

# Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development

Tsugio Seki\*, Kwon-Ho Hong\* and S Paul Oh

Department of Physiology and Functional Genomics, University of Florida College of Medicine, Gainesville, FL, USA

Transforming growth factor  $\beta$  (TGF- $\beta$ ) transmits signals through a heterotetrameric cell-surface complex of type II (TGFBR2) and type I (activin receptor-like kinase 5, ALK5; TGFBR1) serine/threonine kinase receptors, as well as Smad2/3. We have previously shown that another type I receptor, ALK1 (ACVRL1), can also mediate TGF- $\beta$  signals via BMP-activated Smads in vascular endothelial cells (ECs). Our group and others have proposed the hypothesis that two TGF- $\beta$  signaling pathways via ALK1 and ALK5 in vascular ECs may play a balancing role for controlling the proliferation and migration of ECs during angiogenesis. To address *in vivo* roles of this balance in vascular development, we have created a knockin mouse line that carries a *lacZ* reporter in the *Alk5* gene locus (*Alk5<sup>lacZ</sup>*). Throughout development, a well-defined, nonubiquitous expression pattern of *Alk5* expression was observed in multiple tissues, and organs. Overall, a high level of *Alk5* expression was found in perichondria, periosteal, and the mesenchymal layers underlying epithelia in the kidney, lung, and gallbladder. In blood vessels, contrasting to predominant *Alk1* expression in arterial endothelium, *Alk5* expression was localized in the medial and adventitial layers of blood vessels, but was undetectable in the intimal layer. In addition, although *Alk5*-null embryos exhibit a defect in the formation of vascular smooth muscle layers, the lumens of blood vessels are generated properly, which stands in contrast to the severe dilation of the vascular lumens in *Alk1*-null mice. These mutually exclusive expression patterns of *Alk1* and *Alk5* in blood vessels, as well as the undisturbed formation of the vascular lumens in *Alk5*-null embryos, suggest that each type I receptor has its own unique functions in vascular development. The *Alk5<sup>lacZ</sup>* mice will be a valuable resource in identifying the *in vivo* cellular targets of TGF- $\beta$  family signals mediated by *Alk5*, both during embryonic development as well as in diverse pathological conditions.

Laboratory Investigation (2006) 86, 116–129. doi:10.1038/labinvest.3700376; published online 12 December 2005

**Keywords:** ALK1; ALK5; TGF- $\beta$ ; angiogenesis; endothelium; smooth muscle cells

The transforming growth factor- $\beta$  (TGF- $\beta$ ) family members include TGF- $\beta$ s, bone morphogenic proteins (BMPs), activins, and growth and differentiation factors (GDFs). These signaling proteins play critical roles in a wide variety of biological processes, including proliferation, differentiation, pattern formation, and apoptosis. The TGF- $\beta$  family cytokines exert their effects by binding to the heteromeric complexes of two types of transmembrane serine/threonine kinase receptors.<sup>1–3</sup> Type II receptors function primarily as binding receptors.

Upon binding their ligands, the type II receptors associate with and phosphorylate type I receptors which, in turn, activate downstream Smad proteins.

Eight Smad protein have been identified in mammals.<sup>2,4</sup> The activated type I receptor phosphorylates receptor-regulated Smads (R-Smads). R-Smads then form a complex with a common partner Smad4 (Co-Smad), and enter into the nucleus. Once there, the Smad complex interacts with diverse transcriptional coactivators or corepressors, according to genetic makeup and cellular contexts. Depending on which R-Smad is utilized, TGF- $\beta$  signaling can be largely separated into two pathways: BMP signals via Smad1, 5, or 8, and activin/TGF- $\beta$  signals via Smad2 or 3.

For more than 27 known TGF- $\beta$  family ligands, only five type II receptors and seven type I receptors have been identified,<sup>5</sup> indicating that each receptor mediates multiple signals. Biochemical studies have

Correspondence: Dr SP Oh, PhD, Department of Physiology and Functional Genomics, University of Florida, 1600 SW Archer Rd, D5-36, Gainesville, FL 32610, USA.  
E-mail: ohp@phys.med.ufl.edu

\*These authors contributed equally to this work.  
Received 10 October 2005; revised and accepted 7 November 2005; published online 12 December 2005

determined that activin receptor-like kinase 3 (ALK3; BMPR1) and ALK6 (BMPR1B) interact primarily with the BMP type II receptor (BMPR2), and mediate BMP subfamily signals; and that activin type II receptors (ACVR2A and ACVR2B) utilize ALK2 (ACVR1), ALK4 (ACVR1B), and ALK7 (ACVRL1C) depending on their interacting ligands including activins (INHBA, INHBB, INHBC, and INHBE), Nodal, BMP7, and GDF11.<sup>6–8</sup> Activin receptor-like kinase 5 (ALK5) (T $\beta$ RI; TGFBR1) has been shown to interact with the TGF- $\beta$  type II receptor (T $\beta$ RII; TGFBR2), and to mediate TGF- $\beta$  subfamily signals.<sup>5</sup> Recent studies have implicated that ALK5 may also mediate other TGF- $\beta$  family signals, such as GDF8 and GDF11.<sup>9,10</sup>

Activin receptor-like kinase 1 (ALK1) (ACVRL1) was initially considered to be an orphan receptor, because its binding specificities were obscured.<sup>11</sup> We have previously shown that ALK1 in human umbilical vein endothelial cells (HUVECs) binds to TGF- $\beta$ 1.<sup>12</sup> In contrast to the signal transduction of ALK5 via Smad2/3, activated ALK1 phosphorylates Smad1/5/8.<sup>12,13</sup> This observation led us to postulate that both ALK1 and ALK5 mediate the TGF- $\beta$  signal in endothelial cells (ECs) and that one of the two type I receptors is activated depending on the cellular context, such as TGF- $\beta$  concentration.<sup>12</sup> Based on the vascular dilation/fusion and molecular characterization of *Alk1*<sup>-/-</sup> embryos, we suggested that ALK1 may play an important role in the transition of ECs to the resolution phase, whereas ALK5 may stimulate their activation phase during angiogenesis.<sup>12</sup>

Two independent groups investigated this hypothesis *in vitro* by overexpressing the constitutively active (ca) form of ALK1 in cultured ECs using recombinant adenoviruses, and have reported their respective data, which were in disagreement.<sup>14,15</sup> Lamouille *et al*<sup>14</sup> found that ca-ALK1 inhibits the proliferation and migration of ECs, whereas Goumans *et al*<sup>15</sup> observed the opposite phenomenon. Goumans *et al*<sup>16</sup> recently reported that ALK5 kinase activity is required for optimal ALK1 activation by TGF- $\beta$  in ECs. Another report suggested that one of the TGF- $\beta$  type III receptors, Endoglin (ENG), is the key mediator for favoring TGF- $\beta$ /ALK1 over the TGF- $\beta$ /ALK5 pathway.<sup>17</sup>

TGF- $\beta$  is one of the most extensively studied growth factors. The TGF- $\beta$  subfamily members have been implicated in angiogenesis from the emergence of modern vascular biology, and, since this time, innumerable papers have described the diverse cellular functions of TGF- $\beta$  in ECs. However, the precise *in vivo* functions of TGF- $\beta$  in vascular development remain undefined. The existence, as well as the function, of the balance of the TGF- $\beta$  signal via ALK1 and ALK5 *in vivo* is also yet to be investigated.

Coexpression of ALK1 and ALK5 in the vascular ECs of embryos should be a precedent for the balance of TGF- $\beta$  signals through these two

type I receptors. Using *Alk1-lacZ* knockin mice, we have previously shown that *Alk1* gene is predominantly transcribed/expressed in the arterial endothelium. Several reports have described expression patterns of *Alk5* transcript and protein in developing mouse embryos using *in situ* hybridization and immunohistochemistry, but *Alk5* expression in the vascular endothelium has been obscured.<sup>18–20</sup>

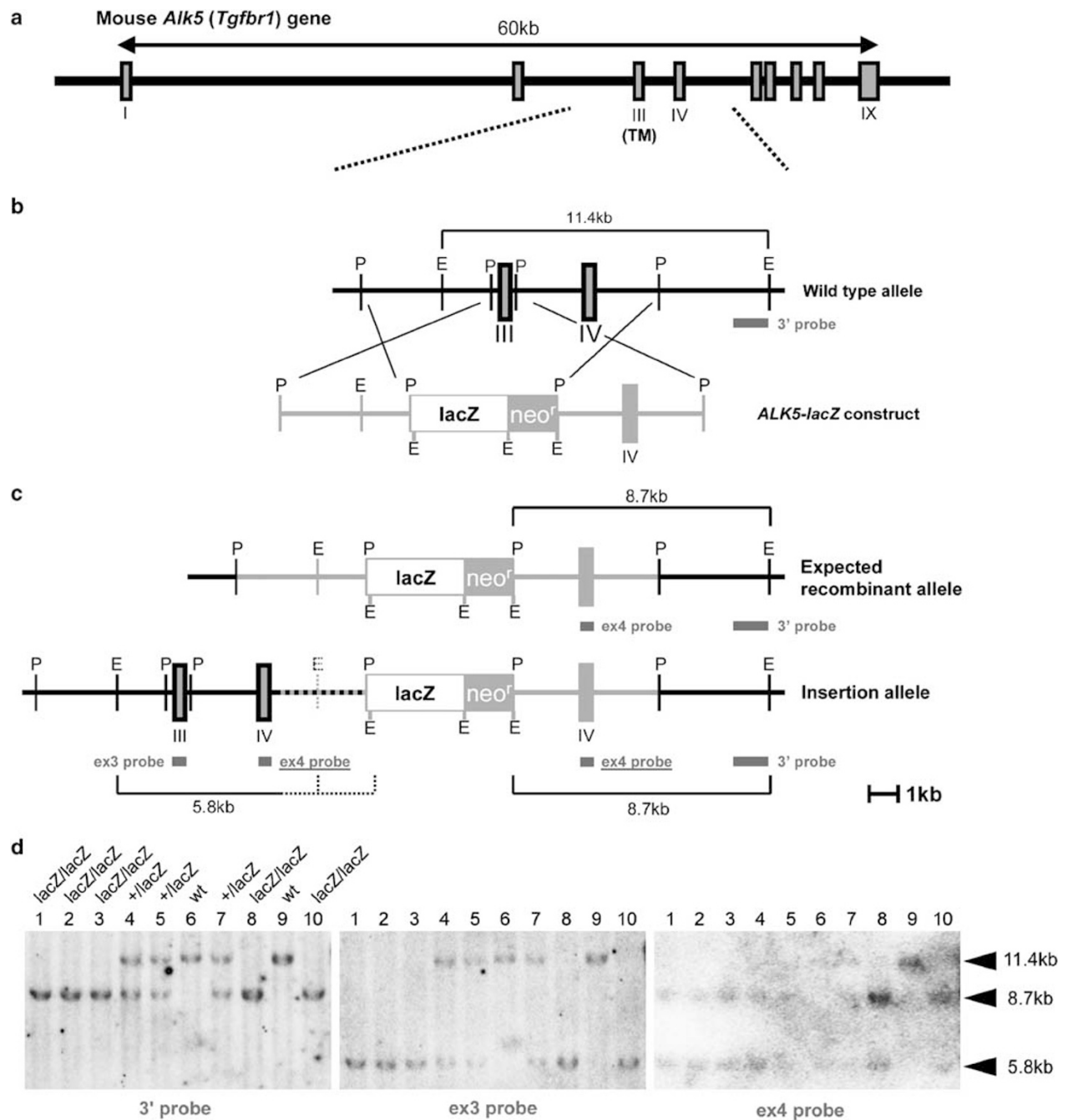
In order to investigate *Alk5* expression in developing mouse embryos, and to compare it with that of *Alk1*, we have generated a novel *Alk5-lacZ* knockin mouse line. The *lacZ* reporter expression was detected in multiple tissues, and was generally consistent with the *Alk5*-expression pattern previously reported. In blood vessels, the *lacZ* expression was strongly detected in smooth muscle cells (SMCs), but not in the ECs. These mutually exclusive *in vivo* expression patterns of two type I receptors suggest that TGF- $\beta$  is involved in vascular morphogenesis utilizing two distinct type I receptors: ALK1 in ECs and ALK5 in vascular SMCs.

## Materials and methods

The *Alk5<sup>lacZ</sup>* strain was generated at the University of Florida. C57BL/6J wild-type mice were purchased from Harlan (Indianapolis, IN, USA), and *Flk<sup>+</sup>/lacZ* mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice have been maintained under standard specific-pathogen-free (SPF) conditions, and the procedures performed on the mice were reviewed and approved by the University of Florida Institution of Animal Care and Use Committee (IACUC).

### Generation of the *Alk5<sup>lacZ</sup>* Mouse Line

A mouse ES-129/SvJ BAC clone containing the *Alk5* gene (Figure 1a) was identified by PCR-based screening and was purchased from Incyte Genomics (St Louis, MO, USA). A *PstI*-*PstI* 4.4 kb fragment containing a part of intron 2; a SIBN cassette; and a 4.8 kb *PstI*-*PstI* fragment containing a part of intron 3, exons 4, and a part of intron 4 were subcloned into pBluescript II vector (Figure 1b). The SIBN cassette contains SV40 splice donor and acceptor signals; internal ribosomal entry sequences; a bacterial  $\beta$ -galactosidase cDNA (*lacZ*) with a poly(A) signal; and a neomycin-resistant gene driven by a PGK promoter. Electroporation, G418 selection, and the Southern hybridization screening using the 3' probe (Figure 1c and d) were performed as previously described.<sup>21</sup> A total of 12 positive embryonic stem (ES) cell clones were obtained from 192 of the G418-resistant clones. ES cells from the positive clone #44 were injected into the blastocysts of the C57BL/6 (B6) strain. The male chimeric offspring were mated with females on either B6 or 129S4/



**Figure 1** Analyses of the *Alk5<sup>lacZ</sup>* allele. **(a)** A schematic diagram of mouse *Alk5* gene structure. Boxes indicate exons. The translation start site resides in the first exon, and the transmembrane (TM) domain of *Alk5* gene is encoded by the third exon (III). **(b)** A diagram of the *Alk5* wild-type allele (in black) and the targeting construct that replaces the third exon with a *lacZ* reporter cassette (in gray). The *lacZ* cassette consists of splice donor and acceptor sites; an internal ribosome entry site; a bacterial  $\beta$ -galactosidase gene (*lacZ*); and poly A signal sequences. The *neo<sup>r</sup>* cassette consists of a PGK promoter; a neomycin-resistant gene; and the poly A signal sequences. **(c)** Diagrams of the expected recombinant allele from the targeting construct and of the insertion allele found in the *Alk5<sup>lacZ</sup>* mouse line. Note that the exon 3 encoding transmembrane domain was still present, and that a part of the genomic region containing exon 4 (IV) was duplicated due to the insertion in the *Alk5<sup>lacZ</sup>* allele. The length and organization between the 5' exon 4 and the *lacZ* gene are yet to be determined (indicated by a dotted line). **(d)** Genomic Southern analyses of embryos from *Alk5<sup>+/lacZ</sup>* mice mating. Genotypes were as follows: *Alk5<sup>lacZ/lacZ</sup>* (lanes 1, 2, 3, 8, and 10); *Alk5<sup>+/lacZ</sup>* (lanes 4, 5, and 7); and wild type (lane 9). Genomic DNA from embryonic day 9.5 (E9.5) mice was digested with *EcoRI*, separated on agarose gel, and blotted on a nylon membrane. This membrane was then subjected to three-probe hybridization and deprobing cycles with the various probes indicated at the bottom. The 3' probe was the one used for the initial screening. The probe location (dark gray bars) and the fragments detected by the probes are shown in **(b)** and **(c)**.

SvJae (129) backgrounds. Heterozygous mice from the 129 strain were kept as a 129 inbred line. We found that there was no difference in expression

patterns and mutant phenotypes between the 129 inbred and 129/B6 hybrid lines. We used the 129/B6 hybrid line for expression studies.

## X-Gal Staining and Immunohistochemistry

Mouse embryos and 3-month-old adult tissues were stained with a  $\beta$ -galactosidase substrate X-gal and were photographed before and/or after clearing as described.<sup>22</sup> Paraffin-embedded whole-mount X-gal-stained samples were sectioned and counterstained with nuclear fast red (NFR) (Vector Laboratory, CA, USA). We also observed the sections without counterstaining to identify the weak X-gal staining, which may be masked by the NFR color. The following antibodies were used to identify specific proteins on the sections using the Vectastain immunohistochemical staining kits (Vector Laboratory, CA, USA): monoclonal anti-smooth muscle  $\alpha$ -actin (clone 1A4, Sigma-Aldrich, MO, USA) for detecting SMCs; polyclonal anti-CD68 (Santa Cruz Biotechnology, CA, USA) for macrophages; and anti-OMP serum (a gift from Dr Margolis at the University of Maryland) for olfactory neurons.<sup>23,24</sup>

## Whole-Mount *In Situ* Hybridization

To examine the SMC development in *Alk5<sup>lacZ/lacZ</sup>* mouse embryos, embryos at 9.5 days post coitus (E9.5) were stained for SMC-specific marker SM22 $\alpha$  (transgelin; *Tagln*). An antisense probe against SM22 $\alpha$  mRNA was generated using degoxygenin UTP-labeling kit (Roche, IN, USA), and whole-mount *in situ* hybridization was performed as described.<sup>25</sup>

## Results

### Generation of the *Alk5<sup>lacZ</sup>* Mouse Line

In order to study the functions and expression patterns of the *Alk5* gene, we targeted mouse *Alk5* locus, and inserted a *lacZ* cassette as a reporter. An *Alk5*-targeting construct was created in order to replace the *PstI*-*PstI* 0.9 kb fragment containing the third exon, which encodes a transmembrane domain, with a cassette containing the *lacZ* reporter gene. The linearized construct was electroporated into ES cells, and the G418-resistant ES cell clones were screened by genomic Southern blot using the 3' probe (Figure 1). One of the targeted clones was randomly chosen to establish an *Alk5<sup>lacZ</sup>* mouse line.

Some of the embryos at 9.5 days post coitus (E9.5) from *Alk5<sup>+/lacZ</sup>* matings appeared to be retarded, and the genomic Southern blot results using the 3' probe consistently confirmed that these retarded embryos were homozygous for the *Alk5<sup>lacZ</sup>* allele (Figure 1d, left panel). When we genotyped these embryos with PCR primers within the *PstI*-*PstI* 0.9 kb fragment containing the exon 3; however, PCR amplifications occurred, indicating that the exon 3 might not be deleted in the *Alk5<sup>lacZ</sup>* allele. To investigate this possibility, we stripped the Southern blot membrane, and rehybridized it with the *PstI*-*PstI* 0.9 kb fragment (ex3 probe) as a probe. As shown in Figure

1d (middle panel), the exon 3 was indeed retained in the *Alk5<sup>lacZ</sup>* allele. To further characterize the *Alk5<sup>lacZ</sup>* allele, we performed one more Southern analysis using the same membrane with an exon 4 probe. The hybridization result showed that exon 4 was duplicated in the *Alk5<sup>lacZ</sup>* allele (Figure 1d, right panel). These sequential Southern blot results revealed that the *lacZ* reporter cassette did not replace the exon 3 but was actually inserted into a 3' region of exon 4 in the *Alk5* locus, which consequently duplicated part of the genomic sequence in these mice (Figure 1c). Although the targeting fashion in this mouse line was not as initially planned, this insertion event kept this allele from losing regulatory elements in the 0.9 kb fragment, if any were present, and made this mouse line even more suitable for the *Alk5* expression study.

### *Alk5<sup>lacZ/lacZ</sup>* Mice Showed a Null Phenotype

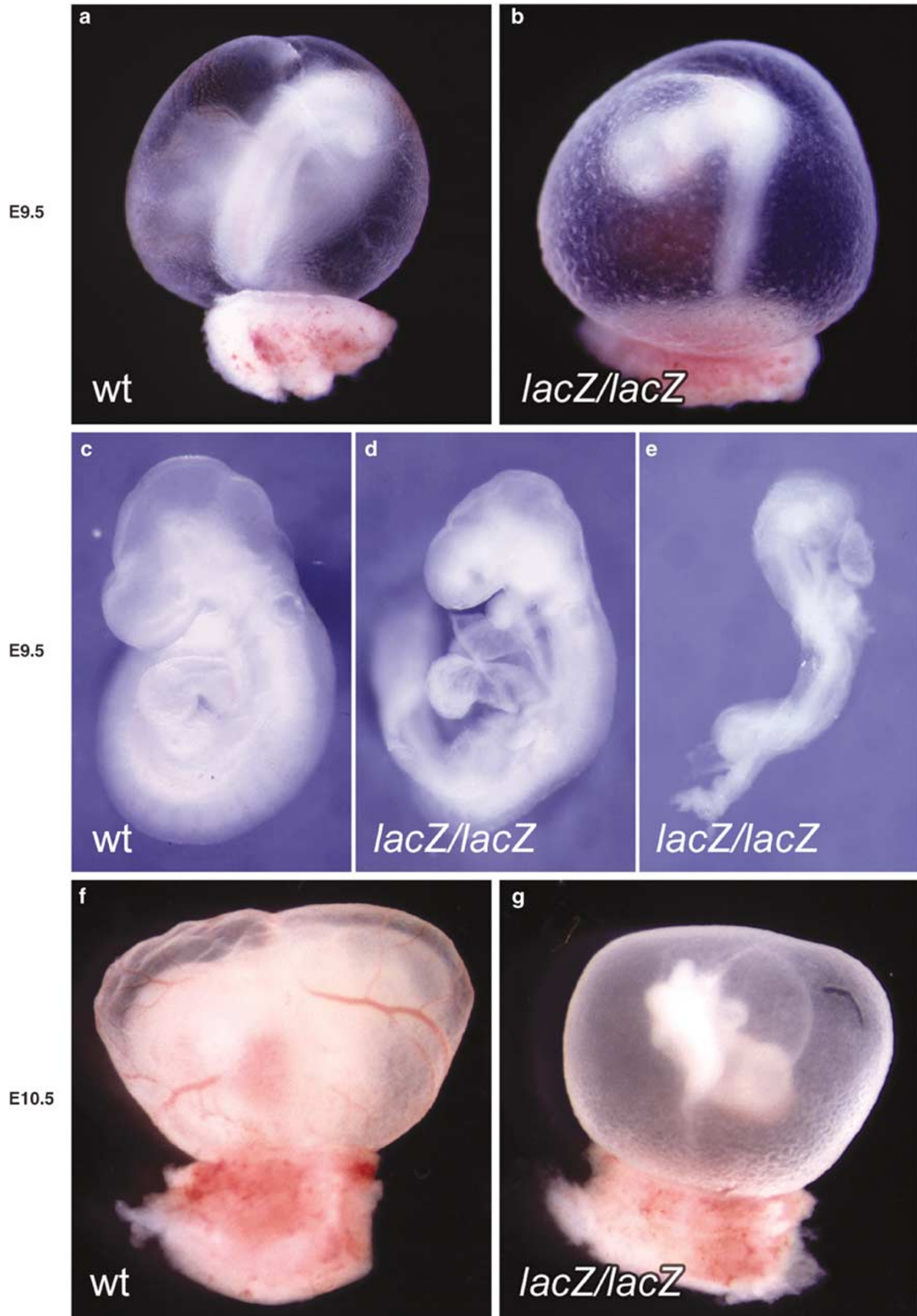
The homozygous *Alk5<sup>lacZ/lacZ</sup>* embryos died around embryonic day 9.5 or 10.5 (E9.5 or E10.5), and showed severe growth retardation (Figure 2a and b). The yolk sacs of homozygous mutant embryos at E9.5 had a wrinkled appearance and no visible blood-filled vessels. The embryo proper was also mildly or severely retarded (Figure 2c–e). At E10.5, the homozygous mutant yolk sac lacked mature vasculatures (Figure 2f and g). These phenotypes are morphologically indistinguishable from the *Alk5*-null embryos previously reported,<sup>26</sup> suggesting that the *Alk5<sup>lacZ</sup>* allele is null.

### X-Gal Staining Patterns of *Alk5<sup>+/lacZ</sup>* Embryos Represent Endogenous *Alk5<sup>lacZ</sup>* Expression Patterns

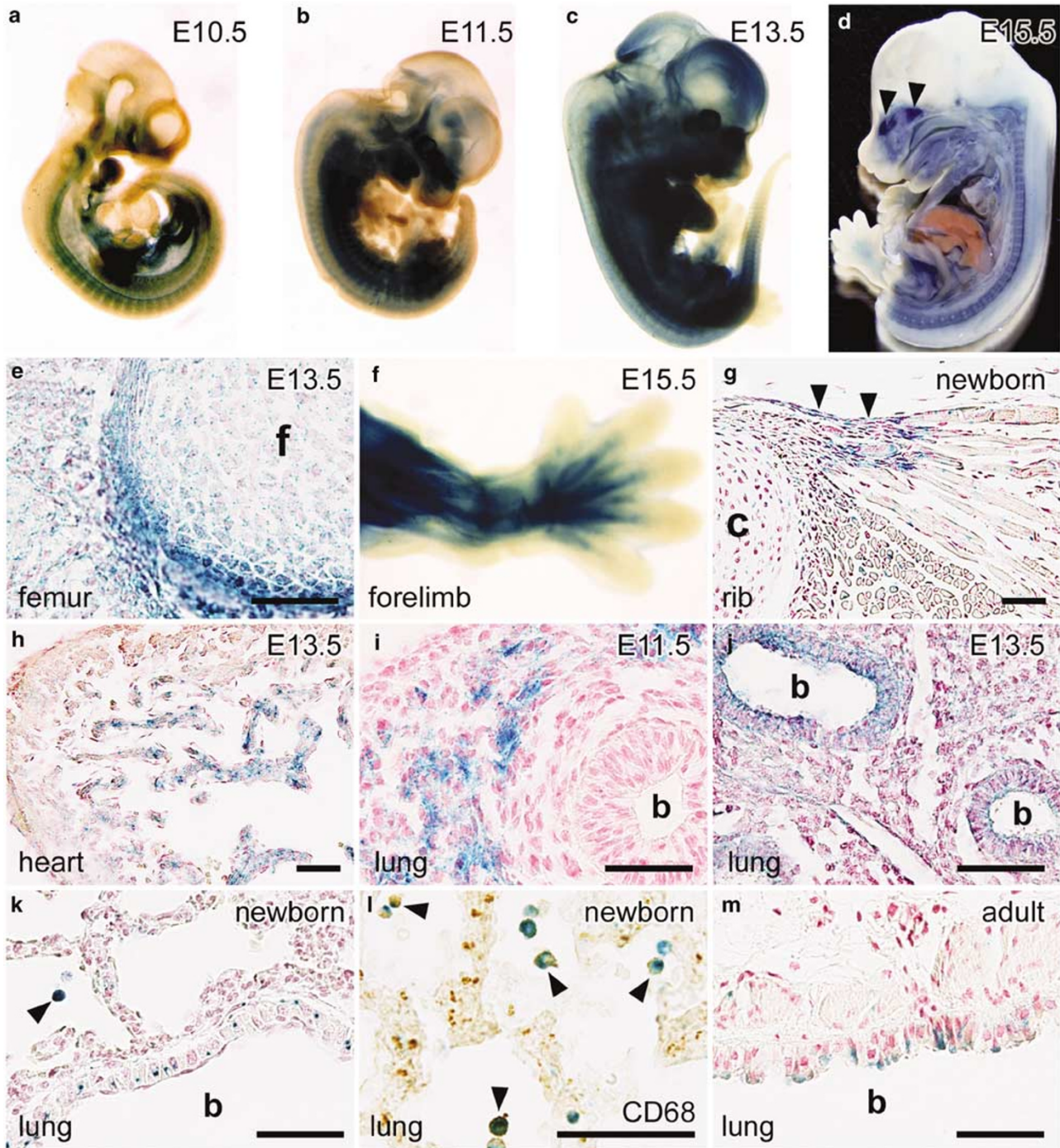
By exploiting the reporter gene under the control of the endogenous *Alk5* promoter, we analyzed *Alk5* gene expression in *Alk5<sup>+/lacZ</sup>* embryos at various developmental stages. As shown in Figure 3a–d, the whole-mount X-gal staining revealed that *lacZ* is expressed in a variety of tissues and organs during embryonic development. The X-gal staining was particularly strong in connective tissues surrounding developing skeletons (Figure 3d).

In order to identify the cell types expressing *lacZ*, we have analyzed the histological sections of the X-gal-stained embryos and tissues. In the limbs, intense X-gal staining was found in the perichondrium and tendons (Figure 3e–g); and weaker staining was detected in the skeletal muscles and chondrocytes. The X-gal staining was also detected in the cardiac myocytes, especially in the trabecular muscles (Figure 3h). In the liver, a strong X-gal staining was detected in the mesenchymal layers surrounding biliary epithelium (data not shown). Weak or undetectable staining was found in neuronal tissues such as brain and spinal cord, except for a strong staining in cortices of the frontal lobe of the brain (data not shown).





**Figure 2** *Alk5<sup>lacZ/lacZ</sup>* mice exhibited phenotypes identical to those of the previously reported *Alk5<sup>-/-</sup>* mice. (a, b) In comparison with the yolk sac of the wild-type embryo (a), that of the *Alk5<sup>lacZ/lacZ</sup>* embryo (b) shows a wrinkled appearance and has no apparent vasculature at E9.5. (c–e) E9.5 *Alk5<sup>lacZ/lacZ</sup>* embryos show either mild (d) or severe (e) retardation. (f, g) At E10.5, the mutant yolk sac lacks visible blood vessels and the embryo is severely retarded.



**Figure 3** *Alk5* expression pattern revealed by X-gal staining of *Alk5*<sup>+/lacZ</sup> embryos. (a–c) Whole-mount X-gal staining of *Alk5*<sup>+/lacZ</sup> embryos at E10.5 (a), E11.5 (b), and E13.5 (c). X-gal-stained embryos were cleared with organic solvents for whole-body observation. (d) A mid-body sagittal section of an E15.5 embryo stained with X-gal. The most intense staining was observed in the olfactory area (arrowhead). (e) Perichondral staining of the developing femur (f) at E13.5. (f) Whole-mount view of cleared forelimb displays X-gal staining in developing bones, tendons, and muscles of E15.5 embryos. (g) X-gal staining in a pre-tendon area (arrowheads) on the rib cartridge (c). (h) Trabecular muscles in the heart express  $\beta$ -gal at E13.5. (i–m) X-gal staining patterns in the lungs throughout development. At E11.5, X-gal staining was initially observed in mesenchymal tissue, but not in the bronchial epithelium (i). As development proceeds, the epithelial cells lining the bronchi (b) express  $\beta$ -gal (j). Epithelial expression persists at the adult stage while mesenchymal expression diminishes (m). At the newborn stage, infiltrated macrophage lineage cells show  $\beta$ -gal expression (arrowheads in k and l). Double staining with a tissue macrophage-specific antigen CD68 confirms the cell type (l). Scale bars in (e), (g), and (h–m) are equal to 50  $\mu$ m.

In the E11.5 lungs, the X-gal staining was detected in the mesenchymal tissue of future parenchyma, but not in the epithelial lining of developing airways

(Figure 3i). Zhao *et al* reported that *Alk5* is expressed in both mesenchymal tissue and the bronchial epithelium, but is more intense in the



bronchial epithelium of the developing rat lungs at gestational day 14 (Gd14).<sup>20</sup> Comparable expression patterns—with higher expression in the airway epithelium than in the mesenchyme—were observed in the mouse lungs at E13.5, which corresponds to rat Gd15 (Figure 3j). The bronchial epithelial staining was detected throughout the adult stage (Figure 3k and m). In addition, intense staining was found in CD68-positive macrophage cells in the alveolar cavities at the newborn stage (Figure 3k and l).

These X-gal staining patterns in various organs of *Alk5*<sup>+/*lacZ*</sup> embryos were consistent with the previously reported *Alk5* expression patterns observed by *in situ* hybridization and immunohistochemical methods,<sup>18–20</sup> suggesting that the X-gal staining patterns of the *Alk5*<sup>+/*lacZ*</sup> mice represent the *in vivo* *Alk5* expression patterns, and thus we refer to the *lacZ* expression in *Alk5*<sup>+/*lacZ*</sup> mice as ‘*Alk5* expression’ hereafter.

### *Alk5* Expression in the Olfactory Epithelium (OE)

The strongest *Alk5* expression in E15.5 embryos was observed in the olfactory area (Figure 3d). *Alk5* expression in the OE of E14.5 mouse embryos was reported,<sup>10,19</sup> but no detailed expression patterns of *Alk5* in the developing OE have been shown. We detected *Alk5* expression in the OE as early as E11.5 (Figure 4a). The neuronal lineage cells in the OE become fully established, and the active neurogenesis begins at E14.5.<sup>27</sup> Around this stage, *Alk5* expression begins to be restricted in the basal layer of the OE (Figure 4b and c). Several cell types reside in the OE proper: olfactory receptor neurons (ORNs) and their progenitors; sustentacular cells (supporting cells found in a single apical layer); as well as horizontal basal cells (cells that lie immediately atop the basal lamina) (Figure 4f). The expression of *Alk5* was detected in ORNs and basal cells, but not in sustentacular cells at E13.5 and E15.5. Double staining of X-gal and anti-olfactory marker protein (OMP) antibodies<sup>23</sup> revealed that ORNs and their progenitor cells express *Alk5* (Figure 4d and e). The *Alk5* staining was detected in a subset of the olfactory nerve axons as well (Figure 4d and g). The whole-mount X-gal staining of the E18.5 olfactory cavity showed that *Alk5* is expressed in the OE, but not in the respiratory epithelium (Figure 4h). Since *Alk5* expression in neuronal tissues was generally undetectable (Figure 3d), the ORN staining in the OE is unique in its high *Alk5* expression. We speculate that

ALK5 may mediate a TGF- $\beta$  family signal important for the development of ORN in the OE.

### *Alk5* is Expressed in the Vascular Smooth Muscle Cells, but not in the ECs of the Embryonic Aorta

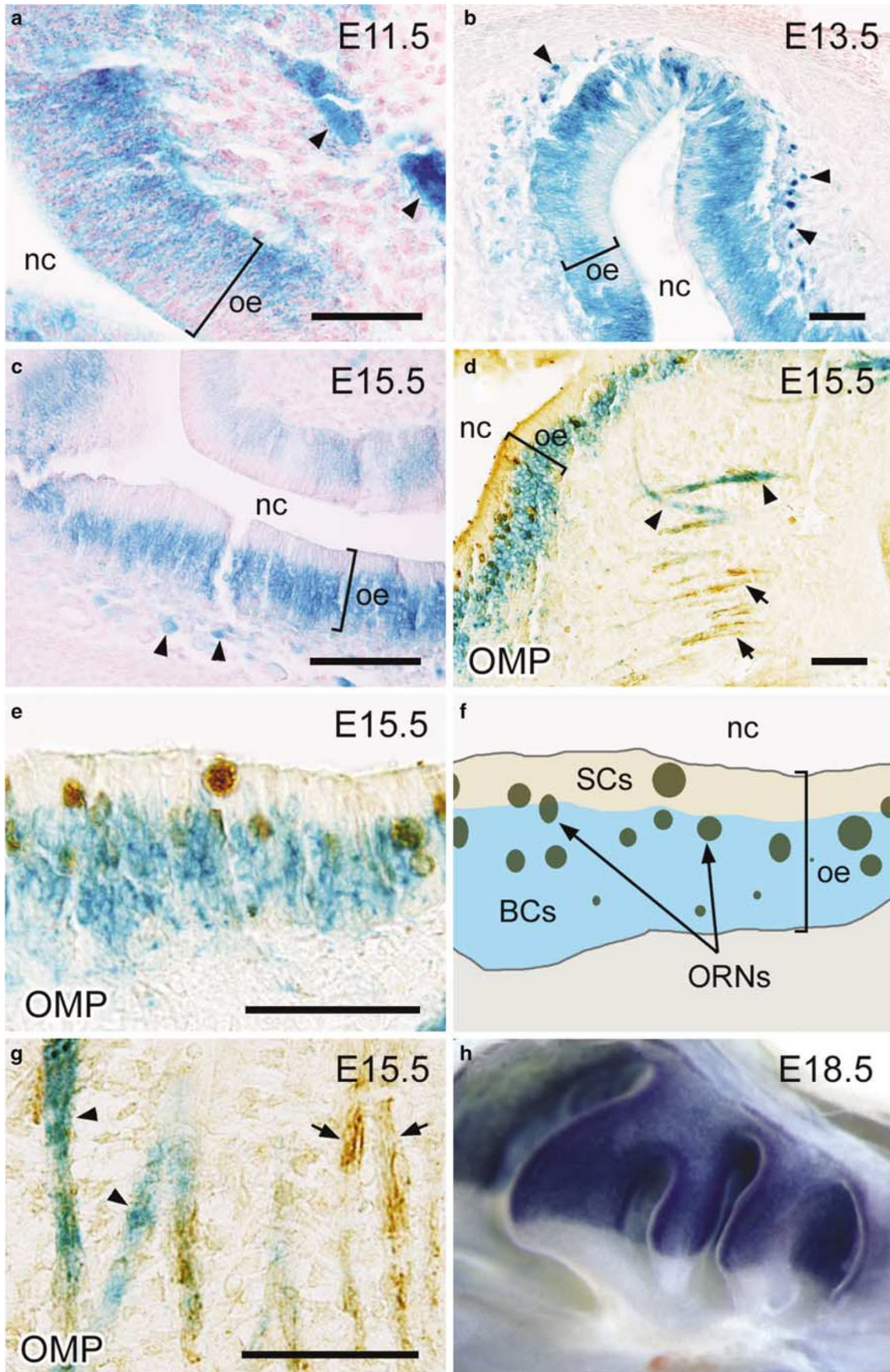
Multiple mechanistic roles of ALK1 and ALK5 as TGF- $\beta$  type I receptors in vascular ECs have been proposed. The premise of such proposals is that both ALK1 and ALK5 are expressed in vascular ECs. We have shown that ALK1 is predominantly expressed in arterial ECs during the embryonic and neonatal stages.<sup>22</sup> However, comprehensive ALK5 expression studies on blood vessels have not been reported. Using the *Alk5*<sup>+/*lacZ*</sup> embryos, we investigated *Alk5* expression in developing blood vessels and compared it with *Alk1* expression in the *Alk1*<sup>+/*lacZ*</sup> embryos (Figure 5).

In low-power magnification views, both *Alk1* and *Alk5* were expressed in the dorsal aortas of the *Alk1*<sup>+/*lacZ*</sup> and *Alk5*<sup>+/*lacZ*</sup> embryos (Figure 5a and b). In high-power views, however, these two type-I receptors displayed mutually exclusive expression patterns: *Alk1* was detected in a single layer of the ECs, whereas *Alk5* was in multiple layers of the cells surrounding the ECs, but not in the ECs themselves (Figure 5c and d). Immunostaining with anti-smooth muscle  $\alpha$  actin antibodies showed that the X-gal-positive cells in the *Alk5*<sup>+/*lacZ*</sup> embryo were SMCs (Figure 5e and f). The weak *Alk5* expression was also detected in mesenchymal tissue surrounding the aorta, but there was no detectable *Alk5* expression in the ECs. In fact, regardless of arterial, venous, or capillary, no *Alk5* positive ECs was found in any of the over 100 histological sections, which produced from the embryos proper at various developmental stages.

### *Alk5* Expression is Undetectable in any Endothelial Lineage Cells

We further analyzed *Alk5* expression in various endothelia in *Alk5*<sup>+/*lacZ*</sup> mice at several postnatal stages (Figure 6). In order to detect weak X-gal staining, the histological sections were also examined without the counterstaining. The intense *Alk5* expression was observed in the vascular SMCs, but not in the ECs, of both newborn and adult aortas (Figure 6a and b). The ECs in both the arterioles and the venules of the newborn tongues were also negative for *Alk5* expression (Figure 6c and d). The ECs on semilunar valves, which transdifferentiate

**Figure 4** *Alk5* expression in the olfactory epithelium (oe). (a–c) At E11.5, the entire oe has staining (a), which is gradually restricted to the base of the epithelium along with the development progress by E15.5 (b, c). nc: nasal cavity. (d, e, and g) Double staining of the olfactory tissue with X-gal and the antibody against olfactory marker protein (OMP) at E15.5. High-power magnification shows that basal cells and developing olfactory cells (shown in e), as well as certain olfactory axons (shown in g), are stained with X-gal. (f) Schematic drawing of the olfactory tissue shown in (e). Sustentacular cells (SCs) at the luminal surface were X-gal negative, whereas basal cells (BCs) expressed *lacZ*. The brown circles indicate olfactory receptor neurons (ORNs), some of which were X-gal positive. (h) Whole-mount X-gal staining of the nasal cavity at E18.5. Note that the olfactory, but not the respiratory, region expresses *Alk5*. Scale bars in a–f are equal to 50  $\mu$ m.



ate to produce valvular mesenchymal cells,<sup>28</sup> were negative for *Alk5* expression, whereas the valvular mesenchymal cells expressed *Alk5* (Figure 6e and f). Similarly, *Alk5* was expressed in ventricular muscles, but not in the endocardium (Figure 6e). Overall, these expression studies show that *Alk5* is predominantly expressed in muscle and mesenchymal cells, but not in the adjacent endothelial lineage cells.

### Vascular Morphogenesis in *Alk5<sup>lacZ/lacZ</sup>* Mice

As shown in Figure 2, *Alk5<sup>lacZ/lacZ</sup>* embryos die around E9.5. Morphologically, the *Alk5<sup>lacZ/lacZ</sup>* phenotype is indistinguishable from the *Alk5*-knockout (*Alk5<sup>-/-</sup>*) embryos previously published by an independent group.<sup>26</sup> Since the vascular phenotype of the *Alk5<sup>-/-</sup>* embryo proper was not described in the *Alk5<sup>-/-</sup>* report, we have investigated whether *Alk5* deficiency affects embryonic vascular development. In order to visualize the ECs, we crossed *Alk5<sup>+ /lacZ</sup>* mice with *Flk1<sup>+ /lacZ</sup>* mice in which the *lacZ* gene is inserted into vascular endothelial growth factor receptor 2 (*Kdr*, *Vegf-r2*, *Flk1*) locus.<sup>29</sup> Additional heterozygous *Flk1* mutation in the *Alk5<sup>lacZ/lacZ</sup>* mice did not appear to alter their phenotypes, and the same approach was successfully used for the characterization of *Alk1<sup>-/-</sup>* embryos in our previous study.<sup>12</sup> Therefore, this approach enabled us to compare the vascular phenotype of *Alk5<sup>lacZ/lacZ</sup>* directly with that of the *Alk1<sup>-/-</sup>* embryos. As shown in Figure 7a–f by whole-embryo X-gal staining, the vascular networks in E9.5 *Alk5<sup>lacZ/lacZ</sup>* embryos appeared unaffected in spite of severe growth retardation. The histological sections of these embryos revealed that the shapes and sizes of the blood vessels in *Alk5* mutant embryos were comparable to those of the wild-type embryos (Figure 7g and h). Since *Alk5* was expressed in vascular SMCs, we examined SMC development in *Alk5* mutant mice. An antisense SM22 $\alpha$  probe detected SM22 $\alpha$  transcripts in the heart tube, but not in the dorsal aorta of *Alk5<sup>lacZ/lacZ</sup>* embryos, indicating defects in the vascular smooth muscle development of these mutant embryos (Figure 7i and j).

### Discussion

In this study, we demonstrated *Alk5* expression patterns during development and postnatal life using a novel knockin mouse line that carries *lacZ* reporter in the *Alk5* gene locus (*Alk5<sup>lacZ</sup>*). We showed that the expression patterns of *lacZ* recapitulated those of previously reported *Alk5*, and that *Alk5* was expressed in a nonubiquitous and dynamic manner during development. In the blood vessels, *Alk5* was predominantly expressed in the smooth muscle layer, but was undetectable in the endothelial layer. We also revealed that *Alk5*-

deficient embryos had impaired vascular smooth muscle layer development. Unlike *Alk1*-deficient embryos, however, *Alk5*-deficient embryos formed relatively normal capillary networks. Based on these data, we propose that TGF- $\beta$  is involved in vascular morphogenesis utilizing two distinct type I receptors: ALK1 in ECs and ALK5 in vascular SMCs (Figure 8).

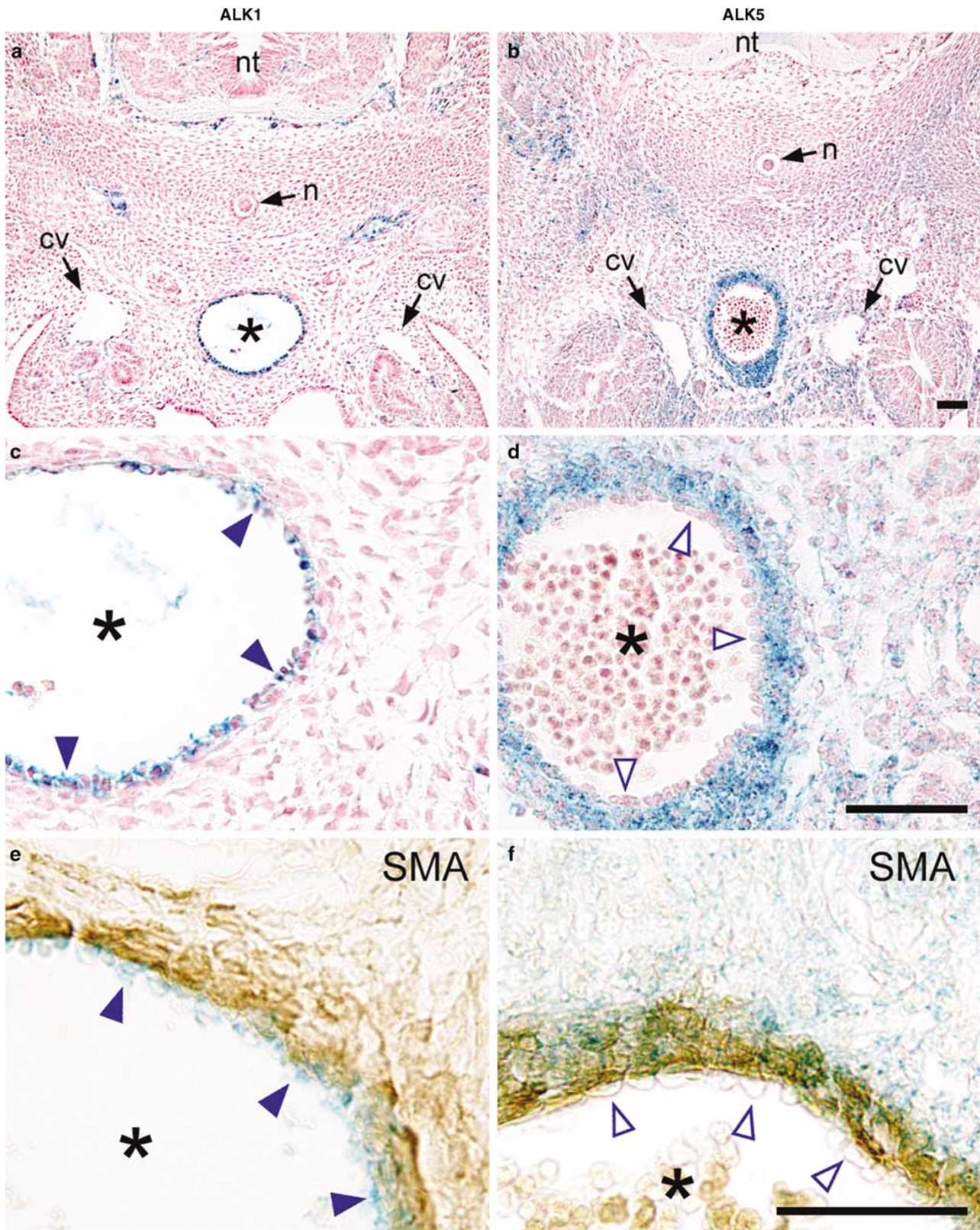
Throughout developmental stages, a high expression of *Alk5* was detected in fiber-rich connective tissues, such as the periosteum, perichondrium, tendons, and ligaments, as well as in the mesenchymal layers supporting the epithelia. These observations are consistent with one of the key functions of TGF- $\beta$ —the production of extracellular matrix (ECM) proteins—and with the previous reports showing the *Alk5*-expression patterns using *in situ* hybridization with *Alk5* antisense probes.<sup>18–20</sup> In addition, we found dynamic changes in *Alk5* expression patterns during development of specific epithelial layers. In the lung, *Alk5* expression was detected in the mesenchymal tissue of future parenchyma, but not in the epithelial lining of the developing airways of E11.5 lungs. As development proceeded, the airway epithelium of E13.5 lungs showed a high level of *Alk5* expression.

Another dynamic expression pattern was observed in the OE. At E11.5, uniform *Alk5* expression was found in olfactory epithelial cells. At around E14.5, when the active neurogenesis occurs in the OE, the *Alk5* expression became restricted in the basal layer of the OE and in the olfactory neurons, but not in the sustentacular cells located in the apical layer of the OE. Growth differentiation factor 11 (GDF11) has been implicated in the determination of the size of the OE.<sup>10</sup> A recent report showed that GDF8, the closest TGF- $\beta$  family member of GDF11, can transduce its signaling via ALK5 and ACVR2B.<sup>9</sup> The dynamic expression pattern of *Alk5* in OE development may suggest that ALK5 is the type I receptor for the GDF11 signaling in OE development.

Angiogenesis refers to the processes by which new vessels form by sprouting or splitting from pre-existing vessels.<sup>30</sup> Angiogenesis can be separated into two distinct phases: activation and resolution.<sup>31</sup> In the activation phase, ECs degrade their vascular basement membranes, migrate into extracellular spaces, proliferate, and form vascular lumens. During the resolution phase, ECs cease the migration, stop proliferation, and instead reconstitute their ECM proteins. The maturation and remodeling of the vessels also occur in this phase, as the mesenchymal cells are recruited for endothelial-tube encasement. Such cells include pericytes for small capillaries and vascular SMCs for larger vessels.

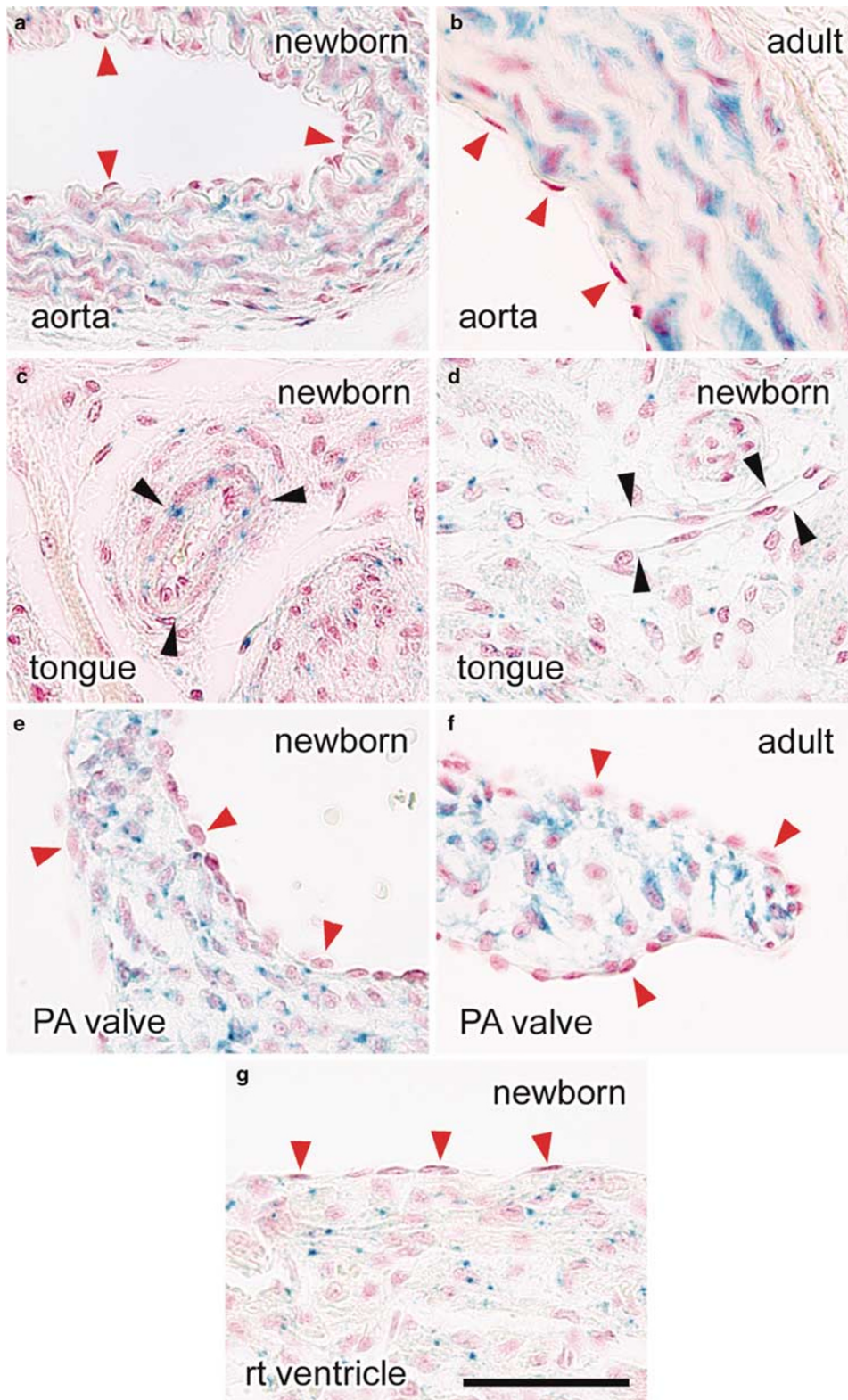
Several growth factors are reported to play important roles in the resolution phase of angiogenesis, which include angiopoietin-1 (ANG1),<sup>32</sup> platelet-derived growth factor B (PDGFB),<sup>33,34</sup> and



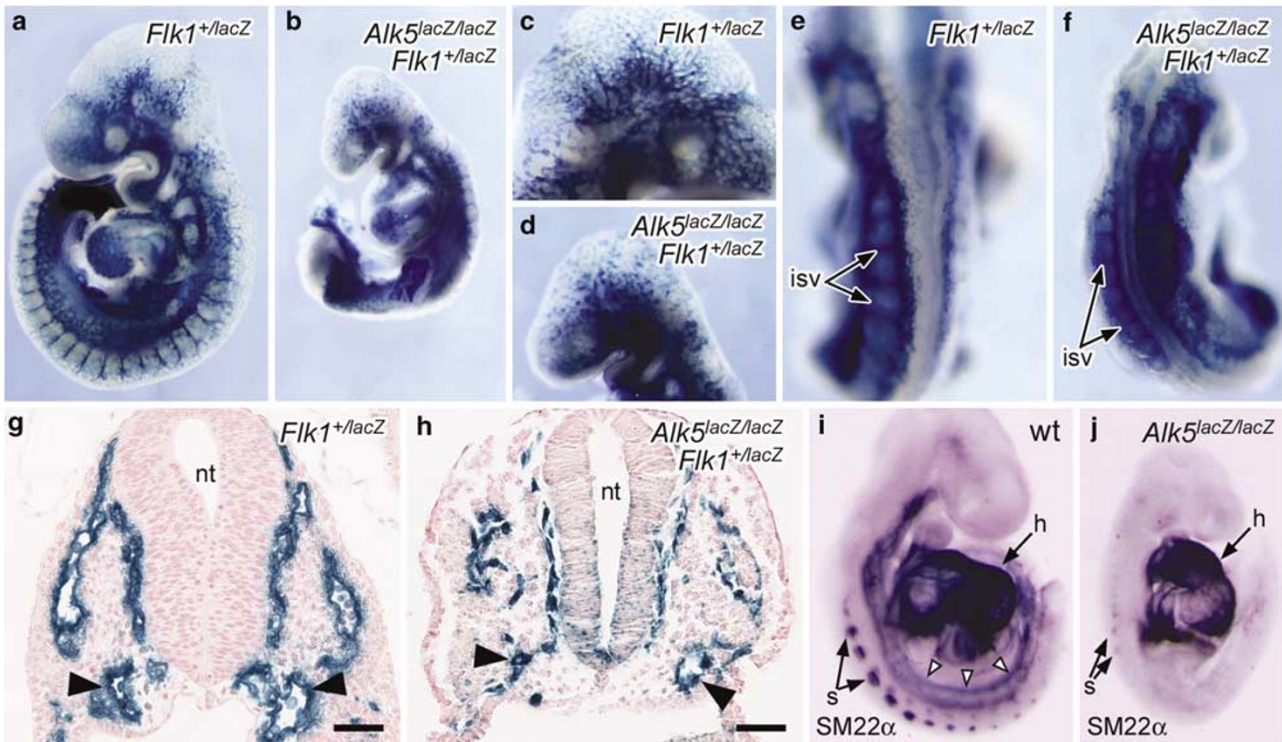


**Figure 5** Vascular expression patterns of *Alk1* and *Alk5* in E11.5 embryos. (a, b) Low-power magnification reveals that both *Alk1* (a) and *Alk5* (b) are expressed in dorsal aortas (asterisk). (c, d) Magnified view of the dorsal aortas in (a) and (b). *Alk1* is expressed in vascular ECs as a single intimal layer of the dorsal aorta (c), whereas *Alk5* is expressed in the multiple layers of the smooth muscle cells surrounding the ECs, but not in the ECs (d). (e, f) Double staining with X-gal and the antibody against smooth muscle  $\alpha$  actin (SMA) further confirms that *Alk1* is expressed in ECs (e) and that *Alk5* is expressed in smooth muscle cells (f). Panels of (a and b), (c and d), as well as (e and f) in individual pairs have identical magnifications. Scale bars in (b), (d), and (f) are equal to 50  $\mu$ m.

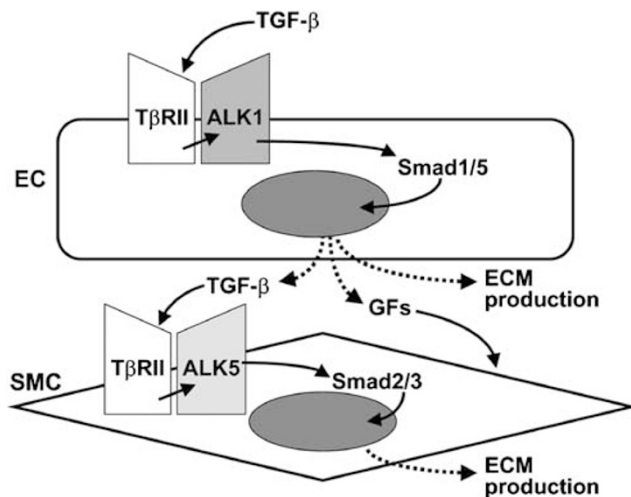




**Figure 6** *Alk5* expression is undetectable in any endothelial lineage cells in various developmental and postnatal stages. (a–d) Endothelial cells (red arrowheads) in newborn and adult aortas, as well as small arteries and veins in newborn tongues (black arrowheads) show no detectable *Alk5* expression. Note intense *Alk5* expression in the vascular SMCs (a–c). (e, g) Endothelial cells on the pulmonary artery valve of newborn (e) and adult (f) mice, as well as on the endocardium in the right ventricle of a newborn mouse (g) are negative for X-gal staining (red arrowheads). All panels are in the same magnification. Scale bar in (g) is equal to 50  $\mu$ m.



**Figure 7** Vascular morphogenesis in the E9.5 *Alk5<sup>lacZ/lacZ</sup>* embryo proper. The *lacZ* gene inserted into *Flik1* locus was utilized to visualize endothelial cells in embryos. (a–f) Although the *Alk5<sup>lacZ/lacZ</sup>* embryo (b, d and f) is retarded compared with *Alk5<sup>+/+</sup>* (a, c, and e), the network structure of blood vessels in the body seems unaffected. Note that the intersomitic vessels are properly formed (f). (g and h) Histological analysis shows that the lumen size and shape of the dorsal aortas (arrowheads) of the *Alk5<sup>lacZ/lacZ</sup>* embryos (h) are comparable to those found in wild-type mice (g). (i and j) *In situ* hybridization with an anti-SM22 $\alpha$  antisense probe revealed a defect in smooth muscle cell development in the aortas of *Alk5<sup>lacZ/lacZ</sup>* embryos. Scale bars in (g) and (h) are equal to 50  $\mu$ m.



**Figure 8** A model of TGF- $\beta$  signaling for the vascular maturation and remodeling. TGF- $\beta$  signal is mediated by ALK1 in ECs and ALK5 in vascular SMCs for the growth regulating as well as extracellular matrix (ECM) protein synthesis for the maturation of the vessel wall. TGF- $\beta$  signal in ECs produces growth factors (GFs) including TGF- $\beta$  itself, which regulate growth, differentiation, and ECM production of the neighboring SMCs.

TGF- $\beta$ .<sup>35</sup> In fact, TGF- $\beta$  signaling has been considered as a key component in the resolution phase of angiogenesis and maturation of blood vessels. Based

on coculture studies of ECs and SMCs, it was postulated that interactions of perivascular cells and ECs can activate the latent form of TGF- $\beta$ , which in turn induces differentiation of mesenchymal cells into perivascular cells, inhibits EC proliferation, and stimulates ECM deposition.<sup>36</sup> In terms of mitogenic activities, TGF- $\beta$  has been shown to either promote or inhibit the proliferation of ECs, depending on the concentration of TGF- $\beta$ , cell types, culture conditions, or presence of other regulators.<sup>31</sup> The previously proposed balance models of the TGF- $\beta$  signals in ECs via two type I receptors might explain for the mechanism underlying these biphasic effects of TGF- $\beta$ : the concentrations of TGF- $\beta$  could selectively activate ALK1 or ALK5 signaling pathway which may be a determinant of the proliferation status of ECs.<sup>12,15</sup> More complex interactions between these two pathways have also been reported.<sup>16,17</sup> Paradoxically, our expression studies using the *lacZ* knockin mice indicate the mutually exclusive expression of *Alk1* and *Alk5* in blood vessels: ALK1 in ECs and ALK5 in vascular SMCs (Figure 5).

These expression data raise the question of whether the proposed *in vitro* roles of the two TGF- $\beta$  signaling pathways via ALK1 and ALK5 are valid for *in vivo* vascular development. One explanation for this discrepancy is that our knockin



system might not be sensitive enough to detect a low level of *Alk5* expression in the ECs. Since we have used the same *lacZ* expression cassette in both the *Alk1* and *Alk5* knockin lines, however, it is indisputable that *Alk1* is predominantly expressed over *Alk5* in ECs, and *vice versa* in the vascular SMCs. An alternative explanation is that similar levels of *Alk1* and *Alk5* expression in cultured ECs may not be representing a normal *in vivo* situation, but rather they occur in the cells under a non-physiological condition. In other words, the balance mechanism in ECs may exist only in very limited circumstances *in vivo*.

With these in view, we propose a model for the role of the TGF- $\beta$  signal in vascular development. The TGF- $\beta$  signal is mediated by ALK1 in ECs and by ALK5 in vascular SMCs, and the TGF- $\beta$  signal in ECs via ALK1 modulates the proliferation and migration of vascular SMCs and/or the matrix protein synthesis by vascular SMCs (Figure 8). The modulation mechanism may include autoregulation of TGF- $\beta$  secretion from ECs, which can be relayed to the adjacent vascular SMCs. This model further implicates the role of TGF- $\beta$  in the maturation, stabilization, and remodeling of blood vessels. It has previously been shown that *Alk1* is predominantly expressed in arterial ECs, and is upregulated by shear stress. Such a specific regulation of the receptor expression is one possible reason why two different receptors exist for the TGF- $\beta$  signaling in blood vessels.

TGF- $\beta$  has been implicated in a diverse range of pathological conditions, including inflammation, wound healing, regeneration, aging, and cancer to list a few. The *Alk5<sup>lacZ</sup>* mice together with previously reported *Alk1<sup>lacZ</sup>* mice will be valuable animal models in identifying the *in vivo* cellular targets of TGF- $\beta$  family signals during various pathogenesises.

## Acknowledgements

We thank Dr Margolis at the University of Maryland for anti-OMP serum, and Marya Park for editorial assistance. This work was supported by NIH (HL64024) to SPO.

## References

- 1 Massague J. TGF-beta signal transduction. *Annu Rev Biochem* 1998;67:753–791.
- 2 Massague J. How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* 2000;1:169–178.
- 3 Wrana JL, Attisano L. The Smad pathway. *Cytokine Growth Factor Rev* 2000;11:5–13.
- 4 Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425:577–584.
- 5 Piek E, Heldin CH, ten Dijke P. Specificity, diversity, and regulation in TGF-beta superfamily signaling. *FASEB J* 1999;13:2105–2124.

- 6 Lee SJ, McPherron AC. Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci USA* 2001;98:9306–9311.
- 7 Yeo C, Whitman M. Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. *Mol Cell* 2001;7:949–957.
- 8 Oh SP, Yeo CY, Lee Y, *et al*. Activin type IIA and IIB receptors mediate Gdf11 signaling in axial vertebral patterning. *Genes Dev* 2002;16:2749–2754.
- 9 Rebbapragada A, Benchabane H, Wrana JL, *et al*. Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. *Mol Cell Biol* 2003;23:7230–7242.
- 10 Wu HH, Ivkovic S, Murray RC, *et al*. Autoregulation of neurogenesis by GDF11. *Neuron* 2003;37:197–207.
- 11 ten Dijke P, Yamashita H, Ichijo H, *et al*. Characterization of type I receptors for transforming growth factor-beta and activin. *Science* 1994;264:101–104.
- 12 Oh SP, Seki T, Goss KA, *et al*. Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci USA* 2000;97:2626–2631.
- 13 Chen YG, Massague J. Smad1 recognition and activation by the ALK1 group of transforming growth factor-beta family receptors. *J Biol Chem* 1999;274:3672–3677.
- 14 Lamouille S, Mallet C, Feige JJ, *et al*. Activin receptor-like kinase 1 is implicated in the maturation phase of angiogenesis. *Blood* 2002;100:4495–4501.
- 15 Goumans MJ, Valdimarsdottir G, Itoh S, *et al*. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J* 2002;21:1743–1753.
- 16 Goumans MJ, Valdimarsdottir G, Itoh S, *et al*. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. *Mol Cell* 2003;12:817–828.
- 17 Lebrin F, Goumans MJ, Jonker L, *et al*. Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *EMBO J* 2004;23:4018–4028.
- 18 Iseki S, Osumi-Yamashita N, Miyazono K, *et al*. Localization of transforming growth factor-beta type I and type II receptors in mouse development. *Exp Cell Res* 1995;219:339–347.
- 19 Tomoda T, Shirasawa T, Yahagi YI, *et al*. Transforming growth factor-beta is a survival factor for neonate cortical neurons: coincident expression of type I receptors in developing cerebral cortices. *Dev Biol* 1996;179:79–90.
- 20 Zhao Y, Young SL, McIntosh JC, *et al*. Ontogeny and localization of TGF-beta type I receptor expression during lung development. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L1231–L1239.
- 21 Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 1992;69:915–926.
- 22 Seki T, Yun J, Oh SP. Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling. *Circ Res* 2003;93:682–689.
- 23 Keller A, Margolis FL. Immunological studies of the rat olfactory marker protein. *J Neurochem* 1975;24:1101–1106.
- 24 Margolis FL. Olfactory marker protein (OMP). *Scand J Immunol Suppl* 1982;9:181–199.
- 25 Wilkinson DG (ed). *In Situ Hybridization: A Practical Approach*. IRL Press at Oxford University Press: Oxford, UK, 1992, pp 75–85.

- 26 Larsson J, Goumans MJ, Sjostrand LJ, *et al*. Abnormal angiogenesis but intact hematopoietic potential in TGF- $\beta$  type I receptor-deficient mice. *EMBO J* 2001; 20:1663–1673.
- 27 Calof AL, Chikaraishi DM. Analysis of neurogenesis in a mammalian neuroepithelium: proliferation and differentiation of an olfactory neuron precursor *in vitro*. *Neuron* 1989;3:115–127.
- 28 Paranya G, Vineberg S, Dvorin E, *et al*. Aortic valve endothelial cells undergo transforming growth factor- $\beta$ -mediated and non-transforming growth factor- $\beta$ -mediated transdifferentiation *in vitro*. *Am J Pathol* 2001;159:1335–1343.
- 29 Shalaby F, Rossant J, Yamaguchi TP, *et al*. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 1995;376:62–66.
- 30 Risau W. Mechanisms of angiogenesis. *Nature* 1997; 386:671–674.
- 31 Pepper MS. Transforming growth factor- $\beta$ : vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev* 1997;8:21–43.
- 32 Yancopoulos GD, Davis S, Gale NW, *et al*. Vascular-specific growth factors and blood vessel formation. *Nature* 2000;407:242–248.
- 33 Leveen P, Pekny M, Gebre-Medhin S, *et al*. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev* 1994;8:1875–1887.
- 34 Soriano P. Abnormal kidney development and hematological disorders in PDGF  $\beta$ -receptor mutant mice. *Genes Dev* 1994;8:1888–1896.
- 35 Folkman J, D'Amore PA. Blood vessel formation: what is its molecular basis? *Cell* 1996;87:1153–1155.
- 36 Antonelli-Orlidge A, Saunders KB, Smith SR, *et al*. An activated form of transforming growth factor  $\beta$  is produced by cocultures of endothelial cells and pericytes. *Proc Natl Acad Sci USA* 1989;86:4544–4548.