Published in final edited form as:

Genesis. 2018 March ; 56(3): e23093. doi:10.1002/dvg.23093.

Compound mutations in *Bmpr1a* and *Tak1* synergize facial deformities via increased cell death

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Abstract

BMP signaling plays a critical role in craniofacial development. Augmentation of BMPR1A signaling through neural crest-specific expression of constitutively active *Bmpr1a* (*caBmpr1a*) results in craniofacial deformities in mice. To investigate whether deletion of *Tak1* may rescue the craniofacial deformities caused by enhanced Smad-dependent signaling through *caBMPR1A*, we generated embryos to activate transcription of *caBmpr1a* transgene and ablate *Tak1* in neural crest derivatives at the same time. We found that deformities of the double mutant mice showed more severe than those with each single mutation, including median facial cleft and cleft palate. We found higher levels of cell death in the medial nasal and the lateral nasal processes at E10.5 in association with higher levels of p53 in the double mutant embryos. We also found higher levels of pSmad1/5/9 in the lateral nasal processes at E10.5 in the double mutant embryos. Western analyses revealed that double mutant embryos showed similar degrees of upregulation of pSmad1/5/9 with *caBmpr1a* or *Tak1*-cKO embryos while the double mutant embryos showed higher levels of phospho-p38 than *caBmpr1a* or *Tak1*-cKO embryos at E17.5, but not at E10.5. It suggested that deletion of *Tak1* aggravates the craniofacial deformities of the *caBmpr1a* mutants by increasing p53 and phospho-p38 at different stage of embryogenesis.

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Authors' roles: Study design: XL, SH, HP, HS and YM. Study conduct, data collection, and data analysis: XL, SH, HP and YM. Provide critical materials: HP and YM. Drafting manuscript: XL, SH, HP and YM. Approving final version of manuscript: XL, SH, HP, HS, and YM. HP and YM take responsibility for the integrity of the data analysis.

Keywords

facial prominence; apoptosis; Smad signaling; MAP kinase; nasal septum

INTRODUCTION

BMPs are the members of the transforming growth factor- β (TGF- β) superfamily, first identified the ability to induce ectopic bone formation in muscles (Urist 1965). BMPs bind to cell surface receptor serine/threonine kinases and then trigger intracellular signaling cascades (Shi and Massague 2003). BMPs signal through two types of receptors, type I receptor (ACVR1 (ALK2), BMPR1A (ALK3) and BMPR1B (ALK6)) and type II receptor (BMPRII, ACTRIIA and ACTRIIB). Upon ligand binding, the type II receptors phosphorylate the type I receptors to activate their kinase activity. In turn, two intracellular downstream pathways, Smad-dependent and Smad-independent pathway are activated. In Smad-dependent pathway, type I receptors phosphorylate and activate receptor-Smads (rSmads), primarily Smad1, Smad5 and Smad9. Phosphorylated Smads associate with common-Smad (Smad4) to form a complex that is able to translocate into the nucleus where it regulates the transcription of downstream targets gene. In Smad-independent pathways, several signaling cascades including mitogen-activated protein kinase (MAPK) pathway are activated (Yamaguchi et al., 1995; Lou et al., 2000).

BMP signaling plays a critical role in embryogenesis and development of organisms including craniofacial development (Kishigami and Mishina 2005; Graf et al., 2016; Wu et al., 2016). Malfunction of components of BMP signaling causes the congenital disorders and deformities in craniofacial region. BMP4 regulates lip fusion, BMP4 deficiency in the ectoderm of the nasal processes and maxillary process results in isolated cleft lip (Liu et al., 2005). Deficient *Bmpr1a* in oral ectoderm leads to cleft lip and palate in mouse model (Liu et al., 2005). Lacking of *Acvr1* in the neural crest causes craniofacial defects including cleft palate probably owing to a hypotrophic mandible, failure of frontal and zygomatic bone formation (Dudas et al., 2004). Deletion of *Msx1* or *Msx2*, downstream of BMP signaling, leads to the skull defects (Antonopoulou et al., 2004; Chung et al., 2010). Tissue specific inactivation of *Bmpr1a* in palatal mesenchyme leads to short snout and cleft palate (Baek et al., 2011). A gain-of-function mutation in *Bmpr1a* leads to cleft palate and delayed tooth differentiation when activated in neural crest derived tissues (Li et al., 2013). We previously reported that augmentation of BMPR1A signaling through neural crest-specific expression of constitutively active *Bmpr1a* (*caBmpr1a*) results in craniofacial deformities in mice such as craniosynostosis, short, broad snouts and orbital hypertelorism (Yamauchi et al., 1999; Komatsu et al., 2013). About half of the mutant mice show neonatal lethality with abnormal nasal cartilage structures (Komatsu et al., 2013; Hayano et al., 2015).

In addition to the Smad-dependent pathway, BMP receptors can also activate Smad-independent pathways including MAPK pathways. TGF- β activated kinase 1 (TAK1, aka MAP3K7), a member of the MAPKKK family, is a key regulator of MAPK kinase activation upon ligand binding to BMP type I receptors (Yamaguchi et al., 1995). Upon activation, TAK1 directly phosphorylates MKK3/6 leading to activation of downstream kinases

including p38 and Jun kinases. TAK1 also activates ERK1/2 (aka p44/42 MAPK) (Wu et al., 2016). Phospho-ERK1/2 (pERK1/2) can phosphorylate Smad1 in the linker region, which inhibits the nuclear translocation of pSmad1 (Kretzschmar et al., 1997). These facts suggest that deletion of *Tak1* could increase BMP Smad-dependent signaling activity (Aubin et al., 2004).

TAK1 plays multiple roles in mouse development (Jadrich et al., 2003; Kajino-Sakamoto et al., 2008; Morioka et al., 2012; Takaesu et al., 2012). Global knockout of *Tak1* results in embryonic lethality around E9.5 due to failure of vasculogenesis (Jadrich et al., 2006). Chondrocyte-specific disruption of *Tak1* leads to chondrodysplasia and defects in formation of the elbow and tarsal joints (Shim et al., 2009). Osteoblast-specific disruption of *Tak1* leads to osteopenia due to insufficient activation of p38 MAPK pathway that is required for RUNX2 activation (Greenblatt et al., 2010b). Contrasting to the prediction based on biochemical data (Kretzschmar et al., 1997), decreased levels of BMP Smad-dependent signaling are also observed in these mutants (Greenblatt et al., 2010a). Neural crest-specific disruption of *Tak1* results in a round shaped skull, cleft palate, hypoplastic maxilla and mandible (Song et al., 2013; Yumoto et al., 2013). In these mice ablation of *Tak1* leads to decrease of BMP Smad-independent pathways as well as suppression of the Smad-dependent signaling pathway.

Taken together these facts, we hypothesized that the craniofacial deformities caused by augmented BMPR1A-Smad signaling in neural crest may be rescued by introducing a *Tak1* mutation. We bred several mouse lines to generate embryos to activate transcription of *caBmpr1a* transgene and ablate *Tak1* in neural crest derivatives at the same time by *P0-Cre* transgene. We compared morphological abnormalities, levels of cell proliferation and cell death as well as levels of BMP Smad-dependent and Smad-independent signaling. Unexpectedly, we found that deformities of mice with compound mutations of gain of function in BMPR1A signaling and ablation of *Tak1* were more severe than those with gain of function in BMPR1A signaling, including median facial cleft and cleft palate associated with higher levels of cell death in facial processes and higher levels of phospho-p38 during late gestation. It suggested that deletion of *Tak1* aggravates the craniofacial deformities caused by increased BMP Smad-dependent pathway through BMPR1A through several different mechanisms.

RESULTS

Increased expression of *caBmpr1a* and ablation of *Tak1* in neural crest cells causes the craniofacial abnormalities in newborn mice

First, we analyzed external morphology of 5 different genotypes of mice at newborn stage (Fig. 1A–O). *caBmpr1a;P0-Cre* (*caBmpr1a* mut hereafter) mice showed a shorter and broader snout, a domed shape skull and hypertelorism as we reported previously (Komatsu et al., 2013; Hayano et al., 2015). As reported previously, approximately half of the *caBmpr1a* mut mice displayed gradual abdominal distension and died within 24 hours after birth (Hayano et al., 2015). Among 59 *caBmpr1a* mut mice that we examined here, 31 pups displayed distension and died within 24 hours whereas 26 pups showed no early lethality to develop craniosynostosis at a later stage (Fig. 1P). Most of the *caBmpr1a* mut mice also

carrying neural crest-specific heterozygosity for *Tak1* (*caBmpr1a* mut;*Tak1*-cHet hereafter) mice have similar craniofacial external morphology to *caBmpr1a* mut mice (Fig. 1C, H, M). Among 44 pups examined here, 17 displayed distension and 21 pups displayed craniosynostosis at a later stage. In addition to them, 2 pups displayed median facial cleft, which died within 24 hours after birth (Fig. 1P). Among 47 of double mutant (DM) mice for *caBmpr1a* and *Tak1* (*caBmpr1a;Tak1fx/fx;P0-Cre*), 17 displayed a median facial cleft (Fig. 1D, I, N, P). *Tak1*-cKO (*Tak1fx/fx;P0-Cre*) mice did not show aforementioned craniofacial abnormalities (Fig. 1E, J, O). Many of the *Tak1*-cKO mice died soon after birth (36/45, Fig. 1P) without overt craniofacial morphological defects (Fig. 1E, J, O and data not shown).

To investigate the effect of *caBmpr1a* and deletion of *Tak1* on the calvarial bone formation, skull morphology of newborn mice was assessed after Alizarin Red and Alcian Blue staining. At the newborn stage, the interfrontal suture is patent in control mice (Fig. 1AA). All mutants carrying *caBmpr1a* displayed a wider suture and defects in frontal bone (Fig. 1BB, CC, DD). We measured the area of the calvarial defects found in the forehead region of each genotype (n=5). DM mice appeared the most severe and largest defects ($p<0.05$) (Fig. 1Q). Moreover, nasal bones displayed defects in DM mice (Fig. 1DD, II). The skull base did not show overt abnormalities in five groups at this stage (Fig. 1KK–OO). The results show that increased BMP-Smad signaling through *caBMPR1A* impairs development of the anterior part of skull and ablation of *Tak1* enhances the impairment.

Compound mutations of *caBmpr1a* and ablation of *Tak1* lead to cleft palate and abnormal nasal septum

We next investigate morphology of palate at newborn stage. Control, *caBmpr1a* mut and *Tak1*-cKO mice did not display obvious abnormal palate (Fig. 2A, B, E). Some *caBmpr1a;Tak1*-cHet mut had a complete cleft of secondary palate (Fig. 2C). At an anterior level, the primary palate was formed (Fig. 2C'), however, the secondary palate did not fuse at the midline and nasal septum was short at middle and posterior levels (Fig. 2C'', C'''). Two out of 8 DM mice displayed a complete cleft of secondary palate and 3 out of 8 showed a complete cleft of secondary and primary palates and lips (Fig. 2D, D', D'', D'''). Three out of 8 also displayed a median facial cleft, absence of the nasal septum with an abnormal nasal cavity (Fig. 2D–D'''). Notably, the nasal cavities in the DM mice were smaller and apart to both sides due to the presence of large frontal lobes (Fig. 2D').

Next we made histologic observation at E15.5. Control, *caBmpr1a* mut and *Tak1* cKO mice showed fusion of the nasal septum with bilateral palatal shelves at nasal chondral level (Fig. 2F, G, J). Bilateral palatal shelves were elevated to the position above the tongue but did not fuse each other at the midline and with nasal septum in *caBmpr1a;Tak1*-cHet and DM mice (Fig. 2H, I). At E12.5, there was obvious and continuous precartilaginous primordium of nasal septum and nasal capsule in control and *Tak1*-cKO embryos (Fig. 2K, K', O, O'). Precartilaginous primordium of the nasal septum and nasal capsule were not obvious or disappeared at anterior level in *caBmpr1a* mut, *caBmpr1a;Tak1*-cHet and DM embryos (Fig. 2L, L', M, M', N, N'). The nasal region in DM embryos was separated into two halves (Fig. 2N). The bottom of septum was not integrated at nasal chondral level (Fig. 2N').

Higher levels of apoptosis at E10.5 may lead to more severe craniofacial abnormality

To investigate cellular defects contributing to the median facial cleft, we measured levels of apoptosis with TUNEL assay and cell proliferation with anti-Ki67 immunohistochemistry staining at E10.5 and E12.5. Since growth and subsequent fusion of several facial prominences are critical for facial development including nasal process growth and formation of the nasal septum (Warbrick 1960), we focused on these areas (Fig 3P, Q).

In the anterior region of the nasal cavity of E12.5 embryos (an anterior level, Fig. 3P), proportions of TUNEL-positive cells in the mesenchyme of the midline and the nasal septum in *caBmpr1a* mut, *caBmpr1a* mut;*Tak1*-cHet, and DM embryos were higher than those in control and *Tak1*-cKO embryos (Fig. 3A–E, R). The DM embryos showed the highest levels of cell death, however, it is not significantly different from *caBmpr1a* mut;*Tak1*-cHet mice. At a level of the nasal cavity where formation of the nasal septum is prominent (a posterior level, Fig. 3P), there were more dead cells in all mutants than that in control. Among the mutant groups the level in *caBmpr1a* mut showed the highest proportion of TUNEL-positive cells (Fig. 3F–J, S). At E10.5 in a plane that includes facial processes (Fig. 3Q), there are higher levels of apoptosis in the mesenchyme of the lateral nasal processes and the medial nasal process in *caBmpr1a* mut, *caBmpr1a* mut;*Tak1*-cHet and DM mice compared with control and *Tak1*-cKO mice ($p < 0.01$) (Fig. 3K–O, T). TUNEL assay revealed a higher level of apoptosis in DM mice than *caBmpr1a* mut ($p < 0.05$) at E10.5 (Fig. 3T).

To identify if impairment of cell growth lead to the median facial cleft, we examined levels of Ki67 positive cells in the mesenchymal regions of the lateral and medial nasal process or the nasal septum at E10.5 and E12.5 using the adjacent sections used in Fig. 3. We did not observe significant difference among the five groups ($n = 3$ or more, $p > 0.05$ at E10.5 for all combinations, counting data not shown) (Fig. 4A–4O, Fig. 4K'–O' for high powered images of E10.5 embryos, and Supplementary Fig. 1 for high powered images of E12.5 embryos).

We previously reported that augmentation of BMP-Smad signaling results in higher levels of cell death through increased p53 activity (Komatsu et al., 2013; Li et al., 2013; Hayano et al., 2015), thus we next investigated levels of p53 and pSmad1/5/9 in the craniofacial region. At an anterior level of the craniofacial region of E10.5 embryos (Fig. 3Q), higher levels of p53 immunosignals were detected in mesenchyme of the lateral nasal and the medial nasal processes of *caBmpr1a* mut embryos (Fig. 5B, B', B'', respectively), although those in *Tak1*-cKO embryos were comparable with control embryos (Fig. 5E, E', E''). In *caBmpr1a* mut;*Tak1*-cHet and DM embryos, higher levels of p53 immunosignals than those in *caBmpr1a* mut embryos were detected in the lateral nasal and the medial nasal processes (Fig. 5C', C'', D', D'').

For pSmad1/5/9, higher levels of immunosignals were detected in mesenchyme of the lateral nasal processes of *caBmpr1a* mut embryos (Fig. 5G, G'), *caBmpr1a* mut;*Tak1*-cHet embryos (Fig. 5H, H') and DM embryos (Fig. 5I, I'). Those in *Tak1*-cKO embryos were comparable with control embryos (Fig. 5J, J'). Levels of pSmad1/5/9 immunosignals in the medial nasal processes were comparable among the five groups (Fig. 5F''–J'').

Compound mutations of *caBmpr1a* and ablation of *Tak1* did not lead to global alteration of Smad-dependent signaling but higher levels of pp38 at late gestation stages

To further gain mechanistic insight, we prepare protein lysate from the cranial region of embryos at E17.5 to quantify signaling levels by western blotting. We found higher levels of pSmad1/5/9 in *Tak1*-cKO embryos compared with controls, but the levels are comparable among *caBmpr1a* mut, *Tak1*-cKO and DM embryos (Fig. 6). These results suggest that TAK1 negatively regulates BMP-Smad signaling in neural crest derived tissues, but when BMPR1A has the constitutively active mutation, loss of TAK1 does not further increase BMP-Smad signaling when measured as a whole head, at least during late gestation.

We also prepared protein lysate from the cranial region of E10.5 embryos. Unlike the results from E17.5, all 4 genotypes of embryos showed comparable levels of pSmad1/5/9 (Fig. 7A, B). Taken together with the data in Fig. 5 showing that loss of TAK1 synergistically enhances BMP-Smad signaling in limited tissues such as the lateral nasal processes, an involvement of TAK1 in BMP-Smad pathway may be highly tissue-specific, but not in a global manner at E10.5.

Regarding the MAPK pathway components, no overt changes were found in levels of pERK1/2 (Fig. 6). Interestingly, levels of phospho-p38 (pp38) were highly increased in all *Bmpr1a* mut, *Tak1*-cKO and DM compared with the Control group when normalized by Vinculin (Fig. 6). Moreover, DM showed significantly higher levels of pp38 compared with *Bmpr1a* mut and *Tak1*-cKO (Fig. 6). When normalized by total p38, all three mutant groups showed tendency of increase compared with control ($p=0.086$, 0.069 , and 0.061 , respectively), suggesting that the increase of pp38 in the mutant groups were largely due to increases of p38 production during late gestation. In contrast, protein lysates from E10.5 embryos did not show overt changes in pp38 levels among 4 different genotypes (Fig. 7A, C). We also failed to detect overt changes in levels and distributions of immunosignals of pp38 in facial processes (Fig. 7D).

DISCUSSION

BMP signaling plays a critical role in the embryogenesis and development of organs, thus its subtle alteration leads to the abnormalities. Previous studies have illustrated that increased BMP Smad-dependent signaling levels through BMPR1A (*caBmpr1a* mutant mice) causes craniofacial deformities in mice (Komatsu et al., 2013; Li et al., 2013; Hayano et al., 2015). Here we report that ablation of *Tak1* combined with *caBmpr1a* in neural crest derivatives aggravates the craniofacial anomalies caused by *caBmpr1a*. Double mutant mice for *caBmpr1a* and *Tak1*-cKO displayed more severe phenotype and deformities such as more neonatal lethality and larger skull defects at newborn stage, and developed cleft palate and median facial cleft in association with elevated cell death in facial prominences during early to mid-gestation. In the double mutant embryos, higher levels of cell death were observed in the medial nasal and the lateral nasal processes at E10.5 in association with higher levels of p53. Double mutant embryos showed higher levels of pSmad1/5/9 in the lateral nasal processes at E10.5. Western analyses revealed that *Tak1*-cKO embryos showed similar levels of upregulated pSmad1/5/9 with *caBmpr1a* mutant or double mutant embryos while the double mutant embryos showed higher pp38 than *caBmpr1a* or *Tak1*-cKO embryos.

BMP type 1 receptors interact with TAK1, one of MAP3Ks, via XIAP to regulate its activity (Yamaguchi et al., 1999). TAK1 activates the MAPK pathway, one of the Smad-independent pathways. pERK1/2 can phosphorylate amino acids in the linker region of Smad1 to inhibit nuclear translocation of pSmad1/5/9 (Kretzschmar et al., 1997). Therefore, it is believed that deletion of *Tak1* can increase BMP Smad-dependent signaling activity. However, recent studies reported that TAK1 not only is a component of BMP Smad-independent pathway but also involves in BMP Smad-dependent pathway directly, at least in chondrocyte and osteoblast lineages (Shim et al., 2009; Greenblatt et al., 2010a; Greenblatt et al., 2010b; Gunnell et al., 2010). We also recently showed that *Tak1* deletion in neural crest derivatives using *Wnt1*-Cre results in decreases of BMP-Smad signaling activity (Yumoto et al., 2013). Thus, our initial prediction was that the double mutant (DM) mice (*P0*-Cre(+);*caBmpr1a*(+);*Tak1^{fx/fx}*) show milder phenotypes than *P0*-Cre(+);*caBmpr1a*(+) mice by reduction of BMP-Smad signaling activity. Opposite to the prediction, DM mice showed more severe phenotypes including cleft palate and median facial cleft. *Tak1*-cKO embryos showed elevated pSmad1/5/9 levels compared with those in control embryos, but no overt differences compared with those in *caBmpr1a* mut or DM mice. These facts suggest that the cause of more severe phenotypes in DM mice is not simply further elevated levels of BMP Smad-dependent signaling activity.

We found higher levels of cell death in the medial nasal process and the lateral nasal processes of DM embryos at E10.5 in association with an increase of p53. TAK1 inhibits apoptosis via repressing caspase activity (Mihaly et al., 2014). We found higher levels of apoptosis in the nasal septum region of *Tak1*-cKO mice compared with control mice at E12.5. As we recently reported, enhanced BMP-Smad signaling mediated by *caBMPR1A* stabilizes p53 to increase expression of caspase 3 (Hayano et al., 2015). Although *Tak1*-cKO mice do not show overt histological differences, it is possible to speculate that ablation of *Tak1* further increases elevated cell death in *caBmpr1a* mice by increase of caspase activity as shown in intestinal epithelium and blood vessel endothelium (Kajino-Sakamoto et al., 2008; Morioka et al., 2012). For the lateral nasal processes at E10.5, the DM embryos showed higher pSmad1/5/9 than each single mutant embryos and control embryos suggesting a mechanism that the deletion of *Tak1* in this tissue may further increase BMP-Smad signaling resulting in a higher level of p53-induced cell death as a cause of the midline cleft.

In the mesenchyme of the medial nasal process of the DM embryos at E10.5, however, we failed to detect higher levels of pSmad1/5/9 despite higher levels of p53 and cell death. Unexpectedly, we found increased levels of pp38 in *Tak1*-cKO embryos and those were increased further in the DM embryos at E17.5. In mouse hybridoma cell line MH60, BMP2 treatment induces apoptosis via the TAK1-p38 cascade (Kimura et al., 2000). In mouse embryonic fibroblasts (MEF), TGF- β -induced p38 phosphorylation requires both MLL2 (aka MAP3K10) and MEKK4 (aka MAP3K4), but not TAK1 (Sapkota 2013). No report is available for the mechanisms of p38 phosphorylation by BMPs, but it is possible to speculate that the increased p38 phosphorylation found in *Tak1*-cKO and DM embryos was TAK1-independent. It is known that TAK1-binding protein 1 (TAB1) induces phosphorylation of p38 α to phosphorylate p53 N-terminal sites. TAB1 stabilizes p53 by inhibition of MDM2 ubiquitin ligase activity (Zhu et al., 2013). Based on the stage-

dependent alterations on p38 levels in DM mutants (Figs. 6, 7), it is possible to speculate that in the DM embryos apoptosis is induced by increased p38 activity during late gestation stages to develop more severe phenotypes than each single mutant embryos.

Epithelium-specific disruption of *Bmpr1a* results in secondary palate cleft likely due to defects in proliferation (Liu et al., 2005). In contrast, increased apoptosis in the edge epithelium of the medial nasal process is believed to be a cause of cleft lip in the same mouse model (Liu et al., 2005). It is also reported that BMP signaling promotes cell proliferation of maxillary process mesenchyme (Liu et al., 2005). Our data show increased BMP-Smad signaling mediated by caBMPRIA does not influence proliferation of nasal process mesenchymal cells in the medial nasal and lateral nasal processes.

In this study, we generated the transgenic mouse line with deletion of *Tak1* and gain of function of *Bmpr1a*. This compound mutation leads to more severe craniofacial deformities than each single mutation. Newly developed phenotypes including midline cleft and cleft palate are highly associated with increased cell death with increased p53 that may be stabilized by increased BMP-Smad signaling activity.

MATERIALS AND METHODS

Generation of mutant mice and embryos

Generation of the mutant mouse lines, *P0-Cre*, *caBmpr1a*, and *Tak1* floxed mouse lines, was reported previously (Yamauchi et al., 1999; Kamiya et al., 2008; Yumoto et al., 2013). We set up different combinations of breeding to generate following mice for comparisons; *P0-Cre*(+)(-)(named control, CT), *caBmpr1a*(+)(-);*P0-Cre*(+)(-)(named *caBmpr1a* mut), *caBmpr1a*(+)(-);*Tak1*fx/+;*P0-Cre*(+)(-)(named *caBmpr1a* mut;*Tak1*-cHet), *caBmpr1a*(+)(-);*Tak1*fx/fx;*P0-Cre*(+)(-)(named double mutant, DM) and *Tak1*fx/fx; *P0-Cre*(+)(-)(named *Tak1*-cKO). Timed mating was determined by noting the presence of a vaginal plug as day 0.5 for staging embryo collections and estimating date of birth. The tail of each embryo or newborn mouse was subjected to DNA extraction for genotyping. All mouse experiments were performed in accordance with University of Michigan guidelines covering the humane care and use of animals in research. All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan (Protocol #PRO00005716 and #PRO00007715).

External morphology, and Skeleton-cartilage staining Histology

Newborn pups were sacrificed on ice and photographed with a stereomicroscope with camera (Olympus SZX16 and Olympus DP72). Heads were skinned, fixed in 95% alcohol and stained with Alcian blue 8GX and Alizarin Red S by standard methods. Samples were photographed under the microscope. The area of skull defects was measured with CellSens Software (Olympus). Statistical analysis was performed using Student's T-test. Significance was accepted at $p < 0.05$.

Embryonic day 10.5 (E10.5), E12.5, E15.5 and newborn mice were fixed in 4% paraformaldehyde (PFA), overnight at 4°C. Head samples of E15.5, E12.5 were dehydrated in ethanol with gradual concentration and then embedded in paraffin. 7-μm serial sections

were made for conventional staining methods with hematoxylin and eosin (H&E) or immunohistochemistry staining. After fixation, E10.5 embryos were incubated in 30% sucrose in PBS overnight at 4°C and embedded in O.C.T. compound (Fisher healthcare, USA). 10-µm serial sections were made for cell proliferation and cell death studies (see below).

Cell apoptosis and proliferation assays, immunohistochemistry

Apoptosis in nasal septum at E12.5 stage and nasal processes at E10.5 stage was evaluated via Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay according to the instruction of In Situ Cell Death Detection Kit (Roche Mannheim, Germany). In brief, paraffin sections were rehydrated. Sections were heated in citrate buffer in microwave for 4 min and then were immersed in Tris-HCl. 0.1 M pH7.5, containing 3% BSA and 20% normal bovine serum for 30 min. Slides were incubated with TUNEL reaction mixture at 37°C, 60min in dark and mounted with ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) mounted the sections. Sections were detected under a fluorescence microscope (Olympus) with a FITC set filter.

Cell proliferation in E12.5 nasal septum and E10.5 nasal processes was detected via examining presence of Ki67. Slides were incubated with an anti-ki67 antibody (1:50, M7249, Dako) for overnight at 4°C, and incubated with an anti-rabbit IgG conjugated with Alexa Fluor 488 (1:100, A21206, Invitrogen) for 1h at room temperature. Sections were mounted with ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen).

The number of TUNEL-positive or Ki67-positive cells and total cells in the nasal processes at E10.5 and in the midline mesenchyme and the nasal septum at E12.5 were counted. Results were presented as percentages of TUNEL-positive or Ki67-positive cells against total cells. At least three embryos were used for each group at each stage. Over 500 cells and 2,000 cells for each embryo at E10.5 and E12.5, respectively, were examined. Comparable levels among embryos were defined according to the shape of nasal processes for E10.5 samples and the shapes of nasal capsules and nasal conchas for E12.5 samples. Statistical analysis was performed using Student's T-test. Significance was accepted at $p < 0.05$.

For immunohistochemistry, embryos were fixed in 4% PFA at 4°C for 2 hours, incubated in 30% sucrose/PBS at 4°C overnight, embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan), and serially sectioned at 10 µm. The samples were incubated with rabbit anti-p53 (1:100, sc-6243, Santa Cruz) and rabbit anti-pSmad1/5/9 (1:100, #13820, Cell Signaling) and rabbit anti-phospho-p38 MAPK (#4631, Cell Signaling) at 4°C overnight. Alexa Fluor 488 donkey anti-rabbit IgG (1:100, A21206, Invitrogen) was used as secondary Ab. Sections were mounted with ProLong Gold antifade reagent with DAPI (P36935, Invitrogen).

Western Blot Analysis

Nasal portions (from nose point to eye) of each embryo (E10.5 or E17.5) were lysed in NP40 lysis buffer (20mM pH 8.0 Tris-HCl, 137mM NaCl, 1% NP40, 10% Glycerol, 1mM Na₃VO₄) using a Bio-Gen Pro200 homogenizer (Pro Scientific, Oxford, CT, USA). Resulted lysates were run on 10% Mini-Protean TGX gels (Bio-Rad, Richmond, CA, USA)

and transferred to PVDF membrane (Millipore, IPVH00010). Following antibodies were used for immunodetection: rabbit anti-phospho-SMAD1/5/9 (pSmad1/5/9) (#13820, Cell Signaling), rabbit anti-phospho-p38 MAPK (#4631, Cell Signaling), rabbit anti-p38 MAPK (#9212, Cell Signaling), mouse anti-Vinculin antibody (V4505, Sigma-Aldrich), rabbit anti-phospho-p44/42 MAPK (pERK1/2) (#9101, Cell Signaling), rabbit anti-p44/42 MAPK (ERK1/2) (#9102, Cell Signaling). HRP substrate ECL (Millipore, WBKL S0500) was used to detect a bound antibody against pSmad1/5/9, pERK1/2, pERK1/2, pp38, p38 and Vinculin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

YM is supported by NIH (R01DE020843), and JNT is supported by NIH (R01GM068812). XL is supported by China Scholarship Council.

We thank Dr. Kenichi Yamamura for providing *P0-Cre* mice. We would like to express our appreciation to Calvin Gee, Amanda Beaune and Xixi Wang for their excellent technical assistance, Drs. Vesa Kaartinen, Yoshihiro Komatsu and Honghao Zhang for helpful discussions. YM is supported by NIH (R01DE020843), and JNT is supported by NIH (R01GM068812). XL is supported by China Scholarship Council. SH thanks Natsuko, Toru and Kaoru Hayano.

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Figure 1. Increased expression of *Bmpr1a* and decreased *Tak1* in neural crest cells causes the craniofacial abnormalities in newborn mice
A–O, Gross morphology, top (A–E), lateral (F–J) and frontal (K–O) views. *caBmpr1a* mut and *caBmpr1a* mut;*Tak1*-cHet have similar phenotype such as short snout, widened orbital distance. *caBmpr1a* mut;*Tak1*-cKO (Double mut, DM) have median facial cleft besides the phenotypes in *caBmpr1a* mut and *caBmpr1a* mut;*Tak1*-cHet (white arrow). **AA–OO**, Alcian Blue and Alizarin Red staining of craniofacial regions, top (AA–EE), lateral (FF–JJ) and bottom (KK–OO) views. Mutants have skull defects in the forehead area (black asterisks). **P**, Distribution of the phenotype. The rate of lethality with craniofacial deformities and median facial cleft in DM is higher than *caBmpr1a* mut and *caBmpr1a* mut;*Tak1*-cHet. **Q**, Measurement of the skull defect size shown in AA–EE. Skull defects in DM are more severe than the other mutants. (n=5; *, $p<0.05$; **, $p<0.01$). Scale bar: 1mm.

Figure 2. Compound mutation led to cleft palate and abnormal nasal septum at newborn and embryo stage
A–E, Gross morphology of palate at newborn. Some *caBmpr1a* mut;*Tak1*-cHet mice display cleft palate (black asterisk). Some DM mice display cleft palate (black asterisk) and a failure of lip fusion (white asterisk). A'–O', Histologic observation of the craniofacial region at newborn and embryonic stages (E15.5 and E12.5); frontal section, H&E staining. P–R, approximate positions of each section at newborn; anterior (levels of the primary palate), middle (levels of the nasal concha), and posterior (levels of upper first molar), E15.5 and E12.5 (anterior and posterior), respectively. At newborn, *caBmpr1a* mut;*Tak1*-cHet display short septum and cleft palate at a nasal chondral level and an eye level (black arrow heads in C'' and C'''). DM mice display cleft palate and no septum from anterior to posterior levels (black arrow heads in D'–D'''). At E15.5, *caBmpr1a* mut;*Tak1*-cHet and DM mice display unfused palate shelf and nasal septum at the nasal chondral level (black arrows in H and I).

At E12.5, at an anterior level nose in DM group is separated into two parts (black asterisk in N) and at a posterior level nasal septum is not integrated (black asterisk in N'). Cartilage primordia of nasal capsule and septum are obvious and continuous in control and *Tak1*-cKO embryos (black arrows in K and O). Those structures are not obvious in other mutants. Scale bar: 200 μ m for A–E, 100 μ m for A'–E'', F–O'.

Figure 3. More apoptosis at embryonic stage may lead to more severe craniofacial deformities Apoptosis in the nasal septum at E12.5 (A–E, R and F–J, S) and in medial and lateral prominence at E10.5 (K–O, T) in five different groups as shown in the top. Approximate positions of each section are shown in P (E12.5) and Q (E10.5). White arrowhead in K, the lateral nasal process, white arrow in K, the medial nasal process. Scale bar: 100 μ m.

Figure 4. Craniofacial abnormalities were not attributed to cell proliferation
Cell proliferation in nasal septum at E12.5 (A–J) and in medial and lateral nasal prominence at E10.5 (K–O, K'–O') is detected by immunohistochemistry staining for Ki67. Approximate positions of each section are shown in Fig. 3P (E12.5) and Fig. 3Q (E10.5). White arrowhead in **K**, the lateral nasal process, white arrow in **K**, the medial nasal process. Scale bar: 100 μm for A–O, 10 μm for K'–O'.

Figure 5. Increased levels of p53 and pSmad1/5/9 were found in facial processes in the double mutant embryos
Immunohistochemical detections (green) of p53 (A–E) and pSmad1/5/9 (F–J) of the anterior cranial regions of five different genotype embryos at E10.5. Enlarged images of the lateral nasal processes (A'–J') and the medial nasal processes (A''–J'') are also shown. Tissues are counterstained with DAPI (blue). Orange staining is a background noise caused by blood cells. Scale bar: 100 μ m.

Figure 6. Higher levels of pSmad1/5/9 and pp38 were found in the double mutant heads
Levels of phospho-Smad1/5/9 (pSmad1/5/9), phospho-ERK1/2 (pERK1/2) and phospho-p38 (pp38) are examined by Western blotting. Protein lysates are prepared from an anterior head region of E17.5 embryos. Signal levels are normalized by vinculin (pSmad1/5/9, pp38) or total forms of ERK1/2 or p38. $n \geq 3$, * $p < 0.005$, ** $p < 0.05$, *** $p < 0.01$

Figure 7. No overt changes in pp38 levels and distributions were found in the double mutant heads at E10.5

A, Levels of phospho-Smad1/5/9 (pSmad1/5/9) and phospho-p38 (pp38) are examined by Western blotting. Protein lysates are prepared from an anterior head region of E10.5 embryos. **B**, **C**, Signal levels are normalized by vinculin (pSmad1/5/9, pp38). $n \geq 4$, no significant changes are observed among 4 genotypes. **D**, Distribution of pp38 (green) is detected by immunohistochemistry in the facial processes at E10.5 embryos. Tissues are counterstained with DAPI (blue). Orange staining is a background noise caused by blood cells. Scale bar: 100 μ m.