Vascular Gene Expression and Phenotypic Correlation during Differentiation of Human Embryonic Stem Cells

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Abstract

The study of the cascade of events of induction and sequential gene activation that takes place during human embryonic development is hindered by the unavailability of post-implantation embryos at different stages of development. Spontaneous differentiation of human embryonic stem cells (hESCs) can occur via the formation of embryoid bodies (EBs) which resemble, certain aspects of early embryos to some extent. Embryonic vascular formation, vasculogenesis, is a sequential process which involves complex regulatory cascades. In this study, changes of gene expression along the development of human EBs for four weeks were studied by large-scale gene screening. Two main clusters were identified - one of downregulated genes such as *POU5, NANOG, TDGF1/Cripto, LIN28, CD24, TERF1, LEFTB,* and a second of upregulated genes such as *TWIST, WNT5A, WT1, AFP, ALB, NCAM1*. Focusing on the vascular system development, genes known to be involved in vasculogenesis and angiogenesis were explored. Upregulated genes include vasculogenic growth factors such as *VEGFA, VEGFC, FIGF (VEGFD), ANG1, ANG2*, *TGFβ3* and *PDGFB*, as well as the related receptors *FLT1, FLT4, PDGFRB, TGFβR2*, and *TGFβR3*, other markers such as *CD34, VCAM1, PECAM1, VE-CAD,* and transcription factors *TAL1*, *GATA2* and *GATA3*. The reproducibility of the array data was verified independently, and illustrated that many genes known to be involved in vascular development are activated during the differentiation of hESCs in culture. Hence, the analysis of the vascular system can be extended to other differentiation

pathways, allocating human EBs as an *in vitro* model to study early human development.

Introduction

The developmental processes which take place during gastrulation and early organogenesis in vertebrates establish the basic body plan and the subsequent pattern of development. The analysis of these processes in humans has been hindered by the limited availability of human post-implantation embryos at different stages of development. Recent cloning of human embryonic stem cells (hESCs) allows the experimental approach to some of these processes *in vitro*. HESCs are pluripotent cells derived from the inner cell mass of embryos at the blastocyst stage. They can be induced to enter a course of differentiation *in vitro* through the formation of embryoid bodies (EBs), in which they differentiate as tissue-like spheroids in suspension culture (Itskovitz-Eldor et al., 2000). Human EB differentiation has been shown to recapitulate aspects of early embryogenesis in sequential stages (Itskovitz-Eldor et al., 2000; Dang et al., 2004). 48 hours after initiation of cellular aggregation, human EBs complete agglomeration (Dang et al., 2004) which results in the formation of simple EBs by day 3, followed by cavitated EBs after 7-10 days and cystic EBs after two weeks of differentiation (Itskovitz-Eldor et al., 2000). Consequently, during human EB development, the formation of a complex three-dimensional architecture takes place, where cell-to-cell and cell-to-matrix interactions are thought to support the development of the three embryonic germ layers and their derivatives (Itskovitz-Eldor et al., 2000). Thus, differentiation of hESCs in suspension culture provides a powerful *in vitro* system for studying early lineage commitment during mammalian development.

Since the means to study human vasculogenesis and embryonic angiogenesis are limited, the mechanisms involved in their regulation remain unclear. Hence, hESCs may provide a tool to explore genetic and molecular events that take place during early stages of human vascular development. Previous studies demonstrated the successive appearance of some specific vascular markers during the development of human EBs (Levenberg et al., 2002; Gerecht-Nir et al., 2003). Such markers included endothelial cell (EC) adhesion molecules PECAM1/CD31, vascular endothelial-cadherin (VE-cad) and CD34, as well as transcription factors GATA-2 and GATA-3 (Levenberg et al., 2002). Moreover, as in the developing human embryo, vascular structures and networks could be detected in the developing human EBs by both CD34 and PECAM1 (Gerecht-Nir et al., 2003). Early vascular smooth muscle cell (v-SMC) markers include alpha smooth muscle actin (α-SMA), calponin and smooth muscle myosin heavy chain (SM-MHC), which were found to be involved in thick tubes and voids but rarely in the formation of network-structures (Gerecht-Nir et al., 2003).

The present study aimed at the analysis of gene expression along the development of human EBs with a focus on vascular system differentiation. Microarray hybridization and clustering analysis revealed two main trends along the development of the human EBs: down-regulation of pluripotency and self-renewal genes, and up-regulation of germ layers generating genes. Focusing on the vascular system development, kinetic pattern of specific markers for ECs and v-SMCs were demonstrated and verified. Altogether, human EBs were found to

recapitulate early changes in gene expression that occur during human vasculature development and these may serve as a useful tool for exploring the mechanism of early development.

Results

Global gene expression upon hESC differentiation to EB

To assess the progression of gene expression with differentiation, we studied a total of 12 samples - three of each culture time point: undifferentiated hESCs, 1 week-old EBs, 2 week-old EBs and 4 week-old EBs. Gene expression was measured by hybridization to Affymetrix U133A DNA microarrays, containing 22,215 probe sets (PS). 13461 PS with at least one “present” call, obtained from MAS 5.0, were selected, expression levels <30 were thresholded to 30, and $\log_2$ was taken to generate the final gene expression matrix. The genes were filtered using *ANOVA* (Sokal & Rohlf, 1995). False discovery rate (FDR) (Benjamini, 1995) was controlled at 0.10 to overcome the multiplicity problem. This left 2574 PS (2308 genes) that varied significantly over the four culture time points.

In exploring gene expression kinetic changes along the development of human EBs, it was noticed that the number of differentially regulating genes increased along the development of human EBs as compared to hESCs (data not shown).

In order to identify distinct differentiation-induced variations of the expression profiles, the clustering method (Blatt et al., 1996) was used to assign genes to clusters of similar patterns of expression. Fig. 1 shows the expression matrix after clustering of the genes along the development of human EBs. Two main expression patterns could be identified: cluster 1, which contains genes with high

expression in hESCs that were down-regulated along the development of the human EBs, and cluster 2 which contain genes that were up-regulated along the human EB development.

Cluster 1 contains many previously identified genes related to the undifferentiated state of hESCs which take part in signal transduction pathways and are known to play a key role in early embryogenesis and cell fate decisions (Sato et al., 2003) such as:

1) FGF pathway - including the ligand *FGF13*, the receptors *FGFR1 FGFR4*, and other regulatory molecules such as Sprouty2,

2) The TGFβ/BMP pathway - including the ligand *GDF3*, the receptors *BMPR1A* and *TDGF1*/cripto, secreted inhibitor *LEFTB*, and related molecules such as *TGFβ*-induced factor,

3) The Wnt pathway – including the receptor *FZD7*, the transcription factor TCF-3 and related molecules such as *FRAT2*.

In order to identify the genes that show the greatest changes in expression during development of human EBs, both clusters were ordered according to the fold change relative to 4 weeks, and genes that were above 5-fold change were considered. In cluster 1, 88 genes were identified (selected genes are shown in Table 1, full list available in supplementary data Table 1) and included genes relevant to pluripotency and self-renewal genes reported to be associated with the undifferentiated hESCs such as: *POU5 (OCT4)*, a transcription factor that is expressed in ESC and down-regulated upon differentiation (Niwa et al., 2000), *NANOG,* a homeobox transcription factor involved in pluripotency and

suppression of differentiation (Chambers et al., 2003; Mitusi et al., 2003), *LIN28* and *CD24*, both of which were recently shown to be highly expressed in different hESC lines (Bhattacharya et al., 2003), Galanin, a 30 amino acid peptide associated with LIF and *STAT* signaling, is highly expressed in ESCs and has been suggested to be involved in ES biology (Tarasov et al., 2002) , and *TERF1*, a telomeric DNA binding factor involved in the regulation of telomerase-dependent telomere length maintenance (Iwasno et al., 2004).

In cluster 2, 139 genes were identified as the most up-regulated after four weeks of human EB developments (selected genes are shown in Table 2, full list available in supplementary data Table 2) and contain genes associated with early stages of development of the three germ layers, such as: *TWIST,* a basic-helix loop-helix protein that plays a central role in cell type determination and differentiation and is required for the development of normal mammalian embryos (Rose & Malcolm, 1997), *WNT5A* which is known to play an essential role in various developmental processes (Yamaguchi et al., 1999), *WIF1*, a secreted protein that binds to *WNT* proteins and inhibits their activities, presumably to fine-tune the spatial and temporal patterns of *WNT* activity (Hsieh et al., 1999), *PITX1* , a homeobox protein, which plays a wide role during early development, in agreement with its early expression in the posterior lateral plate mesoderm and the stomodeum (Lanctot et al., 1999), and *WT1*, a germ cell regulator (Natoli et al., 2004). Key growth factors were also observed, such as mesodermal inductive factor *TGFβ1* (Schuldiner et al., 2000) and endodermal inducing growth factors *IGF1* and *HGF* (Schuldiner et al., 2000). In addition,

extracellular matrix genes, such as Reelin, a large extracellular matrix type protein, which have an important role in neuronal migration (Kubasak et al., 2004), as well as different types of collagen, such as *COL3A1, COL6A3, COL15A1*, were also detected. Among cellular proteins observed there was the neuronal adhesion molecule *NCAM1*, the early neural crest specific gene snail2 (Thisse et al., 1995), Lumican (osteoblasts; Raouf & Seth, 2002), *AFP*, and *ALB* (hepatocytes; Schuldiner et al., 2000).

Vascular system gene expression: large-scale screening

We wished to know whether human EBs could be used as a model to study early gene expression for vascular development. Several genes involved in the development of ECs were found in cluster 2, including *PECAM1*, an early marker of developing human vasculature (Oberlin et al., 2002), *VCAM1*, an endothelial adhesion molecule (Bagley et al., 2003), and the growth factors *VEGF* and *ANG1* that are known to take part in early vasculogenesis (Suri et al., 1996; 1998). Transcription factors involved in the development of both ECs and hematopoietic precursor cells were also observed and included *TAL1*, a basic helix-loop-helix protein, and *LMO2*, a *LIM-Zn* finger protein, both of which are expressed in early haematopoietic and endothelial progenitors and interact with each other during the development (Gering et al., 2003). Only a few developmental-related markers for v-SMCs could be detected, including the mesodermal regulator *TGFβ1* (Schuldiner et al., 2000) and its receptors *TGFβR2* and *TGFβR3*. No genes

related to the development of the vascular system could be observed within the 139 most down-regulated genes of cluster 2.

In order to analyse the expression kinetics of vascular related genes along the development of human EBs, we calculated the fold change of expression in hESCs compared with their expression in human EBs. A wide range of vascular related genes were examined and those that showed increased expression of at least 2-fold along human EB development were included (Table 3). Specific EC and EC/hematopoietic markers which were up-regulated along the development of human EB included *PECAM1, CD34* (Oberlin et al., 2002), *VCAM1*, *VE-CAD* (Bach et al., 1998; Hur et al., 2004), *CD41* (Mikkola et al., 2003; Ferkowicz et al., 2003), *CD45* (Mikkola et al., 2003; Ferkowicz et al., 2003), and transcription factors *TAL1, LMO2, GATA1, GATA2*, and *GATA3*, although up-regulation of the expression of *VEGFR2* (*KDR*), well-established marker for vascular progenitors derived from mouse ESCs (Yamashita et al., 2000), could not be detected. Up-regulation in the expression of early v-*SMC* markers like *LMOD1* (Conley, 2001) and *MYH11*- smooth muscle myosin heavy chain (SM-MHC; Gittenberger-de Groot et al., 1999) was also observed.

Using this large-scale gene expression screening, up-regulation of cytokines known to be involved during vasculogenesis setting and remodelling was detected and included *VEGFA, VEGFC, FIGF (VEGFD), ANG1, ANG2, TGFβ3* and *PDGFB* and some related receptors *FLT1, FLT4, PDGFRB, TGFβR2,* and *TGFβR3*. Other angiogenic growth factors, such as *VEGFB, PIGF,* and *TGFβ1*, were not detected to be up-regulated during human EBs development.

Furthermore, *bFGF*, although a known angiogenic factor, showed down-regulation along the development of human EBs, indicative of its role in the undifferentiated state of hESCs (Amit et al., 2000).

Fig. 2 summarizes the expression kinetics of these genes. Expression profiles of most of the vascular genes were found to be similar with continuous up-regulation along the development of human EBs. The expression of *ANG2* and *GATA3* peaked after one week of differentiation and decreased along human EB development. There was a peak in the expression of both TAL1 and VEGF in the first week, followed by a slight decrease in expression (after two weeks) that increased towards the fourth week of development. The expression of *LMOD1, VE-CAD*, *LMO2, FIGF, ANG1, VEGFC* and, to some extent, CD34 peaked after two weeks of differentiation (Fig. 2).

Vascular system gene expression: single gene/protein event

In order to verify gene expression, semi-quantitative RT-PCR was performed for several vascular genes (Fig. 3A). The expression of *VEGFR2* in both hESC and EBs was detected corresponding to the array results which could not detect any change in expression along the developing human EBs. As was evident by the array assay, up-regulation kinetics of the expression of *PECAM1* and *CD34,* and down-regulation in the expression of *bFGF* along the development of human EBs were detected by the semi-quantitative RT-PCR. However, *VWF*, which showed up-regulation less than 2-fold and could not be detected by the array, was found to up-regulated during human EB development using semi-quantitative RT-PCR.

To verify the array kinetic expression along the developing human EBs, real-time quantitative RT-PCR was also performed. *PECAM1* showed up-regulation along the developing human EBs, whereas *CD34* expression peaked after two weeks of human EB development (Fig. 3Bi). The pattern of expression of both of these genes was found to be the same as that demonstrated by the array and semi-quantitive RT-PCR. The expression of *SMA* in both hESC and EBs was found to correspond to the array results which could not detect any change in expression along the developing human EBs (Fig. 3Bii). *ANG2*, *VEGFC*, and *VEGFR3* showed to be up-regulated in the same manner as demonstrated in the array (Fig. 3Biii). *However, TIE2*, which showed up-regulation less than 2-fold and could not be detected by the array, was detected to vary along the EB development using real-time quantitative RT-PCR, peaking after one week of differentiation (Fig. 3Biii). The magnitude of fold difference detected by real-time RT-PCR tended to be greater than those found by the microarrays, most likely due to greater sensitivity of PCR (also demonstrated by Georgantas et al., 2004)

VE-CAD expression pattern was further verified using immunoblot assay which showed peaking after two weeks of human EB culture (Fig 4A). However, unlike the array, immunoblot assay detected up-regulation in calponin expression along the development of human EBs (Fig. 4A).

Histology sections revealed that both *CD34*+ and *PECAM1*+ cells generated tube-like structures within 2w-old human EBs (Figs. 4B-D). The diameter of these tubes was found to range from 10-350 μm. Confocal examination revealed depth

organization of vessel-like structures formed by *PECAM1*$^{+}$ cells within human EBs as well as void formation by SM-MHC (Fig. 4E-G).

In order to examine the integrity and complexity of the vasculature formed within the developing human EBs along the culture period, a time-course confocal analysis was performed. As PECAM1 expression was found to up-regulate along the 4 weeks while CD34 expression was found to peak after 2 weeks, we examined both of these proteins. Fig. 5A demonstrated vessel-like structures at different time-points of developing human EBs. After one week of differentiation, clear tube-like structures were found to be formed by both CD34+ and PECAM1+ cells. After 2 weeks, both CD34+ and PECAM1+ cells continued and formed complex vessel-like structures. However, after 4 weeks of development, PECAM1+ vasculature structures could be obtained, and a decrease in the organization of CD34+ cells was detected. In addition, clusters of CD34+ cells were frequently obtained within 4 week developing human EBs (Fig. 5B).

Discussion

Beginning at the time of implantation and throughout gastrulation, early mammalian development is characterized by rapid cellular proliferation and migration that placed in the endoderm, ectoderm and mesoderm at their respective positions for organogenesis. The derivation of hESC lines opened many experimental approaches to the study of early human development. This study demonstrated the use of large-scale screening of gene expression in the exploration of gene regulation along the differentiation of human EBs. It has

shown that global gene regulation increases consistently along the 4-week development of human EBs. The alteration in gene expression in the human EB system was found to be superior compared to that previously shown using adherent differentiation system of hESCs (Sato et al., 2003). These results provide genomic verification that EB formation is accompanied by efficient differentiation of human ESCs. Overall gene patterning reveals two main clusters of gene expression. Cluster 1 contains genes involved in the undifferentiated state of hESCs, their pluripotency and self-renewal capabilities that are down-regulated. Cluster 2 contains genes involved in the early stages of human development throughout the generation of the three germ layers which are up-regulated.

In our study, vascular differentiation was explored in particular, using large-scale gene screening. Cluster 2 was found to contain key markers of EC and EC/hematopoietic progenitors (including *PECAM1, VCAM1, TAL1* and *LMO2*), as well as key cytokines known to participate in the early stages of vascular development in mammals (including *VEGF*, *Ang1, TGFβ1*). In some cases, the analysis of fold-change of gene expression in the clusters was found to be more sensitive and revealed wide-ranging changes in vasculogenic genes along human EB development. Most of these genes were found to have a tendency of increasing expression throughout the four weeks, while a few showed peaking in the first or second week of development. Previous studies demonstrated an increase in expression of several EC genes during human EB differentiation, reaching a maximum at 13–15-day-old human EBs by means of semi-

quantitative RT-PCR (Levenberg er al., 2002). Correspondingly, using microarray regulation, these genes either peaked after two weeks of differentiation or remained constant along human EB development. The large-scale gene screening used in the present study demonstrates the importance of a genome-wide gene expression study to analyze the mechanisms underlying human vascular development. Furthermore, deviations in patterns of gene expression during vasculogenesis within the developing human EBs indicated complex regulatory cascades and autoregulatory loops of coordinated signaling during vasculature early development. For example, it appears that VEGF and ANG1 play coordinated and complementary roles during embryonic development, with VEGF required early in development and Ang-1 required for vascular remodeling and sprouting (Suri et al., 1996; 1998). The pattern of expression found for both of these growth factors corresponded to embryonic expression kinetics in which *VEGF* peaked in the first week of differentiation while *ANG1* peaked only in the second week of human EB development. From the point of hematopoietic/EC intersection, *TAL1*, which is a marker for both endothelial and hematopoietic progenitors (Ema et al., 2003), peaks after one week of development whereas *LMO2*, one of the earliest markers for commitment (Minko et al., 2003), peaks after two weeks of differentiation. The expression of more cell commitment markers, such as *CD41, CD45* and *GATA1,* continues to increase along the four weeks of EB development. Another example is the up-regulation of *VEGFC*, which was recently shown to activate *VEGFR3* for the sprouting of lymphatic vessels (Karkkainen et al., 2003). In humans, the lymphatic vessels appear later

than the blood vessels of the arterial and venous system (Wilting et al., 1999). Both VEGFC and VEGFR3 were up-regulated only in the second week of developing human EBs while vasculogenesis initiating factors were up-regulated in the first week of differentiation.

Examination of vasculature integrity and complexity along the culture period of human EBs demonstrated correlation with array data. Cells expressing PECAM1 showed continuous increase in complexity while cells expressing CD34 form vessel-like structures whose organization decreased after 4 weeks of development and were replaced by cluster of cells. These results are consistent with the findings that both PECAM1 and CD34 are expressed during early human vascular development (Oberlin et al., 2002; Gerecht-Nir et al., 2004), while CD34 expression in adults is maintained mainly by hematopoietic cells (and in low levels in ECs).

Reproducibility of the array data was confirmed independently. However, regulation in the expression of *VWF*, *TIE2*, and calponin could not be detected using array. Considering that ESCs were shown to contain high concentrations of nontranslated mRNAs (Notingher et al., 2004; Golan-Mashiach et al., 2004), and that human EBs are not a homogeneous population, some genes may change more than detectable by the array.

In conclusion, this study shows correlation between changes in gene expression during the differentiation of hESCs to EBs in culture and known changes during embryonic development, suggesting that human EBs recapitulate many aspects of embryonic differentiation. Hence, analysis of the vascular system can be

extended to other differentiation pathways, allocating human EBs as a model for the study of early stages of human development.

Experimental Procedures

HESC culture and EB formation

Non-differentiating hESC lines H9.2 were grown as previously described (Gerecht-Nir et al., 2003). In brief, the cells were grown on mouse embryonic fibroblasts and passaged every four to six days using 1 mg/ml type IV collagenase (Gibco Invitrogen Co., San Diego CA). For EB formation, a half confluent six-well plate (60 cm^2) of undifferentiated hESC was used. hESCs were removed from the feeder layers using 1 mg/ml type IV collagenase, further dissociated into small clumps using 1000 μl Gilson pipette tips, and cultured in suspension in 50 mm non-adherent Petri dishes (Ein-Shemer, Israel). EBs were grown in medium consisting of 80% KO-DMEM (Gibco Invitrogen Co.), supplemented with 20% defined fetal bovine serum (FBSd; HyClone, Utah), 1 mM L-glutamine, and 1% non-essential amino acid stock (all from Gibco Invitrogen Co.). For analysis hESC were separated from the feeder layer by type IV collagenase treatment followed by microscopic inspection for the absence of contamination by feeder cells. Human EBs were harvested after 1 week (1W), 2 weeks (2W) and 4 weeks (4W) of cultivation.

RNA extractions and microarray

Cell samples were collected from undifferentiated hESCs, 1W-, 2W- and 4W-human EBs. For microarray hybridization, three samples were collected from each differentiation stage (n=12). For RT-PCR and real-time quantitative RT-PCR, two samples were collected from each differentiation stage (n=6). RNA extractions were performed by total RNA isolation reagent TRIReagent (Gibco Invitrogen Co.), following the manufacturer's protocol, and RNA integrity was assessed by gel electrophoresis. 10 μg total RNA was used to prepare biotinylated target cRNA, used to hybridize HG-U133A GeneChip® according to Affymetrix™ procedure (Affymetrix, Santa Clara, CA). Microarrays were scanned by Affymetrix™ scanner.

Bioinformatics

All microarray data are MIAME compliant. For probe-level data analysis of the raw expression levels, the MAS 5.0 Affymetrix array analysis software was used. 13461 probe sets (PS) with at least one "present" call were selected. Expression levels <30 were set to 30 and $\log_2$ was taken. For clustering analysis, the PS were filtered out on the basis of the variance of their $\log_2$ expression values; we used *ANOVA* to calculate for each PS the P-value for significant variation *between* four kinds of samples, first for ESC, 1WEB, 2WEB and 4WEB. For clustering analysis, the Super Paramagnetic Clustering method (Blatt et al., 1996) was used to cluster the data. Prior to clustering, the expression levels for

each PS were centered and normalized. Fold change of gene expression was calculated to determine the genes that were up-regulated along human EB development.

Reverse Transcription (RT)-PCR Analysis

Total RNA was extracted using TriReagent (Gibco Invitrogen Co.), according to manufacturer's instructions. Total RNA was quantified by a UV spectrophotometer and the samples were negated for DNA contamination. RNA (1 μg per sample) was reversed transcriptase with M-MLV (Promega Co., Madison WI) and oligo (dT) primers (Promega Co., Madison WI) according to manufacturer's instructions. PCRs were performed with BIOTAQ™ DNA Polymerase (Bioline Ltd., London UK) using 1 μl RT product per reaction, according to manufacturer's instructions. To ensure semi-quantitative results of the RT-PCR assays, the number of PCR cycles for each set of primers was verified to be in the linear range of the amplification. In addition, all RNA samples were adjusted to yield equal amplification of GAPDH as an internal standard. RT reaction mix was used for negative controls. PCR conditions consisted of 5 min at 94ºC (hot start), 30-40 cycles (actual number noted below) of 94ºC for 30 sec, annealing temperature (*Ta*, noted below) for 30 sec, 72ºC for 30 sec. A final 7 min extension at 72ºC was performed at the end. The amplified products were separated on 2% agarose gels with ethidium bromide.

The following specific oligonucleotide primers were used:

PECAM1: 5'CAACGAGAAAATGTCAGA3' and

5’GGAGCCTTCCGTTCTAGAGT3’ (*Ta* 60°C; 30 cycles ; 260bp);

CD34: 5’TGAAGCCTAGCCTGTCACCT3’ and

5’CGCACAGCTGGAGGTCTTAT3’ (*Ta* 60°C, 30 cycles; 200bp);

VEGFR2: 5’CTGGCATGGTCTTCTGTGAAGCA3’ and

5’AATACCAGTGGATGTGATGGCGG3’ (*Ta* 60°C, 26 cycles; 790bp);

bFGF: 5'GCCACTTCAAGGACCCCAAG3' and

5'TCAGCTCTTAGCAGACATTGG3' (*Ta* 60°C, 32 cycles; 397bp);

VWF: 5’ATGTTGTGGGAGATGTTTGC3’ and 5’GCAGATAAGAGCTCAGCCTT3’ (*Ta* 55°C, 33 cycles; 656bp)

GAPDH: 5’AGCCACATCGCTCAGACACC3’ and

5’GTACTCAGCGGCCAGCATCG3’ (*Ta* 60°C, 27 cycles; 302bp).

Real-time quantitative RT-PCR

Two step RT-PCR was performed on undifferentiated hESCs, 1W-, 2W-, and 4W- EBs. First strand cDNA was synthesized as described above. TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression Probes (Applied Biosystems, Foster City CA) for *ANG2, TIE2, VEGFC, VEGFR3, PECAM1, CD34, SMA,* and β-actin were used according to manufacturer's instructions. TaqMan PCR step was done with an Applied Biosystem 7000 DNA Sequence Detection System (Applied Biosystems) according to manufacturer's instructions. The relative expression of*, ANG2, TIE2 VEGFC,* and *VEGFR3* was normalized to the amount of β-actin in the same cDNA by using the standard curve method described by the manufacturer. The relative standard curve

method (Applied Biosystems) was used to calculate amplification differences between undifferentiated hESCs and 1W-, 2W- or 4W human EBs, for each primer set. The values for three experiments were averaged and graphed with SD.

Immunoblot analysis

Total protein was measured after lysis of the samples (hESCs, human EBs, and rat uterus which served as a positive control) by the Bradford Protein assay (BioRad, Hercules CA) according to manufacturer's instructions. For western analysis, samples were separated on SDS/PAGE on 4-20% gradient SDS-PAGE gels (Gradipore, Frenchs Forest, Australia) and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After 1 hour blocking with 1xTBS, 0.1% Tween-20, 5% non-fat milk, membranes were incubated with mouse anti-human calponin (both from Dako, Denmark) or goat anti-human VE-cad (Santa Cruz Biotechnology, Santa Cruz CA) or mouse anti-human actin (Chemicon Int., Temecula, CA) . Membranes were then rinsed and incubated for 30 min with suitable peroxidase-conjugated secondary antibody (Dako, Denmark). Detection was performed using the ECL Western blotting analysis system (Amersham Pharmacia Biotech, Piscataway NJ) and visualized using ImageMaster VDS-CL (Amersham Pharmacia Biotech, Bucks, England).

Confocal microscopy

Human EBs were seeded overnight to 48 hours on slides pre-coated with gelatine and fixed *in situ* with 4% paraformaldehyde (Sigma) in PBS (Gibco-BRL)

for 30 min at room temperature. After blocking with 3% serum, the cells were stained with one of the following primary antibodies: anti-*human PECAM1,* and anti-human anti-SM-MHC, anti-*human CD34* (all from Dako). Cells were then rinsed and incubated for 30 min with a suitable Cy3-conjugated secondary antibody (Sigma). For nuclei visualization, To-Pro 3 (Molecular Probes) was added (1:500) to the last rinse. IgG isotype-matching served as control, including mouse, goat and rabbit (all from R&D systems). The immuno-labeled cells were examined using a confocal laser scanning system (BioRad Laboratories Ltd.).

Immunohistochemistry

For histological analyses, the 2W-old human EBs were fixed in 10% neutral-buffered formalin, dehydrated in graduated alcohols (70-100%), and embedded in paraffin for routine histology. 6-8 μm sections were stained with hematoxylin/eosin. Immunostaining was performed using a Dako LSAB®+ staining kit with specific anti-human PECAM1 and CD34 (both from Dako, Denmark). Mouse IgG isotype-matching (R&D systems, Minneapolis MN, USA) or secondary antibody alone (from Dako LSAB®+ staining kit) served as controls.

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Table 1: *Selected genes that showed down-regulation of expression (cluster1) during development of human EBs in culture.* Gene expression in undifferentiated human embryonic stem cells (ESCs), 1 week-old human EBs (1wEBs), 2 week-old human EBs (2wEBs), 4 week-old human EBs (4wEBs), and fold change relative to 4 weeks of development (ESCs/4wEBs) is presented.

Symbol	Title	ESCs	1wEBs	2wEBs	4wEBs	ESCs/4wEBs
GAL	Galanin	796.93	107.50	13.23	7.93	100.45
NANOG	NANOG	394.10	70.63	6.20	5.37	73.43
TDGF1	teratocarcinoma-derived growth factor 1	1494.87	326.20	118.07	45.17	33.10
POU5F1	Oct-04	1319.00	279.63	68.50	60.47	21.81
LIN-28	RNA-binding protein	1759.90	1663.23	662.87	99.57	17.68
ALPPL2	alkaline phosphatase, placental-like 2	125.00	157.57	27.03	8.63	14.48
LEFTB	left-right determination, factor B	749.30	59.80	69.43	51.83	14.46
FOXH1	forkhead box H1	248.93	162.17	53.63	24.10	10.33
CD24	CD24 antigen	1486.67	847.03	173.03	155.47	9.56
TERF1	telomeric repeat binding factor 1	722.90	295.40	136.07	90.23	8.01
COL4A1	collagen, type IV, alpha 1	198.30	221.13	101.33	26.47	7.49
EPHA1	EphA1	192.10	93.53	25.50	25.73	7.47
FGFR4	fibroblast growth factor receptor 4	54.60	71.13	24.93	7.57	7.22
GDF3	growth differentiation factor 3	207.57	52.57	20.20	39.90	5.20
FGFR1	fibroblast growth factor receptor 1	640.07	579.07	186.47	126.23	5.07

Table 2: *Selected genes that showed up-regulation of expression (cluster2) during development of human EBs in culture.* Gene expression in undifferentiated human embryonic stem cells (ESCs), 1 week-old human EBs (1wEBs), 2 week-old human EBs (2wEBs), 4 week-old human EBs (4wEBs), and fold change relative to 4 weeks of development (4wEBs/ ESCs) is presented.

Symbol	Title	ESCs	1wEBs	2wEBs	4wEBs	4wEBs/ESCs
IGF2	insulin-like growth factor 2	5.43	55.17	392.40	405.77	74.68
IGFBP3	insulin-like growth factor binding protein 3	50.27	177.70	1949.97	3140.23	62.47
DKK2	dickkopf homolog 2	2.43	3.87	13.67	148.57	61.05
WT1	Wilms tumor 1	2.40	6.30	78.10	114.67	47.78
IGF1	insulin-like growth factor 1	4.40	28.63	45.73	127.47	28.97
WNT6	wingless-type MMTV integration site	9.37	19.70	57.60	214.13	22.86
TGFBI	transforming growth factor, beta-nduced	87.63	337.77	969.23	1988.83	22.69
OSF-2	osteoblast specific factor 2	92.03	111.37	2493.13	2075.03	22.55
LUM	lumican	64.57	292.20	1630.23	1104.00	17.10
HGF	hepatocyte growth factor	5.43	11.47	76.87	79.00	14.54
COL3A1	collagen, type III, alpha 1	495.47	1333.73	6872.17	7118.40	14.37
TWIST	twist homolog	44.27	87.50	379.57	513.30	11.60
WIF1	WNT inhibitory factor 1	7.83	8.60	14.50	57.30	7.31
RELN	reelin	40.30	232.70	455.17	262.70	6.52
WNT5A	wingless-type MMTV integration site	26.27	57.53	129.50	158.23	6.02
AFP	alpha-fetoprotein	280.97	1216.13	3130.37	1550.23	5.52
NCAM1	neural cell adhesion molecule 1	148.63	163.77	443.07	796.03	5.36
ALB	albumin	12.17	20.00	49.87	63.60	5.23

Table 3: Genes identified by up-regulation of at least 2 fold during EB development and are known to be important for vascular differentiation.

	Symbol	Title
v-SMC		
1	MYH11	myosin, heavy polypeptide 11, smooth muscle
2	LMOD1	leiomodin 1
3	PDGFB	platelet-derived growth factor
4	PDGFRB	PDGF receptor,
5	TGFB3	transforming growth factor,
6	TGFBR2	TGF receptor II
7	TGFBR3	TGF receptor III
ECs		
1	PECAM1	CD31 antigen
2	VCAM1	vascular cell adhesion molecule 1
3	PCDH12	protocadherin 12
4	CDH5	VE-cadherin
5	VEGF	vascular endothelial growth factor
6	VEGFC	vascular endothelial growth factor C
7	FIGF	VEGF D
8	EPAS1	endothelial PAS domain protein 1
9	FLT1	vascular endothelial growth factor)
10	FLT4	fms-related tyrosine kinase 4
11	ANGPT1	angiopoietin 1
12	ANGPT2	angiopoietin 2
13	GATA2	GATA binding protein 2
14	GATA3	GATA binding protein 3
Hematopoietic/ECs		
1	CD34	CD34 antigen
2	TAL1	T-cell acute lymphocytic leukemia 1
3	BMI1	B lymphoma insertion region
4	LMO2	LIM domain only 2
5	TIE	tyrosine kinase
6	GATA1	GATA binding protein 1
7	RUNX1	runt-related transcription factor 1
8	216966_at	CD41(GpIIb)
9	PTPRC	CD45

Figure Legends

Figure 1: Clustering analysis of the genes that changed expression during EB differentiation. Two main clusters were observed through human EBs development- (A) Cluster 1 **–** expression matrix of down-regulated genes and (B) Cluster 2 **–** expression matrix of up-regulated genes.

Figure 2: Large-scale vascular gene expression kinetics. The relative change in the expression of genes involved in the vascular system was explored and those that showed an increase of at least 2-fold along human EB development were included. Genes are presented according to their relevant roles in (A) v-SMCs, (B) ECs, (C) hematopoietic/ECs, and (D) vasculogenic growth factors.

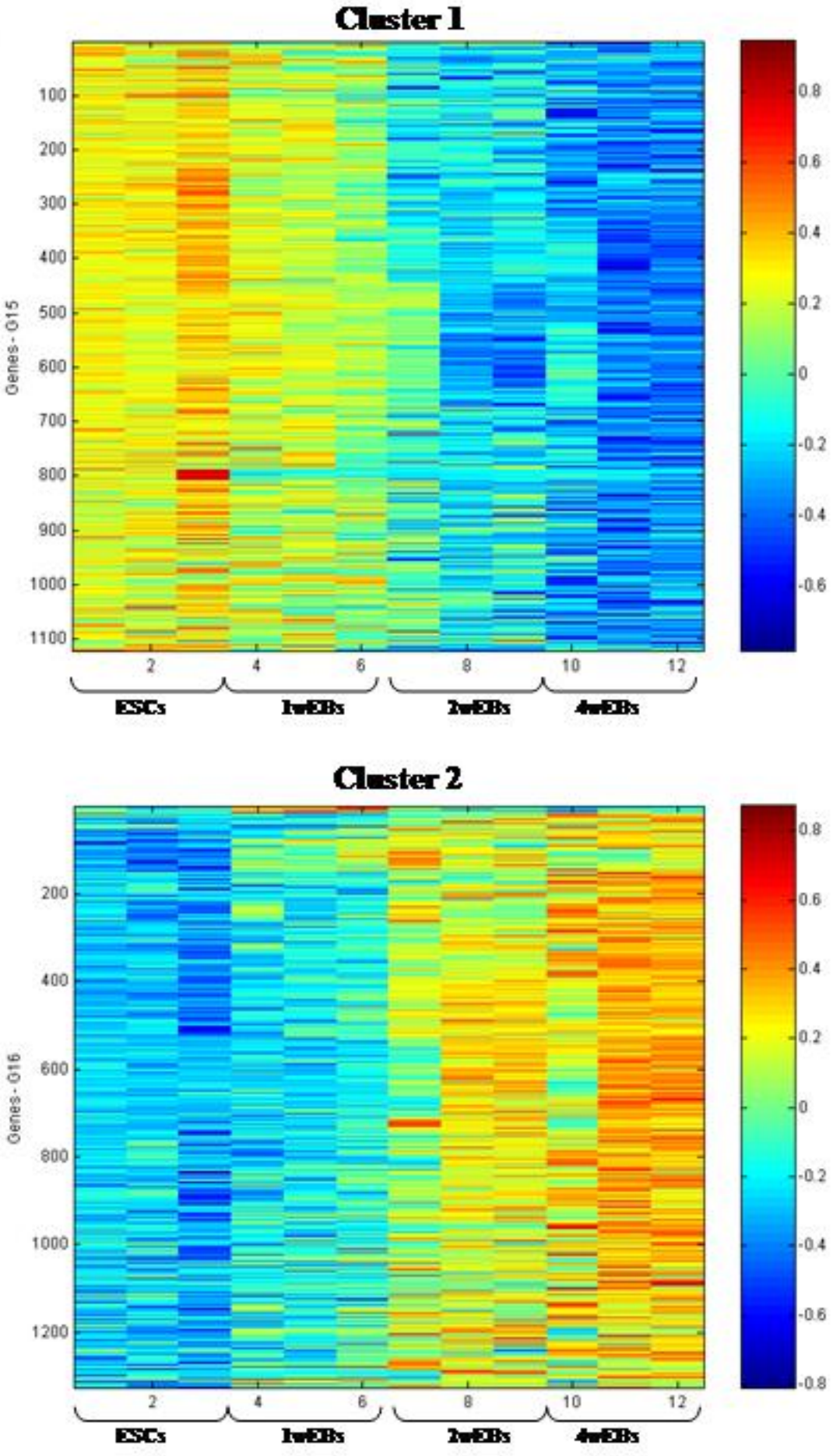
Figure 3: Expression changes of selected vascular genes. The expression of several vascular genes was verify using (A) semi-quantitative RT-PCR in: **1** - hESCs; **2** - 1wEBs; **3** - 2wEBs; **4** - 4wEBs and **5** - control without template, and (B) real-time quantitative RT-PCR for- (i) *PECAM1, CD34, (ii) SMA* (iii) *ANG2*, *TIE2*, *VEGFC*, and V*EGFR3*.

Figure 4: Protein expression of vascular specific markers. The protein expression of several vascular genes was verify using (A) Immunoblot assay in: **1** - hESCs; **2** - 1wEBs; **3** - 2wEBs; and 4 - 4wEBs. Vasculature arrangement within developing human EBs shown by staining of 2W-old human EBs histology

sections with (B) *CD34* and (C) *PECAM1* (D) Isotype control. Confocal microscopy examination further revealed vessel-like arrangement of (E) PECAM1+ cells (red; nuclei in blue) as well as (F) SM-MHC (red; nuclei in blue) (G) isotype control (nuclei in blue) within 2W-old human EBs. Bar = 100 μm.

Figure 5: Vasculature structures along human EB development. (A) low-resolution confocal microscopy analysis of vasculature within different time points of human EB development reveal progress in arrangement of PECAM1+ cells and pick in arrangement of CD34+ cells. (B) After 4 week of human EB growth, CD34+ cells were frequently observed in small clusters.

Figure 1


Cluster 1
Genes - G15
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200
300
400
500
600
700
800
900
1000
1100
2
4
6
8
10
12
ESCs
1wEBs
2wEBs
4wEBs
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
Cluster 2
Genes - G16
200
400
600
800
1000
1200
2
4
6
8
10
12
ESCs
1wEBs
2wEBs
4wEBs
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0.4
0.2
0
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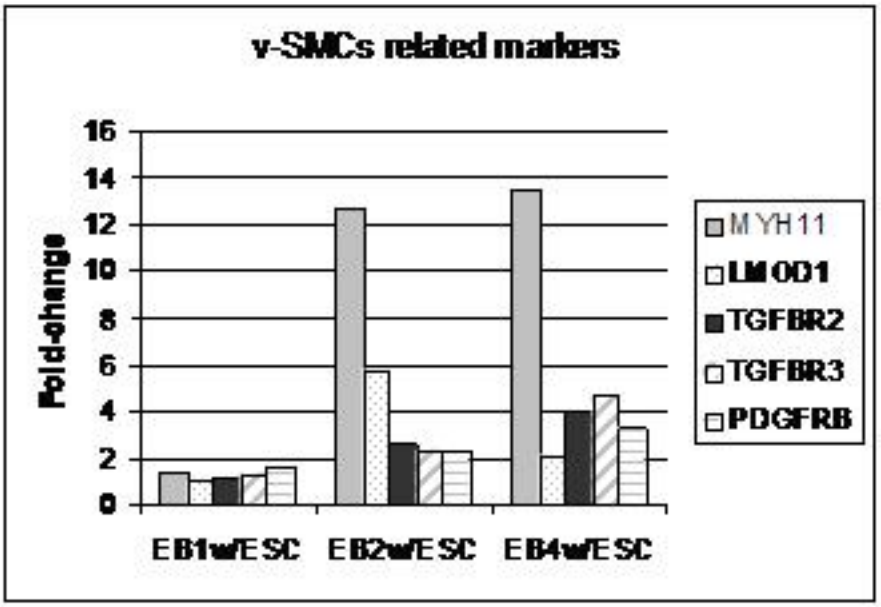
-0.4
-0.6
-0.8

Figure 2

A


v-SMCs related markers
Fold-change
16
14
12
10
8
6
4
2
0
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EB2w/ESC
EB4w/ESC
MYH11
LMOD1
TGFBR2
TGFBR3
PDGFRB

B

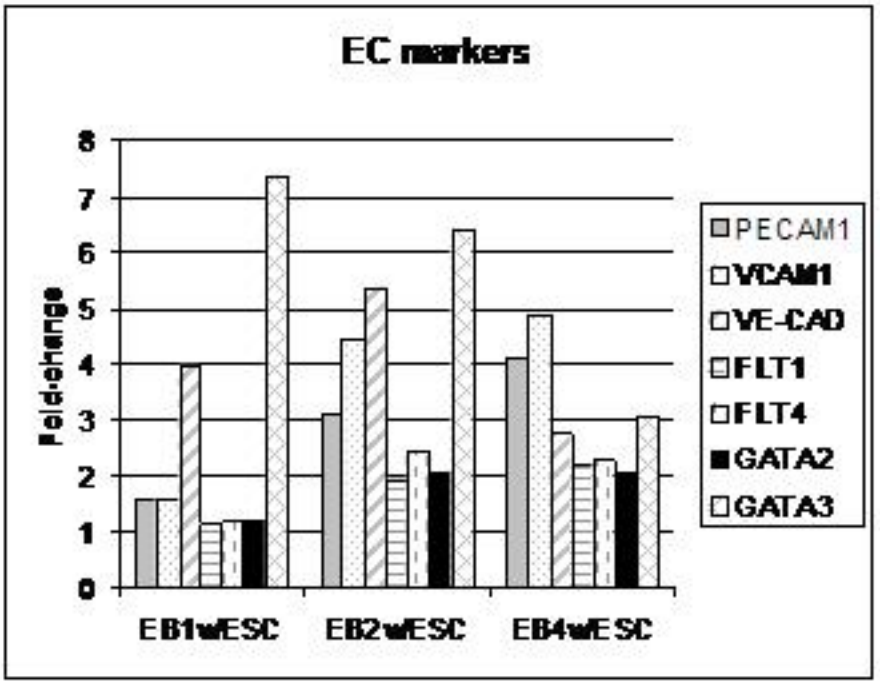
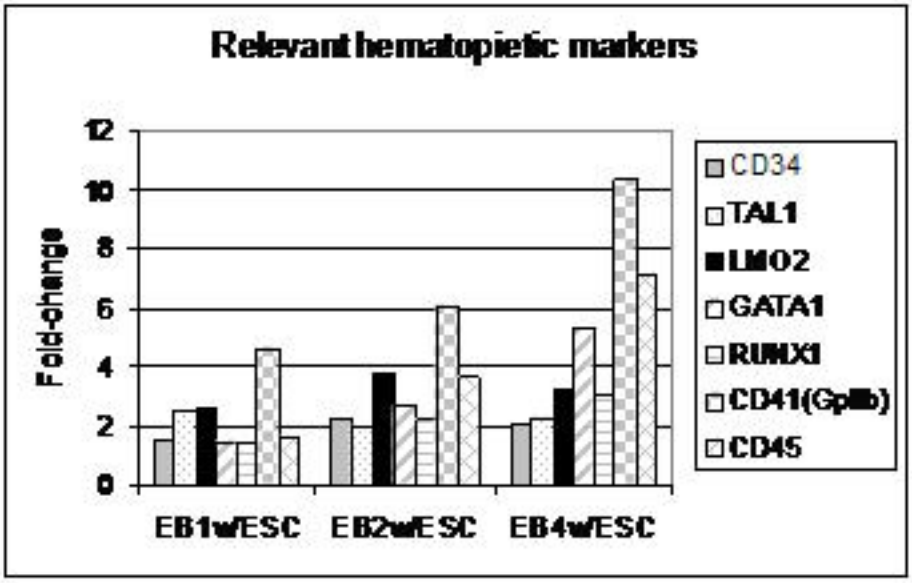
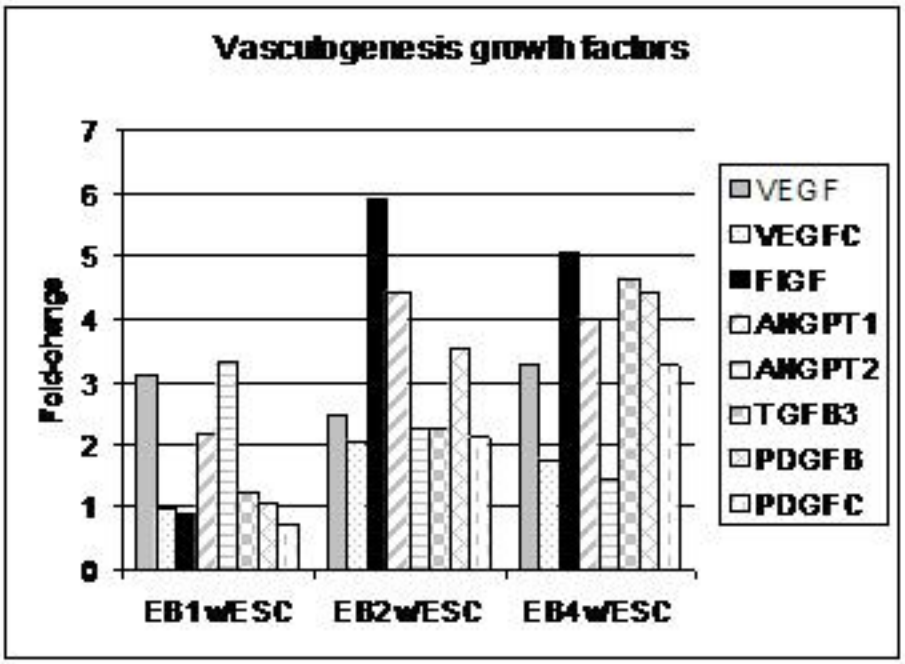

EC markers
Fold-change
8
7
6
5
4
3
2
1
0
EB1w/ESC
EB2w/ESC
EB4w/ESC
PECAM1
VCAM1
VE-CAD
FLT1
FLT4
GATA2
GATA3

Figure 2

C


Relevant hematopietic markers
Fold-change
12
10
8
6
4
2
0
EB1w/ESC
EB2w/ESC
EB4w/ESC
CD34
TAL1
LMO2
GATA1
RUNX1
CD41(GpIIb)
CD45

D


Vasculogenesis growth factors
Fold-change
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6
5
4
3
2
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0
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EB2w/ESC
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VEGF
VEGFC
FIGF
ANGPT1
ANGPT2
TGFB3
PDGFB
PDGFC

Figure 3

A

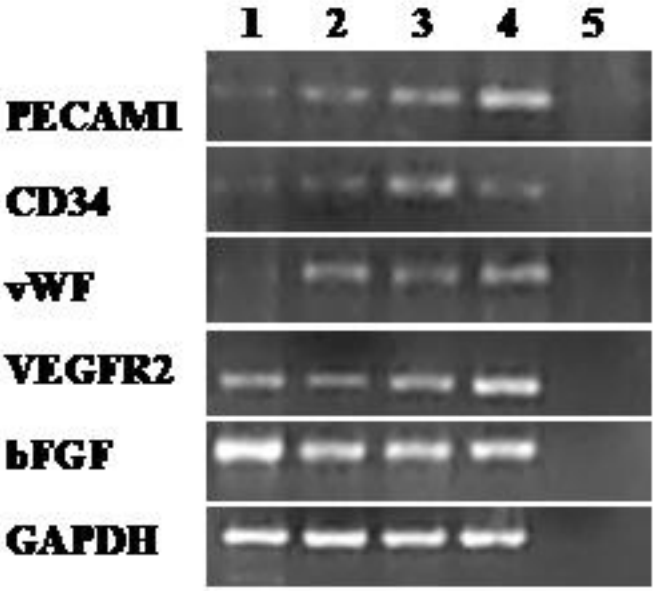
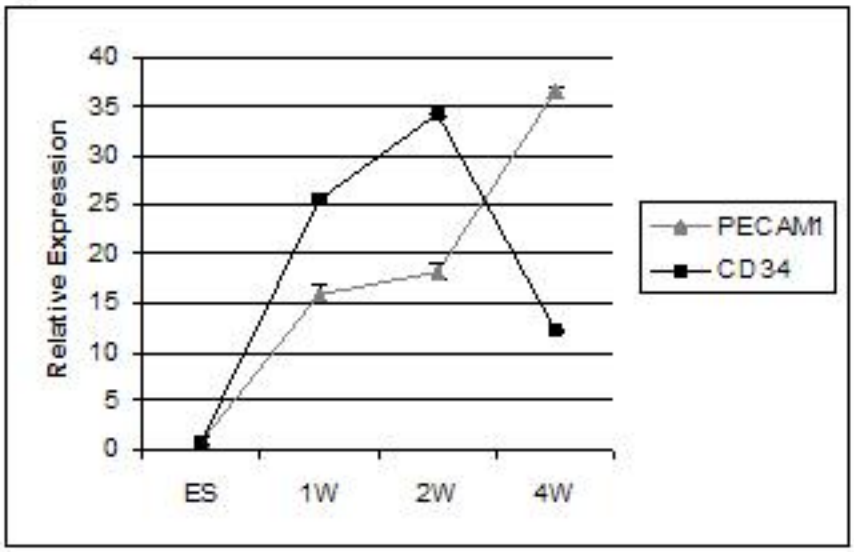


Figure 3

B

i



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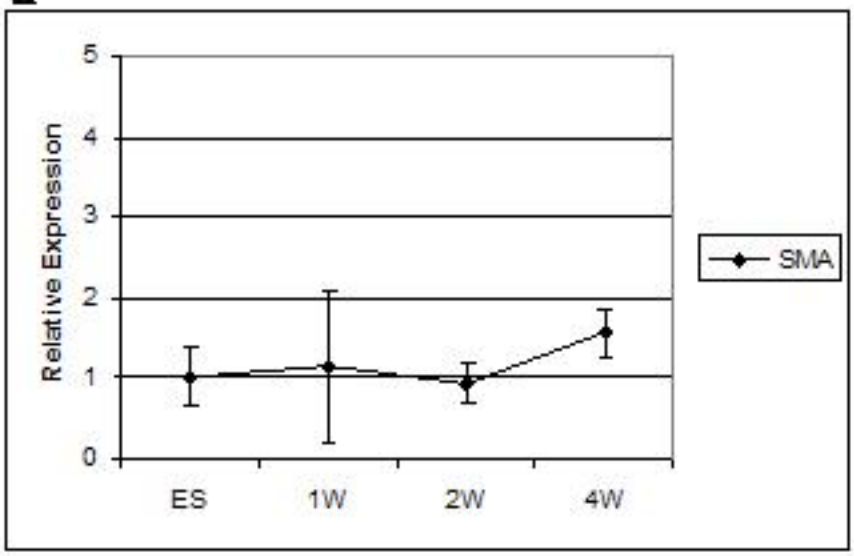

iii

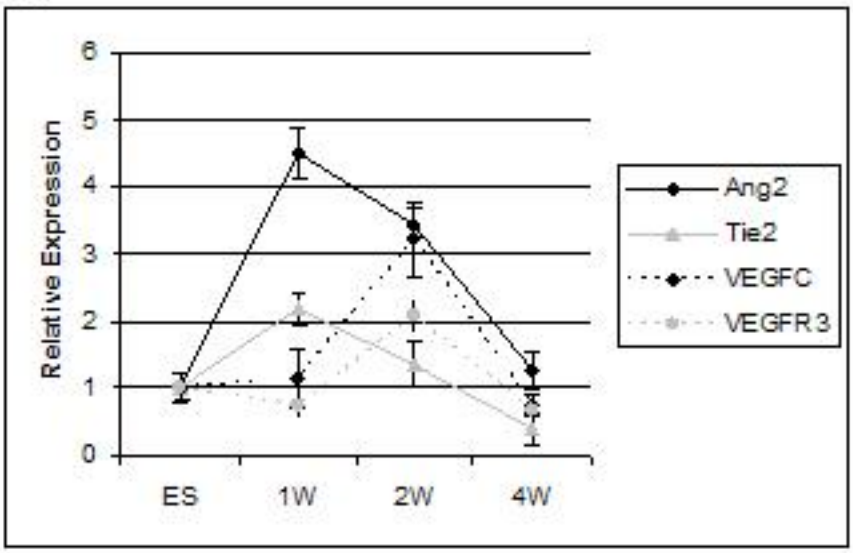

Figure 4

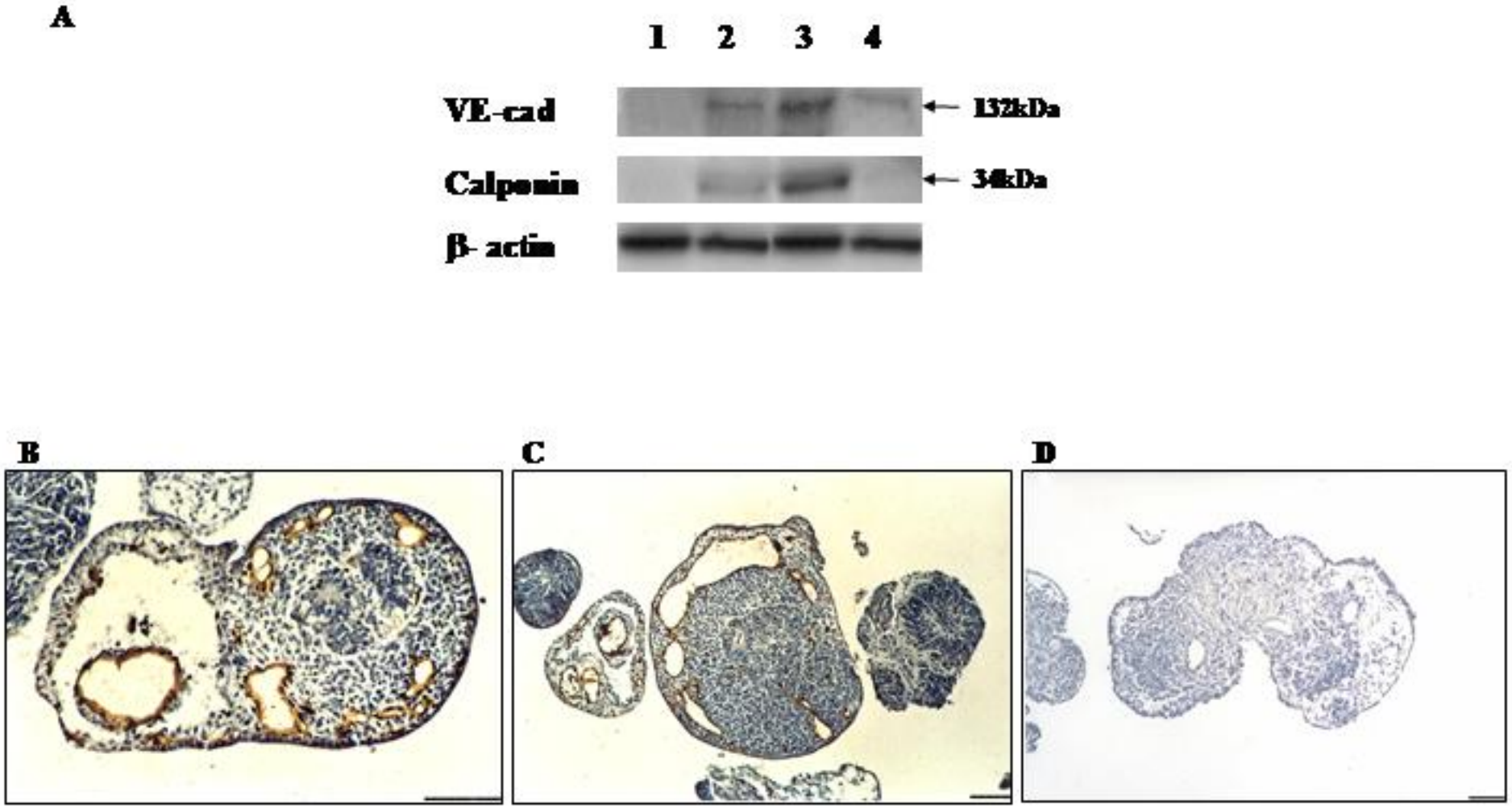

Figure 4

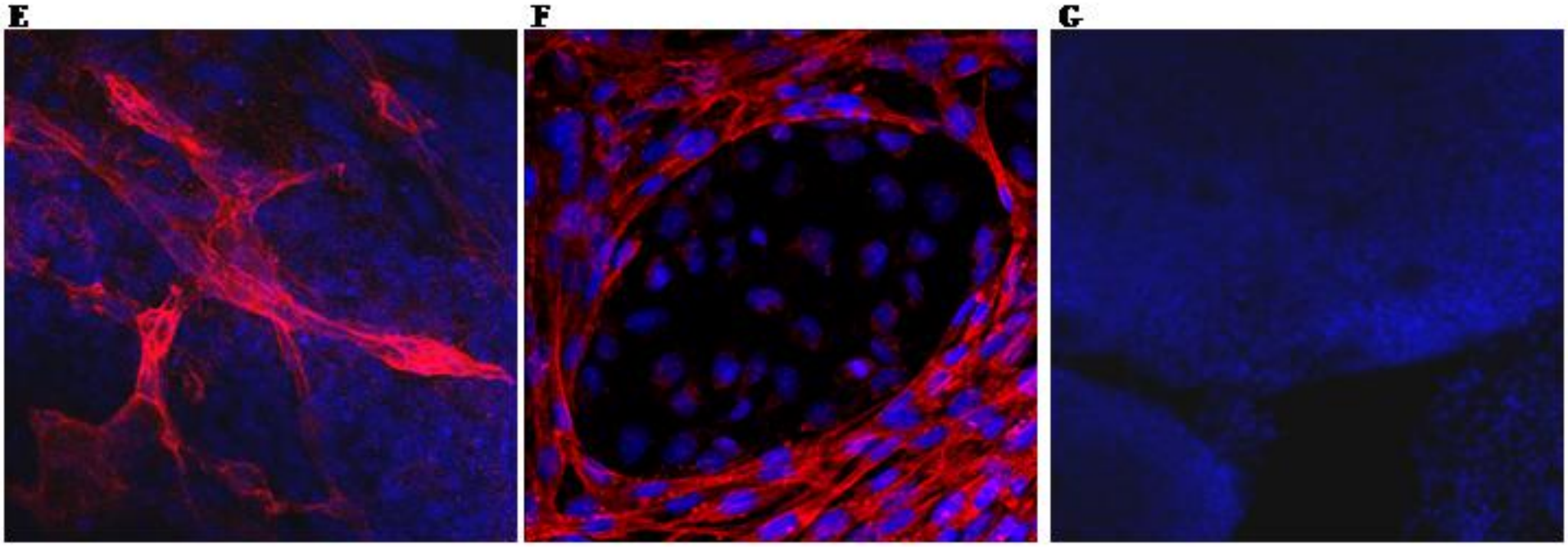

Figure 5