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Differential gene expression by endothelial cells in distinct angiogenic states

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Angiogenesis is a complex process that can be regarded as a series of sequential events comprising a variety of tissue cells. The major problem when studying angiogenesis *in vitro* is the lack of a model system mimicking the various aspects of the process *in vivo*. In this study we have used two *in vitro* models, each representing different and distinct aspects of angiogenesis. Differentially expressed genes in the two culture forms were identified using the suppression subtractive hybridization technique to prepare subtracted cDNA libraries. This was followed by a differential hybridization screen to pick up overexpressed clones. Using comparative multiplex RT-PCR we confirmed the differential expression and showed differences up to 14-fold. We identified a broad range of genes already known to play an important role during angiogenesis like Flt1 or TIE2. Furthermore several known genes are put into the context of endothelial cell differentiation, which up to now have not been described as being relevant to angiogenesis, like NrCAM, Claudin14, BMP-6, PEA-15 and PINCH. With ADAMTS4 and hADAMTS1/METH-1 we further extended the set of matrix metalloproteases expressed and regulated by endothelial cells.

Keywords: differential gene expression; MVEC; angiogenesis; suppression subtractive hybridization.

Angiogenesis, the formation of new capillaries from preexisting blood vessels, plays a crucial role in a wide range of normal and pathological processes, and is necessary for the continous growth of solid tumors [1,2]. Angiogenesis takes not place in a single step, but is a complex sequential process that relies on a controled cross-talk between endothelial cells and the surrounding avascular environment [2,3]. Upon activation by growth factors or cytokines, endothelial cells start to degrade the surrounding extracellular matrix and invade the avascular tissue. The tight endothelial cell-cell adhesion is disrupted, the cells start to proliferate and migrate into the avascular environment. Finally they stop proliferating and differentiate to tubular structures (reviewed in [3]). Most recent studies have focused on the effect of specific growth factors and cytokines secreted by non endothelial cells on angiogenesis, but little is known about the sequential events taking place in the activated endothelial cells during the formation of new blood vessels.

In this work we chose a model system where human microvascular endothelial cells (MVEC) are cultured on a gel composed of extracted basement membrane derived from mouse Engelbreth–Holm–Swarm sarcoma (matrigel) [4]. When seeded at a certain density the cells stop proliferating and virtually all cells are induced rapidly to form capillary-like, lumen containing structures [4,5]. These cells were compared against nondifferentiating, proliferating MVEC again representing an important step during the formation of new blood vessels [2,3]. The advantage of these culture systems is that they can be performed with one and the same cell type resulting in two different homogenous populations within 7 h. We considered this as a prerequisite for a successful subtraction experiment.

As both *in vitro* models used represent different aspects of angiogenesis, we were interested in genes upregulated differentially in either cell population. In order to identify those genes, we chose the suppression subtractive hybridization technique [6] and produced subtractive cDNA libraries enriched for genes upregulated in the two *in vitro* models. The differential expression was checked by a discriminating hybidization experiment and only genes with a differential hybridization result were analyzed further.

The aim of this work was to identify genes expressed by endothelial cells implicated in distinct aspects of angiogenesis. In the present study, we demonstrate that the *in vitro* models used can lead to the identification of genes involved in the process of angiogenesis *in vivo*. Furthermore we implicate several known genes as relevant for angiogenesis that have not been associated with the process before.

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Abbreviations: MVEC, microvascular endothelial cells; X-Gal, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside.

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EXPERIMENTAL PROCEDURES

Cell culture

Primary human MVEC were prepared from human foreskin as described elsewhere [7]. MVEC were cultured in flasks coated with 10 $\mu g \cdot mL^{-1}$ collagen and in M199 medium (Biochrom) containing 10% fetal bovine serum, 10% human serum, 2 mM glutamax, penicillin (100 $U \cdot mL^{-1}$), streptomycin (100 $\mu g \cdot mL^{-1}$), ascorbic acid (1.27 mM), pyruvic acid (1 mM), 1% nonessential amino acids (Biochrom), 6 $\mu g \cdot mL^{-1}$ endothelial growth factor from bovine brain (Sigma) and 7.5 $\mu g \cdot mL^{-1}$ heparin (Sigma).

To induce the differentiating phenotype, MVEC were cultured on a gel composed of extracellular matrix proteins, Matrigel (Beckton Dickinson). Matrigel was diluted 1 : 1 with

Table 1. Primer sequences used for PCR.

Name	Sequence	Temperature (°C)
SH3P18for	AGATGGAGAGTGGTGGACA	62
SH3P18rev	AACAGCAGGGAAAGCCACA	62
23 kDafor	CCACCGCCCTATGACAAGA	64
23 kDarev	ACTTCCAGCCAACCTCGTGA	64
CYR61for	CCAGGGCACACCTAGACAAACAAG	64
CYR61rev	GTCATCAACTCCACAAGCTCCAA	64
PAI-1for	CCTTGCCCTTGAGTGCTTGTT	64
PAI-1rev	CATCAAGGGGAAAAAGAGGGG	64
tPAfor	GGAGTGTGAGCTCTCCGGCTACG	64
tPArev	AAGTCATGCGGCCATCGTTCA	64
MMP-2for	TACACCGGGCCTGGAGAACTA	64
MMP-2rev	CTCTGAGGGTTGGTGGGATTG	64
hADAMTS1for	GGAGATGGACCTGCTACCCCT	64
hADAMTS1rev	CCATGTAACCCTGCTTTGGGA	64
CLDN14f	ATCTCCTTCGTCCCTCTCGCTCATT	64
CLDN14r	CAGGCTGTGGGGACTCACACGTAG	64
CON37f	TTCTCGCCGAGACAGGTAAATGAC	64
CON37r	GGAGCACTCGACCGTGGTGGGTAA	64
LIMK2f	AACCAGCCACACAATGCTGAA	64
LIMK2r	ATTGGAGCAGGGGGAATTGAT	64
CTGFf	GCCTGCCATTACAACTGTCCC	64
CTGFr	TGGGAATCTTTTCCCCCAGTT	64
Numbf	GCAATCCTCAGACGCCTCACT	64
Numbr	GAAGCTACATTTTCCGGTGCG	64
SMRTf	GACCCAAAGCAGGATGACCAC	64
SMRTr	CACCTGGCCTGACTTGGTTTC	64

M199 medium and the gel was poured at 60 $\mu L \cdot cm^{-2}$ in the cold. The gel solidified for 30 min at 37 °C and MVEC were seeded at 1×10^4 $cells \cdot cm^{-2}$. MVEC on Matrigel were cultured in full medium.

RNA preparation

Cells were lysed directly in the culture vessel using GTC-buffer [8] and total RNA was isolated by ultracentrifugation through a CsCl cushion [8]. Poly(A)$^+$ RNA was selected by two rounds of purification over an oligo(dT)-cellulose column using mRNA Purification Kit (Pharmacia Biotech). The amount of poly(A)$^+$ RNA was determined by measurement of absorbance and checked for integrity on a 1% agarose/formaldehyde gel.

Preparation of subtracted cDNA libraries

Subtracted cDNA libraries were prepared using the PCR-Select^{TM} cDNA Subtraction Kit (Clontech) according to manufacturer's recommendations, except the tester to driver ratio was shifted from 1 : 30 to 1 : 60. By doubling the amount of driver in the hybridizations, amplification of non differentially expressed sequences in the following PCR reactions was kept to a minimum. All PCR and hybridization steps were performed in an UNO-Thermoblock (Biometra). After PCR amplification the adaptor sequences in the subtracted cDNA pools were removed by *Rsa*I digestion and subsequent purification using the PCR-Purification Kit (Qiagen). The blunt ended PCR products were ligated in pUC 18 *Sma*I/BAP (Pharmacia Biotech) and transformed in *E. coli* DH5α (Gibco BRL). Transformed bacteria were plated on 2 × YT agarose containing 100 $\mu g \cdot mL^{-1}$ ampicillin, 625 μM isopropyl thio-β-D-galactoside, 0.005% 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) and grown overnight at 37 °C. On 20 randomly selected white colonies insert sizes were checked by colony PCR using M13 standard primers. For each hybridization 1536 clones were arrayed in 384 well plates (Genetix) containing 50 μL 2 × YT, 10% glycerol, grown overnight at 37 °C and stored at −80 °C.

Differential hybridization

For each subtraction 1536 arrayed clones were transfered on a Hybond N$^+$ nylon membrane (Amersham) sitting on a 2YT agarose plate and grown overnight at 37 °C. The clones on the filter were alkaline-lysed, neutralized and proteinase K treated. Filters were baked at 80 °C for 3 h and used for hybridization. Hybridization was performed in Express Hyb Solution (Clontech) at 72 °C for 18 h with ^{32}P-labeled double-stranded cDNA pools of equal specific activity derived from forward and reverse subtraction, respectively. Filters were washed four times for 20 min at 68 °C in 2 × NaCl/Cit, 0.5% SDS and twice for 20 min at 68 °C in 0.2 × NaCl/Cit, 0.5% SDS. Washed filters were exposed to Phosphorimager plates (Molecular Dynamics) for 3 days and hybridization results were quantified using IMAGEQUANT software (Molecular Dynamics).

Sequencing and sequence analysis

Differentially expressed clones were sequenced from plasmid DNA using Big Dye technology (PerkinElmer) on an ABI377 automated sequencer (PerkinElmer). Sequence files were exported to a UNIX workstation and analyzed and stored using the STADEN software package [9]. Remaining vector sequences bits were clipped. The complete set of sequences were elongated in a combined search and assemble procedure using dbEST [10] as described elsewhere [11]. Finally all assemblies were checked manually for inconsistencies and the elongated and assembled data set was screened against the GenEMBL nucleotide database using the BLASTN algorithm [12].

Comparative multiplex RT-PCR

Reverse transcription was performed using Ready-to-go T-Primed first strand Kit (Pharmacia Biotech). For each total RNA, 3.3 μg were reverse transcribed in a total volume of 33 μL. To quantify differential gene expression comparative multiplex RT-PCR was performed as described elsewhere [13]. All primers used in this study are shown in Table 1. The forward primers were labeled at the 5′-end with a fluorescence marker, FAM for the gene of interest and JOE for the internal standard 23-kDa highly basic protein (GenBank accession no. AF039843). Comparative multiplex RT-PCR was performed using 1 μL RT product and Ready-To-Go PCR Beads (Pharmacia Biotech) on a Master Cycler (Eppendorf). After amplification 1 μL of PCR product was diluted with 4 μL loading cocktail (1 μL GS-350 TAMRA, 1 μL 50 $ng \cdot mL^{-1}$ 25 mM EDTA, 2 μL deionized formamide). One microliter of this dilution was denatured and seperated on a 6% sequencing gel using an ABI377automated sequencer (PerkinElmer). The samples were analyzed and quantified using GENSCAN software version 2.1 (PerkinElmer). The PCR products were checked for correct size according to the size standard GS-350 TAMRA (PerkinElmer) included in each sample and the amount of product was quantified by measuring the fluorescence intensity. For further analysis the area below the fluorescence peak of each PCR product was integrated and normalized to the internal standard. The optimal cycle number was determined for each gene of interest/standard combination before comparative analysis was performed.

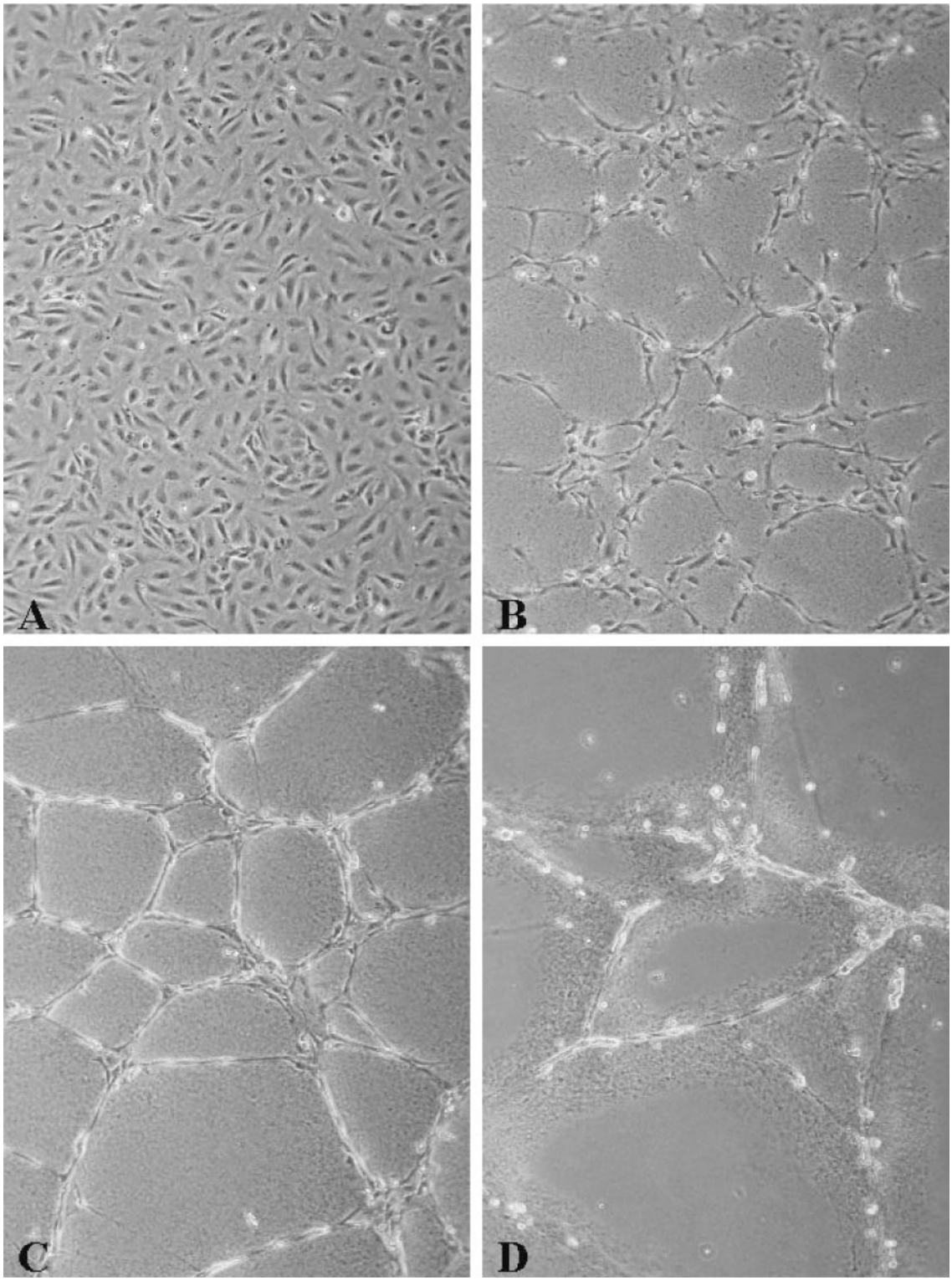

Fig. 1. Cell culture. (A) Confluent MVEC grown on a collagen-coated culture vessel. The cells show the 'cobblestone' morphology typical for confluent endothelial cells. To induce the tubular phenotype MVEC were cultured on matrigel in a density of 1×10^4 cells·cm^{-2} for 2 h (B), 8 h (C) and 24 h (D). Photographs were taken with a camera on a Zeis Axiovert 25 microscope at 80× magnification.

Northern blot

Twenty micrograms of each total RNA was electrophoresed on a 1% agarose/formaldehyde gel, photographed, capillary blotted onto nylon membranes and UV crosslinked. CYR61-PCR-probe was labeled with a random primer labeling kit (Stratagene) using [^{32}P]dCTP. The filter was hybridized in ExpressHyb (Clontech) for 18 h at 68 °C and washed extensively at high stringency. Filter was exposed to X-ray film (Kodak).

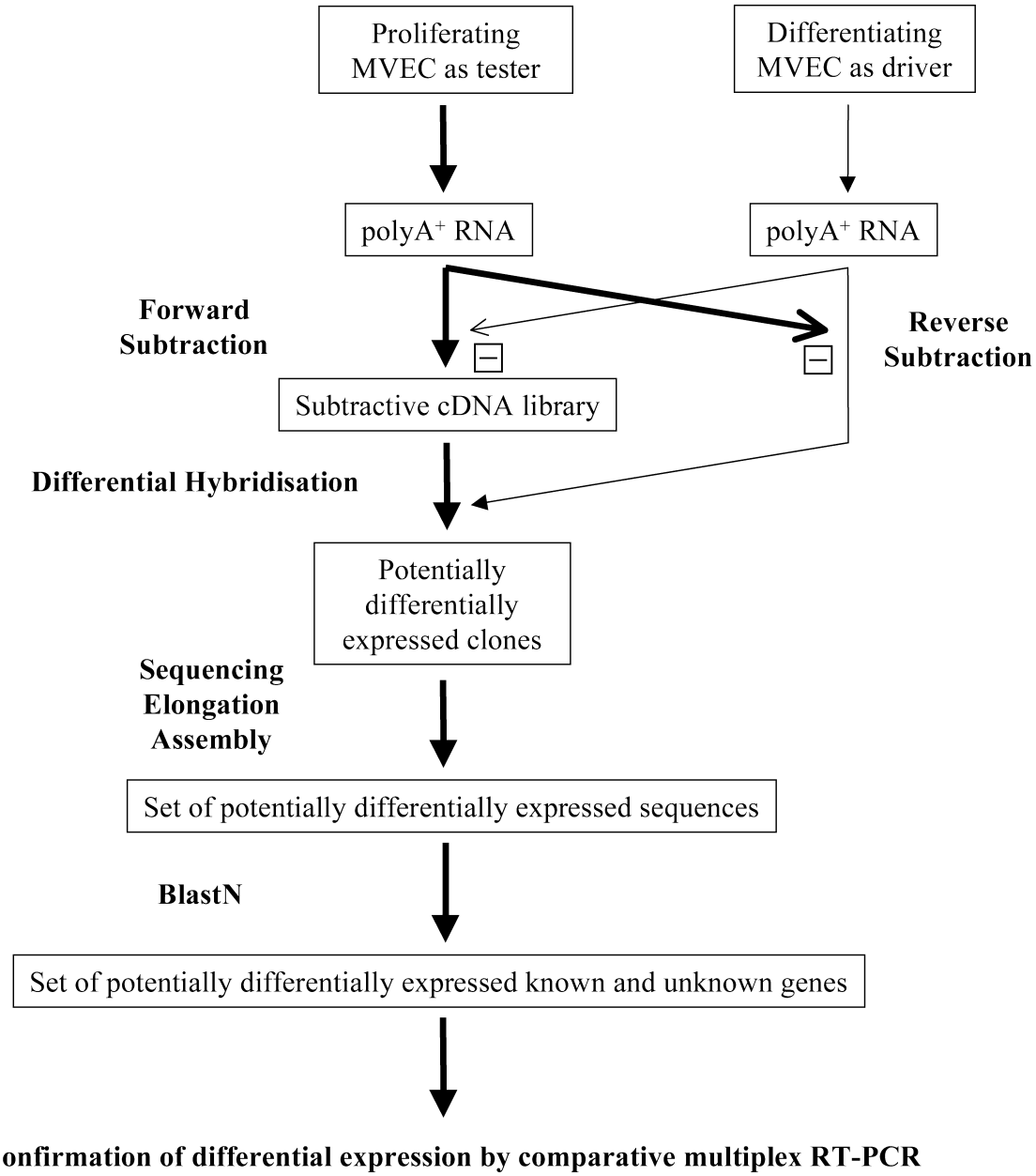

Fig. 2. Schematic representation of the strategy. The strategy to isolate regulated genes is shown as an example for the subtraction enriching for genes upregulated in proliferating MVEC

RESULTS

Endothelial cell model systems:

Human dermal MVEC, when seeded on collagen coated dishes will spread and attach to the surface. Cell patches form within 2 h and the cells proliferate until reaching confluency. MVEC form a cobblestone morphology with cells firmly attached to the substratum and the neighbouring cells (Fig. 1). To avoid apoptosis and to obtain a high rate of proliferation, the cells have to be seeded at a density of 1–5 $\times$ 10^4 cells·cm^{-2} [14–16]. This culture form is called 'proliferating MVEC'.

In contrast, when MVEC of the same batch are seeded at a density of 1 $\times$ 10^4 cells·cm^{-2} in the same medium on a gel composed of extracellular matrix proteins extracted from the Engelbreth–Holm–Swarm mouse sarkoma (matrigel), they stop proliferating and form capillary like, lumen containing structures [4]. The differentiation process can be blocked with 10 μg·mL^{-1} actinomycin D or 50 μg·mL^{-1} cycloheximide (data not shown). As can be seen in Fig. 1, after 2 h the cells on matrigel orient themselves to each other and start to build up intercellular connections. After 8 h complete networks have formed and virtually no isolated cells are left. Extracellular matrix proteins in the gel start to become degraded between the capillary like structures. After 24 h the extracellular matrix not covered by cells is strongly degraded. Control matrix treated with serum containing medium only is not degraded even after several days of incubation (data not shown). This culture system is called 'tubular MVEC'. For subsequent analysis, a time point of 7 h was chosen, when capillary formation is almost completed but matrix degradation is not obvious.

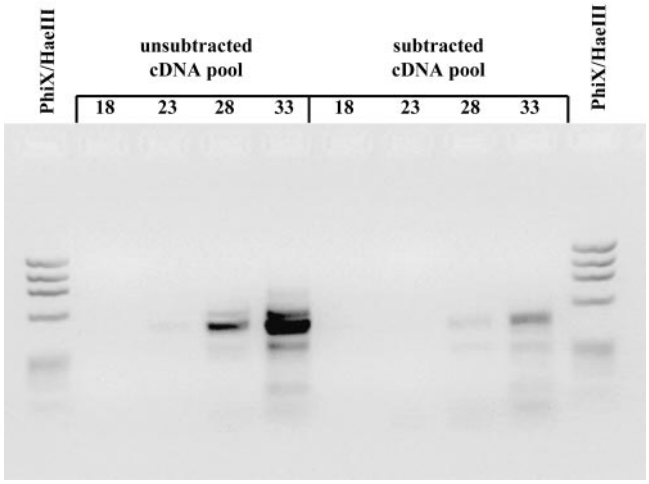

Fig. 3 Monitoring of subtraction efficiency. The subtraction efficiency was determined by PCR using primers specific for SH3P18, a gene present in both differentiation states of MVEC in roughly equal amounts, yielding a PCR product of 573 bp in size. As an example for the subtraction with proliferating MVEC as tester, a negative image of an agarose gel is shown, loaded with 20 μL PCR product from the unsubtracted cDNA pool and the subtracted cDNA pool after an increasing number of cycles. *Phi*X/*Hae*III was used as size standard.

Preparation of subtractive cDNA libraries

For the preparation of two subtractive cDNA libraries, MVEC of the same passage were seeded in a density of 1×10^4 cells·cm^{-2} either on collagen coated culture vessels or matrigel and incubated for 7 h. The cells were lysed directly on the culture vessels and poly(A)$^+$ RNA was prepared. The strategy used to identify differentially expressed genes in each mRNA population is summarized in the flow chart in Fig. 2. This is the example for the subtraction intended to enrich for genes upregulated in proliferating MVEC. The two polyA$^+$ RNA populations to be subtracted are given at the top. The driver is defined as the population of mRNA that will be eliminated during subtraction, whereas the tester population contains in addition the differentially expressed genes of interest. Both mRNA populations have been reverse transcribed and subjected to the suppression subtractive hybridization procedure as described by Diatchenko *et al*. [6]. The subtraction efficiency was monitored by PCR using primers for SH3P18, a gene present in both populations in approximately equal amounts. Figure 3 shows that SH3P18 transcript is significantly reduced in the subtraction with mRNA of proliferating MVEC as tester, called 'forward' subtraction, compared to unsubtracted control. Comparable results were achieved for the subtraction using mRNA of tubular MVEC, called 'reverse' subtraction. As the subtraction efficiency was satisfactory, the subtracted cDNA pools were cloned and 1536 white colonies were picked for each subtraction, arrayed in 384-well plates and stored at −80 °C.

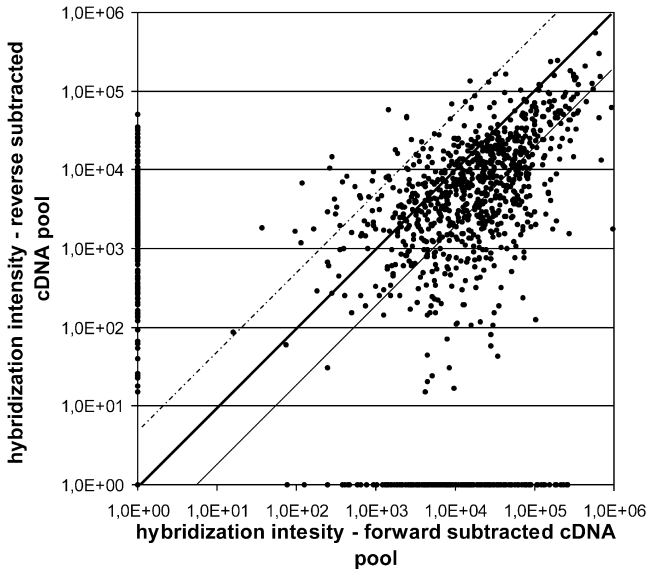

Fig. 4. Comparison of intensity values for single clones after differential hybridization. Colony filters were prepared from the subtracted cDNA library with proliferating MVEC as tester and hybridized with equal amounts of specific activity of either the forward subtracted cDNA pool (itself) or the reverse subtracted cDNA pool. In this log × log plot, each clone is represented by its hybridization intensity to the two probes. The thick line represents equal hybridization to both probes, the thin line marks the decision criteria of at least fivefold stronger expression with the forward probe, the dotted line represents fivefold stronger hybridization with the reverse subtracted cDNA pool. As expected for a successful subtraction experiment, the majority of the clones hybridized more strongly with the forward subtraction than with the reverse subtracted cDNA pool.

Differential hybridization

In order to detect rare messages we used the forward and reverse subtracted cDNA pools as differential screening probes instead of the original mRNA populations. Earlier attempts to identify rarely transcribed, differentially expressed genes by conventional differential screening methods often failed due to detection threshholds [17]. The suppression subtractive hybridization technique enriches not only for differentially transcribed genes, it also leads to normalized cDNA pools and thereby enriches for rare messages [6]. Colony filters were prepared for each subtracted cDNA library. Each filter was hybridized with equal amounts of ^{32}P-labeled forward and reverse subtracted cDNA pools (Fig. 2) and the results were quantified. In Fig. 4 the results for the subtraction with proliferating MVEC as tester are presented. Each clone is plotted by its hybridization intensity to the forward and reverse subtracted cDNA pool. As can be seen in Fig. 4, the majority of the clones hybridized more strongly with the forward subtracted probe (itself) compared to the reverse subtracted probe, as would be expected for a successful subtraction experiment. In total, 85% of the clones showed a detectable hybridization result with one of the probes. Approximately 30% of the clones hybridized at least five times more strongly with the forward subtracted cDNA pool than with the reverse subtracted probe and 15% of the clones were detectable only with the forward subtracted cDNA probe. For the subtractive cDNA library prepared with mRNA from tubular MVEC as tester, similar results were achieved. Clones hybridizing only with the forward subtraction or hybridizing at least five times more strongly with the forward subtraction than with the reverse cDNA pool were selected for further analysis.

Sequence analysis of differentially expressed clones

Three-hundred and fifty clones with a differential hybridization result from each subtractive cDNA library and 400 randomly selected clones from the subtraction with tubular MVEC as tester were sequenced. Of the 400 randomly selected clones, 81 were also present in the set of 350 clones with a differential

Table 2. Selection of differentially expressed genes. Tub, overexpressed in tubular MVEC; Prol , overexpressed in proliferating MVEC.

Gene	AccNo	Expression	Category
PECAM-1/CD31	M28526	> Tub	Adhesion
Integrin alpha v	M14648	> Tub	Adhesion
Integrin alpha 2	X17033	> Tub	Adhesion
ALCAM	L38608	> Tub	Adhesion
CD44	M24915	> Tub	Adhesion
NRCAM	NM_005010	> Tub	Adhesion
MUC18/CD146	X68271	> Tub	Adhesion
Integrin alpha 5	X06256	> Tub	Adhesion
Claudin-14	AJ132445	> Tub	Tight junction
Connexin37	M96789	> Tub	Gap junction
FIN13	U42383	> Prol	Cell cycle
MPP4	X98264	> Prol	Cell cycle
p21	U09579	> Tub	Cell cycle
CD164	D14043	> Tub	Proliferation
Gas5	X59728	> Tub	Proliferation
CYR61	Y12084	> Prol	Proliferation
CTGF	U14750	> Prol	Growth factor
Follistatin	M19481	> Prol	Growth factor
Beta A-inhibin/activin	M13436	> Prol	Growth factor
BMP-6	M60315	> Tub	Growth factor
IL8	M28130	> Prol	Growth factor/cytokine
UFO/Axl	X66029	> Prol	Receptor
BMP receptor type2	U25110	> Tub	Receptor
ALK-2	Z22534	> Tub	Receptor
TIE2	L06139	> Tub	Receptor
Flt1	X51602	> Tub	Receptor
MMP-1	X54925	> Tub	Matrix protease
MMP-2	J03210	> Tub	Matrix protease
hADAMTS1	AF207664	> Tub	Matrix protease
ADAMTS4	AF148213	> Tub	Matrix protease
t-PA	X07393	> Tub	Protease
PAI-1	M16006	> Prol	Protease/inhibitor
Collagen V	M76729	> Prol	Extracellular matrix
Biglycan	J04599	> Prol	Extracellular matrix
Fibronectin	X02761	> Tub	Extracellular matrix
Gla/MGP	M55270	> Tub	Extracellular matrix
Bamacan rat	U82626	> Tub	Extracellular matrix
LIMK2	D45906	> Prol	Signal transduction
LAR	Y00815	> Prol	Signal transduction
Ly-GDI	L20688	> Prol	Signal transduction
HRS	D50050	> Prol	Signal transduction
PEA-15	NM_003768	> Tub	Signal transduction
Caveolin-1	Z18951	> Tub	Signal transduction
hCOX-2	U04636	> Tub	Signal transduction
SPRY2	AF039843	> Tub	Signal transduction
PYST1	X93920	> Tub	Signal transduction
PINCH	U09284	> Tub	Signal transduction
JAK1	M64174	> Tub	Signal transduction
AP-2	X77343	> Prol	Transcription factor
HIF-2/MOP2	U51626	> Tub	Transcription factor
ERM	X96375	> Tub	Transcription factor
MITF-2B	U16322	> Tub	Transcription factor
SMRT	U37146	> Prol	Transcription factor/repressor
NRSF	U22680	> Tub	Transcription factor/repressor
NUMB	AF015040.1	> Tub	Cell fate decision

hybridization result from the library with tubular MVEC as tester and were used as positive controls for the following assembly. All 1100 sequences were assembled using GAP4 [9]. As expected, the 81 sequences from the 400 randomly selected clones clustered together with their identical counterparts in the subtractive library derived from tubular MVEC. Comparing the 350 differentially expressed clones from each subtractive library with each other, only two sequences were identified in both libraries, one being a ribosomal RNA and the second vimentin. Finally, comparing the 400 randomly selected clones

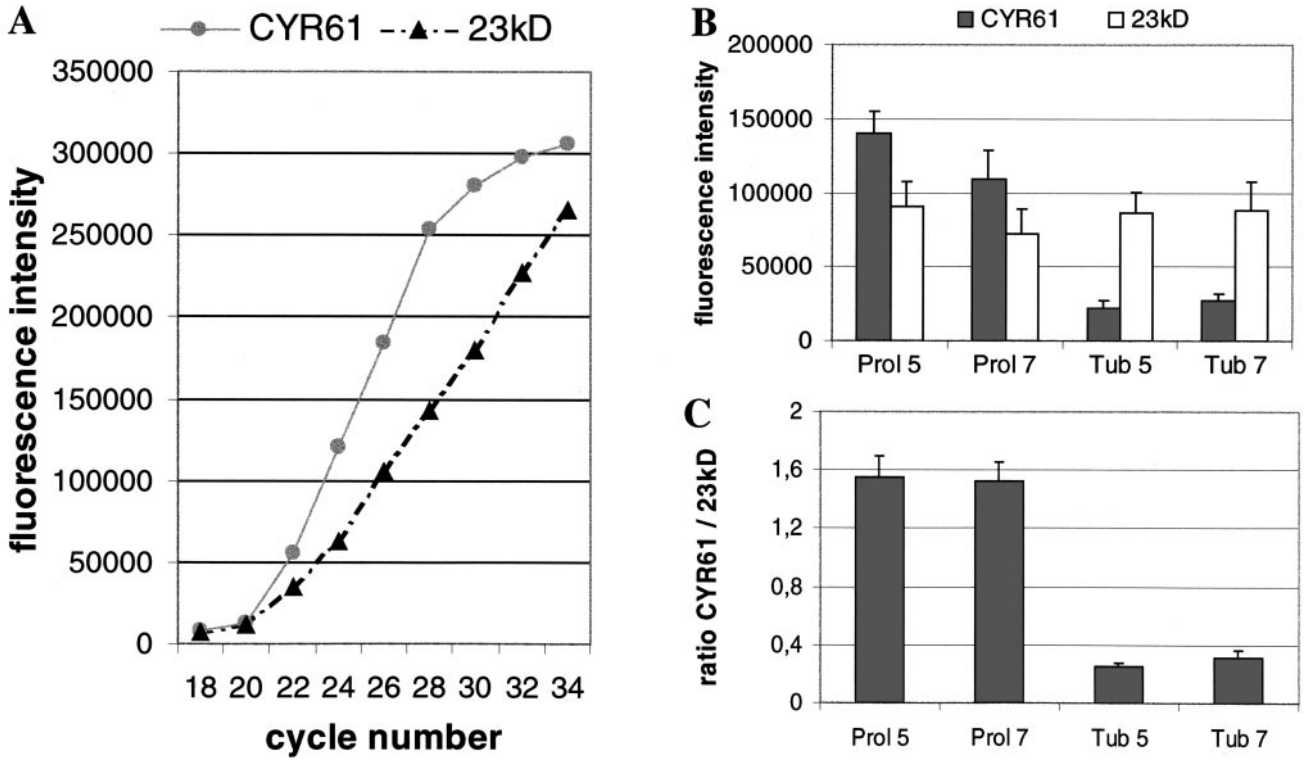

Fig. 5. Comparative multiplex RT-PCR was used to confirm for differential gene expression. In a single PCR reaction, the gene of interest and the internal standard 23-kDa highly basic protein were amplified and the amount of PCR product after an optimized number of cycles was determined. (A) The optimal cycle number is chosen in the range of linear amplification, in the case of CYR61 25 cycles were chosen. To illustrate the reproducibility of this method, two independent experiments, with regard to passage number and date of experiment, were performed. (B) The absolute amounts of CYR61- and 23-kDa-protein PCR products are given for two populations of proliferating MVEC, Prol 5 and Prol 7, and tubular MVEC, Tub 5 and Tub 7. (C) After normalization to the internal standard the ratio of CYR61 to 23-kDa highly basic protein is presented for each cell population.

from the tubular MVEC with the 350 positive clones of the proliferation library, 10 sequences clustered together. For nine of them this fits well with their differential hybridization results. As can be seen in Fig. 4, in the subtracted cDNA libraries there were some clones which hybridized more strongly with the reverse subtracted cDNA pool than with the forward subtraction. After this first assembly, the 400 randomly selected sequences were removed again from the set of potentially differentially expressed sequences.

Because the supression hybridization subtraction technique produces only *Rsa*I fragments of cDNAs, we applied a newly developed iterative elongation and assemblation strategy [11] to reduce redundancy of genes in the set of potentially differentially expressed sequences. The number of 700 initially single sequences with a differential hybridization result was scaled down to 492 single sequence contigs. These 492 contigs were compared to the GenEMBL nucleotide database (nt, NCBI) using the BLASTN algorithm [12]. Out of the 492 single sequences, 263 represented known genes, whereas for the remaining 229 contigs only EST data were available. In the set of known genes 15% have already been implicated in the process of angiogenesis, 12% were categorized as housekeeping genes and 22% represented genes of unknown function.

Known genes identified in this study

In Table 2 a selection of known genes with differential expression is presented and ordered in groups. The tubular cells preferentially express growth inhibitory factors (TGF-β, p21, Gas5), whereas the stimulatory factors are overexpressed in proliferating MVEC (CTGF, CYR61, follistatin). All members of matrix metalloproteases found are upregulated in tubular MVEC, for example MMP-1 and -2, and hADAMTS1/METH-1 and ADAMTS4. Tubular MVEC also show an increased expression of adhesion molecules like integrin subunit α2, α5 and αv. The pattern of extracellular matrix proteins expressed appears to differ, with more fibronectin, Gla and bamacan in tubular but more collagen V and biglycan in proliferating MVEC. A variety of growth factors and growth factor receptors are differentially expressed. So we find TIE2 and Flt1 more strongly expressed in tubular MVEC than in proliferating cells.

Confirmation of differential gene expression

To confirm the differential expression of selected sequences from the subtracted cDNA libraries, we performed comparative multiplex RT-PCR [13]. The gene of interest and a standard gene were amplified in a single reaction. The PCR conditions and cycle numbers were carefully adjusted for each primer combination to assure an amplification in the linear range as shown in Fig. 5A, which shows data for CYR61. For further analysis in the case of CYR61, 25 cycles were used, and the expression level was determined using equal amounts of cDNA derived from proliferating and tubular MVEC of two independent experiments. The absolute expression values for CYR61 and 23-kDa highly basic protein differ between the two experiments as shown in Fig. 5B. After normalization of CYR61 expression to the internal standard, 23-kDa highly basic protein, the two experiments show nearly identical results (Fig. 5C) with CYR61 being overexpressed in proliferating MVEC by a

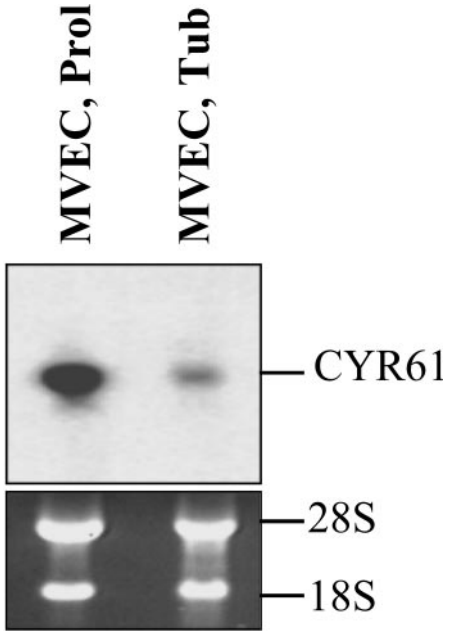

Fig. 6. Confirmation of differential gene expression for CYR61. Twenty micrograms of total RNA from proliferating (Prol) and tubular-forming (Tub) MVEC were hybridized with a CYR61-specific radiolabeled probe. As can be seen in the upper panel CYR61 is overexpressed in the proliferating MVEC. The lower panel shows the samples before blotting to demonstrate equal loading and integrity of the RNA.

factor of ≈ 5; this suggests the method appears to be highly reproducible. Furthermore the results of the comparative multiplex RT-PCR for CYR61 are in agreement with the result of a conventional Northern blotting experiment (Fig. 6).

We performed comparative multiplex RT-PCR for further 10 known genes and confirmed differential expression for nine out of 10 sequences in the expected orientation with a difference greater than a factor of 2. As summarized in Table 3, claudin14 (CLDN14) appears to be overexpressed in tubular MVEC by a factor of ≈ 3, connexin37 (CON37) by a factor of ≈ 2.5 and NUMB by a factor of ≈ 6. Proliferating MVEC upregulate CTGF by a factor of ≈ 2, SMRT by a factor of ≈ 7 and PAI-1 appears to be upregulated in proliferating MVEC by a factor of 14. The proteases t-PA, MMP-2 and hADAMTS-1 are overexpressed in MVEC cultured on matrigel by a factor of 4–5. LIMK2 originally identified as upregulated in proliferating MVEC could not be confirmed as an overexpressed gene. This leads to a rate of ≈ 90% truly differentially expressed sequences in the set of sequences with a differential hybridization result.

Table 3. Differential gene expression of selected genes by RT-PCR in proliferating and tubular MVEC.

	Overexpression (fold)	
Gene	Proliferating MVEC	Tubular MVEC
CLDN14	–	3
Connexin37	–	2.5
CTGF	2	–
CYR61	5	–
hADAMTS1	–	5
LIMK2	1.1	–
MMP-2	–	4
Numb	–	6
PAI-1	14	–
SMRT	7	–
t-PA	–	4

DISCUSSION

The formation of new blood vessels by sprouting angiogenesis is a coordinated multistep process performed by different cell types. There is no *in vitro* model available that represents all aspects of angiogenesis *in vivo*. In order to identify genes that are regulated in certain stages of angiogenesis we cultured human MVEC in an activated, proliferating state [15], and on matrigel where they stop proliferating and form capillary like structures [4].

Differentially expressed genes in the two culture forms were isolated using the suppression subtractive hybridization technique [6] modifying the tester to driver ratio from 1 : 30 to 1 : 60; overexpressed clones were picked up by a differential hybridization approach. Ninety percent of the clones with a differential hybridization result were truly differentially regulated as monitored by comparative multiplex RT-PCR.

Because we compared proliferating to nonproliferating MVEC, we expected to find different sets of cell-cycle regulators and proliferation-associated genes. As summarized in Table 2, in proliferating MVEC we found CYR61 and CTGF to be upregulated. CYR61 and CTGF have been shown previously to stimulate endothelial cell proliferation *in vitro* [18–20] and CYR61 has also been shown to promote angiogenesis *in vivo* [21]. In contrast p21, a negative regulator of cell cycle, and TGF-β1, known to inhibit endothelial cell proliferation *in vitro* [22], were found to be overexpressed in tubular MVEC.

The proliferation- or differentiation-stimulating factors follistatin, beta A-inhibin/activin, BMP-6 and their receptors ALK-2 and type II BMP receptor deserve special attention in this context, as the signal transduction cascade of these factors emerged strongly and differentially under both endothelial cell culture conditions. Follistatin and beta A-inhibin/activin were found to be upregulated in proliferating MVEC in this screen, whereas BMP-6 and the receptors ALK-2 and type II BMP receptor were overexpressed on tubular MVEC.

Recently it has been reported that follistatin is upregulated in proliferating endothelial cells compared to quiescent cells and induces endothelial cell proliferation by binding the proliferation repressor beta A-inhibin/activin [23,24]. Furthermore, follistatin was shown to be a mild inducer of angiogenesis in the rat cornea model [23]. In this screen, the receptors for follistatin/activin, ALK-2 and type II BMP receptor, were found to be overexpressed on tubular MVEC. Another ligand of these receptors, the TGF-β family member BMP-6 [25,26], was found to be overexpressed on tubular MVEC; BMP-6 signaling has been reported to be mediated by SMAD5 [27]. Although BMP-6 knock-out mice are viable and fertile [28], mice knocked out for SMAD5 show a vascular phenotype [29]. SMAD5 $^{-/-}$ mice die between days 10.5 and 11.5 of gestation due to defects in angiogenesis [29]. These findings, which correspond with our expression data, indicate an important role for BMP-6/SMAD5 signaling during endothelial cell differentiation and organization of capillary-like structures.

Cell adhesion is an important aspect of sprouting angiogenesis [3,30]. All adhesion molecules identified in this screen appeared to be overexpressed in tubular MVEC. CD44 and CD146 have been reported recently as markers for angiogenically

active tumor endothelium [31,32]. Antagonism of PECAM-1 function with a blocking antibody leads to inhibition of *in vitro* tube formation of endothelial cells on collagen gels [33].

Surprisingly, we found NRCAM to be expressed on tubular MVEC. NRCAM is a member of the Ig superfamily that mediates neuronal adhesion and neurite outgrowth [34] in cooperation with integrin β1 [35], an integrin subunit also present on endothelial cells. Possibly, NRCAM on endothelial cells exerts a similar function in mediating capillary outgrowth as antibodies against integrin α2β1 strongly interfered with endothelial cell tube formation *in vitro* [36]. Furthermore, we found the integrin subunits α2, α5 and αv overexpressed on tubular MVEC. All three integrin subunits are regulated by the angiogenic factor bFGF [37]. The importance of integrin αvβ3 in sprouting angiogenesis has been shown previously *in vitro* and *in vivo* [36,38–40]. This fits well with PEA-15 and PINCH, two proteins involved in integrin mediated intracellular signaling [41,42], being upregulated in tubular MVEC. This concerted regulation suggests integrin-mediated intracellular signaling events following cell attachment to allow for tube formation.

One gene possibly involved in the formation of tight junctions, an outstanding structural function of endothelial cells [43,44], is claudin-14, a gene found to be overexpressed on tube-forming MVEC in this screen. Claudin-14, predicted from the genomic sequence, shows 52% and 46% sequence identitiy to mouse claudin-2 and human claudin-3, respectively, two proteins already known to be involved in the formation of tight junctions [45–47]. The identification of claudin-14 as a regulated gene in MVEC extends the set of tight junction proteins expressed by endothelial cells and provides evidence for its involvement in the formation or stabilization of tubular structures.

During formation of new blood vessels, endothelial cells have to invade avascular environments [48]. This invasion step is crucial for successful angiogenesis, as has been shown *in vitro and in vivo* [49–52]. Proteases expressed by endothelial cells can degrade the extracellular matrix and allow the cells to migrate into the avascular tissue [51–53]. The plasminogen activator–plasmin proteolytic system has been implicated in these processes [51,54–55]. We found PAI-1 upregulated in proliferating MVEC, whereas tubular MVEC expressed elevated levels of tissue type plasminogen activator (t-PA). This is in agreement with Schnaper *et al*. [56], who showed that PAI-1 is down regulated by endothelial cells on matrigel and addition of active PAI-1 protein to cells on matrigel prevented tube formation [56].

Further, we identified members of the matrix metalloprotease family to be differentially expressed. As shown in Tables 2 and 3, we found MMP-1, MMP-2, ADAMTS4 and hADAMTS1 to be upregulated by MVEC on matrigel. The role of MMP-1 and MMP-2 during angiogenesis, and the regulation of expression in endothelial cells, has already been reported *in vitro* and *in vivo* [52,53]. The identification of hADAMTS1 and ADAMTS4 on MVEC further extends the panel of matrix metalloproteases expressed and regulated by endothelial cells. ADAMTS4 and hADAMTS1 encode secreted metalloproteases, not directly anchored to the plasma membrane like other MMPs [57–59]. Recently hADAMTS1/METH-1 was shown to exert an anti-proliferative and angio-inhibitory function on endothelial cells *in vitro* and *in vivo* [58].

Taken together, our results demonstrate that the model systems used in this work are useful to study several aspects of angiogenesis. We identified a broad range of genes already known to have an important function in angiogenesis. Each of these genes was overexpressed in the expected model system at a location where the proposed function of the respective gene product is relevant. Some known genes not previously associated with the process, have been implicated in the context of endothelial cell differentiation and angiogenesis. Similar to the results presented for the known genes, we identified fragments of unknown genes that include protein domains characteristic for putative transmembrane proteins, signaling proteins and transcription factors. We are confident that among the unknown sequences regulated by MVEC, there are some with important functions in angiogenesis.

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