

Hepatocyte Growth Factor–Regulated Tyrosine Kinase Substrate (*Hgs*) Is Involved in BMP Signaling Through Phosphorylation of SMADs and TAK1 in Early Mouse Embryo

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Hepatocyte growth factor–regulated tyrosine kinase substrate that is encoded by *Hgs* promotes degradation of ubiquitinated signaling molecule in the early endosome. We previously reported that a targeted mutation in *Hgs* results in embryonic lethality soon after gastrulation in the mouse. Here, we report that downstream target genes for BMP signaling were highly down-regulated in the *Hgs* mutant embryos. We also showed that *Hgs* is required for phosphorylation of SMAD1/5/8 and TAK1/p38 to transduce BMP signaling. Furthermore, we found that HGS functions to localize TAK1 in early endosome for its activation. These results suggest that HGS is critical to localize TAK1 to early endosome for transducing BMP signaling for proper development. Our data revealed a new mechanism to modify BMP signaling by *Hgs* during early mouse development. *Developmental Dynamics* 240:2474–2481, 2011. © 2011 Wiley Periodicals, Inc.

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INTRODUCTION

Members of the Transforming growth factor-beta (TGF- β) superfamily, such as Activin, Nodal, and Bone morphogenetic proteins (BMPs) bind to their specific cell surface receptors, which are composed of two distinct subfamilies retaining serine/threonine kinases and are known as the type I and type II receptors (Kishigami and Mishina, 2005). Upon ligand binding, the type II receptor kinase phosphorylates and activates the type I receptor kinase, which induces phosphorylation and activation of signal-transducing effector molecules known as SMAD1, 2,

3, 5, and 8; subsequently, these receptor-regulated SMAD proteins form complexes with SMAD4, a collaborating Smad protein. The TGF/Activin and BMP receptor kinases activate SMAD2 or SMAD3 and SMAD1 or SMAD5, respectively, followed by formation of a complex of each SMAD with SMAD4, which transmits signals from the receptor complex in the plasma membrane to the nucleus.

TGF- β -activating kinase 1 (TAK1) is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family and is activated by cytokines such as interleukin-1 (Ninomiya-Tsuji et al., 1999). TAK1 also plays impor-

tant roles in signaling downstream of a variety of other molecules, including BMPs. TAK1 functions to transduce BMP signaling in dorsoventral patterning and neural induction of Xenopus embryo (Shibuya et al., 1998; Yamaguchi et al., 1999). TAK1 is a ubiquitin-dependent kinase. Its activation requires ubiquitin ligase, deubiquitinating enzymes inhibit TAK1 activation, and polyubiquitin plays an important role as a scaffold protein that facilitate autophosphorylation of TAK1 (Adhikari et al., 2007). Despite these reports, little is known about involvement of TAK1 in BMP signaling during mouse embryogenesis as

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well as other mechanism(s) of TAK1 phosphorylation occurring in vivo.

Hepatocyte Growth factor-regulated tyrosine kinase Substrate (HGS) is an evolutionarily conserved vesicular sorting protein belonging to the endosomal-sorting proteins required for transport (ESCRT) (Komada et al., 1997). HGS possesses a FYVE domain, which enables the molecule to anchor to the internal surface of the lipid bilayer (Raiborg et al., 2001), and a UIM domain, through which HGS binds monoubiquitinated receptors (Komada et al., 1997). By associating with ubiquitinated receptors such as MET or EGFRs, HGS promotes their degradation through the endosomal/lysosomal pathway and thus regulates Hepatocyte Growth Factor or Epidermal Growth Factor signaling (Lloyd et al., 2002; Toyoshima et al., 2007).

Targeted mutations in *Hgs* result in early embryonic lethality soon after gastrulation (Komada and Soriano, 1999; Miura et al., 2000). We also reported that HGS enhance the association of ACTR1 and SMAD2 for ACTIVIN signaling (Miura et al., 2000). Here we report that the phosphorylation of SMAD1 is decreased, and that of TAK1/p38 is abrogated in *Hgs*^{-/-} embryos at E7.5. As a consequence, the expression of the target genes of BMP signaling is lost or strongly down-regulated. Results from selective inhibitions of TAK1 indicate that TAK1 is responsible for *Flk1* expression. Subcellular localization of TAK1 in the early endosome is largely displaced in the *Hgs*^{-/-} embryos, which might be a cause of loss of phosphorylation of TAK1. Together, these results suggest that HGS is a critical component to localize TAK1 to the early endosome for transducing BMP signaling for proper development.

RESULTS

Hgs^{-/-} Represents Null Allele of *Hgs* Gene

We previously reported that homozygous embryos for the targeted *Hgs* allele (*Hgs*^{-/-}) show severe gastrulation defect and die around E9.5 (Miura et al., 2000). Primitive streak formation is not observed or strongly deteriorated as judged by down-regu-

lated expression of *Brachyury* (Fig. 1A, B). Also, *Hgs*^{-/-} embryos develop no mesoderm, as evidenced by the lack of *Mox1* expression (Fig. 1C, D). The *Hgs* mutant allele is not transcribed as shown by RT-PCR (Fig. 1E), indicating that the targeted allele for *Hgs* is likely functionally null.

Target Genes of BMP Signaling Are Down-Regulated in *Hgs*^{-/-} Embryos

During the examination of the expression of markers of embryonic development at the time of gastrulation, we noticed that target genes of BMP signaling were strongly down-regulated in *Hgs*^{-/-} embryos (Fig. 2). *Id2* and *Id3* are target genes of BMP signaling during mouse development (Miyazono and Miyazawa, 2002; Ying et al., 2003). In *Hgs*^{-/-} embryos, the expressions of both genes were highly reduced (Fig. 2A–F). *Msx2* expression was also significantly decreased in *Hgs*^{-/-} embryos (Fig. 2G, H) along with *Flk1* (Fig. 2I, J). We observed the same results in *Bmpr1a*^{-/-} embryos, homozygous null embryos for BMP type IA receptor (Mishina et al., 1995) (Fig. 2K, L). *Wnt3* is also positively regulated by BMP signaling in early mouse embryos (Ben-Haim et al., 2006). The expression of *Wnt3* (Fig. 2M, N) and its target gene *Brachyury* was largely down-regulated in *Hgs*^{-/-} embryos (Fig. 1A, B for E8.5, Fig. 2O, P for E7.5). *Nodal* was apparently expressed in *Hgs*^{-/-} embryos (Fig. 2Q, P). Expression of *Bmp4* (Winnier et al., 1995) and *Eomes* (Russ et al., 2000) in the extraembryonic ectoderm or *Oct4* (Nichols et al., 1998) in epiblast was detected in *Hgs*^{-/-} embryos (Fig. 2T, V, X), suggesting that the extraembryonic ectoderm and epiblast were formed in the mutant embryos. These expression data indicate that down-regulation of *Id2*, *Id3*, *Msx2*, *Flk1*, or *Wnt3* in *Hgs*^{-/-} embryos was not due to the loss of these tissues. Despite the comparable levels of *Bmp4* expression (Fig. 2T), the reductions of these target genes for BMPs in the mutant embryos suggests that *Hgs* is involved in BMP signaling in early mouse embryo.

Hgs Is Required for Phosphorylation of SMAD1/5/8 and TAK1

The above data prompted us to examine the status of phosphorylation of SMAD1/5/8 and TAK1/p38 as a measurement for levels of BMP signaling pathway. In control embryos, pSMAD1/5/8 was observed in proximal epiblast/extraembryonic tissues such as extraembryonic ectoderm and extraembryonic visceral endoderm (Fig. 3A), consistent with the previous report (Yang and Klingensmith, 2006). In mutants, an apparent decrease of pSMAD1/5/8 staining was observed in these tissues (Fig. 3B). HGS binds to TAK1 and overexpression of *Hgs* increases TAK1 activity (Sasaki and Sugamura, 2001). Thus, we speculated that *Hgs* was also involved in BMP signaling through TAK1 in early embryogenesis. The total level of TAK1 showed no apparent difference between WT and *Hgs*^{-/-} embryos (data not shown). However, levels of pTAK1 were markedly decreased in *Hgs*^{-/-} embryos (Fig. 3D). Moreover, phosphorylation of p38, a MAPK downstream of TAK1 (Ninomiya-Tsuji et al., 1999), was strongly down-regulated in *Hgs*^{-/-} embryos (Fig. 3F). Phosphorylation of ERK1/2, another MAPK, was apparently normal in *Hgs*^{-/-} embryos (Fig. 3G, H). These results showed that *Hgs* is involved in phosphorylation of SMAD1/5/8 and TAK1/p38.

Bmp4 Is Critical for Phosphorylation of TAK1

TAK1 is a MAPKKK that transduces variety of signals (Adhikari et al., 2007). Importantly, TAK1 is a potential transducer of BMP signaling in vertebrate embryogenesis (Shibuya et al., 1998; Yamaguchi et al., 1999). However, the status of TAK1 was not reported in mutant mouse embryos for BMPs. Therefore, we examined the total or phosphorylation level of TAK1 in mouse mutant embryos for *Bmp4* (Winnier et al., 1995). Total level of TAK1 observed by whole mount immunohistochemistry was seemingly normal in *Bmp4*^{-/-} embryos (data not shown). However, phosphorylation of TAK1 (pTAK1) is strongly down-regulated (Fig. 3J). The expression of *Id2*,

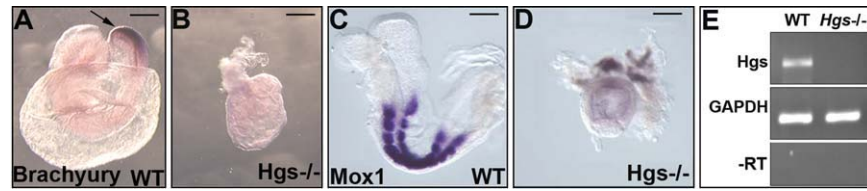


Fig. 1. *Hgs*^{-/-} is a null mutation of *Hgs* gene. **A–D:** Whole mount in situ analysis of E8.5 embryos. *Brachyury* was expressed in the regressing primitive streak in WT (A, arrow). In *Hgs*^{-/-} embryos, the expression was strongly decreased (B). *Mox1*, a somite marker (C), was not expressed in *Hgs*^{-/-} embryos (D). Scale bar = 300 μ m. **E:** Semi-quantitative RT-PCR analyses. The expressions of *Hgs* or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control of mRNA in WT or *Hgs*^{-/-} embryos are shown. –RT is a negative control sample without reverse transcriptase.

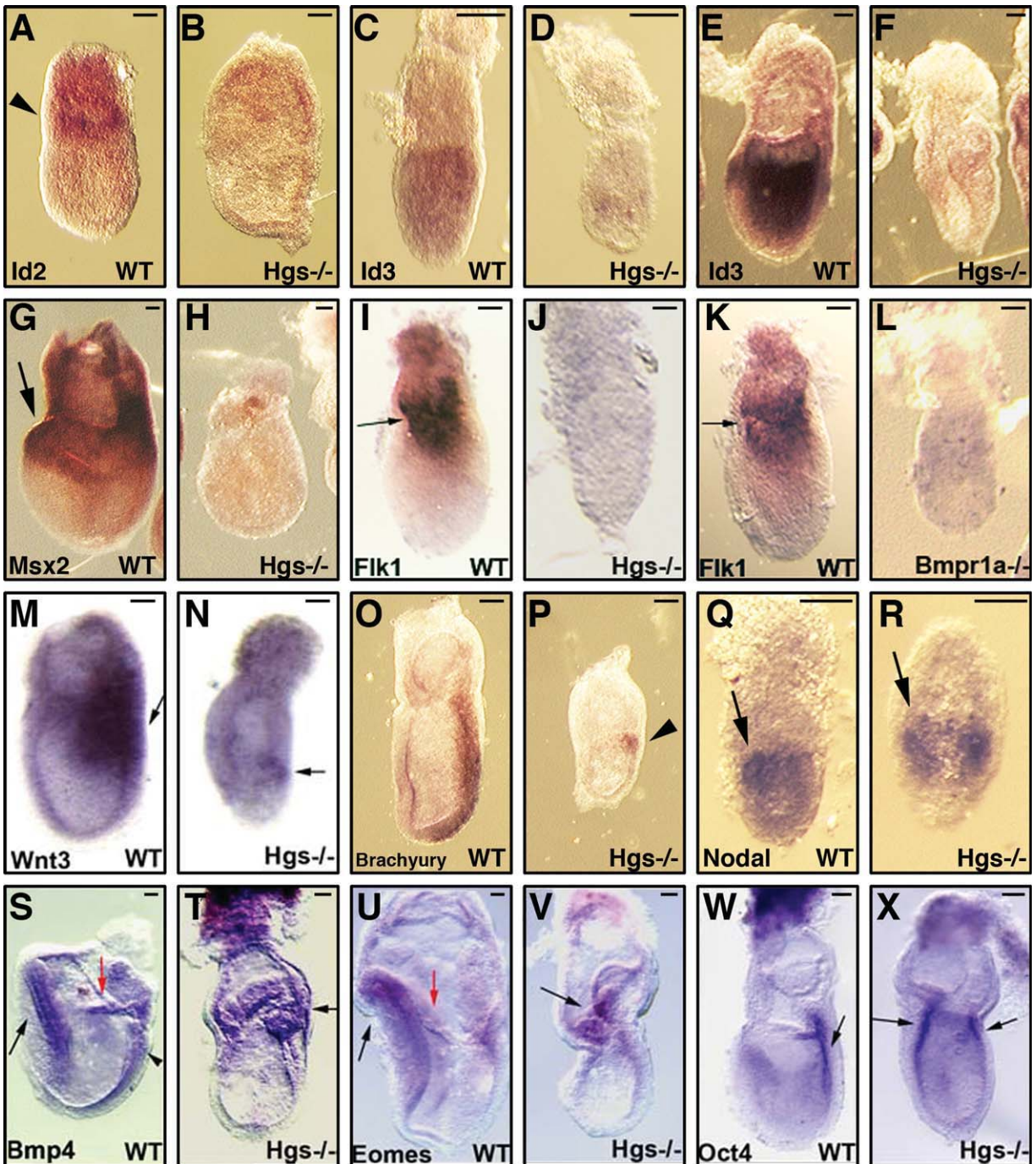


Fig. 2.

Id3, and *Msx2* was also decreased in *Bmp4*^{-/-} embryos (Fig. 3L, and data not shown). These results also support the idea that TAK1 may play a role in BMP signaling in early mouse embryo.

TAK1 Is Involved in Regulation of *Flk1*, Along With Smad Signaling

The aforementioned observations suggest that HGS is involved in BMP signaling by modulating both SMAD signaling and TAK1-p38 signaling pathways. To further investigate this possibility, we employed chemical inhibitors specific for individual pathways. We chose *Flk1*, *Id2*, and *Id3* for readout of BMP signaling because they are transcriptionally activated by SMAD1 when tested using cell lines (Park et al., 2004; Chen et al., 2006; Du and Yip, 2010).

E6.5 embryos were incubated with different concentrations of Dorsomorphin (Yu et al., 2008), an inhibitor for phosphorylation of BMP SMADs (SMAD1/5/8), for 20 h and examined for the expression levels of *Flk1*, *Id2*, and *Id3*. *Flk1* showed about an 80% reduction and *Id2* and *Id3* showed about a 90% reduction, indicating these genes are dependent on SMAD-mediated BMP signaling in vivo as well as in vitro (Fig. 4A). Treatment with 11,12-dihydro-5Z7-oxo-zeaenol, an inhibitor for TAK1 kinase activity (Yao et al., 2007), resulted in more than a 99% reduction of *Flk1* expression, in contrast to the 2–3-fold increase in *Id2* and *Id3* expressions (Fig. 4B). These results suggest that Id genes are regu-

lated mostly by Smad signaling, whereas, *Flk1* expression is regulated by both SMAD and MAPK signaling pathways.

Subcellular Localization of TAK1 Is Abnormal in *Hgs*^{-/-} Embryos

As HGS is normally localized in the early endosome (Komada et al., 1997), we hypothesized that a large part of TAK1 is also localized in the early endosome, and such subcellular localization might not be maintained in *Hgs*^{-/-} embryos. We observed that TAK1 and transferrin, an early endosomal marker (Gorvel et al., 1991), partially co-localize in WT E7.5 embryos (Fig. 5A–C, G–I). However, in *Hgs*^{-/-} embryos, such a distinct pattern was not observed (Fig. 5J–L). These data suggest that *Hgs* is required to maintain normal TAK1 subcellular localization in early endosome and this may be critical for TAK1 phosphorylation and subsequent signal transduction.

In *Bmp4*^{-/-} embryos, subcellular localization of TAK1 appears to be the same with WT embryos (Fig. 5M–O). This indicated that BMP signaling is not required for localization of TAK1 into the early endosome, suggesting that HGS is critical to anchor TAK1 to early endosome.

DISCUSSION

In this study, we demonstrate that HGS is required for phosphorylation of both TAK1 and SMAD1/5/8. We

also show that levels of faithful downstream target genes of BMP signaling are highly down-regulated in the *Hgs* mutant embryos. These results are similar to those found in the mutant embryos for BMP4 and BMP type 1A receptor, indicating that HGS contributes to BMP signaling. We also demonstrate that subcellular localization of TAK1, which is found in early endosome in WT embryos, is altered in *Hgs* mutant embryos, but not in *Bmp4* mutant embryos. Based on these notions, we propose a model of *Hgs* function in BMP signaling (Fig. 6). In normal development, SMAD1/5/8 and TAK1 interact with HGS, and at least in part, these molecules are co-localizing in the early endosome. BMP signaling and HGS function for phosphorylation of SMAD1/5/8 and of TAK1. pSMAD1/5/8 are required for the transcription of *Id2*, *Id3*, and *Flk1* whereas TAK1/p38 is also required for the transcription of *Flk1* (Fig. 6A). In *Hgs*^{-/-} embryos, phosphorylation of SMAD1/5/8 decreased significantly but partly, probably because other molecules such as SARA (Itoh et al., 2002) compensate the loss of HGS. *Id1* and *Id2* expression is also significantly decreased. Phosphorylation of TAK1/p38 is abrogated and accumulation of TAK1 in the early endosome is apparently less significant. In sum, *Flk1* expression is highly downregulated in *Hgs*^{-/-} embryos (Fig. 6B). It is noteworthy that the cells in *Hgs*^{-/-} embryos seem to have an altered apical-basal polarity (Fig. 5J–L). Localization of TAK1 in the early endosome may be disrupted as a consequence of this phenomenon. Further studies are needed to clarify this issue.

Generation of a different mutant allele of *Hgs* gene, *Hrs* allele, was reported previously and shows that *Hrs*^{-/-} embryos develop mesoderm (Komada and Soriano, 1999), which is a milder phenotype than that of *Hgs*^{-/-} described here. Trans-heterozygote embryos for both alleles (*Hgs*⁻/*Hrs*⁻) showed a phenotype between that of *Hrs*^{-/-} and *Hgs*^{-/-} embryos (our unpublished data). Furthermore, transcription from the *Hgs* mutant allele is not detected by RT-PCR (Fig. 1E). Taken together these data suggest that the *Hgs* mutant allele we used here probably represents a null allele of *Hgs* gene and the *Hrs* allele is a hypomorphic allele.

Fig. 2. BMP signaling is down-regulated in *Hgs*^{-/-} embryos. A–V: Whole mount in situ analysis of E6.5 (C,D,Q,R) or E7.5 (A,B,E–P, S–X) WT and *HGS*^{-/-} embryos. *Id2* is expressed in the extraembryonic tissues of WT embryos (A, arrowhead), but not in *Hgs*^{-/-} embryos (B). *Id3* expression in the extraembryonic and/or epiblast of WT embryos (C, E) was highly reduced in *Hgs*^{-/-} embryos (D, F). *Msx2*, which is expressed in the extraembryonic tissues and proximal epiblast (G, arrow), was largely down-regulated in *Hgs*^{-/-} embryos (H). *Flk1*, which is expressed in the extraembryonic and proximal-lateral embryonic mesoderm (I, K, arrow) was highly down-regulated in *Hgs*^{-/-} embryos (J) and *Bmpr1a*^{-/-} embryos (L). The expression of *Wnt3* in the primitive streak and overlying visceral endoderm (M, arrow) was also decreased in *Hgs*^{-/-} embryos (N, arrow). The expression of *Brachyury* in the primitive streak (O) was also decreased in *Hgs*^{-/-} embryos (P, arrowhead). *Nodal* expression was observed normally in the *Hgs*^{-/-} embryos (Q, R, arrows). *Bmp4* expression is observed in the extraembryonic membrane (S, red arrow), neurectoderm (S, arrow), and proximal primitive streak (S, arrowhead) of WT embryos. In *Hgs*^{-/-} embryos, the expression was observed in the extraembryonic ectoderm (T, arrow). In WT embryos, *Eomes* is expressed in the extraembryonic membrane (U, red arrow) and neurectoderm (U, arrow). In *Hgs*^{-/-} embryos, expression is observed in the extraembryonic ectoderm (V, arrow). The expression of *Oct4* is observed in the posterior epiblast of WT embryos (W, arrow). In *Hgs*^{-/-} embryos, the expression was also observed in the epiblast (X, arrows). At least 3 embryos are used for each probe. Scale bar = 100 μm.

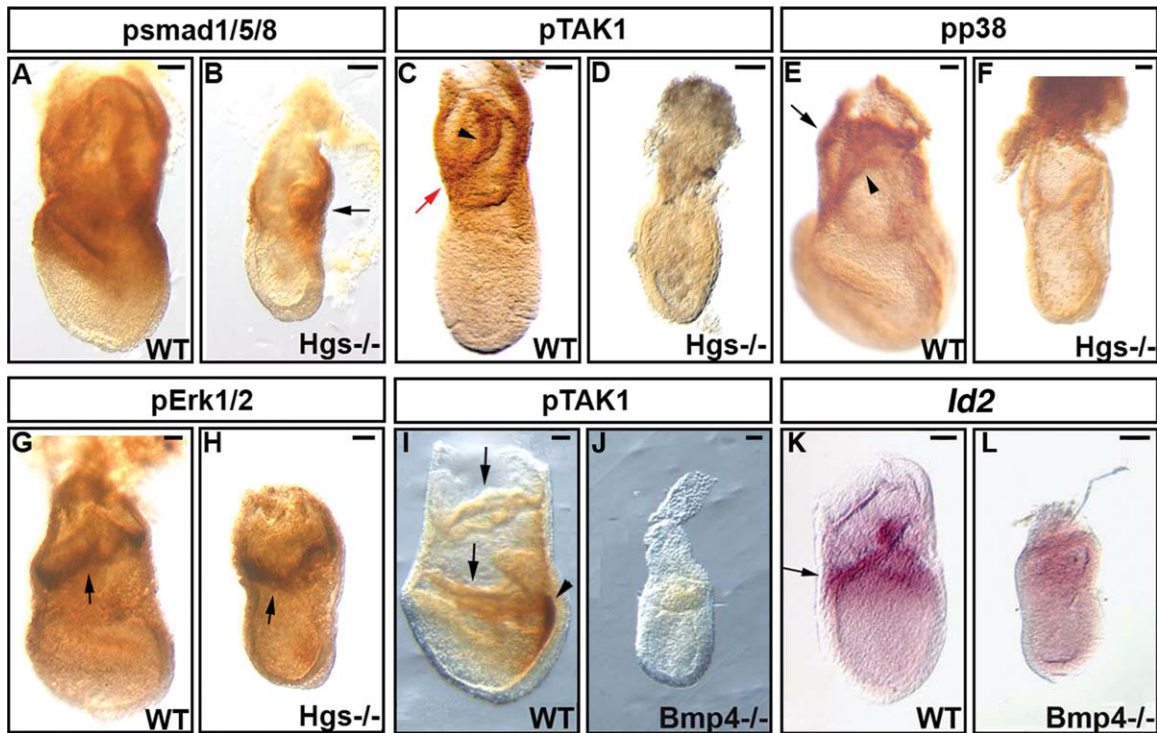


Fig. 3. Phosphorylation of signaling molecules for BMPs is highly down-regulated in *Hgs*^{-/-} embryos. **A–J:** Whole mount immunohistochemistry at E7.5. Presence of pSmad1/5/8 is detected mainly in the extraembryonic tissues of WT (A) and *Hgs*^{-/-} embryos (B, arrow). Presence of pTAK1 is detected in the extraembryonic visceral endoderm (C, red arrow) and chorion (C, arrowhead) at the bud stage. In *Hgs*^{-/-} embryos, pTAK1 expression is not detected (D). Presence of pp38 is detected in the extraembryonic visceral endoderm (E, arrow) and chorion (E, arrowhead). In *Hgs*^{-/-} embryos, pp38 expression is not detected (F). pErk1/2 is mainly detected in the chorion of E7.5 of WT (G, arrow) and *Hgs*^{-/-} embryos (arrow, H). pTAK1 detected in the extraembryonic tissues (I, arrow) and proximal part of the primitive streak (I, arrowhead) at the head-fold stage was highly reduced in *Bmp4*^{-/-} embryos (J). **K,L:** Whole mount in situ analyses at E7.5. Expression of *Id2* was strongly decreased in *Bmp4*^{-/-} embryos (L) compared to WT embryos (K, arrow). At least 3 embryos are used for each antibody. Scale bar = 100 μ m.

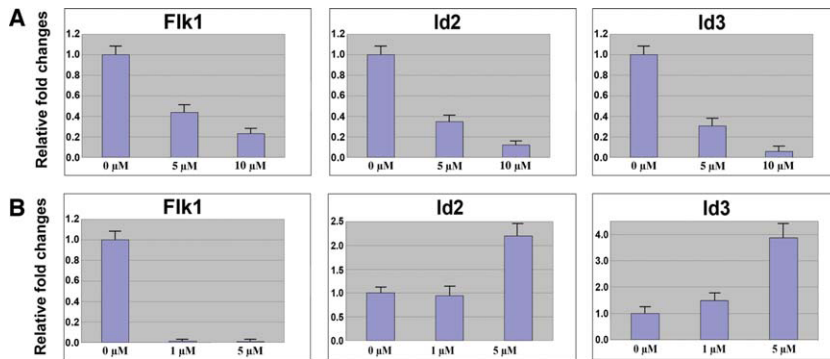


Fig. 4. Expression levels of BMP target genes are differentially regulated by SMAD and TAK1 pathways. **A,B:** Quantitative RT-PCR of downstream target genes for BMP signaling. WT embryos harvested at E7.5 are cultured with Dorsomorphin (A) or dihydro-5Z7-oxo-zeaenol (B) with indicated concentration for 20 h and levels of gene expression are measured. At least 6 embryos are analyzed for each concentration.

BMP signaling is transduced by SMAD1/5/8 as receptor-regulated SMADs and SMAD4. A complex of each receptor-regulated SMAD and SMAD4 translocates to the nucleus and transcribes a number of genes (Mishina, 2003). However, although *Smad4* mutant embryos in the epiblast

still form rudimentary heart (Chu et al., 2004), no heart mesoderm remains in E8.5 embryos with *Bmpr1a* deleted in the epiblast (Miura et al., 2006). This suggests that not all BMP signaling is transduced by the SMAD pathway, but there are non-SMAD-mediated pathways existing to

transduce BMP signaling. In *Xenopus* embryos, a dominant-negative form of TAK1 reverses phenotypes caused by ectopic expression of SMAD1 and SMAD5, such as ventralization or loss of neutralization (Shibuya et al., 1998), suggesting that TAK1 and SMAD1/5 cooperate to transduce BMP signaling. Our data also indicate that TAK1 functions as a component of BMP signaling in the mouse embryo. As phosphorylation of pTAK1-p38 as well as that of SMAD1/5/8 is affected in *Hgs*^{-/-} embryos, HGS contributes to both the SMAD and non-SMAD-mediated pathway of BMP signaling. It is interesting that the reduction of phosphorylation of TAK1 and SMAD1/5/8 is largely found in the extraembryonic regions (Fig. 3). It has been reported that BMP signaling in the extraembryonic tissues is critical for appropriate development of the epiblast (Davis et al., 2004; Kishigami et al., 2004; Miura et al., 2006, 2010; Di-Gregorio et al., 2007; Yamamoto et al., 2009). These results suggest that HGS in the

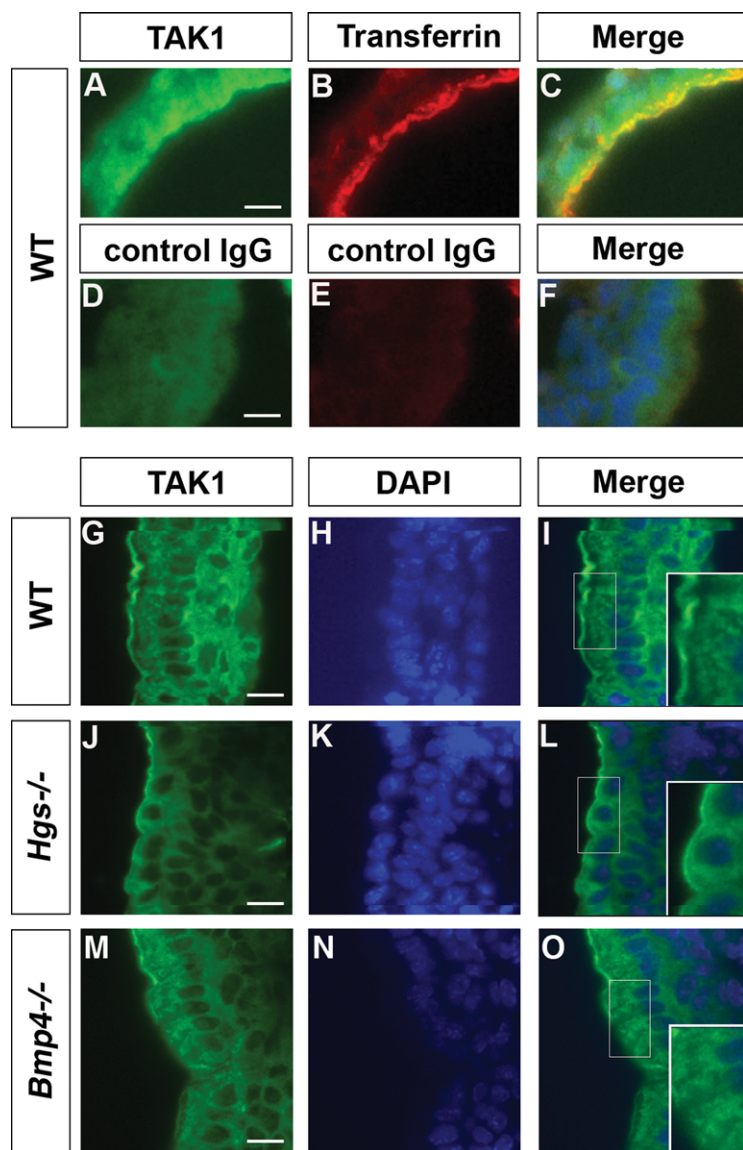


Fig. 5. Localization of TAK1 in the early endosome is not maintained in *Hgs*^{-/-} embryos. **A–F:** Double immunostaining for TAK1 and Transferrin, an early endosome marker, in the E7.5 WT embryos. **G–O:** Immunostaining of TAK1 of E7.5 WT (G–I), *Hgs*^{-/-} (J–L), and *Bmp4*^{-/-} embryos (M–O). Insets in I, L, O are enlarged images of boxed region in each panel. In *Hgs*^{-/-} embryos, TAK1 is homogeneously expressed in the cytoplasm (L). In *Bmp4*^{-/-} embryos, TAK1 was expressed in dot-like structures (O, inset), as observed in WT embryos (I, inset). Scale bar = 10 μ m.

extraembryonic tissues is involved in non-cell-autonomous function of BMP signaling towards the epiblast.

We previously reported that *Hgs*^{-/-} embryos show a significant reduction in responding to ACTIVIN stimulation at E8.5 (Miura et al., 2000), suggesting that the ACTIVIN signaling pathway is also disrupted in *Hgs*^{-/-} embryos. However, *Nodal* expression, which is regulated by NODAL signaling (Gu et al., 1998), is apparently expressed at E6.5 in *Hgs*^{-/-} embryos. Because both ACTIVIN and NODAL share the

same receptor system to signal, such as ACVR1B (ALK4), these results suggest that machinery required for ACTIVIN signaling is mostly not affected at E6.5 in *Hgs*^{-/-} embryos. As BMP signaling is required for the maintenance of *Nodal* expression and ACTIVIN signaling (Di-Gregorio et al., 2007), we believe that the observed defect in ACTIVIN signaling at E8.5 is an indirect outcome caused by the down-regulation of BMP signaling during gastrulation in *Hgs*^{-/-} embryos.

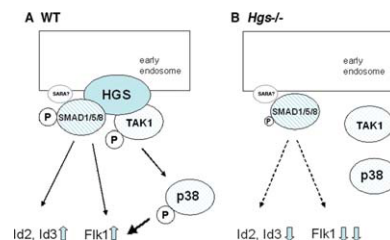


Fig. 6. Proposed model. **A:** In normal development, TAK1 binds to HGS, ensuring its localization in the early endosome. HGS probably acts as a scaffold to facilitate both phosphorylation of SMADs and autophosphorylation of TAK1 to transduce non-SMAD BMP signaling. **B:** In *Hgs*^{-/-} embryos, TAK1 is not localized in the early endosome, thus BMP signaling mediated by TAK1 and subsequent MAK kinase cascades are largely compromised. The canonical BMP signaling mediated by phosphorylation of SMADs is less affected probably due to compensation by other molecules.

In this study, we have revealed that HGS is required for phosphorylation of TAK1 as well as SMAD1/5/8, thus showing the specific contribution of HGS to BMP signaling. *Tak1*^{-/-} embryos die around E12.5 by defects in vasculogenesis (Jadrich et al., 2006). Thus, phenotypes of *Hgs*^{-/-} embryos cannot be only attributed to a failure of activation of the TAK1-p38 MAPK pathway. A PI3K-specific inhibitor wortmannin induces expansion of early endosome in cultured cell lines (Spiro et al., 1996). Because the expansion of early endosome is a well-recognized phenotype found in the *Hgs*^{-/-} embryos (Komada and Soriano, 1999), it is an interesting possibility that PI3K pathway is abrogated by mutation of *Hgs* gene. Recent reports suggest that BMP signaling is, in part, transduced via PI3K pathway (Mandal et al., 2009). Thus, it is reasonable to speculate that HGS may play a critical role in BMP signaling pathways mediated by SMAD, TAK1, and PI3K.

EXPERIMENTAL PROCEDURES

Mouse

Mouse strains and genotyping have been described elsewhere (Winnier et al., 1995; Komada and Soriano, 1999; Miura et al., 2000). All mouse experiments were performed in accordance with NIEHS/NIH guidelines covering the humane care and use of animals in research.

Whole Mount In Situ Hybridization

Whole mount in situ hybridization was performed as described previously (Belo et al., 1997). Probes used were *Mox1* (Candia et al., 1992) and *Nodal* (Conlon et al., 1994), *Wnt3* (Liu et al., 1999), *Foxf1* (Mahlapuu et al., 2001), *Id2* (Jen et al., 1996), *Id3* (Ellmeier and Weith, 1995), *Msx2* (Catron et al., 1996), *Bmp4* (Winnier et al., 1995), *Eomes* (Russ et al., 2000) and *Oct4* (Yang and Klingensmith, 2006). Three or more embryos were used for each probe.

Immunohistochemistry

E7.5 embryos were fixed in 4% paraformaldehyde, dehydrated through graded alcohol embedded in paraffin. Sections (7 μ m) were deparaffinized, and stained with anti-TAK1 rabbit antibody (2.5 μ g/ml) (Abgent, San Diego, CA) and Alexa anti-rabbit IG according to the manufacturer's protocols. Whole-mount immunohistochemistry was performed as described (Miura et al., 2006). Briefly, primary antibodies were used at a dilution of 1:100 for anti-phospho-TAK1 antibody (Cell Signaling, Danvers, MA), 1:200 for anti-phospho-p38 antibody (Sigma, St. Louis, MO), 1:200 for anti-phospho-Smad1/5/8 antibody (Cell Signaling), and 1:100 for anti-phospho-Erk1/2 antibody (Cell Signaling) in blocking solution. After primary antibody incubation, embryos were washed and then incubated with biotinylated goat anti-rabbit antibody (Vector Lab, Burlingame, CA), diluted 1:200 in blocking solution. After several washes in PBS with 0.1% TritonX-100 (Fisher Scientific, Pittsburgh, PA), embryos were incubated with HRP-conjugated biotin-streptavidin system (Vector Lab). Staining was then developed with the ImmunoPure Metal-enhanced DAB substrate kit (Pierce, Rockford, IL). Stained embryos were embedded in paraffin and sectioned and counterstained with eosin. Three or more embryos were used for each antibody.

RT-PCR Analysis

Total RNA was extracted from each embryo using PicoPure RNA isolation

kit (Applied Biosystems, Foster City, CA). Total RNA harvested was measured with Nanodrop 1000 (Thermo Scientific, Waltham, MA) and the same amount of total RNA was used for each RT-PCR reaction. RT-PCR was performed using One step RT-PCR kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. *Hrs* primes are Hrs-F (5'-ctacagatctgcgacctgat ccgt-3') in exon 1 and Hrs-R (5'-tcctcagcatccaccagtcagg-3') in exon 6, yielding a 410-bp product.

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