Differential gene expression during capillary morphogenesis in 3D collagen matrices: regulated expression of genes involved in basement membrane matrix assembly, cell cycle progression, cellular differentiation and G-protein signaling

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SUMMARY

We have performed a screening analysis of differential gene expression using a defined in vitro model of human capillary tube formation. Gene array, differential display and cDNA library screening were used to identify both known and novel differentially expressed genes. Major findings include: the upregulation and functional importance of genes associated with basement membrane matrix assembly; the upregulation of growth factors, transcription factors, anti-apoptotic factors, markers of endothelial cell differentiation, JAK-STAT signalling molecules, adhesion receptors, proteinase inhibitors and actin regulatory proteins; and expression changes consistent with inhibition of cell cycle progression, increased cholesterol biosynthesis, decreased ubiquitin-proteasome mediated degradation, and activation of G-protein signaling pathways. Using DNA microarray analysis, the most induced genes at 8, 24 and 48 hours compared with those at 0 hours were jagged-1, stanniocalcin and angiopoietin-2, whereas the most repressed genes were connective tissue growth factor, fibulin-3 and RGS-5. In addition, the full length coding sequence of two novel regulated capillary morphogenesis genes (CMGs) are presented. CMG-1 encodes a predicted intracellular 65 kDa protein with coiled-coil domains. A CMG-1-green fluorescent protein (GFP) chimera was observed to target to an intracellular vesicular compartment. A second novel gene, CMG-2, was found to encode a predicted intracellular protein of 45 kDa containing a transmembrane segment and a CMG-2-GFP chimera was observed to target to the endoplasmic reticulum. A recombinant portion of CMG-2 was found to bind collagen type IV and laminin, suggesting a potential role in basement membrane matrix synthesis and assembly. These data further elucidate the genetic events regulating capillary tube formation in a 3D matrix environment.

Key words: Endothelium, Morphogenesis, Differential gene expression, Collagen matrices

INTRODUCTION

Studies on the molecular control of endothelial cell (EC) morphogenesis during angiogenesis or vasculogenesis have revealed many insights into how blood vessels participate in complex biological processes such as development, wound repair and tumorigenesis (Hanahan, 1997; Carmeliet and Jain, 2000; Yancopoulos et al., 2000; Conway et al., 2001). Although considerable work has been performed identifying factors that promote or inhibit angiogenic responses (Folkman, 1997; Carmeliet and Jain, 2000; Yancopoulos et al., 2000; Conway et al., 2001), considerably less effort has focused on how individual and groups of ECs assemble into capillary tubes during these events. Identifying new molecular targets that block specific steps in EC morphogenesis may become crucial in efforts to inhibit angiogenesis in human diseases where angiogenesis is a pathogenic component (i.e. cancer, diabetic retinopathy, arthritis, atherosclerosis) (Folkman, 1995; Carmeliet and Jain, 2000).

One experimental approach to address these questions has been to use in vitro models of EC morphogenesis where many of the steps observed in vivo can be mimicked (Montesano et al., 1992; Vernon and Sage, 1995; Nicosia and Villaschi, 1999). The most promising assays for elucidating relevant molecules and pathways necessary for EC morphogenesis are those using 3D extracellular matrices (ECM) composed of collagen type I or fibrin (Montesano and Orci, 1985; Nicosia and Ottinetti, 1990; Davis and Camarillo, 1996; Ilan et al., 1998; Vernon and Sage, 1999; Yang et al., 1999; Bayless et al., 2000; Davis et al., 2000). These matrices represent the major matrix environments where angiogenic or vasculogenic events take place (Vernon and Sage, 1995; Senger, 1996; Nicosia and Villaschi, 1999). In some of these assays, particularly where ECs are suspended as individual cells in 3D matrices, most of the ECs undergo

morphogenesis simultaneously, which allows for an analysis of differential gene expression in large numbers of ECs. This is a critical aspect of EC morphogenic or regression microassays developed by our laboratory (Davis and Camarillo, 1996; Bayless et al., 2000; Davis et al., 2000; Davis et al., 2001). In these systems, differential gene expression can be directly correlated with distinct events in the EC morphogenic or regression cascade (Salazar et al., 1999; Davis et al., 2001).

Many studies over the years have shown that differential gene expression controls complex biological phenomena (Brown and Botstein, 1999). Recently, the development of gene array technology has revealed how classes of differentially regulated genes control processes such as yeast responses to glucose deprivation, fibroblast responses to serum mitogens and tumor development and apoptosis (DeRisi et al., 1997; Iyer et al., 1999; Perou et al., 2000; Maxwell and Davis, 2000). These approaches have been useful to characterize the role of previously identified genes in a given process. To identify relevant differentially expressed novel genes, additional techniques were developed including differential display, subtraction cDNA cloning and serial analysis of gene expression (SAGE) (Velculescu et al., 1995; Martin and Pardee, 1999). Using this latter technology, differentially regulated genes (known and novel) were identified in colon carcinoma-derived endothelium versus normal colonic endothelium (St. Croix et al., 2000).

Here, we present data using a number of the above technologies to investigate differential gene expression during human capillary morphogenesis in 3D collagen matrices. Using gene array analysis, differential display and cDNA library screening, hundreds of differentially expressed genes have been identified. We have examined the expression patterns of thousands of genes and have observed variable modes of gene upregulation as well as gene downregulation during a 48 hour time course of this process. In addition, the full length sequence of two new genes is presented. This survey analysis of differential gene expression serves as a basis for future studies concerning the molecular regulation of capillary morphogenesis in 3D extracellular matrices.

MATERIALS AND METHODS

The GFP-N2 vector was from Clontech, while the pAdEasy adenoviral system was kindly provided by Bert Vogelstein and Tong-Chuan He (Johns Hopkins School of Medicine, Baltimore, MD). A monoclonal antibody directed to hsp47 (Cates et al., 1987) was the kind gift of B.D. Sanwal (University of Western Ontario, London, Canada). Oligonucleotide primers were synthesized by Sigma-Genosys (The Woodlands, TX). The pQE30 vector and Ni/Cd-sepharose were from Qiagen (Valencia, CA). Antibodies to laminin were obtained from Sigma (St Louis, MO), anti-Id1 was from Upstate Biotechnologies (Lake Placid, NY), anti-collagen type IV from Chemicon (Temecula, CA) and anti-α2 macroglobulin from ICN (Costa Mesa, CA). Peroxidase-conjugated rabbit anti-mouse IgG and goat anti-rabbit IgG antibodies were from Dako (Carpinteria, CA) and chemiluminescence reagents were from Amersham (Piscataway, NJ). Rhodamine-conjugated rabbit anti-mouse IgG and fluorescein-conjugated goat anti-rabbit IgG were from Dako.

Capillary morphogenesis assay

Human umbilical vein endothelial cells (Clonetics, San Diego, CA) were cultured as described (Maciag et al., 1979). EC cultures in 3D collagen matrices were performed as described (Davis and Camarillo, 1996; Salazar et al., 1999) except that ECs (passages 2-5) were seeded at 2×10^6 cells per ml of gel.

DNA microarray analysis

DNA microarray analysis (DeRisi et al., 1997; Iyer et al., 1999) was used to study genomic-scale gene expression comparing four time points during capillary morphogenesis. Total RNA was extracted (Chomczynski and Sacchi, 1987) from ECs in the collagen gel, after collagenase treatment, using TRIzol reagent (Life Technologies, Grand Island, NY) at 0, 8, 24 and 48 hour time points. Approximately 360 gels for each time point were needed to obtain enough mRNA for this experiment. Total RNA was passed twice through Oligotex beads to obtain mRNA (Qiagen). The poly-A RNA was eluted in DEPC-H_2O and sent to Incyte Genomics (St Louis, MO), who performed the differential hybridization to a Unigem V chip containing 7,075 genes comparing 0 hour with 8 hour, 24 hour and 48 hour RNA samples. The data presented are ratios of hybridization between these time points.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was used to create cDNA templates and was equalized between the time points by spectrophotometry and formaldehyde agarose gel electrophoresis. Total RNA (5 μg) was used for reverse transcription (Stratagene, La Jolla, CA) to create random-primed cDNA at 0, 8, 24 and 48 hours of culture progression. RT-PCR amplification parameters used were typically 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 2 minutes; this was cycled 25 to 35 times, depending upon the gene, with a final extension at 72°C for 5 minutes using an PTC-100 thermal cycler (MJ Research, Watertown, MA). Primer sequences are shown in Table 1.

Northern and western blot analyses

Northern blot analyses were performed using total RNA equalized by spectrophotometry (3 μg per lane) from 0, 8, 24 and 48 hour time points as described (Salazar et al., 1999). Collagen gels were removed from wells, placed directly into boiling SDS sample buffer and heated to 100°C for 10 minutes, and stored at −20°C until use. Cell extracts were run on standard SDS-PAGE gels or 7% Blattler SDS-PAGE gels (collagen IV, laminin) (Blattler et al., 1972), and blots were incubated and developed as described previously (Salazar et al., 1999).

Differential display analysis

Differential display protocols (GenHunter, Nashville, TN) were used to identify genes that are differentially regulated during capillary morphogenesis (Liang and Pardee, 1998; Martin and Pardee, 1999). A cDNA copy of the total RNA was created using random primers from 0, 8, 24 and 48 hour time points. This cDNA amplification was performed using combinations of three downstream oligo dT primers, G-T_{11}M, C-T_{11}M and A-T_{11}M, and a series of random 10 mer upstream primers, AP-1 to AP-80 (ten sets of eight). We used sets one and two of the random upstream primers (AP-1 to AP-16) in combination with oligo dT downstream primers (GenHunter), which represent about 20% of the total primer combinations. Forty cycles of amplification, incorporating [γ-^{33}P]-dCTP, was used to create the random fragments. The fragments were then run on a 6% polyacrylamide sequencing gel using 1×TAE as the running buffer, and resolved by autoradiography. Differentially expressed bands were excised, boiled to extract DNA, and ethanol precipitated using glycogen as a carrier. Individual fragments were then amplified using the appropriate differential display primers appropriate for that band and purified for further use. DNA fragments were TA cloned into pGEM-T-Easy (Promega, Madison, WI) and sequenced by automated sequencing (Lonestar Laboratories, Houston, TX).

Endothelial cell morphogenic cDNA libraries

cDNA libraries were made using mRNA isolated from 8 and 24 hour cultures. Library production required 1 mg of total RNA isolated from 13 ml of collagen gel containing 2×10^6 cells/ml, which was aliquoted

Table 1. Primer sequences used for RT-PCR

Gene	Upstream primer	Downstream primer
Jagged-1	5′-TTCTCACTCGGGCATGATC-3′	5′-TTACAGCTGCCTCTGTTGTG-3′
Stanniocalcin	5′-TCCACATCTTCACTCAAGCC-3′	5′-CCTTTGGAAAGTGGAGCAC-3′
Egr-1	5′-TGAAGCTTCAGCAGTCCCATTTACTCAG-3′	5′-TGAAGCTTGACTGGTAGCTGGTATTG-3′
Sprouty	5′-AACAGTGTGGGAAGTGCAAG-3′	5′-GCTGAAGATCATCCATGAGG-3′
Myosin I-C	5′-TGGGAAGCAAAGGTGTCTAC-3′	5′-CACCACTGATAATGACGCAC-3′
Angiopoietin-2	5′-ACTGTTCTTCCCACTGCAATC-3′	5′-TATCATCACAGCCGTCTGG-3′
Id-1	5′-CGACATGAACGGCTGTTAC-3′	5′-GACACAAGATGCGATCGTC-3′
CTGF	5′-TTCCAAGACCTGTGGGATG-3′	5′-ATGCCATGTCTCCGTACATC-3′
RGS5	5′-AGTTGGGAATTCTCCTCCAG-3′	5′-AGCTGCTGTGGGAAGATATG-3′
Fibulin-3	5′-ACATTGTCCCAGACGCTTG-3′	5′-GCAATATGGAGGGATGGTAC-3′
α_2-Macroglobulin	5′-AGTCTTCATCCAATGAGGAGG-3′	5′-CAAATGCTCACAGTCACATGTC-3′
Caveolin-1	5′-CGGAGTTTTCATCCAGCCAC-3′	5′-TGGCACCAGGAAAATTAAAAG-3′
p16/ INK4A	5′-ACCCTCACATCAAGCGAAAC-3′	5′-GGAGTGAACGCATCGACTG-3′
α_1 integrin	5′-TATTCTTCCACCGAAGAGGTAC-3′	5′-TCCATTTCAAATGAAGCTGC-3′
α_2 integrin	5′-CGGATCCCCTGATTTTCAGCTCTCAGCC-3′	5′-ATGCTGAAAATTTGTTCTCC-3′
α_3 integrin	5′-GGACTGGTGCTGTGTACCTG-3′	5′-TTGGTCCTCTGGGTCCTTA-3′
α_5 integrin	5′-TTCAGTGGTGATGACACAGAAG-3′	5′-GTCAAGGAGCTGCCAAATC-3′
α_6 integrin	5′-CACGTCAGAAAGCAAGGAAG-3′	5′-CTCAGGGAGGAGATGTGCAG-3′
α_v integrin	5′-CCCGGATGTTTCTTCTCG-3′	5′-TGAAATCTCCGACAGCCAC-3′
α_1 integrin	5′-TACCCCAGCCGGTCCAACCTGA-3′	5′-ATGAATTATCATTAAAAGCTTC-3′
α_3 integrin	5′-GCTGTGACCTGAAGGAGAATC-3′	5′-GTGACACACTCTGCTTCTTCAC-3′
α_5 integrin	5′-AGATTGCCGTGAACCTCC-3′	5′-TCCATCCAATGCGATGTG-3′
Collagen IV α1 chain	5′-CCTGGCTTGAAAGGTGATAAG-3′	5′-CCCGCTATCCCTTGATCTC-3′
Collagen IV α2 chain	5′-TACATCAGCCGCTGTTCTG-3′	5′-TTTGGTTGGCACCTGAGG-3′
Laminin α4 chain	5′-TTGATGCCGTACTCTGCTG-3′	5′-CTGGGTCTTCTCCGTCAGTAG-3′
Laminin α5 chain	5′-CGCATCAGCTTCGACAGTC-3′	5′-TTGAGGTCCACATACTTGCC-3′
Laminin β1 chain	5′-TTTCAAGACATTCCGTCCAG-3′	5′-CCTCCACTTCTTCATTGAATC-3′
Laminin γ1 chain	5′-ACTCTTAATCGCCTGAACAC-3′	5′-CTTAGAGAGCCCACAGGACTAC-3′
Perlecan	5′-ACTCGGGCCAGTACATCTG-3′	5′-CACAGCACAGTGGAACTCAAC-3′
Nidogen	5′-GACATCGACGCAGTCTACG-3′	5′-TTTCCTTCTTTGAGAATGTCG-3′
Fibronectin	5′-CAACATTGATCGCCCTAAAG-3′	5′-CAACCACGGATGAGCTGTC-3′
SPARC	5′-ACTGTGGCAGAGGTGACTG-3′	5′-GGCGCTTCTCATTCTCATG-3′
Thrombospondin-1	5′-ATCGAGGATGCCAACCTG-3′	5′-AGGGCTGGAACCATTCAC-3′
Thrombospondin-2	5′-CTTCGTGCGCTTTGACTAC-3′	5′-CCTTTGGCCACGTACATC-3′
CMG1 (upstream)	5′-GCCAGCAATCACAAATCTTC-3′	5′-CGCTTGTCTTTCAGTAAATATG-3′
CMG1 (downstream)	5′-GGCTGGTAATCACTCTTGAC-3′	5′-TTTGACTTTCAGACATTCAACG-3′
CMG2	5′-TCCTGCAGAAGAGCCTTG-3′	5′-TGCATATGATGGCACCAAG-3′
Fte-1	5′-AGGGAGCCAAGAAGAAAGTG-3′	5′-ATCGGTAGTCTTGACATCAAC-3′
NADH Ubiq. Oxid.	5′-GGATTTGCTGCATTTGTG-3′	5′-TGTAAGGGATAAACCAGAC-3′
Prothymosin-α	5′-CCGAAATCACCACCAAGG-3′	5′-CATCCTCGTCGGTCTTCTG-3′
Melanin conc. horm.	5′-CATCAAACTAAGAATGGCAAAG-3′	5′-CCAACAGGGTCGGTACACTC-3′
FGF-2	5′-ACTCCGGCCTCTATGCTTG-3′	5′- TCCACAATGCAGGTGTAGTTG-3′
TFPI-2	5′-TTGGACTCTGGCATACCTAAC-3′	5′-GGAAGATTGAGCTGGAGTTC-3′

into 25 μl aliquots in 96-well A/2 microplates. After poly-A selection, first- and second-strand cDNA synthesis was performed with oligo dT primers. The cDNA was fractionated by size, and mass cloned into the ZAP-XR vector using the Uni-Zap XR Stratagene system. The cloning was performed unidirectionally, based on opposing *Eco*RI and *Xho*I restriction sites at the 5′ and 3′ ends, respectively. Each fraction was then packaged, and the first fraction was used for amplification while the remaining fractions were left unamplified using standard methodologies from Stratagene. These libraries were screened using using ^{32}P-labelled partial cDNAs to obtain larger clones (Wahl and Berger, 1987).

Construction of recombinant adenoviruses

Recombinant adenoviral constructs were prepared essentially as described (He et al., 1998). Full length CMG-1 and CMG-2 were cloned into the GFP-N2 vector (Clontech) and were then amplified as GFP fusion protein constructs and then further cloned into the pAdShuttle-CMV vector. CMG-1 and CMG-2 were amplified and cloned into pEGFP-N2 (Clontech) using *Xho*I and *Bam*HI and *Xho*I and *Eco*RI restriction sites, respectively. The following primer sets were used to amplify CMG-1 or CMG-2 inserts: CMG-1 5′-AGCTCGAGACAAT-GGCCAGCAATCAC-3′; 5′-AGGGATCCGGTTTCCGCTGGTGCT-ATG-3′. CMG-2 5′-AGCTCGAGAGGATGGTGGCGGAGCGGT-3′; 5′-AGGAATTCAGCAGTTAGCTCTTTCTC-3′.

For cloning of these cDNAs into the pShuttle-CMV vector, *Bgl*II-*Xba*I and *Xho*I-*Xba*I were used for CMG-1 and CMG-2, respectively. The common downstream primer 5′-AGTCTAGATTATGATCTA-GAGTCGCGGC-3′ was used with the upstream primer 5′-AGAGATCTACAATGGCCAGCAATCAC-3′ for CMG-1 and 5′-AGCTCGAGAGGATGGTGGCGGAGCGGT-3′ for CMG-2. These pShuttle-CMV clones were then recombined with pAdEasy-1 and transfected into 293 cells to produce recombinant viruses. The viruses were then amplified through three passages in 293 cells before use. Extracts of 293 cells infected with these viruses were tested on SDS-PAGE gels and western blots with anti-GFP antibodies, showing that the fusion proteins had a molecular weight indicative of intact CMG-1-GFP and CMG-2-GFP fusion proteins. Endothelial cell monolayers were infected on gelatin-coated coverslips for 4-5 hours in serum-free media and then this media was replaced with complete growth media overnight. After 24 hours, cultures were fixed with 3% paraformaldehyde and were either directly examined by fluorescence microscopy or processed further for immunofluorescence staining as described (Salazar et al., 1999).

Recombinant CMG-2 production and extracellular matrix protein binding assays

A portion of recombinant CMG-2 (residues 34-214) was produced in *E. coli* as a recombinant His-tagged protein. A CMG-2 cDNA was

unidirectionally cloned into pQE30 through *Bam*HI and *Hind*III sites. Primers used to amplify CMG-2 were: 5′-AGGGATCCCAGG-AGCAGCCCTCCTGC-3′; 5′-AGAAGCTTAGAAGAATTAATTAT-TCC-3′. The recombinant protein was purified using Ni/Cd-sepharose as described (Bayless and Davis, 2001) and approximately 3 mg of protein was obtained from 400 ml of IPTG-induced bacteria. Control GFP was produced as a His-tagged protein and purified in the same way. Both proteins were adsorbed to plastic microwells at 10 μg/ml and, after detergent blocking (0.1% Tween-Tris-saline, pH 7.5) for 30 minutes, biotinylated extracellular matrix proteins were added (1 μg/ml) in 0.1% Tween-Tris-saline containing 1% BSA for 1 hour. The biotinylated matrix proteins were prepared as described (Davis and Camarillo, 1993). After washing, the wells were further incubated with avidin-peroxidase at 1 μg/ml for 30 minutes in Tween-Tris-saline-BSA and, after washing, were developed for peroxidase activity and read at 490 nm.

RESULTS

DNA microarray analysis of differential gene expression during capillary morphogenesis in 3D collagen matrices

To determine patterns of gene expression during EC morphogenesis and identify differentially regulated genes during this process we performed DNA microarray analysis with mRNA isolated from cultures at 0, 8, 24 and 48 hours of EC morphogenesis (Fig. 1). Cy3 and Cy5-labeled cDNAs were used to probe microarray chips containing 7,075 gene elements. A total of three chips were hybridized for the time course analysis by combining Cy5-labeled 0 hour cDNA with either Cy3 labeled 8 hour, 24 hour, or 48 hour cDNA. Tables 2 and 3 show gene expression differences between different time points of EC morphogenesis of either upregulated or downregulated genes, respectively. In both tables, genes are grouped into functional categories such as growth factors, ECM-related genes, signaling molecules, EC differentiation markers, cell cycle and apoptosis regulators and miscellaneous genes. This data is presented as a ratio of the difference in fluorescence for each given gene element, showing changes between experimental versus reference cDNA (0 hour). The six genes showing the greatest changes in expression of the more than 7,000 genes assayed are shown in Tables 2 and 3 and are labeled with an asterisk for each of the 8, 24 and 48 hours time points. The Notch ligand, Jagged-1 (Lindsell et al., 1995), was induced 11.1-fold at 8 hours, the extracellular inorganic phosphate regulator, stanniocalcin (Olsen et al., 1996), was induced 9.8-fold at 24 hours, and the angiogenic regulator, angiopoietin-2 (Yancopoulos et al., 2000), was induced 7.8-fold at 48 hours. By contrast, the EC-derived mesenchymal cell growth factor, connective tissue growth factor, CTGF (Grotendorst, 1997), was downregulated 11.3-fold at 8 hours, the extracellular matrix gene, fibulin-3 (Giltay et al., 1999), was downregulated 27.2-fold at 24 hours, and a member of the regulator of G-protein signaling family, RGS5 (Seki et al., 1998), was downregulated 34-fold at 48 hours.

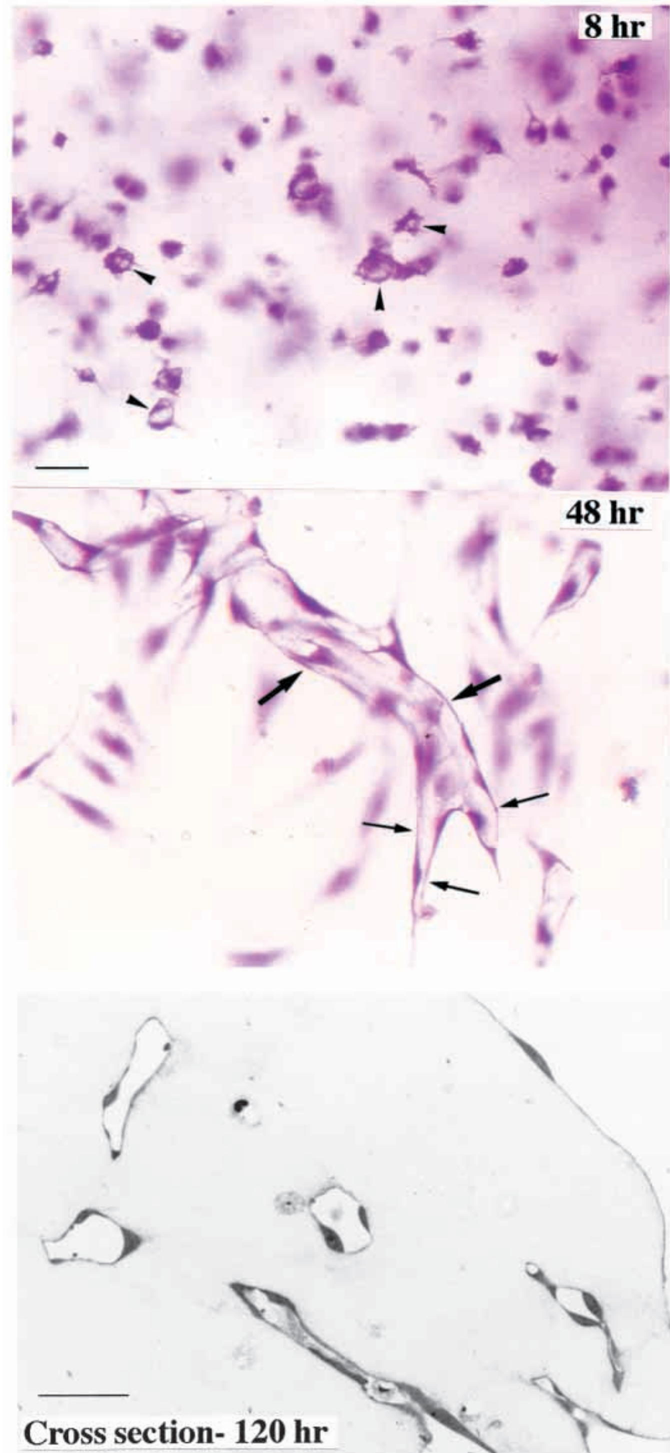

Fig. 1. Capillary morphogenesis in 3D collagen matrices. Human ECs were suspended in 3D collagen matrices and, at varying times of cultures, were fixed, stained with toluidine blue and photographed. Some cultures were embedded in plastic and thin sectioned to obtain cross-sectional views of the capillary tubes. Times of the analysis are indicated. Arrowheads show ECs with intracellular vacuoles and arrows indicate an EC-lined capillary tube. Bar, 50 μm (8 hour panel); bar, 50 μm (120 hours panel).

Based on the sampling of the genes represented in the microarray and additional gene expression data, the differentially regulated mRNAs appeared to fall primarily into one of five general patterns of gene expression (Fig. 2). These patterns of gene expression were confirmed for selected genes using semiquantitative RT-PCR (Fig. 3A), northern blot (Fig. 3B) and western blot analyses (Fig. 4).

Table 2. Upregulation of distinct classes of genes during capillary morphogenesis in 3D collagen matrices as determined by DNA microarray analysis

	Reference sample	Experimental sample		
Gene category	0 hour	8 hour	24 hour	48 hour
Growth factors/hormones, receptors/modulators				
Jagged-1*	1	11.1*	5.6	−1.1
Angiopoietin-2*	1	1.3	3.4	7.8*
Stanniocalcin*	1	9.2	9.8*	4.0
Placental growth factor	1	3.1	2.4	2.9
Melanin concentrating hormone	1	2.5	1.4	1.0
PDGF-b	1	3.7	3.1	2.8
IGF-2	1	1.5	1.8	2.6
Midkine	1	1.2	2.2	1.5
Sprouty	1	1.4	2.0	3.4
Flt-1 (VEGF-R1)	1	1.8	1.5	2.0
Eph A1	1	1.8	1.5	1.1
Eph A7	1	1.4	1.6	1.5
ECM/ ECM receptors, cytoskeleton				
Collagen type IV, α1 chain	1	2.3	3.6	4.0
Laminin, γ1 chain	1	1.9	2.3	1.7
Laminin, α4 chain	1	1.2	1.6	1.6
Heparan sulfate N-deacetylase N-sulfotransferase I	1	2.0	2.3	2.5
Chondroitin sulfate/keratan sulfate sulfotransferase	1	1.1	1.3	3.2
Lysyl oxidase	1	−1.5	1.0	3.7
Lysyl hydroxylase	1	1.2	1.3	1.9
α1 integrin subunit	1	1.8	2.1	2.4
α2 integrin subunit	1	4.6	3.0	1.1
αv integrin subunit	1	2.5	1.1	1.1
GARP	1	3.9	3.1	1.9
ICAM-1	1	4.1	1.7	1.0
Podocalyxin	1	3.0	1.5	1.5
Myosin IC	1	1.7	3.1	3.1
Myosin VI	1	1.3	1.7	2.3
Gelsolin	1	1.1	1.5	2.2
WASP-interacting protein	1	1.4	1.4	1.9
RhoGAP-5	1	1.9	1.8	2.4
Diaphanous 2	1	1.5	1.9	2.6
Coronin 2B	1	1.9	2.1	1.6
Coronin 2A	1	1.8	1.3	1.3
Proteinase inhibitors				
Alpha-2 macroglobulin	1	1.4	3.1	6.1
Signaling molecules/ transcription factors				
Egr-1	1	6.5	4.7	4.7
HLX-1 homeobox gene	1	2.8	3.1	3.4
Gp130 (oncostatin M receptor)	1	2.5	1.7	−1.1
JAK-1	1	−1.1	1.2	2.6
JAK binding protein (SOCS-1)	1	3.6	4.1	3.0
IP-3 kinase A	1	2.6	1.6	1.5
Diacylglycerol kinase δ	1	2.1	3.0	2.9
RGS-3	1	1.1	1.2	2.0
EC differentiation markers				
Angiotensin converting enzyme	1	1.3	1.7	2.6
Von Willebrand Factor	1	1.1	1.3	2.0
Thrombomodulin	1	1.0	1.4	1.9
Protein S	1	−1.1	1.3	2.2
CD39	1	1.0	3.1	7.2
Cell cycle/apoptosis regulators				
Cdc 14	1	1.7	2.0	3.1
Bcl-2	1	1.3	1.6	2.0
A20 zinc-finger protein	1	2.6	1.9	1.8
Pim-1	1	1.5	2.8	1.6
Miscellaneous				
KIAA0212	1	2.3	3.6	4.0
KIAA0758	1	1.0	2.4	5.5
FLJ 20641	1	2.5	1.4	1.0
Ox-2 antigen	1	3.3	2.5	1.9
CD26	1	1.8	2.9	2.9
Sodium bicarbonate cotransporter-2	1	3.9	4.2	2.4
HMG CoA reductase	1	1.2	1.8	3.0
Mevalonate (diphospho) decarboxylase	1	1.0	1.4	2.0
Mevalonate kinase	1	1.5	1.5	1.6
Farnesyl-diphosphate farnesyltransferase-1	1	1.5	1.5	2.4

Patterns A-E illustrate observed trends in gene expression (Fig. 2A-E). Genes showing these patterns are listed to the right and become grouped into expression patterns, which may reflect functional importance at different stages of EC morphogenesis.

Induction and role of basement membrane matrix and integrin genes during endothelial cell morphogenesis in 3D collagen matrices

It is interesting that a series of genes related to basement membrane synthesis and assembly are induced (pattern C), including the collagen type IV α1 and laminin γ1 chains. These inductions were confirmed at the protein level by marked induction of collagen type IV and laminin synthesis and secretion during morphogenesis (Fig. 4A,C). In addition, collagen type IV deposits extracellularly around developing capillary tubes (Fig. 4B). Also induced is the α1 integrin subunit, which forms a heterodimer, $\alpha1\beta1$, with affinity for both type IV collagen and laminin (Senger et al., 1997). The α2 integrin subunit, which forms the heterodimer, $\alpha2\beta1$, is markedly upregulated (pattern B) and may similarly contribute to the appropriate assembly of basement membrane matrix. Blocking antibodies to the α2 integrin subunit, but not control blocking antibodies to the α5 integrin subunit, cause complete collapse of pre-existing tubes in the 3D collagen matrix environment (Davis et al., 2001), suggesting a crucial role for $\alpha2\beta1$ in the maintenance of forming capillary tubes. We have previously shown that $\alpha2\beta1$ is required for the initiation of lumen formation through EC intracellular vacuole formation and coalescence in 3D collagen matrices (Davis and Camarillo, 1996).

We have expanded this list of genes by performing differential expression analysis of an additional set of extracellular matrix and integrin genes (Fig. 5). As shown in Fig. 5, the RT-PCR data supports the gene array data showing inductions of the collagen type IV α1 chain, laminin γ1 and the α2 and α1 integrin subunits. Some of the genes examined show stable expression throughout the time course, whereas others are differentially expressed. Such differentially expressed genes include the laminin α4 subunit,

Table 3. Downregulation of distinct classes of genes during capillary morphogenesis in 3D collagen matrices as determined by DNA microarray analysis

	Reference sample	Experimental sample		
Gene category	0 hour	8 hour	24 hour	48 hour
Growth factors/hormones, receptors/modulators				
Connective tissue growth factor (CTGF)*	1	−11.3*	−8.9	−5.9
Cyr61	1	−3.9	−1.5	1.0
Bone morphogenetic protein-6	1	−2.3	−3.0	−3.3
Frizzled related protein-1	1	−1.6	−2.6	−3.2
Eph B4	1	−1.3	−1.4	−1.5
Eph B1	1	−1.1	−1.4	−1.5
Ephrin A1	1	−1.7	−1.9	−1.3
ILGFBP-7/angiomodulin	1	−2.6	−4.4	−3.9
Signaling molecules/ transcription factors				
RGS-5*	1	−2.6	−13.9	−34.0*
RGS-4	1	−5.4	−7.5	−7.8
RGS-2	1	−2.0	−2.9	−3.2
Transglutaminase-2 (Gh)	1	−4.4	−10.3	−9.5
Axl receptor tyrosine kinase	1	−1.7	−2.1	−2.9
Id-1	1	−9.3	−8.6	−9.8
Id-2	1	−2.1	−2.5	−2.9
Id-3	1	−5.0	−4.5	−6.0
HES-1	1	−4.1	−3.6	−2.7
Coup-TFII	1	−2.4	−2.5	−3.0
ECM/ECM receptors, cytoskeleton				
Fibulin-3 (S1-5 protein)*	1	−5.4	−27.2*	−24.7
Thrombospondin-1	1	−1.8	−2.4	−1.5
Collagen type XIII	1	−2.6	−3.2	−3.5
β3 integrin subunit	1	1.0	−1.8	−4.4
ICAM-2	1	−5.0	−3.9	−2.1
CD44	1	−1.5	−2.6	−3.2
RHAMM	1	1.0	−1.1	−2.5
Syntenin	1	−2.3	−4.6	−5.6
Multimerin	1	−1.7	−2.4	−2.2
Proteinase inhibitors				
PAI-1	1	2.1	−2.3	−6.6
PAI-2	1	1.1	−2.3	−3.7
EC differentiation markers				
Endothelin-1	1	−10.3	−5.6	−2.0
Cell cycle/apoptosis regulators				
Cdc2 (Cdk1)	1	−2.8	−3.4	−3.5
Cdc20	1	−2.1	−2.3	−3.0
Cdc25B	1	−1.8	−2.2	−2.2
Cdc46 (mcm5)	1	−1.9	−2.1	−1.6
Mcm2 (mitotin)	1	−2.6	−3.1	−3.5
Mad-2	1	−1.5	−2.1	−2.4
Cyclin A1	1	−2.4	−2.8	−4.2
Cyclin B2	1	−2.2	−2.5	−2.2
PRC-1	1	−1.6	−2.1	−2.3
Replication protein A1	1	−1.7	−2.2	−2.8
Proliferation assoc. 2G4	1	−1.7	−1.6	−2.3
PCNA	1	−1.7	−2.1	−1.6
Thymidylate synthetase	1	−3.9	−5.1	−3.2
Kinesin-like 4 (Kid)	1	−1.4	−1.8	−2.2
Kinesin-like 5	1	−1.9	−2.5	−2.4
Ran	1	−2.2	−2.3	−2.5
Ran-binding protein-1	1	−1.8	−2.0	−2.1
Caspase-4	1	−2.0	−3.1	−2.8
Proteasome subunit, α-1	1	−2.4	−2.2	−2.8
Proteasome subunit, β-1	1	−2.2	−2.2	−2.2
Proteasome subunit, β-3	1	−1.6	−1.8	−1.9
Proteasome subunit, β-4	1	−1.5	−1.6	−2.3
Proteasome subunit, β-7	1	−1.9	−2.5	−2.5
Proteasome 26S, non-ATPase subunit 11	1	−1.6	−2.0	−1.8
Proteasome 26S, non-ATPase subunit 4	1	−1.6	−1.5	−1.8
Ubiquitin B	1	−1.7	−2.2	−1.4
Ubiquitin C	1	−1.8	−2.6	−1.6
Ubiquitin C-terminal esterase L1	1	−2.1	−3.3	−3.0
Miscellaneous				
Pentaxin	1	−8.2	−10.8	−15.2
Flotillin-1	1	−2.7	−2.1	−3.0
VAMP-5 (myobrevin)	1	−2.1	−2.3	−2.7
Annexin A1	1	−5.2	−4.1	−1.9

thrombospondin-2, and αv integrin subunit, which are upregulated while the β3 integrin subunit, thrombospondin-1 and the interstitial matrix protein, fibronectin are downregulated. Interestingly, differential expression was observed for the α1, α2, αv and β3 integrin subunits which are known to regulate angiogenesis in vivo (Brooks et al., 1994; Senger et al., 1997). The laminin α5 chain was expressed in a stable fashion but we have detected little or no expression of the laminin α1 chain (not shown) during morphogenesis. This suggests that two laminin isoforms, laminin-8 (α4, β1, γ1) and laminin-10 (α5, β1, γ1), are particularly relevant during this process. Recent studies support this conclusion showing a role for the laminin-8 and -10 isoforms in endothelial cell function (Miner et al., 1998; Lefebvre et al., 1999; Kortesmaa et al., 2000).

Other induced genes involved in basement membrane matrix assembly are heparan sulfate deacetylase/ sulfotransferase (i.e. an enzyme that regulates the rate-limiting step in heparan sulfate synthesis) (Kakuta et al., 1999), lysyl oxidase, lysyl hydroxylase, α_2-macroglobulin and melanoma-associated antigen (Table 2; Fig. 3; Fig. 4). Lysyl oxidase and lysyl hydroxylase contribute to matrix stability through protein-protein crosslinking (Uzawa et al., 1999), whereas α_2-macroglobulin is a broad-spectrum proteinase inhibitor with growth factor binding domains (Jensen, 1989; Borth, 1992; Gonias et al., 2000). α_2-macroglobulin may contribute to ECM stability by inhibiting proteolysis and/or regulate EC morphogenesis by controlling growth factor availability. It is markedly induced both at the level of mRNA (Fig. 3A) and protein (Fig. 4C). As shown in Fig. 4C, two induced α_2-macroglobulin bands are observed, which represent the intact protein (upper band) and a proteolytically cleaved form (lower band) that is consistent with proteolytic cleavage in its bait region (Jensen, 1989; Borth, 1992). Previously, we have observed marked inductions of matrix metalloproteinase 1 (MMP-1), MMP-9, MT1-MMP, and uPA during EC morphogenesis (Davis et al., 2001) that may account for this α_2-macroglobulin cleavage. Melanoma-associated antigen is a putative extracellular matrix protein with an RGD sequence and peroxidase-like domains (Mitchell et al., 2000) (Fig. 3B). A highly homologous *Drosophila* protein named peroxidasin contains

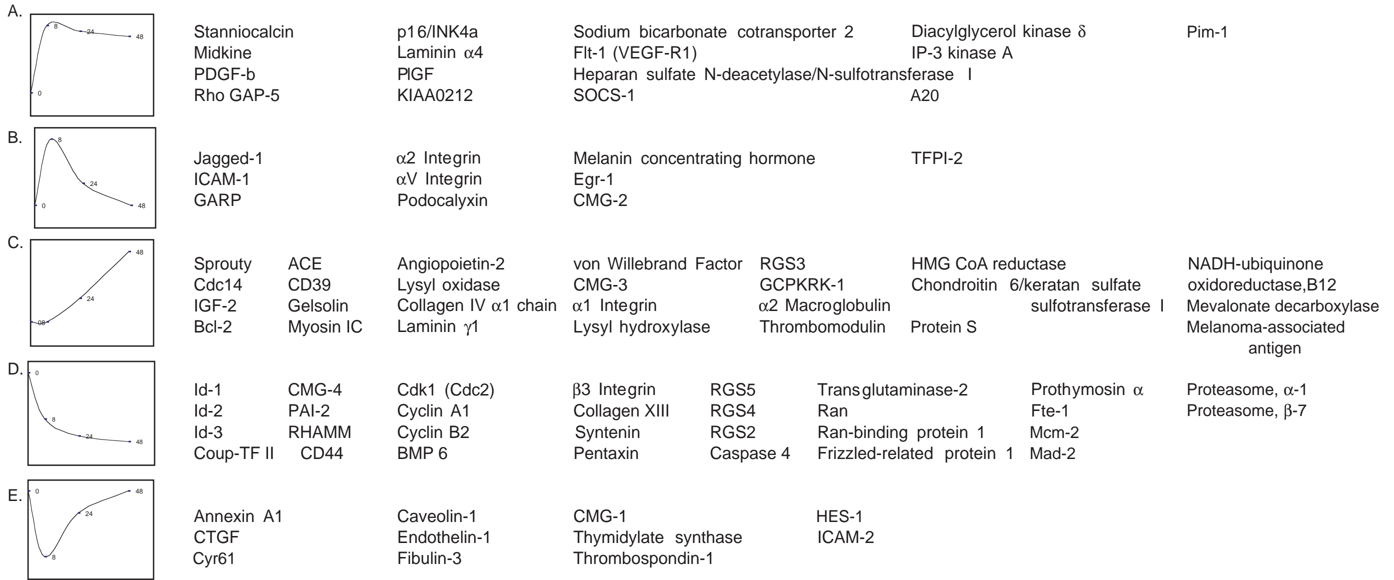

Fig. 2. Patterns of gene expression observed during capillary morphogenesis in 3D collagen matrices. Five different major patterns of gene expression were observed in this system, indicated as patterns A-E. Genes, whose expression patterns most closely fit these patterns, are indicated next to each pattern.

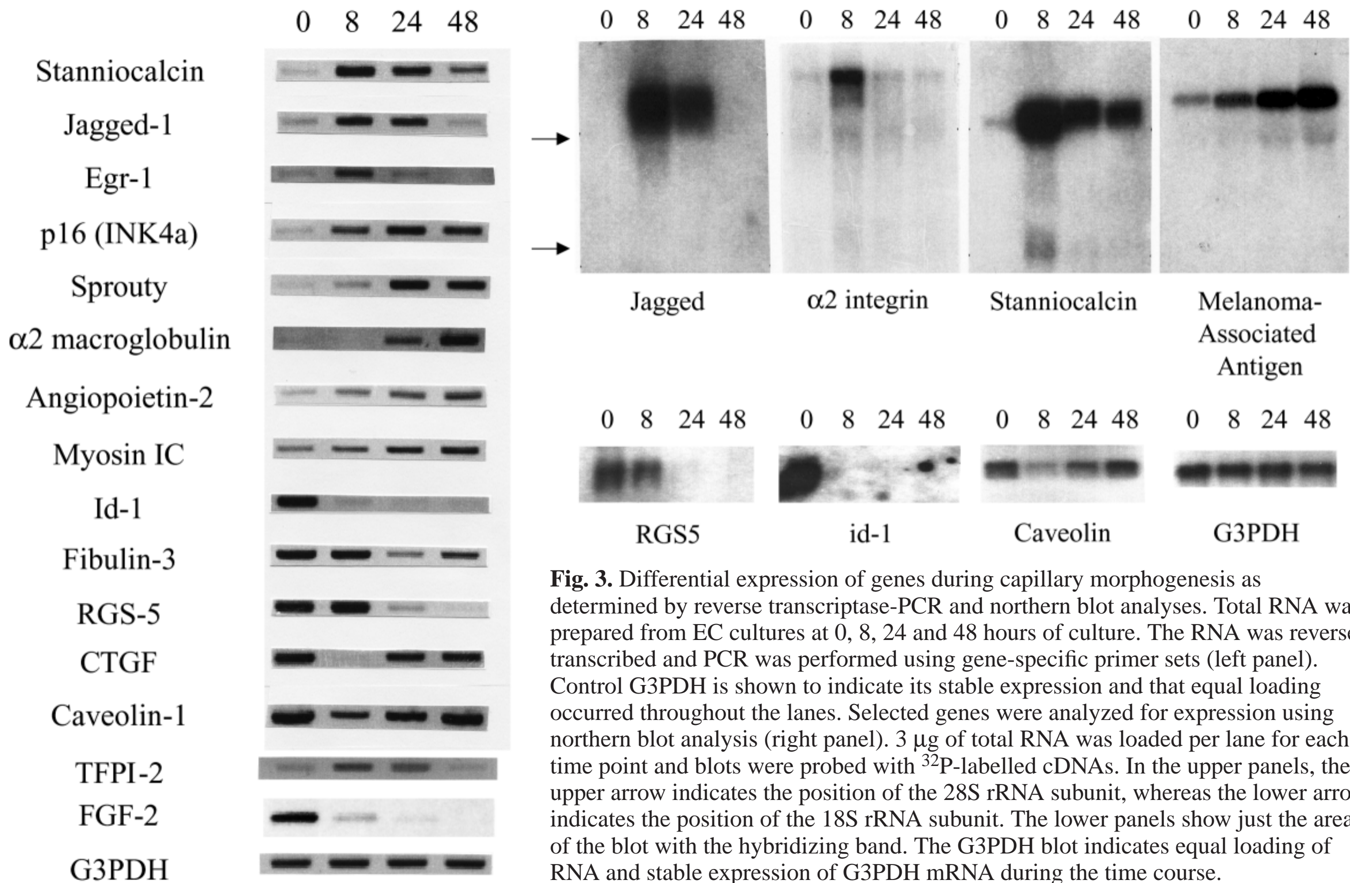

Fig. 3. Differential expression of genes during capillary morphogenesis as determined by reverse transcriptase-PCR and northern blot analyses. Total RNA was prepared from EC cultures at 0, 8, 24 and 48 hours of culture. The RNA was reverse transcribed and PCR was performed using gene-specific primer sets (left panel). Control G3PDH is shown to indicate its stable expression and that equal loading occurred throughout the lanes. Selected genes were analyzed for expression using northern blot analysis (right panel). 3 μg of total RNA was loaded per lane for each time point and blots were probed with ^{32}P-labelled cDNAs. In the upper panels, the upper arrow indicates the position of the 28S rRNA subunit, whereas the lower arrow indicates the position of the 18S rRNA subunit. The lower panels show just the area of the blot with the hybridizing band. The G3PDH blot indicates equal loading of RNA and stable expression of G3PDH mRNA during the time course.

peroxidase and extracellular matrix motifs and has been found to incorporate into basement membranes (Nelson et al., 1994). Overall, these data indicate that ECs are synthesizing basement membrane components such as collagen type IV, laminin and heparan sulfate for the purpose of differentiation during morphogenesis. By contrast, other ECM proteins such as fibronectin, which are more typically expressed by mesenchymal cells, are downregulated.

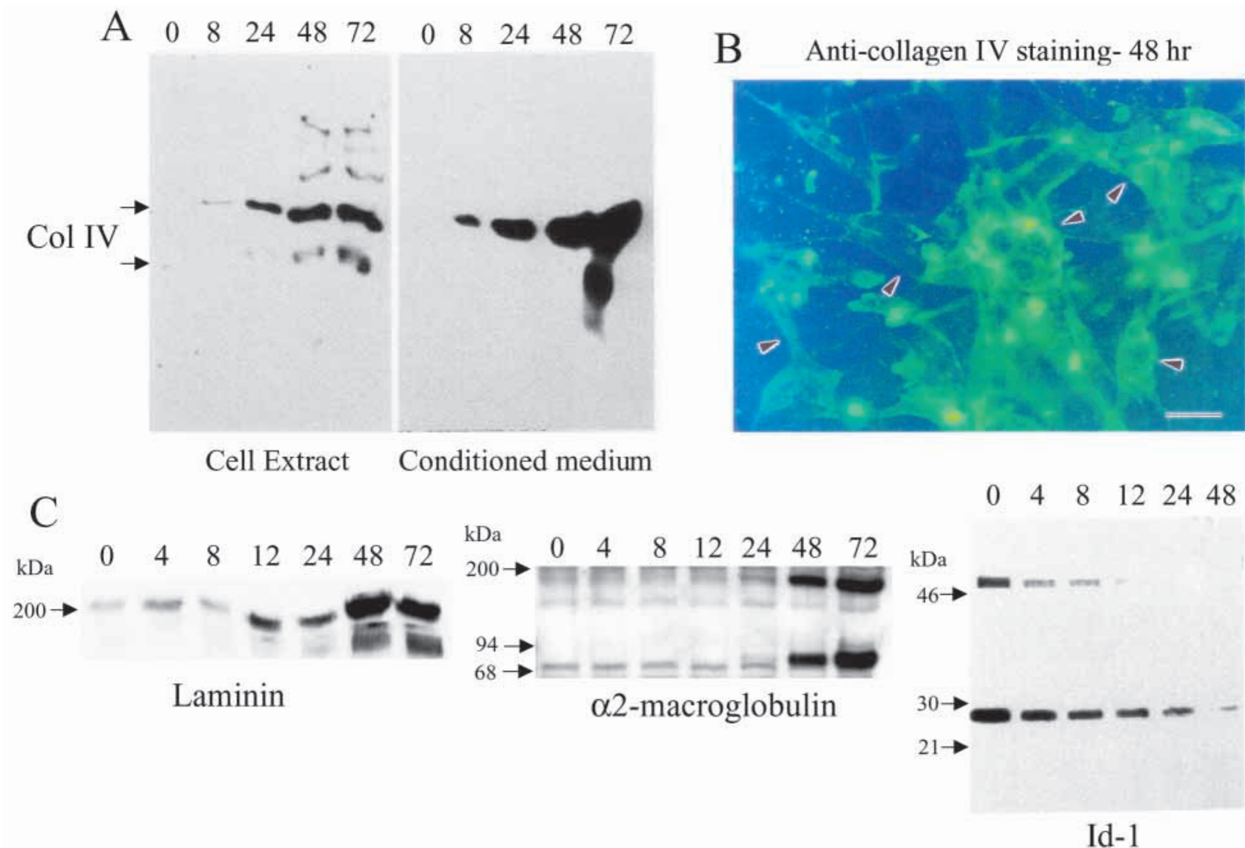

Fig. 4. Differential gene expression during capillary morphogenesis as determined by western blot analysis and immunohistochemistry. EC-collagen gel extracts or conditioned medium were prepared or collected at the indicated time points. Samples were run on SDS-PAGE and, after blotting, were probed with antibodies directed to various proteins. Cell extracts were probed with antibodies directed to collagen type IV, α_2-macroglobulin and Id-1. Conditioned medium samples were probed with antibodies directed to collagen type IV or laminin. The arrows in the collagen type IV blots represent the α1 and α2 chains of collagen type IV. Arrows indicate the position of molecular weight markers on the blots. In B, a 48 hour culture was stained with antibodies to collagen type IV under non-permeabilizing conditions. No staining was observed without the addition of the primary anti-collagen type IV antibody. Bar, 50 µm.

Blockade of collagen type IV synthesis interferes with endothelial cell morphogenesis in 3D collagen matrices

To address a potential functional role for collagen type IV in EC morphogenesis, cultures were treated with ethyl 3,4-dihydroxybenzoate (edb), a prolyl hydroxylase inhibitor that blocks collagen synthesis (Sasaki et al., 1987; Nandan et al., 1990). As shown in Fig. 6A and B, blockade of collagen type IV induction prevents progression of tube formation. Furthermore, edb blocks collagen type IV synthesis during capillary morphogenesis (Fig. 6C). Providing support for our findings are previous studies showing that blockade of collagen or basement membrane synthesis can interfere with angiogenic responses (Ingber and Folkman, 1988; Maragoudakis et al., 1988; Iruela-Arispe et al., 1991; Sephel et al., 1996). The addition of edb to proliferating ECs at the doses used in the above experiment on plastic substrates had no toxic effect and previous investigators have shown no toxic effect of edb on different cell types (Nandan et al., 1990). These data show that collagen type IV synthesis, which is upregulated by increases in the α1 collagen IV subunit mRNA, appears to be necessary for capillary tube formation in collagen matrices. We are unable to rule out the possibility that other triple-helical collagens are involved in morphogenesis, since edb can block the synthesis of other collagen types as well.

Upregulation of cytoskeletal proteins during endothelial cell morphogenesis in 3D collagen matrices

Additional upregulated genes (pattern C) include cytoskeletal and cytoskeletal regulatory proteins that mediate ECM-mediated signalling pathways. These are myosin IC (Swanson et al., 1999), myosin VI, diaphanous-2, WASP-interacting protein (WIP), gelsolin and RhoGAP-5. WIP is known to bind the actin regulatory protein, profilin (Anton et al., 1998), a gene that shows a similar expression pattern (Salazar et al., 1999). We have previously reported that gelsolin, VASP and profilin are coordinately upregulated during capillary morphogenesis (Salazar et al., 1999). It is interesting that upregulation of myosin IC as well as coronins 2A and 2B (Table 2) occur during EC morphogenesis since they have been observed associated with phagosome membranes (Morrissette et al., 1999; Swanson et al., 1999). We have previously suggested that phagosomes show similarities with the intracellular vacuole compartment that controls EC lumen formation (Davis and Camarillo, 1996; Bayless et al., 2000).

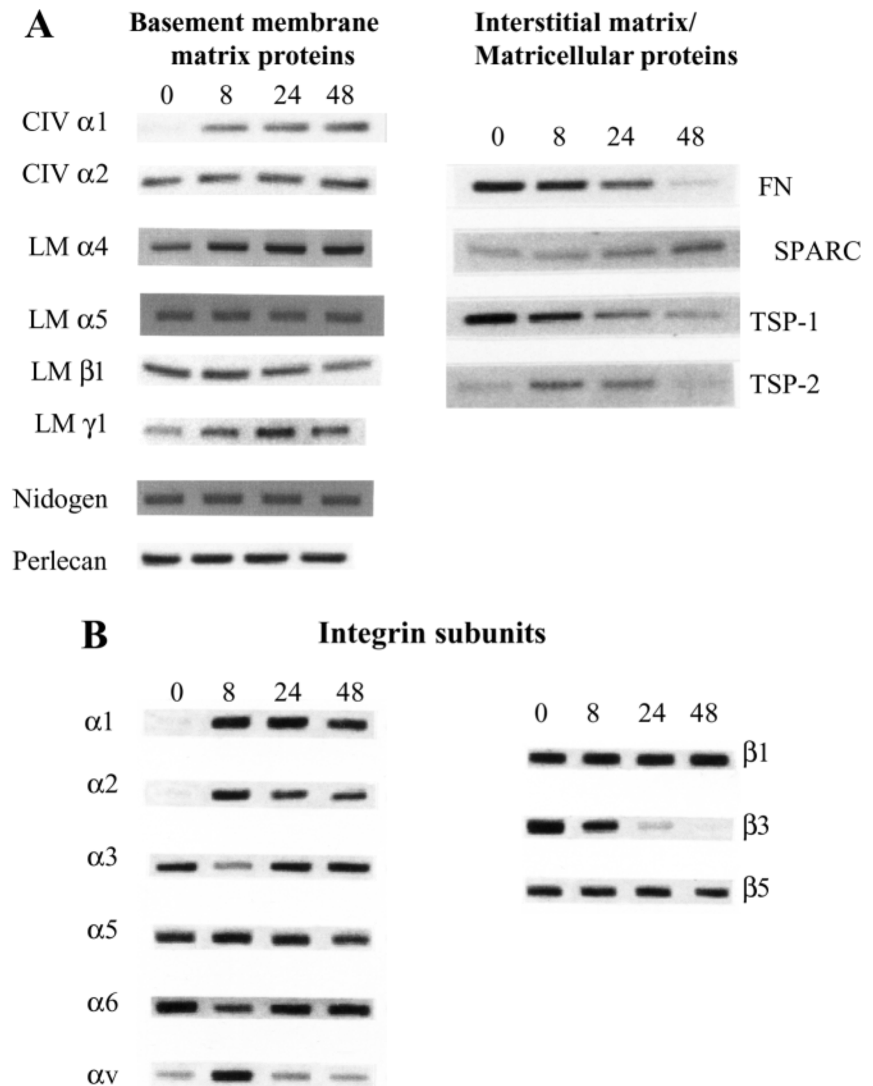

Fig. 5. Differential expression of extracellular matrix and integrin genes during capillary morphogenesis. RT-PCR was performed to assess the mRNA expression patterns of various extracellular matrix proteins (A) and integrin subunits (B) over a 48 hour time course. Total RNA was prepared from EC cultures at 0, 8, 24 and 48 hours of culture. The RNA was reverse transcribed for RT-PCR and PCR was performed using gene specific primer sets.

Induction of genes associated with endothelial cell differentiation and cholesterol biosynthesis occur during endothelial cell morphogenesis in 3D collagen matrices

In addition to the induction of ECM- or cytoskeleton-related genes, there is interesting evidence that EC differentiation genes are upregulated (Table 2). Induced EC differentiation markers include von Willebrand factor, angiotensin converting enzyme (ACE), thrombomodulin, protein S and CD39 (an EC cell surface ecto ATP/ADPase) (Saijonmaa et al., 2000; Goepert et al., 2000). These data suggest that phenotypic changes are occurring with increasing expression of EC-specific genes. Other induced genes include HMG CoA reductase, mevalonate decarboxylase, and mevalonate kinase, which are genes associated with cholesterol biosynthesis (Olivier and Krisans, 2000). Interestingly, these three enzymes have recently been found to associate with peroxisomes (Olivier and Krisans, 2000). In addition, CD26, a dipeptidyl peptidase that regulates hormone and chemokine activities, is also upregulated (Mentlein, 1999). Additional induced genes (pattern B) include ICAM-1, GARP (Ollendorff et al., 1994), jagged-1 (Lindsell et al., 1995), melanin concentrating hormone (also identified by differential display) (Hawes et al., 2000), the transcription factor, egr-1 (Silvermann and Collins, 1999) and the proteinase inhibitor, tissue factor pathway inhibitor-2 (TFPI-2) (Rao et al., 1996) (Table 2; Fig. 3).

Regulated expression of growth factors, hormones and genes regulating the JAK-STAT pathway during endothelial cell morphogenesis in 3D collagen matrices

A series of hormones and growth factors are induced (pattern A) such as stanniocalcin, PDGF-b, placental growth factor (PlGF) (Carmeliet and Jain, 2000; Conway et al., 2001) and midkine, which suggests that ECs produce a series of autocrine factors during morphogenic events. Interestingly, a VEGF receptor with affinity for PlGF, VEGF-R1 (Park et al., 1994), is upregulated along with PlGF. In addition, signalling molecules in the JAK-STAT pathway are induced including the common gp130 subunit of a number of growth factor receptors (e.g. oncostatin M, leukemia inhibitory factor, IL-6 receptors) (Funamoto et al., 2000; Imada and Leonard, 2000). Oncostatin M and leukemia inhibitory factor have been reported to inhibit EC proliferation (Takashima and Klagsbrun, 1996). In addition, JAK-1 and the JAK-1 inhibitor, SOCS-1 are induced (Krebs and Hilton, 2000) (Table 2). Interestingly, three genes whose transcription is dependent on this pathway, α_2-macroglobulin, protein S and angiotensin-converting enzyme, are upregulated along with these genes, suggesting a functional upregulation of the JAK-STAT signal transduction pathway (Hooper et al., 1997; Zhang et al., 1999b; Saijonmaa et al., 2000). In addition, the anti-apoptotic gene, Pim-1, which is a downstream target of STAT3 following gp130 receptor ligation (Shirogane et al., 1999), is upregulated (Table 2). Two other anti-apoptotic genes, bcl2 (Nor et al., 1999) and A20 (Lee et al., 2000), were upregulated along with Pim-1 (Table 2), whereas a proapoptotic gene, Nip2, was downregulated (Brusadelli et al., 2000) (Table 4).

Sprouty, a negative regulator of FGF-signalling and tyrosine kinase receptors, is upregulated during endothelial cell morphogenesis in 3D collagen matrices

Further upregulated genes include receptor tyrosine kinase

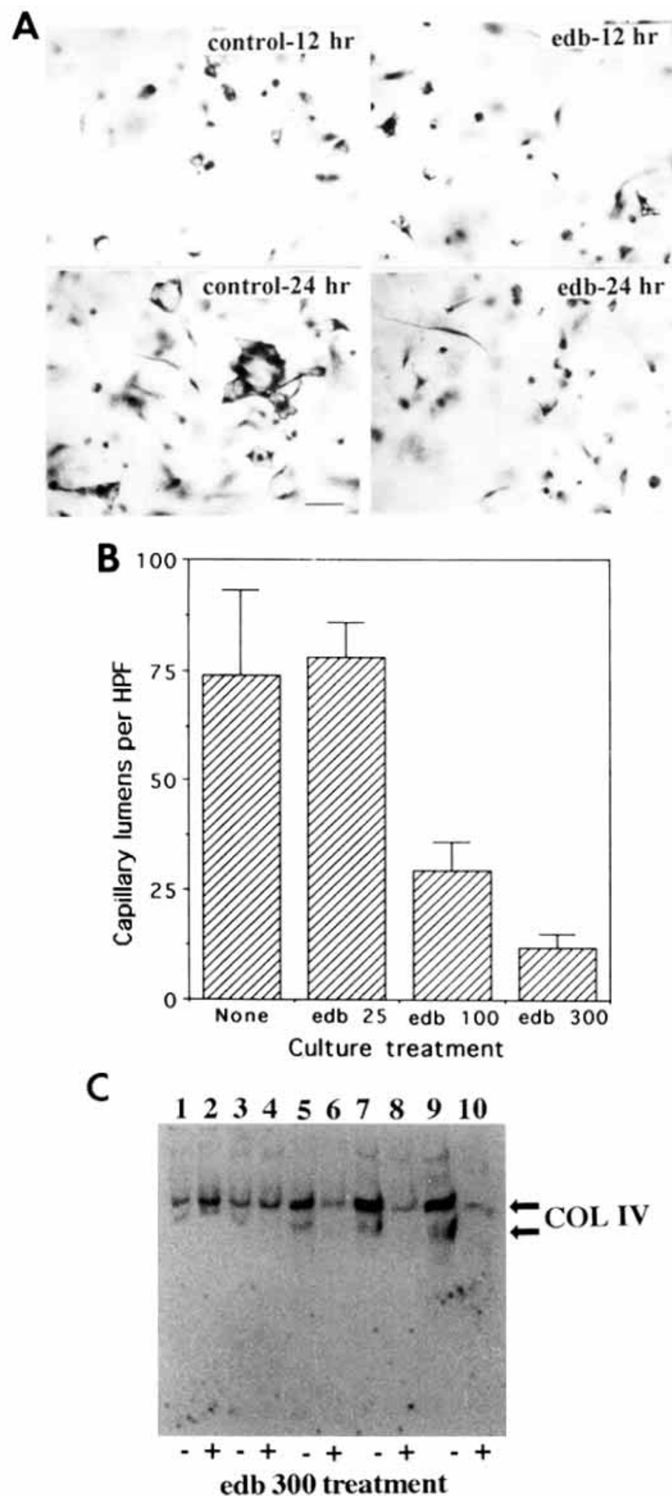

Fig. 6. Interference with collagen type IV synthesis and secretion blocks capillary morphogenesis in 3D collagen matrices. EC cultures were treated with or without ethyl 3,4-dihydroxybenzoate (edb), an inhibitor of prolyl hydoxylase. Cultures were fixed at varying times, stained, quantitated for lumen formation (B) and photographed (A). Bar, 50 μm (A). Cell extract samples (from cultures with or without edb at 300 μM) and at different times of culture were collected and run on 7% SDS-PAGE gels (C). Lanes 1 and 2, 0 hours; lanes 3,4, 8 hours; lanes 5,6, 24 hours; lanes 7,8, 48 hours; lanes 9,10, 72 hours. Proteins were blotted to PVDF membranes and were probed with antibodies to collagen type IV. Arrows indicate the position of the α1 and α2 chains of collagen type IV.

ligands or regulators of tyrosine kinases such as sprouty, angiopoietin-2, and IGF-2 (Pattern C). Interestingly, sprouty has previously been reported to inhibit FGF-mediated signalling as well as other receptor tyrosine kinase-mediated pathways (Reich et al., 1999; Wong et al., 2000) and is important in the regulation of EC and epithelial branching morphogenesis (Hacohen et al., 1998; Metzger and Krasnow, 1999; Lee et al., 2001). In support of this finding is the observation that the FGF-2 gene is markedly downregulated (Fig. 3A) as well as syntenin (Table 3), a cytoplasmic regulator of the FGF-binding cell surface heparan sulfate proteoglycan, syndecan (Grootjans et al., 1997). These data suggest that FGF-2-mediated signaling pathways are downregulated during EC morphogenesis. Another notable downregulated growth factor related gene is frizzled-related protein-1, which is a Wnt antagonist protein (Rattner et al., 1997). Genes that are known to be constitutively or heavily expressed by ECs such as caveolin-1, endothelin-1, ICAM-2, CTGF and Cyr61 are downregulated at the 8 hour time point, and then return to baseline or near baseline expression by 48 hours (pattern E).

Marked downregulation of Id and RGS family genes during endothelial cell morphogenesis in 3D collagen matrices

Additional downregulated genes (pattern D) include Id-1, 2 or 3, which are inhibitory transcription factors of the helix-loop-helix family (Norton, 2000). Recently, knockout mice with combined defects in Id-1 and Id-3 were shown to have a defect in tumor angiogenesis (Lyden et al., 1999). Other downregulated genes include RGS5, RGS4 and RGS2, which are members of the regulator of G-protein signalling (RGS) family (Druey et al., 1996; DeVries et al., 2000) that inhibit G-protein signalling through GTPase activating activity. Transglutaminase 2, which is also referred to as G-protein G_h, is also downregulated (Vezza et al., 1999; Zhang et al., 1999a). Downregulation of RGS genes suggests that activation of G-protein signaling pathways may be occurring. Interestingly, this downregulation occurs during a period of extensive branching morphogenesis in our system (Fig. 1) and RGS proteins are known to block cell migration by inhibiting G-protein-linked chemoattractant receptors (Bowman et al., 1998). Interestingly, a farnesyl transferase enzyme that participates in the functional activation of G-proteins by stimulating membrane targeting is induced during the time course (Dietrich et al., 1996) (Table 2).

Marked downregulation of cell cycle and ubiquitin-proteasome genes during endothelial cell morphogenesis in 3D collagen matrices

An interesting observation in the array analysis is that a large series of genes associated with cell cycle regulation show expression changes. A series of genes known to play a positive role in cell cycle progression such as cyclin B2, cdc2, cyclin A1, cdc20, cdc25B, cdc46, mcm2, mad2, PRC-1, PCNA, ran, thymidylate synthetase, transglutaminase-2 (G_h) and the Id genes are downregulated (Nurse et al., 1998; Jiang et al., 1998;

Table 4. Differentially regulated genes identified by differential display and cDNA library screening

Gene name	Expression pattern				Gene function
	0 h	8 h	24 h	48 h	
CMG-1	+++	+	++	+++	Novel, 65 kDa, unknown, vesicular compartment
CMG-2	+	+++	++	+	Novel, 45 kDa, unknown, endoplasmic reticulum, LM, CIV-binding
CMG-3	+	+	++	+	Novel, unknown, alternatively-spliced forms
CMG-4	+++	++	+	−	Novel, unknown, nuclear localization
CMG-5	+/−	+++	+	+/−	Novel, unknown, 328 bp fragment
CMG-6	+/−	+++	+	+/−	Novel, unknown, 198 bp fragment
Melanoma-associated antigen (MG50)	+	+	++	+++	Extracellular matrix-like protein with RGD site/peroxidase-like domains
Tissue factor pathway inhibitor-2	+	+++	++	+	Extracellular matrix-associated serine proteinase inhibitor
Germinal center protein kinase-related kinase-1	+/−	+	+	+++	In gene family of proteins with functions in MAP kinase, Rho GTPase family signalling
Melanin-concentrating hormone	+	+++	++	+	Regulates ion/water transport across membranes
Sodium bicarbonate cotransporter-3	+/−	+	++	+	Regulates intracellular/extracellular pH
Prothymosin-α	+++	+	−	−	Nuclear protein, implicated in cell proliferation
NADH ubiquinone oxidoreductase, B12 subunit	+/−	+	+	+++	Enzyme in electron-transport chain
NIP-2	+++	++	+	+/−	bcl2-interacting protein
Fte-1	+++	+	+/−	−	v-fos transformation effector gene

Tye, 1999; Heald and Weis, 2000; Howell et al., 2000; Norton, 2000; Nilsson and Hoffmann, 2000; Rudner and Murray, 2000; Nigg, 2001) (Table 3). In addition, a large number of genes associated with ubiquitin-mediated protein degradation through proteasomes are downregulated, which is consistent with this inhibition of the cell cycle (Bounpheng et al., 1999; Koepp et al., 1999; Page and Hieter, 1999) (Table 2). These data strongly support the concept that inhibition of cell cycle progression of ECs is occurring. It is interesting that many of the genes regulate the G_2-M checkpoint of the cell cycle by regulating the function of the cdc2/cyclin B complex (Nurse et al., 1998; Nigg, 2001). In support of this conclusion is the additional finding that a cell cycle inhibitor, cdc14, a phosphatase that inactivates cdc2 kinase (Vistin et al., 1998), is upregulated (Table 2). Also, the cell cycle inhibitor, p16/INK4, which inhibits cyclin dependent kinase 4 (Serrano et al., 1993), is upregulated (Fig. 3A).

Isolation and identification of novel capillary morphogenesis genes (CMGs) by differential display and cDNA library screening

Novel genes, whose messages were differentially regulated during EC morphogenesis, were isolated using differential display and cDNA library screening. We have termed the novel genes identified by these techniques, capillary morphogenesis genes (CMGs), which are defined as novel genes that are differentially expressed during the process of EC morphogenesis. A partial list of the genes identified by this analysis are shown in Table 4 with expression patterns, sequence identities and functional significance, if known. A number of known differentially regulated genes were identified in this analysis including melanoma-associated antigen, TFPI-2, germinal center protein kinase related kinase, melanin concentrating hormone, prothymosin-α, NADH-ubiquinone oxidoreductase-B12 subunit, sodium bicarbonate cotransporter-3 (Pushkin et al., 1999), NIP-2 (Brusadelli et al., 2000) and Fte-1. Known functions for these genes are listed in Table 4. Melanoma-associated antigen (MG50) (Mitchell et al., 2000) is markedly upregulated (pattern C) during morphogenesis, whereas the plasmin and serine proteinase inhibitor, TFPI-2, is strongly upregulated early in the time course (pattern B). Interestingly, melanin concentrating hormone and another member of the sodium bicarbonate cotransporter family (cotransporter-2 versus cotransporter-3) were identified as being differentially regulated by DNA microarray analysis.

To confirm the expression patterns for genes isolated by differential display analysis, RT-PCR, northern blot and western blot analyses were performed (Fig. 7). These results show that the CMGs and other genes are differentially expressed during EC morphogenesis. Note that we have currently identified the full length sequence of four novel genes, CMG-1, CMG-2, CMG-3 and CMG-4 and have small cDNA fragments of two additional genes (CMG-5 and CMG-6) that currently do not match the GenBank databases. The full-length sequences of CMG-3 and CMG-4 will be published elsewhere (A.M. et al., unpublished).

A novel differentially expressed capillary morphogenesis gene, CMG-1, contains coiled-coil domains and targets to an intracellular vesicular compartment

Here, we present the full-length sequence of CMG-1, which encodes a putative intracellular 65 kDa protein. The sequence shown in Fig. 8A reveals a series of coiled-coil domains (from residues 96 to 560) that, in other proteins, have been reported to participate in protein-protein binding, protein multimerization, vesicular fusion and other functions (Burkhard et al., 2001). CMG-1 also contains several consensus motifs for phosphorylation including two for tyrosine phosphorylation at residues 96/97 and 572, one for cAMP/cGMP protein kinases at residue 260, and multiple protein kinase C and casein kinase II sites. Homology searches revealed the greatest similarity (24% identity, 46% positives from residues 9-591) with a putative *C. elegans* protein, C18H9.8. It also shows 24% identity from residues 104-592 (in the coiled coil domain) with myosin heavy chain sequences from various species. The sequence also matches human genome sequences and maps to human chromosome 9q. Interestingly, the pattern of CMG-1 gene expression (pattern E) during EC morphogenesis mirrors that of caveolin, and other major EC genes (Figs 2, 3). Both genes show a marked downregulation at 8 hours of morphogenesis followed by a return to baseline by 48 hours. To examine the

expression pattern of CMG-1 in adult versus fetal human tissues, RT-PCR was performed (Fig. 8C). As shown in Fig. 8, the strongest expression was observed from adult and fetal kidney cDNAs with detectable expression in adult heart, placenta, lung, liver and pancreas. Minimal to no expression was observed in the adult brain or skeletal muscle cDNA samples. Detectable expression was observed in fetal skeletal muscle as well as fetal heart, lung and liver, while minimal to no expression was observed from fetal brain, thymus and spleen cDNAs.

To reveal possible functions for CMG-1, a CMG-1-green fluorescent protein (GFP) chimera was constructed to assess where the protein targets intracellularly. Transfection of 293 epithelial tumor cells revealed targeting of the CMG-1 fusion protein to an intracellular vesicular compartment (Fig. 9A-C). To accomplish this experiment in human ECs, a recombinant adenovirus was constructed in the pAdEasy system carrying the CMG-1-GFP fusion protein. Infection of ECs resulted in an apparent intracellular distribution identical to that observed in 293 cells with targeting to multiple intracellular vesicles (data not shown). By contrast, control GFP distributes throughout 293 cells or ECs with a cytoplasmic staining pattern (Fig. 9D). Coimmunostaining of ECs expressing CMG-1-GFP by using antibodies to various known intracellular compartments such as endosomes, Weibel-Palade bodies, caveolae, mitochondria, Golgi apparatus (GM130) and lysosomes failed to reveal any colocalization. More work is necessary to identify this intracellular compartment.

A novel differentially expressed capillary morphogenesis gene, CMG-2, contains a putative transmembrane domain, targets to the endoplasmic reticulum and shows affinity for the basement membrane matrix proteins, collagen type IV and laminin

Here, we also present the full-length sequence of a second novel gene, CMG-2, which is markedly upregulated at 8 hours during EC morphogenesis, as revealed by both RT-PCR and northern blots (Fig. 8B). This gene reveals a putative 45 kDa protein with a putative transmembrane segment and a potential signal peptide (residues 1-33) (Fig. 8B). Polyclonal antibodies directed to recombinant CMG-2 were prepared, affinity purified and probed on western blots of ECs undergoing morphogenesis. As shown in Fig. 7C, induced protein bands migrating at the predicted size of 45 kDa are detected using this antibody. This antibody also specifically detects CMG-2-GFP or CMG-2-myc epitope-tagged fusion proteins by immunoprecipitation or immunoblotting (data not shown), demonstrating specificity for CMG-2. By contrast, G3PDH or actin antibodies show stable expression during the time course (not shown).

The CMG-2 gene maps to the human genome sequence and is located on chromosome 4q. Using the PSORT II program, the protein was predicted to have a 44% probability of targeting to the endoplasmic reticulum membrane with lesser probabilities to the Golgi apparatus or plasma membrane. Proximal to the potential transmembrane segment, homology searches reveal a von Willebrand Factor A domain (a matrix-

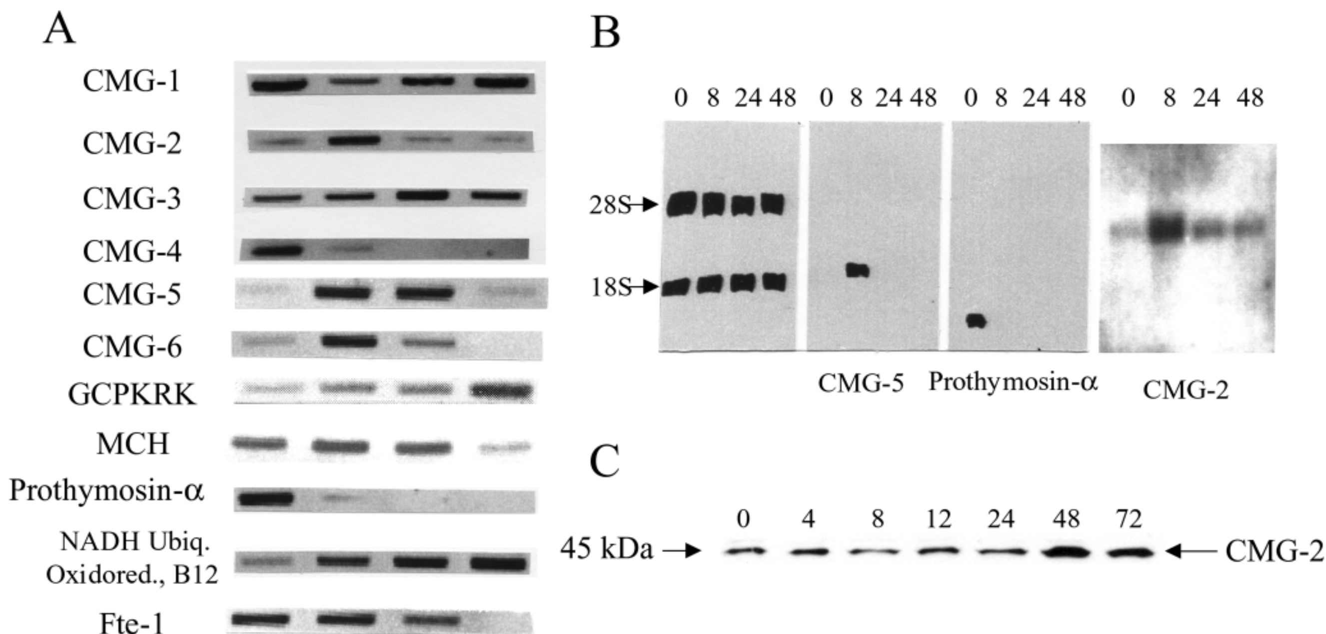

Fig. 7. Differential expression of novel capillary morphogenesis genes (CMGs) during morphogenesis in 3D collagen matrices. Differential display and cDNA library screening were used to identify differentially expressed genes during capillary morphogenesis. DNA sequencing revealed whether genes were known or novel and gene-specific primers were prepared to assess differential expression by RT-PCR (A). RT-PCR was performed to assess the mRNA expression patterns of various CMGs and known genes over a 48 hour time course. Total RNA was prepared from EC cultures at 0, 8, 24 and 48 hours of culture. The RNA was reverse transcribed for RT-PCR and PCR was performed using gene-specific primer sets. In addition, cDNA probes were made in select cases to perform northern blots (B). The far-left panel in B represents a methylene blue-stained blot indicating the position of the 28S and 18S rRNA bands and showing equal loading of 3 μg of total RNA per lane. (C) A western blot of cell extracts from a time course of capillary morphogenesis is shown using affinity purified CMG-2 antibodies. MCH, melanin concentrating hormone; GCPKRK, germinal center protein kinase related kinase-1.

binding domain) from residues 44-213. In addition, WH-1 block homologies were detected to WASP, a cdc42-binding protein that regulates the actin cytoskeleton (Anton et al., 1998) (from residues 250-259 and 315-334). The human tissue distribution of CMG-2 was assessed by RT-PCR (Fig. 8C). As shown in Fig. 8C, CMG-2 was detected in placenta but was not detected in the other adult or fetal tissues examined.

To address where CMG-2 may target within ECs, the same approach described above was performed using a recombinant adenovirus carrying a CMG-2-GFP fusion protein. ECs were infected revealing that CMG-2-GFP primarily targets to endoplasmic reticulum (ER) using fluorescence microscopy. This is shown in a double staining experiment using the ER protein, Hsp47, which is a chaperone protein for collagens type I and IV (Fig. 9E-G) (Clarke et al., 1991; Hendershot and Bulleid, 2000; Nagai et al., 2000). The staining pattern does not overlap with a Golgi-specific marker (not shown). In addition, we have observed CMG-2-GFP to be present within intracellular vesicles in some cells, suggesting that it may be capable of cycling from the ER to intracellular vesicles or that it can target to more than one compartment. We have not yet observed targeting of CMG-2-GFP to the plasma membrane.

A 20 kDa portion of the CMG-2 protein with sequence homology to the Von Willebrand factor A domain was expressed in bacteria and tested for its ability to bind extracellular matrix proteins (Fig. 10A,B). The recombinantly expressed protein along with a control GFP recombinant protein were purified using their histidine tags. These proteins were adsorbed to plastic and were incubated with biotinylated collagen type IV, laminin, fibronectin, osteopontin and control albumin. As shown in Fig. 10C, the CMG-2 protein but not the control GFP protein showed strong binding to the basement membrane proteins, collagen type IV and laminin, but showed little or no binding affinity for the other ECM proteins. This data suggests that CMG-2 has affinity for matrix proteins, which implies a potential role in basement membrane matrix synthesis or assembly due to its localization within the endoplasmic reticulum of ECs.

CMG-1

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             5         10        15        20        25        30
  1 M A S N H K S S A A R P V S R G G V G L T G R P P S G I R P
 31 L S G N I R V A T A M P P G T A R P G S R G C P I G T G G V
 61 L S S Q I K V A H R P V T Q Q G L T G M K T G T K G P Q R Q
 91 I L D K S Y Y L G L L R S K I S E L T T E V N K L Q K G I E
121 M Y N Q E N S V Y L S Y E K R A E T L A V E I K E L Q G Q L
151 A D Y N M L V D K L N T N T E M E E V M N D Y N M L K A Q N
181 D R E T Q S L D V I F T E R Q A K E K Q I R S V E E E I E Q
211 E K Q A T D D I I K N M S F E N Q V K Y L E M K T T N E K L
241 L Q E L D T L Q Q Q L D S Q N M K K E S L E A E I A H S Q V
271 K Q E A V L L H E K L Y E L E S H R D Q M I A E D K S I G S
301 P M E E R E K L L K Q I K D D N Q E I A S M E R Q L T D T K
331 E K I N Q F I E E I R Q L D M D L E E H Q G E M N Q K Y K E
361 L K K R E E H M D T F I E T F E E T K N Q E L K R K A Q I E
391 A N I V A L L E H C S R N I N R I E Q I S S I T N Q E L K M
421 M Q D D L N F K S T E V Q K S Q S T A Q N L T S D I Q R L Q
451 L D L Q K M E L L E S K M T E E Q H S L K S K I K Q M T T D
481 L E I Y N D L P A L K S S G E E K I K K L H Q E R M I L S T
511 H R N A F K K I M E K Q N I E Y E A L K T Q L Q E N E T H S
541 Q L T N L E R K W Q H L E Q N N F A M K E F I A T K S Q E S
571 D Y Q P I K K N V T K Q I A E Y N K T I V D A L H S T S G N
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CMG-2

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             5         10        15        20        25        30
  1 M V A E R S P A R S P G S W L F P G L W L L V L S G P G G L
 31 L R A Q E Q P S C R R A F D L Y F V L D K S G S V A N N W I
 61 E I Y N F V Q Q L A E R F V S P E M R L S F I V F S S Q A T
 91 I I L P L T G D R G K I S K G L E D L K R V S P V G E T Y I
121 H E G L K L A N E Q I Q K A G G L K T S S I I I A L T D G K
151 L D G L V P S Y A E K E A K I S R S L G A S V Y C V G V L D
181 F E Q A Q L E R I A D S K E Q V F P V K G G F Q A L K G I I
211 N S S N G I A A I I V I L V L L L L L G I G L M W W F W P L
241 C C K V V I K D P P P P P P P A P K E E E E E P L P T K K W
271 P T V D A S Y Y G G R G V G G I K R M E V R W G D K G S T E
301 E G A R L E K A K N A V V K I P E E T E E P I R P R P P R P
331 K P T H Q P P Q T K W Y T P I K G R L D A L W A L L R R Q Y
361 D R V S L M R P Q E G D E V C I W E C I E K E L T A
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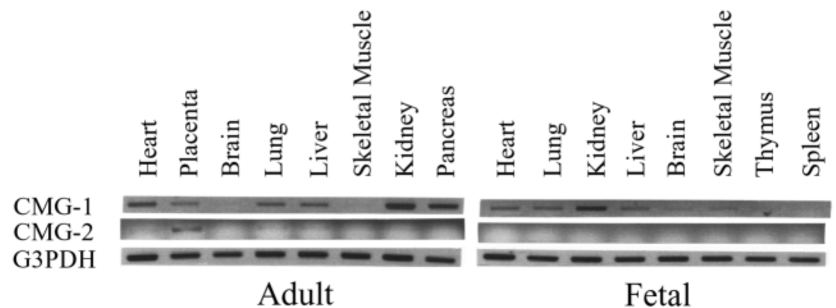

Fig. 8. Amino acid sequences, domain analyses and mRNA tissue distribution of two novel differentially expressed genes, CMG-1 and CMG-2. (A) Amino acid sequence of CMG-1. Bold letters indicates a coiled-coil domain structure and underlined letters indicate potential tyrosine phosphorylation sites. The nucleotide sequence of CMG-1 has been submitted to GenBank (accession no. AY040325). (B) Amino acid sequence of CMG-2. The bold letters proximal to the transmembrane segment show homology to a von Willebrand Factor A domain and the putative transmembrane domain is underlined. The nucleotide sequence of CMG-2 has been submitted to GenBank (accession no. AY040326). (C) RT-PCR analysis of mRNA tissue (adult versus fetal) distribution of CMG-1 and CMG-2. The tissue distribution of control G3PDH is also shown.

DISCUSSION

DNA microarray analysis of differential gene expression during human capillary morphogenesis in 3D collagen matrices

It has been shown that microarray technology has the ability to accurately represent global changes in gene expression (Brown and Botstein, 1999). A time course analysis of EC morphogenesis during tube formation provides information on potentially important genes that regulate this process. We have shown that dramatic regulation of many genes occurs as tube formation progresses. Analysis of gene expression revealed that jagged-1, stanniocalcin and angiopoietin-2 were the most induced genes, whereas CTGF, fibulin-3 and RGS-5 were the most repressed genes at 8, 24 and 48 hours, respectively. Previously, increased jagged expression was observed during FGF-2-mediated EC morphogenesis in fibrin matrices and was reported to inhibit invasion and tube formation, thus implicating it as a negative regulator of the process (Zimrin et

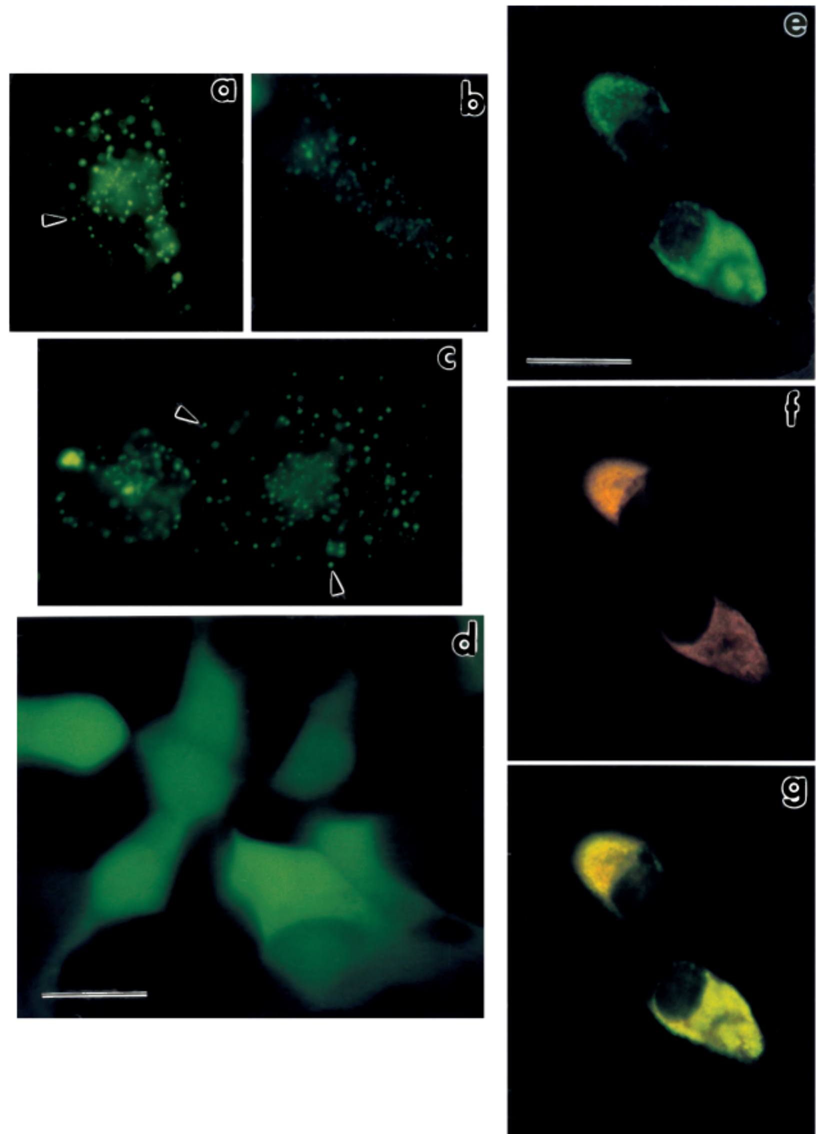

Fig. 9. Intracellular targeting of CMG-1-GFP and CMG-2-GFP fusion proteins. CMG-1-GFP and CMG-2-GFP fusion protein vectors were created by preparing recombinant adenoviral vectors encoding the fusion proteins. A control GFP-only virus was also constructed. Recombinant adenoviruses were infected into 293 cells (a-d) or endothelial cells (e-g). In a-c, individual cells were photographed 24 hours after infection with CMG-1-GFP virus. Arrowheads represent fluorescent vesicular structures. In d, individual cells were photographed 24 hours after infection with GFP only virus. Bar, 20 μm (a-d). In e-g, endothelial cells were infected with CMG-2-GFP virus for 24 hours and were fixed and permeabilized for immunofluorescence staining. Panel e shows cells expressing the CMG-2-GFP protein. Panel f shows cells stained with anti-Hsp47 antibodies followed by staining with rhodamine-conjugated rabbit anti-mouse antibodies. No background staining was observed by the addition of the secondary antibodies alone. In g, a double exposure of the same field photographed in e and f is shown, illustrating the colocalization of CMG-2-GFP and Hsp47. Bar, 20 μm (e-g).

al., 1996). A recent study implicated a positive role for jagged-1 and its receptor, Notch-4 (which shows a slight mRNA induction in our system) in EC morphogenesis (Uyttendaele et al., 2000). Angiopoietin-2 has also been implicated in angiogenesis in a number of contexts (Yancopoulos et al., 2000) and has previously been reported to be produced by ECs (Mandriota and Pepper, 1998). It has been reported to be an inhibitor of angiogenesis by competing with angiopoietin-1 binding to tie-2, a receptor tyrosine kinase (Yancopoulos et al., 2000). By contrast, angiopoietin-2 may disrupt stable pericyte-EC interactions and initiate angiogenesis along with VEGF (Holash et al., 1999). Stanniocalcin, a hormone that regulates extracellular inorganic phosphate concentrations in vivo by affecting renal tubular reabsorption of phosphate, is known to be upregulated in some cell types in response to elevations of extracellular calcium (Olsen et al., 1996). A recent report identified stanniocalcin as being upregulated during EC morphogenesis and expressed by in situ hybridization in tumor-associated blood vessels (Kahn et al., 2000). In addition, stanniocalcin was found to be upregulated in terminally differentiated neurons (Zhang et al., 1998) and to protect neurons from ischemic injury by decreasing the toxicity of calcium influx through increased influx of phosphate ions (Zhang et al., 2000). Stanniocalcin may function similarly during EC morphogenesis to promote survival.

CTGF was originally described as an EC-derived growth factor for mesenchymal cells and a modulator of fibroblast ECM production through synergism with TGF-β (Grotendorst, 1997). Recent reports show the production of fibulin-3 by blood vessels and endothelial cells in vivo (Giltay et al., 1999). Interestingly, fibulin-3 was reported to bind to a growth factor, NOVH, which is in a family of growth factors including CTGF and Cyr61 (Perbal et al., 1999). Marked downregulation (34-fold) of RGS-5 was observed during the time course. This gene, as well as two others in the same family, RGS-4 and RGS-2, were also downregulated. These genes accelerate GTPase activity of heterotrimeric G proteins, resulting in inactivation of specific signalling pathways (DeVries et al., 2000). Downregulation of these genes may indicate that activation of G protein-mediated pathways may be important during EC morphogenesis. RGS-5, and -4 have been reported to interact with G_i family members, whereas RGS-2 and -4 also interact with G_q (DeVries et al., 2000). By contrast, RGS-3 was modestly upregulated during the time course and has been reported to interact with G_i and G_q family members and to inhibit endothelin-1-mediated signalling (Dulin et al., 1999).

Differential expression of basement membrane matrix and integrin genes during EC morphogenesis in 3D collagen matrices

Our previous work and the work presented here strongly implicate the ECM- integrin-cytoskeletal axis as being one of the key regulators of EC morphogenesis in a 3D extracellular matrix. Previously, we have shown the involvement of

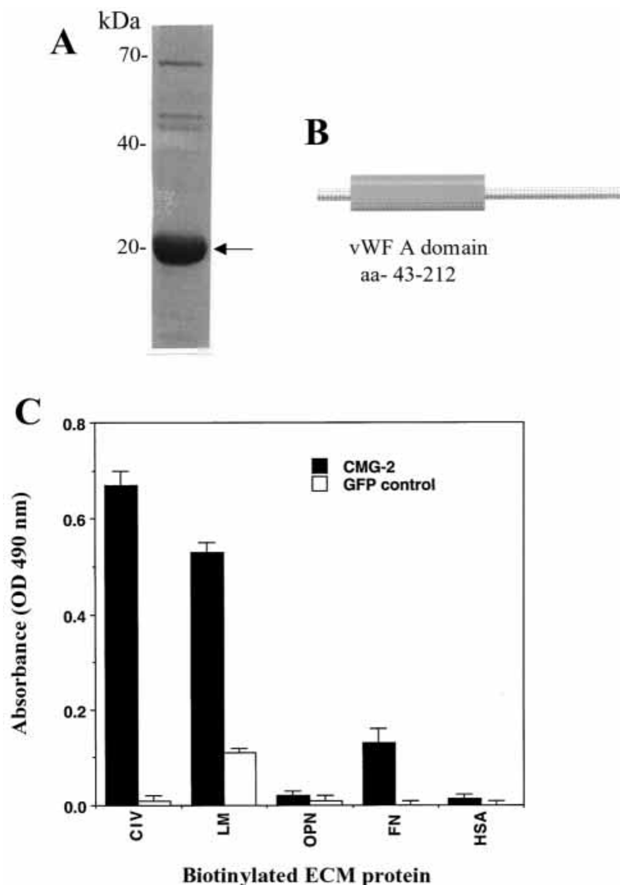

Fig. 10. Recombinant CMG-2 binds to the basement membrane matrix proteins, collagen type IV and laminin. The 20 kDa domain proximal (residues 34-214) to the putative transmembrane domain was expressed as a histidine-tagged fusion protein in E.coli and purified by Ni/Cd-sepharose chromatography. Five µg of the protein was run on a 12% SDS-PAGE gel and was stained by Coomassie blue (A). A schematic diagram of CMG-2 is shown with the von Willebrand Factor A domain homology noted from residues 43-212 which is within the 20 kDa recombinantly produced protein (B). A control protein, histidine-tagged GFP, was similarly purified as a control. These proteins were adsorbed to plastic at 10 µg/ml and after blocking with detergent, were incubated with 1 µg/ml of biotinylated collagen type IV, laminin, osteopontin, fibronectin and human serum albumin in 0.1% Tween-20 in Tris-buffered saline, pH 7.5. After a 1 hour incubation at 25°C, the wells were washed and then incubated with 1 µg/ml of avidin-peroxidase in the same Tween-20-Tris buffer for 30 minutes. The wells were then washed, developed for peroxidase activity and the plate read in an ELISA plate reader at 490 nm. The values shown are derived from triplicate wells + s.d. (C).

particular integrins in EC morphogenesis and the integrin(s) involved are dictated by the ECM environment where morphogenesis occurs (Davis and Camarillo, 1995; Davis and Camarillo, 1996; Bayless et al., 2000; Davis et al., 2000). In addition, we have previously shown that actin cytoskeletal regulatory proteins such as gelsolin, vasodilator-stimulated phosphoprotein (VASP) and its binding partner, profilin, are coordinately upregulated during EC morphogenesis (Salazar et al., 1999). Here, both α2 and α1 integrin subunits were upregulated, and have affinity for basement membrane proteins such as collagen type IV and laminin. In addition, basement membrane matrix genes such as collagen type IV α1 chain, laminin γ1 chain, laminin α4 chain and heparan sulfate deacetylase/sulfotransferase were upregulated. The marked upregulation of collagen type IV synthesis, which was observed during EC morphogenesis, appears to play an important role in these events. Interference with collagen type IV synthesis blocked EC morphogenesis. Further support of these conclusions is the identification of a novel gene, CMG-2, which may participate in these events. It is induced in a pattern similar to that of α2β1 and possesses a von Willebrand factor-like A domain with affinity for collagen type IV and laminin. Both collagen type IV and laminin are markedly induced during EC morphogenesis and deposit around developing tubes in a time-frame coincident with CMG-2 induction. The colocalization of CMG-2 with Hsp47, a chaperone for collagen type IV synthesis (Hendershot and Bulleid, 2000), within the endoplasmic reticulum suggests that it participates in these events. More work will need to be performed to investigate the role of CMG-2 in basement membrane matrix synthesis/assembly and EC morphogenesis.

Interestingly, melanoma-associated antigen, which is markedly upregulated during EC morphogenesis in a time-frame consistent with other basement membrane-related molecules, may be a basement membrane matrix protein (Mitchell et al., 2000). It contains ECM-like domains and strong homology with *Drosophila* peroxidasin, a protein that localizes to basement membranes (Nelson et al., 1994). In addition, within the melanoma antigen protein sequence, is encoded the complete sequence of IL-1 receptor antagonist, suggesting that it is the precursor protein for this inhibitor of IL-1-mediated signalling (Mitchell et al., 2000). Interestingly, the peroxidase domains of the melanoma antigen protein may degrade extracellular H_2O_2 and, along with IL-1 receptor antagonist protein generation, might facilitate the development of EC quiescence during the differentiation process (i.e. H_2O_2 and IL-1 are both EC activators). It is also interesting that CD39, which degrades ATP and ADP (Goepfert et al., 2000), and CD26, which can inactivate biologically active peptides (Mentlein, 1999), are both markedly upregulated in a similar fashion and might perform similar functions to inhibit EC activation and stimulate EC quiescence.

A recent study comparing endothelial cell gene expression from tumor versus normal colonic tissue in vivo also revealed many changes in extracellular matrix genes (St Croix et al., 2000). Interestingly, some of the most prominently differentially regulated genes in tumor endothelium compared with normal endothelium were the interstitial matrix genes, collagen type I, α1 and α2 chains, collagen type III, α1 chain and nidogen, a basement membrane matrix gene. This data implies that endothelial cells may be undergoing an epithelial-mesenchymal transition during tumor angiogenesis since the collagen proteins are more typically synthesized by mesenchymal cells. By contrast, in our system, the ECs are inducing the synthesis of basement membrane matrix molecules such as collagen type IV, laminins and heparan sulfate, while they are downregulating the synthesis of interstitial matrix proteins such as fibronectin.

Novel capillary morphogenesis genes (CMGs) are differentially expressed during EC morphogenesis in 3D collagen matrices

One of the clear advantages of our published system (Davis

and Camarillo, 1996) is its use for the identification of differentially expressed known and novel genes in capillary morphogenesis (Salazar et al., 1999; Kahn et al., 2000; Davis et al., 2001). Other EC morphogenic models have been used to study differential gene expression (Glienke et al., 2000). Here, we have used a combination of experimental approaches to screen large numbers of genes for differential expression patterns. In addition, we present data showing our initial characterization of a number of genes that were isolated using differential display and cDNA library screening (see Table 4 and Fig. 7A).

Here, we report the full length sequences of CMG-1 and CMG-2, proteins with coding sequences predicting proteins of 65 kDa and 45 kDa, respectively. CMG-1 is predicted to be intracellular and to contain a series of coiled-coil domains involving ~500 amino acids of sequence. A CMG-1-GFP construct was observed to target to an intracellular vesicular compartment (Fig. 9A-C). Interestingly, it has an expression pattern (pattern E; see Figs 2, 7) that mirrors that of caveolin-1, endothelin-1 and ICAM-2. RT-PCR analysis of tissue expression reveals its mRNA expression in a number of tissues, with the most abundant being adult and fetal human kidney (Fig. 8C). CMG-2 contains a putative transmembrane domain and signal peptide and was predicted to target to the endoplasmic reticulum, which was confirmed using a CMG-2-GFP fusion protein vector (Fig. 9E-G). As discussed above, its affinity for basement membrane ECM proteins (Fig. 10C) suggests a potential role in basement membrane matrix synthesis and assembly in ECs during morphogenesis. CMG-2 mRNA was detected in placenta and was essentially undetectable in the other adult and fetal tissues examined (Fig. 8C). Thus, CMG-2 appears to have a much more restricted tissue distribution than CMG-1.

Control of EC morphogenesis by differential gene expression in 3D collagen matrices

The patterns of differential gene expression identified a number of known positive and negative regulators of angiogenesis whose differential expression may dictate the molecular control necessary to form networks of EC-lined tubes. It is interesting that three genes that have been reported as possible inhibitors of angiogenic events, angiopoietin-2, jagged-1 and sprouty (Zimrin et al., 1996; Hanahan, 1997; Yancopoulos, 2000; Lee et al., 2001), were found to be markedly induced during EC morphogenesis in this system. These genes could be viewed as negative control genes that suppress specific pathways during morphogenesis to finely control EC migration, branching, lumen formation and proliferation. Alternatively, they may perform stimulatory functions during EC morphogenesis as is reflected in a recent report concerning jagged-1 (Uyttendaele et al., 2000)

Overall, the expression changes reported here suggest that ECs are undergoing major changes during morphogenesis. There is a clear indication that endogenous basement membrane matrix synthesis is a prominent event as well as increased expression of integrins and cytoskeletal regulatory pathways. Upregulation of EC differentiation markers and genes that may facilitate EC quiescence are observed. There is marked downregulation of positive cell cycle regulators and upregulation of negative cell cycle regulators. In addition, there is upregulation of genes encoding cytokines, indicating the potential relevance of autocrine factors in EC morphogenesis. Marked changes in genes regulating signal transduction cascades are observed including possible upregulation of G-protein mediated pathways (via decreased expression of RGS proteins), upregulation of JAK-STAT pathways, possible downregulation of FGF-mediated pathways, upregulation of antiapoptotic pathways and upregulation of cholesterol biosynthetic pathways. These changes reflect the major regulated gene expression events during EC morphogenesis in 3D collagen matrices in our system. Further work will be necessary to compare the gene expression profiles of ECs under different conditions of morphogenesis and to assess the role of individual genes during morphogenic events in vitro and in vivo.

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