonic development occurs in conditions of low $O_2$, we postulated that the culture of hESC under hypoxia might facilitate endothelial differentiation. Thus, we examined the effects of varying $O_2$ levels (1%, 5%, and 21%) on the differentiation potential of hESC. Indeed, culture of hESC in the presence of low $O_2$ (1% and 5%) selectively facilitated differentiation to EPC as evidenced by the upregulation of genes implicated in angiogenesis and vasculogenesis. Differentiated cells obtained from this method formed vascular tubes in vitro and, when transplanted in vivo, improved cardiac function and enhanced angiogenesis in a rodent model of myocardial infarction. Thus, our results demonstrate that functional EPC can be efficiently generated from hESC by simple manipulation of the $O_2$ supply in cultures.

## Materials and Methods

### Culture of hESC

Undifferentiated hESC (H9 line passages 35-38) were cultured on extracellular matrix (ECM) from human foreskin fibroblasts under different $O_2$ levels (21%, 5%, and 1% $O_2$) using foreskin-fibroblast conditioned medium composed of the following: DMEM-F12 (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 100 $\mu$M ß-mercaptoethanol (Sigma, St. Louis, http://www.sigmaaldrich.com), 1 mM L-glutamine (Invitrogen), 100 mM nonessential amino acids, and 20% serum replacement (Invitrogen). To confirm that culture in hypoxia induced similar effects in another line of hESC, we used the VAL9 line (passages 51-53) available from the Spanish stem cell bank (http://www.isciii.es). The cells were collected mechanically at different time points (0, 12, and 24 hours and 5, 10, and 15 days) and stored frozen for subsequent extraction of RNA.

### Microarray Assays

Total RNA was extracted from hESC using the High Pure RNA Isolation kit (Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com) and was quantified by spectrometry (NanoDrop ND1000, NanoDrop Technologies, Wilminton, DE, http://www.nanodrop.com). Quality was confirmed by RNA 6,000 Nano Bioanalyzer (Agilent Technologies, Palo Alto, CA, http://www.agilent.com) assay. Eight hundred ng of total RNA were used to produce cyanine 3-CTP-labeled cRNA using the Low RNA Input Linear Amplification Kit, PLUS, One-Color (Agilent p/n 5188-5339) according to the manufacturer's instructions. On the basis of the protocol for One-Color Microarray-Based Gene Expression Analysis Version 5.5 (Agilent p/n 5188-5977), 3 $\mu$g of labeled cRNA was hybridized with Whole Human Genome Oligo Microarray Kit (Agilent p/n G4112F) containing 41,000+ unique human genes and transcripts. Arrays were scanned in an Agilent Microarray Scanner (Agilent G2565BA) according to the manufacturer's protocol and data extracted using Agilent Feature Extraction Software 9.5.1. Hybridization was performed by the microarray core facility of the Centro de Investigation Principe Felipe.

Statistical analysis of microarray data was done using the platforms R, Matlab, and GEPAS/Babelomics [24]. Functional analyses used Gene Ontology annotations http://www.geneontology.org/ obtained from the Rosseta module of Babelomics [24, 25]. First, normalized data was analyzed using principal component analysis (PCA) [26] to reveal major trends in gene expression data. PCA models were obtained and genes were projected on the composite direction of variability defined by the first two principal components. This projection represents a ranking of genes according to the major patterns of variability. The ranked list was analyzed by gene set enrichment analysis (GSA) to identify specific molecular pathways that were either activated or repressed in the PCA model. The FatiScan [24] function of GSA was used to generate a list of genes that were changed by the experimental conditions. To visualize the functional results, we used the Combined Graph (CG) function of the Blast2GO software [27]. The CG function displays multiple GO terms within the Gen Ontology structure, thereby providing a representation of functional categories within their biological context.

Genes assigned to the functional classes by the PCA–FatiScan approach were used to investigate the dynamics of gene expression changes induced by $O_2$ treatments and to identify differentially expressed genes. Thus, rather than first identifying differentially expressed genes and then using these to investigate functional enrichment, we searched for relevant functional classes using the powerful GSA strategy and then only analyzed genes that were differentially expressed within these classes. This approach proved to be more sensitive because it reduced considerably the requirement for multiple testing correction. Differential expression analysis was performed with the time-course specific program maSigPro [25], an inferential methodology based on regression. Briefly, maSigPro fits a regression model by considering time as a continuous variable and creating specific variables for each treatment, thereby adjusting a temporal profile for each $O_2$ concentration. The method assesses the significance of the global model (i.e., if there are significant differences with respect to time or treatment) and of each variable (i.e., which specific time or treatment change is present). Genes significantly changed were selected and divided into clusters of similar profiles for visualization of results [25].

### Real-Time Polymerase Chain Reaction

Total RNA was prepared as described above. cDNA was synthesized using RT2 First Strand Kit from SuperArray. Real time polymerase chain reaction (RT-PCR) was performed using STBR Green PCR Master Mix (S.A. Biosciences, http://www.sabiosciences.com/) with the LC 480 (Roche, http://www.roche.es/portal/eipf/Spain/spain_portal/roche-spain/inicio) according to the manufacturer's instructions. Quantitative PCR employed the following

conditions: 95°C at 10 minutes, 40 cycles at 95°C, 15 seconds; 55°C, 1 minute; 72°C, 30 seconds. The quality of the primers was tested by analysis of the melting curve using the following conditions: 95°C, 5 seconds; 60°C, 1 minute; and 97°C continuous, one cycle. We used the Human Hypoxia Signaling Pathway RT Profiler PCR array PAHS-032 from SuperArray (SABiosciences, http://www.sabiosciences.com/) that profiles the expression of 84 genes involved in hypoxia-related signaling. The primers for ANGPTL4 and vascular endothelial growth factor (VEGF) were included in this gene panel. The following VEGFR-2 primers were designed with Primer 3 software: VEGFR-2 Left (GTGACCAACATGGAGT CGTG) and VEGFR-2 Right (TGCTTCACAGAAGACCATGC). The CD34 primers were a gift from Dr. A. Ayuso (CIPF, Valencia, Spain, http://www.cipf.es).

## Immunohistochemistry

The medium was removed from the cell cultures, and they were washed carefully with phosphate buffered saline (PBS). Cultures were then fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) for 30 minutes. Subsequently, cells were washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) at room temperature. Blocking of non-specific binding was with normal goat serum in PBS. Primary antibodies to VEGFR-2, Angptl4, CD34, and vWF (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) were diluted in blocking solution and incubated overnight at 4°C. The following day, preparations were washed three times with PBS and incubated with secondary antibodies. Preparations were mounted using Prolong Gold Anti-Fade Reagent with Dapi (Invitrogen).

## Western Blot Analysis

Whole extracts from hESC maintained at 21%, 5%, and 1% $O_2$ were prepared in ice-cold lysis buffer (Sigma). Briefly, each sample was lysed in 60 $\mu$l of buffer, incubated on ice at 4°C for 30 minutes, and centrifuged at 14.000 rpm for 15 minutes (4°C). Protein concentration was determined using the Bradford assay (BioRad Laboratories, Hercules, CA, http://www.bio-rad.com). Fifty $\mu$g of total protein were separated on 8% SDS-polyacrylamide gels. PVDF membranes were incubated with anti-ANGPTL4 (1:200, Santa Cruz) and anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (1:200, Chemicon, Temecula, CA, http://www.chemicon.com) antibodies. Detection was carried out using an enhanced chemiluminescence (ECL) detection kit according to the manufacturer's (Pierce, Rockford, IL, http://www.pierce net.com) recommendations. Relative signal intensity was determined by densitometry using the software Alpha-Imager 2,200.

## Fluorescence-Activated Flow Cytometry

To characterize the expression of endothelial markers in hESC cultures subjected to hypoxia, fluorescence-activated flow cytometry (FACS) was performed. A suspension of endothelial-like cells was harvested by enzymatic digestion with Accumax (Sigma Aldrich Spain, http://www.sigmaaldrich.com/spain.html), centrifuged, washed in PBS, and fixed in 4% PFA during 15 minutes at 4°C. Subsequently, cells were incubated for 1 hour at 4°C with the appropriate antibody dilution. As a negative control, hESC were incubated with the corresponding isotype-matched IgG. Cellular fluorescence was detected in a Beckman Coulter FC500, and statistics were performed with the CXP software from Beckman Coulter (Fullerton, CA, http://www.beck mancoulter.com). All antibodies were from Santa Cruz, http://www.scbt.com/.

## Matrigel Assays of Vessel Formation

Human endothelial-like cells derived from hESC cultured under 5% $O_2$ were transferred to Matrigel-coated plates and monitored for their capacity to form vascular cords. Briefly, Matrigel (Sigma) was thawed on ice overnight and 320 $\mu$l (9 mg/ml) were added to 16 mm wells followed by incubation for 60 minutes at 37°C. Endothelial-like cells generated by the exposure of hESC to 5% $O_2$ for seven days were harvested with Acumax, rinsed with PBS, and seeded on Matrigel according to the manufacturer's instructions. Cultures were maintained with the medium as described above for an additional 7-10 days at 5% $O_2$. Immunohistochemistry was performed to detect VEGFR-2, CD34, CD31, and vWF markers.

## Animals

A total of 60 male nude rats weighing 200-250 g (HIH-Foxn1 rnu, Charles River Laboratories Inc., Wilmington, MA, http://www.criver.com) were included in the study. Animals with fractional shortening (FS) above 35% after MI were excluded. Mortality in all groups due to surgical procedures was 30%. All animal experimentation was approved by the institutional committee for animal welfare and was performed in accordance with national and European legislation.

## Preparation of Cell Cultures for Transplantation

Undifferentiated hESC were cultured as described above under 5% $O_2$ level during 8 days. Subsequently, colonies were disaggregated to single cells using Acumax (Invitrogen) and labeled with Dil according to manufacturer's recommendations (Invitrogen). Finally, $3 \times 10^5$ Dil-labeled EPC/EC were resuspended in PBS. For use as control transplants, human bone marrow mesenchymal stem cells (BM-MSC) ($n = 3$; Inbiomed, San Sebastian, Guipuzcoa, Spain, http://www.inbiomed.org) were cultured following manufacturer's instruction. Single cells, obtained by trypsin (Invitrogen) treatment, were resuspended in PBS ($1.2 \times 10^6$ BM-MSC) just prior to transplantation as described previously [28].

## Induction of Myocardial Infarction and Cell Transplantation

Permanent ligation of the left coronary artery was performed as previously described [29]. Rats were intubated and anesthetized (mixture $O_2$/Sevorane: rate 100 cycles/minute, tidal volume 2.5 mL, Harvard Apparatus small animal ventilator model 683). After thoracotomy, the acute myocardial infarction (MI) was induced by permanent ligation of the left descending coronary artery with 6-0 prolene suture. The infarcted area was identified after the ligation by the development of a pale color in the distal myocardium. The incision was closed with a 3-0 silk suture, and Nolotil (Boehringer Ingelheim Spain, S.A., http://www.boehringer-ingelheim.es/index.jsp) (0.4 g/ml) and Buprenorphin (Schering-Plough, S.A. Spain, Reckitt Benckiser Healthcare (UK), http://www.msd.com/nm/es/) (0.05 mg/kg) were administered intraperitoneally to reduce postoperative pain. Animals were randomly divided into three experimental groups (saline, human endothelial-like cells, or BM-MSC). Seven days after the induction of MI, rats were again subjected to surgery in order to transplant the cellular preparations into the myocardium. Rats received either saline, $1.2 \times 10^6$ BM-MSC cells per animal, or $3 \times 10^5$ Dil-labeled EPC/EC per animal (five injections of 5 $\mu$l with a Hamilton syringe at five distinct points of the infarct border zone).

## Functional Assessment of Transplants by Echocardiography

Rats were anesthetized (Sevorane, Abbott Laboratories, S.A. Spain, http://www.abbott.es/index.asp), the chest was shaved, and animals were placed in the supine position. Transthoracic echocardiography was performed using a system equipped with a 10-MHz linear-array transducer (General Electric, http://www.ge.com). Measurements were taken at the onset of study (basal), post-infarction, and post-transplantation (2 and 4 weeks). The echocardiographs were performed blind since the researcher did not know which animals were assigned to specific experimental groups. Mode-M and two-dimensional (2D) echocardiography at the level of the papillary muscles in the parasternal short axis

view were performed. Functional parameters were calculated by using standard methods in three consecutive cycles [30]. Left ventricular (LV) dimensions in end diastole (LVDd) and end systole (LVDs), anterior and posterior wall (AW and PW) thickness in diastole and systole, end-diastolic area (EDA) and end-systolic area (ESA) were measured. Fractional area change (FAC) was calculated as [(EDA – ESA)/EDA] × 100. Area was measured in $mm^2$. FS was calculated as [(LVDd – LVDs)/LVDd] × 100. Changes in AW were calculated as [(AWs/AWd)/AWs] × 100. The diameter of the LV and the thickness of the AW were measured in mm. One month after transplantation, the infarct size in left ventricle was measured in 8-12 transverse sections of 14 $\mu$m (one slice = 200 $\mu$m of tissue) from apex to base in hearts that had been fixed with 2% PFA and stained with Masson's trichrome. The fibrotic zone was assessed by Image Proplus 5.1 software.

### Analysis of Cell Engraftment by Immunohistochemistry and Confocal Microscopy

Two and four weeks after transplantation, animals were sacrificed and the hearts were removed, washed with PBS, and fixed in 2% PFA. Subsequently, hearts were cryopreserved with 20% sucrose and embedded in Optimal Cutting Temperature (O.C.T.) (Sakura Finetek, http://www.sakura.eu/), and sections of 14 $\mu$m were prepared. To assess the differentiation of the transplanted human cells in the damaged tissue, serial sections were stained with $\alpha$-Sma (Sigma) and anti-troponin (Santa Cruz Biotechnology). Tissue preparations were analyzed by confocal microscopy (Leica TCS-SP2-AOB5, http://www.leica.com).

## RESULTS

### Global Transcriptome Profiling of hESC Cultured under Normoxic (21% $O_2$) Versus Hypoxic (1% and 5% $O_2$) Conditions

To test the effects of hypoxia on the properties of hESC, undifferentiated colonies of two different hESC lines (H9 and VAL9) were plated on extracellular matrix (ECM) from human foreskin feeder cells matrix [31] and maintained in fibroblast conditioned medium. At the onset of the study, the undifferentiated status of the cultures was verified by the presence of pluripotency markers as detected by RT-PCR and immunohistochemistry (supporting information Fig. 1A, 1B). Daily monitoring and phase-contrast images of these cultures demonstrated that, after 5-8 days, cells grown under hypoxic conditions displayed a differentiated morphology similar to cells of the endothelial phenotype (Fig. 1A). In contrast, the morphology of hESC cultures maintained under 21% $O_2$ changed little if any during this same period of time (Fig. 1A). Culture in hypoxia did not alter chromosomal stability since analysis of sub-microscopic copy number did not reveal aberrations in hESC maintained under 5% $O_2$ for 15 days (supporting information Fig. 1C).

Therefore, we examined gene expression changes in the H9 line maintained under different levels of $O_2$. Human embryonic stem cells were cultured for different time periods in the various levels of $O_2$ (1%, 5%, and 21%) without a change of media. In two independent experiments, samples were taken at five time points for microarray analysis: 0 (control), 24 hours after the onset of growth conditions, and at days 5, 10, and 15. It is important to note that the extreme culture conditions of 1% $O_2$ for 15 days produced a high level of cell death in hESC, coincident with low-quality RNA. Thus, this experimental parameter was excluded from the microarray analysis. After normalization, data were subjected to principal components analysis (PCA), which confirmed that gene expression changes could be attributed to the different $O_2$ concentrations as well as to the length of time under the given concentrations of $O_2$ (Fig. 1B). The two components of the PCA collected 40.71% of the gene expression variation and defined a line of variability that was associated with the low $O_2$ condition (Fig. 1C), thereby serving to order genes based on their response to hypoxia. Further bioinformatics analysis with the FatiScan tool was performed to determine whether genes at the extreme positions of these hypoxia-associated changes had specific cellular functions. This analysis revealed that exposure of hESC to hypoxia has a clear impact on the cell-fate decisions of hESC and on cellular functions essential for normal development and cell survival. Growth under low $O_2$ activated genes associated with very specific Gene Ontology (GO) categories including developmental processes such as angiogenesis, blood vessel morphogenesis, and myeloid cell differentiation, as well biological process related with development like negative regulation of programmed cell death (supporting information Fig. 2). Growth under 21% of $O_2$ was associated with regulation of genes implicated in metabolic processes specifically related to cellular division and to ATP production using $O_2$ as the last acceptor of electrons (supporting information Fig. 3).

The PCA–FatiScan analysis highlighted cellular functions potentially modified by low levels of $O_2$. To obtain further insights regarding the specific genes that were regulated under hypoxia, we performed genewise inferential analysis of the genes assigned to these functional classes and other potentially relevant protein families. To this end, we used the maSigPro tool, which is particularly suited for the analysis of time-course data [25]. Specifically, we analyzed expression changes for genes associated with angiogenesis, fatty-acid metabolism, and pluripotency. Of these 1,297 genes, 170 were significantly changed by our experimental conditions (supporting information Table 1). Cluster analysis and visualization revealed the pattern of gene variation with respect to the variable of time; a large proportion of genes (95) were upregulated in hESC cultured under 1% and/or 5% $O_2$, with a strong differential expression at early time points, in comparison to hESC maintained under normoxia (supporting information Fig. 4A and supporting information Table 1 clusters 1, 3, 4, 6, and 8). These upregulated genes were associated with processes such as development, signal transduction, metabolism, cell differentiation, cell adhesion, proteolysis, electron transport, positive regulation of cell proliferation, response to hypoxia, and cell–cell communication (supporting information Fig. 4B). Since the biological process of angiogenesis is one of the most relevant for the assessment of our hypoxia-based differentiation protocol, further analysis of the genes annotated in this GO suggested that they are related with (i) angiogenesis, (ii) vasculogenesis from progenitor cells and de novo formation of blood vessels, (iii) blood vessels formation from the proliferation of pre-existing blood vessels, (iv) regulation of vascular permeability, (v) regulation of vasodilatation and vasoconstriction, and (vi) negative regulation of apoptosis (supporting information Fig. 4C1–C6).

### Characterization of Specific Gene Expression Changes Induced by Exposure of hESC to Hypoxia

Vascular endothelial growth factor (VEGF) A and B modulate vascular biology by regulating vasculogenesis and angiogenesis during embryogenesis and in adult life [32]. These factors exert their biological effects by binding to the structurally homologous receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) [32, 33]. The physiological role of these receptors has been studied in genetic models; VEGFR-1 null mice are lethal because the EC are not able to organize blood vessels,

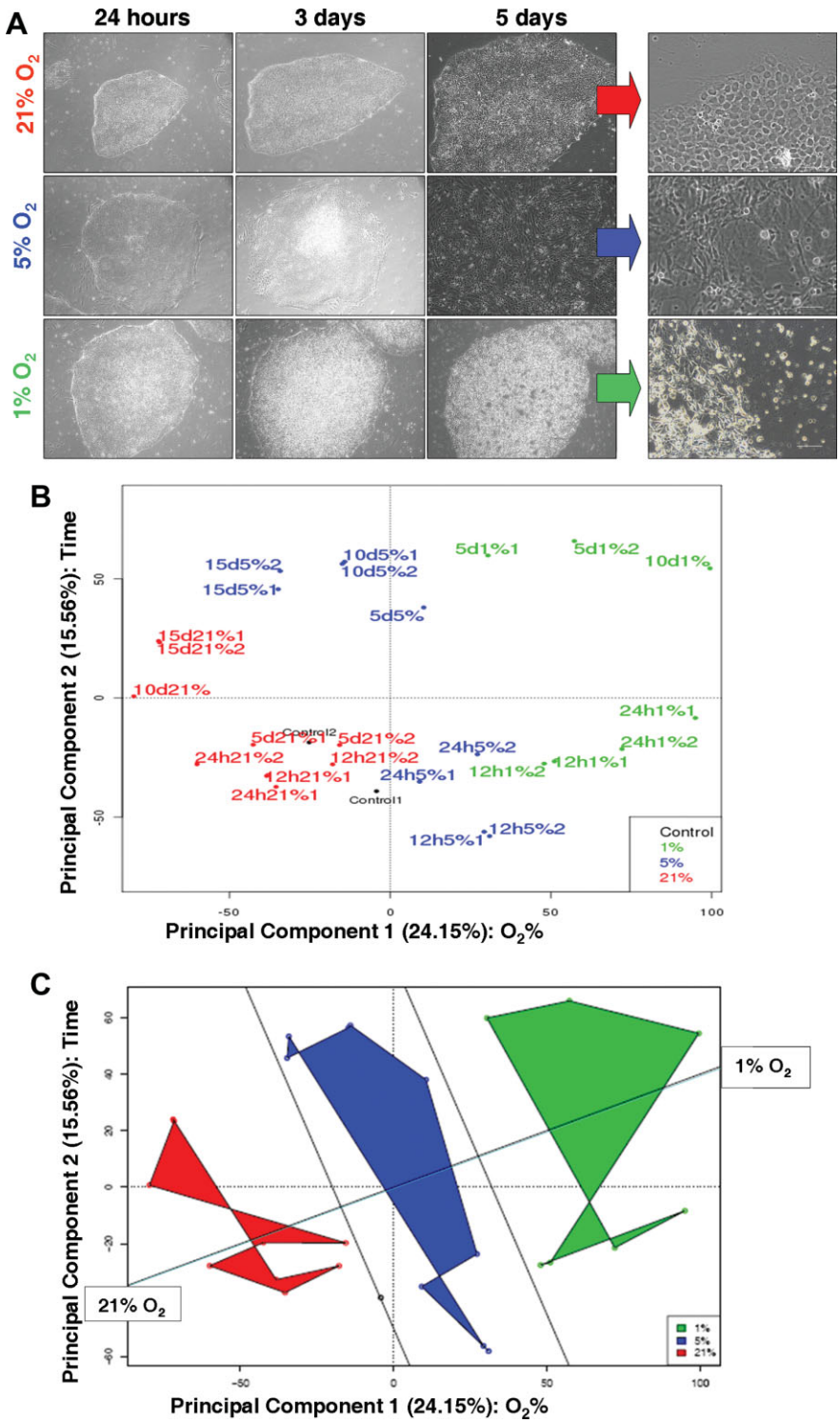



**Figure 1.** Morphological and genetic changes induced by culture of human embryonic stem cells (hESC) under hypoxia. (**A**): Phase contrast images (5×) of hESC cultured at varying concentrations of $O_2$ for 24 hours, 3 days, and 5 days. hESC were plated on human feeder-derived matrix. The panels indicated by arrows on the right represent higher magnifications (20×) of hESC cultured for 5 days under the indicated conditions and reveal that areas of differentiation were observed in the hESC cultured for this time period in 1% or 5% but not in 21% $O_2$ (**B**) and (**C**) principal component analysis (PCA) of gene expression profiling data. Using microarrays, we analyzed the gene expression in hESC cultured for defined time periods (12 hour, 24 hour, 5 days, 10 days, and 15 days) under normoxic (21% $O_2$, red) and hypoxic (5% $O_2$, blue, and 1% $O_2$, green) conditions. In (**B**), the PCA revealed that the two major patterns of variability associated with the $O_2$ level (PC1) and the duration of culture (PC2) explain the differential gene expression: 24.15% (PC1) and 15.56% (PC2). Moreover, the samples could be grouped according to the $O_2$ treatment. In (**C**), the solid blue line indicates the composite direction of variability defined by PC1 and PC2 that represents the $O_2$ gradient. PCA loadings were projected on this line to obtain a ranking of genes based on their response to $O_2$ concentration.

whereas VEGFR-2-deficient mice display defects in the development of endothelial and hematopoietic precursors [34–36]. During development, VEGF binds to VEGFR-2 in the angioblast to stimulate EC formation and proliferation while concomitantly inducing capillary formation by binding to the VEGFR-1 [37–39]. The maSigPro statistical analyses, used to evaluate the temporal expression of genes in hESC maintained under different $O_2$ concentrations, revealed a significant upregulation ($p < .05$) of *VEGFA* and *VEGFB* expression in the cells that were cultured under 5% and 1% $O_2$ during five days as compared to hESC under normoxic conditions (Fig. 2A–C). Additionally, the expression of the gene for *VEGFR-2* was significantly increased by culture in hypoxic conditions ($p < .05$) (Fig. 2D). RT-PCR was used to confirm that *VEGF* expression was upregulated by 9.44-fold after 24 hours at 5% $O_2$, reaching a peak of 47.18-fold at 5 days under these hypoxic conditions. Expression of *VEGFR-2* increased 2.8-fold in cells cultured under 5% $O_2$ for 24 hours, 7.73-fold at 5 days, and 12.8-fold at 15 days in comparison to hESC maintained at 21% $O_2$ (Fig. 2E).

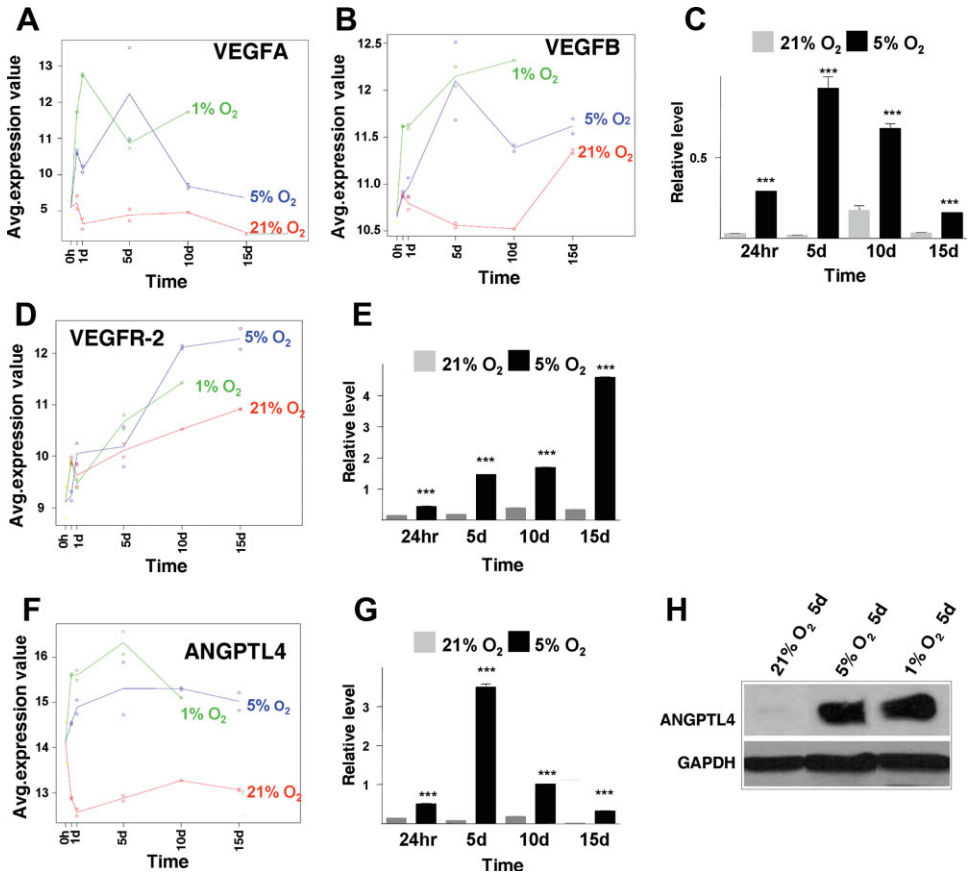



**Figure 2.** Characterization of specific gene expression changes promoted by hypoxia. (**A, B**): maSigPro statistical analyses, used to evaluate the temporal expression of genes in hESC maintained under different $O_2$ concentrations, revealed a significant upregulation ($p < .05$) of VEGFA and VEGFB expression in the cells that were cultured under 5% $O_2$ (blue) and 1% $O_2$ (green) during five days as compared to human embryonic stem cells (hESC) under normoxic conditions (red). (**C**): Quantification of VEGFA transcript levels by RT-PCR analysis in cultures of hESC maintained under different concentrations of $O_2$ versus time. (**D**): maSigPro prediction for the temporal expression of VEGFR-2 in hESC cultures maintained under different $O_2$ concentrations for distinct time periods ($p < .05$). (**E**): Quantification of real time polymerase chain reaction (RT-PCR) analysis of VEGFR-2 transcripts in hESC cultured under different conditions. (**F**): maSigPro prediction of ANGPTL4 expression in hESC cultured under different conditions ($p < .05$). (**G**): Quantification of RT-PCR analysis of ANGPTL4 expression. (**H**): Western blot analysis of ANGPTL4 expression in hESC cultured under 21, 5, and 1% $O_2$ during 5 days. Fifty $\mu$g of total cell lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Anti-GAPDH was used as a loading control. Data are presented as mean ± SD.

Angiopoietin-like (ANGPTL) molecules constitute a novel protein family with seven members. Several studies [40, 41] have demonstrated that ANGPTL proteins have distinct roles in angiogenesis and metabolism. Statistical analysis of our results revealed a significant upregulation of *ANGPTL4* in hESC exposed to low $O_2$ levels ($p < .05$) (Fig. 2F). ANGPTL4 has been detected in placenta, liver, adipose tissue, human ischemic, and tumoral tissue, as well as in EC grown under hypoxia [41], suggesting that this molecule exerts a VEGF-independent pro-angiogenic effect. The temporal expression pattern of *ANGPTL4* under hypoxic conditions was confirmed by RT-PCR (Fig. 2G) and western blotting (Fig. 2H). Quantification of Western analysis revealed that ANGPTL4 was 8.1-fold higher in cultures maintained in 5% $O_2$ for five days and 11-fold higher under 1% $O_2$ when compared with the cells cultured in the presence of 21% $O_2$ for the same period of time.

Fluorescence-activated cell sorting analysis demonstrated that approximately 53% of the cells cultured under 5% $O_2$ expressed VEGF (Fig. 3A), whereas only a small proportion of the cells maintained in 21% $O_2$ during five days expressed this receptor (Fig. 3A, small graph). Additionally, FACS analyses showed that ≥80% of the cells express ANGPTL4 when differentiated for 5 days under 5% $O_2$ (Fig. 3B).

## Characterization of Endothelial Phenotype Resulting from the Culture of hESC Under 5% $O_2$

Our results demonstrate that hESC cultured under 5% $O_2$ express the EC markers VEGFA, VEGFB, and VEGFR-2 and that they adopt an endothelial-like morphology under hypoxic conditions. We next characterized the phenotype of these cells by analyzing the expression of typical endothelial markers including CD34, CD31, and vWF.

CD34 is a heavily glycosylated, transmembrane protein that is expressed in common progenitors for EC and HSC and is frequently employed to isolate EC from a range of sources [10]. CD34 expression is high in the most primitive precursors but is gradually lost as lineage-committed progenitors differentiate. Whereas most EC maintain CD34 expression, the hematopoietic lineage loses this marker when they differentiate to mature blood cells [42]. Our bioinformatics analysis revealed that hESC cultured under hypoxia for 24 hours displayed significant upregulation of CD34 expression, whereas cells maintained with 21% $O_2$ did not express this marker. RT-PCR analysis confirmed that, after 24 hours in 5% $O_2$, the expression of CD34 was 6-fold higher than the levels detected in hESC cultured with 21% $O_2$ and was 4-fold higher after

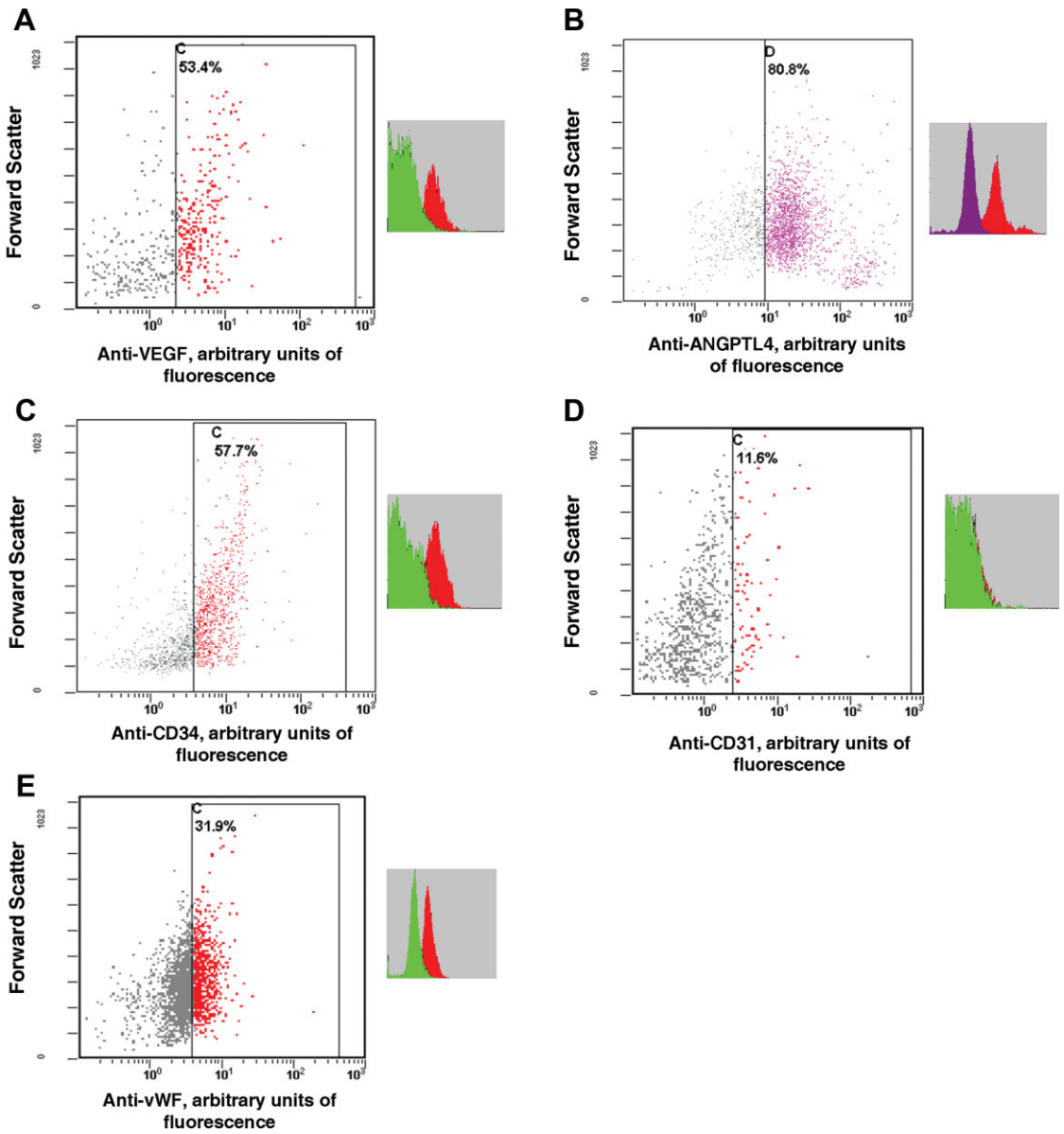



**Figure 3.** Flow cytometry analysis of the expression of endothelial markers in cultures of human embryonic stem cells (hESC) under 5% $O_2$. (**A–E**): hESC cultures were maintained in either 5% or 21% $O_2$ for 5-8 days. Subsequently, cells were collected and incubated with primary antibodies for either VEGF, ANGPTL4, CD34, CD31, or vWF. Overlay plots shown to the right of each flow cytometry assay depict the levels of relative fluorescence detected in cells of 21% $O_2$ (green or purple) versus 5% $O_2$ (red).

5 days in hypoxia (supporting information Fig. 5A). By flow cytometry analysis, 57.7% of cells cultured under 5% $O_2$ for 24 hours expressed CD34 (Fig. 3C), whereas cells grown under 21% $O_2$ during 24 hours did not express CD34. Assessment of cultures by immunohistochemistry also confirmed the presence of $CD34^+$ cells after 5 days at 5% $O_2$, but hESC maintained under 21% $O_2$ did not stain positive for this endothelial marker (supporting information Fig. 5B).

Although CD34 is a marker for the early stages of EC maturation, PECAM-1 and vWF are expressed in more mature EC. The adhesion molecule-1 PECAM-1 (also know as CD31) is a transmembrane protein expressed primarily in endothelial and hematopoietic lineages such as platelets and leukocytes [43]. In our study, 11.6% of cells expressed CD31 when cultured for 8 days in the presence of 5% $O_2$ (Fig. 3D). vWF is a glycoprotein produced in the Weibel-Palade bodies of EC as well in platelets and in subendothelial tissue [44]. vWF expression was detected by confocal microscopy after 8 days of exposure to 5% $O_2$ (supporting information Fig. 5C), and flow cytometry analysis confirmed that 31.9% of the cells cultured under hypoxia express this marker (Fig. 3E).

## Analysis of Vessel Formation In Vitro

To determine whether the changes in gene expression, induced by culture under hypoxia, were sufficient to drive vasculogenesis, the potential of the endothelial-like cells to form vessels was tested using a 3D culture model. This model promotes the formation of vessel-like structures from EC [45]. Thus, after 5-7 days of culture in the presence of 5% and 21% $O_2$, single cells were obtained and plated on Matrigel and subjected to further culture. Following 24 hours under 5% $O_2$ conditions, more than 95% of CD34-positive cells displayed sprouting of cellular aggregates (Fig. 4A). In contrast,

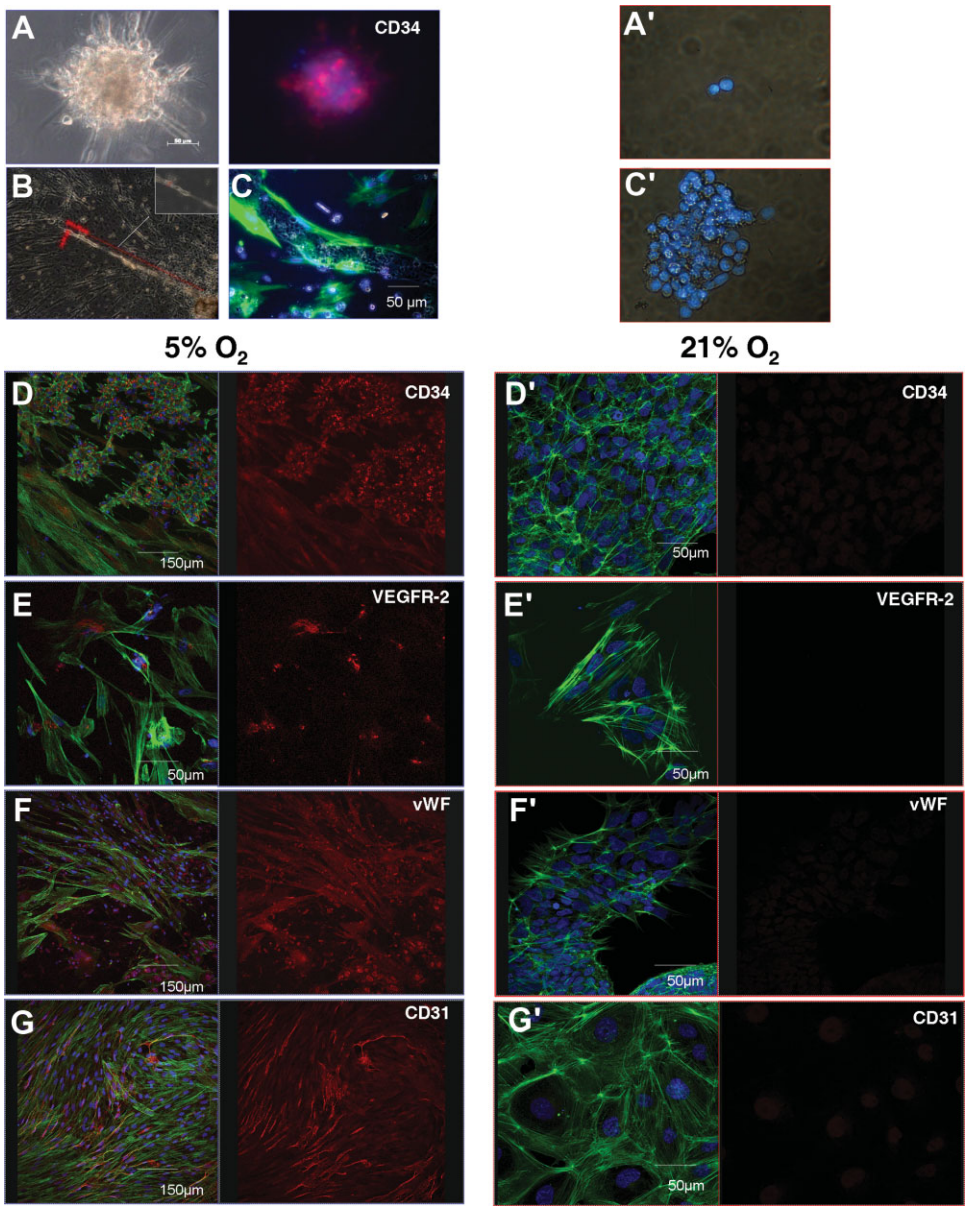



**Figure 4.** Analysis of vessel formation in vitro and expression of endothelial cell markers. After culture in either 5% (**A**) or 21% $O_2$ (**A′**) for 5-7 days, cells were plated on Matrigel and subjected to further culture. Following 24 hours under 5% $O_2$ conditions, more than 95% of CD34-positive cells (red) in (**A**) displayed sprouting of cellular aggregates (×40). In contrast, human embryonic stem cells (hESC) maintained in 21% $O_2$ (**A′**) did not form aggregates nor did they display sprouting. (**B**): Cordlike structures appeared in these cultures of endothelial cells after 3 days (×10). The square represents a magnification (×40) of the indicated area. (**C, C′**): After 7 days on Matrigel in the presence of 5% $O_2$ (**C**), the cordlike structures were denser, with a layer of cells supporting these vessels (×40). However, hESC cultured at 21% (**C′**) under identical conditions did not display this morphology. (**D–G, D′–G′**): Immunohistochemical analysis of hESC cultures in the presence of 5% (**D–G**) or 21% $O_2$ (**D′–G′**) as described above for 3 and 7 days. Primary antibodies (CD34, VEGFR-2, vWF, CD31), red (Alexa 647); Phalloidin, green (Alexa 488); and Dapi (blue). The left panels reveal the staining obtained with the specified endothelial markers, whereas composite images are presented in the right panels.

hESC maintained in 21% $O_2$ did not form aggregates nor did they display sprouting (Fig. 4A′). The cells grown under 5% $O_2$ were also able to initiate the formation of cordlike structures within 3 days (Fig. 4B), which expressed CD34 and VEGFR-2 (Fig. 4D, 4E). However, cells grown under 21% $O_2$ did not form cordlike structures nor did they express CD34 and VEGF-2 (Fig. 4D′, 4E′). More dense cordlike structures (Fig. 4C) expressing CD31 and vWF were observed after 7 days (Fig. 4F, 4G). However, hESC grown at 21% $O_2$ under identical conditions did not display this morphology (Fig. 4C′) and did not express the mature EC markers (Fig. 4F′, 4G′).

## Transplantation of Endothelial-like hESC-Derived Cells Improves Cardiac Function and Reduces Fibrotic Scar Tissue in a Rat Model of Myocardial Infarction

To test the functional capacity of the endothelial-like cells generated by differentiation in hypoxia, we transplanted these cells into the myocardium of rats that had been previously subjected to myocardial infarction. Given that adult MSC from various sources have been shown to facilitate repair of damaged cardiac tissue in rodent models [28], we employed human BM-MSC as a positive control for these transplantation experiments. Cellular doses were selected based on previous observations [46]. The extent of tissue injury induced by MI was comparable between all animals since the echocardiograph parameters were similar for the three groups at this time point (Fig. 5A–5C). Two and four weeks after transplantation, various cardiac function parameters were measured to assess the degree of functional recovery achieved by the presence of human EC-like cells in the infarct border zone. When compared with values obtained shortly after induction of MI, treatment with either EPC/EC or BM-MSC improved various cardiac parameters, whereas no such improvements were observed in the control group, which received only saline (Fig. 5 and supporting information Tables 2-4). When analyzed two weeks after the transplant, the EPC/EC and BM-

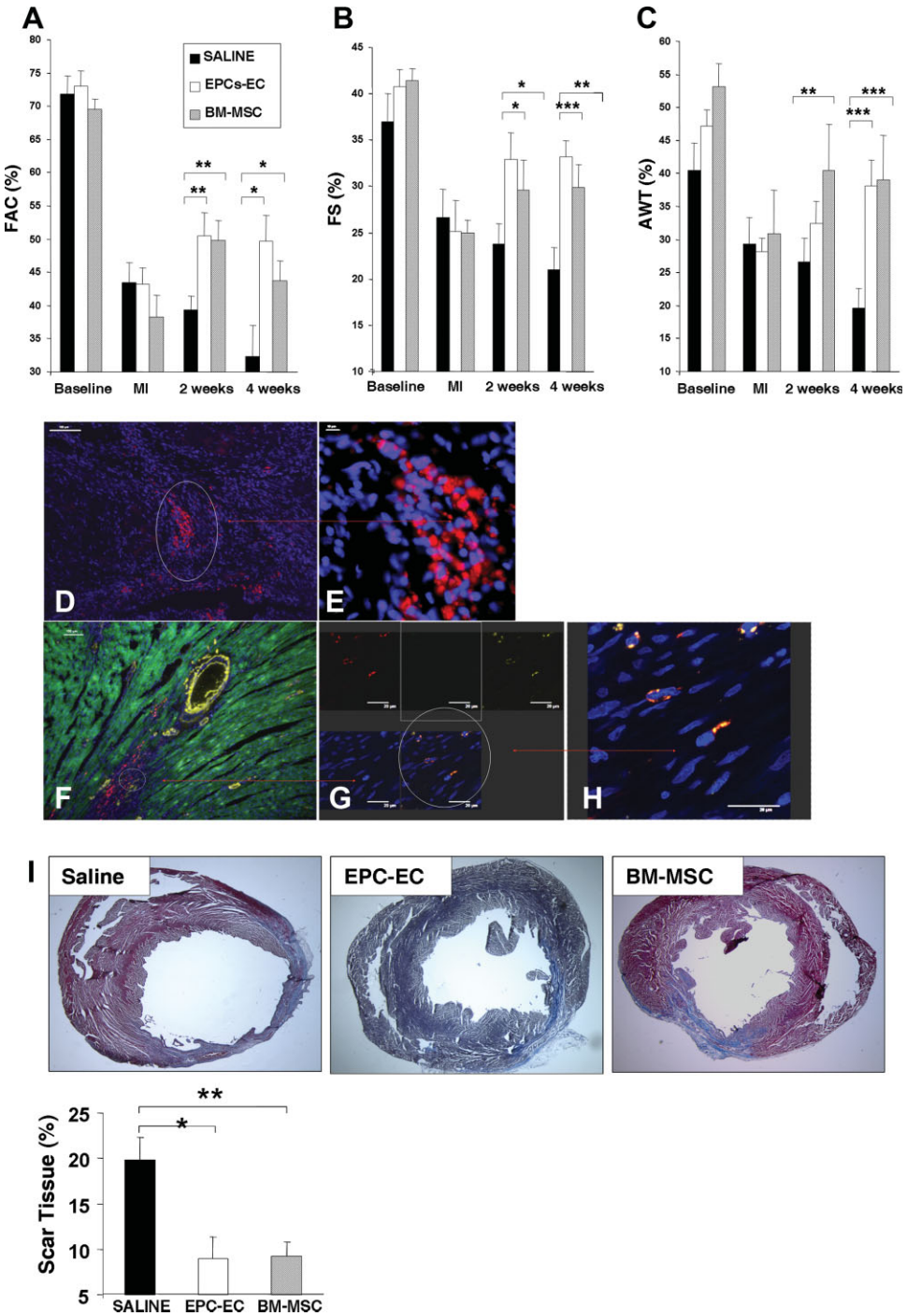



**Figure 5.** Evaluation of transplanted EPC/EC-derived cells in a rat model of acute myocardial infarction. (**A**): Ecocardiograph values were measured one day before (basal) and 7 days after the induction of myocardial infarction in rats. Subsequently, human embryonic stem cells (hESC)-derived EPC/EC ($3 \times 10^5$ cells/animal) or BM-MSC ($1.2 \times 10^6$ cells/animal) were transplanted into the infarcted zone of the myocardium ($n = 6$ controls treated only with saline, six rats transplanted with EPC/EC, and six animals treated with BM-MSC). After 2 and 4 weeks, ecocardiograph parameters at the level of the papillary muscle were again measured to assess functional recovery. FAC was calculated as [(EDA – ESA)/EDA] $\times$ 100. *, $p < .05$, **, $p < .02$. (**B**): FS values were obtained as described above. *, $p < .05$, **, $p < .02$, ***, $p < .001$. (**C**): AWT was measured at the indicated time points. **, $p < .02$, ***, $p < .001$. (**D, E**): Immunohistochemical analysis of cellular engraftment. Two and four weeks post-transplantation animals were sacrificed and hearts were removed for histological studies. Transplanted hESC-derived EPC/EC in the border of the infarcted zone were visualized (**D, E**) by prior labeling of these cells with DiI (red) and Dapi (blue). (**F–H**): Sections of the engrafted area were stained with antibodies against troponin (green, Alexa 488) and the vessel marker αSma (Cy3). These studies detected cells that were positive for both DiI (red) and αSma (yellow, far right panel). (**I**): Effect of cell transplantation on infarct size. One month after transplantation, animals were sacrificed and the heart was processed for histological analysis. Images represent sections stained with Masson's trichrome. Scale bars: 100 $\mu$m. Size of the infracted zone is expressed as percentage of total left ventricular area and as a mean of all slices from each heart, *, $p < .02$, **, $p < .01$. Data are presented as mean $\pm$ SD. Abbreviations: AWT, anterior wall thickening; BM-MSC, bone marrow mesenchymal stem cells; EPCs-EC, endothelial progenitor cells–endothelial cells; FAC, fractional area change; FS, fractional shortening.

MSC groups displayed a significant recovery of both systolic function (Fig. 5A) as calculated by percentage of fractional area change (FAC: 50.47 ± 3.46% in transplanted rats versus 39.34 ± 2.08% in saline group, $p < .02$) and of fractional shortening (FS: 32.94 ± 2.87%, in transplanted rats versus 23.75 ± 2.26% in saline group, $p < .05$). The improvements to fractional shortening were more pronounced after four weeks of transplantation (FS: 33.14 ± 1.77% in EPC/EC transplanted rats and 29.90 ± 2.41% in BM-MSC transplanted rats versus 21.01 ± 2.34% in saline group), and additionally, a beneficial effect was observed on anterior wall thickening (AWT) at this time point (AWT: 38.07 ± 3.92% in transplanted animals and 38.98 ± 6.78 in BM-MSC transplanted rats versus 19.55 ± 2.97% in saline group) (Fig. 5B, 5C). Thus, the effects we obtained in the current study with the transplantation of hESC-derived EPC/EC were similar to the functional recovery achieved by BM-MSC. Analysis of cell engraftment was facilitated by prior labeling of transplanted hEPC/EC with DiI, a long-chain dialkylcarbocyanine which binds to phospholipid bilayer of the plasma membrane and can be visualized at 553 nm. After two weeks, animals were sacrificed and the infarcted area was subjected to histological studies. Immunochemistry analysis revealed the presence of DiI-labeled EPCs-EC in the peri-infarcted regions (Fig. 5D, 5E). Engraftment of these cells in the vasculature was demonstrated by immunostaining for troponin (marker of cardiac muscle) and α-Sma (vessel marker) (Fig. 5F–5H). Quantification of the infarcted area revealed that transplantation of either hESC-derived EPC/EC or BM-MSC significantly ($p < .02$) reduced the area of fibrous scar tissue (Fig. 5I); percentage of scar tissue was 19.82 ± 2.49% in saline group and 8.94 ± 2.40% in EPC-EC group. A similar reduction in the area of the fibrous scar tissue was detected in BM-MSC group 9.23 ± 1.56% ($p < .01$).

## Discussion

Human embryonic stem cells provide a unique tool to study early development and represent a potential source of therapeutic quantities of transplantable material for the regeneration of damaged human tissue. Although the early stages of human development occur in a hypoxic environment, little is known about how low $O_2$ levels modulate the pluripotency and differentiation capacity of established hESC lines. Here we demonstrate for the first time that culture in hypoxia is sufficient to promote efficient differentiation of hESC to an endothelial phenotype. Development of differentiation conditions for the efficient production of endothelial-like cells may circumvent adult stem cell sources for regenerative strategies aimed at repair of damaged vasculature and provides the additional benefit of patient-specific tailoring [11–19].

Previous studies [42, 45, 47–53] have demonstrated the spontaneous differentiation of various hESC lines (H9, H1, and CHA3) to EC by formation of EB, a process known as 3D differentiation [45], or by use of a monolayer system. For the 3D protocols, EB were dissociated after 13-15 days and $CD31^+$ cells were isolated and seeded in plates with endothelial cell grow medium [45].

Under the 2D culture conditions, endothelial differentiation of the H1 and H9 lines was induced by various types of supporting cells or matrices including stromal cells [42], MEF [49], OP9, MS-5 [50, 51], and collagen IV [52]. The major limitation of these published methods is the low efficiency. Spontaneous differentiation of EB yielded only 1-3% EC [45, 53], whereas with the methods based on Matrigel and conditioned media [48], 5-10% of the resulting cultures were $CD34^+$ precursors. Here we demonstrate that culture of hESC in conditions of 5% $O_2$ for five days promotes expression of endothelial markers in a significant proportion of the cells; on the basis of FACS analysis, VEGF and CD34 were detected in more than 50% of the cells cultured under hypoxia, whereas 80% were positive for ANGTPL4. When these cultures were analyzed at later time points (8-15 days), the expression of markers characteristic of more mature endothelial cells was detected including CD31 (≤11.6%) and vWF (≥31.9%). Thus, these data suggest that our hypoxia-based differentiation method is much more efficient at generating EPC/ EC than existing protocols.

The bioinformatics tools employed in the present work allowed us to integrate the high-throughput data, thereby demonstrating that the culture microenvironment plays a critical role in regulating the hESC transcriptome profile. These data reveal that different $O_2$ levels differentially regulate expression of genes related with distinct biological processes in cultures of hESC. Furthermore, our studies demonstrate that exposure of hESC to hypoxia clearly modulates lineage specification decisions by regulating genes that are essential for normal embryo development including (i) angiogenesis, (ii) vasculogenesis from progenitor cells and de novo formation of blood vessels, (iii) blood vessels formation from the proliferation of pre-existing blood vessels, (iv) regulation of vascular permeability, (v) regulation of vasodilatation and vasoconstriction, and (vi) negative regulation of apoptosis. These classes of genes are related with the formation of the circulatory network, one of the first events to occur during normal development of the human embryo. Moreover, the differential gene expression patterns elucidated in hESC by the distinct culture conditions were associated with an endothelial cell phenotype. Interestingly, our bioinformatics analysis also revealed that HIF-1α and EPAS-1 are significantly upregulated in hESC cultured under low $O_2$ levels, suggesting a potential role for these transcription factors in regulating expression of genes associated with vasculogenesis.

The genes regulated by culture of hESC in hypoxia include ANGPTL4, a member of the novel family of angiopoietin-like proteins [55]. Various studies have demonstrated that these molecules have different roles in angiogenesis and metabolism [56, 57]. Our studies demonstrate that expression of ANGPTL4 was 8.1-fold higher in cultures maintained at 5% $O_2$ for five days and 11-fold higher under 1% $O_2$ when compared with the cells cultured in the presence of 21% $O_2$ for the same period of time. ANGPTL4 has been identified in numerous tissue including ischemic and tumoral tissue, as well in EC grown under hypoxia [39], suggesting that ANGPTL4 exerts a VEGF-independent proangiogenic effect.

## Conclusion

In summary, we have developed a very efficient, simple, and rapid method for directing endothelial differentiation that is based on the manipulation of the $O_2$ levels under which hESC are grown. Our strategy generates EPC/EC at much higher efficiency than the conventional 3D and 2D models of differentiation used for endothelial differentiation. Finally, we demonstrate that the EPC/EC derived from this hypoxia-based protocol are functional because, when transplanted into a rat model of myocardial infarction, they facilitated recovery of cardiac function. Thus, our findings have important implications not only for understanding the basic biology of hESC

and early human development but also as the rational basis for the design of novel cellular therapies and drugs aimed at repair of damage vascular tissue.

## ACKNOWLEDGMENTS

This project was supported by the Regenerative Medicine Program of Valencia with funding from the Instituto Carlos III and the Valencian Health Ministry. Other sources of funding include Spanish Ministry for Science and Innovation (MICINN) project SAF2008-00011, Red de Terápia Celular (TerCell), CIBERDEM (ISCIII), and ISCIII project EMER07/012. We are also grateful to Alberto Hernandez Cano and Eva Lafuente Villarreal of the CIPF Confocal Service for their invaluable technical assistance. We would also like to thank Petra Stojkovic for helpful discussions and suggestions at the onset of this project.

## REFERENCES

1 Harvey AJ. The role of oxygen in ruminant preimplantation embryo development and metabolism. Anim Reprod Sci 2007;98:113–128.
2 Genbacev O, Zhou Y, Ludlow JW et al. Regulation of human placental development by oxygen tension. Science 1997;277:1669–1672.
3 Pabon JE, Jr., Findley WE, Gibbons WE. The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. Fertil Steril 1989;51:896–900.
4 Semenza GL. Hydroxylation of HIF-1: Oxygen sensing at the molecular level. Physiology 2004;19:176–182.
5 Iyer NV, Kotch LE, Agani F et al. Cellular and developmental control of $O_2$ homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev 1998;12:149–162.
6 Peng J, Zhang L, Drysdale L et al. The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling. Proc Natl Acad Sci U S A 2000;97:8386–8391.
7 Carmeliet P. Angiogenesis in life, disease and medicine. Nature 2005;438:932–936.
8 Kovacic JC, Moore J, Herbert A et al. Endothelial progenitor cells, angioblasts, and angiogenesis—Old terms reconsidered from a current perspective. Trends Cardiovasc Med 2008;18:45–51.
9 Ribatti D. Hemangioblast does exist. Leuk Res 2008;32:850–854.
10 Asahara T, Murohara T, Sullivan A et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964–967.
11 Beltrami AP, Barlucchi L, Torella D et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003;114:763–776.
12 Camargo FD, Green R, Capetanaki Y et al. Single hematopoietic stem cells generate skeletal muscle through myeloid intermediates. Nat Med 2003;9:1520–1527.
13 Ichim TE, Solano F, Brenes R et al. Placental mesenchymal and cord blood stem cell therapy for dilated cardiomyopathy. Reprod Biomed Online 2008;16:898–905.
14 Ingram DA, Mead LE, Tanaka H et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood 2004;104:2752–2760.
15 Jie KE, Goossens MH, van Oostrom O et al. Circulating endothelial progenitor cell levels are higher during childhood than in adult life. Atherosclerosis 2009;202:345–347.
16 Larrivée B, Karsan A. Involvement of marrow-derived endothelial cells in vascularization. Handb Exp Pharmacol 2007;180:89–114.
17 Planat-Benard V, Silvestre JS, Cousin B et al. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 2004;109:656–663.
18 Wurmser AE, Nakashima K, Summers RG et al. Cell fusion-independent differentiation of neural stem cells to the endothelial lineage. Nature 2004;430:350–356.
19 Zuk PA, Zhu M, Mizuno H et al. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng 2001;7:211–228.
20 Cervera RP, Stojkovic M. Human embryonic stem cell derivation and nuclear transfer: Impact on regenerative therapeutics and drug discovery. Clin Pharmacol Ther 2007;82:310–315.
21 Ezashi T, Das P, Roberts RM. Low $O_2$ tensions and the prevention of differentiation of hES cells. Proc Natl Acad Sci U S A 2005;102:4783–4788.
22 Niebruegge S, Bauwens CL, Peerani R et al. Generation of human embryonic stem cell-derived mesoderm and cardiac cells using size-specified aggregates in an oxygen-controlled bioreactor. Biotechnol Bioeng 2009;102:493–507.
23 Koay EJ, Athanasiou KA. Hypoxic chondrogenic differentiation of human embryonic stem cells enhances cartilage protein synthesis and biomechanical functionality. Osteoarthritis Cartilage 2008;16:1450–1456.
24 Al-Shahrour F, Arbiza L, Dopazo H et al. From genes to functional classes in the study of biological systems. BMC Bioinformatics 2007;8:114.
25 Conesa A, Nueda MJ, Ferrer A et al. maSigPro: A method to identify significantly differential expression profiles in time-course microarray experiments. Bioinformatics 2006;22:1096–1102.
26 de Haan JR, Wehrens R, Bauerschmidt S. Interpretation of ANOVA models for microarray data using PCA. Bioinformatics 2007;23:184–190.
27 Conesa A, Gotz S, Garcia-Gomez JM et al. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 2005;21:3674–3676.
28 Gandia C, Arminan A, Garcia-Verdugo JM et al. Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction. Stem Cells 2008;26:638–645.
29 Friedrich J, Apstein CS, Ingwall JS. 31P nuclear magnetic resonance spectroscopic imaging of regions of remodeled myocardium in the infarcted rat heart. Circulation 1995;92:3527–3538.
30 Litwin SE, Katz SE, Weinberg EO et al. Serial echocardiographic-Doppler assessment of left ventricular geometry and function in rats with pressure-overload hypertrophy. Chronic Angiotensin-Converting Enzyme Inhibition Attenuates The Transition To Heart Failure. Circulation 1995;91:2642–2654.
31 Escobedo-Lucea C, Stojkovic M. Growth of human embryonic stem cells using derivates of human fibroblasts. Methods Mol Biol 2009;584:55–69.
32 Ylä-Herttuala S, Rissanen TT, Vajanto I et al. Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. J Am Coll Cardiol 2007;49:1015–1026.
33 Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. Nature 2005;438:937–945.
34 Ng YS, Ramsauer M, Loureiro RM et al. Identification of genes involved in VEGF-mediated vascular morphogenesis using embryonic stem cell-derived cystic embryonic bodies. Lab Invest 2004;84:1209–1218.
35 Ferrara N. Role of vascular endothelial growth factor in the regulation of angiogenesis. Kidney Int 1999;56:794–814.
36 Carmeliet P, Moons L, Dewerchin M et al. Insights in vessel development and vascular disorders using targeted inactivation and transfer of vascular endothelial growth factor, the tissue factor receptor, and the plasminogen system. Ann N Y Acad Sci 1997;811:191–206.
37 Fong GH, Rossant J, Gertsenstein M et al. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 1995;376:66–70.
38 Shalaby F, Ho J, Stanford WL et al. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell 1997;89:981–990.
39 Shalaby F, Rossant J, Yamaguchi TP et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995;376:62–66.
40 Hato T, Tabata M, Oike Y. The role of angiopoietin-like proteins in angiogenesis and metabolism. Trends Cardiovasc Med 2008;18:6–14.
41 Le Jan S, Amy C, Cazes A et al. Angiopoietin-like 4 is a proangiogenic factor produced during ischemia and in conventional renal cell carcinoma. Am J Pathol 2003;162:1521–1528.
42 Kaufman DS, Hanson ET, Lewis RL et al. Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2001;98:10716–10721.
43 Newman PJ, Newman DK. Signal transduction pathways mediated by PECAM-1: New roles for an old molecule in platelet and vascular cell biology. Arterioscler Thromb Vasc Biol 2003;23(6):953–964.
44 van Mourik JA, Boertjes R, Huisveld IA et al. von Willebrand factor propeptide in vascular disorders: A tool to distinguish between acute and chronic endothelial cell perturbation. Blood 1999;94:179–185.
45 Levenberg S, Golub JS, Amit M et al. Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2002;99:4391–4396.
46 Behfar A, Perez-Terzic C, Faustino RS et al. Cardiopoietic programming of embryonic stem cells for tumor-free heart repair. J Exp Med 2007;204:405–420.

47 Wang L, Li L, Shojaei F et al. Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. Immunity 2004;21:31–41.
48 Wang ZZ, Au P, Chen T et al. Endothelial cells derived from human embryonic stem cells form durable blood vessels in vivo. Nat Biotechnol 2007;25:317–318.
49 Jezierski A, Swedani A, Wang L. Development of hematopoietic and endothelial cells from human embryonic stem cells: Lessons from the studies using mouse as a model. ScientificWorldJournal 2007;7: 1950–1964.
50 Yamahara K, Sone M, Itoh H et al. Augmentation of neovascularizaiton in hindlimb ischemia by combined transplantation of human embryonic stem cells-derived endothelial and mural cells. PLoS One 2008;3(2):e1666.
51 Vodyanik MA, Bork JA, Thomson JA et al. Human embryonic stem cell-derived CD34+ cells: Efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. Blood 2005;105:617–626.
52 Gerecht-Nir SZA, Cohen S, Itskovitz-Eldor J. Human embryonic stem cells as an in vitro model for human vascular development and the induction of vascular differentiation. Lab Invest 2003;83(12): 1811–1820.
53 Li Z, Suzuki Y, Huang M et al. Comparison of reporter gene and iron particle labeling for tracking fate of human embryonic stem cells and differentiated endothelial cells in living subjects. Stem Cells 2008;26: 864–873.
54 Al-Shahrour F, Carbonell J, Minguez P et al. Babelomics: Advanced functional profiling of transcriptomics, proteomics and genomics experiments. Nucleic Acids Res 2008;36:W341–346.
55 Morisada T, Kubota Y, Urano T et al. Angiopoietins and angiopoietin-like proteins in angiogenesis. Endothelium 2006;13:71–79.
56 Gealekman O, Burkart A, Chouinard M et al. Enhanced angiogenesis in obesity and in response to PPARgamma activators through adipocyte VEGF and ANGPTL4 production. Am J Physiol Endocrinol Metab 2008;295:E1056–1064.
57 Lee EC, Desai U, Gololobov G et al. Identification of a New Functional Domain in Angiopoietin-like 3 (ANGPTL3) and Angiopoietin-like 4 (ANGPTL4) Involved in Binding and Inhibition of Lipoprotein Lipase (LPL). J Biol Chem 2009;284:13735–13745.


See www.StemCells.com for supporting information available online.