TGF-β signaling inhibits canonical BMP signaling pathway during palate development

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Abstract

During early palate development, gene expression and regulation exhibit heterogeneity along the anterior-posterior axis. Transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) signaling pathways play essential roles in secondary palatal formation but the exact relationship between the TGF-β and BMP pathways in palate development remains unknown. Here, we demonstrate that, during early secondary palate development, phospho-(p)Smad1/5/8 is highly expressed in the anterior palate but relatively lowly expressed in the posterior palate. Conversely, pSmad2/3 has a lower expression level in the anterior palate than in the posterior palate. With the *BRE-Gal* reporter, we found that the canonical BMP signaling pathway was not activated in the anterior palate but exhibited a moderate level in the posterior palate. Co-immunoprecipitation revealed that Smad4 bound to pSmad1/5/8 only in the posterior palate and not in the anterior palate. Knocking-out of *Tgfbr2 (Wnt1-Cre;Tgfbr2^{f/f};BRE)* in the palatal mesenchyme enhanced canonical BMP activity in the posterior palate but not in the anterior palate, because of decreased pSmad2/3. pSmad1/5/8-Smad4 complexes were found to be dramatically increased in posterior palatal mesenchymal cells at embryonic day 13.5 cultured in the presence of SB525334. Proximity ligation assay also showed pSmad1/5/8-Smad4 complexes were increased in the posterior palate of *Wnt1-Cre;Tgfbr2^{f/f}* mice. Therefore, the reduction of pSmad2/3 level in the palatal mesenchyme of *Wnt1-Cre;Tgfbr2^{f/f};BRE* mice contributes primarily to the increase of pSmad1/5/8-Smad4 complexes leading to enhanced canonical BMP activity in the posterior palate. Moreover, during early development, canonical BMP signaling operates in the posterior palate but is completely absent in the anterior palate. Canonical TGF-β signaling suppresses canonical BMP signaling activity in the posterior palate by competing limited Smad4.

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Keywords

Bone morphogenetic protein; Smad1/5/8; Smad2/3; Transforming growth factor-β; Palate development

Introduction

Signaling by the transforming growth factor β (TGF-β) superfamily, including bone morphogenetic proteins (BMP), is transduced into cells by the binding of ligands to the type I and type II transmembrane serine/threonine kinase complex. With the binding of the ligand, the type II receptor activates the type I receptor by phosphorylation of the type I receptor. The activated type I receptor phosphorylates receptor-activated Smads (R-Smads, including Smad-1, –2, –3, –5, and –8) in the cytoplasm. Canonical (or Smad-dependent) TGF-β or BMP signaling occurs via the phosphorylation of Smad-2/3 or Smad1/5/8, respectively. The phosphorylated Smad-2/3 (pSmad2/3) or Smad1/5/8 (pSmad1/5/8) binds to common Smad (Smad4) and enters the nucleus to interact with other transcription factors and regulates downstream gene expression. In addition to the canonical Smad pathway, TGF-β/BMP receptors can also activate members of the mitogen-activated protein kinase (MAPK) pathway, known as the noncanonical pathway, including the TGF-β activated kinase 1 (TAK1; Yamaguchi et al. 1995). TAK1, a member of the MAPKKK family, was originally identified as a key regulator of MAPK kinase activation in TGF-β signaling pathways (Yamaguchi et al. 1995). Upon activation, TAK1 phosphorylates MKK3/6 directly, leading to the activation of a number of downstream kinases including P38 and JNK kinases (Zhang 2009).

The development of the palate requires a series of interactions between pharyngeal ectoderm-derived epithelium and the cranial neural crest (CNC)-derived mesenchyme. The palate comprises two structures: the primary and the secondary palates. In mice, the development of the secondary palate begins at embryonic day 11.5 (E11.5) with the emergence of bilateral outgrowths, called palatal shelves, on the oral side of the maxillary process. At E12.5-E13.5, the palatal shelves initially grow vertically flanking the developing tongue. Around E14.0, the bilateral palatal shelves reorient to the horizontal positon above the tongue. Subsequently, two palatal shelves grow towards and finally fuse with each other at the midline to form the intact roof of the oral cavity (Liu et al. 2008).

The mammalian secondary palate is divided anatomically into the anterior bony region (hard palate) and the posterior muscular region (soft palate), each of which have specialized functions in occlusion, speech and swallowing. Consistent with the morphological differences in the anterior and posterior palate, previous studies have clearly demonstrated the molecular heterogeneity along the anterior-posterior (AP) axis of the developing secondary palate (Hilliard et al. 2005; Liu et al. 2008). During early palate development, several critical signaling molecules and transcription factors exhibit regional differential expression along the AP axis. For example, the expression of the *Shox2, Msx1* and *Fgf10* genes is restricted to the anterior palatal mesenchyme, whereas the *Bmp3, Barx1, Mn1, Meox2* and *Tbx22* genes are preferentially expressed in the posterior palatal mesenchyme

(Alappat et al. 2005; Barlow et al. 1999; Li and Ding 2007; Liu et al. 2008; Nie 2005; Rice et al. 2004; Yu et al. 2005; Zhang et al. 2002). In addition, anterior and posterior palatal mesenchymal cells exhibit distinct responses in terms of gene expression and cellular behavior to growth factors. For example, exogenous BMP is able to induce *Msx1* and *Bmp4* expression in the anterior palate but not in the posterior palate, whereas FGF8 induces *Pax9* expression in the posterior but not in the anterior palatal mesenchyme (Hilliard et al. 2005; Zhang et al. 2002). All these investigations indicate that the regulation of palate development is the result of distinct signaling and genetic networks in the anterior and the posterior regions of the palate.

Gene inactivation studies in mice have demonstrated that the TGF-β superfamily plays essential roles in secondary palatal formation, including *Bmp4, Bmpr1a, Tgfb2, Tgfb3* and *Tgfbr2*, indicating that the TGF-β and BMP pathways interact to regulate palate development (Baek et al. 2011; Ito et al. 2003; Kaartinen et al. 1995; Li et al. 2011; Liu et al. 2005; Rice et al. 2004; Zhang et al. 2002). However, the exact relationship between the TGF-β and BMP pathways during secondary palate development remains unknown.

Here, we provide evidence that, during early secondary palate development, the canonical BMP signaling pathway is not activated in the anterior palate but is involved in posterior palate development. TGF-β inhibits the canonical BMP signaling pathway in the posterior palate region but not in the anterior region.

Materials and methods

Animals and embryo collection

The generation of BRE-Gal (BRE), Wnt1-Cre, Tgfbr2$^{f/+}$ and *Tak1*$^{f/+}$ lines have been described previously (Chytil et al. 2002; Danielian et al. 1998; Javier et al. 2012; Liu et al. 2006). *Wnt1-Cre;Tgfbr2*$^{f/f}$*;BRE* mice and *Wnt1-Cre;Tak1*$^{f/f}$*;BRE* mice were generated by mating *Wnt1-Cre;Tgfbr2*$^{f/+}$ with *Tgfbr2*$^{f/+}$*;BRE* mice and by mating *Wnt1-Cre;Tak1*$^{f/+}$ with *Tak1*$^{f/+}$*;BRE*, respectively. All wild-type mice were on a CD-1 or C57BL6/J background. Embryos were collected from timed-mated females and subjected to genotyping based on the polymerase chain reaction.

Immunofluorescence and X-gal staining

For immunofluorescence, samples were fixed with 4% paraformaldehyde at 4 °C overnight, dehydrated through a graded ethanol series and then processed for paraffin sectioning. Sections were subjected to standard immunofluorescence staining as described previously (He et al. 2010). The following primary antibodies were used: anti-pSmad1/5/8 (Cell Signaling), anti-pSmad2/3 (Santa Cruz Biotechnology), anti-Smad4 (Abcam) and anti-phosphorylated P38 (pP38; R&D Systems). Alexa Fluor 568 or Alexa Fluor 488 (Invitrogen) acted as labels on secondary antibodies. Nuclei were counter-stained with 4,6-diamidino-2-phenylindole (Invitrogen). For X-gal staining, samples were fixed in 4% paraformaldehyde, washed in ice-cold phosphate-buffered saline (PBS), subsequently dehydrated with 30% sucrose/PBS, embedded in O.C.T. (Tissue-Tek) and cryo-sectioned. Standard X-gal staining

was conducted as described previously (Ito et al. 2003). The tissue anterior or posterior to the first molar tooth bud was considered as the anterior or posterior palate, respectively.

Co-immunoprecipitation

Primary cultured palatal mesenchymal cells were lysed in RIPA buffer (Cell Signaling Technology) with protease inhibitor for 15 min at 4 °C. For organs, tissues were digested with 0.25% trypsin (Life Technologies) at 37 °C for 10 min prior to lysis. Lysates were centrifuged at 12,000 rpm at 4 °C for 20 min to remove cellular debris. The supernatant was precleared with 40 μl Dynabeads protein A/G (Life Technologies) at 4 °C for 30 min. Anti-Smad4 antibody or negative control IgG (Santa Cruz Biotechnology, 3 μg each) was added to the precleared lysates following incubation at 4 °C overnight and then with 50 μl Dynabeads protein A/G for 2 h to precipitate immunocomplexes. Beads were washed three times with wash buffer (10 mM TRIS-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40 and 2 mM EDTA). Of the sample buffer, 50 μl were added to the beads and samples were heated to 95 °C for 10 min. pSmad1/5/8 or pSmad2/3 were detected in the protein complex by Western blot.

Western blot

Proteins were separated with SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk for 30 min at room temperature and then incubated with antibodies against pSmad1/5/8 or aD-glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology) overnight at 4 °C with constant rocking. The membrane was then washed three times and incubated with IRDye 800 secondary antibody (Li-COR) for 1 h. Immunoreactive bands were visualized with the Odyssey imaging system (Li-COR).

Primary palatal mesenchymal cell culture

E13.5 embryonic posterior palate was dissected out and incubated with 2 units/ml Dispase (Life Technologies) in PBS for 30 min at room temperature. Palatal epithelium was removed from mesenchyme with the aid of fine forceps. The isolated mesenchyme was then digested with 0.25% trypsin at 37 °C for 10 min and dissociated into a single-cell suspension by gentle pipetting. Cells were plated onto a 6-well plate and cultured in DMEM (Life Technologies) supplemented with 20% FBS (Life Technologies) at 37 °C. For the blocking of TGF-β/pSmad2/3 signaling, cells were treated with SB525334 (Tocris Bioscience) at a final concentration of 10 μM and were harvested after 24 h after culture.

In situ proximity ligation assay

Endogenous pSmad1/5/8-Smad4 and pSmad2/3-Smad4 complexes in the posterior palate were detected by using an in situ proximity ligation assay (PLA) kit (Duolink kit, Sigma-Aldrich) as described previously (Yang et al. 2014). Briefly, tissue sections were blocked with blocking reagent and incubated with primary antibodies (anti-Smad4 and anti-pSmad1/5/8 or anti-pSmad2/3 antibodies) overnight at 4 °C. Then, sections were incubated with secondary antibodies conjugated with oligonucleotides (anti-mouse PLA probe Minus and Anti-rabbit PLA probe Plus). Ligation and amplification were performed according to

the protocol provided by the kit. PLA signals were detected under a fluorescence microscope.

Data quantification and statistical analysis

All experiments were repeated at least three times and at least three samples were collected for each transgenic or mutant mouse embryo. Histological immunofluorescence intensity was analyzed from three continuous sections of each sample with Image J software. Statistical analysis was performed by using Student's *t*-test.

Results

Expression pattern of down-stream mediators of TGF-β/BMP signaling pathway in early developing palate

To investigate the differential engagement of TGF and BMP signaling in palate development, we first examined the expression of TGF-β/BMP signaling down-stream mediators, including pSmad1/5/8, pSmad2/3, Smad4 (Smad-dependent) and pP38 (Smad-independent), in early developing palatal shelves along the AP axis. Immunofluorescent results showed that pSmad1/5/8 and pP38 were highly expressed in the anterior palate but were expressed at a relatively low level in the posterior palate (Fig. 1a–h). Conversely, pSmad2/3 showed a lower expression level in the anterior palate but a higher level of expression in the posterior palate (Fig. 1c, d). For Smad4, comparable expression levels were detected in the anterior and the posterior palatal shelves (Fig. 1e, f).

Canonical BMP signaling is operating in developing posterior palate but not in anterior palate

We next attempted to map the exact activity of the canonical BMP signaling pathway in the developing palate. We took advantage of a reporter mouse line harboring seven copies of *BRE* and the *Id3* minimal promoter with LacZ reporter *(BRE-Gal)*, which has been shown to serve as a faithful indicator of BMP canonical signaling activity in developing mouse embryo (Javier et al. 2012). To our surprise, this *BRE-Gal* reporter failed to show canonical BMP activity in the anterior palate but revealed a moderate level of canonical BMP activity in the posterior palate (Fig. 2a–d), suggesting that canonical BMP signaling is not operating in the anterior palate.

Because activation of the BRE reporter requires the formation of the pSmad1/5/8-Smad4 complex (Javier et al. 2012), we then set out to determine whether the failed activation of *BRE-Gal* in the anterior palate was attributed to the lack of pSmad1/5/8-Smad4 complex formation by conducting a co immunoprecipitation assay. Eye tissue was used as a positive control, as it exhibited a high level of canonical BMP activity (Yang et al. 2014). Indeed, using Smad4 antibody, we were able to precipitate abundant pSmad1/5/8 in posterior palate and eye tissue but pSmad1/5/8 precititated from the anterior palate were barely detectable by Western blot (Fig. 2e). These results indicate that Smad4 binds to pSmad1/5/8 only in the posterior palate but not in the anterior palate. Furthermore, we found amounts of pSmad2/3-Smad4 complex both in the anterior and in the posterior palate (Fig. 2e).

TGF-β signaling inhibits canonical BMP signaling activity in posterior palate

We reported previously that TGF-β signaling suppressed canonical BMP signaling activity in the early developing tooth germ (Yang et al. 2014). In view of the expression of pSmad2/3 and the abundance of pSmad2/3-Smad4 complex in both the anterior and posterior palates, we tested whether the same mechanism exists in the developing palate. To do this, we generated mice carrying compounded *BRE-Gal* and *Wnt1-Cre;Tgfbr2^{f/f}* alleles. We examined *BRE-Gal* activity in the palatal mesenchyme in the absence of *Tgfbr2*. Surprisingly, although canonical BMP activity remained undetectable in the anterior palatal shelves, significantly enhanced canonical BMP activity was indeed found in the posterior palate (Fig. 3a, b), indicating that TGF-β signaling attenuates canonical BMP signaling activities. To confirm further that the enhanced canonical BMP activity in the posterior palatal shelves of *Wnt1-Cre;Tgfbr2^{f/f};BRE* mice was correlated with the absence of TGF-β signaling, the expression levels of pSmad1/5/8, pSmad2/3, Smad4 and pP38 were examined in the mutant mice by immunofluorescent staining. pSmad1/5/8 and Smad4 showed no obvious changes either in the anterior or in the posterior palate as compared with wild-type controls (Fig. 3c–h). However, as expected, the level of pSmad2/3 was remarkably downregulated in the mesenchyme of both the anterior and the posterior palate (Fig. 3e, f). The expression level of pP38, a downstream target of TGF-β signaling, was also dramatically decreased in both the anterior and the posterior palates (Fig. 3i, j).

TAK1, a key kinase activator of P38, is recognized as an important regulator of Smad-independent TGF-β signaling in palate development and is expressed in the anterior and posterior domains of E13.5 palatal shelves (Rice et al. 2004; Song et al. 2013). To exclude the possibility that the elevated canonical BMP activity in the posterior palate was the consequence of a dramatically reduced pP38 level in *Wnt1-Cre;Tgfbr2^{f/f};BRE* mice, we generated *Wnt1-Cre;Tak1^{f/f};BRE* mice and examined *BRE-Gal* activity in the palatal shelves. As shown in Fig. 4, the deletion of *Tak1* in the palatal mesenchyme did not result in an increased canonical BMP activity such as that observed in *Wnt1-Cre;Tgfbr2^{f/f};BRE* mice (Fig. 4a–d).

We thus assumed that reduced/absent pSmad2/3 in the palatal mesenchyme of *Wnt1-Cre;Tgfbr2^{f/f};BRE* mice contributed primarily to increased pSmad1/5/8-Smad4 complexes leading to enhanced canonical BMP activity in the posterior palate. To test this hypothesis, we first examined and detected the co-localization of pSmad15/8 and pSmad4 in primary posterior palatal mesenchymal cells (Fig. 4e–e"). Then, we used in vitro primary palatal cell culture with SB525334, a widely used selective inhibitor of TGF-β receptor I (ALK5) that can specifically block phosphorylation of Smad2/3 induced by TGF-β1 (Grygielko et al. 2005). E13.5 posterior palatal mesenchymal cells were isolated and placed in cell culture in the presence/absence of SB525334. The inhibition efficiency was assessed by detecting the expression of pSmad2/3 (data not show). As determined by the co-immunoprecipitation assay shown in Fig. 4f, with the decreased pSmad2/3-Smad4 complex, the amount of pSmad1/5/8-Smad4 complex dramatically increased in the cells treated with SB525334 as compared with the control cells, demonstrating that the enhanced canonical BMP activity in the posterior palate of *Wnt1-Cre;Tgfbr2^{f/f};BRE* mice is indeed attributable to increased pSmad1/5/8-Smad4 complexes. To confirm this deduction further, we detected the

pSmad1/5/8-Smad4 and pSmad2/3-Smad4 complexes in a tissue section by using in situ PLA. As expected, no pSmad2/3-Smad4 complexes but an increased level of pSmad1/5/8-Smad4 complexes was detected in the posterior palate of *Wnt1-Cre;Tgfbr2^{f/f}* mice compared with wild-type mice (Fig. 4g–j). Taken together, our results demonstrate that canonical TGF-β signaling suppresses canonical BMP signaling activity in the posterior palate by competing limited Smad4.

Discussion

In the present study, we revealed the differential regulatory mechanisms of TGF-β/BMP signaling in the anterior and the posterior portions of the developing palate. Canonical BMP signaling operates in the posterior palate, although at a moderate level but is completely absent in the anterior palate during early development. Inactivation of TGF-β signaling is able to enhance canonical BMP signaling in the posterior palate but surprisingly not in the anterior palate.

In the developing palate, several *Bmp* genes are expressed in dynamic and differential patterns along the AP axis and BMP signaling has been shown to regulate cell proliferation in the anterior palatal mesenchyme and to maintain palatal epithelial integrity in the posterior portion (Bush and Jiang 2012; He et al. 2010; Hilliard et al. 2005; Zhang et al. 2002). In the early developing palatal shelves, both *Bmp2* and *Bmp4* transcripts are present and *Bmp3* is moderately expressed at the posterior palate at E13.5 (Nie 2005). At E12.5 and E 13.5, *Bmpr1a* is highly expressed in the anterior palate and weakly expressed in the posterior palate, whereas *Bmpr1b* is only expressed in the anterior palate but not in the posterior palate (Baek et al. 2011; Li et al. 2011). The presence of BMP ligand or BMP signaling components is not an unequivocal indicator of BMP signaling activity (Javier et al. 2012). BRE has been identified as a regulatory sequence activated by canonical BMP signaling in various BMP target genes, including the *Xeno-pus id3* and *ventx2* genes and the *Drosophila brk* gene (von Bubnoff et al. 2005; Yao et al. 2006). BRE is recognized by Smad1/5-Smad4 complex in response to the activated BMP type I receptor (Katagiri et al. 2002), which makes BRE a faithful indicator for the transcriptional response of cells to the canonical BMP signaling. In the present investigation, we found no BRE activation in the anterior palate because of the absence of pSmad1/5/8-Smad4 complexes. However, abundant pSmad1/5/8-Smad4 complexes have been detected in the posterior palate, which is capable of activating BRE. The reason for these different responses to BMP signaling might be the different expression levels of BMP and pSmad1/5/8 in the anterior and the posterior palates. High levels of BMP and pSmad1/5/8 have been determined in the anterior palate as compared with those in the posterior palate (Nie 2005; Parada and Chai 2012; this study). In some cellular processes that depend on low levels of BMP signaling, Smad4 is essential for R-Smads to maximize their full function, probably by forming an obligate heterotrimer with R-Smads. However, in other cellular processes that have high levels of BMP signaling, abundant phosphorylated R-Smads can be formed. Under these conditions, R-Smads can still activate their downstream targets and Smad4 appears to be less important (Tong and Kwan 2013). In the posterior palate, pSmad1/5/8 are at a lower expression level and need the help of Smad4 to transduce BMP signaling, which activates the canonical BMP signaling. Furthermore, the lower level of pSmad1/5/8 makes this canonical BMP signaling more

sensitive to TGF-β signaling activity. Inactivation of TGF-β signaling reduces the pSmad2/3 level and releases Smad4 from pSmad2/3-Smad4 complexes facilitating the formation of Smad1/5/8-Smad4 complexes. This explains the enhanced X-gal staining in the posterior palate of *Wnt1-Cre;Tgfbr2^{f/f};BRE* mice. In the present investigation, we observed that canonical TGF-β signaling suppresses canonical BMP signaling activity in the mesenchymal cells of the posterior palate by competing limited Smad4. The antagonism between TGF-β and BMP signaling has also been reported in several other cells, such as dermal papilla cells, cardiomyocytes and breast cancer cells (Bin et al. 2013; Grönroos et al. 2012; Wang et al. 2012).

We have shown previously that an atypical canonical BMP signaling (Smad1/5/8-dependent but Smad4-independent) pathway operates in the dental mesenchyme during early craniofacial development (Yang et al. 2014). Indeed, this atypical canonical BMP signaling pathway has been implicated in several other developing organs, such as nervous system, lens, bone and heart (Chu et al. 2004; Rajagopal et al. 2009; Tan et al. 2007; Tong and Kwan 2013); it might also operate in the anterior palatal shelves, as evidenced by the presence of high levels of pSmad1/5/8 and the absence of BRE activity and Smad1/5/8-Smad4 complexes. The *Msx1* homeobox gene, a well-established downstream target of BMP signaling, is expressed in developing facial primordia, particularly at the sites where epithelial-mesenchymal interactions occur during organogenesis, including of the tooth and palate (Zhang et al. 2002). *Msx1*-gene-deficient mice exhibit cleft secondary palate and an arrest of tooth development at the bud stage (Houzelstein et al. 1997; Satokata and Maas 1994). In the early developing dental mesenchyme, Msx1 is activated by BMP via the atypical canonical BMP (Smad4-independent) signaling pathway (Yang et al. 2014). Our preliminary data also show that the formation of pSmad1/5/8-Smad4 complexes by the overexpression of *Smad4* suppresses *Msx1* expression in dental mesenchymal cells (unpublished data). This might explain the observation that exogenous BMP4 is able to induce *Msx1* expression in the anterior palatal mesenchyme but not in the posterior palatal mesenchyme (Zhang et al. 2002). Thus, in the anterior palate, BMP probably transduces signals through the atypical canonical pathway, which is able to induce *Msx1* transcription. However, in the posterior palate, BMP signaling is transduced by the canonical pathway via the pSmad1/5/8-Smad4 complex, which suppresses the *Msx1* expression in the posterior palate.

The present investigation thus reveals different regulation mechanisms of BMP signaling in the anterior and posterior portions of the early developing palate. In the anterior palate, an atypical canonical BMP signaling pathway may control palate development, because of the absence of the pSmad1/5/8-Smad4 complex. In the posterior palate, the canonical BMP signaling pathway operates and TGF-β signaling attenuates and modulates canonical BMP signaling activity by competing Smad4.

Acknowledgments

Abbreviations

**AP**   Anterior-posterior

BMP   Bone morphogenetic protein

CNC   Cranial neural crest

E   Embryonic day

MAPK   Mitogen-activated protein kinase

p   Phospho

PLA   Proximity ligation assay

R   Receptor-activated

TAK-1   TGF-β activated kinase 1

TGF-β   Transforming growth factor-β

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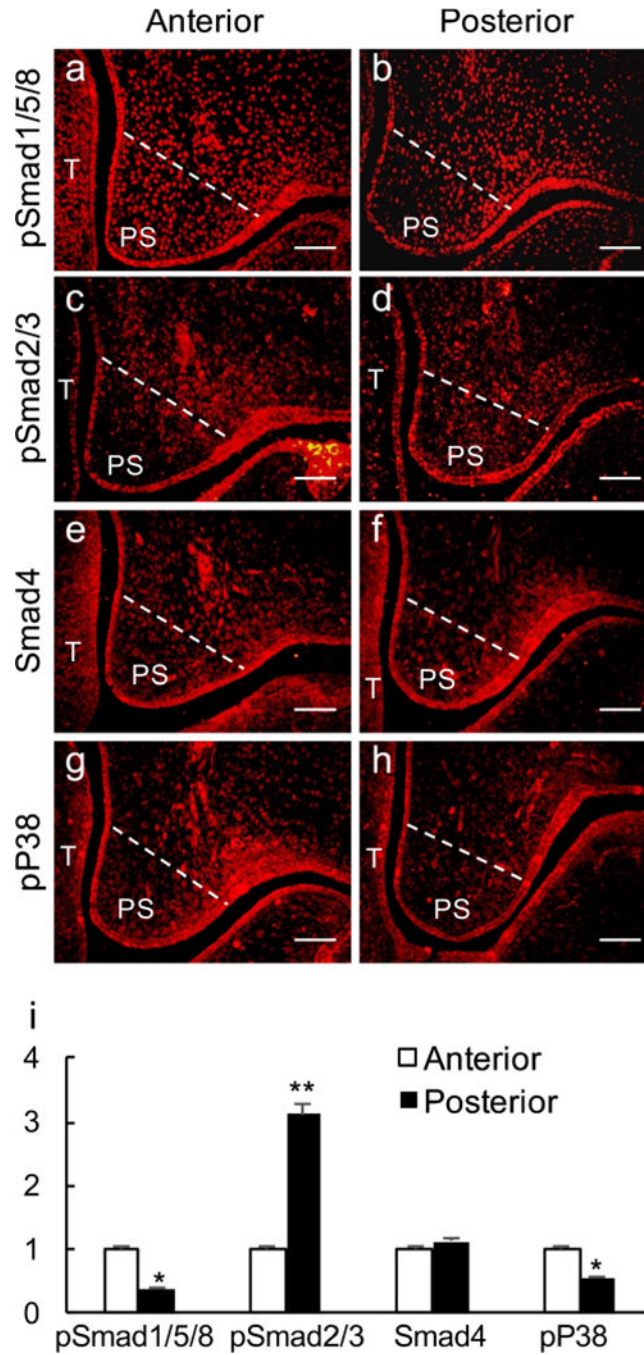
Fig. 1.
Immunofluorescent staining of down-stream mediators of transforming growth factor α (TGF-β)/bone morphogenetic protein (BMP) signaling pathway in the developing palate at E13.5. **a**, **b** pSmad1/5/8; **c**, **d** pSmad2/3; **e**, **f** Smad4; **g**, **h** pP38. **a**, **c**, **e**, **g** Anterior palate; **b**, **d**, **f**, **h** posterior palate. *White lines* demarcate the palatal region for quantifying the immunofluorescence intensity (*T* tongue, *PS* palatal shelf). *Bars* 50 μm. **h** Statistical comparison of immunofluorescence intensity. *$P < 0.05$, **$P < 0.01$
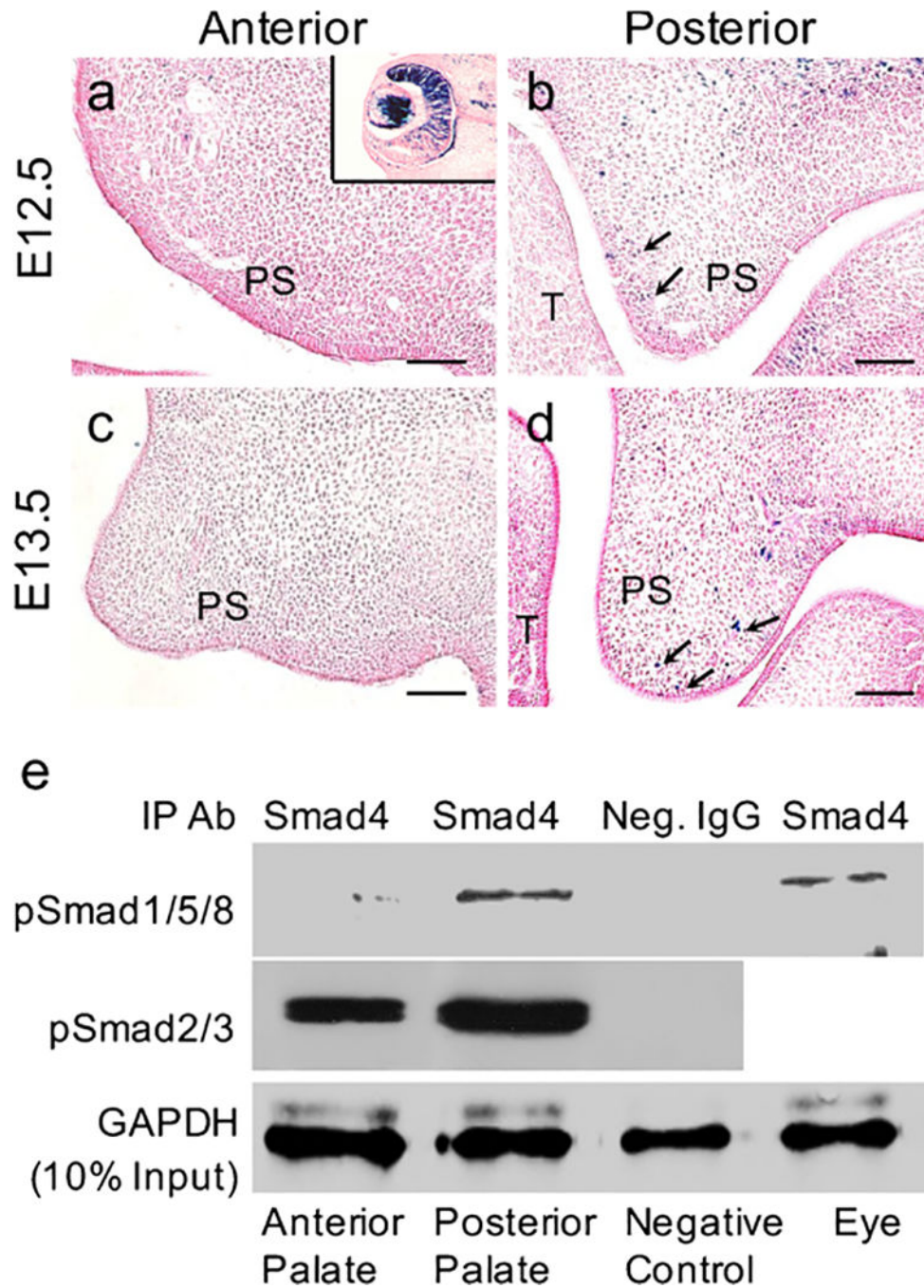
Fig. 2.
Canonical BMP signaling is operating in the posterior but not in anterior portion of the developing palatal shelves. **a-d** X-gal staining of the anterior (**a, c**) and posterior (**b, d**) palate from *BRE-Gal* reporter mice at embryyonic day 12.5 (E12.5; **a, b**) and E13.5 (**c, d**). *Black arrows* indicate positive X-gal Staining (*T* tongue, *PS* palatal shelf). *Insert* in **a** Eye tissue as a positive control for X-gal staining. *Bars* 50 μm. **e** Western blot showing pSmad1/5/8-Samd4 and pSmad2/3-Samd4 complexes in palatal shelves. Eye tissues were included as positive controls for pSmad1/5/8-Samd4 complexes (*IP Ab* immunoprecipitation

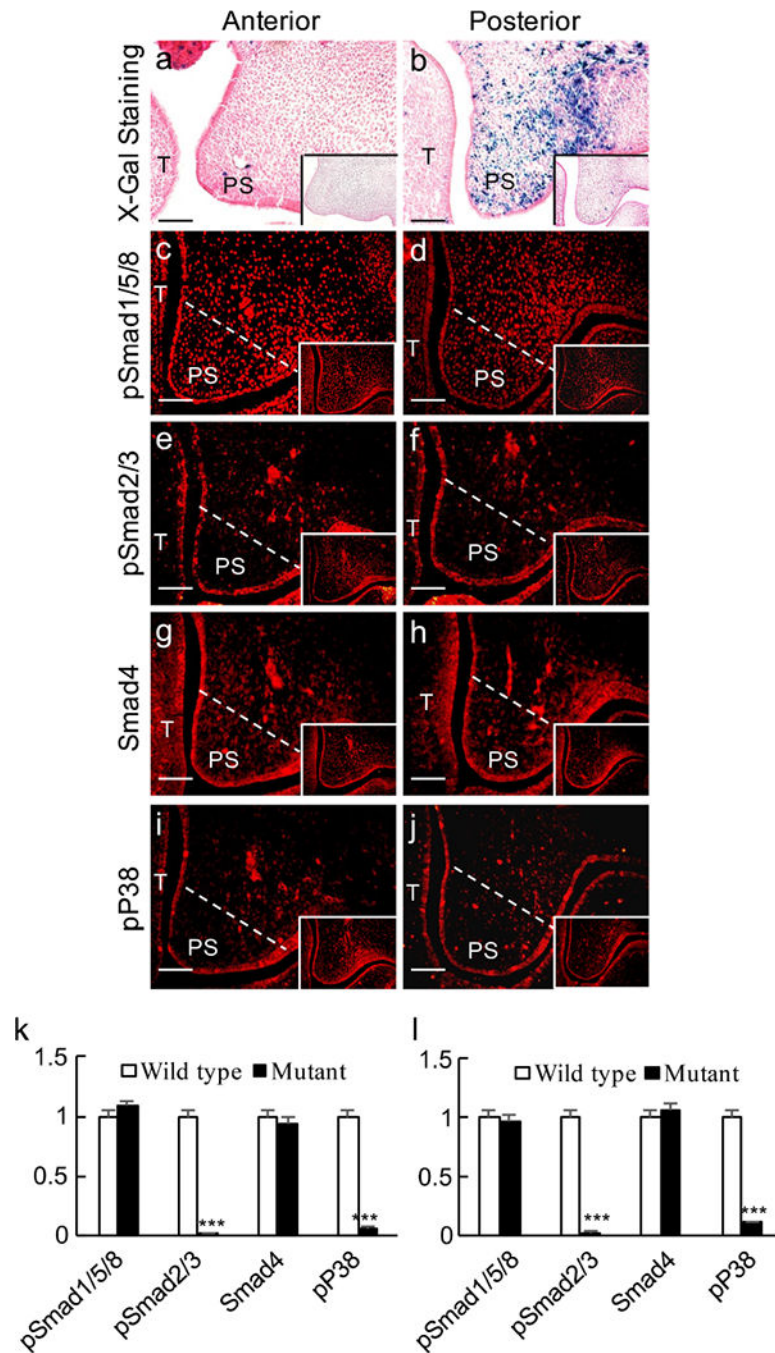antibody, *Neg. IgG* negative control IgG, *GAPDH* D-glyceraldehyde-3-phosphate dehydrogenase)

Fig. 3.
Inactivation of *Tgfbr2* in the palatal mesenchyme enhances canonical BMP activity. **a, b** X-gal staining of the anterior (**a**) and posterior (**b**) palate from an E13.5 *Wnt1-Cre;Tgfbr2^f/f^;BRE* embryo. **c-j** Immunofluorescence staining of pSmad1/5/8 (**c, d**), pSmad2/3 (**e, f**), Smad4 (**g, h**) and pP38 (**i, j**) in the anterior (**a, c, e, g, i**) and posterior (**b, d, f, h, j**) palate from E13.5 *Wnt1-Cre;Tgfbr2^f/f^;BRE* embryos. *White lines* demarcate the palatal region for quantifying the immunofluorescence intensity (*T* tongue, *PS* palatal shelf).
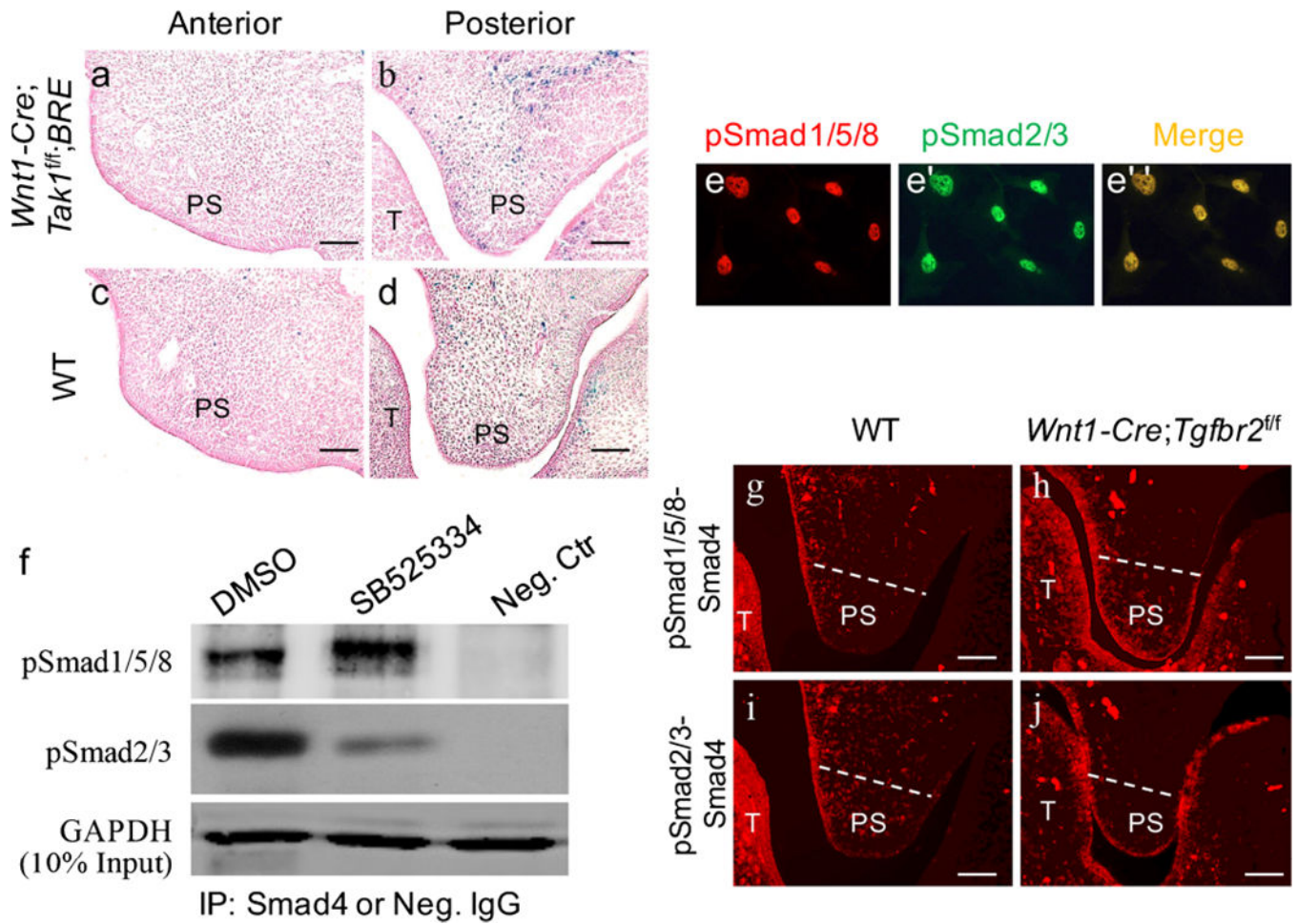
Inserts Wild-type controls. *Bars* 50 μm. **k, l** Statistical comparison of immunofluorescence intensity. ***P < 0.001

Fig. 4.
Inhibition of TGF-β/Smad2/3 signaling induces pSmad1/5/8-Smad4 complex formation. **a-d** X-gal staining of the anterior (**a**, **c**) and posterior (**b**, **d**) palatal shelves from E12.5 *Wnt1-Cre;Tak1^{f/f};BRE* (**a**, **b**) and wild-type (**c**, **d**) embryos (*T* tongue, *PS* palatal shelf). **e–e"** Immunofluorescence showing the co-localization of pSmad1/5/8 and pSmad2/3 in cultured primary posterior palatal mesenchymal cells from E13.5 wild-type embryo. **f** Western blot showing a significantly increased level of pSmad1/5/8-Smad4 complexes after treatment with SB525334 (*Neg. Ctr* negative control, *IP* immunoprecipitation, *Neg. IgG* negative control IgG). **g-j** In situ proximity ligation assay shows pSmad1/5/8-Smad4 (**g**, **h**) and pSmad2/3-Smad4 (**i**, **j**) complexes in the posterior palate of E13.5 wild-type mice (**g**, **i**) and *Wnt1-Cre;Tgfbr2^{f/f}* mice (**h**, **j**). *Bars* 50 μm