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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.

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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 (Lim 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 Tgfβ-activated kinase 1 (Tak1; Map3k7 – Mouse Genome Informatics) by Tgfβ 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. $Alk3^{+/-}$ (Mishina et al., 1995), $Smad4^{fx/fx}$ (Yang et al., 2002), and $Tak1^{fx/fx}$ (Liu et al., 2006), were crossed with *Wnt1-Cre* to obtain $Alk3^{+/-}$; *Wnt1-Cre*, $Smad4^{fx/+}$; *Wnt1-Cre*, and $Tak1^{fx/+}$; *Wnt1-Cre* males. The males were crossed with $Alk3^{fx/fx}$ (Mishina et al., 2002), $Smad4^{fx/fx}$ and $Tak1^{fx/fx}$ lines, respectively, to obtain embryos. For lineage tracing of NC cells, $Alk3^{fx/fx}$, $Tak1^{fx/fx}$ and $Smad4^{fx/fx}$ lines were crossed with *R26R* (*Gt*(*ROSA*)*26Sor*^{tm1Sho}) (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 $Ascl1^{-/-}$ background, $Ascl1^{+/-}$ mice (Guillemot et al., 1993) were mated with *Wnt1-Cre* and *R26R/R26R* lines to obtain $Ascl1^{+/-}$; *Wnt1-Cre* and $Ascl1^{+/-}$; *R26R/R26R* lines.

In situ hybridization

In situ hybridizations were performed as described previously (Morikawa et al., 2007; Morikawa et al., 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 Signaling); 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. F. 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.

Primary neural crest culture

Cells for primary NC cultures cells were isolated from 9.5 dpc embryos, as previously described (Shah et al., 1996; Sommer et al., 1995) with modifications. In brief, the thoracic region containing the last ten somites was dissected in 1% FBS in D-MEM using tungsten needles. To isolate the neural tubes, the tissues were treated with 1.5 mg/ml Dispase (Invitrogen) and 0.04% DNase I (Sigma) in dissecting medium for 20 minutes, followed by 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% MEDII medium (Rathjen and Rathjen, 2002) and 20 ng/ml Fgf2 (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.

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 closure, and the NC cells that become SNS precursors reached the dorsal aorta by 9.5 dpc (Fig. 1A). NC cells began to aggregate at the dorsal aorta at 10.0 dpc and formed compact ganglia-like clusters of cells by 10.5 dpc (Fig. 1C,E). In embryos lacking *Alk3* in the NC lineage, migration of the NC cells to the dorsal aorta was unaffected (Fig. 1B), showing that deletion of *Alk3* by *Wnt1-Cre* does not affect migration of NC cells nor their ability to recognize the correct migratory pathway. The *Alk3*-null NC cells populated the region in which SNS is formed by 10.0 dpc; however, the cells failed to aggregate (Fig. 1D). By 10.5 dpc, the region normally populated by forming sympathetic ganglia was devoid of NC cells (Fig. 1F). This shows that BMP signaling for survival of the NC precursor cell is a cell-autonomous requirement.

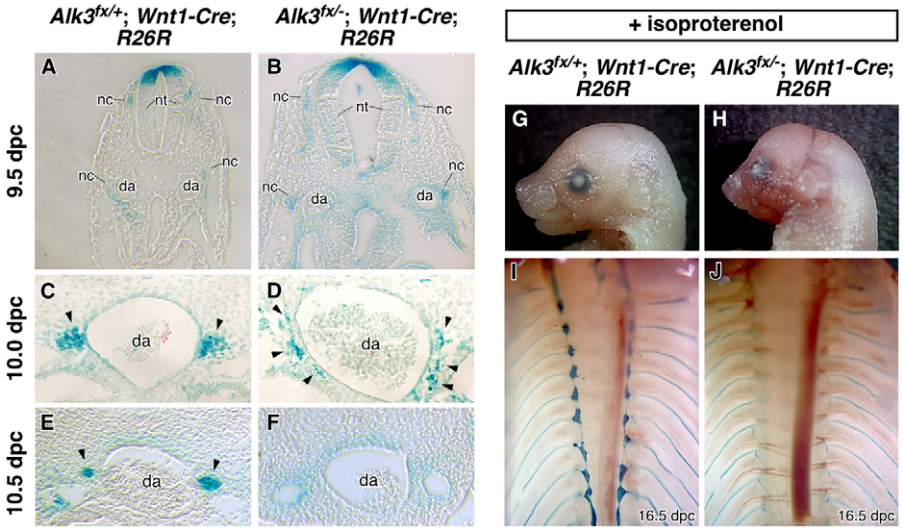

Fig. 1. BMP signaling is required for formation of sympathetic ganglia. *Alk3* was deleted in the NC lineage using *Wnt1-Cre*, and NC cells were simultaneously traced by genetically marking by β-galactosidase expression from the *R26R* knock-in allele. (**A-F**) β-Galactosidase expression in *Alk3*$^{fx/+}$; *Wnt1-Cre*; *R26R*/+ which are phenotypically wild-type (A,C,E), and *Alk3*$^{fx/-}$; *Wnt1-Cre*; *R26R*/+ cKO (B,D,F) 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 *Alk3*$^{fx/-}$; *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.

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, 2008; Morikawa et al., 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* cKOs in 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 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

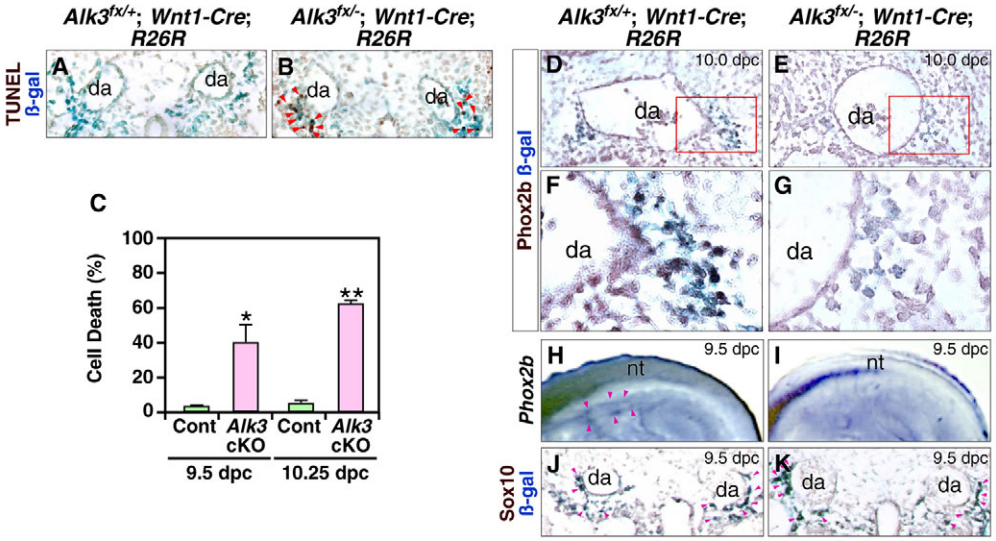

Fig. 2. BMP signaling is required for the survival of sympathetic precursors and for the expression of Phox2b. To determine if sympathetic precursor cells were dying by apoptosis, 9.5 dpc embryos were analyzed by TUNEL. (**A-C**) Immunohistochemical analysis of apoptosis in *Alk3*$^{fx/+}$; *Wnt1-Cre*; *R26R*/+ (A) and *Alk3*$^{fx/-}$; *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). *, $P \leq 0.022$; **, $P \leq 0.000014$. (**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 *Alk3*$^{fx/-}$; *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 *Alk3*$^{fx/-}$; *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 dpc, 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 dpc 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).

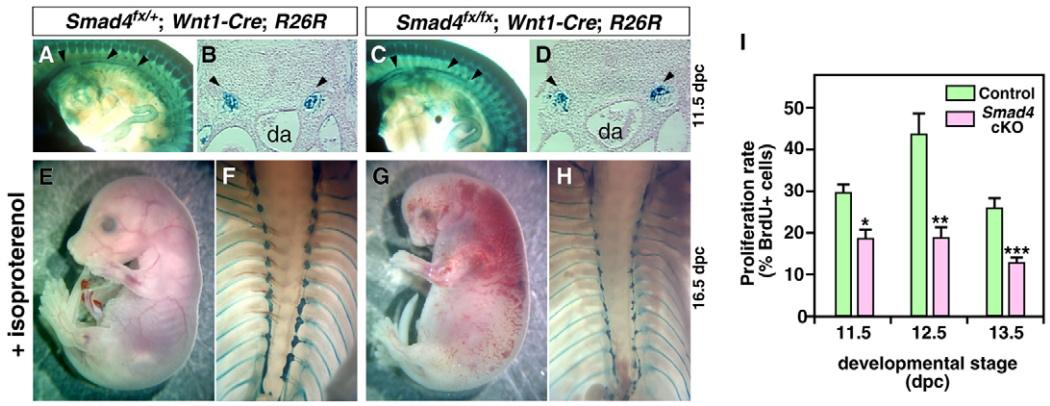

Fig. 3. Smad4 is not required for formation of the SNS but is required for proliferation. (**A-D**) *Smad4* was deleted in the NC lineage using *Wnt1-Cre*, and the NC lineage was simultaneously traced using the *R26R* allele. NC-derived cells in *Smad4*$^{fx/+}$; *Wnt1-Cre*; *R26R*/+ (A,B), and *Smad4*$^{fx/fx}$; *Wnt1-Cre*; *R26R*/+ cKO (C,D) embryos at 11.5 dpc were stained for β-galactosidase activity and cleared (A,C) or sectioned (B,D). The sympathetic ganglia (arrowheads), DRG, and enteric nervous system form in the absence of Smad4. (**E-H**) Pharmacological rescue of *Smad4* cKO embryos. Dams were administered isoproterenol, and *Smad4*$^{fx/+}$; *Wnt1-Cre*; *R26R*/+ (E,F) and *Smad4*$^{fx/fx}$; *Wnt1-Cre*; *R26R*/+ cKO (G,H) embryos were collected at 16.5 dpc. *Smad4* cKO embryos showed gross morphological defects of the head (G) and a hypoplastic SNS (H). (**I**) The proliferation rate of cells in the SNS was quantified by BrdU incorporation from 11.5 to 13.5 dpc of development, showing that loss of Smad4 results in reduced proliferation. *, $P \leq 0.03$; **, $P \leq 0.01$; ***, $P \leq 0.007$. da, dorsal aorta.

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. 4A,B). Loss of *Smad4* did not affect the 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

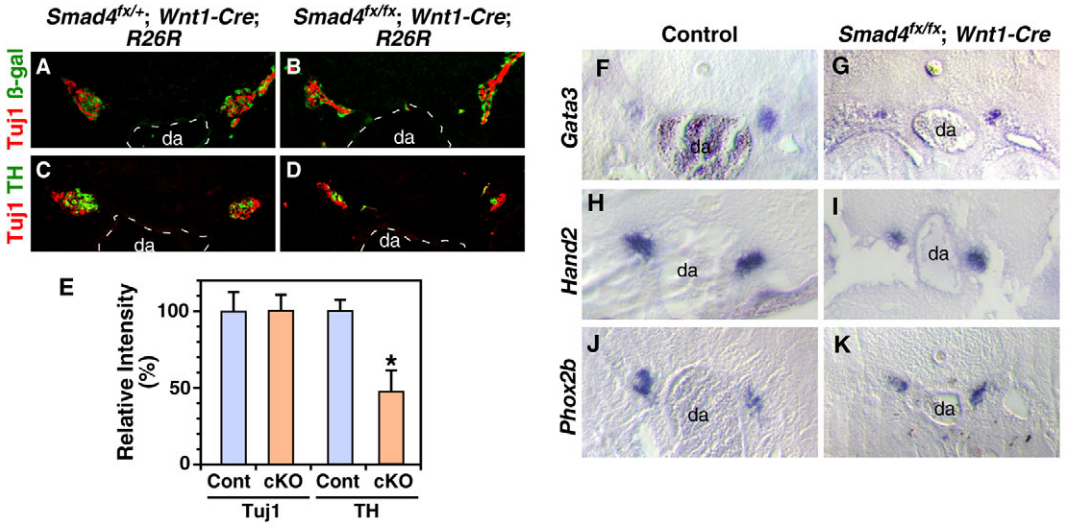

Fig. 4. The Smad4-dependent pathway is required for noradrenergic differentiation but not for neurogenesis. (**A-E**) Immunofluorescent analysis of *Smad4*$^{fx/+}$; *Wnt1-Cre*; *R26R*/+ (A,C, control) and *Smad4*$^{fx/fx}$; *Wnt1-Cre*; *R26R*/+ cKO (B,D) embryos at 12.5 dpc for expression of the pan-neuronal marker Tuj1 (A,B) and the noradrenergic marker TH (C,D). The levels of Tuj1 were quantified by the level of immunofluorescence relative to β-galactosidase, and the levels of TH were quantified relative to Tuj1 immunofluorescence per ganglia (E). (**F-K**) Effect of Smad4 loss on expression of transcriptional factors regulating TH expression. In situ hybridization of control (F,H,J) and *Smad4*$^{fx/fx}$; *Wnt1-Cre* cKO (G,I,K) embryos at 12.5 dpc. Zinc finger factor *Gata3* (F,G), bHLH factor *Hand2* (H,I) and homeodomain factor *Phox2b* (J,K) were not affected by loss of Smad4. *, $P \leq 0.031$.

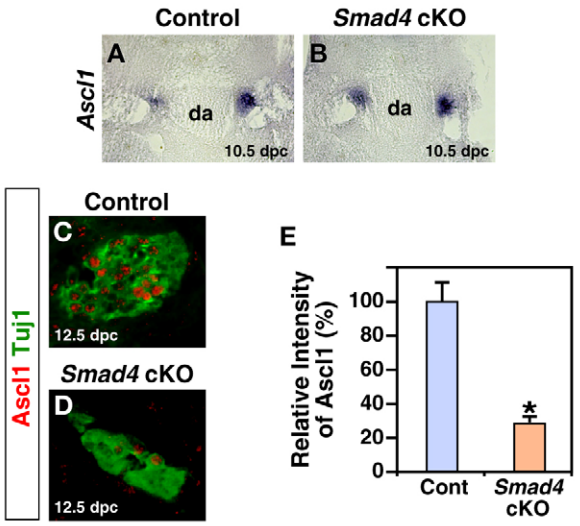

Fig. 5. Continued Ascl1 expression depends on Smad4. (**A**,**B**) Expression of *Ascl1* in control (A) and *Smad4*$^{fx/fx}$; *Wnt1-Cre* cKO (B) embryos was analyzed at 10.5 dpc by in situ hybridization. The activation of *Ascl1* expression is not affected by loss of Smad4. (**C-E**) Immunofluorescent analysis of control (C) and *Smad4*$^{fx/fx}$; *Wnt1-Cre* cKO (D) embryos at 12.5 dpc shows that Ascl1 expression decreases in the absence of Smad4. The expression of Ascl1 was quantified relative to the expression of the pan-neuronal marker Tuj1 (E). Continued expression of Ascl1 depends on expression of Samd4. *, $P \leq 0.043$. da, dorsal aorta.

expressed in *Smad4* cKO embryos at levels comparable to control embryos (data not shown). The level of noradrenergic differentiation was determined by the expression of the norepinephrine biosynthetic enzyme tyrosine hydroxylase (TH) (Fig. 4C,D). Quantification of the immunoreactivity of TH relative to Tuj1 levels showed that the levels of TH decreased 50% in the SNS of *Smad4* cKO embryos (Fig. 4E). The area expressing TH also decreased 50%, suggesting that loss of TH expression is due to a decrease in the number of TH-expressing cells. The loss of Smad4 in the NC lineage resulted in a reduced SNS and in the decreased number of cells expressing norepinephrine-synthesizing enzymes.

Noradrenergic differentiation is regulated by the transcription factors Gata3 (Lim et al., 2000), Hand2 (Morikawa et al., 2007) and Phox2b (Pattyn et al., 1999), the expression of which depend on BMP signaling (Howard et al., 2000; Lo et al., 1998; Schneider et al., 1999). To determine whether expression of these transcription factors is regulated by Smad4, we examined their expression by in situ hybridization (Fig. 4F-K). In the embryos lacking *Smad4* in the NC lineage, *Gata3* (Fig. 4F,G), *Hand2* (Fig. 4H,I) and *Phox2b* (Fig. 4J,K) were expressed at normal levels in the SNS, showing that the canonical BMP pathway does not regulate these genes.

The canonical BMP pathway regulates expression of Ascl1

Loss of Smad4 in the NC lineage resulted in a hypoplastic SNS (Fig. 3), resembling the phenotype of embryos carrying a deletion of the gene encoding the bHLH factor Ascl1 (Guillemot et al., 1993; Morikawa et al., 2005). The expression of Ascl1 can be activated by BMPs (Lo et al., 1997; Shah et al., 1996), and the similarity of the hypotrophic SNS phenotype between *Smad4* and *Ascl1* mutant embryos suggests that Ascl1 is a target of the canonical BMP pathway. To determine if *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*$^{-/-}$ 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*$^{-/-}$ embryos was dramatically reduced (Fig. 6D). To determine if the reduced ganglia size in *Ascl1*$^{-/-}$ 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*$^{-/-}$ 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

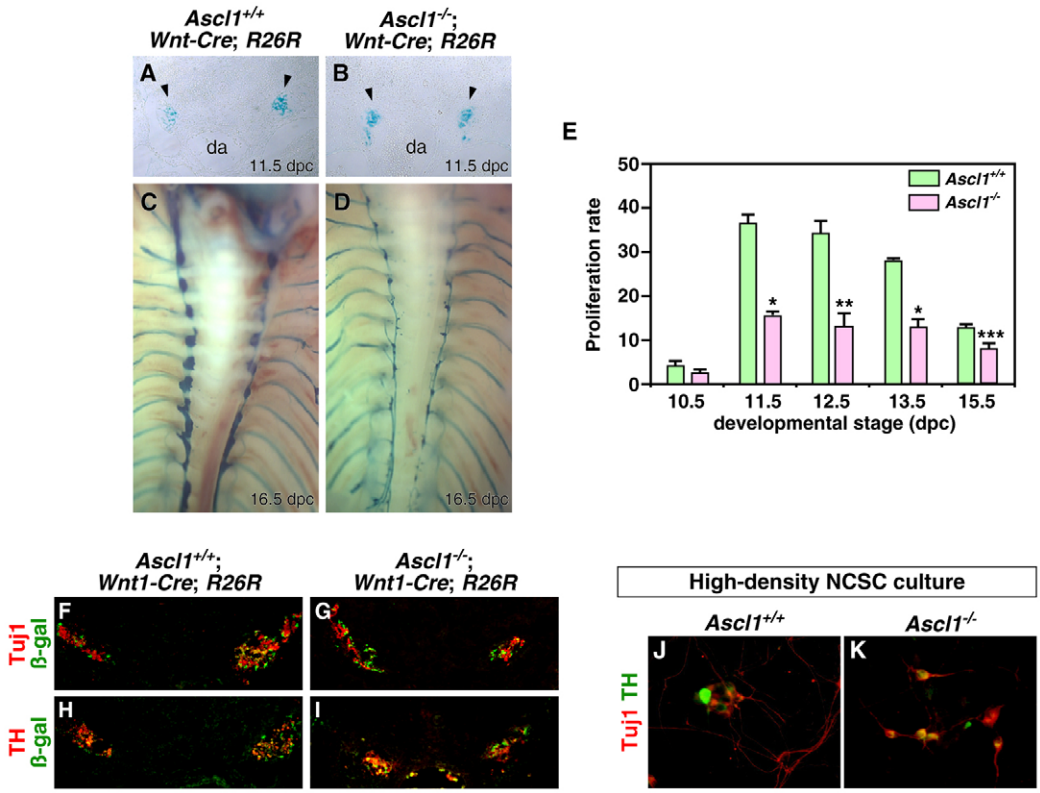

Fig. 6. Ascl1 regulates proliferation of sympathetic neuroblasts. (**A-D**) Sympathetic ganglia (arrowheads) were traced using *Wnt1-Cre* and the *R26R* locus in *Wnt1-Cre*; *R26R* (A,C) and in *Ascl1*$^{-/-}$; *Wnt1-Cre*; *R26R* (B,D) embryos at 11.5 dpc (A,B) and 16.5 dpc (C,D) embryos. The sizes of ganglia were comparable between control and mutant embryos at 11.5 dpc but were dramatically reduced by the loss of Ascl1 by 16.5 dpc. (**E**) To determine if a lower neuroblast proliferation rate accounted for the decreased size of the SNS in *Ascl1*$^{-/-}$ embryos, the proliferation rate was quantified by BrdU incorporation. (**F-K**) Ascl1 is not required for neurogenesis or noradrenergic differentiation. Immunofluorescent analysis of control (F,H) and *Ascl1*$^{-/-}$ (G,I) embryos at 12.5 dpc shows that Tuj1 (F,G) and TH (H,I) expression in the mutant is comparable to that in the control. Sympathetic ganglia were delineated using *Wnt1-Cre* to activate β-galactosidase expression from the *R26R* locus and visualizing using immunofluorescent analysis. To analyze the potential of *Ascl1* null NCSCs to differentiate in vitro, NCSCs from control (J) and *Ascl1*$^{-/-}$ (K) embryos were isolated and plated at high density. Neuronal differentiation was determined by expression of the pan-neuronal marker Tuj1, and noradrenergic differentiation by TH expression. The NCSCs from *Ascl1*$^{-/-}$ embryos are able to differentiate into noradrenergic neurons in vitro. *, $P \leq 0.0003$; **, $P \leq 0.007$; ***, $P \leq 0.03$. da, dorsal aorta.

competent to undergo neurogenesis and neuronal phenotype selection in the absence of Ascl1, we examined whether NCSC 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* or *Smad4* 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

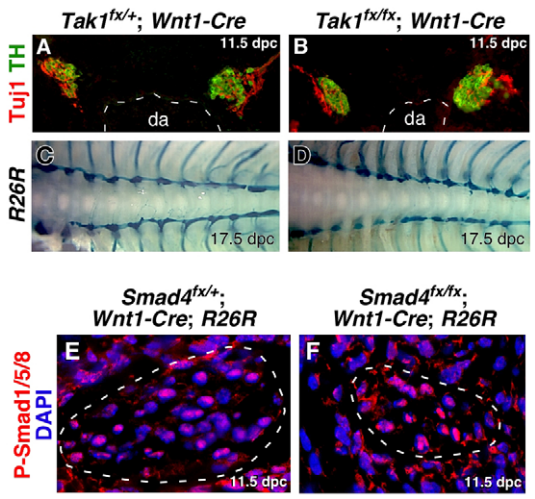

Fig. 7. BMP signaling is Tak1 independent, but R-Smads are nuclear localized during SNS development. (**A-D**) The Tak1 pathway does not play a role in SNS development. Differentiation of the SNS was analyzed by expression of TH and Tuj1 in control (A) and *Tak1*$^{fx/fx}$; *Wnt1-Cre* (B) embryos at 11.5 dpc. Expression of TH and Tuj1 was not affected by the loss of Tak1. NC-derived cells in *Tak1*$^{fx/+}$; *Wnt1-Cre*; *R26R*/+ (C), and *Smad4*$^{fx/fx}$; *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 phopho-Smad1/5/8 in the SNS was Smad4 independent. The distribution of phospho-Smad1/5/8 was examined by immunofluorescent analysis in *Smad4*$^{fx/+}$; *Wnt1-Cre*; *R26R*/+ (E) and *Smad4*$^{fx/fx}$; *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.

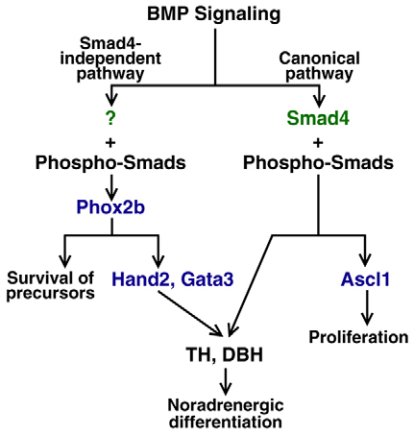

Fig. 8. The roles of BMP signaling pathways during SNS development. Both Smad4-dependent and -independent pathways are involved in SNS development. The Smad4-independent pathway activates the paired homeobox factor Phox2b, which regulates the survival of precursors and the expressions of bHLH factor Hand2 and zinc finger factor Gata3. The Smad4-dependent pathway is not required for *Ascl1* gene activation but is required for continued expression. Ascl1 regulates proliferation of neuroblasts but not their noradrenergic differentiation. Both pathways converge to regulate noradrenergic differentiation of sympathetic neurons.

nucleus (Fig. 7E), showing that BMP signaling activates R-Smads. In *Smad4* cKO embryos (Fig. 7F), phospho-Smad1/5/8 was transported to the cell nuclei of the developing sympathetic ganglia. This result shows that R-Smads can locate in the nucleus independently of Smad4, suggesting that they function to regulate survival of the SNS in a Smad4- and Tak1-independent manner.

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 Tak1 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; Moriguchi et al., 2006; Morikawa et al., 2007), 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-dependent 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.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/21/3575/DC1

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