BMP signaling in the development of the mouse esophagus and forestomach

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SUMMARY

The stratification and differentiation of the epidermis are known to involve the precise control of multiple signaling pathways. By contrast, little is known about the development of the mouse esophagus and forestomach, which are composed of a stratified squamous epithelium. Based on prior work in the skin, we hypothesized that bone morphogenetic protein (BMP) signaling is a central player. To test this hypothesis, we first used a BMP reporter mouse line harboring a BRE-lacZ allele, along with in situ hybridization to localize transcripts for BMP signaling components, including various antagonists. We then exploited a Shh-Cre allele that drives recombination in the embryonic foregut epithelium to generate gain- or loss-of-function models for the Bmpr1a (Alk3) receptor. In gain-of-function (Shh-Cre;Rosa26CAG-loxpstoploxp-caBmpr1a) embryos, high levels of ectopic BMP signaling stall the transition from simple columnar to multilayered undifferentiated epithelium in the esophagus and forestomach. In loss-of-function experiments, conditional deletion of the BMP receptor in Shh-Cre;Bmpr1aRasfllox embryos allows the formation of a multilayered squamous epithelium but this fails to differentiate, as shown by the absence of expression of the suprabasal markers loricrin and involucrin. Together, these findings suggest multiple roles for BMP signaling in the developing esophagus and forestomach.

KEY WORDS: BMP signaling, Esophagus, Forestomach, Stratification, Differentiation, Mouse

INTRODUCTION

Similar to the stratified squamous epidermis, the epithelium of foregut-derived tissues, including the esophagus, arises from a layer of simple columnar cells that stratify and differentiate. In the epidermis, these processes are associated with multiple signaling pathways, including those driven by bone morphogenetic protein (BMP) and Wnt (Botchkarev, 2003; Botchkarev and Sharov, 2004). However, it remains unknown whether these pathways are also active in the embryonic foregut during development and in the steady state.

The canonical BMP signaling pathway is activated by a secreted ligand (e.g. Bmp4, Bmp7) interacting with a complex of type I and type II transmembrane receptors. This initiates signal transduction through phosphorylation of Smads 1/5/8 (R-Smads), which interact with two co-Smad (Smad4) to regulate downstream target genes (Massague et al., 2005). In the epidermis, conditional deletion of Bmpr1a leads to increased epithelial proliferation (Kobielak et al., 2003; Qiao et al., 2006; Yuuki et al., 2004). Deletion of Smad4 in the epithelium also results in basal cell hyperproliferation and leads to squamous cell cancer (Qiao et al., 2006).

The mouse esophagus separates from the foregut tube between embryonic day (E) 9.5 and E11.5. This separation process is regulated by multiple signaling pathways, including Wnt, BMP and Shh, which may act upstream of Bmp4 (Litingtung et al., 1998; Morrisey and Hogan, 2010; Roberts et al., 1998). Analysis of Nog-null mutants has shown that BMP signaling regulates the initial dorsal-ventral patterning of the single foregut tube and the separation into dorsal esophagus and ventral trachea (Li et al., 2007; Que et al., 2006). Here, we show that Nog also regulates the transition from simple columnar to stratified squamous epithelium in the developing esophagus. Combining gain- and loss-of-function studies, we further show that BMP signaling plays multiple roles in the development of the esophagus and forestomach, the epithelium of which appears histologically as a continuation of the esophagus.

MATERIALS AND METHODS

Mouse strains

Heterozygous BRE-lacZ (Blank et al., 2008), NogloxZ/lox (Brunet et al., 1998) and Bmp7loxZ/lox (Godin et al., 1998) reporter mice were maintained on the C57Bl/6 × 129/SvEv background. To conditionally activate BMP signaling in foregut endoderm, Shh-GFP-Cre (Shh-Cre) mice (Harfe et al., 2004) were crossed with Rosa26CAG-loxpstoploxp-caBmpr1a (Bmpr1aCA) mice. In this line, a constitutively active Bmpr1a (caBmpr1a) was inserted into the Rosa26 locus preceded by a CMV early enhancer/chicken β-actin (CAG) promoter and a floxed neo cassette (see Fig. S2A in the supplementary material). The caBmpr1a construct has a single amino acid substitution (Q to D) within the glycine-serine (GS) domain, giving the protein strong kinase activity even in the absence of ligand or type II receptor (Zou et al., 1997). To conditionally delete Bmpr1a, Shh-Cre;Bmpr1aRasfllox embryos were generated (Yuuki et al., 2004).

In situ hybridization

In situ hybridization was performed as previously described (Que et al., 2006; Que et al., 2007). Digoxigenin (DIG)-labeled antisense cRNA probes against mouse Bmpr1a, Bmp7, Nog, Fst, Fstl1, Chrd, Grem1 and Cerl were synthesized using T3, T7 or SP6 RNA polymerases. Grem1 was a kind gift from Dr Clifford Tabin (Harvard University).

Immunohistochemistry, immunostaining and X-gal staining

Tissues were fixed in 4% paraformaldehyde for 3–4 hours at 4°C and embedded in paraffin for sectioning. Cryosections were also used for immunostaining with phospho (p) Smad1/5/8 antibody (1:1000; kind gift
from Dr Edward Laufer, Columbia University). The following primary antibodies were used on paraffin sections: mouse anti-p63 (1:200; Santa Cruz Biotechnology); mouse anti-Krt14 (1:200; Thermo Scientific); rat anti-Krt8 (1:100; DSHB); rat anti-phospho-histone H3 (1:1000, Upstate Biotechnology); rabbit anti-loricrin (1:100) and anti-involucrin (1:10,000) (kind gifts from Dr Terry Lechler, Duke University); and rabbit anti-Krt5 (1:100; Covance). Whole embryos, isolated esophagi and stomachs were stained with X-gal and processed as previously described (Que et al., 2006).

RESULTS AND DISCUSSION
Dynamic BMP signaling in the developing mouse esophagus and forestomach

The epithelium of the embryonic esophagus and forestomach starts to stratify at early E11.5. At E10.5, the esophagus is lined by keratin 8 (Krt8)-positive simple columnar epithelial cells, a subset of which express p63 (Trp63 – Mouse Genome Informatics) (Fig. 1A,B), a transcription factor that is required for epithelial stratification (Daniely et al., 2004). By E11.5, the epithelium is 1-2 cells thick (Yu et al., 2005), with all cells expressing p63 and Krt8, but not Krt5 and Krt14, which only starts to be expressed from E14.5 (Fig. 1C,D; data not shown). Similarly, the epithelium in the forestomach starts to stratify between E10.5 and E11.5 (Fig. 1E-G).

To investigate canonical BMP pathway activation in the developing esophagus and forestomach, we used the BRE-lacZ transgenic reporter line, which expresses β-galactosidase (β-gal) under the control of BMP response elements (BREs) from Idl (Blank et al., 2008). At E9.5, β-gal-positive cells were observed in the epithelium and mesenchyme of the ventral foregut (see Fig. SIA in the supplementary material), which generates the trachea at E11.5 (see Fig. S1B in the supplementary material). Although the simple columnar epithelium of the hindstomach and mesenchyme showed active BMP signaling at E13.5 (Fig. 1H), the epithelium of the esophagus and forestomach remained negative (Fig. 1H-L). By E14.5, β-gal activity was seen in a limited number of suprabasal cells in both the esophagus and forestomach, and this increased by E15.5 (see Fig. S1C-H in the supplementary material). At postnatal day (P) 0, all suprabasal cells exhibited β-gal activity (Fig. 1M-R) and ~75% of these cells were also positive for pSmad1/5/8. Interestingly, ~5% of basal cells were positive for both activities (Fig. 1O,R).

Expression of BMP ligands and inhibitors in the developing esophagus and forestomach

In situ hybridization showed that between E9.5 and E15.5, Bmpr1a is ubiquitously expressed, whereas Bmp7 transcripts are enriched in the esophageal and forestomach epithelium (Fig. 1S-X; data not shown). The expression of Bmp7 in the epithelium of the esophagus and forestomach was confirmed by analysis of the Bmp7-lacZ ‘knock-in’ allele (see Fig. S1I,J in the supplementary material). At E15.5, low levels of Bmp6 were detected in the differentiating suprabasal cells (see Fig. S1K,L in the supplementary material).

Although the epithelial cells express Bmp7 and Bmpr1a from at least E10.5 to E13.5, BRE-lacZ-positive cells were only observed from E14.5. We hypothesized that the absence of BMP signaling in the epithelium is due to extracellular BMP inhibitors that block ligand-receptor interaction. Nog is expressed in the dorsal E9.5 foregut (Li et al., 2007; Que et al., 2006). This expression pattern was maintained as the separation proceeds at ~E10.5-11.5, and Nog-lacZ expression was restricted to a subpopulation of epithelial cells in the esophagus and in the dorsal forestomach (Fig. 2A-C). Although at E13.5 Nog-lacZ expression was very low in the esophageal epithelium, Nog transcripts could still be detected by in
of the E12.5 forestomach at a moderate level, which contrasts with the very low levels found in the esophagus (see Fig. S1O,P in the supplementary material).

**Ectopic activation of BMP signaling in the epithelium**

Previously, we reported esophageal atresia and tracheoesophageal fistula (EA/TEF) in nearly 60% of Nog-null mutants (Que et al., 2006). In the remaining 40% of mutants, the foregut separates normally. However, in the E18.5 dorsal esophagus we found multiple abnormal gland-like pits lined with simple columnar epithelium (Fig. 2L-Q; n=12). The epithelial cells lining the pits were positive for Alcian Blue staining, which is indicative of mucus production (Fig. 2M,M'), and the number of p63-positive cells was reduced compared with the wild type (Fig. 2P-Q'). No expression of the tracheal transcription factor Nkx2.1 or the Clara cell marker Scgb1a1 was detected (data not shown), excluding the possibility that the cells have acquired a respiratory identity. It is noteworthy that there were no abnormalities in the forestomach of Nog-null mutants at any stage. Here, other BMP inhibitors in the forestomach, such as Fst, might play a compensatory role in the event of Nog deletion (see Fig. S1P in the supplementary material).

Our results suggest that the formation of a multilayered epithelium from a simple columnar epithelium between E10.5 and E14.5 is inhibited by the loss of Nog and by the resultant increase in canonical BMP signaling. To further explore this idea, we used a combination of a new conditional Rosa26 allele that incorporates constitutively active Bmpr1a (Bmpr1aCA) (see Fig. S2A in the supplementary material) and a Shh-Cre allele that is preferentially expressed in the ventral esophagus and forestomach between E10.5 and E11.5 (Harris-Johnson et al., 2009) (Fig. 3A). By E13.5, most of the epithelial cells in these tissues of Shh-Cre;R26R embryos were labeled by X-gal staining, suggesting that the recombination later extends dorsally (Fig. 3B). All of the Shh-Cre;Bmpr1aCA/+ mutants died before P5, probably owing to respiratory distress (J.Q. and B.L.M.H., unpublished). The activation of BMP signaling was confirmed by pSmad1/5/8 staining, which showed nuclear signals in most epithelial cells of the E15.5 mutant esophagus and forestomach, as compared with the control (see Fig. S2B-E in the supplementary material).

Examination of the E15.5 Shh-Cre;Bmpr1aCA/+ mutant revealed clusters of simple columnar epithelial cells along the esophagus, preferentially in the ventral region (Fig. 3C-3; n=9). These simple epithelial cells maintained a high level of Krt8, whereas the number of p63-positive cells was apparently reduced (Fig. 3F). Moreover, the simple columnar epithelium was negative for Fst, Krt14, and by the resultant increase in BMP signaling. Our results suggest that the formation of a multilayered epithelium from a simple columnar epithelium between E10.5 and E14.5 is inhibited by the loss of Nog and by the resultant increase in canonical BMP signaling. To further explore this idea, we used a combination of a new conditional Rosa26 allele that incorporates constitutively active Bmpr1a (Bmpr1aCA) (see Fig. S2A in the supplementary material) and a Shh-Cre allele that is preferentially expressed in the ventral esophagus and forestomach between E10.5 and E11.5 (Harris-Johnson et al., 2009) (Fig. 3A). By E13.5, most of the epithelial cells in these tissues of Shh-Cre;R26R embryos were labeled by X-gal staining, suggesting that the recombination later extends dorsally (Fig. 3B). All of the Shh-Cre;Bmpr1aCA/+ mutants died before P5, probably owing to respiratory distress (J.Q. and B.L.M.H., unpublished). The activation of BMP signaling was confirmed by pSmad1/5/8 staining, which showed nuclear signals in most epithelial cells of the E15.5 mutant esophagus and forestomach, as compared with the control (see Fig. S2B-E in the supplementary material).

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The simple columnar epithelium was present only on the ventral side of the esophagus of Shh-Cre;Bmpr1aCA/+ mutants, which correlated with the ventral activation of Shh-Cre before E12.5 (Fig. 3A). At E13.5, even though Shh-Cre was expressed on the dorsal side of the esophagus (Fig. 3B), the resultant ectopic BMP signaling was incapable of reversing the stratified epithelium back to a monolayer (Fig. 3D,H), suggesting that there is a limited time...
period during which the fate of the epithelium can be influenced by ectopic signals. Similar observations have been reported in the chick gizzard, where ectopic Bmp2 can only switch the luminal to glandular epithelium within a certain time window (Yasugi and Mizuno, 2008).

**Conditional deletion of Bmpr1a in the forestomach epithelium**

After E14.5 the BMP pathway is active in the differentiating suprabasal cells of the esophagus and forestomach (see Fig. S1C-H in the supplementary material). To address whether BMP signaling is necessary for the differentiation of the epithelium, we deleted Bmpr1a by generating Shh-Cre;Bmpr1a<sup>flox/</sup><sup>fl</sup> and Shh-Cre;Bmpr1a<sup>flox/null</sup> mutants. Since similar phenotypes were observed in the Shh-Cre;Bmpr1a<sup>flox/</sup><sup>fl</sup> and Shh-Cre;Bmpr1a<sup>flox/null</sup> mutants, subsequent analyses employed the former. Most mutants died in utero or at birth, presumably owing to heart defects, but a few did survive to P9 \((n=3)\). The loss of Bmpr1a transcripts in the mutant esophagus and forestomach at E15.5 was confirmed by in situ hybridization (see Fig. S2H-K in the supplementary material).

In the esophageal epithelium of the E18.5 and surviving P9 mutants, expression of p63 persisted in the upper layers (Fig. 4A,B; data not shown), suggesting that these cells are not fully differentiated. There was no involucrin or loricrin immunostaining in the mutant epithelium, but even the wild-type epithelium expressed low levels of these proteins at this stage (Fig. 4C,D; data not shown). By contrast, the loss of Bmpr1a in the forestomach led to a more obvious defect in the differentiation of suprabasal cells (Fig. 4E-L). The affected regions remained p63 positive, but lacked involucrin and loricrin (Fig. 4I-L). In addition, some of these p63-positive cells remained proliferative, as indicated by phosphohistone H3 staining (see Fig. S2L,M in the supplementary material). These results suggest that BMP signaling is required for the differentiation of the suprabasal cells in the esophagus and forestomach. Interestingly, other studies have shown that the inhibition of BMP signaling by overexpression of Nog under a Krt5 promoter in the epidermis also results in alterations in eyelid epithelial differentiation, and the levels of involucrin and loricrin are reduced (Sharov et al., 2003). Conversely, the addition of Bmp4 to cultured human oral epithelium increases the expression of involucrin and loricrin (Kim et al., 2006). These studies suggest that BMP signaling regulates the differentiation of suprabasal cells in stratified epithelium.

In conclusion, our results suggest that BMP signaling has two distinct functions in the development of the mouse esophagus and forestomach (Fig. 4M). Initially, between E10.5 and E14.5, the inhibition of BMP signaling by Nog is necessary for epithelial stratification to occur. The deletion of Nog or expression of constitutively active BMP receptor maintains a simple columnar epithelium. Then, starting at ~E14.5, BMP signaling is required for the differentiation of the suprabasal cells. In the absence of Bmpr1a, the epithelial stratification does occur but the suprabasal cells do not fully differentiate. In this study, we have only investigated the role of BMP signaling in the embryonic and early development of the mouse esophagus and forestomach.
epithelium. Then, after E14.5-15.5, active BMP signaling is required to allow the stratification of simple columnar to multilayered epithelium. BMP signaling, as mediated by an inhibitor such as noggin, is necessary to prevent abnormal epithelial stratification. Related esophagitis have been associated with multiple pathways, including abnormal BMP and Shh signaling. In humans, the pathological condition Barrett’s esophagus and the related esophagitis have been associated with multiple pathways, including abnormal BMP and Shh signaling.

BMP in esophagus/forestomach development

**Fig. 4. Deletion of Bmpr1a inhibits differentiation of the esophageal and forestomach epithelium.** (A-D) Longitudinal sections of the E18.5 Shh-Cre control and Shh-Cre:Bmpr1a^floxed^ mutant mouse esophagus show a multilayered stratified epithelium with p63-positive cells in the upper layers of the mutant. Note that involucrin (Inv) is absent from both control and mutant esophagus. (E-H) Longitudinal sections of E18.5 Shh-Cre:Bmpr1a^floxed^ mutant and control forestomach stained with H&E. G and H are high magnifications of the boxed regions in E and F, respectively. (I-L) Immunohistochemistry shows that the mutant forestomach maintains a multilayered p63-positive epithelium that fails to express the suprabasal markers loricrin (Lor) and involucrin, whereas the control restricts p63 staining to the basal layer and expresses loricrin and involucrin in the suprabasal layers. (M) Model proposing that between E10.5 and E14.5 the inhibition of BMP signaling, as mediated by an inhibitor such as noggin, is necessary to allow the stratification of simple columnar to multilayered epithelium. Then, after E14.5-15.5, active BMP signaling is required for differentiation of the suprabasal cells. Scale bars: 50 μm.

postnatal development of the esophagus and forestomach. In humans, the pathological condition Barrett’s esophagus and the related esophagitis have been associated with multiple pathways, including abnormal BMP and Shh signaling (Chang et al., 2007; Milano et al., 2007; Wang et al., 2010). In the future, it will be important to determine the role of the BMP signaling pathway in the steady-state maintenance of the normal esophageal epithelium and in various injury and repair models.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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**References**


