# Genetic and molecular pathogenesis of hereditary hemorrhagic telangiectasia

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**Abstract : Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder characterized by vascular dysplasia and hemorrhage. The pathogenesis regarding heterogeneity of vascular malformations in patients with HHT has been obscure, although it has become possible to partially explain the pathogenesis from the identification of two distinct genes, *endoglin* and *ALK-1*. Endoglin and ALK-1 are type III and type I TGF-β receptors, respectively, and are exclusively expressed on vascular endothelial cells. Binding of TGF-β to the type II TGF-β receptor on endothelial cells, which is accelerated in the presence of endoglin, phosphorylates type I TGF-β receptors, ALK-5 and ALK-1, and phosphorylated ALK-5 and ALK-1 activate the downstream proteins Smad2/3 and Smad1/5, respectively. These activated Smad proteins dissociate from the type I TGF-β receptor, bind to Smad4, and enter the nucleus to transmit TGF-β signaling by regulating transcription from specific gene promoters involved in angiogenesis. Therefore, a balance between these two signaling pathways via ALK-5 and ALK-1 plays an important role in determining the properties of endothelial cells during angiogenesis.**

**Mutations of *endoglin* and *ALK-1* genes are genetic pathogenesis of HHT type 1 and HHT type 2, respectively. To date, at least 29 and 17 different kinds of mutations in *endoglin* and *ALK-1*, respectively, have been found, including missense, nonsense, frameshift, and deletion mutations. The precise mechanisms of vascular abnormalities elicited by these mutations observed in HHT patients are still uncertain, although elucidation of the mechanism of intracellular signal transduction and the change in targeted gene expressions using mutant recombinant endoglin or ALK-1 proteins and knockout mice will enable us to understand the genetic and molecular pathogenesis of HHT and to effectively treat patients with HHT. J. Med. Invest. 47 : 81-90, 2000**

***Key words*** **:** *Hereditary hemorrhagic telangiectasia, endoglin, ALK, TGF-β, Smad*

## INTRODUCTION

Hereditary hemorrhagic telangiectasia (HHT) or Osler-Weber-Rendu (OWR) disease is an autosomal dominant disorder characterized by vascular dysplasia and hemorrhage (1, 2). Recent progress in elucidation of the pathogenesis of HHT has led to the identification of disease loci on chromosome 9q (designated *OWR1*) and 12q (*OWR2*), and at least one other HHT locus has been predicted (3) (Table 1). McAllister *et al*. explored candidate genes linked to the *OWR1* locus and investigated the *endoglin* gene, which encodes a type III TGF-β receptor. In three unrelated families with HHT, they successfully identified three different mutations (4). With regard to *OWR2*, a second locus for HHT, Johnson *et al*. have shown that the *activin receptor-like kinase 1* gene (*ALK1*), which is also a type I TGF-β receptor family protein, was included in *OWR2*, and they identified three different missense mutations in the

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Table 1. Summary of types, loci, and candidate genes for HHT

| type | locus name | chromosome | candidate gene |
|---|---|---|---|
| I | OWR1 | 9q33-34 | endoglin (type III TGF-β receptor) |
| II | OWR2 | 12q | ALK-1 (type I TGF-β receptor) |
| III | not known but independent of linkage with 9q and 12q | | |

coding sequence of the *ALK1* gene in three unrelated families with HHT (5). In addition, mutations of tyrosine kinase with Ig and EGF homology (TIE) 2 have been discovered in families with congenital venous malformation (6). However, the molecular mechanisms, including signal transduction pathways inside the cells, for the development of aberrant vascular formation (angiogenesis) by these gene abnormalities remain to be identified.

The present study reports recent growing evidence for the pathophysiological roles of endoglin and ALK-1 in the development of HHT.

## Hereditary hemorrhagic telangiectasia (HHT)

HHT generally shows a triad representing diverse vascular abnormalities in the nose, skin, lung, brain, and gastrointestinal tract ; repeated bleeding from the above regions ; and an autosomal dominant inheritance. The prevalence of the disease is reported to be from 1/2,351 to 2/100,000 (1), and the penetrance is age-dependent, being almost complete by the age of 40 years (7-10). Arteriovenous malformations in the lung, brain, and liver are frequently observed in HHT patients. In the lung, pulmonary arteriovenous malformations (PAVMs) are a common finding, occurring in approximately 20% of HHT patients and giving rise to dyspnea, cyanosis, polycythemia, hemoptysis, and hemothorax (11-14). It has also been reported that a diagnosis of HHT is plausible in 60% of patients with manifestating PAVMs. In the brain, cerebral arteriovenous malformations (CAVMs) are complicated in 0.6-5% of patients with HHT (1, 9). This heterogeneity has been explained, in part, by the identification of two distinct genes, as described above. Recent advances in diagnostic technologies using high-resolution helical CT scanning, MR imaging, or angiography should enable HHT to be diagnosed more accurately and at an earlier stage, and this would mean that the prevalence of the disease would become higher than the findings reported so far.

Histologically, arterioles in the papillary dermis in normal skin are connected to post-capillary venules through multiple capillaries. These vessels arise from larger arteioles and venules at the junction of the dermis and fat. Pathologically, during the earliest stage of the disease a single post-capillary venule becomes dilated, and during a more developed stage the post-capillary venules and those branches become markedly dilated and convoluted (1). Vascular walls of dilated post-capillary venules lack elastic fibers and have excessive layers of smooth muscle cells. Finally, during the fully developed stage the dilated post-capillary venules often connect directly to dilated arterioles (arteriovenous shunt).

## Genetic analysis of HHT

With regard to the genes responsible for HHT, Iannuzzi *et al*. performed genetic linkage analysis in families with members affected by both von Willebrand disease (vWD) and HHT using two RFLPs within the *vWF* gene (12p12) ; however, this failed to demonstrate a linkage between the *HHT* and *vWF* genes (15-17). In 1994, McDonald *et al*. demonstrated that the gene responsible for HHT was linked to 9q33-34 and this locus was designated *OWR1* (18-22). Taking advantage of these findings, McAllister *et al*. attempted to search for the *endoglin* gene, a type III TGF-β receptor that is present in this locus, and they found three different mutations in three unrelated families among the 68 families with HHT that they analyzed (4). In 1995, Johnson *et al*. and Vincent *et al*. both showed that a second locus for HHT resides at 12q (designated *OWR2*), and subsequent analysis of the *ALK-1* gene, which belongs to the type I TGF-β receptor family protein, revealed three different missense mutations in three unrelated families with HHT (23-25). At present, HHT is classified into two types according to the responsible gene ; namely, HHT type 1 for an abnormality of the *endoglin* gene and HHT type 2 for an abnormality of the *ALK-1* gene. Furthermore, Piantanida *et al*. predicted the presence of HHT type 3, of which the responsible gene is indepndent of linkage of 9q and 12q (3). Therefore, HHT is thought to be a genetically and clinically heterogeneous disorder.

## TGF-β superfamily and angiogenesis

The TGF-β superfamily consists of TGF-β isoforms (β1, β2, β3, and β5), activins, and bone morphogenetic proteins (BMPs), and possesses a variety of functions, including embryogenesis, organogenesis, morphogenesis of tissues like bone and cartilage, vasculogenesis, wound repair and angiogenesis, hematopoiesis, and immune regulation (26-28). TGF-β is secreted as an inactive latent form from cells *in vivo* and binds to smooth muscle cells or pericytes at vascular walls (29). Activation of the latent form of TGF-β to the active form takes place at urokinase-type plasminogen activator receptors on endothelial cells. Plasmin generated by digestion of plasminogen with a plasminogen activtor is prerequisite for this process (Fig. 1). The mechanisms underlying physiological angiogenesis are not yet fully understood. Nevertheless, it is thought that quiescent endothelial cells of post-capillary venules become mitogenic and chemotactic in response to growth and chemotactic factors such as VEGF that are produced in a condition of oxygen depletion or by tumor cells, leading to the formation of a new three-dimensional tube.

TGF-β and activin A are reported to inhibit the proliferation of endothelial cells and accelerate the production of extracellular matrix from endothelial cells. In addition, BMP-2 and BMP-7 suppress the proliferation of smooth muscle cells. Through these actions, the TGF-β superfamily is thought to modulate the interaction of endothelial and smooth muscle cells in the process of physiological and pathological angiogenesis (30).

## TGF-β signaling and Smad

Smad proteins play a critical role in transmitting the TGF-β superfamily signals from the cell surface to the nucleus. At present, eight kinds of Smad proteins have been cloned, and they have been subdivided into three classes according to differences in their functions : the receptor-regulated Smads (R-Smads), the common Smads (Co-Smads), and the inhibitory Smads (I-Smads) (31-33). R-Smads have two conserved domains in their amino-and carboxy-terminal regions, termed the MH (Mad homology)1 and MH2 domains, respectively, and are phosphorylated on a carboxy-terminal SSXS motif by specific type I TGF-β receptors. Co-Smads possess MH1 and MH2 domains but not an SSXS motif. I-Smads have an MH2 domain, although alignment of amino acid residues in the MH1 domain is not as conserved as that in other species and an SSXS motif is not present. Type I receptors for both TGF-β and activin activate R-Smads such as Smad2 and Smad3, whereas ALK 1 and type I receptors for BMP (ALK2, 3, and 6) activate other R-Smads, including Smad1, Smad5, and Smad8. A basic pocket that is present in R-Smads is important for the interaction with activated type I receptors. Since this basic pocket is not found in Co-Smads (Smad4), it is possible to explain the observation that Smad4 does not associate with activated type I receptors. I-Smads contain Smad6 and Smad7, and they are believed to inhibit the activation of R-Smads by binding to type I receptors activated by type II receptors. Smad7 can inhibit the signals of TGF-β, activin, and BMP, whereas Smad6 strongly sup-

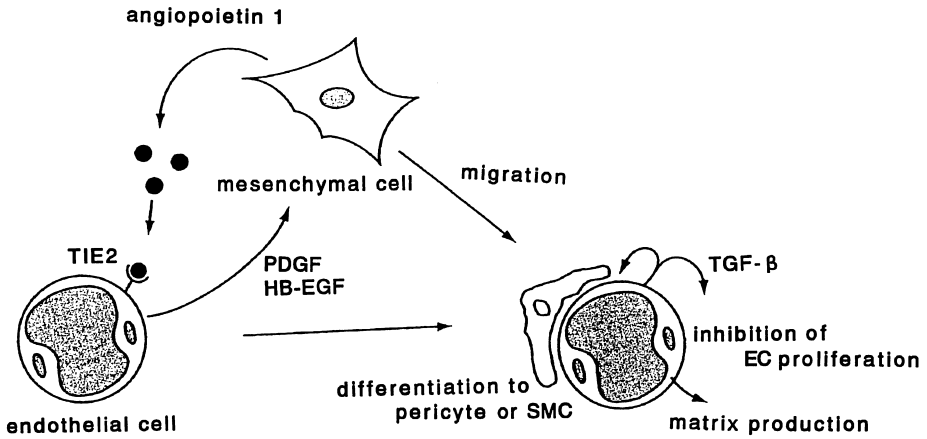


Fig.1. Process of normal angiogenesis (modified from ref. 52). Angiopoietin 1 produced by mesenchymal cells binds to TIE2 receptors on endothelial cells, whereby the secretion of migratory and growth factors such as PDGF or HB-EGF from endothelial cells are elicited, leading to the recruitment of mesenchymal cells to the place of angiogenesis. Attachment between endothelial cells and mesenchymal cells causes the activation of TGF-β. Activated TGF-β differentiates mesenchymal cells into vascular smooth muscle cells or pericytes, inhibits the growth of endothelial cells, and enhances the production of extracellular matrix.

presses the signal of BMP and weakly suppresses the signals of TGF-β and activin.

Once the receptors have been activated by the TGF-β superfamily, R-Smads are phosphorylated and dissociate from type I receptors, bind to Smad4, and enter the nucleus. In the nucleus, heteromeric complexes of Smads function as effectors of the TGF-β superfamily signaling by regulating transcriptions from specific gene promoters (33).

## Endoglin and HHT type 1

Endoglin is a homodimeric membrane glycoprotein with a molecular weight of 180 kDa and contains large extracellular, one transmembrane, and short intracellular regions (Fig. 2). Alignment of amino acid residues in the transmembrane and intracellular regions is highly conserved between endoglin and betaglycan, another type III TGF-β receptor. Expression of endoglin is restricted to endothelial cells, activated monocytes, syncytiotrophoblasts, and some leukemic cells, with the most prominent expression being in endothelial cells (34-40). Three isoforms of TGF-β (β1, β2, and β3) are produced in mammalian cells. Since TGF-β 1 and -β 3, but not -β 2, bind to endoglin with high affinity (Kd=50 pM) on human endothelial cells, it has been suggested that endoglin presents TGF-β1 and -β3 efficiently to type-I and -II TGF-β receptors that possess a serine/threonine kinase activity, leading to the transduction of TGF-β signals into the cells. However, Letamendia *et al.* have shown by transient transfection experiments that binding of TGF-β to endoglin required the presence of type II TGF-β receptors and that association of these molecules led to a decreased TGF-β response to cellular growth inhibition and plasminogen activator inhibitor-1 synthesis (41). Since this finding is entirely contrary to the present concept of TGF-β's action, further study is needed to define the precise function of endoglin. Therefore, it is of importance to identify the gene mutations in patients with HHT type 1 and to analyze the alteration of TGF-β's action using mutant recombinant endoglin molecules.

Analysis of the *endoglin* gene in patients with HHT type 1 has led to the detection of 29 different kinds of mutations (Table 2) (42). Mutations disclosed to date include 6 missenses, 6 nonsenses, 7 frameshifts, 5 gross deletions, and 5 splicing errors (42). It is most likely that these mutant endoglin proteins are not expressed on the endothelial cell surface or in reduced amounts. Alternatively, it is possible that these mutant endoglin proteins act in a dominant negative fashion against normal endoglin proteins. With regard to the latter possibility, Pece *et al.* transiently expressed 5 different mutated forms of endoglin in COS-1 cells and demonstrated that these mutant endoglin proteins did not act as dominant negative proteins (43).

We reported a missense mutation in the *endoglin* gene in a Japanese family with HHT type 1 in which $Asp^{160}$ (GAT) is substituted for $Ala^{160}$ (GCT) in the extracellular domain of endoglin protein (44). Among the mutant *endoglin* genes identified so far, this mutation is one of only five missense mutations so

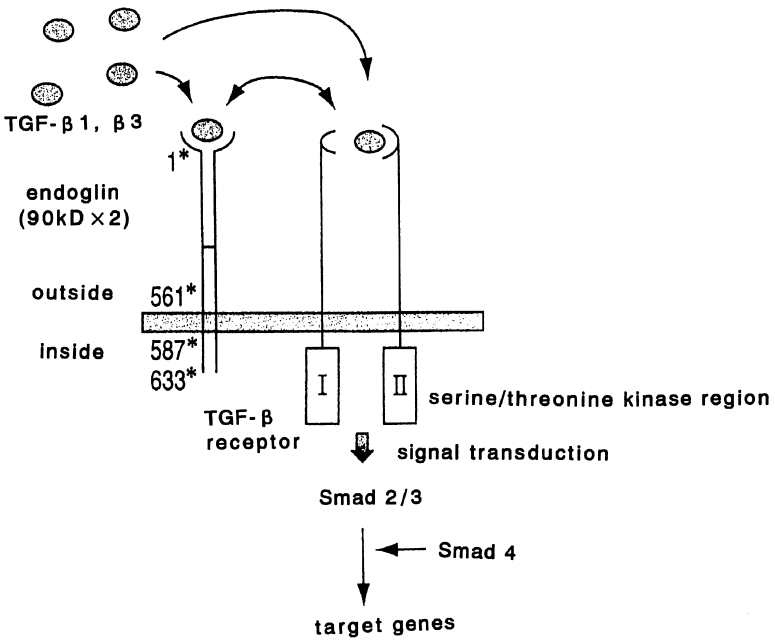


Fig. 2. Model of the interaction between endoglin and TGF-β and the TGF-β signaling in endothelial cells. TGF-β binds to the extracellular region of endoglin directly or via interaction with type II receptor. Activation of type II receptor leads to the phosphorylation of the serine/threonine kinase domain on type I receptor, resulting in the transduction of TGF-β signals inside the cells through Smad proteins (asterisks indicate amino acid residue numbers of mature endoglin).

Table 2. Mutations of the endoglin gene in patients with HHT type 1 (modified from ref. 42)

| exon | | mutation | consequence |
|---|---|---|---|
| 1 | missense | T2C | destroys start |
| 2 | | G155T | G52V |
| 2 | | T157C | C53R |
| 4 | | G447C | W149C |
| 4 | | C479A | A160N |
| 7 | | T917C | L306P |
| 4 | nonsense | C511T | R170X |
| 5 | | G587A | W196X |
| 7 | | C831G | Y277X |
| 8 | | T1050A | C350X |
| 10 | | C1414T | G472X |
| 8 | frameshift | Δ CAGA1078-81 | premature stop in exon 8 |
| 9A | | Δ G1206 | premature stop in exon 9A |
| 9A | | Δ A1267 | premature stop in exon 11 |
| 11 | | Δ AG143203 | premature stop in exon 11 |
| 11 | | Δ TG1550-1 | premature stop in exon 11 |
| 11 | | Δ GC1553-4 | premature stop in exon 11 |
| 11 | | Δ C1655 | prermature stop in exon 12 |
| 2 | gross deletions | Δ 152bp | loss of exon 2, frameshift |
| 5 | | Δ 21bp in exon 5 | loss of 7 amino acids in frame |
| 7 | | Δ 39bp in exon 7 | loss of 13 amino acids in frame |
| 8 | | Δ 15kb | loss of exon 8, frameshift |
| 9A | | Δ < 60kb | loss of exons 9A-1 |
| intron 3 | splicing errors | 5' IVS + 1 g to a | exon 3 skip |
| intron 3 | | 5' IVS + 4 a to g | exon 3 skip |
| intron 4 | | 3' IVS - 2a to g | disrupts 5' splice site |
| intron 8 | | 5' IVS + 1 g to a | exon 8 cryptic splice Δ 24bp |
| 9B | | G1311C | disrupts 3' splice site |

far reported (42), and it is expected that the level of its expression on the cell surface membrane is similar to that of normal endoglin proteins. We therefore analyzed the expression levels of this mutant protein in COS-1 cells, and found that the expression levels of the mutant protein on the cells were similar to those of normal protein (unpublished data). These findings suggest that this mutant endoglin molecule would be useful in terms of a structure and function study of endoglin protein by analyzing the change in intracellular signal transduction via Smad2/3 or Smad1/5 using MCF-7 cells that possess TGF-β type I and II receptors but lack type III receptor.

Recently, Shovlin *et al*. established cell lines from patients' peripheral lymphocytes using the Epstein-Barr virus transformation method and evaluated changes in the amount of endoglin transcripts in lymphocytes (45, 46). They found that a large deletion mutation, extending from exons 9A to 14, produced no endoglin transcripts in lymphocytes, while a nonsense mutation in exon 4, by which a short truncated endoglin may be synthesized, did not produce a stable endoglin transcript in lymphocytes. These findings indicated the possibility that a region extending from exons 4 to 9A contributes to the stability of endoglin transcript. Since all mutations that reduce the stability or block the production of endoglin mRNA can cause HHT type 1, a haplo-insufficiency mechanism is proposed for the pathogenesis of HHT.

More recently, Li *et al*. developed knockout mice lacking endoglin and investigated vascular abnormalities (47). They found that mice lacking endoglin died from defective vascular development by gestational day 11.5 and that they exhibited poor vascular smooth muscle development and arrested endothelial remodeling. However, in contrast to mice lacking TGF-β,

vasculogenesis was unaffected. Bourdeau *et al*. also generated endoglin-deficient mice and found that endoglin is critical for both angiogenesis and heart valve formation (48). These findings clearly demonstrated that endoglin was essential for angiogenesis and that aberrant endoglin could elicit the phenotype of HHT type 1.

## ALK1 and HHT type 2

At present, seven type I TGF-β receptors have been identified and designated as ALK-1 to -7 (49). ALK-1 is capable for binding to TGF-β1 or activins in the presence of either type II TGF-β receptor or type II activin receptor, respectively. ALK-1, like endoglin, is exclusively expressed on endothelial cells. However, ALK-1 does not elicit a specific transcriptional response, implying that ALK-1 may be an orphan receptor (50, 51). Mutations of the *ALK-1* gene in human HHT type 2 patients suggest that ALK-1 may play an important role during vascular development. With regard to this issue, Oh *et al*. demonstrated that a TGF-β1 signal can be mediated by two distinct type I TGF-β receptors, ALK-1 and ALK-5, and that a balance between these two signaling pathways plays an important role in determining the properties of the endothelial cells during angiogenesis (49).

There have been 17 different mutations of the *ALK-1* gene so far identified in patients with HHT type 2 (Table 3) (42). Amino acid residues encoded by exon 3 are located in the extracellular region and are extremely conserved among species, suggesting that this domain is crucial for expressing the authentic function of the ALK-1 protein. Therefore, missense mutations in exon 3 are likely to disrupt the action of ALK-1 protein. Mutations in exon 4 are either nonsense or frameshift mutations. Since exons 6 to 9 encode the intracellular kinase domain, mutations in these regions can decrease the kinase activity. In sequencing analysis of reverse-transcribed cDNA from peripheral lymphocytes of patients with missense and frameshift mutations in exon 7, only wild-type cDNA was detected, suggesting reduced transcriptional efficacy or instability of mutant mRNA.

## DISCUSSION

The most intriguing question is how gene abnormalities of *endoglin* and *ALK-1* result in an identical phenotype by a dominant inherited pattern. It was reported that there was an approximately 100-fold expression ratio of endoglin to type II TGF-β receptor on the surface of normal endothelial cells and that both inhibition of cell proliferation and enhancement of fibronectin production by TGF-β action were not influenced by a 50% decrease in endoglin expression *in vitro* (43). However, since TGF-β functions in a variety of aspects on angiogenesis, it is certain that some of the responses participating in the process of angiogenesis in patients with HHT are altered.

Table 3. Mutations of the ALK-1 gene in patients with HHT type 2 (modified from ref. 42)

| exon | | mutation | consequence |
|---|---|---|---|
| 3 | missense | G150T | W50C |
| 3 | | G152A | C51Y |
| 3 | | G200A | R67Q |
| 3 | | C231G | C77W |
| 3 | | A286G | N96D |
| 7 | | G998T | S331 W |
| 8 | | G1120T | R374W |
| 8 | | T1126G | M376R |
| 8 | | G1232A | R411Q |
| 9 | | C1207A | P424T |
| 4 | nonsense | G423A | W140X |
| 4 | | G475T | E159X |
| 7 | | C924A | C308X |
| 4 | frameshift | Δ G400 | premature stop in exon 4 |
| 4 | | Δ GGTG406-9 | premature stop in exon 4 |
| 6 | | Δ TCC694-6 | deleted serine in frame |
| 7 | | Ins. T865 | premature stop in exon 7 |

During angiogenesis, endothelial cells are in either the activation phase or resolution phase. Endothelial cells during the activation phase degrade the perivascular basement membrane, invade and migrate into the extracellular space, proliferate, and form capillary lumen. During the resolution phase, endothelial cells cease these actions and reconstitute basement membrane. Activation of ALK-1 and ALK-5 transforms endothelial cells into the resolution phase and activation phase, respectively (49). In patients with HHT type I, endoglin mutation disrupts both the ALK-1 and ALK-5 pathways, while only the ALK-1 pathway is disrupted in patients with HHT type 2 (Fig. 3). Therefore, the ALK-5 pathway becomes dominant in patients with HHT type 2, which would lead to an increase in endothelial cells during the activation phase and result in arteriovenous connections between dilated venules and arteriols. However, in patients with HHT type 1 a significant decrease in the TGF-β concentration by endoglin mutation blocks signaling through both ALK-1 and ALK-5. Therefore, if the ALK-5 pathway is not affected due to its higher sensitivity to TGF-β action than that of the ALK-1 pathway, it may be possible to explain why an identical phenotype elicited by mutations of these two genes by a dominant inherited pattern.

Even in knockout mice of the *endoglin* gene, a haplo-insufficiency mechanism for the pathogenesis of HHT has been confirmed (48). Indeed, when 129/Ola $End^{+/-}$ mice were analyzed, clinical signs such as nosebleeds and telangiectases on the ears were observed in half of the mice over 3-9 months. The absence of clinical signs in the remaining half of 129/Ola $End^{+/-}$ mice and variation in age of onset resembled the human disease, suggesting that epigenetic factors such as environment, blood pressure, oxygenation, shear forces, and hormonal levels must influence clinical signs. Therefore, 129/Ola $End^{+/-}$ mice will be useful for clarifying the molecular pathogenesis of HHT.

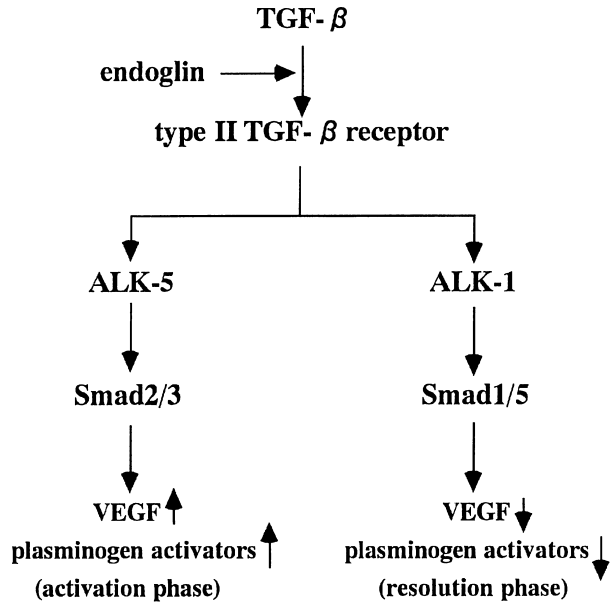


Fig.3. The balance model for TGF-β signaling in regulation of angiogenesis (modified from ref. 49). The TGF-β signal mediated by ALK-5 induces endothelial cells into the activation phase where angiogenic factors and proteinases are produced. While, TGF-β signaling through ALK-1 induces endothelial cells into the resolution phase where angiogenic factors and proteinases are suppressed. The balance between ALK-5 and ALK-1 signalings determines the properties of endothelial cells during angiogenesis.

## CONCLUSION

At present, it is not easy to differentially diagnose the type of HHT accurately on the basis of clinical signs, laboratory findings, or inheritance patterns. However, findings obtained from analysis of the genes in *endoglin* and *ALK-1* and from examination of the functional differences between wild and mutant proteins via activated Smads should enable precise differential diagnosis and typing of HHT.

## ACKNOWLEDGEMENTS

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