BMP signaling regulates sympathetic nervous system development through Smad4-dependent and -independent pathways

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Induction of the sympathetic nervous system (SNS) from its neural crest (NC) precursors is dependent on BMP signaling from the dorsal aorta. To determine the roles of BMP signaling and the pathways involved in SNS development, we conditionally knocked out components of the BMP pathways. To determine if BMP signaling is a cell-autonomous requirement of SNS development, the Alk3 (BMP receptor IA) was deleted in the NC lineage. The loss of Alk3 does not prevent NC cell migration, but the cells die immediately after reaching the dorsal aorta. The paired homeodomain factor Phox2b, known to be essential for survival of SNS precursors, is downregulated, suggesting that Phox2b is a target of BMP signaling. To determine if Alk3 signals through the canonical BMP pathway, Smad4 was deleted in the NC lineage. Loss of Smad4 does not affect neurogenesis and ganglia formation; however, proliferation and noradrenergic differentiation are reduced. Analysis of transcription factors regulating SNS development shows that the basic helix-loop-helix factor Ascl1 is downregulated by loss of Smad4 and that Ascl1 regulates SNS proliferation but not noradrenergic differentiation. To determine if the BMP-activated Tak1 (Map3k7) pathway plays a role in SNS development, Tak1 was deleted in the NC lineage. We show that Tak1 is not involved in SNS development. Taken together, our results suggest multiple roles for BMP signaling during SNS development. The Smad4-independent pathway acts through the activation of Phox2b to regulate survival of SNS precursors, whereas the Smad4-dependent pathway controls noradrenergic differentiation and regulates proliferation by maintaining Ascl1 expression.

KEY WORDS: BMP, Neural crest, Smad4 independent, Mouse

INTRODUCTION
The sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS) constitute the autonomic nervous system; acting together, they maintain homeostatic balance. The postganglionic component of the SNS, a relatively simple model used to study development of the nervous system in mammals, develops independently of other neuronal lineages, with the neurons being predominantly noradrenergic. All neurons and glia of the SNS develop from the neural crest (NC) lineage. The trunk NC cells form at the dorsal aspect of the neural fold and migrate through the ventromedial somitic sclerotome to the dorsal aorta and major blood vessels. Once the NC cells reach the dorsal aorta, they receive an inductive cue, thought to be BMPs, to activate the SNS-specific differentiation program (Reissmann et al., 1996). The dorsal aorta expresses Bmp2, Bmp4 and Bmp7, and all have been shown to activate the SNS developmental program in vitro (Reissmann et al., 1996; Shah et al., 1996; Varley and Maxwell, 1996). In addition, inhibition of BMPs by implantation of noggin-soaked beads by the dorsal aorta prevents NC cells from differentiating into neurons (Schneider et al., 1999), further supporting a role for BMPs in SNS development.

BMP-induced SNS development results in the activation of a number of transcription factors. These include the paired homeodomain factors Phox2a (Lo et al., 1998; Schneider et al., 1999) and Phox2b (Howard et al., 2000; Schneider et al., 1999), the basic helix-loop-helix (bHLH) factors Hand2 (Howard et al., 2000) and Ascl1 (formerly called Mash1) (Lo et al., 1997; Shah et al., 1996), and the zinc finger factors Gata2/3 (Tsarovina et al., 2004). Each of these factors is involved in different aspects of SNS development. Phox2b is essential for the survival of sympathetic neuroblasts (Pattyn et al., 1999), whereas Ascl1 is involved in maintenance of neuroblasts and acceleration of neuronal differentiation (Guillemot et al., 1993; Pattyn et al., 2006; Sommer et al., 1995). Both Hand2 (Lucas et al., 2006; Morikawa et al., 2007) and Gata3 (Lin et al., 2000; Moriguchi et al., 2006) regulate noradrenergic phenotype selection of SNS neurons by regulating the genes that encode the enzymes required for norepinephrine synthesis.

BMP molecules are received by type I and type II receptors, and the signal is transduced by phosphorylation of the BMP-activated receptor Smads (R-Smads). Once activated, the R-Smads bind with the co-Smad Smad4, which facilitates the shuttling of phospho-R-Smads into the nucleus, where they activate target gene expression (Hill, 2009; Massague et al., 2005). More recently, signal transduction pathways independent of Smads have been reported. Most prominent is the activation of Tgfb-activated kinase 1 (Tak1; Map3k7 – Mouse Genome Informatics) by Tgfb and BMP-activated receptors. Tak1 acts by activation of downstream kinases, including P38 and Jun kinases (Zhang, 2009).

Although BMP appears to activate SNS development through a direct activation of the BMP-signaling pathway in NC cells, the mechanisms of BMP signal transduction have not been addressed. In primary NC cultures, addition of Bmp4 results in translocation of...
Smad1 to the nucleus, suggesting that BMP-activated R-Smads are involved in differentiation of NC cells (Wu and Howard, 2001). However, regulation of the R-Smads and the roles of other Smad-independent signal transduction pathways have not been investigated. To address how the BMP signal transduction pathways regulate SNS development, key components of the signal transduction pathways were conditionally deleted in the NC lineage during development. Loss of the type I BMP receptor, Alk3 (BMP receptor IA), and the co-Smad, Smad4, in the NC lineage shows that BMP signaling plays multiple roles in SNS development; a Smad4-independent BMP pathway is involved in the colonization and survival of sympathetic precursors, whereas the Smad4-dependent pathway is required for the proliferation of neuroblasts and for noradrenergic differentiation. Deletion of Tak1, which is regulated by the Tgfβ superfamily receptors and is a Smad-independent component of BMP signaling, shows that this pathway does not play a significant role in SNS development. Analysis of the downstream transcription cascades regulating SNS development shows that the paired-like homeobox transcription factor Phox2b, which is essential for SNS precursor survival, is a target of the Smad4-independent pathway, whereas the bHLH factor Ascl1 regulates SNS neuroblast proliferation through a Smad4-dependent pathway.

MATERIALS AND METHODS
Generation of mutant embryos
Embryos containing Alk3, Tak1 or Smad4 deletions in their NC cells were obtained by crossing conditional lines to the Wnt1-Cre (Danielian et al., 1998) mouse line. Alk3<sup>3/–</sup> (Mishina et al., 1995), Smad4<sup>−/−</sup> (Yang et al., 2002), and Tak1<sup>−/−</sup> (Liu et al., 2006), were crossed with Wnt1-Cre to obtain Alk3<sup>3/–; Wnt1-Cre, Smad4<sup>−/−</sup>; Wnt1-Cre, and Tak1<sup>−/−</sup>; Wnt1-Cre males. The males were crossed with Alk3<sup>3/–</sup> (Mishina et al., 2002), Smad4<sup>−/−</sup> and Tak1<sup>−/−</sup> lines, respectively, to obtain embryos. For lineage tracing of NC cells, Alk3<sup>3/–</sup>, Tak1<sup>−/−</sup> and Smad4<sup>−/−</sup> lines were crossed with R26R (Gt(Rosa)26Sortm1Sho) (Mao et al., 1999).

To overcome the embryonic lethality associated with loss of Alk3 or Smad4, the drinking water of dams was supplemented with 200 µg/ml isoproterenol and 2.5 mg/ml ascorbic acid from 7.5 days post-coitum (dpc), as described previously (Morikawa and Cserjesi, 2008). To trace the NC lineage in the Ascl1<sup>–/−</sup> background, Ascl1<sup>–/−</sup> mice (Guillenot et al., 1993) were mated with Wnt1-Cre and R26R/R26R lines to obtain Ascl1<sup>–/−; Wnt1-Cre and Ascl1<sup>–/−; R26R/R26R</sup> lines.

In situ hybridization
In situ hybridizations were performed as described previously (Morikawa et al., 2007; Morikawa and Cserjesi, 2005). In brief, 25 µm paraffin sections were hybridized with digoxigenin-labeled probes at a concentration of 0.5 µg/ml at 68°C overnight. After washing and RNase treatment, digoxigenin was detected with anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostics). Color development was obtained using alkaline phosphatase substrate BM Purple (Roche Diagnostics). The probes used in this study were Phox2b (Pattyn et al., 1997), Hand2 (Dai et al., 2004) and Gata3 (Morikawa et al., 2007).

Immunohistochemistry and β-galactosidase staining
Immunohistochemistry and β-galactosidase staining were performed as previously described (D’Autreaux et al., 2007; Morikawa and Cserjesi, 2004; Morikawa et al., 2007). Antibodies used in this study were: rabbit polyclonal against phospho-Smad1/5/8 (1:200; Cell Signalling); mouse monoclonal against Tuj1 (1:1000; Chemicon); rabbit polyclonal against TH (1:200; Chemicon); guinea pig polyclonal against Ascl1 (1:10,000; provided by J. Johnson, University of Texas Southwestern, Dallas, TX, USA); rabbit polyclonal against Phox2b (1:1000; provided by J. Brunet, CNRS, École Normale Supérieure, Paris, France); mouse monoclonal against Sox10 (1:5; provided by D. Anderson, Caltech, Pasadena, CA, USA). Secondary antibodies used for immunofluorescent studies were labeled with Alexa Fluor 555 (Invitrogen) or fluorescein (Vector Laboratories), and for colorimetric detection, secondary antibodies used were labeled HRP and detected by DAB (Vector Laboratories). All cross sections were obtained from the abdomen region.

Cell proliferation and TUNEL assay
Proliferating cells were identified by BrdU incorporation. Five milligrams per 100 g body weight of BrdU solution (Invitrogen) was introduced by intraperitoneal injection 2 hours before dissection, and embryos were collected and stained for β-galactosidase activity to identify NC-derived cells. Embryos were sectioned at a thickness of 7 µm, and BrdU incorporation was detected by an anti-BrdU antibody staining kit (Invitrogen). Proliferation rates were measured by counting BrdU-positive cells as a percentage of total NC-derived cells, as monitored by β-galactosidase activity.

Apoptosis was detected using a TUNEL assay kit according to the manufacturer’s manual (Promega). For the TUNEL assay, embryos were stained with β-galactosidase activity, collected and sectioned to 7 µm. Statistical analysis of differences in proliferation and apoptosis used the Student’s t-test.

RESULTS
BMP signaling is required for the formation of the sympathetic ganglia
The type I BMP receptor Alk3 is expressed in premigratory NC cells (Panchision et al., 2001) and continues to be expressed in sympathetic precursors after they reach the dorsal aorta. Overexpression of a constitutively active form of Alk3 increases catecholaminergic differentiation in NC cultures (Varley et al., 1998), suggesting that BMP signaling during SNS development occurs through the Alk3 receptor. To determine if BMP signaling is essential for NC cells during mouse SNS development, we conditionally deleted Alk3 in NC cells, using the Wnt1-Cre deleter line, while simultaneously tracing NC cells using the R26R allele (Mao et al., 1999) (Fig. 1). NC cells started migrating after neural tube isolation in dissecting medium containing 0.1% DNase I. After several washes with dissecting medium, neural tubes were transferred to 24-well tissue culture plates coated with 20 µg/ml fibronectin (Sigma) blocked with 500 µg/ml BSA. Neural tubes were cultured in 300 µl of NC culture medium containing 10% FBS in D-MEM supplemented with 50% MEDI medium (Rathjen and Rathjen, 2002) and 20 ng/ml Fg2 (Chemicon) at 37°C. The neural tubes were removed after 24 hours, and NC cells were collected by scraping followed by replating at high density and were allowed to differentiate for 4 days before analysis.
The dorsal root ganglia (DRG), the sensory division of the PNS, are also derived from the trunk NC. To determine if the NC lineage depends on Alk3 for induction, migration and survival, or if the dependence is restricted to the SNS, we examined the formation of the DRG in Alk3 conditional knockout (cKO) embryos. Consistent with previous reports (Stottmann et al., 2004), the size and shape of DRG in Alk3 cKO embryos and in control embryos were comparable (see Fig. S1 in the supplementary material), suggesting that the loss of Alk3 in the NC lineage affects SNS precursors and is not a general requirement of trunk NC cells.

Deletion of Alk3 in the NC lineage results in cardiac defects and embryonic lethality at 12.5 dpc (Stottmann et al., 2004). The lethality was attributed to severe cardiovascular defects, but the Alk3 phenotype resembles those found in embryos with defects in SNS norepinephrine synthesis (Lim et al., 2000; Morikawa and Cserjesi, 2007). To determine if embryonic lethality is due to the intrinsic cardiovascular defects or is an indirect effect of norepinephrine deficiency, the adrenergic receptors in Alk3 cKO embryos were activated pharmacologically. Dams were administered the β-adrenergic receptor agonist isoproterenol, and embryos were collected at different stages of development. The Alk3 cKO embryos rescued by isoproterenol survived to term, showing that embryonic lethality is due to norepinephrine depletion and providing a means to examine the role of Alk3 throughout development of NC-derived tissues. The most dramatic mutant phenotypes of embryos rescued by isoproterenol administration were craniofacial defects, including smaller head size and reduced projection of facial structures (Fig. 1G,H). To determine if the isoproterenol rescue of embryos was due to the direct activation of adrenergic receptors in the cardiovascular system or to the rescue of SNS development, we examined the SNS of Alk3 cKO embryos at 16.5 dpc embryos (Fig. 1I,J). Rescued embryos with NC deletion of Alk3 showed a complete lack of an SNS, showing that isoproterenol does not rescue the loss of SNS precursors.

**BMP signaling is required for formation of sympathetic ganglia.** Alk3 was deleted in the NC lineage using Wnt1-Cre, and NC cells were simultaneously traced by genetically marking by β-galactosidase expression from the R26R knock-in allele. (A-F) β-Galactosidase expression in Alk3fx/+, Wnt1-Cre; R26R+/ which are phenotypically wild-type (A,C,E), and Alk3fx/+, Wnt1-Cre; R26R/+ cKO embryos at 9.5 dpc (A,B), 10.0 dpc (C,D), and 10.5 dpc (E,F). The loss of Alk3 in the NC lineage (arrowheads) shows that the cells reach the dorsal aorta (B,D) but fail to form sympathetic ganglia in Alk3fx/+; Wnt1-Cre; R26R/+ cKO embryos (F). (G-J) The loss of Alk3 in the NC lineage results in craniofacial defects and the absence of the SNS. The early lethality caused by the lack of embryonic synthesis of norepinephrine was overcome by administering dams with isoproterenol. At 16.5 dpc, relative to control littermates (G), Alk3 cKOs (H,J) exhibit severe craniofacial defects (H) and complete loss of the SNS (J). In panels I and J, organs were removed before photographing from the ventral side. da, dorsal aorta; nc, neural crest; nt, neural tube.

**BMP signaling is required for survival of sympathetic neuroblasts**

Loss of sympathetic precursors in Alk3 cKO embryos after they have migrated to the dorsal aorta suggests that NC-derived cells are lost due to cell death. To determine if cell loss is due to apoptosis, mutant embryos were analyzed using TUNEL analysis (Fig. 2A-C). In 9.5 dpc control embryos, little apoptotic death was found in the developing SNS (Fig. 2A), whereas in Alk3 cKO embryos, a large proportion of sympathetic precursors were undergoing apoptosis (Fig. 2B). Quantification of apoptotic levels in Alk3 mutant embryos showed that by 9.5 dpc, 40% of the cells were undergoing apoptosis, and the proportion increased to 60% of cells by 10.25 dpc (Fig. 2C). These results show that the loss of sympathetic precursors in Alk3 cKO embryos is due to apoptotic cell loss.

The transcription factor Phox2b is required for survival of sympathetic precursors (Pattyn et al., 1999), and its expression is activated by BMP signaling (Schneider et al., 1999). To determine if Phox2b expression depends on Alk3, and if the loss of Phox2b may account for the death of SNS precursors, we examined expression of Phox2b in Alk3 cKO embryos by immunohistochemistry (Fig. 2D-G). Robust expression of Phox2b was found in control sympathetic neuroblasts at 10.0 dpc (Fig. 2D,F) whereas expression of Phox2b could not be detected in sympathetic neuroblasts of cKO embryos (Fig. 2E,G). To determine if the onset of Phox2b expression is dependent on BMP signaling, we examined the expression of Phox2b in 9.5 dpc embryos by in situ hybridization (Fig. 2H,I). Control embryos begin to express Phox2b in the SNS by 9.5 dpc (Fig. 2H) whereas Phox2b was not expressed in SNS of cKO littermate embryos (Fig. 2I), showing that Phox2b is not activated in Alk3 cKO embryos. To eliminate the possibility that the lack of Phox2b expression is due to loss of SNS precursors, NC cells were identified by Sox10 expression (Fig. 2J,K). Immunohistochemical analysis showed
Fig. 2. BMP signaling is required for the survival of sympathetic precursors and for the expression of Phox2b. To determine if sympathetic precursor cells were dying by apoptosis, 9.5 dpc embryos were analyzed by TUNEL. (A-C) Immunohistochemical analysis of apoptosis in Alk3fx/+, Wnt1-Cre; R26R/+ (A) and Alk3fx/+, Wnt1-Cre; R26R/+ cKO (B) embryos at 9.5 dpc. TUNEL-positive cells are highlighted with red arrowheads. The number of TUNEL-positive sympathetic precursor cells were quantified at 9.5 and 10.25 dpc (C). *, P≤0.022; **, P≤0.00014. (D-G) The expression of Phox2b in β-galactosidase-stained sympathetic precursor cells was analyzed in 10.0 dpc embryos by immunohistochemistry using a Phox2b-specific antibody. F and G show higher magnification of the regions enclosed by the red box in panels D and E. In control sections (D,F), Phox2b is expressed in cells of the developing SNS, whereas expression of Phox2b protein could not be detected in Alk3 cKO embryos (E,G). (H-K) Onset of Phox2b expression is not dependent on BMP signaling. The expression of Phox2b was examined by whole mount in situ hybridization in control (H) and Alk3fx/+, Wnt1-Cre (I) embryos at 9.5 dpc. Phox2b is expressed in the SNS forming at the rostral region of control embryos but is not expressed in mutant embryos. Immunohistochemical analysis of the NC marker Sox10 was used to identify cells forming the SNS of control (J) and Alk3fx/+, Wnt1-Cre; R26R/+ cKO (K) embryos at 9.5 dpc. Sox10 is expressed in both control (J) and cKO (K) β-galactosidase-expressing cells showing that loss of Phox2b expression in mutant embryos is not due to the absence of SNS progenitors. Purple arrowheads point to the forming SNS. da, dorsal aorta; nt, neural tube.

that Sox10-expressing cells were present in comparable numbers in SNS of control and cKO embryos, demonstrating that Phox2b is regulated by Alk3 and suggesting that loss of Phox2b could account for the loss of SNS precursors in Alk3 cKO embryos.

**Sympathetic ganglia formation is independent of Smad4**

The canonical Tgfβ superfamily signaling pathway involves the binding of phosphorylated R-Smads with Smad4, the co-Smad that transports the R-Smads into the nucleus, where they subsequently bind target-specific DNA sequences to regulate gene transcription (Hill, 2009; Massague et al., 2005). Deletion of Smad4 in the NC lineage using Wnt1-Cre (Jia et al., 2007; Ko et al., 2007; Nie et al., 2008) showed that Smad4 cKO embryos died at 12 dpf, with pooling of blood in the periphery that resembled defects in SNS development (Lim et al., 2000; Morikawa and Cserjesi, 2008; Morikawa et al., 2007), suggesting Smad4 plays a role in SNS development. To determine the role of the canonical BMP pathway in SNS development, we deleted Smad4 and simultaneously marked the NC lineage to examine how its loss affected early SNS development (Fig. 3A-D). Morphological analysis of control (Fig. 3A) and Smad4 mutant (Fig. 3C) embryos at 11.5 dpc showed that the sympathetic, sensory and enteric nervous systems developed normally. Histological analysis of the sympathetic ganglia showed that their size and location were also comparable in control (Fig. 3B) and mutant (Fig. 3D) embryos, suggesting that Smad4 is not involved in colonization and survival of sympathetic precursors.

The canonical BMP pathway regulates SNS proliferation

To determine whether embryonic lethality in Smad4 mutant embryos is due to a lack of norepinephrine, we fed dams with the adrenergic receptor agonist isoproterenol. Isoproterenol administration overcame the embryonic lethality at 12.5 dpc, letting embryos survive to 16.5 dpc. Analysis of embryos at late gestation showed that, relative to control embryos (Fig. 3E), mutant embryos (Fig. 3G) had greatly reduced face and cranial vaults, both of which are predominantly NC derived.

The ability to use the adrenergic receptor agonists to rescue embryos from the lethality resulting from the loss of Smad4 suggests that Smad4 is required for biosynthesis of embryonic norepinephrine. To investigate the role of Smad4 during late SNS development, the morphology of 16.5 dpf sympathetic trunks was examined by lineage tracing (Fig. 3F,H). Analysis showed that, relative to control embryos (Fig. 3F), the SNS of Smad4 cKO embryos (Fig. 3H) is hypoplastic. To determine the cause of the hypoplastic SNS in Smad4 cKO embryos, we examined the levels of cell proliferation during development by BrdU incorporation (Fig. 3I). In the absence of Smad4, SNS proliferation decreased at all stages of development, showing that Smad4 loss results in decreased proliferation. To investigate whether cell death was contributing to the hypoplastic phenotype, the level of apoptosis was determined by TUNEL assay. Loss of Smad4 did not result in a significant difference in the level of apoptotic cell death (data not shown).
The canonical BMP pathway regulates noradrenergic differentiation

BMP signaling is known to induce NC cells to form sympathetic neurons, but it is not known if signaling is required to promote neuronal and noradrenergic differentiation (Reissmann et al., 1996; Schneider et al., 1999; Shah et al., 1996; Varley and Maxwell, 1996). To determine whether BMP signaling is required for neurogenesis and noradrenergic differentiation, we examined how disruption of the canonical BMP signaling pathway affected SNS differentiation by deleting Smad4 in NC cells (Fig. 4A-D). To determine whether the loss of Smad4 leads to defects in neurogenesis, neurons were identified by expression of the pan-neuronal marker Tuj1 (Fig. 4B). Loss of Smad4 did not affect neurogenic differentiation of the SNS of Smad4 cKO embryos (Fig. 4B). To quantify the levels of Tuj1, sympathetic ganglia were marked by β-galactosidase expression from the R26R locus, and the level of Tuj1 was quantified relative to the level of β-galactosidase. Quantification showed that loss of Smad4 did not affect neurogenic differentiation (Fig. 4E).

Analysis of the pan-neuronal marker HuC/D and the peripheral neuronal marker peripherin showed that both proteins were also.
Ascl1 expression requires Smad4, we examined the expression of Ascl1 in Smad4 cKO SNS by in situ hybridization (Fig. 5A,B). At 10.5 dpc, Ascl1 was expressed in both control and Smad4 cKO embryos (Fig. 5A,B); however, the expression level decreased after 11.5 dpc (data not shown). To quantify the level of Ascl1, we performed immunohistochemical analysis of Ascl1 in sympathetic neurons marked by the expression of TuJ1 in 12.5 dpc embryos (Fig. 5C,D). In control embryos, Ascl1 was expressed in the majority of SNS neurons (Fig. 5C). By contrast, the expression of Ascl1 in the Smad4 cKO SNS was greatly reduced (Fig. 5D). The level of Ascl1 expression in the SNS was quantified, showing a 70% decrease in Smad4 cKO embryos relative to the control littermates (Fig. 5E), suggesting that Smad4 regulates SNS ganglion size through expression of Ascl1.

**The role of Ascl1 in SNS development**

It has been proposed that Ascl1 regulates SNS development through the maintenance of neuroblasts and neuronal differentiation (Guillemot et al., 1993; Pattyn et al., 2006; Sommer et al., 1995), yet the mechanisms by which Ascl1 regulates those functions are not known. To investigate the role of Ascl1 in SNS development, the NC lineage was traced using a Wnt1-Cre line and the R26R locus in Ascl1 mutant embryos, and the development of the sympathetic trunk was examined at 10.5 (data not shown), 11.5 (Fig. 6A-E) and 16.5 dpc. In control embryos, NC-derived cells populated the site of SNS formation and aggregated as they began to form ganglia (Fig. 6A). The size and distribution of the ganglia were not affected by loss of Ascl1 at 11.5 dpc (Fig. 6B), but growth was retarded subsequently. By 16.5 dpc, relative to control embryos (Fig. 6C), the size of the ganglia in Ascl1 mutant embryos was dramatically reduced (Fig. 6D). To determine if the reduced ganglia size in Ascl1 mutant embryos is due to decreased cell proliferation or to increased cell death, we examined the proliferation rate and level of apoptosis in 10.5 to 15.5 dpc embryos. TUNEL analysis showed that the level of apoptosis did not change in the SNS of Ascl1 mutant embryos (data not shown). Cell proliferation in the SNS was quantified by BrdU incorporation of β-galactosidase-expressing cells. Loss of Ascl1 resulted in a reduced level of proliferation beginning at 11.5 dpc (Fig. 6E). These results show that Ascl1 plays a role in cell proliferation during SNS development but is not required for cell viability.

Embryos lacking Ascl1 survive to birth (Guillemot et al., 1993), suggesting that the noradrenergic phenotype is not defective and that the noradrenergic marker dopamine β-hydroxylase (Dbh) is expressed in Ascl1 mutant embryos (Pattyn et al., 2006). However, it has been reported that Ascl1 is involved in neurogenesis and neuronal selection of SNS (Sommer et al., 1995). To address the discrepancy, we determined whether SNS neurons are formed and differentiate into catecholaminergic neurons by tracing the NC lineage with β-galactosidase and examining the expression of the pan-neuronal marker TuJ1 and the catecholaminergic marker TH in Ascl1 knockout embryos (Fig. 6F-I). Although the size of SNS ganglia in Ascl1 mutant embryos was smaller, the levels of TuJ1 (Fig. 6G) and TH (Fig. 6I) expression relative to their size was the same as for control embryos. These results suggest that Ascl1 does not play an essential role in neuronal differentiation or selection of the noradrenergic phenotype.

Wild-type primary NC stem cells (NCSCs) can respond to BMP signaling and form noradrenergic neurons, whereas NCSCs from the embryos lacking Ascl1 do not respond to BMP signaling and fail to differentiate into catecholaminergic neurons (Sommer et al., 1995). However, we show that, in the embryo, sympathetic precursors do not require Ascl1 to form catecholaminergic neurons. To resolve this discrepancy and to determine if NC cells are autonomously
competent to undergo neurogenesis and neuronal phenotype selection in the absence of Ascl1, we examined whether NCSCs obtained from the neural folds of Ascl1–/– embryos had the ability to become noradrenergic neurons in vitro. Differentiation of NC cells into SNS neurons is dependent on cell number and is referred to as a colony effect (Kleber et al., 2005). As BMP signaling regulates proliferation in SNS development, we tested whether the lack of noradrenergic neurons in NCSCs from Ascl1–/– is a secondary effect to the lack of proliferation resulting in too few cells. To bypass the reduced proliferation in NCSCs lacking Ascl1, we plated NCSCs at high density. Both wild-type and Ascl1–/– NCSCs cultured at high density differentiated into noradrenergic neurons without the addition of exogenous BMP (Fig. 6J,K). These results suggest that Ascl1 is a downstream target of the Smad4-dependent BMP signal transduction pathway and that it regulates proliferation but not noradrenergic differentiation of the SNS.

**SNS development is not dependent on Tak1**

BMP signaling is transduced by both Smad-dependent and -independent pathways. A prominent non-Smad-dependent pathway occurs through BMP receptor activation of Tak1, leading to the activation of a number of downstream kinases, including p38 and Jun kinases (Zhang, 2009). To determine if Smad4-independent SNS development occurs through Tak1 and downstream kinases, we deleted Tak1 in NC cells (Liu et al., 2006). To determine if Tak1 is required for SNS neuronal and catecholaminergic differentiation, 11.5 dpc embryos were examined for expression of Tuj1 and TH (Fig. 7A,B). The loss of Tak1 did not effect initial expression of Tuj1 or TH and was not required to maintain their expression during development (see Fig. S2 in the supplementary material). To determine if Tak1 regulates patterning of the SNS, we traced the NC lineage using R26R and examined the morphology of the SNS in 17.5 dpc embryos (Fig. 7C,D). The size and pattern of the sympathetic trunk were not affected by the loss of Tak1, suggesting that the Tak1 pathway does not play a major role in SNS development.

The sympathetic ganglia formed in embryos lacking Tak1 but not in the absence of Alk3-mediated BMP signaling, showing that SNS formation is regulated by a novel BMP pathway. During canonical BMP signaling, Smad4 functions to translocate phosphorylated Smad1/5/8 into the nucleus (Hill, 2009; Massague et al., 2005). To determine if the nuclear localization of phospho-Smads occurs independently of Smad4 during SNS development, phospho-Smad1/5/8 was localized by immunohistochemistry. During SNS formation, phospho-Smad1/5/8 was located in the
During development, BMP acts as the inductive signal for noradrenergic neurogenesis (Reissmann et al., 1996; Schneider et al., 1999; Shah et al., 1996; Varley and Maxwell, 1996). The Tak1 pathway does not play a role in SNS development. Differentiation of the SNS was analyzed by expression of TH and Tuj1 in control (A) and Tak1fx/fox; Wnt1-Cre (B) embryos at 11.5 dpc. Expression of TH and Tuj1 was not affected by the loss of Tak1. NC-derived cells in Tak1fx/fox; Wnt1-Cre; R26R+/− (C), and Smad4fx/fox; Wnt1-Cre; R26R+/− cKO (D) embryos at 17.5 dpc were stained for β-galactosidase activity. Development of sympathetic ganglia was not affected by loss of Tak1. (E,F) Nuclear localization of phospho-Smad1/5/8 in the SNS was Smad4 independent. The distribution of phospho-Smad1/5/8 was examined by immunofluorescent analysis in Smad4fx/fox; Wnt1-Cre; R26R+/+ (E) and Smad4fx/fox; Wnt1-Cre; R26R+/− cKO (F) embryos at 11.5 dpc. The SNS was identified by β-galactosidase staining and is delineated by the white dashed lines. Loss of Smad4 does not affect the ability of activated R-Smads to enter the nucleus. da, dorsal aorta.

**DISCUSSION**

During development of the SNS, BMP acts as the inductive signal for noradrenergic neurogenesis (Reissmann et al., 1996; Schneider et al., 1999; Shah et al., 1996; Varley and Maxwell, 1996). BMP is known to induce transcription factors involved in SNS development (Howard et al., 2000; Lo et al., 1998; Schneider et al., 1999; Shah et al., 1996; Tsarovina et al., 2004), yet the mechanism by which BMP signaling regulates SNS development has not been investigated. Here, we determined the roles of BMP signaling in SNS development, using cKO embryos of Alk3, Smad4, and Tak1. Our results show that BMP signaling regulates multiple events: survival of sympathetic precursors, proliferation of neuroblasts and noradrenergic differentiation. Furthermore, these developmental events are regulated through different transduction pathways by regulation of different transcription factors.

**The roles of Tgfβ/BMP signaling in SNS development**

During SNS development, BMP signaling has been shown to induce noradrenergic neurons (Reissmann et al., 1996; Schneider et al., 1999; Shah et al., 1996; Varley and Maxwell, 1996). Our results now show that BMP signaling regulates numerous aspects of SNS development. We show that BMP signaling is required for the survival of sympathetic precursors and that canonical BMP signaling regulates the proliferation of sympathetic neuroblasts and noradrenergic differentiation. These roles are similar to those of BMP during the development of the enteric nervous system, which has numerous similarities to the development of the SNS. In vitro studies using isolated enteric NC cells has shown that BMP enhances the formation of neurons (Lo et al., 1997) and promotes the aggregation of enteric NC cells to form ganglia (Chalazonitis et al., 2004; Goldstein et al., 2005). In addition, BMP signaling regulates cell cycle exit and neuronal phenotype selection (Chalazonitis et al., 2004; Chalazonitis et al., 2008).

**SNS development is regulated by a Smad4-independent BMP pathway**

In the canonical Tgfβ/BMP pathway, BMP signaling is mediated by phosphorylation of R-Smads and shuttling of the phosphorylated R-Smads into the nucleus by Smad4 (Hill, 2009; Massague et al., 2005). Our results show that survival of sympathetic precursors is dependent on BMP signaling but independent of Smad4. The roles of Smad4-independent Tgfβ signaling during development have recently been reported. During development of the palate, a Smad4-independent Tgfβ pathway, which is mediated by a p38 kinase pathway, is involved in palate fusion (Xu et al., 2008). Smad4-independent Tgfβ signaling also plays a role in cardiac (Song et al., 2007) and sensory nervous system development (Hodge et al., 2007) through unknown pathways.

We observed that nuclear-shuttling of phospho-Smad1/5/8, the BMP-specific R-Smads, occurs in the absence of Smad4 in the SNS, suggesting that phospho-Smads interact with factors other than Smad4 during the translocation process. During tumor progression, Smad2, a Tgfβ-specific R-Smad, functions independently of Smad4 by crosstalk with Wnt/β-catenin and the P300 pathway (Hirota et al., 2008). Furthermore, numerous factors have been identified as
interacting with R-Smad proteins (Guo and Wang, 2009; Jiao et al., 2002; Zwijsen et al., 2003). Factors that are able to transport R-Smads to the nucleus may regulate development of SNS through Smad4-independent shuttling of R-Smads.

The survival of sympathetic precursors depends on Phox2b (Pattyn et al., 1999). Our results show that Phox2b is downstream of the BMP pathway, which is consistent with previous in vitro analysis (Howard et al., 2000; Schneider et al., 1999). However, the mechanism by which BMP signaling regulates Phox2b expression and, thus, survival of precursors remains unknown. Although the major non-Smad-dependent pathway activated by BMP is the Takh pathway, we show that it is not involved in SNS development.

**SNS development is regulated by a Smad4-dependent pathway**

Our results show that Smad4 is involved in the proliferation and noradrenergic differentiation of sympathetic neuroblasts. Smad4 is a common factor involved in all Tgfβ superfamily signaling pathways (Hill, 2009; Massague et al., 2005). Tgfβ signaling does not appear to be involved in SNS development (Dudas et al., 2006; Ito et al., 2003; Shah et al., 1996), suggesting that Smad4 function occurs through the BMP-specific R-Smads. Expression of Ascl1 is regulated by Smad4, which is consistent with previous studies showing that BMP activates transcription of Ascl1 (Shah et al., 1996). Ascl1 has been assigned a number of conflicting roles during SNS development (Guillemot et al., 1993; Pattyn et al., 2006; Sommer et al., 1995). We show that the role of Ascl1 is to promote proliferation of sympathetic neuroblasts, suggesting that Smad4 regulates proliferation of SNS progenitors through Ascl1. Ascl1 is required for the expression of zinc finger factor Insm1, which also regulates proliferation of SNS progenitors (Wildner et al., 2008), suggesting that Ascl1 regulates the proliferation of SNS in parallel with Insm1 or by activating Insm1 expression.

Noradrenergic differentiation on SNS neurons is also regulated by Smad4. The expression of transcription factors Gata3 and Hand2, the known regulators of noradrenergic differentiation (Lim et al., 2000; Lucas et al., 2006; Morikawa et al., 2006), is not affected by the loss of Smad4, indicating that a Smad4-dependent pathway regulates noradrenergic differentiation by a novel mechanism. BMP-induced noradrenergic phenotype selection in vitro is enhanced by protein kinase A (PKA) activity, and phosphorylation of Hand2 may be involved in this pathway (Liu et al., 2005), as phosphorylation of Hand factors by PKA is known to regulate their transcription activities (Firulli et al., 2003). Although the role of PKA in SNS development is not clear, it is possible that Smad4-dependent noradrenergic differentiation is mediated by activation of PKA followed by phosphorylation of Hand2.

**Multiple functions of BMP signaling are mediated by different pathways**

BMPs have been shown to regulate different developmental events in the same cell lineage. However, it remains an enigma how each event is mediated. Here, we show that BMP activates multiple developmental events through different pathways. Based on the current study and other studies, we propose that different components of the BMP pathway regulate different aspects of development. During SNS development (Fig. 8), a Smad4-independent pathway activates the expression of Phox2b, which is essential for the survival of sympathetic precursors. A Smad4-dependent pathway regulates the proliferation of neuroblasts through its regulation of the continued expression of Ascl1. Another role for BMP signaling is the regulation of noradrenergic differentiation through a Smad4-independent pathway. A Smad4-independent pathway regulates the expression of Hand2 and Gata3, two transcription factors required for the expression of the norepinephrine biosynthetic enzymes TH and Dbh through Phox2b; this suggests that two pathways converge to regulate noradrenergic differentiation.
Jiao, K., Zhou, Y. and Hogan, B. L. (2002). Identification of mZnf18, a mouse Kruppel-like transcriptional repressor, as a novel nuclear interaction partner of Snail1. Mol. Cell. Biol. 22, 7633-7644.

Kleber, M., Lee, H. Y., Wurdak, H., Buchstaller, J., Riccamogno, M. M., Itnner, L. M., Suter, U., Epstein, D. J. and Sommer, L. (2005). Neural crest stem cell maintenance by combinatorial Wnt and BMP signaling. J. Cell Biol. 169, 309-320.

Ko, S. O., Chung, I. H., Xu, X., Oka, S., Zhao, H., Cho, E. S., Deng, C. and Chai, Y. (2007). Smad4 is required to regulate the fate of cranial neural crest cells. Dev. Biol. 312, 435-447.

Lim, K. C., Lakshmanan, G., Crawford, S. E., Gu, Y., Grosveld, F. and Engel, J. D. (2000). Gata3 loss leads to embryonic lethality due to noradrenaline deficiency of the sympathetic nervous system. Nat. Genet. 25, 209-212.

Liu, H., Margiotta, J. F. and Howard, M. J. (2005). BMP4 supports noradrenergic differentiation by a PKA-dependent mechanism. Dev. Biol. 286, 521-536.

Liu, H. H., Xie, M., Schneider, M. D. and Chen, Z. J. (2006). Essential role of TAK1 in thymocyte development and activation. Proc. Natl. Acad. Sci. USA 103, 11677-11682.

Lo, L., Sommer, L. and Anderson, D. J. (1997). MASH1 maintains competence for BMP2-induced neuronal differentiation in post-migratory neural crest cells. Curr. Biol. 7, 440-450.

Lo, L., Tiveron, M. C. and Anderson, D. J. (1998). MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. Development 125, 609-620.

Lucas, M. E., Muller, F., Rudiger, R., Henion, P. D. and Rohrer, H. (2006). The BHLH transcription factor Hand2 is essential for noradrenergic differentiation of sympathetic neurons. Development 133, 4015-4024.

Mao, X., Fujiwara, Y. and Orkin, S. H. (1999). Improved reporter strain for examining alkaline phosphatase expression in mouse embryos. Circ. Res. 81, 318-324.

Massague, J., Seoane, J. and Wotton, D. (2005). Smad transcription factors. Genes Dev. 19, 2783-2810.

Mishina, Y., Suzuki, A., Ueno, N. and Behringer, R. R. (1995). Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. Genes Dev. 9, 3027-3037.

Mishina, Y., Hanks, M. C., Miura, S., Tallquist, M. D. and Behringer, R. R. (2000). Gata3 loss leads to embryonic lethality due to noradrenaline deficiency of the sympathetic nervous system. Nature 399, 366-370.

Pattyn, A., Morin, X., Cremer, H., Goridis, C. and Brunet, J. F. (1997). Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. Development 124, 4065-4075.

Pattyn, A., Morin, X., Cremer, H., Goridis, C. and Brunet, J. F. (1999). The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature 399, 2079-2088.

Reissmann, E., Ernsberger, U., Francis-West, P. H., Rueger, D., Brickell, P. M. and Rohrer, H. (1996). Involvement of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons. Development 122, 2079-2088.

Song, L., Yan, W., Chen, X., Deng, C. X., Wang, Q. and Jiao, K. (2007). Myocardial smad4 is essential for cardiac differentiation in mouse embryos. Circ. Res. 101, 277-285.

Stottmann, R. W., Choi, M., Mishina, Y., Meyers, E. N. and Klingensmith, J. (2004). BMP receptor IA is required in mammalian neural crest cells for development of the cardiac outflow tract and ventricular myocardium. J. Cell Biol. 164, 2205-2216.

Tasrovinova, K., Pattyn, A., Stubbusch, J., Muller, F., Van Der Weeke, J., Schneider, C., Brunet, J. F. and Rohrer, H. (2004). Essential role of Gata transcription factors in sympathetic neuron development. Development 131, 4775-4786.

Varley, J. E. and Maxwell, G. D. (1996). BMP-2 and BMP-4, but not BMP-6, increase the number of adrenergic cells which develop in quail trunk neural crest cultures. Exp. Neurol. 140, 84-94.

Varley, J. E., McPherson, C. E., Zou, H., Niswander, L. and Maxwell, G. D. (1998). Expression of a constitutively active type I BMP receptor using a retroviral vector promotes the development of adrenergic cells in neural crest cultures. Dev. Biol. 196, 117-118.

Wildner, H., Gierl, M. S., Strehle, M., Pia, P. and Birchmeier, C. (2008). Insrm1 (IA-1) is a crucial component of the transcriptional network that controls differentiation of the sympathoadrenal lineage. Development 135, 473-481.

Xu, X. and Howard, M. J. (2001). Two signal transduction pathways involved in the catecholaminergic differentiation of avian neural crest-derived cells in vitro. Mol. Cell. Neurosci. 18, 394-406.

Xu, X., Han, J., Ito, Y., Bringas, P., Jr., Deng, C. and Chai, Y. (2008). Ectodermal neural crest stem cell populations by differentiation of embryonic stem cells in vitro. Scientific World Journal 2, 690-700.

Zwijnen, A., Verschuuren, K. and Huylenbroeck, D. (2003). New intracellular components of bone morphogenetic protein5 smad signaling cascades. FEBS Lett. 546, 133-139.