

DEVELOPMENT OF A NOVEL MOUSE MODEL TO STUDY
THE MECHANISM OF HHT PATHOGENESIS

By

EUNJI LEE

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2008

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To my parents, Young-Min Lee and Yi-Soon Kim

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. S. Paul Oh, for all of his support for my graduate career. And I would like to express my gratitude to my committee members, Dr. Naohiro Terada and Dr. Gregory Schultz, for their support during my studies. I also wish to thank the members of Dr. Oh laboratory for their help and advice. I would also like to give my special thanks to my parents, Young-Min Lee and Yi-Soon Kim for their sincere support and encouragement.

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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

DEVELOPMENT OF A NOVEL MOUSE MODEL TO STUDY
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By

Eunji Lee

May 2008

Chair: S. Paul Oh
Major: Medical Sciences

Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber (ORW) syndrome is an autosomal-dominant vascular disorder with an incidence around 1/10000. It is characterized primarily by epistaxis (spontaneous and recurrent nosebleeds), telangiectases and arteriovenous malformations (AVM). Arteriovenous malformations are characterized by direct communication of arterioles to venuoles without intervening capillaries. Blood vessels in AVM are dilated, convoluted, and tortuous, and the vascular wall of these vessels often devoid of smooth muscle layer. Telangiectasia refers to small AVMs that usually form in the mucosal layer of skin. Pulmonary, cerebral, and gastrointestinal AVMs can cause significant morbidity and mortality. The molecular pathogenetic mechanisms underlying AVM formation remain elusive. More than 300 heterozygous mutations have been recognized in *ENG* (*Endoglin*) and *ALK1* (activin receptor-like kinase1 or *ACVRL1*) genes as the cause of HHT1 and HHT2, respectively. Both of these genes are plasma membrane proteins involved in the signal transduction of TGF- β (Transforming Growth Factor- β) superfamily proteins. In addition, mutations in *SMAD4*, a common mediator of TGF- β signaling, are seen in patients with the combined syndrome of juvenile polyposis (JP) and HHT (JP-HHT). Since all three known HHT genes are involved in

TGF- β family signaling, it has been postulated that impaired TGF- β family signaling underlie the pathogenesis of HHT.

Alk1-deficient mice die in utero with severe vascular abnormalities, characterized by hyperdilation of large blood vessels and extreme fusion of the capillary plexus into cavernous vessels. Arterial endothelium-specific ALK1 expression patterns and thin, vein-like vessels in *Alk1*-deficient embryos suggested that ALK1 may play an essential role in arteriogenesis. However, the specific function of ALK1 in this biological process remains to be determined. Our laboratory has recently generated a novel mouse line in which GFP^{Cre} gene—a fusion protein of GFP (Green Fluorescent Protein) and Cre recombinase—was inserted into the *Alk1* gene locus (*Alk1*^{+/^{GFP}Cre}) by the gene targeting method. These mice will enable us to distinguish ECs expressing ALK1 from ECs that do not express ALK1 in vivo using GFP-mediated cell sorting, and thereby we can identify the downstream targets of ALK1 signaling. Another important utility of this mouse strain is to spatiotemporal expression of ALK1 during development, postnatal stages, and pathological processes. The first step toward these goals is to carefully characterize expression of GFP^{Cre} gene during development in order to establish strong foundation of this mouse strain for further use. Since our laboratory has previously generated additional mouse lines which express bacterial β -galactosidase gene (*lacZ*) under the endogenous *Alk1* promoter (*Alk1*^{lacZ}), comparison of expression pattern of transgene between *Alk1*^{+/^{GFP}Cre} and *Alk1*^{lacZ} will provide complete information regarding ALK1 expression in vivo. To monitor GFP^{Cre} expression, I monitored Cre activities by crossing the *Alk1*^{+/^{GFP}Cre} mice with R26R mice (a Cre reporter). The *lacZ* expression pattern in *Alk1*^{+/^{GFP}Cre};R26R bigenic mice should reflect Cre activities, and show cell types expressed ALK1. I found that *lacZ*-positive cells are mostly vascular endothelial cells. Unlike the pattern in *Alk1*^{lacZ}, where *lacZ*-positive cells were

predominantly localized in arterial endothelium, venous endothelial cells were also lacZ-positive in these bigenic lines, indicating that Alk1 is indeed expressed in venous endothelium at a lower level than arterial endothelium. I also found that the mesenchymal cells of the atrioventricular (AV) cushions during heart development were also lacZ-positive, suggesting that *Alk1*^{+/GFPCre}-mediated DNA recombination occurred prior to endothelial-mesenchymal transformation during AV cushion formation. In terms of the temporal expression, lacZ-positive cells were first detected in embryonic day (E) 9.5 in a patch staining pattern, but become uniformly detected in vascular endothelial cells by E10.5. This is one or two days delay compare to endogenous ALK1 expression observed by *Alk1*^{lacZ} mice. Accounting for the fact that the Cre-mediated recombination requires time, these data show that GFPCre expression in this novel mouse strain is consistent with endogenous ALK1 expression in a spatiotemporal manner.

I also present data showing that this novel mouse line can be useful as another endothelial Cre line by characterizing mice in which *Tgfb2* (TGF- β type II receptor) gene is conditionally deleted by *Alk1*^{+/GFPCre} mice. Although due to limited time, I was unable to reach the ultimate goal of this project, my results demonstrate that *Alk1*^{+/GFPCre} line is an important resource with which one can study ALK1 expression patterns in various pathogenic conditions, conditionally delete genes in the ALK1 expressing cells, and sort out cells expressing ALK1.

CHAPTER 1 INTRODUCTION

Clinical Manifestations of HHT

The hereditary hemorrhagic telangiectasia (HHT) is an under-diagnosed disease. Important reason for this is due to the variability of the disease symptoms in terms of the onset, location, severity, and types of clinical manifestations. Epistaxis (recurrent nose bleeds) is the most common symptom of HHT [2]. More than 90% of HHT patients are reported to have epistaxis in their life time. Telangiectasias (focal dilation of postcapillary venule) in varying sizes which usually appear in the mucosal layer of nasal and oral cavity and nail beds are another common sign of HHT. More than 60% of HHT patients possess more than one arteriovenous malformations (AVM—direct connections between arteries and veins) in their brain, lung, liver, and gastrointestinal tract (GI). HHT is a genetic disease, and thus most HHT patients have family history of these symptoms. HHT is diagnosed, if one has three of the four common symptoms--epistaxis, telangiectasia, visceral AVM, family history,

AVMs are associated with high morbidity and mortality in HHT patients. Cerebral AVMs can cause severe headache and stroke, pulmonary AVMs can lead to cerebral abscess, liver AVMs can result in cirrhosis, and GI AVMs often leads to chronic hemorrhages. Many cases AVMs are benign, but having AVMs in a body is like to have a time bomb. Often HHT patients suffer from anemia, blood transfusions due to chronic hemorrhages. Embolism is the best treatment option for CAVM and PAVM when only a few AVMs formed, or transplantations are required for severe cases. Unfortunately, no therapeutic options are available for HHT patients to date. Fortunately, a few genes have been identified as HHT-causing (or predisposing) genes, including *Alk1* as details below. Investigating the function of these genes, therefore, will provide important clues for development of therapeutic options for HHT patients.

Genetics of HHT

HHT is a genetically heterogeneous disorder that has been mapped to areas 9q33-q34.1 on chromosome 9 (HHT1)[9] and 12q11-q14 on chromosome 12 (HHT2). HHT 1 turned out to be *Eng* (*Endoglin*) whereas HHT 2 *Alk1* (activin receptor-like kinase 1 or *ACVRL1*)[10]. Both ENG and ALK1 are plasma membrane protein involved in signal transduction of TGF- β superfamily signals. Haploinsufficiency (diminished levels of functional protein) appears to be the principal mechanism. Recently, it has been reported that a subgroup of people having juvenile polyposis with SMAD4 mutations also displayed HHT symptoms [11]. Furthermore, a new locus for HHT (HHT3) has been mapped to chromosome 5[10], and a fourth locus (HHT4) to chromosome 7[12].

As many as 156 different ENG mutations have been reported[10]. Of 14 coding exons, mutations have been observed in exons 1-12 coding for the extracellular domain, but no mutations have been identified in exon13 and 14 coding transmembrane and cytoplasmic domains [10]. All types of mutations including deletions, missense, splice sites, insertions, nonsense, and insertion+deletion have been found. A large number of ENG mutations would lead to structural unsteadiness, unsuccessful intracellular trafficking and loss of function, suggesting that haploinsufficiency as a mechanism of disease.

The *Alk1* gene covers more than 15kb of genomic DNA and the cDNA encodes a protein of 503 amino acids[13]. ALK1 protein has the characteristics of a TGF- β type I serine-threonine kinase receptor[13]. Like other type I receptors, it has a relatively high extent of similarity in serine/threonine kinase subdomains, a glycine/serine-rich (GS) region heading the intracellular kinase domain, a short C-terminal tail[10] and an N-terminal extracellular domain with 10 conserved cysteine residues[14]. The coding region includes 9 exons, consisting of the start codon in exon 2 and the termination codon in exon 10[10]. As many as 123 mutations in the

Alk1 gene have been reported[10]. Nonsense, deletions, insertions, insertion+deletion and splice site mutations were also identified in ALK1 mutations (the number of frequency is 16, 24, 13, 1 and 4, respectively). Unlike ENG, missense substitution consist the highest portion of the mutations (53%) connected to ALK1. The extracellular domain occupied 20% mutations (n=25), intracellular kinase domain 75% (n=92) and transmembrane 5% (n=6). 65% of all identified mutations were confirmed in exons 3, 7 and 8 [10].

Because the majority of HHT patients have been found to have mutations in ENG or ALK1, it is probable that only a small fraction of the remaining cases will be caused by mutations in these additional genes. Gallione et al screened 30 patients from unselected group of HHT patients, who had been referred for DNA based testing for HHT and verified to be negative for mutations in either ENG or ALK1, but had mutations in the SMAD4 gene, a key downstream effector of TGF- β signaling transduction[11] and present in all cell types[10]. The region of the SMAD4 gene coding for the highly conserved carboxyl terminus accounts for these mutations[16]. The presence of both juvenile polyposis and HHT in an affected individual defines the syndrome of juvenile polyposis and HHT (JPHT)[10]. Juvenile polyposis is distinguished by the presence of five or more ominous gastrointestinal polyps, and was shown to be associated with mutations in either SMAD4 or BMPRIA[11].

In a previously described HHT family no mutations were identified in the ENG, ALK1, or SMAD4 genes. The putative HHT3 gene was recently linked to chromosome 5, and was mapped to regions 5q31.3–5q32. As all three identified HHT genes encoded proteins are involved in TGF- β signaling pathway, the gene responsible for HHT3 is also anticipated to encode a protein involved in SMAD dependent TGF- β signaling[15]. Furthermore, the presence of another gene causing HHT4 has been reported recently. A family displayed classic features of

HHT, but with milder epistaxis and telangiectasia phenotypes. A complete genome linkage investigation and fine mapping suggested this HHT4 gene was on a 7 Mb region on the chromosome 7 (7p14) [12].

Signal Transduction of TGF- β Family Proteins

As described above, all three genes linked to HHT are involved in TGF- β signal transduction (Figure 1-1). TGF- β superfamily members are a large group of pleiotropic secreted proteins, which can be grouped into several subfamilies, such as TGF- β , bone morphogenetic protein (BMP), activins/inhibin, and growth and differentiation factor (GDF). Each member is capable of effecting a diverse range of cellular processes including cell proliferation, migration, differentiation, lineage determination, pattern formation, adhesion, and apoptosis [17]. TGF- β family cytokines control gene expression by binding to heteromeric complexes of two types of transmembrane serine/threonine kinase receptors [18]. Five type II and seven type I receptors are known in mammals. The type I receptor and type II receptor have the same fundamental structural elements: a cysteine-rich N-terminal extracellular domain, which is associated with ligand binding; a single transmembrane domain; and a C-terminal cytoplasmic kinase domain. Type I receptors have distinctive amino acid sequence at its cytoplasmic juxtamembrane area called the GS (glycine/serine-rich domain) domain [19].

TGF- β signaling is commenced by the binding of TGF- β ligands to the type II receptors, which then recruit type I receptors and transphosphorylate at the GS domain of type I receptors. The activated type I receptors send out signals through phosphorylating receptor-regulated SMAD (R-SMAD) proteins, that is, SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8. Phosphorylated R-SMADs form heteromeric complex with the coregulatory SMAD (SMAD4 in mammals), and this complex translocates into the nucleus, where they regulate target gene transcription by interaction with transcriptional co-activators or co-repressors [20]. A few

conserved amino acid residues in the L45 loop of the kinase domain of the type I receptor and the L3 loop of the MH2 domain of R-SMADs determines specific communication between the type I receptors and the R-SMADs [19, 21]. Depending on which R-SMAD is utilized the TGF- β family signals can be divided into two pathways. Generally, BMP signals activate SMAD1, 5, and 8, whereas TGF- β and Activins signal through SMAD2 and 3. There are also inhibitory I-SMADs (SMAD6 and SMAD7), which negatively alter TGF- β signaling by inhibiting the interaction of R-SMADs with SMAD4 or their corresponding type I receptors. [17, 20]. Two type III receptors, TGFBR3 (also called betaglycan) and ENG are known to be involved in TGF- β signaling. The precise roles of these type III receptors are unclear, but generally believed that they act as coreceptors for facilitating (or inhibiting) interactions between ligands and type II receptors.

Arterial Endothelium-Specific Alk1 Expression

ALK1 is one of the seven type I receptors for TGF- β family proteins. The ligand specificity of the type I receptor has been defined by the ability to bind to a given ligand and to stimulate specific downstream genes in the presence of corresponding type II receptors. ALK1 can interact with TGF β -1 or activins in the existence of either TGFBR2 or activin type II receptors (ACVR2 or ACVR2B), respectively[18]. In addition, recent studies showed that BMP9 and its close subfamily member BMP10 can transduce its signal through either BMPRII or ActRIIA [20]. Therefore it is unclear which ligand is the most relevant ALK1 ligand in vivo, especially for pathogenesis of HHT.

Our laboratory previously generated a lacZ reporter line (*Alk1*^{lacZ}), in which the bacterial β -galactosidase gene is inserted into the *Alk1* locus. Using this reporter line, it was demonstrated that ALK1 is primarily expressed in developing arterial endothelium [18]. ALK1-deficient mice die in mid-gestation (E10.5), exhibiting severe vascular abnormalities such as

hyperdilation of large vessels, fusion of capillary plexus into major vessels, and AVMs [24, 55]. Furthermore, ALK1-deficient embryos showed severe defects in the development of vascular smooth muscle cells, demonstrating that ALK1 signaling is necessary for proper differentiation and recruitment of vascular smooth muscle cells [24]. Taken together, these data suggest that ALK1 may play a pivotal role in the development of arterial structure (arterialization), and stabilization and maturation of blood vessels [18].

Role of Alk1 in Vascular Remodeling

The vascular system consists of two largely distinct and separate networks of arterial and venous blood vessels. Arteries and veins are morphologically, functionally and molecularly distinguishable. Recent evidence has revealed that molecular differences do exist between arterial and venous endothelial cells, even before blood vessels are formed and that complex genetic paths are responsible for these early differences. Identification of several molecular signaling offers information regarding molecular regulators of arteriovenous boundaries, configuration, and cell fate[25]. Notch and its ligands have a key function in identifying artery versus vein, as well as determining cell destiny. Notch signaling is suggested to have a significant role in vasculogenesis and angiogenesis. Notch1 and Notch4 are expressed in vascular endothelium and deficient mice in various genes involved in Notch signaling exhibited defects in blood vessel formation [26-28] . Ephrin-B2, an Eph family transmembrane ligand, denotes the destiny of arterial but not venous endothelial cells in the beginning of angiogenesis. Conversely, Eph-B4, a receptor for ephrin-B2, accounts for the fate of veins but not arteries[29]. Notch signaling regulates the differential expression of Ephrin and Eph genes in the vasculature, while Ephrin/Eph signaling helps delineate the borders between arteries and veins, assisting to define the arterial cellular compartment. In addition, the vascular endothelial growth factor (VEGF), an

indispensable vascular signaling factor, also emerged as a critical trigger of Notch pathway in developing vasculature in zebrafish and mice [25].

The fact that ALK1 is specifically expressed in arterial endothelium implies that ALK1 signaling may play an important role in arteriogenesis. As a TGF- β receptor in vascular endothelium, ALK1 signaling may be required for the production of perivascular matrix proteins and the induction of the differentiation and recruitment of periendothelial cells, such as pericytes and smooth muscle cells. In addition, the fact that ALK1 signaling participates in the maturation of arterial vessels accounts for the reason why ALK1 expression is confined to arteries only during the course of fast growth stages[18]. Meanwhile, vascular function and structure are also defined by the profound effects of hemodynamic stimuli[30]. The distinctive characteristics of arteries and veins are destined to satisfy the physiological necessities, such as shear stress originated from blood flow or cyclic circumferential strain from pressure. The response to hemodynamic changes, including the creation of extracellular matrix proteins and proliferation of vascular smooth muscle cells, are exerted by these factors. ALK1 is believed to have a function in the signaling of the vascular remodeling of arteries, which was suggested by Alk1 expression in the mesenteric arteries in accordance with increased blood flow, as well as, in already existing arteries apart from wound and tumor during wound reparation and tumorigenesis [18]. Increased pulmonary blood flow stimulates upregulated TGF- β and ALK1 expression in pulmonary blood vessel [31], in addition, endothelial cells disclosed to high level of shear stress induce the expression of TGF- β 1[30]. Taken together, these data suggest that ALK1 is perhaps a protein that responds to arterial hemodynamic milieu and plays an essential role for the remodeling arteries.

The Cre/loxP Site-Specific Recombination System

The Cre/loxP site-specific recombination system is a powerful system to delete a gene of interest in a specific cell types or stage, and most widely utilized among several conditional knockout systems. Cre is a 38 kDa recombinase protein from bacteriophage P1 which mediates excisive or inversional site specific recombination between loxP sequences. A loxP sequence is made up of two 13 bp inverted repeats separated by an 8 bp asymmetric spacer area (Figure 1-2a). Recombination can take place in the asymmetric spacer area. Cre inverts the intervening part of DNA when two loxP sequences are in an opposite orientation to each other, while it excises the intervening DNA between the sites leaving one loxP site afterward when two sites are in a direct orientation (Figure 1-2b). When a conventional knockout line results in embryonic lethality, this Cre/loxP system can make it possible to overcome embryonic lethality and explore the role of a gene in later stages of development or postnatal life. This conditional approach is also widely used to determine the primary cells (or tissues) where the gene of interest plays [33].

Transgenic Mouse Strains for Expressing Cre Recombinase in ECs

Several transgenic (Tg) and knockin (KI) lines have been developed to express the Cre recombinase in the vascular endothelium. These include; Tg(*Tie1*-Cre) and Tg(*Tie2*-Cre), Tg(*Flk1*-Cre), *Flk1*^{+Cre} and Tg(*VECad*-Cre) [34-37]. Among them, the Tg(*Tie2*-Cre) line has been used most widely for conditional knockout in vascular endothelium [35]. The Cre is expressed in endothelial cells of arteries and veins by the promoter and enhancer regions of the mouse *Tie2* gene [38]. In addition to such a pan-endothelial Cre expression, *Tie2*-Cre-mediated recombination (lacZ activities) was found in the endocardium and the mesenchymal cells of the atrioventricular (AV) cushions [39]. In recent studies it has been shown that conditional elimination of several genes using the Tg(*Tie2*-Cre) line lead to defects in the valve formation as

well as minor or detrimental phenotypes in blood vessels [40-42]. In these studies, the Tg(*Tie2-Cre*) line was suggested to be a practical model to investigate the function of a gene in the whole ECs or to study cell fate-mapping, but is not suitable for studying functions of genes in the ECs of specific vascular beds.

Tg(*Flk1-Cre*) lines, were generated using regulatory sequence of the mouse *Flk1* gene adequate for endothelial cell-specific expression of the lacZ reporter gene. In these mice, intensive endothelium-specific staining of most vascular beds was detected at E11.5 to E13.5[36], but they also demonstrated lacZ expression in muscle lineages[50]. Furthermore, Cre-mediated lineage tracing study using *Flk1*^{+/*Cre*} mice have shown that both vascular endothelium and cardiac muscle arise from *Flk1*-positive mesodermal progenitors during development [43].

Tg(*Tie1-Cre*) mice were directed to endothelial cells by the mouse *Tie1* promoter. In *Tie1-Cre* line intercrossing with *ROSA26R* reporter mice, in which lacZ reporter gene is activated by the Cre-mediated excision, lacZ staining was observed in almost all cells of the forming vasculature in early stage of vascular formation (E8.5-E9.5) and in most endothelial cells within the embryo between E10.5 and birth, suggesting that *Tie1-Cre* transgenic strain can proficiently direct to the deletion of floxed genes in endothelial cells in vivo[34]. However, like the Tg(*Tie2-Cre*) line, the Tg(*Tie1-Cre*) line also displays the Cre-mediated DNA excision in the endocardial cells, mesenchymal cells in the AV cushion and ECs [34, 44], indicating that before epithelial-mesenchymal transition (EMT) during AV cushion formation is complete, the *Tie1-Cre*-mediated DNA recombination might happen [37]. The *VE-Cadherin* (vascular endothelial cadherin)-Cre line exhibits standardized expression in the endothelium of developing and

dormant vessels of all organs, however, it is also expressed within a small compartment of hematopoietic cells[37].

Experimental Scheme using *Alk1*^{+/-GFP^{Cre}} Mice

Primary goal of my thesis project is to characterize *Alk1*^{+/-GFP^{Cre}} mouse line from which spatiotemporal expression of the transgene can be compared with that in *Alk1*^{+/-lacZ} line. To analyze the Cre expression, the *Alk1*^{+/-GFP^{Cre}} mice were intercrossed with the *ROSA26* reporter line (*R26R*). Embryos at various gestational stages from this cross were then subjected to whole mount X-gal staining followed by histochemical analysis. In addition, I was also involved in characterization of a transgenic mouse line, Tg(*Alk1*GFP^{Cre}), in which GFP^{Cre} is expressed under previously identified 9.2-kb *Alk1* regulatory fragment. Both mouse lines are valuable to characterizing the pattern of *Alk1* expression by reciprocally compensating the limitations of each line.

As described above, to date, an exclusive endothelial specific Cre line with constitutive expression is yet to be developed. Therefore, as the second approach to verify *Alk1*^{+/-GFP^{Cre}} line, we examined the activity of Cre recombinase in *Alk1*^{+/-GFP^{Cre}} line by Cre-mediated conditional deletion of *Tgfb β 2* gene to compare this line with already established Cre lines, which expression are driven by other endothelial cell-specific promoters, such as *Tie2*-Cre, *Tie1*-Cre, *Flk1*-Cre, and *Flk*^{+/-cre} lines.

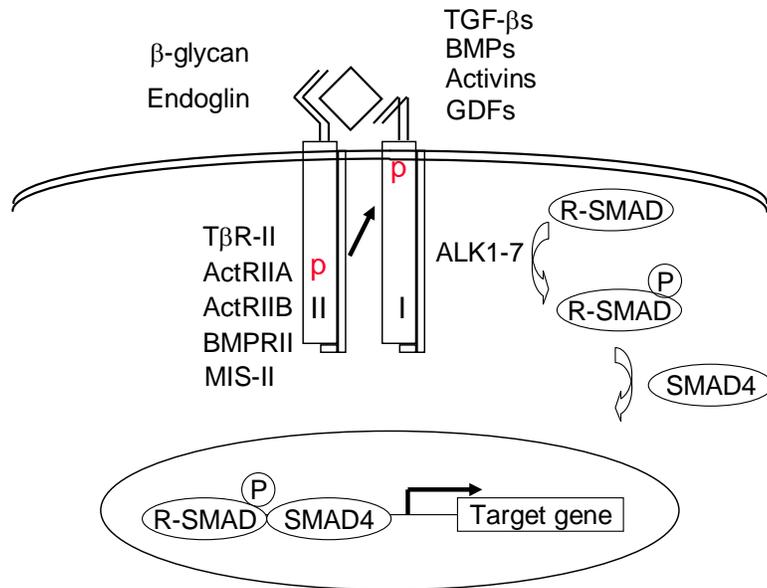
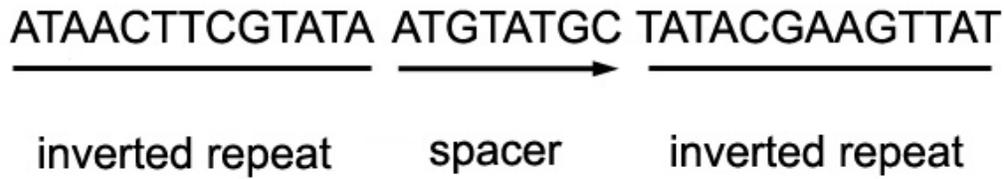


Figure 1-1. Signal transduction of TGF- β superfamily. More than 30 known ligands are known to be contained in the TGF- β superfamily. TGF- β family members include TGF- β s, bone morphogenic proteins(BMPs), activins/inhibin, and growth and differentiation factors (GDFs). Binding of the ligand to its type II receptor activates the receptor, which combines with and phosphorylates the type I receptor. The activated type I receptor subsequently phosphorylates a receptor-regulated SMAD (R-SMAD), permitting this protein to associate with SMAD4 and translocate into the nucleus. This complex binds to specific enhancers in the nucleus of target genes, activating transcription. TGF- β family members are classified into seven type I, five type II and two type III receptors. The type II receptor consists of TGF- β type II receptor (TβRII), activin type II receptors (ActRIIA and ActRIIB), bone morphogenetic protein type II receptor (BMPRII), and Müllerian inhibiting substance type II receptor (MISRII). The type I receptors are comprised of activin receptor-like kinase (ALK) 1-7. The type III receptors include β -glycan and endoglin. At present, eight SMAD proteins have been identified in mammals.

a



b

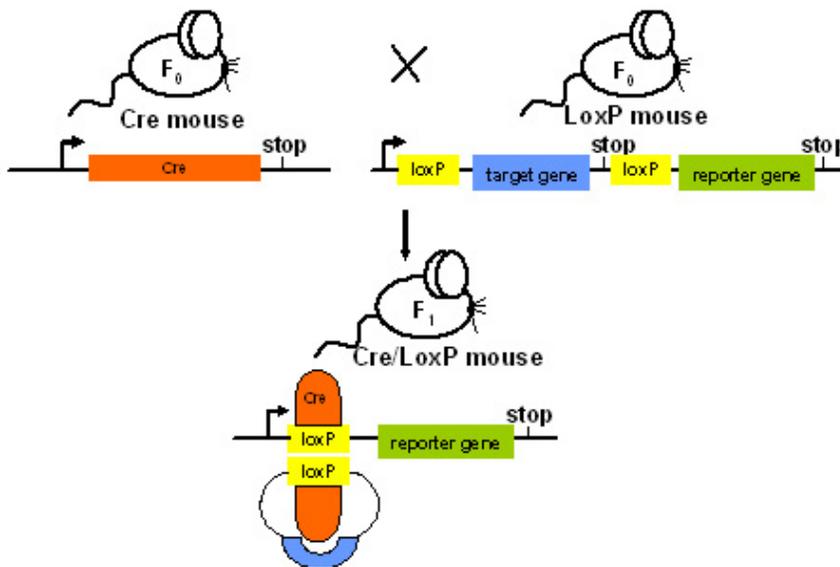


Figure 1-2. The Cre/LoxP Recombination System (a) A detailed structure of LoxP site. LoxP is a site on the Bacteriophage P1 consisting of 34bp. There exists an asymmetric 8bp sequence between two sets of palindromic, 13bp sequences flanking it. (b) A model experiment using the Cre/LoxP system. Two mouse lines are required for conditional gene deletion. First, a conventional transgenic mouse line with Cre targeted to a specific tissue or cell type, and secondly a mouse strain that embodies a target gene flanked by two loxP sites in a direct orientation (“floxed gene”). Recombination, which is an excision and consequently inactivation of target gene, occurs only in those cells expressing Cre recombinase.

CHAPTER 2 MATERIALS AND METHODS

Generation and analysis of *Alk1*^{+/GFPCre} mice

An *Alk1*-eGFPCre-knock-in (*Alk1*^{+/GFPCre}) vector was constructed. SV40 splice donor and acceptor signals (SD/SA), internal ribosomal entry sequence (IRES) and poly(A) signal (pA) were sequentially subcloned into pBluescriptIISK(+), of which XhoI site was deleted, and the eGFPCre fusion gene was subcloned between IRES and pA. This SD/SA-IRES-eGFPCre-pA fragment was replaced with SD/SA-IRES-lacZ fragment in the *Alk1*-SIBN2 knock-in vector previously generated [18]. After electroporation of the linearized knock-in vector, 365 G418-resistant clones were screened by Southern hybridization analysis. Southern hybridization analysis was done by digestion of G418-resistant ES cell DNA with EcoRI, separated on 0.8% agarose gels, transferred to Hybond-XL membrane (Amersham Biosciences) and probed with a [³²P]dCTP labeled 0.4-kb HindIII-HindIII fragment to analyze the homologous recombination. Three of positive ES clones obtained from 365 of G418. Positive ES cells were injected into C57BL/6J (B6) blastocysts to generate chimeric mice. Mating of chimeric mice with B6 females was performed to establish and maintain the *Alk1*^{+/GFPCre} line on a mixed 129/B6 hybrid background.

Mouse Breeding

All procedures performed on animals were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee. To investigate the expression pattern of ALK1 in embryos, male *Alk1*^{+/GFPCre} mice were crossed with female *ROSA26* reporter line. When female mice had a vaginal plug, the stage of the embryo was judged as at embryonic days (E) 0.5. The pregnant female mice were dissected at various embryonic stages such as E9.5, 10.5, 11.5, 12.5, 15.5, and 17.5 embryonic stages and postnatal stage. At E9.5, 10.5 and 11.5,

embryos were harvested, fixed and stained with an X-gal solution. To examine the expression pattern of ALK1 at E12.5, 15.5, 17.5 and postnatal stage, various organs such as lung, liver, heart, intestine, eyes, brain, kidney, and sternum were isolated and used for X-gal staining. At every stage, PCR was performed for genotyping using yolk sac (E9.5 to 11.5) or small piece of tail (E12.5 to postnatal stage).

X-gal staining

To investigate ALK1 expression pattern in the *Alk1*^{+GFP^{Cre}};R26R mice, pregnant R26R female crossed with *Alk1*^{+GFP^{Cre}} male mice were euthanized by cervical dislocation and embryos were removed from uteri. For the embryos older than E11.5, internal organs were isolated. For whole mount staining, dissected embryos of E9.5 to E11.5 were subjected to fixation with a fixative solution containing 1% formaldehyde, 0.2% glutaraldehyde, 2mM MgCl₂, 5mM EGTA, and 0.02% NP-40 for 15 minutes at room temperature. Fixed embryos were then washed with two times 3 minutes each with PBS on a rocker. Then, staining step was performed with solution containing 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆, 2mM MgCl₂, 0.01% Na-Deoxycholate, and 0.5 mg/ml X-gal. Embryos were incubated in staining solution overnight at 37°C. Stained embryos were washed with PBS, fixed in 4% formaldehyde at room temperature for 1 to 2 hours and washed with PBS. To observe the lacZ expression in organs at E12.5 to 17.5 and postnatal stages, each isolated organ was removed, sliced to 1-2 mm thickness and stained with the same X-gal staining solution just as the embryos.

Histology and Immunohistochemistry

Histological analysis was carried out by using standard methods. Embryos and pieces of organs from embryos and postnatal mice were dehydrated with serial concentrations of ethanol (70 %→95 %→100 %) for 15 minutes each and then kept in Citrosolv for 10 minutes, followed

by incubating in paraffin for 3 hours. Paraffin embedded samples were subsequently sectioned into 5 μm thickness, placed on glass slides. After deparaffinizing, the sections were counterstained with nuclear fast red (NFR) to determine the X-gal positive cells. Particularly, sternum piece from newborn was serially dehydrated in methanol (25%→50%→75%100%) for 10 minutes each and then cleared with organic solvent including benzyl benzoate and benzoic acid with 1:1 ratio. For immunohistochemistry, the standard ABC method was used with a Vector staining kit (Vector laboratories, Inc., CA). Briefly, sections were hydrated and endogenous peroxidase activity was blocked by treating them with 3% (v/v) hydrogen peroxide (H_2O_2) at room temperature for 10 minutes. After two times of washes with PBS, the sections were incubated with blocking serums corresponding to their secondary antibodies species for 1 hour at room temperature, followed by an incubation of primary antibodies. Biotinated secondary antibodies were incubated for thirty minutes after being washed with PBS. After secondary antibody incubation, sections were treated with peroxidase-conjugated avidin/biotin complex for thirty minutes followed by two PBS washes. Color development was carried out with a DAB + substrate chromogenic solution (Vector laboratories, Inc., CA). The antibody used for immunohistochemistry is α SMA (clone: 1A4; Sigma, 1:800).

PCR

A small piece of the yolk sac or tail was used for the genotyping of embryonic and postnatal stages. Tissues were lysed in lysis buffer consisting of 50mM Tris (pH 8.0) and 0.5% TritonX-100 with proteinase K (1 mg/ml) for overnight at 55°C. The lysed samples were centrifuged at 10000 rpm for 10 minutes to precipitate tissue fragment. The PCR reaction mixture was made up of the following: 2.5 μl of 10X buffer, 3 μl of MgCl_2 (25mM), 0.5 μl of dNTPs (25pM), 0.5 μl of each primer, 1.8 μl of genomic DNA, and 15 μl of H_2O . To prevent the

PCR mixture from evaporation Mineral oil was overlaid. A “hot start” PCR reaction was set under the following guidance: the mixture was stayed at 94°C for 10 minutes then the temperature was lowered to 72°C. Once the temperature reached 72°C, 2µl of a *Taq* polymerase was added. After adding the *Taq* polymerase, the PCR cycle was proceeded in the following way: 35 cycles at 94°C for 45 seconds, 1 cycle at 60°C for 45 seconds, and 1 cycle at 72°C for 1 minute. The primers used for genotyping are summarized in the Table1. PCR products were analyzed on 2% agarose gel containing ethidium bromide for about 20min at 105V. DNA bands were visualized under UV light and photographed. The primers used for genotyping are summarized in the Table2-1.

Genomic Southern

DNA was extracted from ES cells by adding 250µl of lysis buffer containing 100mM Tris (pH8.0), 5mM EDTA, 0.2% SDS, 200mM NaCl with proteinase K. The plates were wrapped thoroughly and incubated at 55°C over night with shaking at about 210rpm. The next day the plates were cooled down at room temperature and then 250µl of isopropanol was added and mixed. DNA was carefully picked up by forceps and excess isopropanol was removed and then resuspended in 25 µl of deionized water. The tubes with DNA mixture was then incubated at 55°C overnight for complete melting. Digest ion 10µl of genomic DNA was performed with 2µl of EcoRI and H buffer and 6µl distilled water, in 20µl total volume overnight at 37°C. Next day, 2µl loading dye per one digest sample was added and samples loaded into wells of 0.8% agarose gel, and electrophoresis was carried out overnight at about 23voltage until the dye passes off the end of the gel. Gel was, then photographed with a ruler and placed in glass dish followed by shaking gently in the series of solutions for the specified times;

1. 0.25N HCl (depurination solution) : 30minute, two times
2. Distilled water (for rinse)
3. 0.4N NaOH (denaturation solution) : 30minute, two times

4. 20X SSC (transfer buffer for soaking)

The Hybond-XL membrane (Amersham Biosciences), which is positively charged, and blotter papers were prewet in deionized water for 5 minutes, then in 0.4N NaOH, followed by putting on the gel/membrane blotting stack dissembled in the order. The blot was stayed overnight for transfer. The membrane was hybridized with a [³²P]dCTP labeled 0.4-kb HindIII-HindIII fragment to analyze the homologous recombination.

Table 2-1. Primers used in PCR

Genes	Forward primers	Reverse primers
Cre	GCTAAACATGCTTCATCGTCGGTC	CAGATTACGTATATCCTGGCAGCG
LacZ	GTCGTTTTACAACGTCGTGACT	GATGGGCGCATCGTAACCGTGC
GFP	CGATGGGGGTGTTCTGCTGGTAGT	ATGGTGAGCAAGGGCGAGGAG
Tgfr2 (floxed)	TAAACAAGGTCCGGAGCCCA	ACTTCTGCAAGAGGTCCCCT

CHAPTER 3 RESULTS

Screening of *Alk1*^{+GFP^{Cre}} mouse strain

The *Alk1*^{+GFP^{Cre}} mouse line was generated by inserting the GFP^{Cre} fusion gene into the *Alk1* locus by homologous recombination in order to express GFP^{Cre} in cells where endogenous ALK1 is expressed. The *Alk1*-eGFP^{Cre}-knock-in (*Alk1*^{+GFP^{Cre}}) targeting vector consists of SV40 splice donor and acceptor signals (SD/SA), internal ribosomal entry sequence (IRES), eGFP^{Cre} fusion gene, and poly(A) signal (pA) (Figure 3-1), essentially identical (except for the GFP^{Cre} portion) to previous *Alk1*^{+lacZ} targeting vector [18].

Characterization of the Cre Transgene Expression in the *Alk1*^{+GFP^{Cre}} Line

Alk1 is Expressed Primarily in Endothelium of Arteries, and also Detectable in that of Veins, Endocardium and Mesechymal Cell in Artiroventricular Cushions : To monitor the expression of functional Cre activities during development, *Alk1*^{+GFP^{Cre}} mice were intercrossed with the *ROSA26* reporter line, referred to as “R26R”, in which lacZ reporter gene is activated under the ubiquitous *ROSA* promoter, only when Cre excises the STOP cassette flanked by loxP sequences. Therefore, lacZ expression in *Alk1*^{+GFP^{Cre}};R26R mice represents the cells where Cre-mediated recombination had been occurred. The lacZ expression in various tissues/organs from various stages of embryos (from E9.5 to new born) was analyzed by X-gal staining. The same stages of embryos from Tg(*Alk1*GFP^{Cre}) mice were also paralleled for comparison purposes. Littermates of *Alk1*^{+GFP^{Cre}};R26R(-) and *Alk1*^{+/+};R26R(+) were used as a control for endogenous lacZ activity at all stages. Consistent to our previous data from *Alk1*^{+lacZ} mice, the lacZ activity was primarily detected in endothelium of developing blood vessels of *Alk1*^{+CreGFP};R26R embryos throughout development.

In E9.5 embryos, lacZ positive cells were observed in the developing heart, dorsal aorta, head vascular system, however, they appeared to be patchy and spotty at the vascular tree (Figure 3-2a). However, positive lacZ expression was increased throughout development (Figure 3-2a and 2b). In whole mount E10.5 embryos, X-gal staining displayed vascular trees more uniformly (Figure 3-2b, 2e and 2f), whereas these vessel-specific staining was not detectable in the control (Figure 3-2c). At this stage, lacZ expression appeared earlier in artery than in vein, especially in umbilical vessels (Figure 3-2g). Comparable lacZ expression pattern in vasculature was observed in Tg(*Alkl*-GFPCre);*R26R* embryos, though the homogeneous vascular lacZ expression pattern appeared little earlier in the Tg embryos than the knockin embryos (Figure 3-2d and 2h). This difference may be due to higher level of transgene expression in the Tg embryos compared to the knockin embryos. In E11.5 embryos, the pattern of X-gal positivity was similar with that of E10.5, but more specifically detected in capillaries surrounding neural tube and brain (Figure 3-2i and 2j). As the *Alkl*^{+GFPCre} line is supposed to be expressed both GFP and Cre protein, we also examined the GFP expression under the fluorescent microscope, confirming its expression in the lungs of Tg(*Alkl*-GFPCre) newborn mice (Figure 3-2k and 2l). Taken together, these results further confirm that the GFPCre was knocked into the *Alk1* locus, and indicate that expressed Cre becomes active at around E9.5.

Histological analysis of the whole-mount X-gal stained embryos revealed that X-gal-positive cells are mostly vascular endothelial cells (ECs) (Figure 3-3a). Unlike *Alkl*^{+lacZ} embryos in which venous ECs were lacZ-negative, venous as well as arterial ECs were X-gal positive in *Alkl*^{+GFPCre} mice, although X-gal staining intensity in venous ECs was weaker than that in arterial ECs (Figure 3-3b to 3d). This result indicates that a lower level of ALK1 is indeed expressed in venous ECs. Consistent with this view, as described above, when umbilical

vessels were stained in X-gal only for a few hours, umbilical artery but not vein turned to blue (X-gal-positive) (Figure 3-2g). When these vessels were incubated in the X-gal staining solution for overnight, however, both umbilical artery and veins became X-gal positive (data not shown).

I found that non-ECs were also X-gal positive. As shown in Figure 3-3e to 3g, endocardium and mesenchymal cells in the AV cushions were X-gal positive. Similar results were also observed in *Tg(Alk1-GFPCre):R26R* embryos (Figure 3-3h to 3j). Since endogenous ALK1 is not expressed in the AV cushion cells, this data indicate that Cre-mediated activation of the *lacZ* reporter gene occurred before the epithelium-mesenchymal-transition (EMT) in the atrioventricular region, resulting mesenchymal cells to show positive reporter gene expression (Figure 3-3e to 3g)[34]. Immunostaining with anti-smooth muscle α -actin (α -SMA) antibodies for vascular smooth muscle layers further confirmed that *Alk1* expression were limited in ECs but not in smooth muscle layers of blood vessels (Figure. 3-3k).

Strong *lacZ* expression was detected in the vasculature of most organs at E12.5 to E17.5. At these stages, we dissected out organs including the lungs, liver, kidney, intestine and dorsal aorta. At E15.5, noticeable arteries and capillary network of the lung was obviously stained, while the epithelium of the bronchioli and smooth muscle layers of the airways and blood vessels was negative (Figure 3-4a and 4b). Also, the endothelium of dorsal aorta show positive *lacZ* staining (Figure 3-4d and 4e). The vessels of developing kidney glomeruli show positive *lacZ* expression (Figure 3-4g and 4h). In liver, sinusoidal endothelium looked negative, and ECs in large blood vessels rarely showed positive X-gal (Fig. 3-4j and 4k). Interestingly, *lacZ* activity was also detected in the submucosal layer of small intestine (Fig. 3-4m and 4n). Not all of cells showing positive X-gal staining in intestinal submucosal layer appear to be ECs, implying the inclusion of mesenchymal cells. I expect that it can be verified by using cell type specific-

antibodies to examine in terms of a protein level, or by using in situ hybridization regarding of a RNA level. So I observed two areas where non-endothelial cells express lacZ, the mesenchymal cells of AVC and intestinal submucosal layer. Endogenous lacZ activities were detected in the bone, kidney and GI tracts. However, these background staining did not interfere our analysis as shown in Figure 3-4c, 4f, 4i, 4l, and 4o.

At newborn mice, endothelium-specific lacZ activity was still detected in most organ system including hematopoietic organ such as the liver, in which distinguishable lacZ activity was not examined in embryonic stages. In the liver, lacZ activity was detectable in endothelium of large veins and in some dispersed cells that may be possibly originated from hematopoietic cells (Figure 3-5a to 5c). Positive lacZ in venous endothelium may result from ALK1 expression in the process of a hemotopoiesis in liver around E13.5, as I suspect. Intensive lacZ reaction was noted in the lung (Figure 3-5d). Histological examination revealed positive labeling in arteries and in the capillaries. No staining was noted in the epithelia of bronchiolar units (Figure 3-5e and 5f). The lacZ was also contiguous in the endothelium of arteries and in the capillaries as well as glomeruli of the kidney (Figure 3-5g to 5i). In the intestine, mesenteric vessel was positively stained with X-gal (Figure 3-5j) and histological section revealed that lacZ was expressed in the mucosa and submucosa layers (Figure 3-5k and 5l). The strong LacZ expression was still observed in endothelium of dorsal aorta (Figure 3-5m and 5n). We also examined the eye having a very sensitive vessel capillary network, which showed positive staining in the endothelium of vessels around ciliary body, lens and optic nerve (Figure 3-5o and 5p). lacZ expression is also shown in the blood vessels on the thoracic wall (Figure 3-5q and 5r). Figure 3-5r shows X-gal stained internal arteries, but not veins. In addition, the brain vasculature showed positive and specific staining in all vessels observed (Figure 3-5s and 5t).

Investigation of *Alk1*^{+/-GFPCre} Line as a Potential Endothelial Cre Line for Conditional KO Approaches..

Angiogenesis during development as well as pathological conditions such as diabetes retinopathy, tumor angiogenesis, and ischemic insults receive much attention in modern medical sciences. Since endothelium is the most fundamental structural and functional unit of the blood vessels, function of a gene in ECs has been heavily investigated both in vitro and in vivo. The Cre/loxP system has been the most powerful approach to delineate EC-specific functions of a gene. For this reason, numerous transgenic and knock-in mouse lines expressing Cre in ECs have been generated and characterized. Recent studies have shown heterogeneity among ECs, e.g. arterial vs. venous or microvessel vs. large vessels. Spatiotemporal expressions as well as tightness of EC-restricted expression vary among these EC-specific promoters.

As shown in chapter 3, *Alk1*^{+/-GFPCre} mice express Cre mostly in vascular ECs, but the Cre expression appeared relatively late compared to other EC-specific Cre lines. In order to compare the Cre activity of the *Alk1*^{+/-GFPCre} line with that of a known EC-Cre deleter line, I have investigated *Alk1*^{+/-GFPCre}-mediated *Tgfb2* -deleted mice. *Tgfb2*-floxed mice were available in our laboratory, and Tg(*Tie1*-Cre)- and Tg(*Tie2*-Cre)-mediated *Tgfb2*-conditional knockout studies have been reported. Interestingly, Tg(*Tie1*-Cre);*Tgfb2*^{f/f} embryos exhibited abnormalities in the vasculature of the yolk sac at E9.5 and die around E10.5 with almost identical lethal phenotype with *Tgfb2*^{-/-} embryos which is the lacked networks of vessels, retardation, pericardial effusion in heart [58], whereas Tg(*Tie2*-Cre); *Tgfb2*^{f/f} embryos die around E13.5 with idiopathic cardiovascular defects [59].

I crossed *Alk1*^{+/-GFPCre}; *Tgfb2*^{+/-} male mice with *Tgfb2*^{f/f} females to generate *Alk1*^{+/-GFPCre}; *Tgfb2*^{f/f} mice. The resulting embryos of this cross were *Alk1*^{+/-GFPCre}; *Tgfb2*^{+/-}, *Alk1*^{+/-GFPCre}; *Tgfb2*^{f/f}, *Tgfb2*^{+/-}, *Tgfb2*^{f/f} (Figure3-6a). Three embryos having noticeable

hemorrhage in the cranial region among the littermate (Figure3-6c and 6d), whose genotype turned to be $Alkl^{+/GFPCre};Tgfb2^{f/f}$ (Figure3-6a lane2, 4, and 6). And the rest of embryos without a hemorrhage (Figure3-6b) were $Alkl^{+/GFPCre};Tgfb2^{+/f}$, $Tgfb2^{+/f}$ or $Tgfb2^{f/f}$ (Figure3-6a lane1, 3, 5, 7 and 8). Therefore, only mutant embryos in which loxP sequence in both alleles of *Tgfb2* gene was deleted by $Alkl^{+/GFPCre}$ displayed the obvious hemorrhage in the specific region of the head. The histological analysis of this area showed that red blood cells leaked from damaged blood vessels were observed to migrate into the epithelium layer of neural tube (Figure3-7a and 7b), whereas the control mice displayed no hemorrhage sign in the area (Figure3-7c). Concerned with the heart, there was the slight difference in terms of the thickness of the ventricular septum, the density of trabeculation in ventricles between mutant and control mice (Figure3-7d and 7e). The identical phenotype was observed in $Alkl^{+/GFPCre};Tgfb2^{f/f}$ embryo at E13.5 (Figure3-8). More severe hemorrhage than that of E11.5 in the same cranial region was found (Figure 3-8b and 8c), whereas control embryos of $Tgfb2^{f/f}$ still showed the absence of the hemorrhage (Figure 3-8a). Red blood cells in this hemorrhage area penetrating into the epithelium layer of neural tube were confirmed again in the histological analysis (Figure 3-9a and 9b). I also examined the placenta of both mutant and control embryos, in which no significant discrepancy between mutant placenta and that of control was detected (Figure 3-9d to 9f). In addition, hearts of both mutant and control were distinguished in a view of density of the ventricular trabeculation, in which denser and less organized trabecular was displayed in mutant heart (Figure3-9g and 9h). I expanded the stage to examine the consistency of the phenotype. At E15.5, six embryos were generated from the crossing of $Alkl^{+/GFPCre};Tgfb2^{+/f}$ mice with $Tgfb2^{f/f}$ females. Two embryos with normal phenotype turned to be $Alkl^{+/GFPCre};Tgfb2^{+/f}$ and $Tgfb2^{+/f}$. One of embryo was discovered to die about one day before, whose genotype was $Alkl^{+/GFPCre};Tgfb2^{f/f}$, demonstrating

mutant embryos of $Alk1^{+/GFPCre};Tgfb2^{fl/fl}$ displayed the embryonic lethality around E14.5. The rest of three embryos appeared to be dead at earlier stage, but its genotypes were not determined due to their resolved DNA.

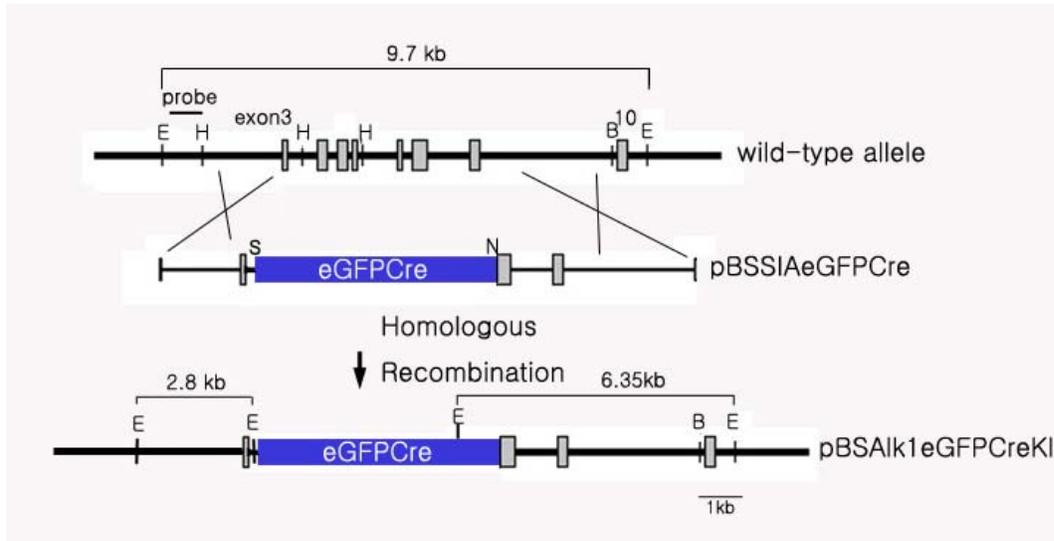


Figure 3-1. Procedures for the generation of *Alk1*^{+/GFP-Cre} construct. Constructed *Alk1*-eGFP-Cre- (*Alk1*^{+/GFP-Cre}) knock-in vector contains SV40 splice donor and acceptor signals (SD/SA), internal ribosomal entry sequence (IRES) and poly(A) signal (pA) that are sequentially subcloned into pBluescriptIISK(+), of which XhoI site was deleted, and the *eGFP-Cre* fusion gene was subcloned between IRES and pA.

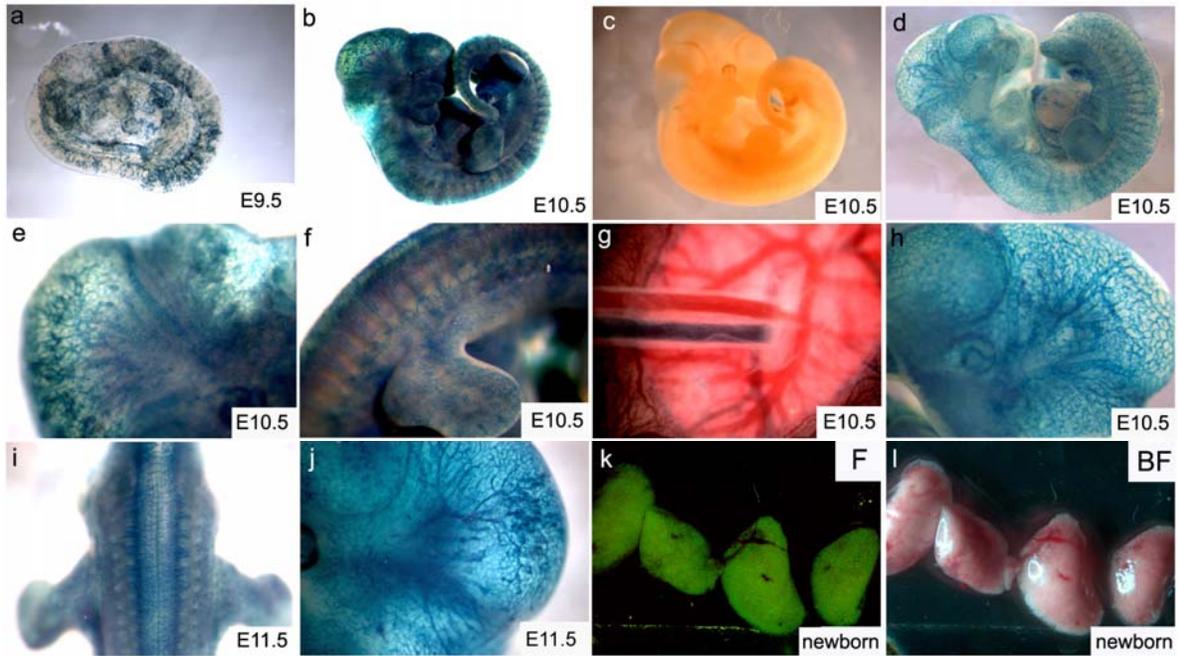


Figure 3-2. The LacZ expression in the blood vessel of $Alk1^{+/GFPCre};R26R$ embryos. (a) At E9.5, lacZ was detected in developing vasculature, but at this time expression was appeared as spotty pattern. (b) Whole mount E10.5 embryo shows positive lacZ in most embryonic vascular system. (c) Cre-negative littermate also stained for lacZ, however, no lacZ activity was detected. (d, h) LacZ expression in $Tg(Alk1GFPCre);R26R$ mice at E10.5 comparable to that of $Alk1^{+/CreGFP};R26R$. (e) At E10.5, lacZ expression was found in developing capillaries covering brain. (f) Intersomatic arteries and capillaries on neural tube and limb of E10.5 embryo show positive lacZ staining. (g) E10.5 umbilical vessel stained with X-gal. Note that lacZ was detected only in umbilical artery soon after X-gal staining. (i) Dorsal view of E11.5 embryo; lacZ-positive capillary-like vessels in the developing neural tube. (j) Higher magnification of the cephalic vascular tree at E11.5. The staining was specifically associated with blood vessels. (k and l) Fluorescent (k) and bright field image (l) of lungs of $Tg(Alk1GFPCre)$ newborn mouse.

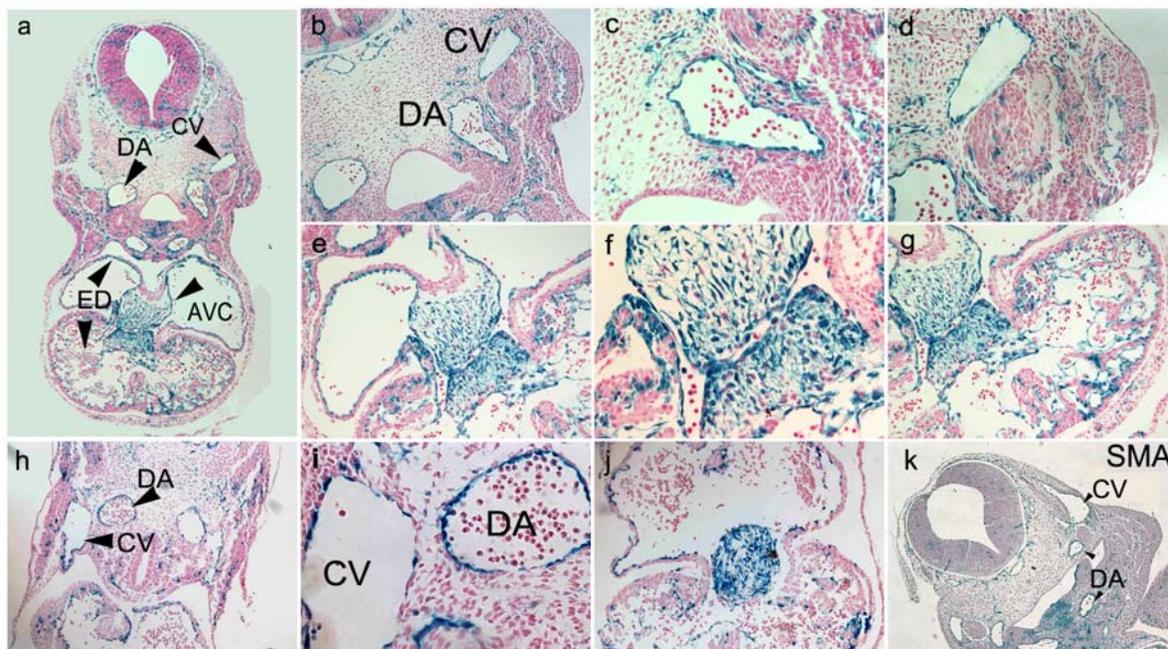


Figure 3-3. Expression pattern of lacZ during embryonic stage E10.5 in $Alk1^{+/GFP-Cre};R26R$ and $Tg(Alk1GFP-Cre);R26R$ embryos. Transverse sections of whole mount X-gal stained embryo of $Alk1^{+/GFP-Cre};R26R$;(a to g), $Tg(Alk1GFP-Cre);R26R$;(h to j). Embryo was counterstained with NFR (a to j) or immunostained with α SMA antibody (k). (a) Note that lacZ expression was detected in endothelium of dorsal aorta, cardinal vein, heart endocardium and in the AV cushions of $Alk1^{+/GFP-Cre};R26R$ embryo. (b to d) The endothelium of dorsal aorta(c) and cardinal vein(d) show positive lacZ staining. (e to g) mesenchyme of AV cushion (e and f) and endocardium (g) are obviously lacZ-positive. (h to j) In $Tg(Alk1GFP-Cre);R26R$ embryo at E10.5, the identical pattern of lacZ expression was observed in dorsal aorta, cardinal vein, endocardium and AV cushion, which were comparable to that of $Alk1^{+/CreGFP};R26R$ embryo. (k) LacZ positive cells were localized in endothelium of dorsal aorta and cardinal vein. DA; dorsal aorta CV; cardinal vein ED; endocardium AVC; atrioventricular cushion

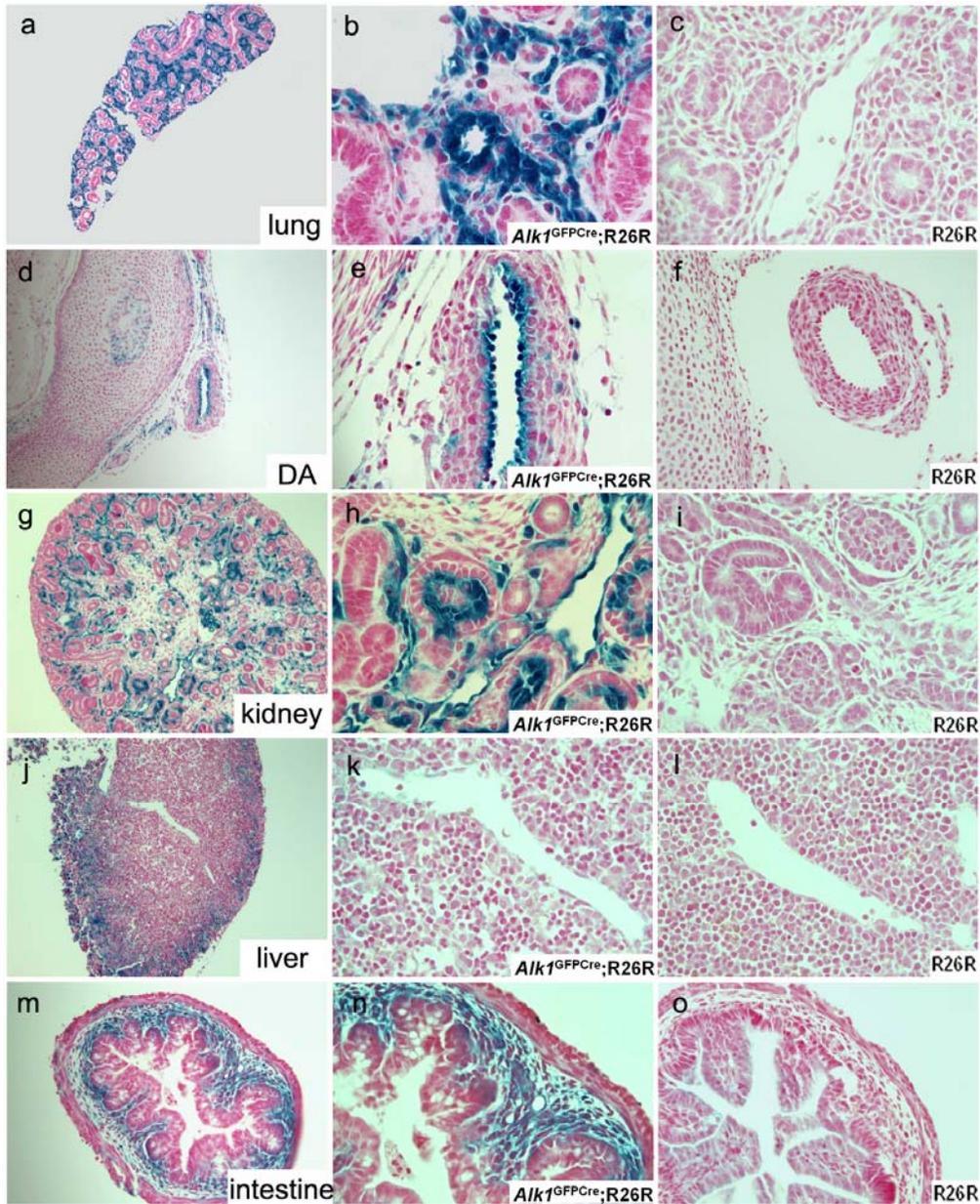


Figure 3-4. The lacZ expression of tissues derived from *Alk1*^{+GFP^{Cre}};R26R embryo at E15.5 (a and b) LacZ staining was detected in alveolar capillaries and arterial endothelium of lung while the epithelium of broncholi are devoid of staining (b) A high magnification view of LacZ stained pulmonary arteries. (d) Endothelium of dorsal aorta show positive staining. (e) Higher magnification of positively stained dorsal aorta (g) Vessels of developing kidney glomeruli show positive lacZ expression. (h) High magnification view of stained glomeruli and endothilum of kidney vessel. (j) Endothelium of liver large blood vessel show negative, lacZ staining. (k) Higher magnified liver blood vessel with devoid of lacZ staining. (m) In the submucosal layer of small intestine, lacZ expression was observed. (n) High magnification view

of mucosal layers show strong lacZ activity. (c,f, i, l, and o) No lacZ expression was shown in all tissues of control.

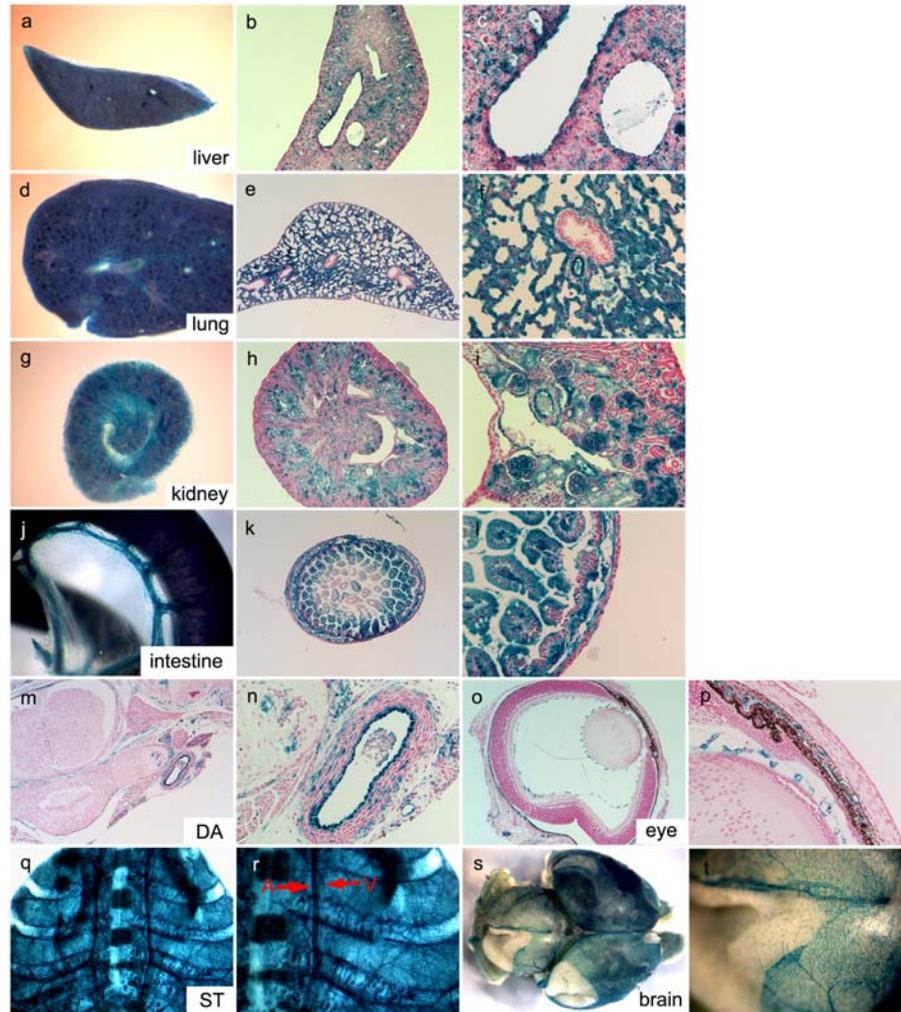


Figure 3-5. The Cre recombinase is active in $Alk1^{+/GFPCre};R26R$ newborn endothelium of blood vessel. X-gal stained newborn organs (a, d, g, j, q, and s) were then counterstained with nuclear fast red (NFR) (b, e, h, k, m, o). (a) Liver (b) Histological section of liver (c) High magnification of the liver with lacZ expression in the endothelium of large veins, in the sinusoidal endothelium with dot-like fashion and in some hematopoietic cells. (d) Lung. (e) Lung histology demonstrating lacZ-positive pulmonary endothelium. (f) Positive lacZ staining in arterial endothelium, while no staining in the epithelia of bronchiolar units. (g) Kindey. (h) Kindey histological section (i) Capillaries in glomeruli are lacZ positive (j) Mesenteric vessels in small intestine show positive lacZ staining. (k) Histological section of the intestine. (l) High magnification of intestine vessels revealed lacZ-positive capillary of submucosal area. (m) Histological section of dorsal aorta. (n) Higher magnified view showed strong lacZ expression in endothelial layer of dorsal aorta. (o) Histological section of eye (p) Eye showed capillary endothelial staining. (q) Blood vessels on the thoracic wall. (r) Magnified view of thoracic wall. Arrows indicate artery (A) showing positive staining and vein (V) showing no lacZ staining. (s) Brain. (t) Brain vasculature showed positive and specific staining in all vessels.

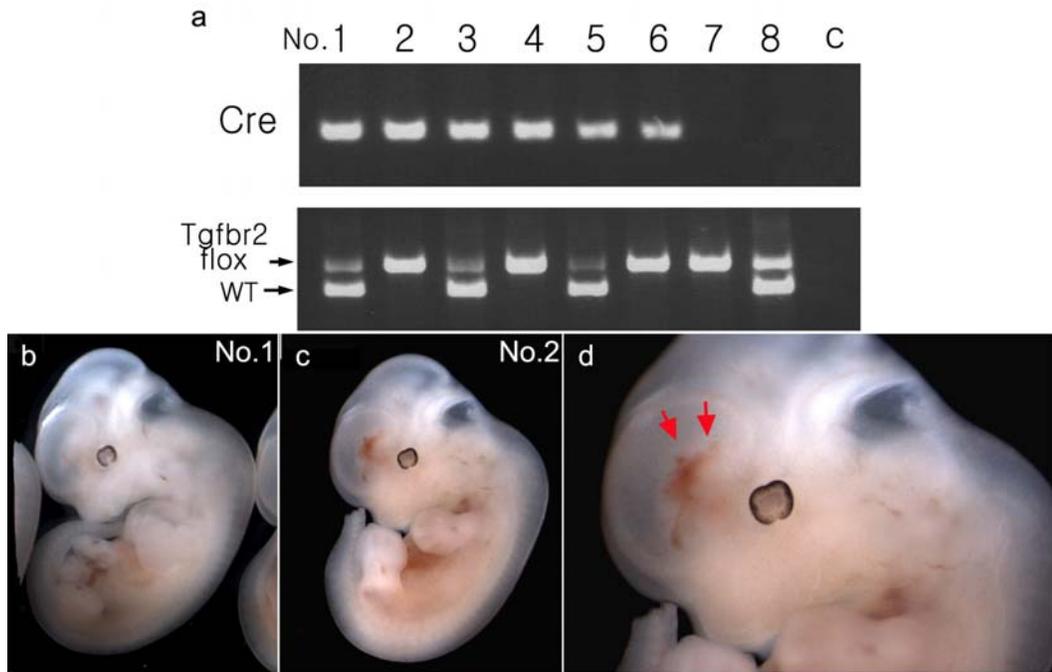


Figure3-6 Conditionl deletion of *Tgfr2* gene by *Alk1*^{+GFP}Cre line showed noticeable hemorrhage in the cranial region at E11.5. (a) The genotype of embryos from the crossing of *Alk1*^{+GFP}Cre;*Tgfr2*^{+f} mice with *Tgfr2*^{f/f} females. The resulting embryos were *Alk1*^{+GFP}Cre;*Tgfr2*^{+f}, *Alk1*^{+GFP}Cre;*Tgfr2*^{f/f}, *Tgfr2*^{+f}, *Tgfr2*^{f/f}. (b) The control embryo of *Alk1*^{+GFP}Cre;*tgfr2*^{+f} shows the absence of hemorrhage. (c) The embryos of *Alk1*^{+GFP}Cre;*Tgfr2*^{f/f} displayed a noticeable hemorrhage in the cranial region of head. (d) High magnification view of the cranial region of head in *Alk1*^{+GFP}Cre;*Tgfr2*^{f/f} embryos. Red arrow indicates the hemorrhage sign.

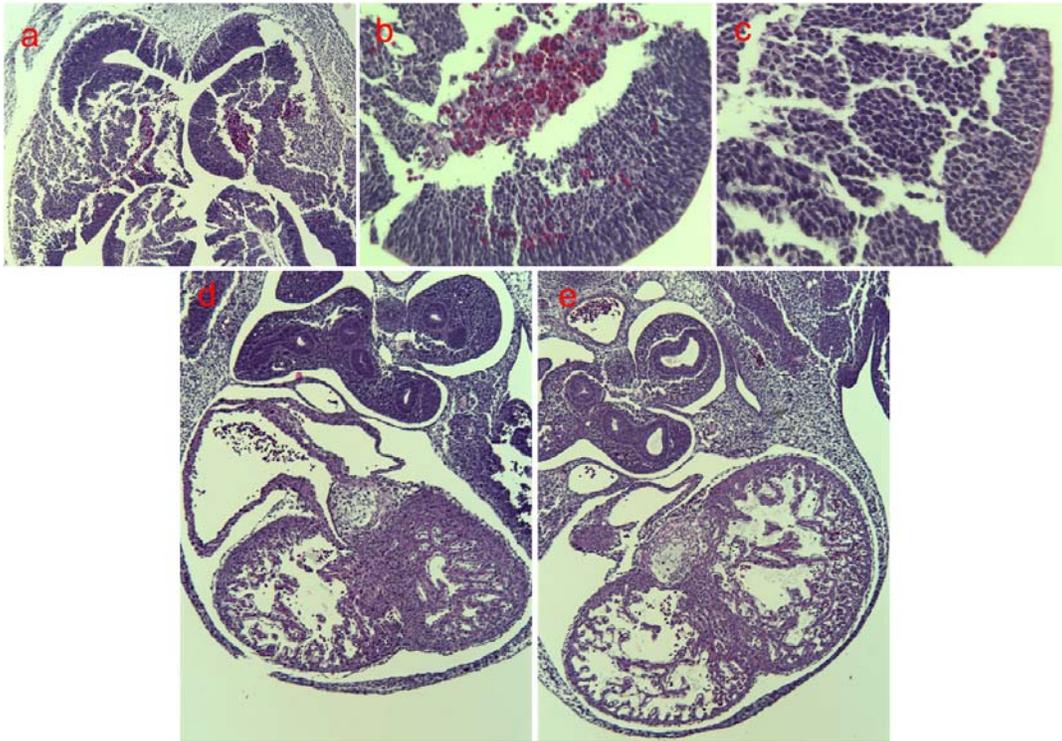


Figure3-7 Histological analysis of *Alk1*^{+/GFPCre}-mediated conditional deletion of *Tgfb2* gene in the brain and heart at E11.5.(a) The transverse section of the cranial region of head having noticeable hemorrhage in *Alk1*^{+/GFPCre};*Tgfb2*^{f/f} embryos. (b) High magnification view shows that red blood cells escaped from damaged blood vessels migrate into the epithelium layer of neural tube. (c) Control mice displayed no hemorrhage sign in the area. (d and e) The transverse section of both mutant (d) and control heart (e). The mutant heart displayed thicker ventricular septum and higher density of trabeculation in ventricles (d) compared to that of control heart (e).

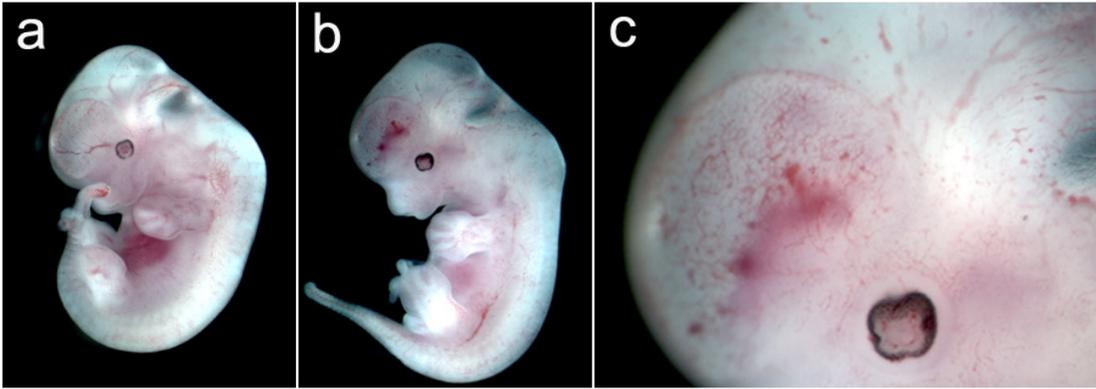


Figure 3-8 More severe but identical pattern of a hemorrhage was observed in E13.5 embryos. (a) *Tgfr2*^{f/f} control embryo displayed no hemorrhage. (b) *Alk1*^{+/GFP^{Cre}};*Tgfr2*^{f/f} embryo showed severe hemorrhage sign in the cranial region of head, consistent with that observed in E11.5. (c) High magnified view showed the hemorrhage spot.

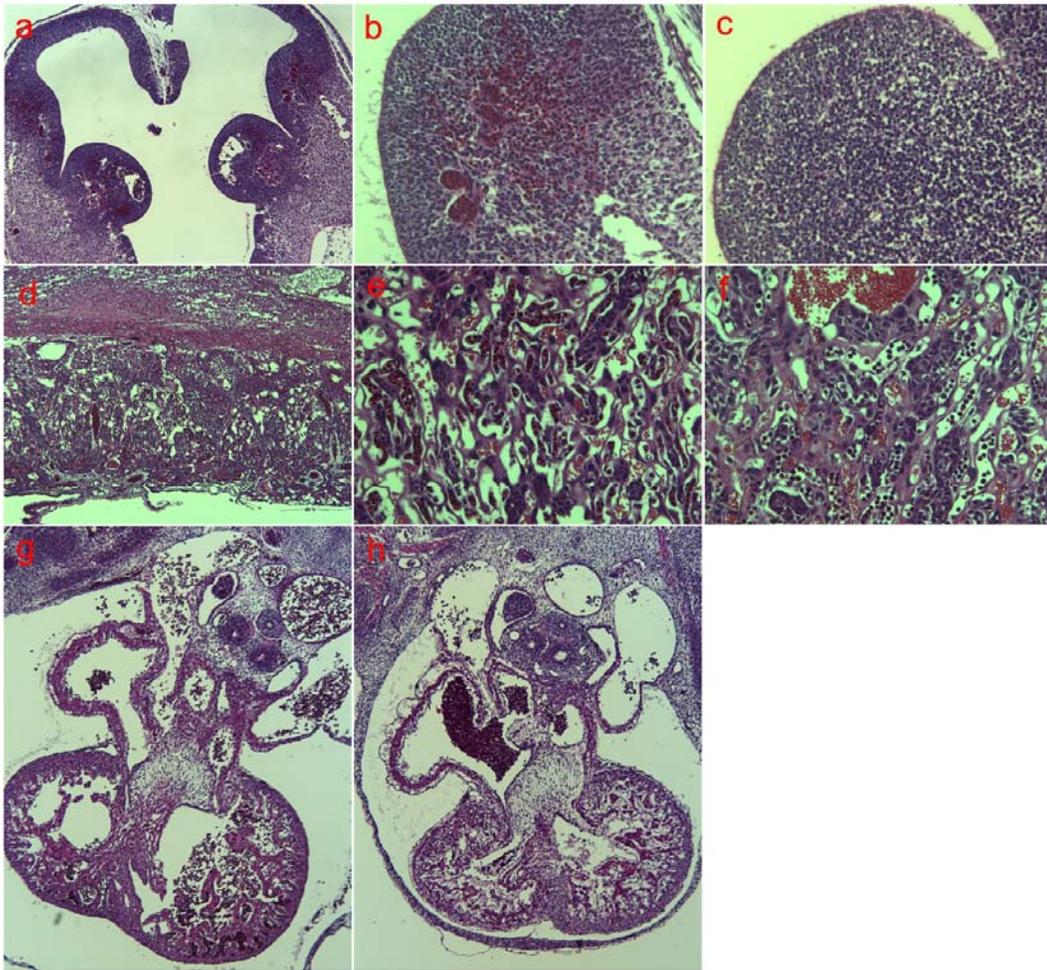


Figure 3-9. Comparison of the phenotype of brain, placenta and heart between *Alk1*^{+/^{GFP}Cre};*Tgfr*^{f/f} embryos and control one in the histological analysis. (a and b) The transverse section of head with the hemorrhage area. (a) The red blood cells were found in the epithelium layer of neural tube. (b) RBCs were observed to penetrate into the neural tube area in high magnification view. (c). The hemorrhage sign was absent in the control embryo brain. (d to f) The transverse section of the mutant placenta (d and e) and control placenta(f). There was no noticeable discrepancy observed between mutant placenta and that of control. (g and h) There was somehow difference in the density of the ventricular trabeculation between mutant heart (g) and control heart (h). Note that denser and less organized trabecular was displayed in mutant heart.

CHAPTER 4 DISCUSSION

In a previous study using a null mutant mouse line $Alkl^{+/lacZ}$ in our laboratory showed a dynamic spatiotemporal expression pattern of ALK1; This ALK1 expression is prevalent in developing arterial endothelium and tumor angiogenesis. The strongest signal is observed in preexisting arteries and newly developed arteries, during wound healing, but it's considerably reduced in adult arteries. These results demonstrated a necessary role of ALK1 in arterialization and remodeling of arteries[18]. However, exploring the complete mechanism of ALK1 function in arteriogenesis is ongoing, and we have considered various approaches to this topic. One approach was to delete ALK1 in arteries (knockout or conditional knockout), then compare ALK1-deleted ECs to normal ECs, and finally analyze their ALK1 function with microarray. To achieve this, the deletion of ALK1 can be implemented in cultured ECs, but it is not guaranteed that *in vitro* condition can reflect *in vivo* environment of ALK1 expression. A better approach might be to distinguish ALK1-expressing ECs from ALK1-nonexpressing cells *in vivo*. Establishment of mouse line directs to the purpose to label positive-ALK1 endothelium with GFP reporter gene by insertion of GFP gene in *Alkl* gene locus or by inducing GFP expression driven by *Alkl* promoter in transgenic mice, which is $Alkl^{+/GFPCre}$ or $Tg(AlklGFPCre)$ line, respectively. Once sorting of ALK1-expressing ECs are complete, we can determine distinctive gene expression profiles of ALK1-expressing ECs and identify the downstream targets of ALK1 signaling. The ultimate goal of this thesis is to elucidate molecular mechanism by which ALK1 regulates arteriogenesis. Since HHT results from a heterozygous mutation in ALK1, the verification of the mechanism that ALK1 functions in arteriogenesis would demonstrate underlying pathogenesis for HHT and to expand a range of therapeutic design for these disorders.

The first approach to this object was to generate and characterize the $Alk1^{+/GFPCre}$ mouse line, which reflected endogenous ALK1 expression by inserting the fusion gene of *GFP* and *Cre* recombinase into *Alk1* gene locus. We observed that ALK1 is mainly expressed in developing arterial endothelium, but was also expressed in venous endothelium, endocardial cells, and mesenchymal cells in the AV cushion. This was detected by the lacZ expression in endothelium of cardinal vein, endocardium lining the chamber of the heart, and the mesenchymal layer in a heart cushion as well as arterial endothelium in $Alk1^{+/GFPCre};R26R$ line. This expression pattern is different from previously defined arterial endothelium specific ALK1 expression in $Alk1^{+/lacZ}$ or Tg(*Alk1-lacZ*) line.

In fact, this distinctive result is regarded as another achievement obtained from $Alk1^{+/GFPCre}$, since this line is sufficient to compensate limitations to characterize ALK1 expression in $Alk1^{+/lacZ}$ or Tg(*Alk1-lacZ*) line. Continuous arterial endothelium specific Alk1 expression and lacZ staining observed in $Alk1^{+/lacZ}$ or Tg(*Alk1-lacZ*) line demonstrates a transcriptional activity of the *Alk1* promoter at a given time point. As lacZ is expressed by endogenous *Alk1* promoter in this line, lacZ can be undetectable, if the promoter is insufficient to visualize the intense expression of the reporter gene, even though given cells or tissues actually express ALK1. Otherwise, in the $Alk1^{+/lacZ}$ line, once a relatively high level of lacZ expression can be observed in the cells or tissues where ALK1 is expressed, it implies that ALK1 is concurrently expressed in that region. That is, lacZ expression in $Alk1^{+/lacZ}$ is detected in a quantitative manner, so that it is difficult to determine whether the endothelium in an examined tissue actually expresses ALK1 or not. In contrast, lacZ expression in $Alk1^{+/GFPCre};R26R$ mice, which is derived from crossing a *R26R* reporter line showing strong promoter activity, would constitutively show the lacZ activity, because once Cre-mediated recombination occurs and

activates lacZ expression, resulting recombined *lacZ* gene would be inherited throughout the cellular lineage. Therefore, ALK1 expression in *Alk1*^{+/*GFP*Cre} might be visualized in a relatively qualitative fashion compared to that in *Alk1*^{+/*lacZ*}. This is also why lacZ expression in an *Alk1*^{+/*GFP*Cre};R26R line cannot be considered to guarantee the concurrent Cre activity. However, due to inserted *GFP* gene expression, *Alk1*^{+/*GFP*Cre} provides an advantage to detect current ALK1 expression as well, which makes this line possible to exert more enhanced detection ability than that of *Alk1*^{+/*lacZ*} line.

As described above, for labeling ALK1-expressing ECs with *GFP* reporter gene, our laboratory also created a Tg(*Alk1GFP*Cre) as well as an *Alk1*^{+/*GFP*Cre} line. Tg(*Alk1GFP*Cre) mouse was generated from a previously characterized 9.2kb fragment of the *Alk1* gene, including a 2.7kb promoter region and the whole homologous sequence in intron 2, which precisely recapitulated the endogenous ALK1 expression. The enhancer elements in a conserved region of intron 2 of this transgenic line regulate arterial endothelium-specific expression [46]. Cre expression in this Tg(*Alk1GFP*Cre), however, may be affected by the circumferential sequence toward the regulatory element, such as a repressor for ALK1 expression in venous ECs or an activator for ALK1 expression in arterial ECs. Consequently, we need a scheme to verify the possible ectopic expression in Tg(*Alk1GFP*Cre). *Alk1*^{+/*GFP*Cre} might be a device to compensate for this limitation observed in transgenic line, since it reflects endogenous ALK1 expression. Histological analysis has shown that *Alk1*^{+/*GFP*Cre}-mediated lacZ activity was found in the endocardium, the mesenchyme of the atrioventricular (AV) cushions as well as vascular ECs (Figure 3-3), suggesting that *Alk1*^{+/*GFP*Cre}-mediated DNA recombination occurred prior to endothelial-mesenchymal transition during the AV cushion formation. The onset of Cre-mediated recombination in the ECs and the mesenchyme of AV cushions of the

Alk1^{+/-GFPCre};R26R mice was observed at E10.5. During early heart valve development, a subset of ECs which specifies the cushion-forming regions divide and enter the cardiac jelly, a gathering of connective tissue, where they subsequently proliferate and fulfill their differentiation into mesenchymal cells [47, 48]. Positive lacZ activity in *Alk1*^{+/-GFPCre};R26R line suggest that it possibly deletes a gene in the AV cushion as well as in ECs, which then conceivably causes defects in cardiac valve development. Consistent with this expectation, widely used pan-endothelial Cre line, Tg(Tie2-Cre), also deletes a gene of interest in the AV cushion as well as in the ECs. In recent studies, elimination of some genes using the Tg(Tie2-Cre) line lead to defects in the valve formation as well as slight or deteriorative phenotypes in the blood vessel [40, 41].

This study proposes the cornerstone to identify genes regulated by ALK1 signaling in ECs and in advance, to characterize downstream targets of ALK1 signaling. The lethality of *Alk1* deletion in mice implies its essential role in arteriogenesis, but exact roles are still largely unknown. To understand the function of ALK1 in arteriogenesis, it is necessary to elucidate its activities in angiogenesis, cell migration, adhesion, vasculature, cytoskeleton establishment and gene regulation. The studies related to this field were performed to determine the role of ALK1 in ECs by using a constitutively active ALK1 adenovirus to infect human umbilical vein endothelial cells (HUVEC), or the human micro-vascular endothelial cell line HMEC-1 [49-51]. HHT is a vascular disorder of impaired angiogenesis caused by mutations in elements of TGF- β signal transduction, such as ALK1, ENG and SMAD4. It implies that ALK1 signaling regulates angiogenesis linked to certain genes. Actually, several recent studies support this theory by identifying genes involved in angiogenesis and arteriogenesis. These genes include IL-8 (interleukin-8) [52], ET-1(endothelin-1) [53], ID1 (Inhibitor of DNA binding 1) [54],

HPTP η (human protein-tyrosine phosphatase η) [55], TEAD4(TEA domain family member4) [56], SMAD6/7, STAT1, SMAD1, CXCR4, Ephrin-A1 [51]. The deletion of some of these genes caused embryonic lethality in mice with defective vascular development, which shared a similar phenotype seen in HHT mice models [56]. ID proteins are helix-loop-helix proteins that stimulate endothelial cell proliferation and migration and are induced to be expressed by TGF- β 1 [48]. In interruption of both ID1 and ID3 genes, vessels were dilated and lacked branching capillaries [49], which was comparable to vascular abnormalities in ALK1-deficient mice. These data suggest that ID1-3 may play a critical role in regulating endothelial cells and possibly vascular functions as downstream mediators of ALK1 [47, 50]. Microarray analysis has enabled us to accomplish a comprehensive investigation of many responsive genes to *Alk1* in ECs. This approach can reveal transcriptional targets and their regulation by ALK1.

In addition, we presented the *Alk1*^{+/*GFPCre*} line as another useful EC-Cre line compared to other known pan-EC specific Cre lines, in which *Tgfb2* gene is conditionally deleted by *Alk1*^{+/*GFPCre*} line. *Alk1*^{+/*GFPCre*};*Tgfb2*^{f/f} embryos at E11.5 and E13.5 displayed the localized hemorrhage in the cranial region of the head on both sides. The severity appeared to be increased throughout development between E11.5 and E13.5. Other than the cranial area, there was no hemorrhage observed. The mechanism by which the hemorrhage in the specific area of the brain appears is still under investigation, but we suspect the rupture of blood vessels may cause hemorrhage as an indirect effect of the deletion of *Tgfb2* gene in vessel development or heart function, because these localized vascular defects were limited to only specific areas of the brain, not leading to systemic vascular abnormality. We will continue monitoring the phenotype of *Alk1*^{+/*GFPCre*}-mediated conditional deletion of *Tgfb2* gene at later developmental stages.

Originally, our lab established the *Alk1*^{+GFP^{Cre}} mouse line to distinguish and characterize the ALK1-expressing endothelium by labeling positive-ALK1 endothelium with *GFP* reporter gene. Once characterization of ALK1-expressing ECs is complete, we can assess distinctive gene expression profiles of ALK1-expressing ECs, which would enable us to identify the downstream targets of ALK1 signaling. Initially, we characterized the ALK1 expression pattern in *Alk1*^{+GFP^{Cre}} mice line. The cumulative developmental and biological data from ALK1-regulated genes suggest that ALK1 is part of an independent network in vascular homeostasis, which might explain the vascular defects observed in HHT patients. Many genes regulated by ALK1 may be potential candidates of study in clinical research of HHT patients. The long-term goal of this thesis is to elucidate the molecular mechanisms by which ALK1 regulates arteriogenesis. Since HHT results from a heterozygous mutation in ALK1, verification of these mechanisms would provide valuable insight to the underlying pathogenesis of HHT as well as contribute to therapeutic approaches to this vascular disease.

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BIOGRAPHICAL SKETCH

Eunji Lee graduated the Chung-Ang University in South Korea where she earned a Bachelor of Science degree in pharmacy in 2005. In 2006, she joined the Department of Physiology and Functional Genomics at the University of Florida to achieve her master's education under the mentorship of Dr. S. Paul Oh.