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Gene Expression Profiling in an *in Vitro* Model of Angiogenesis

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In the present study we have used a novel, comprehensive mRNA profiling technique (GeneCalling) for determining differential gene expression profiles of human endothelial cells undergoing differentiation into tubelike structures. One hundred fifteen cDNA fragments were identified and shown to represent 90 distinct genes. Although some of the genes identified have previously been implicated in angiogenesis, potential roles for many new genes, including OX-40, white protein homolog, KIAA0188, a homolog of angiopoietin-2, ADAMTS-4 (aggrecanase-1), and stanniocalcin were revealed. Support for the biological significance was confirmed by the abrogation of the changes in the expression of angiogenesis inhibitors and *in situ* hybridization studies. This study has significantly extends the molecular fingerprint of the changes in gene expression that occur during endothelial differentiation and provides new insights into the potential role of a number of new molecules in angiogenesis. *(Am J Pathol 2000, 156:1887–1900)*

Angiogenesis, defined as the process whereby new blood vessels are formed from previously existing ones, plays an important role in the development and progression of a number of disease states, including various cancers, diabetic retinopathy, macular degeneration, psoriasis, and rheumatoid arthritis. During the last 10 years there have been many advances in our understanding of the biology of and the molecules that are involved in angiogenesis. A number of different growth factors, including vascular endothelial growth factor (VEGF), fibroblast growth factors, platelet-derived growth factor, hepatocyte growth factor, angiopoietins 1 and 2, as well as various endothelial surface molecules, such as CD31 (PECAM), CD144 (VE-Cadherin), and $\alpha v\beta 3$ integrins, have been implicated in various steps of angiogenesis. These advances have enabled the development of new therapeutic strategies for inhibiting angiogenesis (eg, to inhibit tumor growth) or promoting angiogenesis (coronary and peripheral ischemia, wound healing).

Understanding the molecular events that direct angiogenesis and the order in which they occur and identifying new pathways that are required for this process are of fundamental importance for all researchers who study angiogenesis. The present study was undertaken to identify the alterations in gene expression that occur in an *in vitro* model of angiogenesis. In this model, endothelial cells are suspended in a three-dimensional gel composed of type I collagen and incubated with a mixture of stimuli (phorbol myristate acetate (PMA), basic fibroblast growth factor (bFGF), and vascular endothelial cell growth factor (VEGF)). Previous studies by our laboratory demonstrated that this combination of stimuli resulted in the optimal formation of a three-dimensional tubular network of endothelial cells with interconnecting lumenal structures.[1] In this model, endothelial differentiation into tubelike structures is completely blocked by inhibitors of new mRNA (actinomycin D) or protein synthesis (cycloheximide). Furthermore, the cells progress through this differentiation process in a coordinated and synchronized manner, thus optimizing the profile of gene expression.

The goal of the present study was to identify a molecular fingerprint or transcriptional profile of endothelial differentiation into tubelike structures, using amplification and an imaging approach called GeneCalling.[2] This method was previously shown to provide a comprehensive sampling of cDNA populations in conjunction with the sensitive detection of quantitative differences in mRNA abundance for both known and novel genes.[2] We describe the identification of 115 differentially expressed cDNA fragments, which corresponded to 90 previously identified genes. The identification and differential expression of these genes was confirmed by a second independent method employing real-time quantitative polymerase chain reaction (PCR). Although some of the cDNA fragments identified were genes previously known to play some role in the process of angiogenesis, many other differentially expressed genes were unexpected

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and suggest possible roles for these additional genes in endothelial differentiation and vessel assembly.

Materials and Methods

Materials

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA) and maintained in endothelial growth medium (EGM) media supplemented to a final concentration of 10% fetal bovine serum. Type I rat tail collagen was from Upstate Biotechnology (Lake Success, NY), and recombinant bFGF was purchased from Collaborative Biomedical Products (Becton Dickinson Labware, Bedford, MA). Recombinant VEGF was from Genentech (South San Francisco, CA). Medium 199 (10×) (M199, M0650), PMA, ITS (insulin, transferrin, and selenium-A), trypsin, actinomycin D, and cycloheximide were from Gibco-BRL (Gaithersburg, MD).

Formation of Three-Dimensional Collagen Gels

Collagen gels were formed by mixing together ice-cold gelation solution (10× M199, H_2O, 0.53 mol/L $NaHCO_3$, 200 mmol/L L-glutamine, type I collagen, 0.1 mol/L NaOH, 100:27.7:50:10:750:62.5 v/v) and cells in 1× basal medium (see below) at a concentration of 3×10^6 cells/ml at a ratio of four volumes gelation solution to one volume of cells. The gels were allowed to form by incubation in a CO_2-free incubator at 37°C for 30 minutes to 1 hour. The gels were then overlaid with 1× basal medium consisting of M199 supplemented with 1% FBS, 1× ITS, 2 mmol/L L-glutamine, 50 μg/ml ascorbic acid, 26.5 mmol/L $NaHCO_3$, 100 U/ml penicillin, and 100 U/ml streptomycin. In the tube-forming experiments, the culture medium was supplemented with 80 nmol/L PMA, 40-ng/ml bFGF, and 40 ng/ml VEGF.

mRNA Isolation and cDNA Synthesis

Medium was aspirated from the surface of the collagen gels, and the gels were scraped into a 50-ml polypropylene tube containing three volumes of Tri-Reagent-LS (Molecular Research Center, Cincinnati, OH). The tubes were incubated for 10 minutes at 23°C with intermittent gentle agitation. The tubes were stored at −80°C until all experimental samples had been collected. The tubes were then thawed at room temperature, and the RNA was extracted following the manufacturer's specifications. The RNA pellets were resuspended in diethyl-pyrolidine-carbonate-treated water, and the RNA content was quantified spectroscopically at 260 nm. RNA samples were stored at −20°C. Samples used for GeneCalling analysis were shipped on dry ice to CuraGen (New Haven, CT). Samples from time points of 4, 24, and 48 hours were used for the GeneCalling analysis, and in separate experiments, samples from additional time points of 30 minutes and 2, 4, 8, 16, 24, 38, and 46.5 hours were prepared for TaqMan confirmation. For the quantitative expression analysis, contaminating DNA was removed by treatment of the isolated RNA with DNase I (Promega, Madison, WI). PolyA+ RNA was prepared by fractionation of total RNA with an mRNA purification kit that uses the biotinylated oligo-dT-streptavidin magnetic bead method (MPG, Lincoln Park, NJ), followed by cDNA synthesis by reverse transcription of oligo-dT-primed mRNA (Superscript II; Life Technologies) and second-strand synthesis. Terminal phosphate removal is achieved by treatment with arctic shrimp alkaline phosphatase (Amersham Life Sciences, Piscataway, NJ), followed by purification of cDNA by phenol-chloroform extraction. Yield of cDNA was quantitated by fluorometry using PicoGreen dye (Molecular Probes, Eugene, OR). Double-stranded DNA was digested using pairs of restriction enzymes with 6-bp recognition sites. More than 48 enzyme pairs were used and were chosen such that a representative coverage of most of the possible sequences in a given DNA sample was achieved.[2] PCR amplification using specific linkers was carried out as described previously.[2] The final DNA products were denatured by heating to 96°C and electrophoresed on ultrathin polyacrylamide gels under denaturing conditions in 6 mol/L urea. PCR products were visualized by the presence of 6-carboxy fluorescein (FAM) label on the product, using a multicolor laser excitation (Niagara; CuraGen, New Haven CT) imaging system.

Data Interpretation

The data obtained from Niagara gels were queried (ie, "GeneCalled") against public and proprietary databases.[2] GeneCalling is the process that takes the restriction enzyme pair recognition site information and the cDNA fragment size determined from the migration of the labeled fragment on Niagara gels and uses that information (the size of the fragment and the relative position of the terminal sequences defined by the restriction enzyme pairs) to search public and proprietary databases for likely gene matches, using statistical and mathematical criteria. A GeneCall is defined as the probability of a cDNA fragment belonging to a known gene.[2] The cDNA fragment data were compiled as a list of likely genes to which that cDNA fragment might belong. If a provisional identification of a cDNA fragment could not be obtained by querying databases, the cDNA fragment was designated as belonging to a putative novel gene.

Confirmation of Gene Calls

GeneCalls were confirmed in a competitive PCR reaction, "GeneCall poisoning," in which the known sequence of the likely gene of interest is used to design poisoning primers as previously described.[2] Ablation of the cDNA fragment of interest confirmed that the cDNA fragment belonged to the gene for which the specific poisoning primer was designed.

Novel cDNA Fragments

If no GeneCall was obtained for a cDNA fragment, the cDNA fragment was eluted and subcloned into *Esche-*

richia coli with the standard TA-cloning vector (Invitrogen, Palo Alto, CA). The cDNA fragment was then sequenced, and the resulting sequence was used to design poisoning primers for confirmation as described above.

Validation and Confirmation of Gene Expression by Quantitative Reverse Transcriptase-Polymerase Chain Reaction (TaqMan)

To confirm the expression data from GeneCalling by an independent technique, gene-specific PCR oligonucleotide primer pairs and an oligonucleotide probe labeled with a reporter fluorescent dye at the 5′ end and quencher fluorescent dye at the 3′ end were designed using Oligo 4.0 software (National Bioscience, Plymouth, MN). Table 1 provides the sequences for the primers and probes used in this study. Total RNA (50 ng) was added to a 50 μl reverse transcriptase-polymerase chain reaction (RT-PCR) reaction mixture according to the manufacturer's protocol (Roche Molecular Systems, Branchburg, NJ). The thermal cycling conditions included one cycle at 48°C for 30 minutes, one cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 s, annealing at 60°C for 1 minute, and a final hold at 25°C for 2 minutes. Standard curves for the expression of each gene were generated by serial dilution of a standard preparation of total RNA isolated from quiescent HUVECs grown in monolayer culture. Data are expressed as the fold induction normalized to the same gene from quiescent HUVEC RNA.

Treatment with 15d-PGJ$_2$

mRNA was harvested from endothelial cells incubated 4 and 24 hours in the absence (control) or presence of 10 μmol/L of the PPARγ ligand 15-deoxy-Δ2,14–15-prostaglandin J$_2$ (15d-PGJ$_2$). In both groups the cells were incubated with the mixture of growth stimuli (ie, PMA, VEGF, and bFGF), and the cells were incorporated in the collagen gels as described above.

In Situ *Hybridization of Tissue Specimens*

Formalin-fixed, paraffin-embedded human tissues were investigated for *in situ* mRNA expression. Tissues included first-trimester (14–15-week) placenta, adult adrenal cortex, aorta, muscular artery with atherosclerosis, brain, gall bladder, heart, pancreas, prostate, stomach, eye with age-related macular degeneration (AMD), inflamed appendix, pulmonary adenocarcinoma, ductal mammary adenocarcinoma, kidney with renal cell carcinoma, hepatocellular carcinoma, squamous cell carcinoma, osteosarcoma, and chondrosarcoma. *In vitro* transcription and ^{33}P labeling of sense and antisense riboprobes were performed as described previously.[3] Briefly, stanniocalcin, osteonidogen, podocalyxin, and ADAMTS-4 sequences were PCR-amplified from plasmid DNA, using gene-specific primers that encoded T3 or T7 RNA polymerase initiation sites. Sense and antisense riboprobes were prepared by *in vitro* transcription from the PCR-amplified templates and diluted in hybridization buffer to a specific activity of 1×10^6 cpm/ml. Tissue sections 5 μm thick were deparaffinized, deproteinated in 4 μg/ml of proteinase K for 30 minutes at 37°C, hybridized at 55°C overnight, then washed at high stringency (55°C in 0.1× standard saline citrate for 2 hours). Glass slides were dipped in NBT2 nuclear track emulsion (Eastman Kodak), exposed in sealed plastic slide boxes containing desiccant for 4 weeks at 4°C, developed, and counterstained with hematoxylin and eosin.

Results

cDNA Fragment Selection and Identification

As reported previously,[1] incubation of endothelial cells in 3D gels in the absence of the growth factors resulted in rapid induction of apoptosis. Therefore, no comparison was made of mRNA from cells in 3D gels in the absence of growth factors. Instead we evaluated temporal changes in gene expression in the 3D gel environment in the presence of PMA, VEGF, and bFGF, by comparison of the RNA harvested at 4, 24, and 48 hours. A summary of the differences observed can be found in Table 2. The differentially expressed cDNA fragments in the 24-hour *versus* the 4-hour data set were examined in more detail. As shown in Table 3, the identities of 115 cDNA fragments were determined by oligonucleotide poisoning or cloning of the gene fragments, resulting in the identification of 90 distinct genes. In addition (not shown), 80 cDNA fragments were identified as totally novel or as corresponding to expressed sequence tags (ESTs) of unknown function. Full-length cloning of these genes is currently under way.

Confirmation of cDNA Fragment Identification and Expression

TaqMan probes were prepared to confirm by an independent method the identification of 67 of the genes identified by GeneCalling. As shown in Table 3, there was a reasonable agreement in the direction of the fold induction as predicted by GeneCalling and as analyzed by TaqMan. Disagreement between the GeneCalling and TaqMan results are likely due to small differences in the temporal sequence of events in the two separate experiments (ie, the RNA harvested for the GeneCalling experiment *versus* the RNA harvested for later TaqMan analysis), which could readily account for the discrepancies in the fold induction. A more complete time course analysis of 26 of the genes is depicted in Figure 1, A–D.

Genomic Response of Endothelial Cells in a Tube-Forming Environment

According to the classification schema used, the genes identified fell into most of the major role categories, including cell division, cell signaling, cell adhesion, hormone/growth factors, receptors, cytoskeleton, extracellu-

Table 1. Taqman Primer and Probes Sets

Gene name	Forward primer	Reverse primer	Probe
Hormones/growth factors			
Placental growth factor (PLGF)	GACGTTCTCTCAGCACGTTCG	CACCTTTCCGGCTTCATCTTC	CGAATGCCGGCCTCTGCGG
Stanniocalcin precursor	CGAGTGGCGGCTCAAAA	CCGCAGCCGACCTGTAGA	TCAGCTGAAGTGGTTCGTTGCCTCAA
Fibroblast growth factor 16 (FGF-16)	CCTTAGCTGACTCCCCAGGTT	CTGCAGCTTCCCCTCGATT	CCTGAACGAGCGCCTGGGCC
Angiopoeitin-2 Hlog (ai079861)	GGCCTGCAGCGGGTAGTA	GGCAGAAGCTTAAGAAGGGAATC	CGGCCCCGCCAGGTCTTCC
Connective tissue growth factor	TGCACCGCCAAAGATGGT	GGACTCTCCGCTGCGGTAC	CTCCCTGCATCTTCGGTGGTACGG
Cell cycle/apoptosis			
Human replication factor C	TTGCTTGTAATGCTTCGGATAAGA	TGTGTACCGGAGGACTGCAC	CATCGAGCCCATTCAGTCCCGC
ALG-2 interacting protein	TGAGCAATGGATCTGTTAACCAA	TCCCGTGTCAGTACAGCAGTCT	TCGGCTTTCCTCTGATTATAGGCAGCCA
p120	CCTCCGGACCAATACCTTGA	CCCCACGATTGATTAGAGCCT	CCCGACGCCGAGACCTTGCA
CDEBP (amyloid precursor-like protein 2)	GATGCCTCGTTGGTACTTCGA	CGCCGCAGCCACCATA	AAGCGCACGCACTTTCCCTTGG
BCL-2 related A1 protein	CAGCTCAAGACTTTGCTCTCCA	AGTCCTGAGCCAGCCTGTAAAT	ATCCAAATTCACAGTCTGTCATCTTCTGCCTG
CDC28/CDC2 associate protein CLK	TTGTTGCCTGGGCTGGA	TGAAACGAGAGCGCGAAGA	CGGGCGCAGCAGACAAAACCA
Human binding protein	GGCTACATCGAGGCTCTTGC	GGCTACATCGAGGCTCTTGC	GGCTACATCGAGGCTCTTGC
Polo-like kinase	GGATCACACCAAGCTCATCTTG	CCCGCTTCTCGTCGATGT	CCCACTGATGGCAGCCGTGACC
p53	GAGGTGCGTGTTTGTGCCT	TTCTTGCGGAGATTCTCTTCCT	TGCGCCGGTCTCTCCCAGGA
DNA binding proteins/transcription factors/histones/repair			
OS-9	AAGGCCTCCAAGCAGCATC	GGTCGCACTTGGACCCATT	TCTTAAACGCTACCACAGCCAGACCTATGG
Mel-18 homolog	GCGCTTCCTGCGATGC	TGCGGAGAAACTTGGCAAG	CAGCAGCCATGACCGTCATGCA
RNA synthesis/ribosomes			
Clone 23689	CTCAGCCCTCCGAGACCA	CTCCCGGATAATCTTGAGCACT	CGTGGGCAACATGGTGCGGA
Ribosomal protein L37	TGTCCTGGCTGGACGCTACT	TGAGGTGCCATCATCAATGTTC	TCACGATGACAGCTTTGCGTCCG
Chemokines			
Interleukin-8 (IL-8)	AAGGAACCATCTCACTGTGTGTAAAC	ATCAGGAAGGCTGCCAAGAG	TGACTTCCAAGCTGGCCGTGGC
GRO-1α (MGSA)	TGAGGAGCCTGCAACATGC	CATTGGCCATTTGCTTGGA	CCGCCAGCCTCTATCACAGTGGCT
Tyrosine kinase receptors			
axl	GCATGAAGGAATTTGACCATCC	TCTCTCGTTCAGAACCCTGGA	CAGACACCGATGAGCCTCATGACGTT
Epithelial cell tyrosine kinase (ECK)	GCCTGTTCACCAAGATTGACAC	GCCTCGAAGTCGCTGCTG	TTGCGCCCGATGAGATCACCG
Serine/threonine kinases			
Serum-inducible kinase	GAGGATCGTCCCAGTTTGGA	GAAGACAGTCTGTCCGGAGTGAA	CATCATTCGACATGACTTTTTTTTGCAGGG
Branched chain alpha keto acid Dehydrogenase kinase	CGGTTCCCCTTCATCCCTAT	TGTGGCTCTCATGGCATTCTT	CCACTGGACTACATCCTGCCGGAGCT
Thymidylate kinase	AAGCASTCGAAGCTGTCCATGA	TCTCTGTGGCAGTGCGGAT	TCCGCGTGCTCTCTGAGGACGC
Other receptors/integral membrane glycoproteins			
OX40	CCAACTCTGCACCGTTCTAGG	GGTATGCATGGCATACGTAAGC	CCGATGGCTGCCTCCGGCT
CXCR-4	CGCTACCTGGCCATCGTC	CATAGACCACCTTTTCAGCCAAC	CGCCACCAACAGTCAGAGGCCA
Podocalyxin-like protein	GGGCATGGTGAGGTTTCATCT	TTTACGCCCAGAACGATGG	CCATGGCGAAAGTTCAACATTCCACA
Alpha-2 integrin	TCTGAGACTGCCAAGGTCTTCA	CAGCTGGTATTTGTCGGACATC	AGGACTAGATCAGAAATGCAAAGTCCATCCTCA
JuSo MUC18 glycoprotein	GAACACAGTGGGCGCTATGA	CCTGTGGTTCACTCAGCAGC	CAGGCCTGGAACTTGGACACCATGATAT
MHC class 1 antigen	GCGCTCCGCTACTACAACCA	CGTCGCAGCCAAACATCA	AGGCCGGTTCTCACACCCTCCAG
gp130	ATCCGCGCAAGATGTTGAC	ACCTGTAGATTCAGTGGTGAGGAAA	ACAAGGCTTGCACTACCCAAGTCTGCA
T-cell receptor Beta 2	GAGGGTCTCGGCCACCTT	AGAACTGGACTTGACAGCGGAA	TGGCAGAACCCCCGCAACCA
Protein zero-related protein	TGTGTCATATCAATTTCTGGATTCATAA	TTGATCCAACTGTGTCCAGAATG	TGACTTCGGCATTTATCCTTTGCTAATCTTGCT
Proteases/protease inhibitors			
Tissue factor pathway inhibitor-2 (TFPI-2)	CGATGCTTGCTGGAGGATAGA	ACACTGGTCGTCCACACTCACT	AAAGTTCCCAAAGTTTGCCGGCTGC
Aggrecanase (ADAMTS4; KIAA0688)	ACTGGTGGTGGCAGATGACA	TCACTGTTAGCAGGTAGCGCTTT	ATGGCCGCATTCCACGGTGC
Matrix metalloproteinase-9 (MMP-9)	CCCGGAGTGAGTTGAACCA	CCTAGTCCTCAGGGCACTGC	TGGACCAAGTGGGCTACGTGACCTATG
Matrix metalloproteinase-1 (MMP-1)	CATGAAAGGTGGACCAACAATTT	CCAAGAGAATGGCCGAGTTC	CAGAGTACAACTTACATCGTGTTGCGGCTCA

Table 1. Continued

Gene name	Forward primer	Reverse primer	Probe
Cathepsin B	GAAGCCATCTCTGACCGGATC	TCCGCCGACACCTCCA	CCACACCAATGCGCACGTCAGC
Plasminogen activator inhibitor-2 (PAI-2)	GCAGGCACAAGCTGCAGATA	CCTGTGGATGCATTGATTGC	TCCATTCATCCTTCCGCTCTCTCAGC
KIAA0188			
Transporter/channels			
yLAT1 glycoprotein amino acid	AGGAGGCAATGCCAGGAAG	CTTCATACTCAGTGCTGTCAACCA	TGGTGAAGGGTTTCCTCTCCTCCACC
Transporter			
White protein homolog	CCCTTTCAGATCATGTTCCCA	GGACGGCTGCGACGTC	CCAGTACACGATGCTGCAGTAGGCCA
Cytoskeleton/motility			
Moesin	ACTGGGCCGAGACAAATACAA	AATGCGCTGCTTGGTGTTG	CCCTGCGCCAGATCCGGC
actin bundling protein	CCAGCTGCTACTTTGACATCGA	CCATTGGACGCCCTCAGT	GATGCGCCGGTCACGCCA
T-plastin	AATAAAACAGCCATGCTCCCA	CCTTAAGCCATAAGCACTTCACC	TGCATGATTCGCAGGTCAGCTATTTCC
Brain ankyrin-2	AAGCAGCTTCCTGATGCATTC	CGGACACAGCGCCTTACAT	TCGCAGCCAAGAACAGCCACCA
Intermediate filaments			
Mesothelial keratin K7	CCCAGATCTCCGACACATCTG	GCGATGATGCCGTCCAG	CCATGGACAACAGTCGCTCCCTGG
Extracellular matrix			
Laminin gamma 2 (nicein B2 chain)	GCTGACAGGCAGGTGTTTGAA	CGAAGTAGCCTGCTTTGCACT	TGTATCCACAACACAGCCGGCATCTACTG
Nidogen-2 (osteonidogen)	AAAATCTTAGAACTTTTGTTGGGAAACTA	CCTTGACAGTTGGAGAAGCCA	AAATAATTGGTCCTTTCCCATCAGTTCTGCA
Type IV collagen	CCCCTGGAACCTCCTCTGTT	CATTGTGGTGCATCCGTTGT	CACATGGATTTCTTATTACACGCCACAGCC
Extracellular protein S1-5	GGAACCCAGCTGACCCTCA	CTGCTGCACACTGGATACGG	CGCATTCCCTCCAACCCTTCCC
p137	CAGTGCTCCCCGGGATTACT	CCAGAGCCTCGCTTGAAATT	TGCTGATATCCATCCCGTTGATAGCCA
Metabolic enzymes			
OXA1	ACACGGCTCCTATTCCCAGTAG	AAGCCGCAAGGAAGAGGTAGT	CCCGTGCTGCTGTCGCCCA
thioredoxin peroxidase AOE 37-2-187F	GAGGCATCCCGGGTATCG	GGCTTGGAAATCTTCGCTTTG	CGCCGACCACTCCCTGCACCTAA
Posttranslational protein modification			
Peptidyl-glycine alpha amidating monooxygenase	GAGGGTCTCGGCCACCTT	AGAACTGGACTTGACAGCGGAA	TGGCAGAACCCCCGCAACCA
Signal transduction			
TRAF-1	GGACCCATCTGATGCACCTT	TGTGGTCCTCGGATTGCTTT	TCCCTCACTCGATTCCCCGGG
Lipids and lipid turnover			
Phospholipase A_2 gamma	GAAGGCGGTGAGCCTGAAC	TTCCAGGGAGGTCCTGGTC	TTCTCGAGCATCTCAGTCAGCCAGGTG
Cyclooxygenase-2	GAATCATTCACCAGGCAAATTG	TCTGTACTGCGGGTGGAACA	TCCTACCACCAGCAACCCTGCCA
Coagulation system			
Tissue factor	CACCGACGAGATTGTGAAGGA	CCCTGCCGGGTAGGAGAA	ACCCGTGCCAAGTACGTCTGCTTCA
Clathrin components			
Clathrin heavy chain	GGAGAAAATTGTCCTTGATAACTCTGT	TCAGCCTTAATTGCAGTGAGGAT	TTCAGTGAACACAGGAATCTGCAAAACCTCC
Unknown			
ALG2 hlog	TGAGCAATGGATCTGTTAACCAA	TCCCGTGTCAGTACAGCAGTCT	TCGGCTTTCCTCTGATTATAGGCAGCCA
Sushi-like Repeat Protein	GGGCTTTCGATTGATTGGAAG	GGCAGTTCCAGACCAACGAC	TCGGTGCAATGCCTGCCAAGC

lar matrix, protein turnover, protein modification, and metabolism (Table 3). There was no apparent bias in the identification of any given class of gene.

The mRNA changes were clustered based on four basic patterns of expression. Group I, Early Transient (Figure 1A), which included interleukin-8 (IL-8), binding protein A1, plasminogen activator inhibitor-2 (PAI-2), growth-related oncogene α (GRO-α), and cyclooxygenase-2 (COX-2), was characterized by mRNA levels that were rapidly and highly induced then declined to the initial levels within 24 hours. Peak mRNA levels were observed at 2–4 hours. Not shown in Figure 1A because of the small magnitude of fold induction, is an EST with homology to the fibrinogen domain of angiopoeitin-2 (AI79861). The message levels for this EST increased by twofold by 4 hours then returned to baseline levels between 8 and 12 hours. Group II, Delayed Transient, which included the genes white protein homolog, fibroblast growth factor-16 (FGF16), KIAA0188, ADAMTS-4 (aggrecanase-1), tissue factor pathway inhibitor-2 (TFPI-2), podocalyxin-like protein, cathepsin B, and epithelial tyrosine kinase (ECK), was characterized by mRNA levels

Table 2. Summary of Statistics of the Gene Fragments Found to Be Differentially Modulated in GeneCalling at a Given Fold Difference Threshold

Comparison	Differentially expressed fragments (%)*	
	Difference threshold	
	±2-fold	±4-fold†
24 hrs *vs.* 4 hrs	1343 (4.8)	393 (1.4)
48 hrs *vs.* 24 hrs	1291 (4.7)	367 (1.4)
48 hrs *vs.* 4 hrs	368 (1.3)	27 (0.1)

*Value is the percentage of gene fragments observed to be differentially expressed in the comparison.
†Arbitrary higher cut-off.

that peaked somewhat later than those of Group I (8–12 hours), then fell back to near-baseline levels by 46.5 hours. Group III, Stable Induction (Figure 1C), was characterized by genes whose mRNA levels rise somewhat later than those of Group I or Group II, peaked at 12–46.5 hours, and remained markedly above baseline levels, even at 46.5 hours. This group included placental growth factor, yLAT1, clone 23689, osteonidogen, matrix metalloproteinase 9 (MMP-9), CXC chemokine receptor 4 (CXCR4), and stanniocalcin precursor (STC). Group IV, Rapid Repression (Figure 1D), was quite different from Groups I–III. mRNA levels for genes in Group IV declined from the initial value observed at 30 minutes and, by 16–24 hours, were below the mRNA levels observed in the mRNA controls obtained from quiescent HUVEcs (see Materials and Methods). Genes in Group IV included extracellular protein S1–5 (S1–5), axl, polo-like kinase, and mesothelial keratin.

PPARγ Modulation of Endothelial Gene Expression

Treatment of endothelial cells with 10 μmol/L 15-d-PGJ_2 completely blocks endothelial tube formation in response to bFGF, VEGF, and PMA.[4] As shown in Table 4, 15d-PGJ_2 treatment reduced or, in some cases, abrogated the fold increase (24 *versus* 4 hours) of PLGF, clone 23689, STC, OX-40, TFPI-2, MMP-9, KIAA0188, +yLAT1, laminin γ2, and $PLA_2\gamma$. In addition, 15d-PGJ_2 reduced the fold decrease (24 *versus* 4 hours) of IL-8, axl, PAI-2, white protein homolog, and keratin K-7 observed during tube formation. The ratios of other mRNAs (A1, FGF16, eck, podocalyxin, and osteonidogen, for example) were not markedly affected by 15d-PGJ_2.

In Situ *Hybridization Analyses*

As an additional test of the biological relevance of the genes identified, we evaluated the *in situ* expression of four of the genes identified in this study, ie, STC, podocalyxin, osteonidogen, and ADAMTS-4, by examining their expression in a number of different tumors as well as sections prepared from a variety of human organs (see Materials and Methods). All four genes were detected at sites of endothelial activation or new blood vessel formation. For example, STC demonstrated strong but variable expression in the vasculature in and around mammary adenocarcinoma and squamous cell carcinoma (Figure 2) and, to a lesser extent, in chondrosarcoma and renal cell carcinoma (not shown), but there was no significant expression seen in normal vessels (not shown). Detectable expression of podocalyxin in normal adult tissue was limited to glomerular urinary epithelial cells (podocytes) and some endothelial cells in the adventitia around large vessels. Podocalyxin expression was expressed in the endothelium of small vessels associated with chondrosarcoma, squamous and renal cell carcinomas, and ductal mammary adenocarcinoma. (not shown), as well as in arteriolar endothelium in inflamed appendix (Figure 2). Osteonidogen expression was absent in normal adult vessels but was observed in endothelial cells of inflamed appendix (not shown) and in peritumor stromal (Figure 2) endothelium and nonendothelial cell types, as well as in osteosarcoma, chondrosarcoma, and squamous cell CA tumors. ADAMTS-4 expression in adult tissue was intensely expressed in vascular endothelium and smooth muscle in areas of inflammation (appendices, around tumors, in inflamed lung) (Figure 2), as well as in scleral and corneal limbic endothelium in an age-related macular degeneration eye (not shown). No detectable expression of ADAMTS-4 was observed in blood vessels of normal adult tissues.

Discussion

Many of the genes identified in this experiment have previously been implicated in angiogenesis. For example, the mRNA for PLGF, a member of the VEGF family of growth factors, increased during the initial 8 hours of incubation in the gel environment (Figure 1C). The EST AI79861 identified from the GeneCalling analysis is 78% identical to the COOH-terminal region of human angiopoietin-2, a naturally occurring antagonist for the tie-2 receptor kinase,[5] suggesting that yet another member of the angiopoietin family may play some role in the regulation of new vessel formation.

TFPI-2, which was a highly up-regulated gene in this study (Figure 1B), is a 32-kd serine protease and is associated with the extracellular matrix that inhibits the activation of matrix metalloproteinase zymogens, pro-MMP-1 and MMP-3.[6,7] TFPI has also been reported to be a smooth muscle mitogen.[8] Thus the up-regulation of TFPI-2 by the differentiation of endothelial cells might have a dual role of limiting the extent of matrix degradation and recruiting or promoting the proliferation of mural cells, leading to the assembly of the new vessel wall.

The mRNA levels for a number of proteases increased substantially during the initial 8–10 hours of incubation in the gel, notably the matrix metalloproteinase, MMP-9 (Figure 1C), and cathepsin B (Figure 1B). MMP-9 is an established participant in angiogenesis, playing important roles in the degradation of basement membrane/matrix in both *in vitro* and *in vivo* models of angiogenesis.[9,10] MMP inhibitors reduce the elongation of endothelial cells into tubelike structures *in vitro*[11] and

Table 3. Genes Identified by GeneCalling

Confirmed gene*	Accesion no.†	GeneCalling ratio‡	TaqMan ratio§
Hormones/growth factors			
Placental growth factor	X54936	6	5
Transforming growth factor beta	X02812	11	2
Stanniocalcin precursor	U25997	14	8
FGF-16	AB009391	4	1
Angiopoetin-2 Hlog	AI079861	0.1	0.1
Connective tissue growth factor	U14750	0.1	0.2
Cell cycle/apoptosis			
Human replication factor C	M87338	5	23
ALG-2 interacting protein	sim to AJ005073	5	2
p120 proliferation associated antigen	X55504	4	1
CDEBP (amyloid precursor-like protein 2)	Z22572	4	1
Bcl-2 related protein A1	L19597	4	1
ABC50 ATP binding cassette protein	AF027302	2	ND
cdc28/cdc2 associated protein CLK	L29219	<0.1	0.3
Polo-like kinase	U01038	<0.1	<0.1
p53	AA143745	0.1	1
CDK4 inhibitor (p16-INK4)	L27211	5	ND
DNA binding protein transcription factors histones/repair			
KIAA0192/TRAP230	AF117755	3	1
Mel-18 hlog	AA477595	4	ND
OS-9	AB002806	2	1
DNA nucleotide exotransferase	AA744855	2	ND
Histone HUMGS00579	M37583	0.2	0.3
oriP binding protein (OBP-2)	L29606	<0.1	ND
RNA synthesis/ribosomes			
Clone 23689	AF035280	8	8
Ribosomal protein L37a	L22154	4	ND
Chemokines			
Interleukin-8	M28130	0.3	<0.1
GRO1α (melanoma growth-stimulating activity)	X57019	<0.1	<0.1
Tyrosine kinase receptors			
eck	NM_004431	3	3
axl	P30530	0.2	<0.1
Serine/threonine kinases			
Serum inducible kinase	O60679	7	2
Branched chain alpha keto acid dehydrogenase kinase	AF026548	4	1
Thymidylate kinase	L16991	0.5	0.3
Other receptors/integral membrane glycoproteins			
OX40	S76792	18	18
CXCR4	AF052572	13	6
Alpha 2 integrin	X17033	13	2
Podocalyxin-like protein	U97519	12	2
CD82	D28137	12	4
MUC18	M29277	5	1
MHC class I	M83191 D14343 M20022	3	3
gp130	M57230	3	1
T-cell receptor beta 2	X01411	3	2
MHC class II	L18885	2	ND
Protein zero related protein	R60084	6	6
Proteases/protease inhibitors			
Tissue factor pathway inhibitor-2	L27624	9	7
Aggrecanase (ADAMTS4)	NM_005099	18	2
Type IV collagenase (MMP9)	J05070	6	177

*Gene identified and confirmed by GeneCalling.
†GeneBank accession number of gene from which cDNA fragment was identified.
‡Refers to the median value of ratios for 24 hours/4 hours for the different cDNA fragments identified for a specific gene.
§TaqMan Ratio refers to the ratio of mRNA at 24 hours *versus* 4 hours as determined by Taqman (see Materials and Methods).
ND, not determined.

Table 3. Continued

Confirmed gene*	Accesion no.†	GeneCalling ratio‡	TaqMan ratio§
Type I collagenase (MMP1)	X05231	10	2
Cathepsin B	M14221	3	2
Plasminogen activator inhibitor-2	M31551	<0.1	<0.1
KIAA0188	d80010	3	2
Transporters/channels			
Glycoprotein-associated amino acid transporter LAT1	AJ130718	6	20
White protein Hlog	AF038175	3	0.5
Cytoskeleton/motility			
Brain ankyrin 2	H58696	2	1
Moesin	M69066	2	1
Myosin-IC	U14391	2	ND
Actin bundling protein	U09873	2	2
T-plastin	L05491	2	1
Dynein light chain	U32944	3	ND
Intermediate filaments			
Keratin K7	X03212	0.2	0.1
Extracellular matrix			
Laminin gamma 2 chain	U31201	12	49
Nidogen-2 (osteonidogen)	D86425	4	2
Type IV collagen	Y00706	5	1
S1-5 (EGF-containing fibulin-like extracellular matrix protein-1)	U03877	0.2	<0.1
p137	Z48042	3	1
Signal transduction			
Calmodulin	M27319	4	ND
Ras-related protein RAL-A	H94944	2	ND
TRAF1	U59863	2	1
MT-GRPE precursor	AA989480	2	ND
Nonreceptor tyrosine kinase	AF097738	4	1
Metabolic enzymes			
Z-crystallin/quinone reductase	AA316207	4	ND
S-adenosylmethionine synthase Hlog SAMS2	D11332	4	ND
OXA1 subunit of cytochrome oxidase	X80695	2	1
Antioxidant enzyme A0E37-2	U25182	0.3	0.5
Endoplasmic reticulum ATPase	W38423	2	ND
Posttranslational protein modification			
Peptidylglycine alpha amidating monooxygenase	AF035320	5	4
Ubiquitin 52 amino acid fusion protein	D28425	0.3	ND
Lipids and lipid turnover			
Phospholipase A_2 gamma	AF058921	11	21
Apolipoprotein E	AA087386	0.3	ND
Cyclooxygenase-2	M90010	0.1	<0.1
Coagulation system			
Tissue factor	J02931	0.5	0.1
Endosome/lysosome			
Lysosomal membrane sialoglycoprotein(CD36-2L)	D12676	2	ND
rab 5 interacting protein	S83365	2	ND
Clathrin components			
Clathrin heavy chain	AA100413	3	1
Clathrin assembly protein	U45976	3	ND
Unknown			
PMP41 Hlog (ALG2)	AA226371	6	2
Insulin-induced protein	W37284	5	ND
NK-4	M59807	3	ND
KIAA0726	AB018269	2	ND

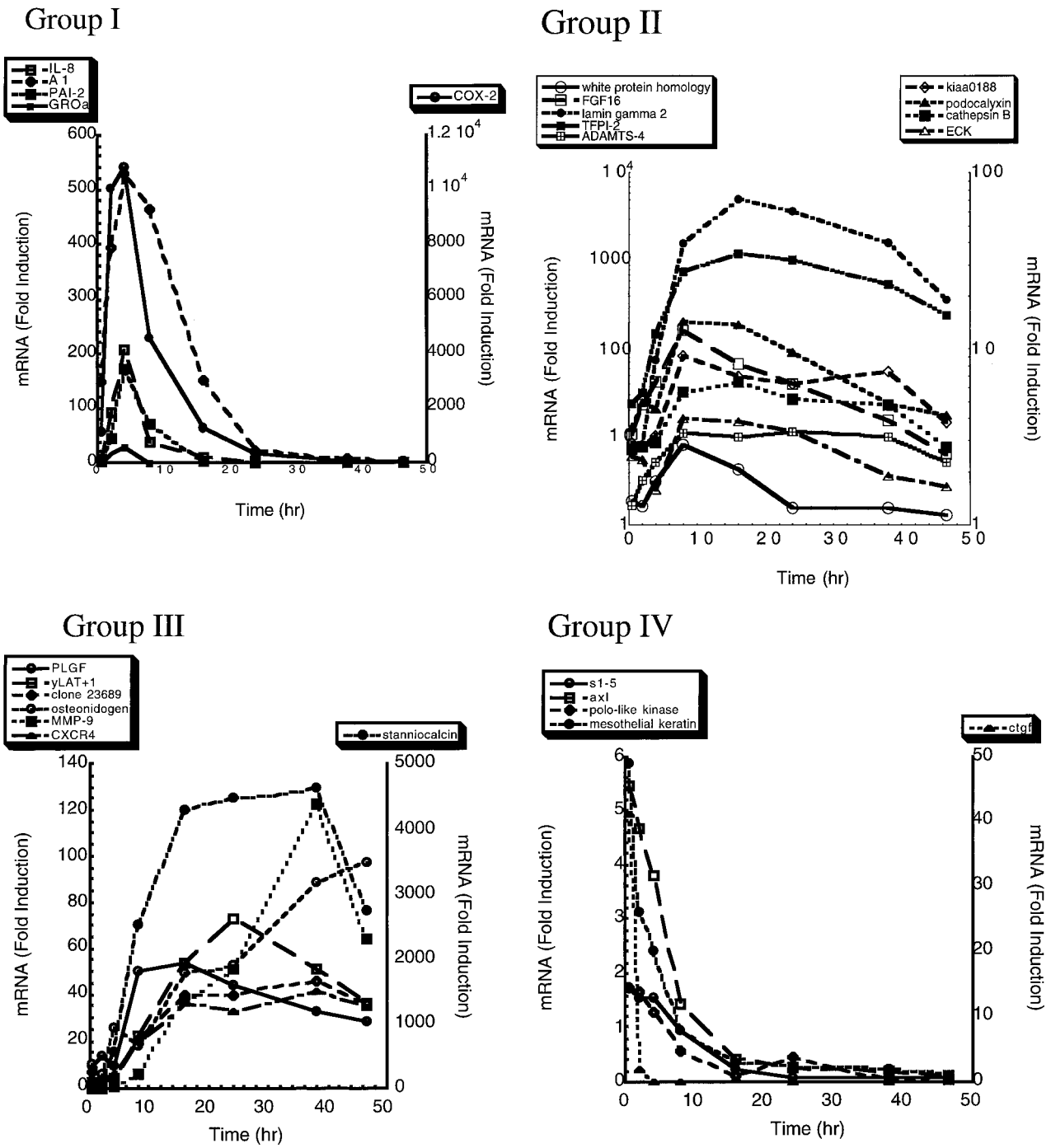

Figure 1. TaqMan analysis of the changes in gene expression of 26 genes identified by the GeneCalling analysis over the time period of 30 minutes to 46.5 hours. The changes in gene expression were grouped into four patterns, shown as Group 1 (rapid elevation in mRNA, peaking at 2–4 hours, then declining to baseline levels by 24 hours), Group II (more delayed elevation in mRNA, peaking at 8–12 hours, then declining to near-baseline levels by 46.5 hours), Group III (mRNA levels rising somewhat later than Group I or II, peaking at 12–46.5 hours, and remaining markedly above baseline levels at 46.5 hours), and Group IV (mRNA levels declining from the initial value observed at 30 minutes, and by 16–24 hours mRNA levels were below those obtained from quiescent HUVECs (see Materials and Methods)).

inhibit tumor angiogenesis in *in vivo* animal models[12] and in cancer patients.[13] Cathepsin B, a lysosomal cysteine protease, has been observed in many different tumor types and is highly expressed in tumor blood vessels as compared to normal vasculature.[14] One of the newest members of "disintegrin and metalloproteinase family members with thrombospondin motifs," ADAMTS-4 was identified as a differentially expressed gene in this study. Although relatively little information is available concerning ADAMTS-4, Tortorella et al[15] recently reported that this protein has aggrecanase activity and suggested that ADAMTS-4 played an important role in the turnover of the proteoglycan aggrecan in diseases such as osteoarthritis. Aggrecan has not previously been reported in vascu-

Table 4. Response of Modulated Genes to PPARγ Ligand 15d-PDJ_2

Confirmed gene*	Accesion no.†	TaqMan ratio‡	TaqMan ratio in 15d-PDJ_2-treated cells§
Hormones/growth factors			
Placental growth factor	X54936	5	0.4
FGF-16	AB009391	1	1
Stanniocalcin precursor	U25997	8	3
Cell cycle/apoptosis			
Bcl-2 related protein A1	L19597	1	1
Polo-like kinase	U01038	<0.1	15.5
RNA synthesis/ribosomes			
Clone 23689	AF035280	8	0.3
Chemokines			
Interleukin-8	M28130	<0.1	14.5
Tyrosine kinase receptors			
eck	NM_004431.1	3	2.3
axl	P30530	<0.1	15
Other receptors/integral membrane glycoproteins			
OX40	S76792	18	0.7
Podocalyxin-like protein	U97519	2	1
Proteases/protease inhibitors			
Tissue factor pathway inhibitor-2	L27624	7	1.3
Type IV collagenase (MMP9)	J05070	177	<0.1
Plasminogen activator inhibitor-2	M31551	<0.1	10.2
KIAA0188	d80010	2	0.5
Transporters/channels			
Glycoprotein-associated amino acid transporter LAT1	AJ130718	20	0.2
White protein Hlog	AF038175	0.5	2
Intermediate filaments			
Keratin K7	X03212	0.1	0.3
Extracellular matrix			
Laminin gamma 2 chain	U31201	49	4.9
Nidogen-2 (Osteonidogen)	D86425	2	1
Lipids and lipid turnover			
Phospholipase A_2 gamma	AF058921	21	0.8

A subset of genes identified as modulated in experiment were tested for response to PPARγ ligand 15d-PDJ2. Data are expressed as the ratio of expression observed at 24 hours/4 hours in collagen gel.

*Gene identified and confirmed by GeneCalling.

†GeneBank accession number of gene from which cDNA fragment was identified.

‡TaqMan ratio refers to the ratio of mRNA at 24 hours *versus* 4 hours.

§The ratio of mRNA at 24 hours *versus* 4 hours in the 15d-PDJ2-treated groups.

lar tissues, and we were unable to detect the expression of aggrecan mRNA in our endothelial cells under a variety of experimental conditions (data not shown). Intriguingly, however, two other members of the ADAM-TS family, METH-1 (human ADAMTS-1) and METH-2 (ADAMTS-8), were recently reported to be potent antiangiogenic agents.[16] The up-regulation of ADAMTS-4 during endothelial tube formation suggests a potential role of this enzyme in the metabolism of vascular proteoglycans, such as versican or other components of the basement membrane.

The mRNAs for the chemokines IL-8 and Gro-α are up-regulated early in the time course of endothelial differentiation into tubelike structures in the 3D gel environment (Figure 1A). These chemokines have previously been reported to have angiogenic activity.[17] mRNA levels for CXCR4, the chemokine receptor for SDF-1α,[18] increased by nearly 40-fold over a time period between 30 minutes and 16 hours, then remained elevated for the duration of the experiment (Figure 1C). Deletion studies have shown that both CXCR4 and SDF-1 null mice have defective formation of large blood vessels supplying the gastrointestinal tract.[19]

The mRNA level for the receptor "ECK" or epithelial tyrosine kinase (EphA2), a Eph receptor kinase family, increased modestly between 4 and 8 hours, and then slowly declined toward the levels observed at 30 minutes (Figure 1B). The ligand for ECK is a gene known as B61 (ephrin A1), initially identified as a tumor necrosis factor-α (TNFα)-induced gene in endothelial cells.[20] Antibodies to

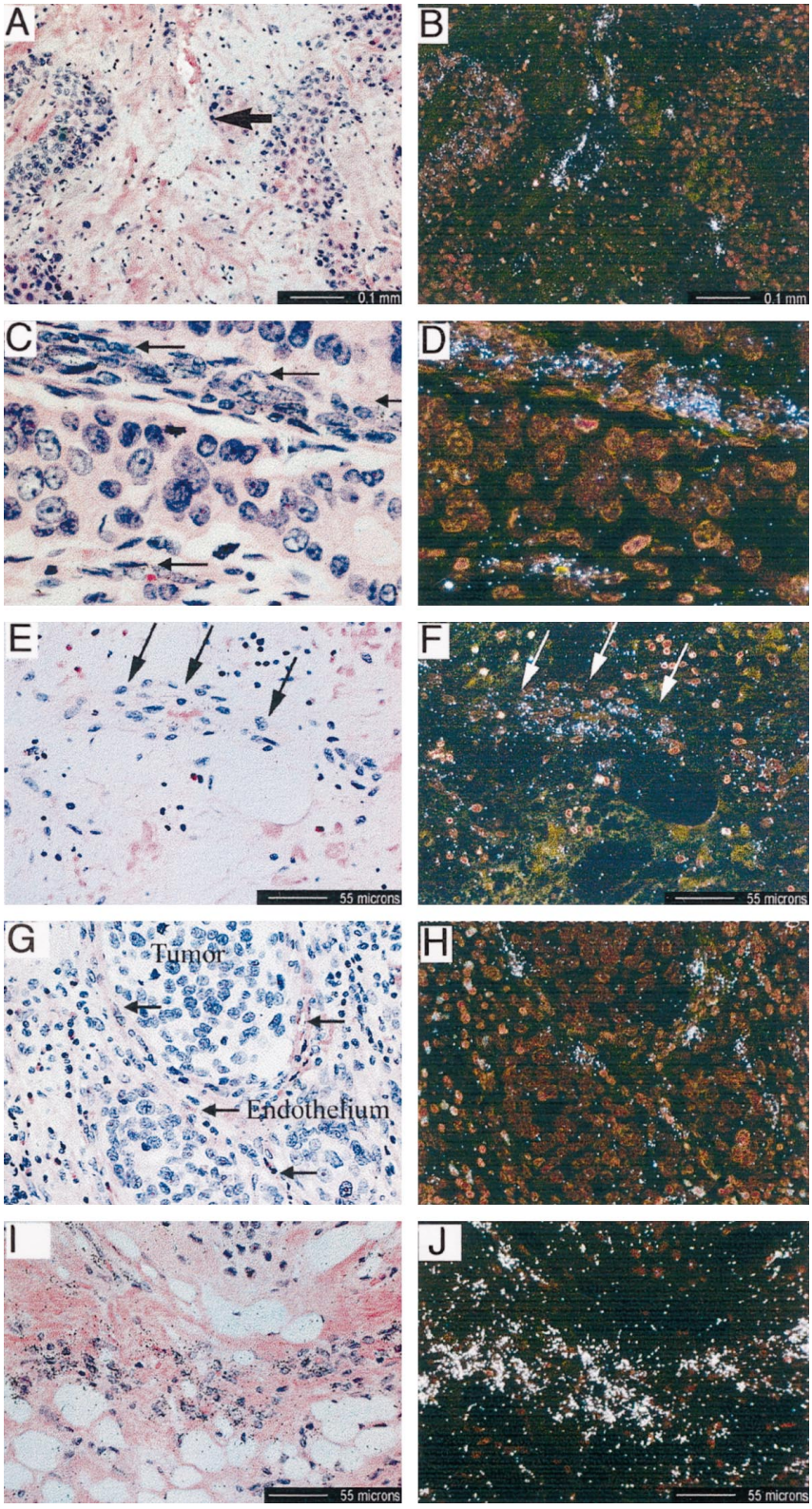

Figure 2. *In situ* hybridization demonstrating expression of genes identified from the differential expression analysis in the vasculature associated with tumors and with inflammatory disease. **A–D:** Hematoxylin-eosin (**A** and **C**) and *in situ* (**B** and **D**) hybridization demonstrating vascular expression (**arrows**) of stanniocalcin precursor mRNA in squamous cell carcinoma (**A** and **B**) and ductal mammary adenocarcinoma (**C** and **D**). **E** and **F:** Hematoxylin and eosin stain (**E**) and *in situ* (**F**) hybridization of osteonidogen mRNA in an arteriole (**arrows**) of inflamed appendix. **G** and **H:** Hematoxylin and eosin stain (**G**) and *in situ* (**H**) hybridization of podocalyxin expression in vessels surrounding lung squamous cell carcinoma (**arrows**). **I** and **J:** Hematoxylin and eosin stain (**I**) and *in situ* (**J**) hybridization of ADAMTS-4 expression adjacent to chondrosarcoma.

B61 block TNFα-induced angiogenesis in a corneal neovascularization assay.[21]

The mRNA for the laminin γ2 increased by almost 1000-fold between 0.5 and 24 hours (Figure 1B). Laminins are known to play key roles in angiogenesis[22] and are an important component of the basement membrane. Although the precise role of laminin in the differentiation process is unclear, laminin might be important for the establishment of the endothelial apical:basal polarity that may be a prerequisite for the formation of a vascular

lumen. The message levels for another basement membrane protein, osteonidogen (also known as Nidogen-2), increased throughout the duration of the HUVEC incubation in the 3D gels (Figure 1C). Nidogens can interact with laminin, collagen, and proteoglycans, and there are suggestions that this family of molecules may play some role in angiogenesis.[23]

In contrast to the genes discussed above, which have previously been implicated in various steps of angiogenesis, the study also identified a number of genes not previously associated with endothelial cell proliferation, differentiation, or angiogenesis.

The mRNA, designated KIAA0188, codes for a novel hypothetical protein. Domain analysis of the predicted amino acid sequence revealed a putative domain, aa 724–732, with homology to the consensus Kunitz-type serine protease inhibitor, and a domain, 455–467, with homology to the subtilase family of serine proteases, suggesting that this gene might code for a proform of a serine protease. KIA0188 mRNA levels increased modestly during the initial 8–10 hours of incubation in the gel, then declined to the initial levels (Figure 1B).

STC was first isolated from bony fishes,[24] where this glycoprotein is synthesized and secreted by the corpuscles of Stannius and regulates blood calcium levels through its inhibitory action on calcium ion uptake in the gill, a highly vascularized tissue.[24] Mammalian cDNAs encoding STC have been reported for the human and mouse and are highly homologous to those of the fish,[25] although the role of this protein in mammals is not known. mRNA levels for STC increased over 100-fold between 30 minutes and 16 hours and remained well above baseline levels out to 46.5 hours (Figure 1C). What role this gene plays in angiogenesis is unknown, but this observation suggests that further study in the context of angiogenesis is warranted.

The mRNA for FGF-16 demonstrated a biphasic profile, increasing substantially during the initial 8 hours and then declining for the remainder of the experiment (Figure 2B. This recent member of the FGF family of growth factors was originally cloned from human heart cDNA.[26] FGF-16 weakly stimulates NIH 3T3 fibroblast proliferation and but is a reasonably potent stimulus of primary rat oligodendrocyte proliferation.[27]

The mRNA levels of a number of membrane receptors were also increased over the time course of endothelial differentiation into tubelike structures, including the signaling component of the IL-6 receptor gp130, the TNFR-related protein OX40,[28] and the sialomucin, podocalyxin-like protein. There is no known role for any of these receptors in angiogenesis. Indeed, this study documents for the first time the expression of OX40 by endothelial cells. Previously this TNFR family member was thought to be restricted to cells of the lymphocyte lineage.[29] Increased protein expression of OX40 was also confirmed by fluorescence-activated cell sorter analysis (data not shown).

Podocalyxin-like protein, a well-known constituent of the endothelial plasma membrane,[30] was recently shown protein to function as an L-selectin receptor in inflamed lymph nodes,[31] suggesting a role in cell-cell interactions or adhesion.

The mRNA levels for the antiapoptotic bcl-2-related protein A1 were elevated at the early time points and then declined (Figure 1A). In the three-dimensional gel environments, HUVECs do not survive well in the absence of growth factors, and they cannot be rescued by supplementation with VEGF or bFGF.[1] However, PMA treatment will induce endothelial survival and tubule formation. The induction of A1 expression may thus be related to inhibition of apoptosis in the 3D gel environment.

The protein designated "white protein homolog" (also known as ATP-binding cassette (ABC) 8) is 84% identical to the *Drosophila* gene white protein, which codes for a transporter protein whose expression results in white eye color. Many members of the ABC family of proteins function as transporters or channels. The mRNA levels for this gene increased by ~10-fold between 0.5 and 8 hours (Figure 1B). There is little information relating to its expression or function in mammalian cells, although ESTs containing white protein sequence from a variety of tissue sources and tumors can be found in GenBank. The mRNA for y+LAT-1, a new member of a family of polytopic transmembrane proteins,[32] increased by about eightfold between 0.5 and 24 hours (Figure 1C). Little is known about the function of this permease, although it undoubtedly plays a role in amino acid transport and protein synthesis, two activities critical to altered endothelial protein expression.

Cyclooxygenase 2 (COX-2), a rate-limiting enzyme in the prostaglandin biosynthesis pathway, was detected in our experiment system. COX2 mRNA levels rose abruptly during the initial few hours of the experiment, then declined (Figure 1A). The transient expression we observed for COX2 is consistent with published reports proposing a role for COX2-regulated prostanoid responses after vascular injury.[33]

Xin et al[4] recently reported that agonists of PPARγ receptors specifically blocked endothelial tube formation *in vitro* and VEGF-driven angiogenesis *in vivo*. Furthermore, Xin et al[4] found that treatment of endothelial cells with the PPARγ ligand 15d-PGJ_2 inhibited the induction of kdr, flt-1, and uPA in a three-dimensional collagen gel model identical to the system used in the present investigation. The effects of 15d-PGJ_2 on mRNA levels for a number of genes identified in this study were therefore examined for the purpose of identifying genes modulated during tube formation specifically responsive to treatment with the PPARγ ligand. Those genes so identified might represent important targets for therapeutic intervention. As shown in Table 4, there were different classes of response to the PPARγ ligand (supermodulation, countermodulation, and no change in modulation), suggesting that the effect of treatment with 15d-PGJ_2 was not simply a general phenomenon. The selective and marked effects of 15d-PGJ_2 treatment on endothelial gene expression in the three-dimensional collagen system provides further support for the potential roles in angiogenesis of many of the genes identified in this study.

In summary, GeneCalling successfully identified 115 differentially expressed cDNA fragments corresponding

to 90 known genes from the study of collagen matrix-driven endothelial cell gene expression. In addition to the known genes identified, 80 fragments considered totally novel, or belonging to ESTs of unknown function, were identified in this study. The identity and expression of 67 of the known genes were confirmed by a second independent method (TaqMan). For the initial confirmations, we focused on membrane proteins and secreted proteins and only confirmed the identity of a few of the other cDNA fragments by this independent technique. However, in every instance the gene identified by the GeneCalling method was confirmed by TaqMan to be expressed by HUVECs in 3D gels, and the magnitude and direction of the changes in expression agreed reasonably well with the GeneCalling estimates. Because the method also identified a number of potentially new genes, we have not, at this time, pursued TaqMan confirmation of the remaining 23 genes, choosing to focus, instead, on the identification of new genes that might play a role in the process of endothelial differentiation into tubelike structures. Most importantly, the biological relevance of many of these newly identified "angiogenesis-associated" genes is strongly supported by the selective abrogation of their differential expression by the PPARγligand, 15d-PGJ_2, as well as by the *in situ* demonstration of selective expression of some of the genes at sites of new blood vessel formation. Although many of the identified genes have previously been associated with angiogenesis or tumor vasculature (eg, cathepsin B, MMP-9, PLGF, IL-8, GRO-α, CXCR4) or have reported roles or expression patterns consistent with a function in the differentiation process (A1, TFPI-2, laminin γ2), a number of genes identified in this study had never previously been associated with angiogenesis (stanniocalcin precursor, OX40, white protein homolog, the angiopoietin-2 homolog, ADAMTS-4, FGF16, KIAA0188), suggesting the need for the further evaluation of the potential biological roles of these genes in the process of new blood vessel formation

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