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• *BASIC RESEARCH* •

Differential gene expression during capillary morphogenesis in a microcarrier-based three-dimensional *in vitro* model of angiogenesis with focus on chemokines and chemokine receptors

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Abstract

AIM: To globally compare the gene expression profiles during the capillary morphogenesis of human microvascular endothelial cells (HMVECs) in an *in vitro* angiogenesis system with affymetrix oligonucleotide array.

METHODS: A microcarrier-based *in vitro* angiogenesis system was developed, in which ECs migrated into the matrix, proliferated, and formed capillary sprouts. The sprouts elongated, branched and formed networks. The total RNA samples from the HMVECs at the selected time points (0.5, 24, and 72 h) during the capillary morphogenesis were used for microarray analyses, and the data were processed with the softwares provided by the manufacturers. The expression patterns of some genes were validated and confirmed by semi-quantitative RT-PCR. The regulated genes were grouped based on their molecular functions and expression patterns, and among them the expression of chemokines and chemokine receptors was specially examined and their functional implications were analyzed.

RESULTS: A total of 1 961 genes were up- or downregulated two-folds or above, and among them, 468 genes were up- or down-regulated three-folds or above. The regulated genes could be grouped into categories based on their molecular functions, and were also clustered into six groups based on their patterns of expression. As for chemokines and chemokine receptors, CXCL1/GRO-α, CXCL2/GRO-β, CXCL5/ENA-78, CXCL6/GCP2, IL-8/CXCL8, CXCL12/SDF-1, CXCL9/Mig, CXC11/ITAC, CX3CL1/fractalkine, CCL2/MCP-1, CCL3, CCL5/RANTES, CCL7, CCL15, CCL21, CCL23, CCL28, and CCR1, CCR9, CXCR4 were identified. Moreover, these genes demonstrated different changing patterns during the capillary morphogenesis, which implied that they might have different roles in the sequential process. Among the chemokines identified, CCL2/MCP-1, CCL5/RANTES and CX3CL1 were specially up-regulated at the 24-h time point when the sprouting characterized the morphological change. It was thus suggested that they might exert crucial roles at the early stage of angiogenesis.

CONCLUSION: The present study demonstrates a global profile of gene expression during endothelial capillary morphogenesis, and the results provide us much information about the molecular mechanisms of angiogenesis, with which further evaluation of individual genes can be conducted.

Key words: Angiogenesis; *In vitro* model; Endothelial cell; Oligonucleotide array

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INTRODUCTION

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, plays a crucial role in a wide range of normal and pathologic processes, and is necessary for the continuous growth and invasion of solid tumors. After initial activation by angiogenic mediators such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), endothelial cells (ECs) degrade the local basement membrane, migrate into the underlying stroma, proliferate, and form capillary sprouts. It is believed that opposing sprouts coalesce and form a new capillary loop[1]. It has become increasingly clear that the angiogenesis is regulated by a balance of pro-and anti-angiogenic forces[2,3], and is a complex sequential process that relies on a controlled cross-talk between ECs and the surrounding environment. In normal adult tissues, anti-angiogenic mediators are dominant and ECs are suppressed and quiescent. During angiog-

enesis, the release of molecules, such as VEGF, overwhelms the inhibitors and ECs undergo the morphological steps necessary to generate a new capillary loop.

The molecular mechanisms underlying these morphological changes are, in large part, unknown. However, the advent of the global analytic techniques of gene expression, such as microarray, differential display, subtraction cDNA cloning and serial analysis of gene expression, has enabled the identification of the crucial angiogenic genes whose expression is regulated under different angiogenic conditions. Using this latter technology, St. Croix *et al*[4], compared the patterns of gene expression in ECs derived from the blood vessels of normal and malignant colorectal tissues. Bell *et al*[5], identified differential gene expressions during capillary morphogenesis in 3-D collagen matrices.

In order to determine the mechanisms controlling the sequential process of angiogenesis, appropriate models are necessary. *In vitro* model represents a rapid, defined and efficient experimental strategy to elucidate the molecular events required for the morphogenesis. Since 2-D angiogenesis models lack the third dimension and obviously do not reflect all physiological steps, the 3-D models are increasingly used for this purpose, because they consider more steps of angiogenesis[6]. In fact, a variety of 3-D angiogenesis models have provided great advances in the understanding of angiogenesis and the investigation of angiogenic and anti-angiogenic factors.

In 3-D angiogenesis models, the ECs are usually reorganized in the following ways: directly overlaid by gels, sandwiched between two gel layers, seeded dispersedly or clustered as spheroids in gels, or attached onto microcarrier (MC) beads. Recently, we have also established an *in vitro* angiogenesis system, which was based on MC, human dermal microvascular endothelial cells (HMVECs) and fibrin matrix[7]. With this system, we have observed all of the angiogenic steps that characterize the *in vivo* angiogenesis, including sprouting, branching, and capillary network formation[7]. The aim of the present study was to identify the gene expression profiles of HMVECs during capillary morphogenesis under stimulation of bFGF in the MC-based *in vitro* angiogenesis system.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was from Gibco Laboratories. Endothelial basal medium (EBM), epidermal growth factor (EGF), bovine brain extract (BBE), hydrocortisone, penicillin and streptomycin were from Clonetics (San Diego, CA). BFGF was from PeproTech EC Ltd (Rocky Hill, NJ). CD31-dynabeads were purchased from Dynal A.S (Oslo, Norway). Immuno-staining kit for factor VIII-related antigens was from Zhongshan Biotechnology Co., Ltd (Beijing, China). Cytodex-3 MCs were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Fibrinogen was from Raas Blood Product Co., Ltd (Shanghai, China).

Cells isolation and culture

HMVECs were isolated from juvenile foreskin as described[8,9]. Briefly, the foreskin was cut into small pieces, washed with PBS and incubated with 15-20 mL of 0.2% dispase in DMEM overnight (18-24 h) at 4 ℃. The tissue pieces were transferred into fresh DMEM and scratched with a scalpel. The resulting cell suspension was centrifuged and re-suspended in 10-15 mL of EGM, containing EBM supplemented with 10 ng/mL EGF, 12 μg/mL BBE, 1.0 μg/mL hydrocortisone, 100 U/mL penicillin, and 100 mg/mL streptomycin in the presence of 150 mL/L normal human serum, and seeded into a gelatin-coated 75-cm^2 tissue culture flask. Culture medium was changed every 2-3 d. Before the culture reached confluence (normally 5-7 d upon seeding), the HMVECs were separated from the other cells in culture by immuno-magnetic isolation with CD31-dynabeads following the manufacturer's instructions. ECs were characterized and determined to be>99% purity on the basis of the formation of typical cobblestone monolayer in culture and positive immunostaining for factor VIII-related antigens. The purified HMVECs were cultured in EGM at 37 ℃ in a humidified atmosphere containing 50 mL/L CO_2 in air on gelatin-coated cell culture surfaces. All assays were conducted with cells in passages 6 to 8.

Microcarrier cell culture

Gelatin-coated cytodex-3 MCs were prepared according to the recommendations of the supplier. Freshly autoclaved MCs were suspended in EGM, and HMVECs were added to a final density of 50-80 cells/MC. The cells were allowed to attach to the MCs in a 1.5-mL Eppendorf tube for 4 h at 37 ℃. The MCs were then resuspended in a larger volume of medium and cultivated for another 24-48 h at 37 ℃ (50 mL/L CO_2). MCs were gently agitated to prevent aggregation of individual MCs. MCs were used for angiogenesis assay when the ECs on the MCs reached confluence[10,11].

Microcarrier-based fibrin gel angiogenesis system

Microcarrier-based fibrin gel angiogenesis was performed as described[10,11] with some modifications. A working solution of fibrinogen was prepared by dissolving the stock solution of fibrinogens (20 mg/mL in PBS) in EBM to a concentration of 1.0 mg/mL, supplemented with 10 ng/mL of EGF, 1.0 μg/mL of hydrocortisone and 40 ng/mL of bFGF. The 24-well plates were used for the assay. The bottom of each well was first covered with 250 μL of fibrinogen solution, and the clotting was induced by addition of thrombin (0.5 U/mL). After 30 min of polymerization, another 250 μL of fibrinogen solution containing about 250 EC-coated MCs was added. The MCs were evenly distributed in the fibrinogen by gently shaking the plates, and polymerization was induced as described above. After complete polymerization, 1.0 mL of EBM supplemented with 10 ng/mL of EGF, 1.0 μg/mL of hydrocortisone, 40 ng/mL of bFGF and 50 mL/L adult human serum were added. To prevent excess fibrinolysis by fibrin-embedded cells, aprotinin was added to the growth media at 200 kIU/mL.

Total RNA isolation

Medium was aspirated from the surface of fibrin gels, and

the gels were scraped into a 50-mL polypropylene tube. After being centrifugated at 8 000 r/min for 3 min to remove the redundant liquid, two volumes of solution D/β-mercaptoethanol were added. The tube was vortexed vigorously for 5 min to resolve the gels, followed by the addition of 0.1 volume of 2 mol/L sodium acetate, one volume of water-saturated phenol and 0.3 volume of chloroform. After vigorously vortexed for 2 min, the tube was placed on ice for 15 min and then centrifuged at 12 000 *g* for 30 min at 4 ℃. A maximum volume of the upper aqueous phase was transferred to a new tube and equal volume of isopropanol was added. The solution was mixed by reiterative inversion, and incubated at -20 ℃ for at least 2 h. Following centrifugation at 8 000 r/min for 30 min at 4 ℃, the supernatant was removed, 2 mmol/L EDTA+0.1% SDS (1.5 mL/24 gels) were added to resuspend the RNA pellets. The suspension was transferred to 1.5-mL Eppendorf tubes and one volume of water-saturated phenol was added. The tubes were vortexed vigorously for 1 min and centrifuged at 12 000 *g* for 5 min. The upper aqueous phase was transferred to new tubes and 0.5 volume of water-saturated phenol and 0.5 volume of chloroform were added, followed by centrifugation at 12 000 *g* for 5 min. The upper aqueous phases were transferred to new tubes, and 0.1 volume of 3 mol/L sodium acetate and one volume of isopropanol were added. After incubation at -20 ℃ for at least 2 h, the tubes were centrifuged at 12 000 *g* for 15 min. After the supernatant was carefully removed and the pellet was washed twice with 750 mL/L ethanol, the pellets were air desiccated and dissolved in RNase-free H_2O. To determine the concentration and purity of RNA, 1-2 μg of RNA was diluted in 50 mL H_2O, absorption at 260 and 280 nm was checked. The $A_{260\ nm}/A_{280\ nm}$ ratio should be above 1.8. The integrity of total RNA was confirmed by 1% agarose/formaldehyde gel electrophoresis and Northern blotting for fibronectins. The samples of total RNA were eluted in DEPC-H_2O and stored at -80 ℃.

DNA microarray analysis

DNA microarray analyses were used to study the genomic-scale gene expression, the samples at different time points (0.5, 24, and 72 h after the beginning of culture) were compared during capillary morphogenesis. The selection of time points was based on the expression patterns of genes reported to be closely related to angiogenesis, by RT-PCR as described below. Approximately 150 gels for each time point were needed to obtain enough total RNA (about 25 μg for each time point) for this experiment. The total RNA was sent to Gene Company, to perform the hybridization to the Affymetrix GeneChip human genome U133 Sets (including A and B chips) containing 33 000 genes, to compare the RNA samples from the selected time points.

Biotin-labeled cDNAs were used to probe microarray chips and the hybridized probe arrays were stained with streptavidin phycoerythrin conjugate and scanned by the Affymetrix ® G2500A genearray scanner. The amount of light emitted at 570 nm was proportional to the bound target at each location on the probe array and was expressed as the signal value. The signal values from each time point were analyzed automatically by Affymetrix® Microarray suite (version 5.0), Affymetrix® MicroDB (Version 2.0), and Affymetrix® data mining tool (Version 2.0). The system processing methods were found at the manufacturer's web site (www.affymetrix.com).

Validation and confirmation of gene expression by semi-quantitative RT-PCR

To confirm the expression data from cDNA microarrays by an independent technique, gene-specific PCR oligonucleotide primer pairs were designed using Genelooper software (Geneharbor Inc.). Two micrograms of total RNA from each time point was used as the template for production of cDNA. Reverse transcriptions were performed using random primers for first-strand cDNA synthesis. Polymerase chain reactions were performed using samples from each time point to show patterns of expression with gene-specific primers. A GAPDH primer set was utilized to normalize the cDNA over the time course. RT-PCR amplification parameters used were typically 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 3 min. This was cycled 30 to 35 times, depending upon the gene, with a final extension at 72 ℃ for 7 min using a PTC-100 thermal cycler (MJ Research, Watertown, MA). Primer sequences used are shown in Table 1.

Table 1 **Primer sequences used for RT-PCR**

Genes	Primer (upstream)	Primer (downstream)	Length (bp) of PCR product	Annealing (temperature)
CYR61	5′-GAATGGAGCCTCGCATCCGATA-3′	5′-GTGGCTGCATTAGTGTCCATCC-3′	703	60
CD31	5′-ATGTGGCTTGGAGTCCTGCTGA-3′	5′-GTGTAGTTGCCACTGTGCTCCA-3′	878	60
RANTES	5′-GGATGGAGAGTCCTTGAACCTG-3′	5′-TTGAGACGGAGTCTCGCTCTGT-3′	441	60
PRSS3	5′-AGCAGCTCACTGCTACAAGACC-3′	5′-AGACCTTGGTGTAGACTCCAGG-3′	518	60
PTGS2	5′-CGGTGAAACTCTGGCTAGACAG-3′	5′-TTGGCTTCCAGTAGGCAGGAGA-3′	941	60
EBI3	5′-CCTTCATTGCCACGTACAGGCT-3′	5′-AGTGACTGGGATTACAGGTGCG-3′	845	60
ANXA1	5′-GTGAAGTCATCCAAAGGTGGTCC-3′	5′-CCTTATGGCGAGTTCCAACACC-3′	811	60
EGR1	5′-AGTGCCATCCAACGACAGCAGT-3′	5′-AGGACTTGGCTCTGAGAACCTC-3′	1 183	60
EDN1	5′-GCTGTTTGTGGCTTGCCAAGGA-3′	5′-GACCTTCGTCAGAAACTCCACC-3′	956	60
ADAM15	5′-TTCCAGCACCTGCTAAACCGCA-3′	5′-CAACTGAGCAGAGACAGGCTGT-3′	708	60
Rac1	5′-TGGTGGGAGACGGAGCTGTA-3′	5′-GGATCGCTTCGTCAAACACTG-3′	791	60
TIMP-1	5′-CACCAGAGAACCCACCATG-3′	5′-GCAGGCTTCAGCTTCCACTC-3′	669	57
TIMP-2	5′-TTTGCAATGCAGATGTAGTG-3′	5′-TCGAGAAACTCCTGCTTGG-3′	534	56
GAPDH	5′-GCCAAAAGGGTCATCATCTC-3′	5′-GTAGAGGCAGGGATGATGTTC-3′	286	60

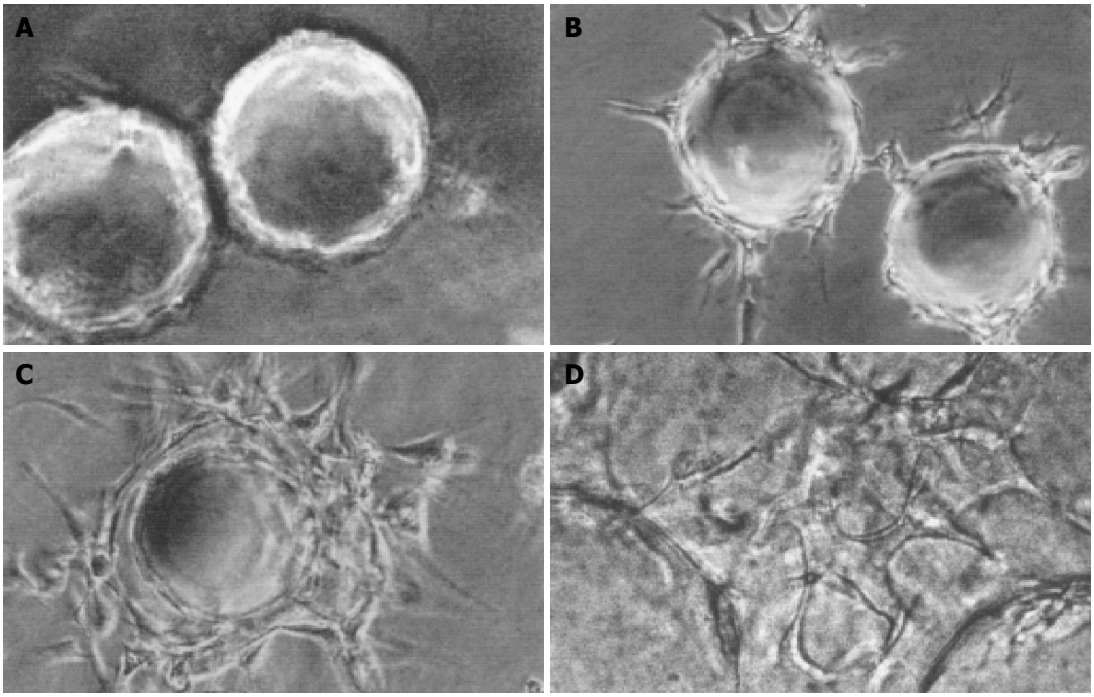

Figure 1 Sequential steps of capillary formation. **A**: MCs coated with HMVECs were embedded in fibrin matrix; **B**: HMVECs on MCs migrated into the matrix and formed sprouts without detectable lumina; **C**: Sprouts elongated, and small intracellular or intercellular lumina formed; **D**: Capillary sprouts anastomosed to each other, and capillary-like network formed (original magnification: A, B, C, ×100; D, ×400).

RESULTS

Sequential steps of capillary formation

In 1995, Nehls and Drenckhan[10] and Bülow[11] described a MC-based *in vitro* model of angiogenesis. In their model, the ECs coated on the MCs invaded the fibrin gel, forming an elongated capillary-like structure as a response to angiogenic factors. However, this model was performed with large vessel ECs of calf pulmonary artery origin[10-12]. In the present study, by modifying the system, we successfully established the MC-based *in vitro* model of angiogenesis with HMVECs in fibrin.

In the early stages of capillary growth (about 16 h after polymerization of the fibrin gel), the HMVECs on MCs migrated into fibrin matrix to form sprouts without detectable lumina. At about 16-48 h, sprouts elongated, and small intracellular or intercellular lumina formed. Usually, broad lumina developed at the base of the sprouts. The lumina frequently contained cellular debris, which could be seen to float by shaking the culture plates, indicating that lumina contents were liquified. The tips of capillary sprouts were generally solid or showed only primitive, slit-like lumina, which in later stages anastomosed to each other, and finally formed capillary-like networks (at about 5-7 d) (Figure 1). This result was not consistent with what was observed by Nehls *et al*[13], who assumed that due to the presence of serum, anastomosis of capillary sprouts occurred rarely. Since our goal was to characterize the gene expression patterns at different steps of angiogenesis including sprouting, branching and networking, our 3-D angiogenesis model gave us a good support.

Determination of the time points for DNA microarray by RT-PCR

From the morphological observation of the model, we found that the angiogenic course in our system could be coarsely divided into three phases as sprouting, branching, and network formation. But this might not give enough information about the changes at gene expression level for our determination of the time points. So some genes such as matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-1 (TIMP1), tissue inhibitor of metalloproteinase-2 (TIMP-2), sprouty, RhoA, Rac1, which were demonstrated to be closely related to angiogenesis by others, were selected and semi-quantitative RT-PCRs were performed to observe their expression patterns during the capillary morphogenesis (9 time points from 0.5 h to 7 d). We demonstrated that the most abrupt changes usually occurred from 24 to 72 h after the beginning of culture. According to the results of RT-PCR (data not shown), we chose 0.5, 24, and 72 h as the time points for the microarray analyses.

DNA microarray analysis of differential gene expression during capillary morphogenesis in the microcarrier-based 3-D fibrin angiogenesis system

Individual genes were represented by 'probe sets' on the Affymetrix oligonucleotide array. To identify differentially regulated genes during the ECs morphogenesis, we performed DNA microarray analyses with the total RNA isolated from cultures at selected time points. Consequently, a total of 1 961 genes were found up- or down-regulated two-folds or above, and among them, 468 genes were regulated three-folds or above. The regulated genes could be grouped into following categories based on their molecular functions such as growth factor and receptor, cell proliferation, extracellular matrix, cell cycle and apoptosis, signaling molecule and transcription factor, EC

Table 2 **Patterns of gene expression observed during capillary morphogenesis in 3-D MC-based angiogenesis system**

Patterns	Gene symbol
1	CDCA8, IRTA2, FLJ23548, ZNF83, ZNF131, ARPC4, COL13A1, LRRFIP1, FLJ90806, FACL4, AZ2, TNFRSF6B, GBP3, FLJ11029, MGC39350, FMO3, POLA2, PLEC1, DHX29, SLC20A1, FLJ21908, WSX1, CIRBP, CDC20, NRCAM, PRC1, LOC85028, TUBGCP5, C1orf33, CDC2, ADAM15
2	LOC51659, CCL5, GLRA3, CCNF, HELLS, WBSCR20A, C14orf80, EBI3, UBD, MCM4, SQLE, CDH2, TAGLN, HYAL3, FLJ22215, CDCA5, S100A3, NAV1, NFKB2, COL22A1, FRA, TP53I5, AURKB, URP2, TRAF1, ARHGDIA, STARD10, C22orf18, CX3CL1, CDT1, PRSS3
3	CAMK2G, PSMD5, MRPL35, RGS4, PXK, UBXD2, CGI-116, NET1, FLJ30707, CPD, DKFZp434L142, TRIP12, MGC11324, MAN2A1, CCL21, PRCP, CDA08, TPK1, SAS10, PTGDS, TRA1, ZNF317, ANGPT2, ADAMTS1, GPR116, GEMIN6, LEREPO4, RBMS1, ANGPT2, CARD10
4	OSR1, SKIL, SPATA1, RASA1, NFATC3, HDAC9, NYD-SP20, MGC8685, DPT, THAP6, BGN, COL4A3, SDCCAG33, KIAA1387, UBE2D3, CHD3, TNRC9, GATM, KERA, KCNF1, LOC90378, AEBP1, MFGE8, WISP3, CDC2L5, FBLN1, KIAA0014, GJA4, CPLX2, KIAA1365
5	FOS, FOSB, DUSP2, EGR4, ATF3, EGR3, EGR1, EGR2, NR4A1, NR4A2, CEBPA, PTGS2, MYLK2, SLC30A3, ZFP36, PTBP2, MYNN, JUNB, CDKN1C, ID2, GADD45B, TM4SF12, GLRA3, KLF4, SOCS5, SKIL, NR4A2, ID2, SUI1, FLJ13615, CYR61, EDN1
6	BHLHB2, KLF4, LOC92906, FXR1, DUSP6, NRP1, MIR, C6orf166, DUSP1, XLKD1, MAF, C11orf15, LRRC1, JUN, DLG1, FLJ90798, SPRY2, NPEPPS, PARVA, MGC9850, DKFZP564B1162, SEPP1, RGS2, HSA9761, ALDH1A1, DKFZP434F0318, LZK1, PDK4, dJ55C23.6, HES1, ANXA1, CD31

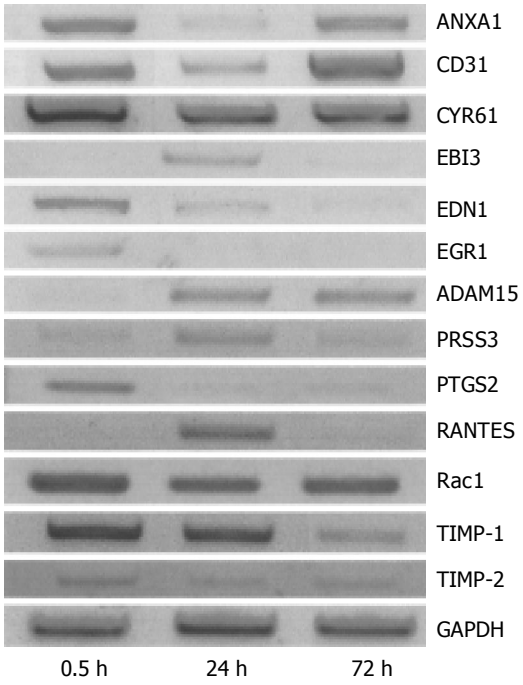

Figure 2 Validation of the expression patterns by RT-PCR.

differen-tiation marker, proteinase inhibitor, *etc*., using the gene ontology mining tool in The NetAffx™ Analysis Center. The regulated genes were also grouped into six patterns based on their changes of expression (Table 2).

To validate the patterns of expression analyzed by the microarrays, some genes were selected and semi-quantitative RT-PCRs were performed. The cDNAs from each time point were normalized to the housekeeping gene GAPDH. As shown in Figure 2, all of the genes evaluated, exhibited alterations in gene expression which was consistent with the data obtained from the oligonucleotide array analyses.

Expression of chemokines and chemokine receptors during the capillary morphogenesis

The chemokines and receptors detected by the oligonucleotide arrays are listed in Table 3, in which the genes were grouped based on their expression patterns (patterns 1-6, pattern 0 represented the genes without significant change during the capillary morphogenesis). Among the genes listed here, CXC and CC group included CXCL1/GRO-α, CXCL2/GRO-β, CXCL5/ENA-78, CXCL6/GCP2, IL-8/CXCL8, CXCL12/SDF-1, CXCL9/Mig, CXC11/ITAC, and CCL2/MCP-1, CCL3, CCL5/RANTES, CCL7, CCL15, CCL21, CCL23, CCL28, respectively. The CX3CL1/fractalkine, a number of CX3C group, were also detected. The expressions of CCL3, CCL28, CXCL9/Mig, and CXC11/ITAC were just detectable level and had no significant change during the process. The chemokine receptors in the list included CCR1, CCR9, CCR10 and CXCR4, but only the significant expression of CXCR4 was detected at all the time points. This implied its critical role in the capillary morphogenesis. Interestingly, by careful perusal of the change of expression, we could find that the CXCR4 ligand CXCL12 was expressed (pattern 4), which was consistent with its hypothetical role in the maintenance of a quiescent endothelium[32].

The different patterns of gene expression in this study gave a complicated context of the chemokines in the angiogenesis process, and implied that different chemokines might have a specific role in the different stages of the capillary morphogenesis. The chemokines that attracted our attention most were of pattern 2, which included CCL2/MCP-1, CCL5/RANTES and CX3CL1/fractalkine. They were specially up-regulated at the 24-h time point, when the sprouting was the morphologic characteristic, and then down-regulated to the basal level. The meaning of the expression in a timely fashion remains to be elucidated.

Since chemokines exerted their roles by recruiting leukocytes, we also examined the expression patterns of adhesion molecules in our assay and found that the adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and fibronectin (FN), which could mediate the interaction between ECs and leukocyte cells, were also regulated in the same pattern as that of CCL2/MCP-1, CCL5/RANTES and CX3CL1/fractalkine.

DISCUSSION

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, plays a crucial role in a wide range of normal and pathologic processes, and is necessary for the continuous growth and invasion of solid tumors[1]. Angiogenesis is regulated by the balance between the pro- and anti-angiogenic factors[2,3]. When the net balance was

Table 3 **Chemokines/chemokine receptors detected during capillary morphogenesis**

Sequence derived from	Gene symbol	24 h/0.5 h log ratio	24 h/0.5 h change	24 h/0.5 h change *P*	72 h/24 h log ratio	72 h/24 h change	72 h/24 h change *P*	72 h/0.5 h log ratio	72 h/0.5 h change	72 h/0.5 h change *P*
Pattern 1										
NM_001511.1	CXCL1	1.1	I	0.00004	-0.1	NC	0.5	0.8	I	0.00002
AK026546.1	CXCL5	1.2	I	0.009292	-0.4	NC	0.506476	0.6	I	0.00249
Pattern 2										
U84487	CX3CL1	1.5	I	0.000389	-1.9	D	0.999562	-0.8	NC	0.911062
NM_002985.1	CCL5	3.4	I	0.00002	-3.9	D	0.99998	-0.4	D	0.998799
S69738.1	CCL2	2	I	0.00002	-1.3	D	0.99998	0.5	I	0.00002
Pattern 3										
NM_002989.1	CCL21	-0.1	NC	0.596188	2.8	I	0.00002	2.1	I	0.000023
U58913.1	CCL23	-0.3	NC	0.5	1.4	I	0.000692	0.9	I	0.000147
Pattern 4										
NM_000609.1	CXCL12	0.4	NC	0.118009	-0.8	D	0.999727	-0.4	NC	0.777251
NM_002993.1	CXCL6	0.3	NC	0.088938	-1	D	0.999226	-0.6	NC	0.930187
NM_013246.1	CLC	-0.1	NC	0.5	-0.1	D	0.996959	-1.2	NC	0.98579
Pattern 5										
NM_006273.2	CCL7	-0.6	NC	0.5	-0.8	NC	0.977068	-0.7	D	0.999759
M57731.1	CXCL2	-1	D	0.99288	-0.5	NC	0.900148	-1.5	D	0.995927
Pattern 6										
AF348491.1	CXCR4	-1.7	D	0.99998	1.8	I	0.00002	0.1	NC	0.094279
NM_000584.1	IL8	-0.9	D	0.999965	1.1	I	0.001486	-0.2	NC	0.5
NM_004166.1	CCL15	-1.4	D	0.99998	0.4	I	0.000552	-0.9	D	0.99998
Pattern 0										
NM_016602.1	CCR10	1.1	NC	0.028766	-0.4	NC	0.62112	0.4	NC	0.019624
NM_002416.1	CXCL9	0.1	NC	0.5	-0.4	NC	0.814019	-0.2	NC	0.692594
AF275260.1	CXCL16	0.5	NC	0.5	-0.4	NC	0.5	0.1	NC	0.013078
AF030514.1	CXCL11	-1	NC	0.692594	0.9	NC	0.018128	0.2	NC	0.078937
AF145439.1	CCR9	0.3	NC	0.5	-0.7	NC	0.973302	-0.9	NC	0.938478
NM_001295.1	CCR1	0.4	NC	0.5	-0.8	NC	0.822588	-0.3	NC	0.881991
NM_002983.1	CCL3	0.3	NC	0.5	-0.5	NC	0.506476	-0.5	NC	0.60871
AF266504.1	CCL28	0.3	NC	0.177412	-0.9	NC	0.987976	-0.6	NC	0.854318

D: decrease; I: increase; NC: no change.

tipped in favor of angiogenesis, the "angiogenic switch" was on[14], and the ECs consequently degraded the local basement membrane, migrated into the underlying stroma, proliferated, and formed capillary sprouts[15].

The basal morphologic process of angiogenesis includes sprouting, branching and network formation. The molecular mechanisms underlying these characteristic morphologic changes are now under deep investigation and a variety of *in vitro* 3-D models have been employed. However, because of different systems employed, the results were usually different, or even controversial. Thus, we must keep in mind that any *in vitro* system can only reflect *in vivo* scenario from one respect, and more steps of the process should be considered when we choose the model system for investigation of molecular mechanisms of angiogenesis. In addition, the EC and the 3-D matrix are also the critical factors, which have great effects on the results. It has thus been emphasized that microvascular ECs are mandatory for the angiogenesis investigations[16].

In the present study, we developed an *in vitro* angiogenesis system based on MCs, HMVECs and fibrin matrix. With this system, and under the stimulation of angiogenic factors, such as bFGF and VEGF, we observed all the characteristic steps of *in vivo* angiogenesis, including sprouting, branching and network formation. As shown in a previous study, the morphological changes were dose-dependent on the growth factor used[7]. Thus, we believe that this system has an advantage over the model employed by Bell *et al*[5], for the mechanism investigation of angiogenesis, because in the latter system, the ECs were suspended in the matrix as single cells, and all cells underwent morphologic changes at the same time, which was assumed to be closer to vasculogenesis as discussed by the authors[5].

For the molecular mechanisms, microarray analysis has proved to be an efficient strategy. Using rigorous and tightly controlled experimental conditions, tightly controlled biological replicates, and multiple comparisons, gene expression profiling using microarray technology can yield reliable, highly validated results. For example, Gerritsen *et al*[17,18], identified over 1 000 differentially expressed genes as regulated in a 3-D collagen gel model of endothelial differentiation using the Affymetrix oligonucleotide technology combined with software analysis packages. Several hundred of these genes were selected for further evaluation by an independent method (RT-PCR) and greater than 95% of the genes identified were shown to be regulated in a manner suggested by the results from the oligonucleotide array. In the present study, we also utilized the Affymetrix oligonucleotide array to compare the gene expression profiles during the capillary morphogenesis and the data analyzed were associated with the morphologic changes. We identified discrete subsets of genes that were up-regulated or down-regulated during the process. We also classified the regulated genes into groups according to their changing

patterns, which enabled us to explain their significance in their complicated context.

Among the data obtained from the array, we focused our interest on the chemokines and chemokine receptors. Chemokines are a family of small secreted proteins that, depending on the spacing or presence of four conserved cysteine residues, have been classified into CC, CXC, CX3C and C chemokines[19]. CXC chemokines can be further divided into two groups of molecules (ELR+and non-ELR) according to the presence or absence of an ELR (Glu–Leu-Arg) motif. It has been demonstrated that many types of malignant cells could express chemokines such as RANTES, MCP-1, IL-8, and the expression of these chemokines by tumors were found to be correlated with the tumor angiogenesis and progression[20-22]. It has also been found that ECs are capable of binding and responding to a number of chemokines[23-28]. For example, CXC chemokines, such as CXCL8/IL-8, CXCL1/Gro-α, CXCL2/Gro-β, CXCL4/PF-4 and CXCL10/IP-10 have been implicated in the regulation of EC functions including the stimulation and inhibition of proliferation, angiogenesis and cell migration[29-31]. A proposed theoretical model about the role of chemokines in the angiogenesis is that the chemokines expressed and secreted from malignant cells (or other type of cells) could interact directly with the special receptors on ECs, or recruit leukocytes, which in turn could produce angiogenic factors that then bind to the special receptors on the ECs, exert their specific roles in regulating the EC functions[32].

However, the roles of chemokines and their receptors in angiogenesis still remain to be further elucidated. In fact, many chemokines have been confirmed to be angiogenic or angiostatic, directly or indirectly by regulating other factors. ECs of different origins can express not only chemokine receptors as targets of chemokines from other cells, but also chemokines themselves, which can improve the adhesion of leukocytes to the endothelium, or regulate endothelial functions in autocrine ways[32-36].

In this study, several genes for chemokines were also identified, including CXCL1/GRO-α, CXCL2/GRO-β, CXCL5/ENA-78, CXCL6/GCP2, IL-8/CXCL8, CXCL12/SDF-1, CXCL9/Mig, CXC11/ITAC, and CCL2/MCP-1, CCL3, CCL5/RANTES, CCL7, CCL15, CCL21, CCL23, CCL28, and CX3CL1. The chemokine receptors identified included CCR1, CCR9 and CXCR4. During capillary morphogenesis, these genes underwent different changing patterns. Although the exact roles of these chemokines and chemokine receptors during angiogenesis need further investigation, the changing patterns have given many hints to their possible functions. For example, as described above, the specific up-regulation of CCL2/MCP-1, CCL5/RANTES and CX3CL1/fractalkine in the sprouting process might suggest their important roles at the early stage of angiogenesis.

In conclusion, the present study demonstrates a global profiling of the gene expression during endothelial capillary morphogenesis, and the results provide us much information about the molecular mechanisms of angiogenesis, with which further evaluation of individual genes can be conducted.

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