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Deregulation of Drosha in the pathogenesis of hereditary hemorrhagic telangiectasia

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Purpose of review

The TGF β (transforming growth factor β) superfamily – a large group of structurally related and evolutionarily conserved proteins – profoundly shapes and organizes the vasculature during normal development and adult homeostasis. Mutations inactivating several of its ligands, receptors, or signal transducers set off hereditary hemorrhagic telangiectasia (HHT), a disorder that causes capillary networks to form incorrectly. Drosha, an essential microRNA-processing enzyme, also interfaces with TGF β signal transducers, but its involvement in vascular conditions had not been tested until recently. This review summarizes current evidence that links mutations of *Drosha* to HHT.

Recent findings

Genetic studies have revealed that rare missense mutations in the *Drosha* gene occur more commonly among HHT patients than in healthy people. Molecular analyses also indicated that Drosha enzymes with HHT-associated mutations generate microRNAs less efficiently than their wild-type counterpart when stimulated by TGF β ligands. In zebrafish or mouse, mutant Drosha proteins cause the formation of dilated, leaky blood vessels deprived of capillaries, similar to those typically found in patients with HHT.

Summary

Recent evidence suggests that Drosha-mediated microRNA biogenesis contributes significantly to the control of vascular development and homeostasis by TGF β . Loss or reduction of *Drosha* function may predispose carriers to HHT and possibly other vascular diseases.

Keywords

bone morphogenetic protein, Drosha, hereditary hemorrhagic telangiectasia, microRNA, transforming growth factor β

INTRODUCTION

Previous review articles on Drosha have focused on its roles in microRNA biosynthesis [1,2], cancer [3], or the TGF β signaling pathway [4–6]. This review summarizes evidence that supports a role of Drosha in vascular biology, especially as an effector of non-canonical signaling by the TGF β superfamily in endothelial cells.

SIGNALING PATHWAYS INSTRUCT VASCULAR DEVELOPMENT

Members of the TGF β superfamily of cytokines deeply shape blood vessel formation, similarly to vascular endothelial growth factors, ephrins, fibroblast growth factors, Notch, and angiopoietins, among others [7^{***}]. At the molecular level, TGF β s and the related bone morphogenetic proteins (BMPs) bind to a receptor complex composed of type I and a type II receptors, both serine/threonine kinases (Fig. 1). Upon ligand binding, the type II

receptor phosphorylates and activates the type I receptor kinase, which then phosphorylates a set of receptor-specific signal transducers, the R-Smads. Once stimulated, R-Smads interact with the common transducer Smad4, translocate to the nucleus, and bind the promoter region of target genes to modulate transcription [8] (Fig. 1). Alternatively, activated R-Smads associate with the microprocessor complex – a multisubunit structure containing the Drosha enzyme – and promote the synthesis of

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KEY POINTS

- More than 95% of HHT patients carry mutations in proteins involved in the signal transduction of the TGF β superfamily of cytokines.
- The activity of Drosha, an essential enzyme in microRNA biogenesis, is regulated by the TGF β pathway.
- Missense mutations of Drosha that are rare in control individuals are present in HHT patients with no known mutations.
- Drosha variants fail to regulate microRNA synthesis in response to TGF β and produce vascular defects similar to HHT *in vivo*.

microRNAs, which inhibit the expression of specific messenger RNAs [9]. In summary, TGF β and BMPs control gene expression both transcriptionally and, via microRNAs, posttranscriptionally (Fig. 1).

FUNCTIONS OF THE DROSHA MICROPROCESSOR

Though just 22 nucleotides (nt)-long, microRNAs can destabilize or inhibit the translation of messenger RNAs by pairing to partially complementary sequences in their 3'-untranslated regions [1,2] (Fig. 2). Biogenesis of a typical microRNA begins with RNA polymerase II (RNA Pol II) transcribing its gene into a long primary transcript (*pri-miRNA*) that contains a stem-loop hairpin structure [1,2] (Fig. 2). To become a mature microRNA, the *pri-miRNA* first undergoes a processing step by the microprocessor complex. Key components of this structure are the ribonuclease (RNase) III enzyme Drosha and its cofactor DiGeorge syndrome critical region gene 8 (*DGCR8*, also called *Pasha*). The microprocessor recognizes the stem-loop structure in the *pri-miRNA*, cleaves it, and releases a 60-to-70-nt-long, hairpin-shaped precursor named *pre-miRNA* (Fig. 2). In the second step of microRNA processing, the *pre-miRNA* exits the nucleus and meets the RNase III Dicer and its cofactor transactivation-responsive RNA-binding protein (TRBP, also known as TARBP2), which then cut it to yield the mature ~22-nt-long *microRNA duplex* in the cytoplasm [1,2] (Fig. 2). The microRNA duplex is loaded onto the Argonaute (Ago) protein to form a RNA-induced silencing complex (RISC) capable to elicit translational repression, mRNA de-adenylation, or mRNA decay through base pairing of the microRNA with the 3'-untranslated region of the target mRNAs [1,2] (Fig. 2). The biogenesis of almost all microRNAs

requires Drosha, besides a few exceptions contained within the introns of protein-coding messenger RNAs [10–12]. In addition to its microRNA-processing function, Drosha can also associate with a hairpin structure inside target messenger RNAs to directly silence their expression [13,14]; or can bind RNA Pol II at the transcription start site of microRNA genes (and a few protein-coding genes) to modulate their transcription [15,16].

MUTATIONS IN DROSHA ASSOCIATE WITH CANCER

Heterozygous mutations in the *Drosha* gene occur in Wilms tumor, a childhood cancer that starts in the kidneys (Fig. 3) [17–22,23^{*}]. More than 60% of *Drosha* mutations in Wilms tumors consist of a single amino acid change in the second RNase domain, from glutamic acid to lysine at position 1147 (E1147K) [17–22,23^{*}] (Fig. 3). Mutant Drosha proteins display reduced metal binding and can dominantly suppress the RNase activity of wild-type Drosha [17–22,23^{*}], which explains why Wilms tumor cells with heterozygous E1147K *Drosha* mutations express fewer microRNAs [17–22,23^{*}]. Some other less frequent *Drosha* variants found in Wilms tumors map to the proline (P)-rich and arginine/serine (R/S)-rich domains, where mutations in some patients with HHT can also be found (more on these later). In this context, the change of Arg 279 into Cys (R279C) holds particular interest as the same amino acid can be found mutated to Leu (R279L) in HHT (Fig. 3).

Although the association of Drosha variants with cancer appears solid, many questions persist, such as the reason why *Drosha* mutations only affect certain human tissues, or how and to what extent they cause Wilms tumors.

GENETICS OF HEREDITARY HEMORRHAGIC TELANGIECTASIA

As an autosomal dominant vascular disease, HHT (also known as Rendu–Osler–Weber syndrome; MIM600376) affects approximately 1 in 5000–8000 people [24]. Patients with HHT suffer recurrent epistaxis (nosebleeds), telangiectasias (dilated blood vessels), and arteriovenous malformations (or AVMs, improper connections between arteries and veins without capillaries) [25^{*}]. All the vascular lesions of HHT stem from abnormalities in how endothelial cells organize and function [24]. Telangiectasias emerge on the lips, hands, and mucosa of the nose and gastrointestinal tract, whereas AVMs occur in the lung (in 40–50% of patients), liver (75%), brain (~10%), and spine (~1%). Although

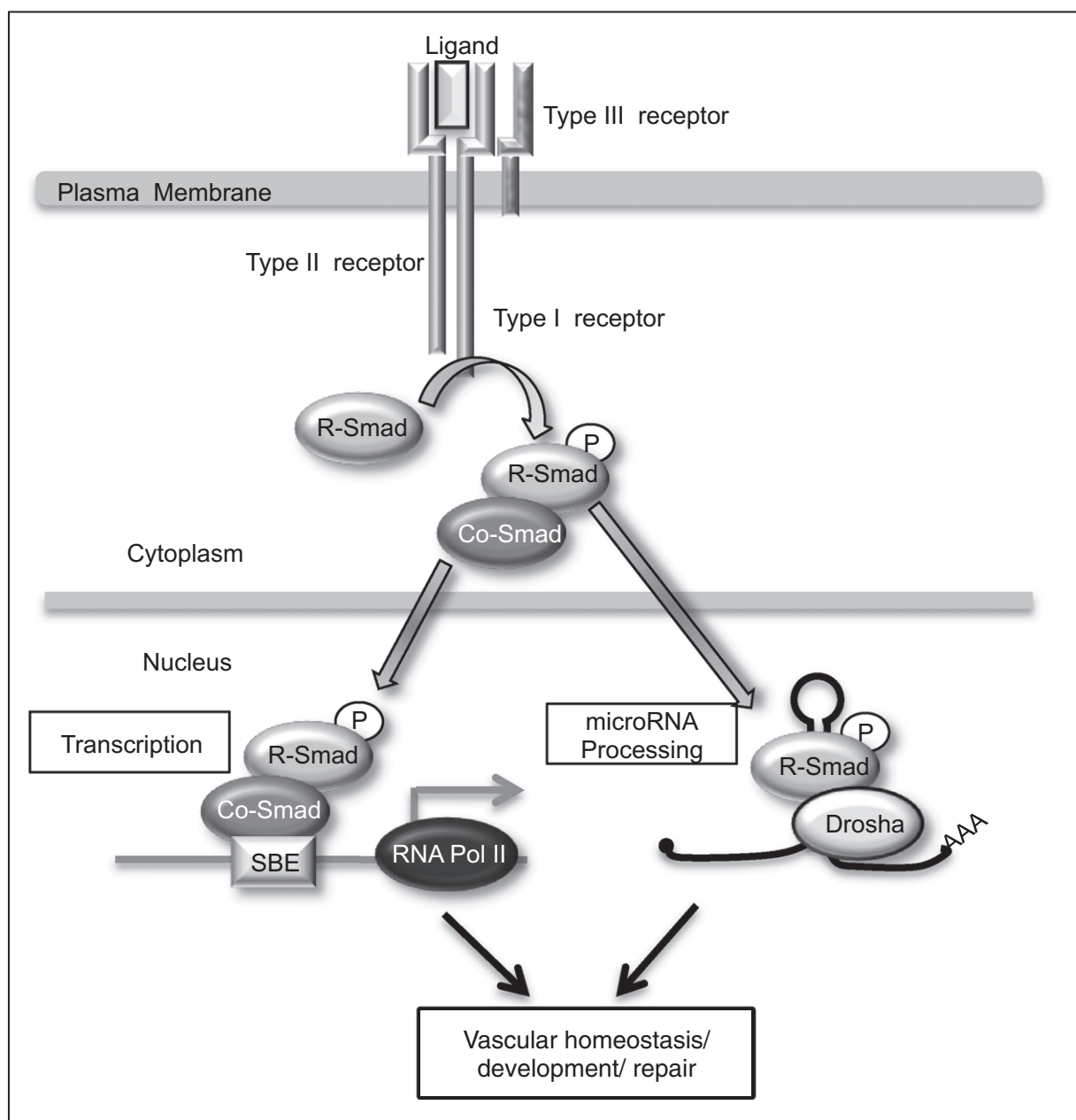


FIGURE 1. Transforming growth factor β and bone morphogenetic protein signaling pathway and Drosha. Ligands of the TGF β superfamily, including TGF β s and BMPs, bind to multisubunit complexes of type I, type II, and type III receptors. After the type III receptors attract them to the cell membrane, two pairs of type I and type II receptors form a heterotetrameric complex with the ligands, initiating intracellular signaling. The serine/threonine kinase domain of the type I receptor phosphorylates receptor-regulated Smad (R-Smad) proteins, which bind Smad4, translocate to the nucleus, and regulate gene expression by two alternative mechanisms: modulating transcription of target genes via binding to a consensus sequence known as Smad-binding element (SBE), or facilitating microRNA processing by Drosha and increasing the production of selected microRNAs.

rarely, AVMs can rupture and cause sudden death in HHT patients [24].

Genetic evidence indicates that when the TGF β or BMP pathways malfunction in vascular endothelial cells, HHT ensues. This paradigm finds support in the discovery of HHT patients with heterozygous loss-of-function and loss-of-expression mutations in

several genes encoding components of the TGF β /BMP signaling pathway: the ligand *BMP9* (also known as *GDF2*); the receptors endoglin (*Eng*) and activin A type II-like 1 (*ACVRL1*); and the signal transducer *Smad4* [24] (Fig. 4). Despite the evident correlation between TGF β /BMP pathway genes and HHT, some of the genetic triggers of HHT still elude

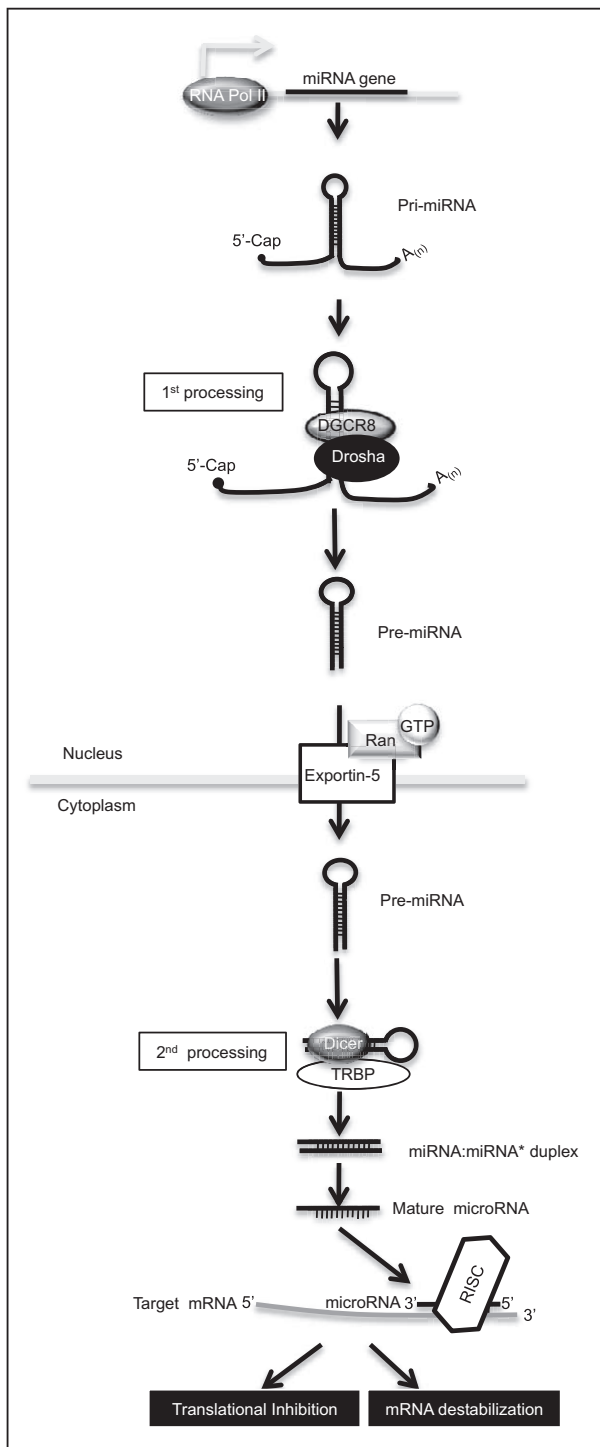


FIGURE 2. MicroRNA biogenesis pathway. MicroRNA (miRNA) biogenesis undergoes at least three steps: transcription of a long primary transcript (pri-miRNA) and production of a precursor (premiRNA) occur in the nucleus, whereas the mature microRNA duplex forms in the cytoplasm. MicroRNA genes are transcribed by RNA polymerase II (RNA Pol II) into pri-miRNAs with a 5'-methylguanosine cap and a 3'-polyadenylated tail. Pri-miRNAs are first processed by the RNase III Drosha and the cofactor DGCR8 in the nucleus, yielding premiRNAs with a

discovery. Approximately 96% of HHT patients carry *Eng* or *ACVRL1* mutations, and about 1% each harbor *Smad4* or *BMP9* mutations, but identifying the cause of the residual 1–2% of cases remains a high priority to ensure early diagnosis and proper monitoring of all HHT patients.

As cell membrane receptors mostly restricted to vascular endothelial cells, both *Eng* and *ACVRL1* can bind *BMP9* molecules coursing in the bloodstream [26,27]. Their expression matches the pattern expected for causative agents of an endothelial dysfunction, such as HHT, with minimum impact on other tissues. Less direct appears the link between *Smad4* – a ubiquitously expressed protein – and HHT. Indeed, individuals with *Smad4* mutations often develop other conditions in addition to HHT, such as juvenile polyposis (the presence of benign polyps in the colon), confirming the importance of *Smad4* also elsewhere in the body [24].

Although we cannot completely explain how changes in signaling by the TGFβ superfamily cause HHT, we can predict that other proteins involved in TGFβ and BMP signaling need to function properly to prevent HHT.

MUTANT ALLELES OF DROSHA ARE ENRICHED IN HEMORRHAGIC TELANGIECTASIA PATIENTS

In the first study to associate Drosha variants to HHT, Bayrak-Toydemir’s group sequenced the exome of 23 affected individuals from 9 families with HHT, and of 75 probands who lacked mutations in known HHT-associated genes (*Eng*, *ACVRL1*, *Smad4*, or *BMP9*). Three nonsynonymous substitutions (P32L, P100L and K226E) occurred in the *Drosha* gene of approximately 7% of HHT patients, whereas only 0.04% of the individuals in the control population carried these mutations (Table 1 and Fig. 3) [28**]. In addition to its presence in probands, P100L also appeared in four HHT patients from a single family (Table 1) [28**]. All the patients carrying *Drosha* mutations (with the exception of P1, who only presented pulmonary AVMs) met the

hairpin-loop structure. PremiRNAs are shuttled by the Exportin-5/Ran-GTP transporter to the cytoplasm, where another RNase III, Dicer, and the cofactor TRBP catalyze the second processing and produce miRNA/miRNA* duplexes. In most cases, only one of the two miRNA strands is incorporated into the RNA-induced silencing complex (RISC), binds to a partially complementary sequence in the 3'-untranslated region of the mRNA targets, and inhibits their expression either by translational repression or mRNA destabilization.

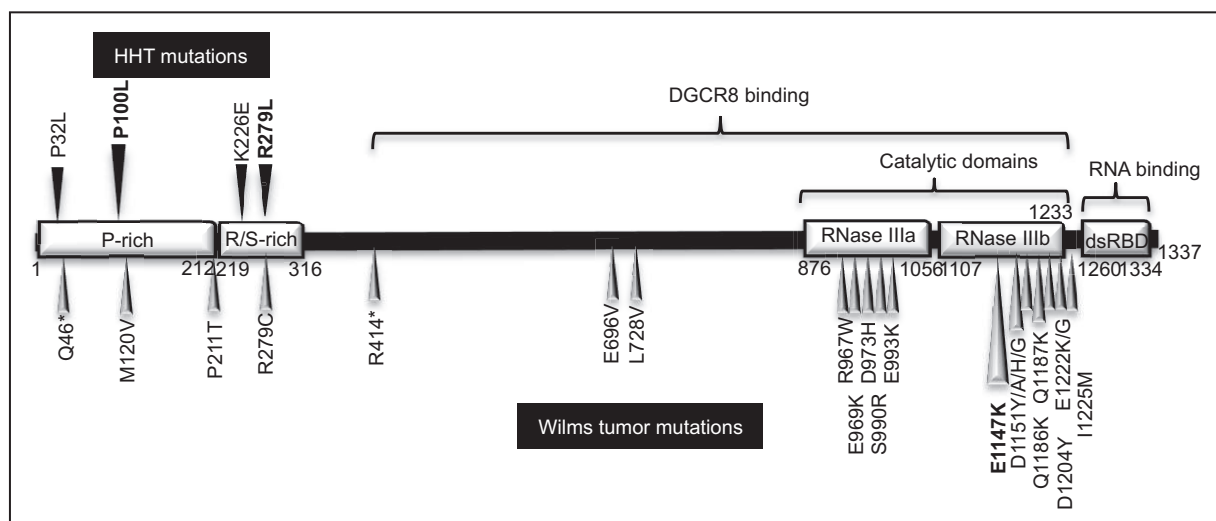


FIGURE 3. Drosha mutations associated with hereditary hemorrhagic telangiectasia and Wilms tumor. The relative locations and the types of amino acid substitution in Drosha identified in HHT (top) and Wilms tumors (bottom) are shown. Asterisks indicate nonsense mutations. HHT, hereditary hemorrhagic telangiectasia; P-rich, proline-rich domain; R/S-rich, arginine/serine-rich domain; dsRBD, double-stranded RNA-binding domain; RNase III, ribonuclease III.

diagnostic criteria of HHT – including recurrent epistaxis and vascular abnormalities (Table 1) – with features indistinguishable from those of patients with *Eng*, *ACVRL1*, *Smad4*, or *BMP9* mutations [29]. This finding established a novel association between Drosha variants and HHT, and confirmed the likelihood that proteins engaged in TGF β and BMP signaling, such as Drosha, may subtend HHT.

In one of the HHT family studied (family 2), a *Drosha* missense mutation in the R/S-rich domain (R279L) was present together with a splice site mutation in *Eng* (c.1311+1G>A) (Table 1). Mosaicism of the *Eng* alleles, previously reported also in HHT patients with *ACVRL1* mutations [30–32], characterized this affected individual in family 2 (F2-I-2). In addition to the severe epistaxis and multiple pulmonary AVMs affecting patients in family 2 (F2-I-2 and F2-II-1, Table 1), the individual with the R279L *Drosha* variant (F2-I-2) also exhibited hepatic lesions. Therefore, *Drosha* alleles, such as R279L, could exacerbate the clinical presentation of HHT in patients with an *Eng* mutation.

EFFECT OF THE HEMORRHAGIC TELANGIECTASIA-ASSOCIATED DROSHA VARIANTS

We and our colleagues sought to investigate the role of the genetically identified Drosha variants in promoting HHT.

Although mouse wild-type Drosha mRNA could rescue the abnormal vascular development of zebrafish in which Morpholino oligonucleotides had

silenced the endogenous gene (*Drosha* morphants), the P100L and R279L variant mRNAs could not. Thus, these two mutations seemed to impair a function of Drosha that is essential to the vasculogenesis of vertebrates, including zebrafish [28^{***}].

When we compared the wild-type Drosha to the P100L or R279L alleles in response to BMP stimulation of mouse embryonic fibroblasts, we detected a reduction of microRNA processing only in cells expressing the Drosha variants [28^{***}]. Notably, this decline concerned only microRNAs regulated by BMP signaling, such as miR-21, whereas other microRNAs did not demonstrate significant differences among cells expressing P100L, R279L, or wild-type Drosha [28^{***}]. Thus, the P100L and R279L alleles seemed to selectively hinder BMP-regulated pri-miRNA processing.

All the variants identified in HHT map to two evolutionarily conserved domains located at the Drosha amino-terminus: the P-rich and the R/S-rich domains (Fig. 3). Unlike the carboxyl-terminus, which contains the domains essential for Drosha's processing activity (such as two RNase III domains and the double-stranded RNA-binding domain), the amino-terminus plays a role hitherto unknown in Drosha's biochemistry (Fig. 3). We propose that the binding of R-Smads (the BMP signal transducers) to the Drosha amino-terminus can explain the confinement of HHT mutations to this region [28^{***}]. Although mutations in the amino-terminus of Drosha would predominantly disrupt its interaction with R-Smads – and thus the modulation of microRNA processing by BMP signals – without severely

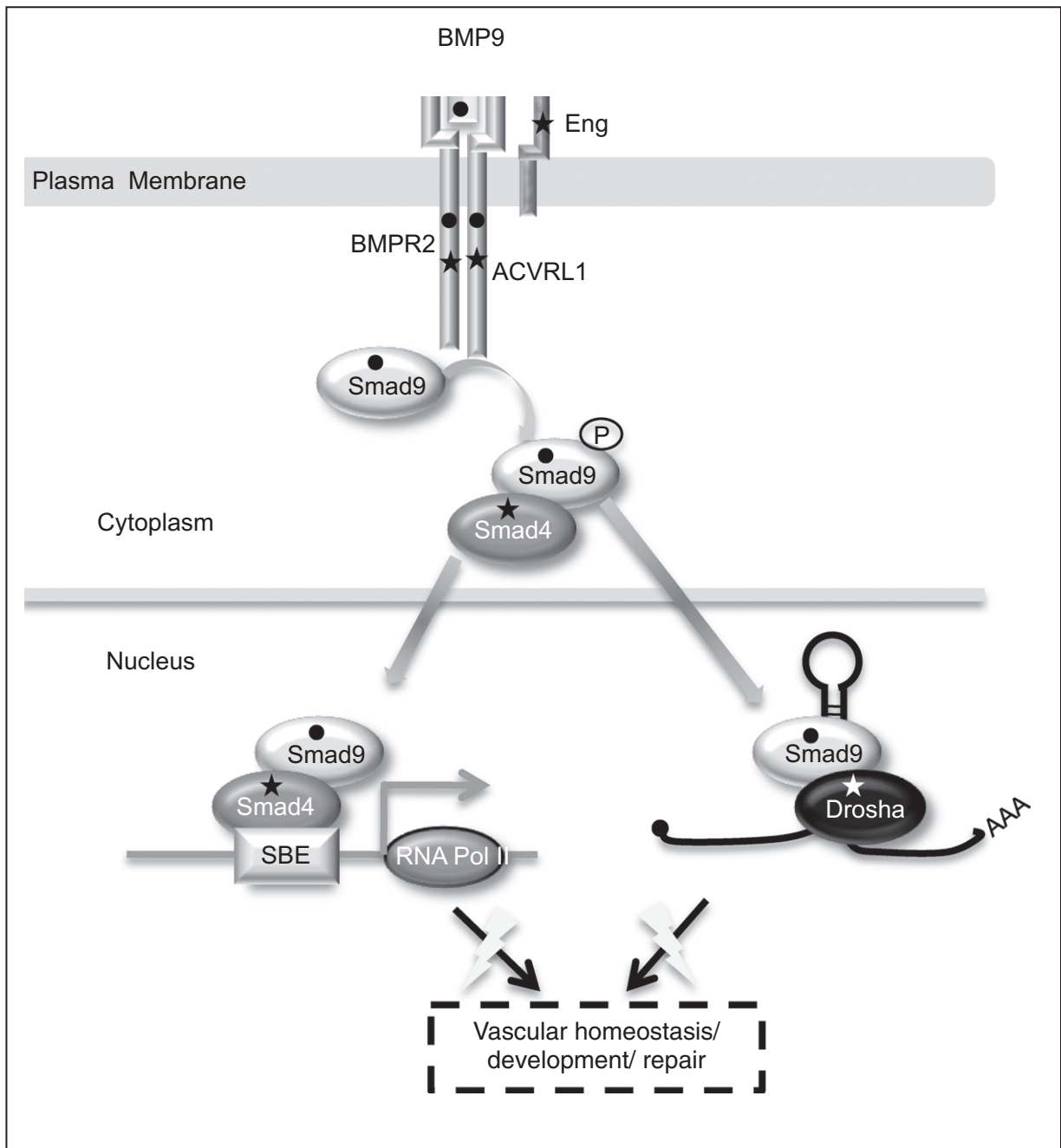


FIGURE 4. Mutations in the bone morphogenetic protein-signaling pathway are linked to hereditary hemorrhagic telangiectasia and pulmonary artery hypertension. Loss-of-function or loss-of-expression mutations of genes involved in BMP signaling correlate with the pathogenesis of HHT. Loci targeted include the ligand (BMP9), the type II receptor (BMPR2), the type I receptor (ACVRL1), the type III receptor (Eng), the R-Smad (Smad9), the co-Smad (Smad4), and Drosha. Mutations in the *BMPR2* gene were also found in patients with pulmonary artery hypertension (PAH). A small number of individuals with *BMP9*, *ACVRL1*, *Eng*, or *BMPR2* mutations develop both HHT and PAH, indicating a genetic overlap between these disorders. Star indicates mutations associated with HHT and circle indicates mutations associated with PAH. BMP, bone morphogenetic protein; HHT, hereditary hemorrhagic telangiectasia; PAH, pulmonary artery hypertension; SBE, Smad binding element.

affecting the enzymatic function of Drosha, mutations elsewhere in Drosha could trigger broader and more extensive damage extending beyond BMP signaling and the vasculature, such as in Wilms tumors.

ROLE OF DROSHA AND MICRORNAS IN ENDOTHELIAL CELLS

Whereas Eng and ACVRL1 are detected mainly in vascular endothelial cells, Drosha is present in all cell types. Why then would *Drosha* mutations only

Table 1. *Drosha* variants found in hereditary hemorrhagic telangiectasia patients

Patient	Clinical characteristics	Nucleotide substitution	Protein substitution	Allele frequency
P1	P, mother with H, sister and grandmother have E	95C>T	P32L	0.0001
F1-I-1	Severe E, (cauterized age 10)	299C>T	P100L	0.0002
F1-II-4	Severe E, T	299C>T	P100L	0.0002
F1-III-1	E, T	299C>T	P100L	0.0002
F1-III-2	E, C (ruptured)	299C>T	P100L	0.0002
P4	E, T	299C>T	P100L	0.0002
P5	E, T, P,	676A>G	K226E	0.00009
F2-I-2 ^a	E, T, GI-T, P, H, liver shunts	836G>T	R279L	absent
F2-II-1 ^a	E, T, GI-T, multiple P	836G>T	R279L	absent

All nucleotide substitutions are heterozygous. Presence in population refers to the presence of *Drosha* variant in the ExAC database.

^aThese individuals carry a mosaic *Eng* mutation (c.1311+1G>A) in addition to *Drosha* variant. C, cerebral arteriovenous malformation (AVM); E, epistaxis; F, family; GI-T, gastrointestinal telangiectasia; H, hepatic AVM; NA, not applicable; P, proband; P1, pulmonary AVM; T, telangiectasia.

cause an endothelial-specific defect? Endothelial cells may be more sensitive than other tissues to changes in the abundance of microRNAs. For example, inactivation of the endothelial-specific *miRNA-126*: alone in zebrafish is sufficient to induce vascular defects similar to those observed in *Drosha* morphants [33]. Although the processing of *miRNA-126* does not require *Drosha* [33] and, therefore, its expression is not affected in zebrafish and mice lacking *Drosha* [34] – and thus cannot explain the effect of *Drosha* mutations – the unusually pronounced effect of *miRNA-126* loss may point to a heightened susceptibility of the endothelium to microRNA imbalances.

Future studies should identify BMP-regulated endothelial microRNAs with essential roles in vascular development or remodeling. They may shed light on the link between *Drosha* and endothelial diseases, and ultimately acquire an important therapeutic value in correcting the vascular lesions of HHT.

DO DROSHA MUTANTS CAUSE HEMORRHAGIC TELANGIECTASIA?

One approach to determine whether *Drosha* mutations cause HHT is to compare what happens when *Drosha* or other HHT-related genes are inactivated. The endothelial-specific loss of *Eng* or *ACVRL1* in mice produces well established animal models of HHT. For example, the endothelium-restricted *ACVRL1* knockout mouse (*Acvrl1^{flox/flox}; L1-cre; ACVRL1 cKO^{EC}*; hereafter referred to as *ACVRL1 cKO^{EC}*) exhibits tortuous, disorganized, and enlarged vasculature; loss of the smallest blood vessels; and abnormal connections between arteries and veins [35,36]. Our study of an endothelium-specific *Drosha* knockout mouse (*Drosha^{flox/}*

flox; Cdh5-cre; hereafter referred to as *Drosha cKO^{EC}*) revealed an enlarged dorsal aorta and disorganized, dilated hepatic and extraembryonic vasculature [28^{***}]. A postnatal, inducible endothelial-specific *Drosha* knockout mouse (*Drosha^{flox/flox}; Cdh5-cre/ERT2⁺* hereafter referred to as *Drosha iKO^{EC}*) showed loss of the smallest blood vessels, enlarged capillaries, and formation of abnormal connections between arteries and veins in the skin vasculature [28^{***}]. Therefore, *Drosha* appears to be essential for normal endothelial development and homeostasis. But unlike the *ACVRL1 cKO^{EC}* mice, which develop AVMs in the yolk sac and in the gastrointestinal and brain vasculature by postnatal day 3 [35,36], neither *Drosha cKO^{EC}* nor *iKO^{EC}* mice presented AVMs. We speculate that the difference in Cre-drivers might partially explain the disparity of vascular phenotypes, as *Cdh5-Cre* is expressed earlier and in a broader area than *L1-Cre* [35,36]. Additionally, the HHT-causing alleles of *Eng*, *ACVRL1*, *Smad4*, or *BMP9* would be expected to cause a more severe vascular phenotype than *Drosha* alone because of their combined deregulation of Smad-dependent transcriptional regulation and microRNA-dependent posttranscriptional control. An additive property of different HHT-causing alleles would find support in the development of severe epistaxis and AVMs by patients who carry both *Drosha* and *Eng* alleles (Table 1).

The currently limited evidence cannot distinguish between a role of *Drosha* as modifier or as causative agent of HHT. We are examining the vascular phenotypes of *Drosha* mutant knock-in mice in which the P100L or R279L alleles are expressed in a tissue-specific manner. These model mice could provide more conclusive answers to the remaining critical questions: Are *Drosha* mutations sufficient to cause HHT? And what is the molecular

mechanism by which *Drosha* regulates endothelial cell function?

DROSHA AND OTHER VASCULAR DISEASES

More than 80% of patients with hereditary pulmonary artery hypertension (PAH) carry mutations in *BMP receptor type 2 (BMPR2)*, an essential gene encoding a receptor for BMPs [37]. Unlike HHT, vascular lesions in PAH are restricted to the distal pulmonary arteries and affect both endothelial and smooth muscle cells [37]. Although these two vascular diseases appeared distinct, recent studies have discovered PAH patients with mutations in HHT genes, such as *BMP9*, *ACVRL1*, or *Smad4* (Fig. 4) [38–42]. The molecular triggers of PAH and HHT might then converge on BMP signaling, although the lesions appear different. At present, the potential genetic link between *Drosha* and PAH remains to be investigated.

A small number of individuals with *ACVRL1*, *Eng*, or *BMPR2* mutations develop both PAH and HHT (PAH-HHT) [39,43[■]]. The hemodynamic profiles and prognosis of PAH-HHT patients are significantly worse than those of patients with PAH alone, underscoring the benefit of closer monitoring and appropriate treatment [44[■],45]. It is unclear whether the current PAH therapies are effective to treat PAH-HHT patients.

CONCLUSION

This review discusses the involvement of the microRNA biogenesis enzyme *Drosha* in the vascular abnormalities of HHT. An imbalance of microRNAs could plausibly explain the effect of *Drosha* in HHT, but studies have also revealed that *Drosha* has microRNA-independent functions [46[■]], such as interacting with RNA Pol II to regulate transcription initiation. This and other alternative mechanistic roles of *Drosha* may contribute to vascular dysfunction.

We expect to see future genetic studies in which other components of the microRNA biogenesis pathway, including *DGCR8*, *Dicer*, *TRBP*, or *Ago*, are linked to HHT and/or other vascular diseases.

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Conflicts of interest

There are no conflicts of interest.

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- of special interest
- of outstanding interest

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