

Distinct regulation of gene expression in human endothelial cells by TGF- β and its receptors

Xiaoping Wu^{a,b,1}, Jing Ma^{a,1}, Jing-Dong Han^c, Nanping Wang^d, Ye-Guang Chen^{a,*}

^a State Key Laboratory of Biomembrane and Membrane Biotechnology, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China

^b Department of Biotechnology, Nanchang University, Nanchang 330047, China

^c Institute of Genetics and Developmental Biology, The Chinese Academy of Sciences, Beijing 100101, China

^d Institute of Cardiovascular Sciences and MOE Key Laboratory of Molecular Cardiology, Peking University Health Science Center, Beijing 100083, China

Received 4 July 2005; revised 24 October 2005; accepted 4 November 2005

Available online 5 January 2006

Abstract

Transforming growth factor beta (TGF- β) and its signaling mediators play essential roles in angiogenesis—formation of new blood vessels, as evidenced by targeted gene disruption in mice and their mutations in human vascular dysplasia. However, little is known about the molecular basis of TGF- β function in vascular formation. To study the function of TGF- β signaling in angiogenesis and to elucidate the signaling specificity of TGF- β receptors at the gene transcriptional level, we analyzed the expression profile of the genes regulated by TGF- β and its type I receptors ALK1 and ALK5 in human microvessel endothelial cells (ECs). Global change of gene expression profiles was examined by microarray and RT-PCR analyses in the ECs treated with TGF- β 1 or by adenoviral expression of the active ALK1 or ALK5. We found that the profiles of the genes regulated by TGF- β , ALK1 and ALK5 are distinct from each other, although some of genes are modulated by all of them. TGF- β regulated far more genes than ALK1 and ALK5 did. ALK1 enhanced the formation of tube-like structures of ECs, while ALK5 stimulates EC aggregation. Our results suggest that ALK1 appears to have important functions in regulating proliferation of ECs, whereas ALK5 tends to modulate cell–cell interaction and cell adhesion and extracellular matrix remodeling.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Angiogenesis; TGF- β ; Receptors; Endothelial cells; Microarray analysis

Introduction

Formation of new blood vessels during angiogenesis is thought to result from preexisting capillaries due to proliferation and remodeling of differentiated endothelial cells. In general, the angiogenic process can be divided into the activation phase and the resolution phase. In the activation phase, the vascular permeability and the basement membrane degradation are increased, which make the endothelial cells (ECs) capable to proliferate and migrate into the extracellular space and form new capillaries. In the resolution phase, proliferation and migration of ECs are terminated, and the basement membrane is reconstituted. Subsequently, mesenchymal cells are recruited onto the endothelial tube and differentiate into pericytes and

smooth muscle cells surrounding the newly formed vessel (Carmeliet, 2000; Folkman and D'Amore, 1996; Jain, 2003).

Transforming growth factor- β (TGF- β) is an important regulator of endothelial cell differentiation, vascular network formation and establishment and maintenance of vessel wall integrity (Marchuk et al., 2003; Pepper, 1997). TGF- β signaling plays a pivotal role in development and normal function of the cardiovascular system (Azhar et al., 2003). The genetic inactivation of genes encoding TGF- β 1, the type II receptor and ALK1 in mice led to embryonic lethality due to defect in the initial formation of the primitive vasculature, along with defective endothelial cell differentiation and inadequate capillary tube formation (Dickson et al., 1995; Goumans et al., 1999; Oh et al., 2000; Oshima et al., 1996; Urness et al., 2000). Smad5 knockout mice also die due to defects in vasculogenesis and angiogenesis (Weinstein et al., 2000; Yang et al., 1999). Moreover, mutations in the human ALK1 gene and in the endoglin gene, which is also specifically

* Corresponding author. Fax: +86 10 62794376.

E-mail address: ygchen@tsinghua.edu.cn (Y.-G. Chen).

¹ These authors contributed equally to this work.

expressed in ECs and encodes a TGF- β -binding protein, are associated with hereditary hemorrhagic telangiectasia (HHT), an autosomal dominant vascular dysplasia with characteristics of nasal and gastrointestinal hemorrhage (Johnson et al., 1996; Marchuk et al., 2003; McAllister et al., 1994; van den Driesche et al., 2003). Endoglin-null mice also show a defective angiogenesis and die at embryonic day 11.5 (Arthur et al., 2000; Bourdeau et al., 1999; Li et al., 1999).

Members of the TGF- β superfamily initiate their signals through heteromeric complexes of type II and type I serine/threonine kinase receptors. Upon ligand binding, the constitutively active type II receptor kinase phosphorylates and then activates the type I receptor. The activated type I receptor phosphorylates downstream receptor-regulated Smads (R-Smads) proteins, which can bind the common partner Smad4 (Co-Smad). The Smad complexes then move into the nucleus and regulate the transcription of target genes. Activin and TGF- β bind to type I receptors known as activin receptor-like kinase (ALK)-4 and -5, respectively. Smad2 and Smad3 transduce signals for TGF- β s and activins, whereas Smad1, 5 and 8 specifically mediate the signaling of bone morphogenetic proteins (BMPs) (Massague, 1998; Massague and Chen, 2000; ten Dijke and Hill, 2004). ALK-1, which is specifically expressed in endothelial cells, binds TGF- β but activates the Smad1/5 pathways (Chen and Massague, 1999; Macias-Silva et al., 1998; Oh et al., 2000).

TGF- β is known to activate two distinct Smad pathways in ECs: the canonical Smad2/3 pathway through ALK5 and the Smad1/5 pathway via ALK1 (Goumans et al., 2002, 2003; Oh et al., 2000). ALK1 and ALK5 may have distinct functions during angiogenesis. It was reported that the TGF- β signal mediated by ALK1 is essential for the transition of ECs from the activation phase to the resolution phase, whereas TGF- β signaling via ALK5 promotes the activation phase. Therefore, it was suggested that the balance between the ALK1 signaling and ALK5 signaling might be crucial in regulating proliferation and migration of endothelial cells during vascular formation (Oh et al., 2000). However, the molecular mechanism underlying their action still remains controversy (Goumans et al., 2002; Lamouille et al., 2002).

To study the function of TGF- β signaling in angiogenesis and to elucidate the specificity of TGF- β receptors at the gene transcriptional level, we analyzed the expression profile of the genes directly regulated by TGF- β , ALK1 and ALK5 in human microvessel ECs. Our results indicate that the profiles of the genes regulated by TGF- β , ALK1 and ALK5 are distinct from each other. Analysis of gene expression profiles suggests that ALK1 appears to promote proliferation of endothelial cells, whereas ALK5 mainly increases cell–cell interaction and cell adhesion and extracellular matrix (ECM) remodeling.

Materials and methods

Reagent and cell culture

Human microvessel endothelial cells (HMVECs) (Clonetics) were maintained at 37°C with 5% CO₂ in growth medium EBM-2 containing 5% fetal

bovine serum (FBS) (Clonetics). TGF- β 1 was obtained from R&D. Antibodies against Smad1 and Smad2 were generated from rabbits with recombinant Smad1 protein and recombinant Smad2 linker region polypeptide. Anti-HA antibodies are from Roche. Antibodies against phospho-Smad1/5 and phospho-Smad2 were from Upstate Biotech.

Generation of recombinant adenoviruses and cell infection

The constitutively active forms of ALK1 (ALK1(AAD)) and ALK5 (ALK5(AAD)) were generated by polymerase chain reaction (PCR) and confirmed by DNA sequencing. The cDNAs encoding HA-tagged ALK1(AAD) and HA-ALK5(AAD) were subcloned into the pAdlox shuttle plasmid and recombined with ψ 5 viral DNA in CRE8 cells, as described previously (Hardy et al., 1997). The recombinant adenoviruses were generated in 293 cells. The expression of the recombinant proteins was controlled in a tet-off system and driven by a 7 \times tetracycline (tet)/minimal cytomegalovirus promoter that was under the control of a tet-responsive transactivator (TTA). Adenovirus expressing TTA was co-infected with the recombinant adenovirus to induce the tet-controllable expression of the receptors (Wang et al., 1999).

The cells were infected with recombinant viruses of 100 multiplicity of infection (MOI) in the presence of 2 μ g/ml tet for 16 h. After washing with PBS, the cells were incubated in 5% FBS medium in the presence or absence of tet for the indicated time to control receptor expression.

Western blotting

Western blot analysis was performed as described (Chen et al., 1998). Cell lysates were separated by SDS-PAGE and proteins of interest analyzed by immunoblotting and ECL (Amersham).

Reporter gene assay

HMVECs were seeded in six-well plates. One day later, they were transfected with different reporter constructs with lipofectamine (Invitrogen) according to the manufacturer's instruction. After 8 h, the cells were infected with various recombinant adenoviruses, as indicated, in the presence of tet for 16 h and then incubated in medium in the absence of tet for another 24 h prior to harvest for luciferase assay.

RNA extraction

HMVECs were treated with or without TGF- β 1 for 2 h or 4 h in the medium containing 5% FBS. The cells infected by ALK1(AAD) virus and ALK5(AAD) virus in the presence of 2 μ g/ml tet for 16 h were incubated in the presence or absence of tet for 10 h or 12 h in the medium containing 5% FBS. Total RNAs were extracted from the cells using TRizol reagent (Invitrogen).

Oligonucleotide microarray and data analysis

Affymetrix HG-U133A oligonucleotide chips were used to monitor the mRNA levels in mock-treated, TGF- β -treated, ALK1(AAD)- and ALK5(AAD)-overexpressed cells. The GeneChip contains about 45,000 probe sets representing more than 39,000 transcripts derived from approximately 33,000 well-substantiated human genes. The HG-U133A Array includes representation of the RefSeq database sequences. The sequence clusters were created from the UniGene database. Chip hybridization was processed in the Microarray Core Facility of the University of California, Irvine.

The relative gene expression levels were obtained by comparing the TGF- β -treated, ALK1(AAD)- and ALK5(AAD)-overexpressed versus mock-treated cell lines using Affymetrix softwares. Then, genes, which were determined as 'Present' and consistently exhibited over 2-fold increase or decrease in both 2-h and 4-h treatments (for TGF- β) or in both 10h and 12 h treatments (for virus-mediated expression of ALK1(AAD) and

ALK5(AAD)) over the control mock-treated samples, were extracted as the ones whose expression levels increase or decrease under a particular treatment. Gene annotations updated by Affymetrix were used to determine gene categories.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

After RNA isolation, contaminating genomic DNA was removed by DNase I (Promega) for 1.5 h at 37°C in the presence of RNasin (Promega). DNase I was removed by phenol/chloroform extraction and ethanol precipitation. First-strand cDNA was reverse transcribed from total RNA by oligo(dT)₁₅ primer using the Reverse Transcription System (Promega) and stored at –20°C until use.

PCR was conducted in a 20 µl system. Samples were first denatured for 3 min at 94°C, and semi-quantitative PCR was performed at 94°C for 30 s, at 55°C for 30 s and at 72°C for 50 s for 30 cycles, with the final extension of 5 min at 72°C. PCR products were separated by electrophoresis in 2% agarose gel. Expression of GAPDH was used to serve as a control.

Tube formation assay

Fifty microliter matrigel (BD) was added into each well of a chilled 96-well plate and incubated for 15 min in 37°C. About 1.5×10^4 HUVECs were placed in 23 µl of M199 plus 2% FBS into each well. Then, 77 µl M199 plus 2% FBS containing growth factors FGF (10 ng/ml) was added. The cells transfected with recombinant adenoviruses of ALK1(AAD) or ALK5(AAD) were used after tet withdrawal for 8 h, and the control cells were those which were transfected with recombinant viruses and incubated with tet (2 µg/ml). After mixing and tipping, the plate was incubated in 37°C. Tube formation was observed with microscopy 4–8 h later.

Results

Expression of TGF-β receptors with a tetracycline-controllable adenoviral expression system

We attempted to dissect the functions of ALK1 and ALK5 in endothelial cells. In order to express exogenous proteins in endothelial cells, a tetracycline (tet)-controllable (tet-off) adenoviral expression system was employed (Hardy et al., 1997; Wang et al., 1999). The recombinant adenoviruses expressing an active ALK1 mutant, ALK1(AAD) and an active ALK5 mutant, ALK5(AAD) were generated. Both ALK1(AAD) and ALK5(AAD) have mutations in the regulatory region of the intracellular domain (the GS domain): Leu190 to Ala, Pro191 to Ala and Gln201 to Asp in ALK1 and Leu193 to Ala, Pro194 to Ala and Thr204 to Asp in ALK5. Leu193Pro194 in ALK5 is important for FKBP12 binding (Chen et al., 1997; Huse et al., 1999), and Thr204 renders ALK5 an activity independent of ligand and type II receptor (Wieser et al., 1995). Combination of these point mutations confers these receptors a high constitutive activity (Siegel et al., 2003) (our unpublished data). Then, primary human microvessel endothelial cells (HMVECs) were infected with the receptor-expressing recombinant viruses in the presence of 2 µg/ml tetracycline for 16 h and then incubated in 5% FBS medium for different times. Receptor expression was analyzed by immunoblotting. The proteins of both ALK1(AAD) and ALK5(AAD) started to be detected at 8 h after tet withdrawal and reached to the

maximal level at 12 h (Fig. 1A). Moreover, 50 ng/ml tet effectively inhibited the expression of these proteins, indicating that receptor expression was tightly controlled by tet (Fig. 1B).

The exogenously expressed active receptors are functional in endothelial cells

To test whether the receptors expressed with this adenoviral system are functional, we examined phosphorylation of endogenous Smad proteins in the presence of ALK1(AAD) and ALK5(AAD). HMVECs were infected with recombinant adenoviruses in the presence of tet for 16 h and then incubated in the medium with or without tet for another 12 h. Total cell lysates were subjected to immunoblotting with anti-phospho-Smad1 and anti-phospho-Smad2 antibodies, respectively. TGF-β1 treatment induced phosphorylation of both Smad1 and Smad2 in HMVECs (Figs. 2A and B), consistent with the previous reports that TGF-β activates both Smad1 and Smad2 in endothelial cells (Goumans et al., 2002). Furthermore, ALK5(AAD) specifically stimulated Smad2 phosphorylation, whereas ALK1(AAD) specifically promoted Smad1 phosphorylation (Figs. 2A and B).

To further confirm the function of ALK1(AAD) and ALK5(AAD) in HMVECs, the receptor activity in activating gene expression was analyzed with the reporters BRE-luciferase and (CAGA)₁₂-luciferase that are specifically responsive to Smad1 and Smad3, respectively (Dennler et al., 1998; Hata et al., 2000). As shown in Fig. 2, ALK5(AAD) stimulated the expression of (CAGA)₁₂-luciferase (Fig. 2C), whereas ALK1(AAD) activated BRE-luciferase (Fig. 2D). These data suggested that the adenovirus-mediated expression of both the active ALK1 and ALK5 are functional in activating the expression of their specific reporters in ECs.

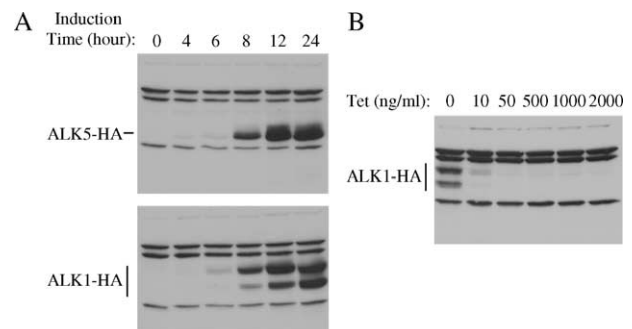


Fig. 1. The expression of receptors mediated by recombinant adenoviruses in HMVECs. The recombinant viruses expressing the active form of receptors ALK1(AAD) and ALK5(AAD) were infected into HMVECs in the presence of 2 µg/ml tetracycline(tet) for 16 h and then incubated in the absence of tet for different times (A) or with different concentrations of tet for 12 h at various concentrations of tet (B). Cell lysates were analyzed by immunoblotting. The bands corresponding to receptors are indicated by bars, and the other bands are nonspecific.

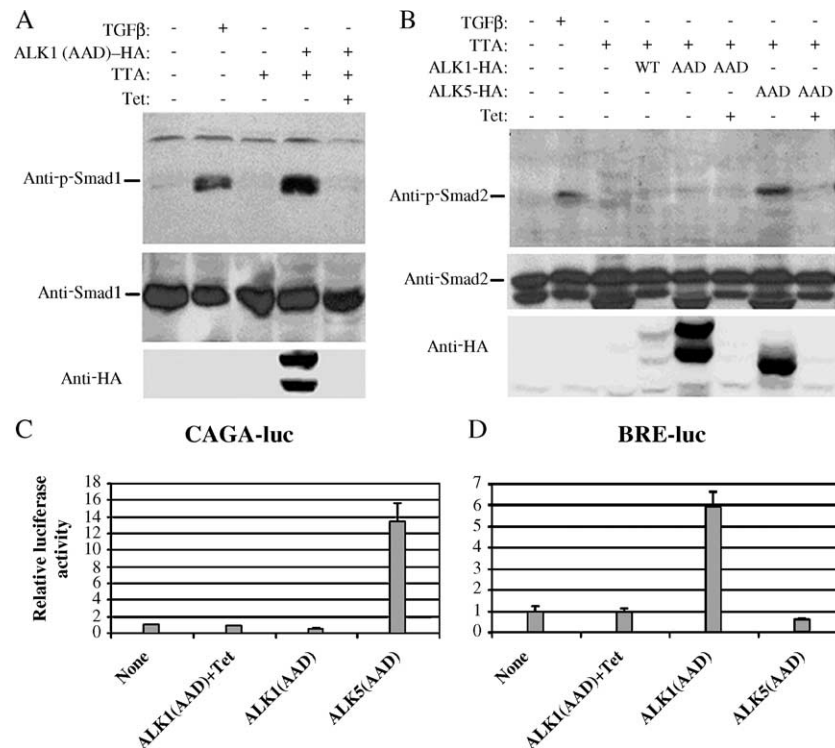


Fig. 2. The active forms of ALK1 and ALK5 expressed with adenovirus are functional in ECs. ALK1(AAD) and ALK5(AAD) induce phosphorylation of Smad1 (A) and Smad2 (B), respectively. After infected with recombinant adenoviruses in the presence of tet for 16 h, HMVECs were incubated in a medium with or without tet for another 12 h. TGF- β 1 was added 1 h before cell harvest. Total cell lysates were subjected to immunoblotting with anti-phospho-Smad1 or anti-phospho-Smad2 antibodies. Protein levels of endogenous Smad proteins and exogenously expressed receptors were shown in the middle and the lower panels. HMVECs were transfected with Smad2-specific reporter (CAGA)₁₂-luciferase (C) or Smad1-specific reporter BRE-luciferase (D). After 8 h, the cells were infected with various recombinant adenoviruses as indicated, in the presence of tet for 16 h, and then incubated in medium in the absence of tet for another 24 h prior to harvest for luciferase assay. TTA: Tet-responsive transactivator-expressing virus; AAD: an active mutant of receptors.

Differential effects of ALK1 and ALK5 on tube formation of ECs

To further investigate the activities of these TGF- β receptors in regulating EC tube formation, the ability of HUVECs to form tube-like structure on matrigel was tested. While the cells infected with the control virus poorly formed tube-like structures, FGF, a well-known angiogenic factor, stimulated tube formation (Fig. 3). Interestingly, the active ALK1(AAD) stimulated the EC tube formation, whereas the active ALK5(AAD) had no inducible effect on tube formation. Furthermore, the cells expressing ALK5(AAD) intended to form tight aggregates. These results suggest that ALK1 induces EC migration and ALK5 mainly promotes in ECs adhesion.

Microarray analysis and RT-PCR confirmation

To dissect the functions of ALK1 and ALK5 in angiogenesis, we attempted to compare the gene expression profiles directly regulated by TGF- β , the active form of ALK1 and the active form of ALK5. For this purpose, total RNA was extracted from HMVECs treated with TGF- β for 2 h and 4 h or from HMVECs which were infected with the recombinant adenovirus expressing ALK1(AAD) and ALK5(AAD) with tet withdrawal for 10 h and 12 h to allow receptor expression. Since the receptor proteins start to appear at 8 h after tet withdrawal (Fig. 1), we reasoned that signals resulted from receptor expression for 10 h and 12 h would be comparable to that initiated from TGF- β treatment for 2 h and 4 h. To eliminate the genes influenced by viral infection, control RNA

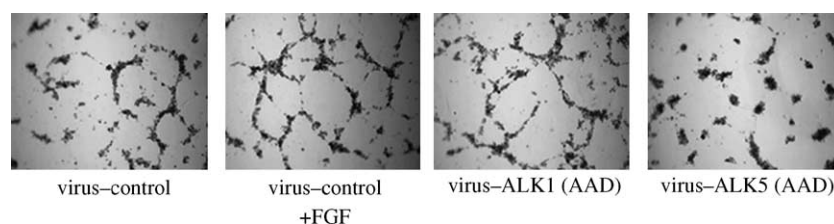


Fig. 3. The effects of ALK1(AAD) and ALK5(AAD) on tube formation of ECs. HUVECs were infected with recombinant adenoviruses expressing ALK1(AAD) or ALK5(AAD) and then placed on matrigels. Tube formation was observed with microscopy 4–8 h later.

was extracted from HMVECs infected with the recombinant adenovirus expressing ALK1(AAD) in the presence of tet. Microarray analyses were performed with Affymetrix Oligonucleotide GeneChip HG-U133A.

Then, genes, which consistently exhibited over 2-fold increase or decrease in both 2 h and 4 h treatments (for TGF- β) or in both 10 h and 12 h treatments (for virus-mediated expression of ALK1(AAD) and ALK5(AAD)) over the control samples, were sorted as the ones whose expression levels increase or decrease under a particular treatment. ALK1 was highly expressed in HMVECs, and the microarray analysis revealed that its mRNA level was further increased upon its exogenous expression mediated by the recombinant adenovirus, verifying the validity of this analysis. Virus-mediated overexpression of ALK5 was not detected in this study as the ALK5 oligo on HG-U133A chip is within the 3' untranslated region (UTR) of the ALK5 mRNA and is unable to detect the exogenous expressed mRNA lacking the 3'UTR. As shown in Fig. 4, 1268, 563 and 363 genes were regulated by TGF- β , ALK1(AAD) and ALK5(AAD) respectively, among which 1048, 428 and 244 genes were upregulated, while 220, 135 and 119 genes downregulated by the three treatments respectively. Unexpectedly, there were a limited number of genes regulated by both ligand and receptors—50 genes influenced by both TGF- β and ALK1, 28 genes by TGF- β and ALK5. On basis of the biological processes in which the genes may be involved (Consortium, 2001), the genes modulated by TGF- β , ALK1 and ALK5 are mainly participated in cell signaling, transcription regulation, protein processing and molecule transport (data not shown). The genes that are involved in angiogenesis are listed in Table 1.

In order to confirm the microarray results, we carried out semi-quantitative RT-PCR to verify expression of several genes. Data were quantitated and normalized to glyceraldehydes-3-phosphate-dehydrogenase (GAPDH). As shown in Fig. 5, endoglin, Smad5, monocyte chemoattractant protein-1 (MCP-1), matrix metalloproteinase-1 (MMP-1), interleukin-8 (IL-8), fibronectin and integrin α 6 identified as TGF- β -upregulated genes from microarray analysis were confirmed by RT-PCR. Expression of MCP-1, placental growth factor (PGF) and EphB2 was stimulated by ALK5, whereas Smad7 and PGF enhanced by ALK1. Platelet endothelial cell adhesion mole-

cule-1 (PECAM-1) was found to be downregulated by TGF- β , as shown by microarray analysis and RT-PCR. It is notable that fold of change in the expression level shown by microarray was not well consistent with the one obtained by RT-PCR. It could be due to different sensitivities of these methods. Nevertheless, the expression profile for most of the studied genes was consistent with the result obtained from gene chip analysis.

Discussion

TGF- β type I receptors ALK1 and ALK5 are thought to have different functions in angiogenesis. But, the molecular mechanisms are not clear. The results of our tube formation assay indicate that ALK1 promotes tube formation, whereas ALK5 increases cell aggregation.

This study is to elucidate the specificity of ALK1 and ALK5 in mediating TGF- β signaling by microarray analysis of the gene expression profiles. The genes regulated by TGF- β function in various aspects of angiogenesis: cell proliferation (VEGF, IL1 α , TRAIL, angiopoietin-2, FGF, FGFR1, MAPK1, TAK1), cytoskeleton organization and cell migration (EphB2, MCP-1, RANTES, IL-8, COX2, integrin α 4, integrin β 4, paxillin, spectrin), ECM remodeling (PLOD2, MMP1, MMP14, PECAM-1, fibronectin 1) and cell–cell interaction (ICAM-1, claudin 1, claudin 11). Some of them were also modulated by ALK1 and ALK5, such as VEGF, IL1 α , COX2 (PTGS2) and TRAIL (TNFSF10). They are important factors involving angiogenesis. In addition, ALK1 and ALK5 regulated expression of other genes that were not influenced by TGF- β : PIGF, C/EBP, ATF3, GADD34, myosin10, etc. PIGF has been implicated to synergize with VEGF in promoting angiogenesis (Carmeliet et al., 2001). GADD34, a regulator of cell growth and apoptosis in response to cell stress and DNA damage, has been recently suggested to attenuate TGF- β signaling by interacting with Smad7 and recruiting the catalytic subunit of protein phosphatase 1 (Shi et al., 2004).

ALK1 and ALK5 also modulated expression of distinct genes. For example, ALK1 upregulated JunD, CDK5, CCT4, CDC6, MMP10, Ephrin-B1 and P4HA1 and downregulated Rac2, integrin α E, ICAM-1 and ICAM-2, whereas ALK5 upregulated ephrin-A1, angiopoietin-like 4, Jun, JunB, MMP2,

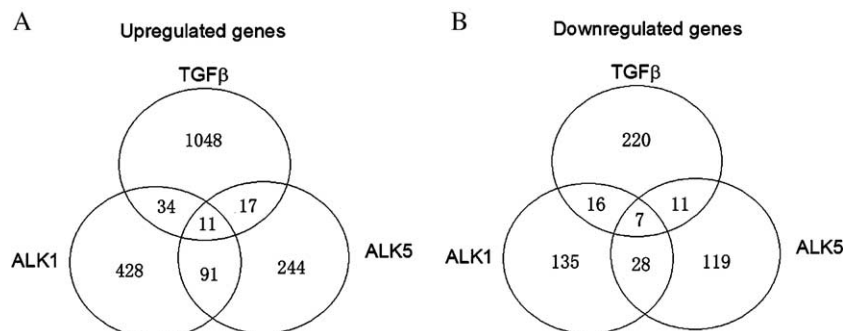


Fig. 4. The number of genes upregulated (A) and downregulated (B) by TGF- β , ALK1(AAD) or ALK5(AAD) are revealed by microarray analysis. The numbers in the overlapped areas indicate the genes co-modulated by different treatments.

Table 1

The genes which are related with angiogenesis and were regulated by TGF- β , ALK5(AAD) and ALK1(AAD) as revealed by microarray analysis

Gene description	Gene access number	Fold of change		
		TGF- β	ALK5(AAD)	ALK1(AAD)
Angiopoietin-2	AF187858	16		
Angiopoietin-like 4	NM_016109.1		2.8	
ATF3	NM_001674	0.4	3.0	8.0
BCL6	NM_001706	0.4		2.1
bFGF2	M27968	3.7		
Biglycan	BC002416.1	0.4	3.5	
C/EBP	AL564683		4.3	6.5
cadherin 2	M34064	2.8	3.5	
CCT4	NM_006430			2.3
CDC27	AI203880	4.6		
CDC5L	AW268817	3.5		
CDC6	U77949		3.3	4.0
CDK5R1	AL567411			11.3
Claudin1	NM_021101.1	13		
Claudin11	NM_005602.3	3.0		
CLK1	AI251890			6.5
collagenIV α 1	NM_001845	4		
collagenIV α 2	AA909035	6.5		
collagenVIII α 1	BE877796	6.5		
collagenV α 1	N30339		2.8	
collagenV α 2	NM_000393	2.1		
CRK	NM_016823	3.7		
CUL3	AU145232			2.5
CyclinB1	BE407516	0.2		
CyclinD1	M73554		0.5	
CyclinD2	AW026491	2.6		
ENG	BE732652	8.6		
EphB2	D31661	12.1	2.6	
Ephrin-A1	NM_004428.1		6.1	
Ephrin-B1	BF001670			2.0
F2R	NM_001992	2.0		
FGF18	NM_003862		8.6	
FGFR1	M63889	5.3		
FKBP1A	BC005147	4.9		0.5
FKBP1B	NM_004116	0.4		
FN1	AJ276395	14.9		
GADD34	U83981		3.7	6.5
GADD45A	NM_001924		2.5	2.6
GADD45B	NM_015675	0.4	2.6	
HYPE	NM_007076			2.3
ICAM1	NM_000201		2.5	0.5
ICAM2	AA126728	0.4		0.5
IL13RA1	U81380	7.5		
IL1RL1	NM_003856	3.5		
IL-1 α	M15329	5.7	3.0	3.0
IL-8	AF043337	3.7		
Integrin α 2	NM_002203	3.5		
Integrin α 4	NM_000885	7.0		
Integrin α 6	AV733308	9.9	0.5	
Integrin α E	NM_002208			0.4
Integrin β 3	M35999	4.0		
Integrin β 4	BF305661	6.5		
JUN	BG491844	0.4		2.8
JUNB	NM_002229.1		2.5	
Junction	NM_021991.1		2.5	
plakoglobin				
JUND	NM_005354.2			2.0
MAP3K2	AF239798	3.5		
MAP3K5	NM_005923		0.5	
MAPK1	AA195999	2.3		
MAPKAPK2	NM_004759	5.7		

Table 1 (continued)

Gene description	Gene access number	Fold of change		
		TGF- β	ALK5(AAD)	ALK1(AAD)
MAPRE1	AI633566	2.3		
MCP-1	S69738	5.7	3.0	
MMP1	NM_002421	2.6		
MMP10	NM_002425			3.5
MMP14	X83535	4		
MMP2	NM_004530		2.3	
Myosin10	NM_012334.1		2.0	2.8
NEDD5	AI191427	3.0		
NEDD9	AL136139	3.3		3.0
Ninjurin	NM_004148.1		2.3	
P4HA1	NM_000917.1			2.1
P53	K03199	24.3		
PAK2	BF796470	3.3		
Paxillin	D86862.1	119.4		
PECAM1	M37780	0.4		
PIGF	AK023843		2	2.8
PLOD2	NM_000935.1	6		2
PPAP2B	AA628586	2.1	2.3	
PPP1R15A	NM_014330		3.7	6.1
PTGS2	NM_000963.1	7.0	2.6	6.5
RAC2	NM_002872	2.1		0.5
RANTES	M21121		2.8	
RBBP6	NM_006910			2.1
Smad3	NM_005902	3.25		
Smad5	AF010601	2.6		
Smad6	NM_005585		0.2	
Smad7	NM_005904			2.1
Spectrin	NM_003128.1	64		
TAK1	AB009358	3.0		
TCF4	AU118026	2.8	2.1	
TGIF	NM_003244		2.6	
THBS1	AV726673	2.3		0.4
TNFRSF6	A164751	3.3		
TNFSF10	NM_003810		0.3	0.4
VEGF	AF022375	3.3	4.3	3.5

Fold of change indicates relative expression levels of the genes at 2 h of TGF- β 1 treatment or at 10 h after tet withdrawal for ALK1(AAD) and ALK5(AAD).

biglycan, ninjurin and junction plakoglobin and downregulated integrin α 6.

ALK1 stimulated the expression of CCT4 and CDC6, both of which function as cell cycle regulators. CCT4 is a cytosolic chaperonin essential for cyclin E maturation and thereby may play an important role in cell cycle progression (Won et al., 1998). Cdc6 promotes the assembly of the prereplicative complexes at the origins of DNA replication and has been suggested to regulate mitotic exit (Archambault et al., 2003). ALK1 specifically induced the expression of JunD, an AP-1 transcription factor. ALK1 also stimulated the expression of MMP10. MMPs hydrolyze components of the ECM and are critical factors regulating many normal biological processes such as embryonic development, organ morphogenesis, wound healing, tissue remodeling and angiogenesis (Visse and Nagase, 2003).

ALK5 upregulated the expression of angiopoietin-like 4, MCP-1, RANTES, cadherin, etc. Angiopoietin-like 4 modulates angiogenesis both in tumors and in ischemic tissues (Le Jan et al., 2003). Both MCP-1 and RANTES belong to the CC-chemokine family and direct leukocyte trafficking into the

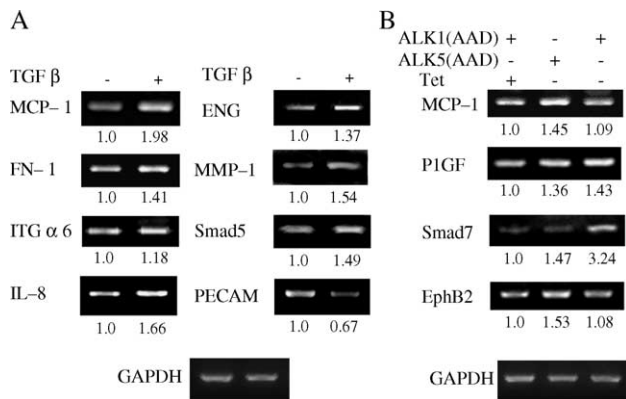


Fig. 5. Confirmation of microarray results by semi-quantitative RT-PCR. Total RNA was extracted from the cells treated with 100 pM TGF- β (A) or expressing the active form of receptors ALK1(AAD) or ALK5(AAD) (B) and subjected to RT-PCR and electrophoresis analysis. The relative expression levels were normalized to GAPDH, and the changing fold was shown under each band.

areas of inflammation. MCP-1 has also been suggested to modulate angiogenesis by inducing EC migration and upregulating VEGF expression (Hong et al., 2005; Salcedo et al., 2000). N-cadherin enhances the adhesion of endothelial cells and mural cells. Both TGF- β and ALK5 induced EphB2 and intercellular adhesion molecule-1. EphB2 functions in guiding cell migration and neural axon (Bruckner and Klein, 1998).

Interestingly, TGF- β regulated much more genes than ALK1 or ALK5 did, indicating that expression of some genes influenced by TGF- β requires cooperation between ALK1 signaling and ALK5 signaling. It is also possible that TGF- β signals via other pathways independent of ALK1 and ALK5. Indeed, Daxx has been suggested to mediate c-Jun N-terminal kinase (JNK) activation and apoptosis by TGF- β (Perlman et al., 2001).

Although some of the genes identified in this study have been found to be the targets of ALK1 or ALK5 (Ota et al., 2002), the genes identified by Ota et al. were from the cells infected with ALK1- or ALK5-expressing recombinant adenoviruses for 48 h. To ensure that all the identified genes are directly modulated by ALK1 or ALK5, we chose 10 h and 12 h after tet withdrawal for microarray analysis as the recombinant adenoviral system began to express the receptors at 8 h after tet withdrawal. These times were corresponding to 2 h and 4 h treatment by TGF- β , respectively. Thus, the genes identified in the present study can be regarded as direct target genes by TGF- β , ALK1 and ALK5. In addition, this study included TGF- β treatment in addition to ALK1 and ALK5 overexpression, which made it possible to compare the gene profiles regulated by TGF- β , ALK1 and ALK5.

In summary, our results, together from analysis of the gene expression profile and tube formation, suggest that ALK1 participates in cell proliferation and ECM disassembly and induces EC proliferation and migration to form new tubes. This is consistent with the previous data that ALK1 enhanced ECs proliferation and migration (Goumans et al., 2003; Goumans et al., 2002). ALK5 appears to control cell adhesion and ECM remodeling, to enhance EC aggregation (Fig. 3) and mural cell

recruitment and reestablishment of the ECM and thus to promote the maturation of blood vessels. This is consistent with the note that ALK1 has more important functions in the activation phase of angiogenesis and ALK5 mainly plays a part in the resolution phase. Detailed dissection of the molecular mechanisms of gene regulation by TGF- β is needed to better understand the function of TGF- β in angiogenesis.

Acknowledgments

We are grateful to the Microarray Core Facility of the University of California, Irvine for their great assistance. This work was supported by grants from The Bugher Foundation (New York), the National Science Foundation (Grant # 30125021, 30270681, 30430360), 973 Program (2004CB720002) and SRFDP of the Ministry of Education of China. Y.G.C. is the recipient of the Li Foundation Heritage Prize.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.mvr.2005.11.004](https://doi.org/10.1016/j.mvr.2005.11.004).

References

- Archambault, V., et al., 2003. Genetic and biochemical evaluation of the importance of Cdc6 in regulating mitotic exit. *Mol. Biol. Cell* 14, 4592–4604.
- Arthur, H.M., et al., 2000. Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev. Biol.* 217, 42–53.
- Azhar, M., et al., 2003. Transforming growth factor beta in cardiovascular development and function. *Cytokine Growth Factor Rev.* 14, 391–407.
- Bourdeau, A., et al., 1999. A murine model of hereditary hemorrhagic telangiectasia. *J. Clin. Invest.* 104, 1343–1351.
- Bruckner, K., Klein, R., 1998. Signaling by Eph receptors and their ephrin ligands. *Curr. Opin. Neurobiol.* 8, 375–382.
- Carmeliet, P., 2000. Mechanisms of angiogenesis and arteriogenesis. *Nat. Med.* 6, 389–395.
- Carmeliet, P., et al., 2001. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat. Med.* 7, 575–583.
- Chen, Y.G., Massague, J., 1999. Smad1 recognition and activation by the ALK1 group of transforming growth factor-beta family receptors. *J. Biol. Chem.* 274, 3672–3677.
- Chen, Y.G., et al., 1997. Mechanism of TGFbeta receptor inhibition by FKBP12. *EMBO J.* 16, 3866–3876.
- Chen, Y.G., et al., 1998. Determinants of specificity in TGF-beta signal transduction. *Genes. Dev.* 12, 2144–2152.
- Consortium, T.G.O., 2001. Creating the gene ontology resource: design and implementation. *Genome Res.* 11, 1425–1433.
- Dennler, S., et al., 1998. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J.* 17, 3091–3100.
- Dickson, M.C., et al., 1995. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* 121, 1845–1854.
- Folkman, J., D'Amore, P.A., 1996. Blood vessel formation: what is its molecular basis? *Cell* 87, 1153–1155.
- Goumans, M.J., et al., 1999. Transforming growth factor-beta signalling in extraembryonic mesoderm is required for yolk sac vasculogenesis in mice. *Development* 126, 3473–3483.

- Goumans, M.J., et al., 2002. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J.* 21, 1743–1753.
- Goumans, M.J., et al., 2003. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. *Mol. Cell* 12, 817–828.
- Hardy, S., et al., 1997. Instruction of adenovirus vectors through Cre-lox recombination. *J. Virol.* 71, 1842–1849.
- Hata, A., et al., 2000. OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. *Cell* 100, 229–240.
- Hong, K.H., et al., 2005. Monocyte chemoattractant protein-1-induced angiogenesis is mediated by vascular endothelial growth factor-A. *Blood* 105, 1405–1407.
- Huse, M., et al., 1999. Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. *Cell* 96, 425–436.
- Jain, R.K., 2003. Molecular regulation of vessel maturation. *Nat. Med.* 9, 685–693.
- Johnson, D.W., et al., 1996. Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. *Nat. Genet.* 13, 189–195.
- Lamouille, S., et al., 2002. Activin receptor-like kinase 1 is implicated in the maturation phase of angiogenesis. *Blood* 100, 4495–4501.
- Le Jan, S., et al., 2003. Angiopoietin-like 4 is a proangiogenic factor produced during ischemia and in conventional renal cell carcinoma. *Am. J. Pathol.* 162, 1521–1528.
- Li, D.Y., et al., 1999. Defective angiogenesis in mice lacking endoglin. *Science* 284, 1534–1537.
- Macias-Silva, M., et al., 1998. al Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. *J. Biol. Chem.* 273, 25628–25636.
- Marchuk, D.A., et al., 2003. Vascular morphogenesis: tales of two syndromes. *Hum. Mol. Genet.* 12, R97–R112 (Spec No 1).
- Massague, J., 1998. TGF-beta signal transduction. *Annu. Rev. Biochem.* 67, 753–791.
- Massague, J., Chen, Y.G., 2000. Controlling TGF-beta signaling. *Genes Dev.* 14, 627–644.
- McAllister, K.A., et al., 1994. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat. Genet.* 8, 345–351.
- Oh, S.P., et al., 2000. Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 97, 2626–2631.
- Oshima, M., et al., 1996. TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev. Biol.* 179, 297–302.
- Ota, T., et al., 2002. Targets of transcriptional regulation by two distinct type I receptors for transforming growth factor-beta in human umbilical vein endothelial cells. *J. Cell. Physiol.* 193, 299–318.
- Pepper, M.S., 1997. Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev.* 8, 21–43.
- Perlman, R., et al., 2001. TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. *Nat. Cell Biol.* 3, 708–714.
- Salcedo, R., et al., 2000. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* 96, 34–40.
- Shi, W., et al., 2004. GADD34-PP1c recruited by Smad7 dephosphorylates TGFbeta type I receptor. *J. Cell Biol.* 164, 291–300.
- Siegel, P.M., et al., 2003. Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc. Natl. Acad. Sci. U. S. A.* 100, 8430–8435.
- ten Dijke, P., Hill, C.S., 2004. New insights into TGF-beta-Smad signalling. *Trends Biochem. Sci.* 29, 265–273.
- Urness, L.D., et al., 2000. Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat. Genet.* 26, 328–331.
- van den Driesche, S., et al., 2003. Hereditary hemorrhagic telangiectasia: an update on transforming growth factor beta signaling in vasculogenesis and angiogenesis. *Cardiovasc. Res.* 58, 20–31.
- Visse, R., Nagase, H., 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ. Res.* 92, 827–839.
- Wang, N., et al., 1999. Adenovirus-mediated overexpression of c-Jun and c-Fos induces intercellular adhesion molecule-1 and monocyte chemoattractant protein-1 in human endothelial cells. *Arterioscler., Thromb., Vasc. Biol.* 19, 2078–2084.
- Weinstein, M., et al., 2000. Functions of mammalian Smad genes as revealed by targeted gene disruption in mice. *Cytokine Growth Factor Rev.* 11, 49–58.
- Wieser, R., et al., 1995. GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. *EMBO J.* 14, 2199–2208.
- Won, K.A., et al., 1998. Maturation of human cyclin E requires the function of eukaryotic chaperonin CCT. *Mol. Cell. Biol.* 18, 7584–7589.
- Yang, X., et al., 1999. Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5. *Development* 126, 1571–1580.