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Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites

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SUMMARY

The secretion of Sonic hedgehog (Shh) from the notochord and floor plate appears to generate a ventral-to-dorsal gradient of Shh activity that directs progenitor cell identity and neuronal fate in the ventral neural tube. In principle, the establishment of this Shh activity gradient could be achieved through the graded distribution of the Shh protein itself, or could depend on additional cell surface or secreted proteins that modify the response of neural cells to Shh. Cells of the neural plate differentiate from a region of the ectoderm that has recently expressed high levels of BMPs, raising the possibility that prospective ventral neural cells are exposed to residual levels of BMP activity. We have examined whether modulation of the level of BMP signaling regulates neural cell responses to Shh, and thus might contribute to the patterning of cell types in the ventral neural tube. Using an in vitro assay of neural cell differentiation we show that BMP signaling markedly alters neural cell responses to Shh signals, eliciting a ventral-to-dorsal switch in progenitor cell identity and neuronal fate. BMP signaling is regulated by secreted inhibitory factors, including noggin and follistatin, both of which are expressed in or adjacent to the neural plate. Conversely, follistatin but not noggin produces a dorsal-to-ventral switch in progenitor cell identity and neuronal fate in response to Shh both in vitro and in vivo. These results suggest that the specification of ventral neural cell types depends on the integration of Shh and BMP signaling activities. The net level of BMP signaling within neural tissue may be regulated by follistatin and perhaps other BMP inhibitors secreted by mesodermal cell types that flank the ventral neural tube.

Key words: Neural patterning, Sonic hedgehog, BMP, Cell signalling, Neural plate, Chick

INTRODUCTION

In vertebrate embryos the specification of neural cell types is initiated by the actions of inductive factors derived from mesodermal and ectodermal tissues that flank the neural plate and neural tube (Tanabe and Jessell, 1996; Lumsden and Krumlauf, 1996). At caudal levels of the neural tube that give rise to the spinal cord and hindbrain, the patterning of ventral cell types depends on the actions of the secreted signaling factor Sonic hedgehog (Shh; Marti et al., 1995; Chiang et al., 1996; Ericson et al., 1996; Hammerschmidt et al., 1997). The long-range diffusion of Shh from the notochord and floor plate has been proposed to establish a ventral-to-dorsal gradient of Shh activity within the ventral neural tube that directs subsequent patterns of neurogenesis (Ericson et al., 1997b; Briscoe et al., 2000).

The possibility that graded Shh signaling activity controls ventral neural fates is supported by several lines of evidence. First, Shh activity can be detected at a distance from ventral sources of *Shh* synthesis (Ericson et al., 1996), suggesting that the active autoproteolytic amino-terminal fragment of Shh, Shh-N (Porter et al., 1995), is transferred over many cell diameters within the ventral neural epithelium. Second, recombinant Shh-N acts at several threshold concentrations in vitro to induce distinct subsets of ventral neurons (Roelink et al., 1995; Ericson et al., 1997a; Briscoe et al., 2000). Third, the *Ptc* gene is expressed in a ventral-to-dorsal gradient within the ventral neural tube (Goodrich et al., 1996; Marigo and Tabin, 1998; Ericson et al., 1997b). *Ptc* encodes a ligand binding subunit of the Shh receptor whose expression appears to be activated as a direct function of the level of hedgehog signaling (Marigo et al., 1996; Ingham, 1998; Murone et al., 1999), and thus its graded expression is indicative of a gradient of Shh activity. Finally, ectopic expression of an activated form of *Smo*, a gene encoding the signaling transducing subunit of the Shh receptor (Stone et al., 1996; Quirk et al., 1997), has been reported to induce ventral cell types in a cell-autonomous manner (Hynes et al., 2000), supporting the idea (Ericson et al., 1996) that Shh acts directly on target cells to specify ventral cell fates.

The gradient of Shh signaling activity evident in the ventral neural tube could reflect the graded distribution of Shh protein itself, or be achieved through a more complex process that involves the action of additional cell surface or secreted

proteins that modify neural cell responses to Shh. One class of secreted proteins that has been shown to exert a critical role in the patterning of the neural tube is the bone morphogenetic protein (BMP) family (Lee and Jessell, 1999). The secretion of BMPs by the epidermal ectoderm and the roof plate is required for the induction of neural crest cells and dorsal interneurons (Basler et al., 1993; Liem et al., 1995, 1997; Lee et al., 1998, 2000; Barth et al., 1999; Nguyen et al., 2000). Although studies on the roles of BMPs in the spinal cord have focused primarily on the control of dorsal cell fates, prospective ventral neural cells may also be exposed to BMP signals prior to or coincident with their exposure to Shh. Cells of the neural plate differentiate from a region of the ectoderm that initially express high levels of *BMPs (*Fainsod et al., 1994; Streit et al., 1998; Streit and Stern, 1999), and thus newly formed neural plate cells may be exposed to low levels of BMP protein at stages after *BMP* gene expression itself has been down-regulated.

BMP and Shh signals appear to have opponent and antagonistic functions in the control of cell fate along the dorsoventral axis of the neural tube. Dorsal neural progenitors normally give rise to neural crest cells and dorsal interneurons but their exposure to Shh inhibits the differentiation of these dorsal cell types (Yamada et al., 1991; Liem et al., 1995). Conversely, the Shh-mediated differentiation of floor plate cells and motor neurons is blocked by exposure to high BMP levels (Basler et al., 1993; Arkell and Beddington, 1997). Thus, the coincident exposure of prospective ventral neural cells to Shh and BMP could regulate the patterning of ventral neural cell types.

BMP signaling is itself subject to regulation by secreted factors that bind to BMPs and block their activity (Smith, 2000). Some of these inhibitory factors have been shown to bind to selective subsets of BMP-related proteins. Noggin binds to and inhibits the BMP2/4 and GDF5-GDF7 subclasses of BMPs (Zimmerman et al., 1996; Lee et al., 1999), whereas follistatin preferentially inhibits members of the BMP5-BMP7 subclass and also inhibits activin signaling (Nakamura et al., 1990; Yamashita et al., 1995; Liem et al., 1997; Iemura et al., 1998). *noggin*, *follistatin* and other BMP inhibitors are expressed by axial and paraxial mesodermal cells that flank the neural tube (Graham and Lumsden, 1996; Connolly et al., 1997; Dale et al., 1999), raising the possibility the secretion of these inhibitors by mesodermal tissues regulates the ambient level of BMP signaling within neural cells.

In this study we have examined whether BMP signaling and its control by secreted BMP inhibitors regulates neural cell responses to Shh, and thus might contribute to the patterning of cell types in the ventral neural tube. Using an in vitro assay of neural cell differentiation we show that BMP signaling markedly alters neural cell responses to Shh signals, eliciting a ventral-to-dorsal switch in progenitor cell identity and neuronal fate. Conversely, exposure of neural cells to the secreted BMP inhibitor follistatin produces a dorsal-to-ventral switch in progenitor cell identity and neuronal fate in vitro and in vivo. These results suggest that the specification of ventral cell types normally depends on the integration of Shh and BMP signaling activities. The net level of BMP signaling, and thus the response of neural cells to Shh signaling, in turn may be regulated by the actions of follistatin and other secreted BMP inhibitors supplied by mesodermal cell types than flank the ventral neural tube.

MATERIALS AND METHODS

Neural plate explant culture

Regions of the intermediate neural plate, ([i]) explants, were isolated from Hamburger and Hamilton (1951) (HH) stage 10 chick embryos as previously described (Yamada et al., 1993). Explants were cultured in a collagen gel (Vitrogen Inc) with Shh-N (Porter et al., 1995; Roelink et al, 1995; Ericson et al., 1996), follistatin (provided by National Hormone and Pituitary Program), noggin (gift from R. Harland), and *BMP7*-, or *BMP4*-transfected COS cell supernatant (see Basler et al., 1993; Lee et al., 1998; Alder et al., 1999). A myc-tagged pMT23-BMP7 was constructed using a mouse *BMP2* (Derynck) proregion fused to the mature region of chick *BMP7* (see Basler et al., 1993). One unit of BMP activity is defined as the threshold dilution of *BMP*-COS cell supernatant sufficient to induce the migration of >10 neural crest cells from [i] explants at 24 hours in vitro (see Liem et al., 1995). All experiments involving BMP7 activity derived from the same batch of *BMP7*-transfected COS cell supernatants.

In ovo electroporation

A cDNA encoding *Xenopus* follistatin (a gift from A. Hemmati-Brivanlou) was cloned into a CMV expression vector, mixed in a ratio of 5:1 with a CMV-GFP expression vector (Clontech) and electroporated in ovo into the neural tube of HH stage 10-12 chick embryos. Embryos were allowed to develop for a further 48 hours, until HH stage 20-22, and then fixed and processed for immunohistochemistry.

Immunohistochemisty

Neural explants were processed as for immunocytochemistry as described by Yamada et al. (1993). Antibodies used were: mouse monoclonal anti-Pax6 and anti-Pax7 (Ericson et al, 1996), rabbit or guinea pig anti-Nkx6.1 (Briscoe et al., 1999), anti-Nkx2.2 (Ericson et al., 1997a), anti-HNF3β (Ericson et al., 1997a), anti-En1 (Ericson et al., 1997a), anti-Otx2 (Nothias et al., 1999), anti-Isl1/2 and anti-Isl2 (Tsuchida et al., 1994; Ericson et al., 1992, 1996, 1997). FITC- and Cy3-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Inc. Images were collected on an MRC 1024 confocal microscope or on a Zeiss fluorescence microscope.

In situ hybridization histochemistry

Whole-mount in situ hybridization histochemistry was performed as described by Thery et al. (1995) on 30 μm cryostat sections. Chick probes used for in situ hybridization were *flik* (Patel et al., 1996*), follistatin* (Graham and Lumsden., 1996), *noggin* (Capdevila and Johnson, 1998), and *chordin* (Dale et al., 1999). A full length *flik* cDNA was cloned by PCR from the published sequence (Patel et al., 1996).

RT-PCR analysis

RT-PCR was performed as described by Tanabe et al. (1995). Primers used were:

S17 ribosomal protein (5′AGAAGGCGGCGCGGGTGATCATCG-3′ and 5′GTTTATTGTAAAAGCAACATAACG3′);

Isl-1 (5′TCAAACCTACTTTGGGGTCTTA3′ and 5′ATCGCCGG-GGATGAGCTGGCGGCT3′);

Ptc (5′TACCACGGGATACACGGGAATA3′ and 5′AATGG-GCTTTGCTCGGTTGC3′).

RESULTS

BMP exposure changes the identity of Shh-induced neural progenitor cells

To begin to address whether the level of BMP activity can influence the response of neural cells to Shh signaling we

monitored the expression of markers that define progenitor cell subclasses generated in the ventral neural tube of chick embryos. The identity of neural progenitor cells was defined on the basis of the expression of four homeodomain proteins, Pax7, Pax6, Nkx6.1 and Nkx2.2. Each protein marks a distinct progenitor (p) domain along the dorsoventral axis of the neural tube (see Fig. 1A; Ericson et al., 1997a; Briscoe et al., 1999, 2000). Pax7 is expressed by dorsal progenitor cells but is excluded from all ventral progenitors. Pax6 is expressed by ventral progenitors within the p0, p1, p2 and pMN progenitor domains. Nkx6.1 is expressed by cells within the p2, pMN and p3 progenitor domains. Nkx2.2 is expressed selectively by cells in the p3 progenitor domain (Fig. 1A). We also monitored the expression of HNF3β, a winged helix transcription factor expressed by floor plate cells (Ruiz i Altaba et al., 1995; Fig. 1A).

Previous studies have shown that the expression of Nkx6.1, Nkx2.2 and HNF3β can be induced in vitro at different Shh-N concentration thresholds. Nkx6.1 induction requires the lowest Shh-N level, Nkx2.2 a two- to three-fold higher level, and HNF3β a further two- to three-fold increase in Shh-N (Roelink et al., 1995; Ericson et al., 1997a; Briscoe et al., 1999; 2000 data not shown). Conversely, the expression of Pax7 is inhibited by Shh-N at a concentration approx. four-fold lower than that required for repression of Pax6 (see Ericson et al., 1997a). Thus, the differential patterns of expression of these five transcription factors serve as indicators of the response of neural progenitor cells to different levels of Shh signaling (Ericson et al., 1997b; Briscoe et al., 2000).

To test the influence of BMP signaling on the Shh-mediated control of neural progenitor cell identity we examined the expression of each of these transcription factors in HH stage 10 chick intermediate neural plate [i] explants (Yamada et al., 1993) grown in a defined concentration of Shh-N, with or without added BMPs. In these assays we routinely used BMP7 as a source of BMP, since all BMPs tested on [i] explants have qualitatively similar inductive activities (Liem et al., 1995; Lee et al., 1998). Consistent with these observations, we obtained similar results upon addition of BMP7 and BMP4 (data not shown).

In [i] explants grown alone for 24 hours, virtually all cells expressed Pax7 and Pax6, (Fig. 1B; data not shown), but expression of Nkx6.1, Nkx2.2 and HNF3β was not detected (Fig. 1C; data not shown; see Ericson et al., 1997b). Exposure of [i] explants to 2 nM Shh-N blocked Pax7 expression completely (Fig. 1D), permitted low level expression of Pax6 by virtually all cells (data not shown; Ericson et al., 1997a) and induced Nkx6.1 expression in ~30% of cells (Fig. 1E). At this Shh-N concentration, no expression of Nkx2.2 and HNF3β was detected (data not shown; Ericson et al., 1997a). In contrast, in the presence of both 1.0 Unit BMP7 and 2 nM Shh-N, virtually all cells in [i] explants expressed Pax7 (Fig. 1F) and high levels of Pax6 (data not shown), and no expression of Nkx6.1 expression was detected (Fig. 1G). Thus, BMP signaling changes the response of progenitor cells to a fixed concentration of Shh, resulting in the conversion of progenitor cells from a ventral to a dorsal identity.

We next examined whether, in addition to causing a complete shift from ventral to dorsal progenitor identity, BMP exposure can also alter the subtype identity of ventral progenitor cells generated in response to a set level of Shh signaling. We defined a concentration of Shh-N (2.4 nM) that suppressed Pax7 expression, generated few Pax6$^+$ cells and many Nkx2.2$^+$ ventral progenitor cells (Fig. 2A-C), but resulted in the generation of few if any HNF3β$^+$ cells (Fig. 2D). Coincident exposure of [i] explants to 0.25 Units BMP7 and 2.4 nM Shh-N resulted in an approx. 20-fold increase in the number of Pax6$^+$ progenitor cells (Fig. 2F), and an approx. ten-fold reduction in the number of Nkx2.2$^+$ progenitor cells (Fig. 2G). With this combination of factors, no Pax7$^+$ cells were detected (Fig. 2E), indicating that progenitor cells have

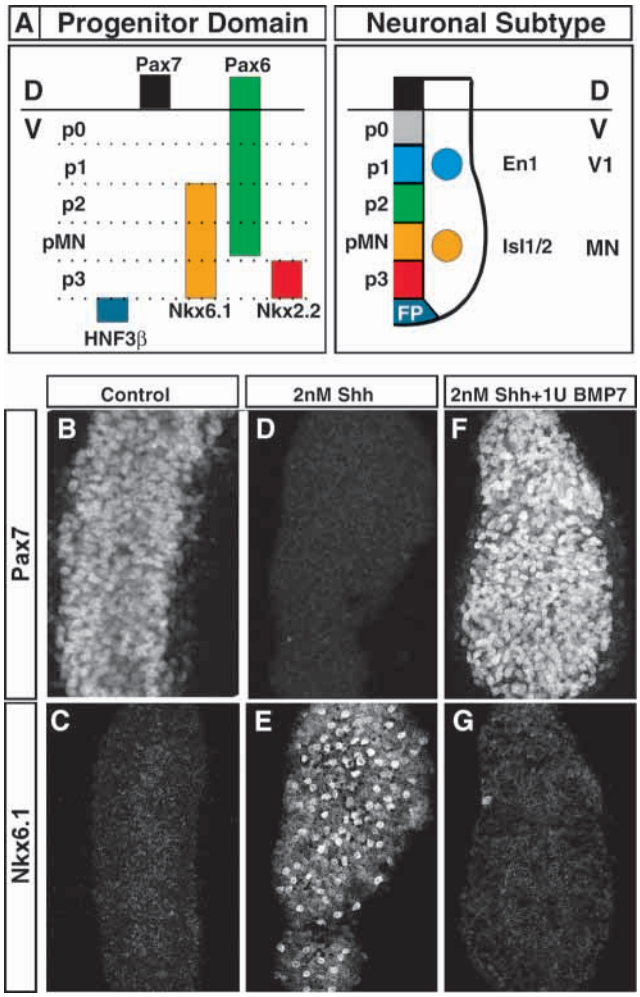

Fig. 1. BMP7 can dorsalize shh-induced ventral progenitor cells. (A) Diagram summarizing the patterns of expression of transcription factors that mark distinct ventral progenitor cell domains, and the location of two ventral neuronal subtypes, V1 interneurons and motor neurons (MN) that emerge from two of these domains. Progenitor domains (p) are marked. D and V indicate dorsal-ventral boundary of the neural tube. (B,C) Cells in neural plate ([i]) explants cultured for 24 hours express Pax7 (>500 cells/explant, *n*=3 explants) (B), but not Nkx6.1 (0 cells, *n*=3 explants) (C). (D,E) Stage 10 [i] explants cultured with 2 nM Shh-N give rise to few Pax7$^+$ cells (4±3 cells, *n*=3 explants) (D), and many Nkx6.1$^+$ cells (112±8 cells, *n*=4 explants) (E). (F,G) Stage 10 [i] explants cultured with 2 nM Shh-N and 1.0 Units BMP7 give rise to many Pax7$^+$ cells (>500 explant, *n*=3 explants) (F) but few Nkx6.1$^+$ cells (2±1 cells, *n*=3 explants) (G). Images and quantitation are representative of >20 explants under each condition.

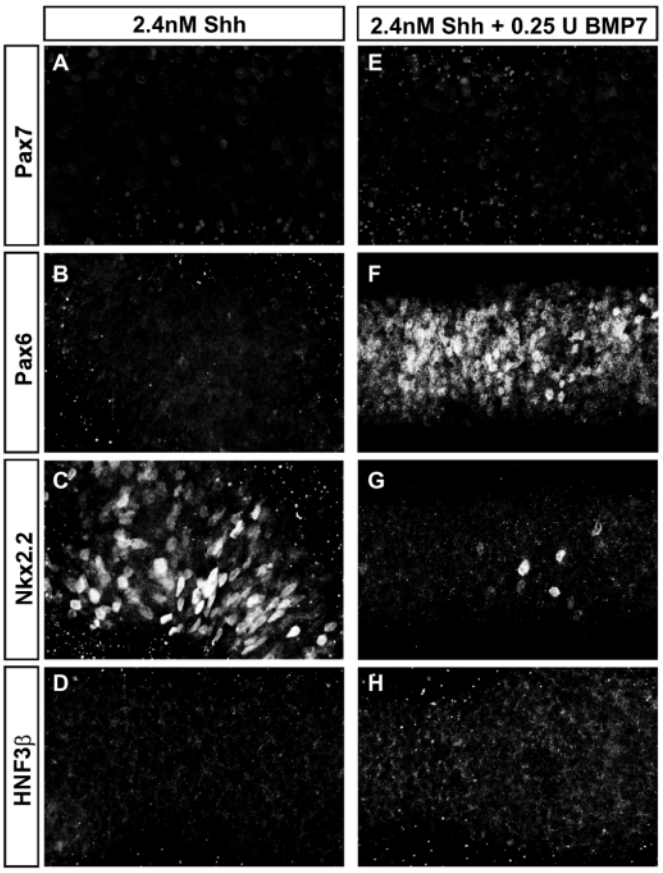

Fig. 2. BMPs alter the subtype identity of Shh-induced ventral progenitor cells. (A-D) Stage 10 [i] explants cultured for 24 h with 2.4 nM Shh-N do not contain Pax7$^+$ cells (0 cells/explant, *n*=4 explants) (A), contain a few cells that express Pax6 at low levels (14±7 cells, *n*=4 explants) (B), contain many Nkx2.2$^+$ (68±24 cells, *n*=4 explants) (C), but few HNF3β$^+$ cells (6±4 cells, *n*=4 explants) (D). (E-H) Stage 10 [i] explants cultured with 2.4 nM Shh-N and 0.25 Units BMP7 contain no Pax7$^+$ cells (0 cells, *n*=4 explants) (E), many cells that express Pax6 at high levels (>500 cells/explant, *n*=4 explants) (F), few Nkx2.2$^+$ cells (20±9 cells, *n*=4 explants) (G) and very few HNF3β$^+$ cells (8±6 cells, *n*=4 explants) (H).

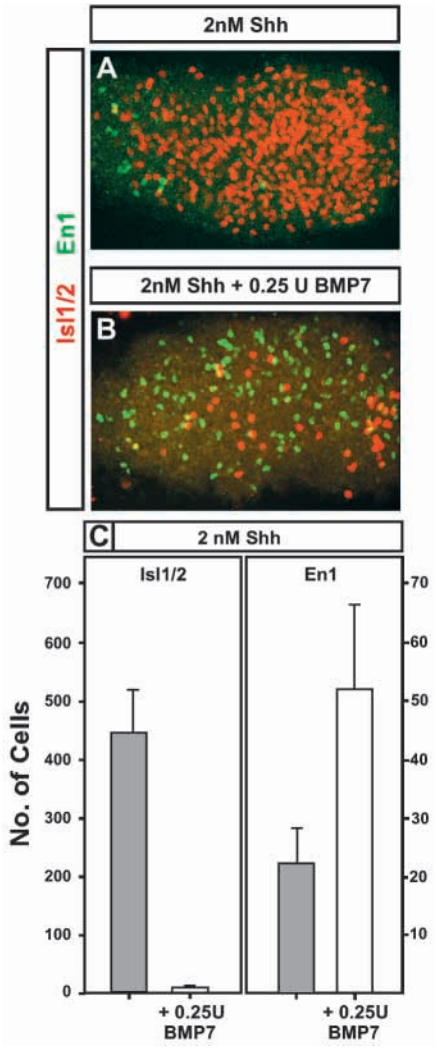

Fig. 3. BMPs alter the identity of Shh-induced ventral neuronal subtypes. (A) Stage 10 [i] explants cultured for 48 hours in the presence of 2 nM Shh-N contain many Isl1/2$^+$ motor neurons (red; 445±75 cells, *n*=5 explants) and few En1$^+$ V1 interneurons (green; 23±6 cells, *n*=5 explants). (B) Stage 10 [i] explants cultured for 48 hours in the presence of 2 nM Shh-N and 0.25 Units of BMP7 contain few Isl1/2$^+$ motor neurons (red; 9±4 cells, *n*=5 explants) and many En1$^+$ V1 interneurons (green; 53±14 cells, *n*=5) explants. (C) Quantitation of motor neuron and En1$^+$ V1 neuron generation in the presence of Shh-N, with or without 0.25 Units BMP7. Histograms show mean ± s.e.m.; *n*=5 explants.

retained a generic ventral identity. These findings show that BMP signaling can change the subtype identity of ventral progenitor cells generated in response to a set level of Shh signaling activity.

Coincident exposure of newly generated caudal neural plate cells to Shh and BMPs has been shown to induce midline forebrain neural fates, at least in rat tissue (Dale et al., 1997). We were concerned therefore that cells induced in HH stage 10 chick [i] explants by coincident BMP and Shh exposure might lose their caudal identity, thus complicating interpretation of the observed changes in neural marker expression. However, coincident exposure of stage 10 chick [i] explants to Shh (4 nM) and BMP (1.0 Unit) did not result in expression of the anterior neural markers Otx2 and Nkx2.1 (Acampora et al., 1999; Sussel et al., 1999) (data not shown). Moreover, the Isl1$^+$ neurons induced by this factor combination also coexpressed Isl2, a LIM homeodomain protein that is restricted to somatic motor neurons generated at caudal levels of the neuraxis (Ericson et al., 1997a; Varela-Echavarria et al., 1996; data not shown). These results indicate that the changes in neural marker expression by cells in stage 10 [i] explants exposed to Shh in the presence of BMPs result from an alteration in dorsoventral and not rostrocaudal identity.

Is the switch in the identity of ventral progenitor cells elicited by coincident BMP and Shh signaling accompanied by a change in neuronal fate? To test this, we assayed the generation of two classes of ventral neurons, motor neurons and V1 interneurons (Fig. 1A). In vitro, the level of Shh activity required to induce motor neurons is four- to six-fold greater than that required to induce V1 neurons (Ericson et al., 1997b). Exposure of [i] explants to 2 nM Shh-N generated approx. 450 Isl1/2$^+$ motor neurons and approx. 20 En1$^+$ V1

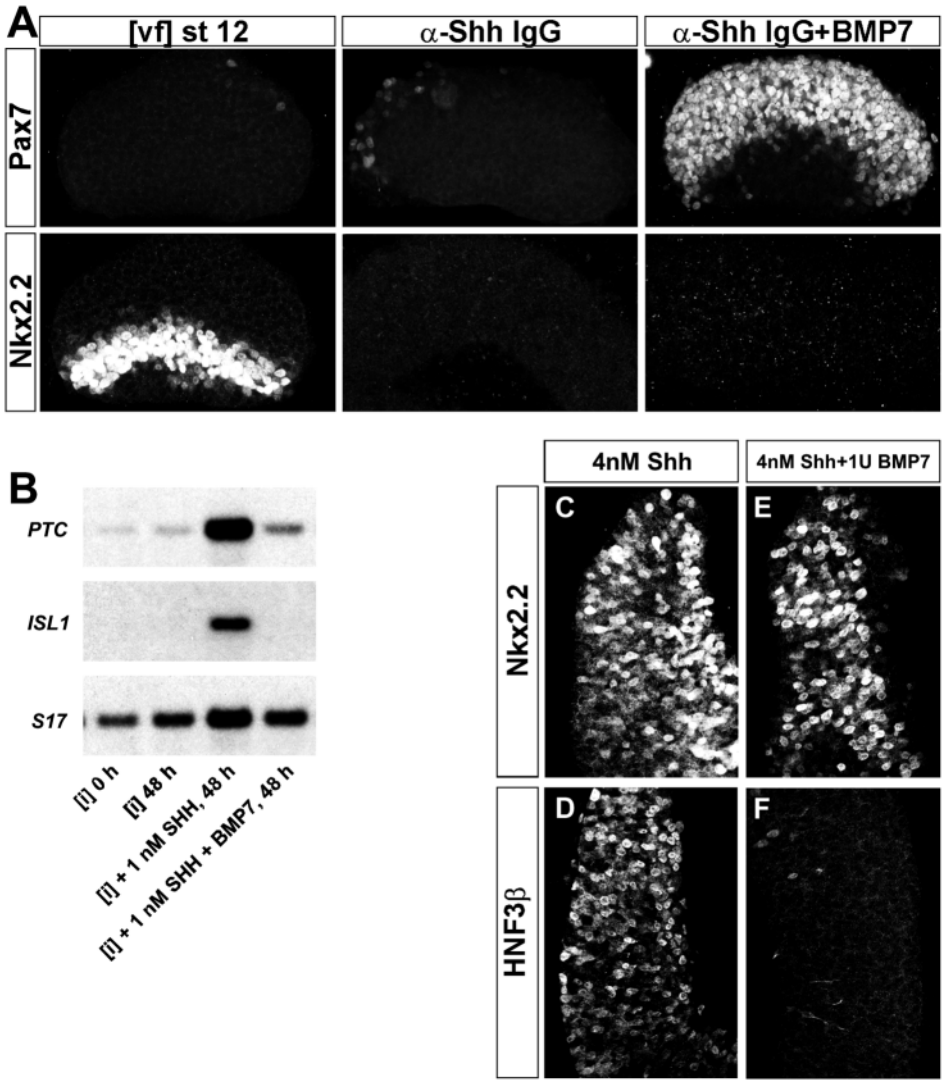

Fig. 4. BMPs modulate a proximal step in Shh signal transduction. (A) BMP7 modifies neural cell responses at a step downstream of the availability and presentation of extracellular Shh protein. Stage 12 floor plate ([vf]) explants cultured alone in vitro for 24 hours express Nkx2.2 but not Pax7. Cells in stage 12 [vf] explants, cultured in the presence of a function-blocking anti-Shh IgG that binds the receptor interaction surface of Shh, lack Nkx2.2 and Pax7 expression. Cells in stage 12 [vf] explants grown with anti-Shh IgG and in addition BMP7, lack Nkx2.2 expression but express Pax7. Similar results were obtained in 12 explants. (B) RT-PCR analysis of *Ptc*, *Isl1*, and ribosomal protein *S17* expression in stage 10 [i] explants at the time of isolation (0 hours), and after 48 hours in culture, alone, in the presence of 1 nM Shh-N, or with 1 nM Shh-N + 1.0 Unit BMP7. (C,D) Stage 10 [i] explants cultured for 24 hours in the presence of 4 nM Shh-N give rise to many Nkx2.2$^+$ (>500 cells, *n*=3 explants) (C) and many HNF3β$^+$ cells (340±51, *n*=4 explants) (D). (E,F) Stage 10 [i] explants cultured for 24 hours in the presence of 4 nM Shh-N and 1.0 Unit BMP7 still give rise to many Nkx2.2$^+$ cells (60±8, *n*=3) (E), but now generate few HNF3β$^+$ cells (33±16, *n*=4) (F). Images representative of >20 explants for each factor combination.

neurons (Fig. 3A,C). At this Shh-N concentration, no HNFβ$^+$ floor plate cells were induced (see Fig. 6R). In the presence of 0.25 Units BMP7 and 2 nM Shh-N, the the number of Isl1/2$^+$ motor neurons was reduced by approx. ten fold and the number of En1$^+$ V1 neurons was increased approx. two to three fold (Fig. 3B,C). We detected no change in overall neuronal generation in the presence and absence of BMP7, as assessed by the number of neurons that express NeuN, a cell-type-independent neuronal marker (Mullen et al., 1992) (2 nM Shh-N: 212±25 NeuN$^+$ neurons/explant; 2 nM Shh-N$^+$ and 0.25 Units BMP7: 218±24 NeuN$^+$ neurons, mean ± s.e.m., *n*=5 explants). Thus, the subtype identity of ventral neurons can also be switched in a ventral-to-dorsal direction by increasing the level of BMP signaling in the presence of a fixed level of Shh activity.

BMP signaling acts on neural cells and at a proximal step in the Shh transduction pathway

The ability of BMPs to switch the identity of ventral progenitor cells and postmitotic neurons in response to Shh signaling raised the question of the site of convergence of these two signaling pathways in responsive neural cells. We first examined whether BMPs modify cell responses to Shh by altering the availability or presentation of the Shh protein itself, or by modifying the response of neural target cells. We reasoned that the ability of BMPs to modify the fate of ventral neural cells under conditions in which Shh is no longer able to interact with its receptor would provide evidence that BMPs act on neural target cells. To test this idea we isolated HH stage 12 ventral neural explants containing a floor plate ([vf] explants) and grew them in the presence of a function-blocking anti-Shh monoclonal antibody (mAb 5E1) directed against the receptor-binding surface of Shh (Fuse et al., 1999; Pepinsky et al., 2000), with or without added BMP (Fig. 4A). Many ventral cells in stage 12[vf] explants grown alone expressed Nkx2.2 but not Pax7, indicating that these cells have been exposed to a high concentration of Shh (Fig. 4A). Stage 12 [vf] explants grown in the presence of anti-Shh IgG, without added BMP, lacked Nkx2.2 expression but did not express Pax7 (Fig. 4A). This result indicates that neural cells have been exposed to a level of Shh signaling sufficient to repress Pax7 but below the level required for the induction of Nkx2.2. Cells in stage 12 [vf] explants grown with anti-Shh IgG, in the additional presence of BMP7, also lacked Nkx2.2 expression, but under these conditions expressed high levels of Pax7 (Fig. 4A). These findings show that BMP7 can modify the response of ventral

neural cells in the absence of available extracellular Shh protein, supporting the idea that BMPs act on neural cells to modify their response to Shh at a step downstream of the availability and presentation of extracellular Shh protein.

We next addressed whether BMPs modify a proximal step in the transduction of Shh signals within target neural cells. To test this possibility we examined the effect of BMPs on the Shh-induced expression of *patched (ptc)*, a gene encoding a Shh receptor subunit that is likely to be induced as a direct response to Shh signaling (Marigo et al., 1996; Goodrich et al.,1996; Ingham, 1998). Low levels of *ptc* were detected in [i] explants both at the time of isolation and after culture for 48 hours (Fig. 4B; data not shown). The addition of 1 nM Shh to [i] explants for 48 hours resulted in an approx. ten-fold elevation in the level of *ptc* expression (Fig. 4A). In contrast, the coincident addition of 1 nM Shh-N and 1.0 Unit BMP7 for 48 hours resulted in a level of *ptc* expression only slightly greater than that obtained in [i] explants grown in the absence of Shh-N (Fig. 4B). Since *ptc* is likely to be a direct target of Shh signaling, the ability of BMPs to block the Shh-induced elevation in *ptc* expression provides one line of evidence that the modulatory action of BMPs on neural cell responses to Shh is exerted at a proximal step in the Shh signal transduction pathway.

The *HNF3β* gene has also been suggested to be a direct transcriptional target of Shh signaling in neural cells (Ruiz i Altaba et al., 1995). Consistent with this idea, the *HNF3β* promoter contains functional binding sites for Gli proteins, a class of zinc finger transcription factors that mediate certain of the signaling functions of Shh (Sasaki et al., 1997). We therefore addressed whether BMP signaling can also block the Shh-induced expression of HNF3β. Exposure of [i] explants to 4 nM Shh-N resulted in the induction of many HNF3β$^+$ floor plate cells (Fig. 4D), and also in the generation of many Nkx2.2$^+$ progenitors (Fig. 4C). Addition of 1.0 Unit BMP7 and 4 nM Shh-N to [i] explants almost completely blocked the induction of HNF3β$^+$ cells (Fig. 4F), whereas many Nkx2.2$^+$ cells were still generated (Fig. 4E). The ability of BMPs to block of the Shh-mediated induction of HNF3β expression provides further evidence that BMP signals modify neural cell responses to Shh through actions at a proximal step in the Shh signal transduction pathway.

Mesodermal tissues express genes encoding secreted BMP inhibitors

The ability of BMPs to switch the fate of neural cells in response to Shh signaling led us to consider whether secreted BMP inhibitors might have a role in regulating the response of neural cells to Shh signaling. To begin to define which secreted BMP inhibitors might influence the initial patterning of ventral cell types, we analyzed the expression of genes encoding four secreted BMP inhibitors, *follistatin*, *flik*, *chordin* and *noggin*, at caudal levels of the neuraxis in stages 10-15 embryos.

follistatin was expressed at high levels by the unsegmented paraxial mesoderm adjacent to the caudal neural plate and neural folds (Fig. 5A) and was detected at high levels in the ventral region of the somitic mesoderm (Fig. 5B-D). The expression of *follistatin* by the somitic mesoderm persisted until at least stage 15 (data not shown). The expression of *follistatin* was also detected in the notochord, from stage 10 onwards (Fig. 5C; data not shown). Expression of *flik*, a follistatin-related gene, was detected at high levels in the notochord at an axial level underlying the closing neural plate (Fig. 5E). *flik* was also expressed transiently by cells at the midline of the neural plate at stage 10 (Fig. 5E), but not at later stages (data not shown; see Patel et al., 1996; Amthor et al., 1996). Both *noggin* and *chordin* were expressed by newly formed notochord (Fig. 5F,G; data not shown) and expression of both genes persisted until at least stage 15 (see Dale et al., 1999). Neither *noggin* nor *chordin* were expressed by cells in the paraxial mesoderm at these stages (data not shown), but *chordin* was expressed in midline cells of the neural plate and ventral neural tube in stage 10 embryos (data not shown; see Dale et al., 1999). These results extend previous observations on the expression of these genes in the chick embryo (Graham and Lumsden, 1996; Dale et al., 1999; Patel et al., 1996; Connolly et al., 1997). Thus, over the period that neural cells acquire their ventral positional identities, the genes encoding secreted factors that bind to and inhibit the signaling activity of BMPs and other TGFβ family members are expressed by both axial and paraxial mesodermal cells.

Regulation of neural responses to Shh by secreted BMP inhibitors

To test the role of secreted BMP inhibitors in ventral neural patterning we examined whether follistatin and noggin, two

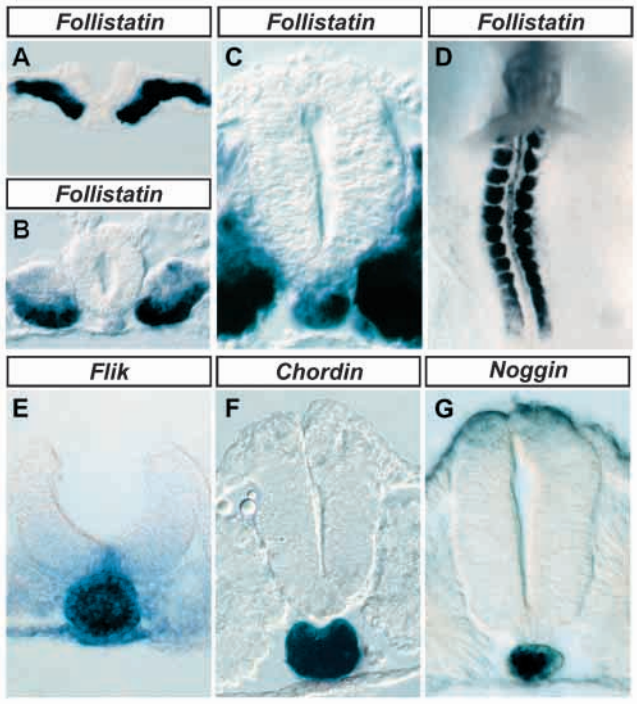

Fig. 5. Mesodermal expression of genes encoding secreted BMP inhibitors. (A-C) *follistatin* is expressed in segmental plate mesoderm at caudal levels of stage 10 chick embryos (A,B). At more rostral levels, *follistatin* is expressed in the ventral somites and in the notochord flanking the closed neural tube (C). (D) Whole-mount image of the pattern of expression of *follistatin* in the notochord and somites of a stage 10 chick embryo. Rostral is upwards. (E) Expression of *flik* in the notochord and in midline neural plate cells at caudal levels of stage 10 chick embryos. (F) Expression of *chordin* in the notochord in a stage 10 chick embryo. (G) Expression of *noggin* in the notochord in a stage 10 chick embryo.

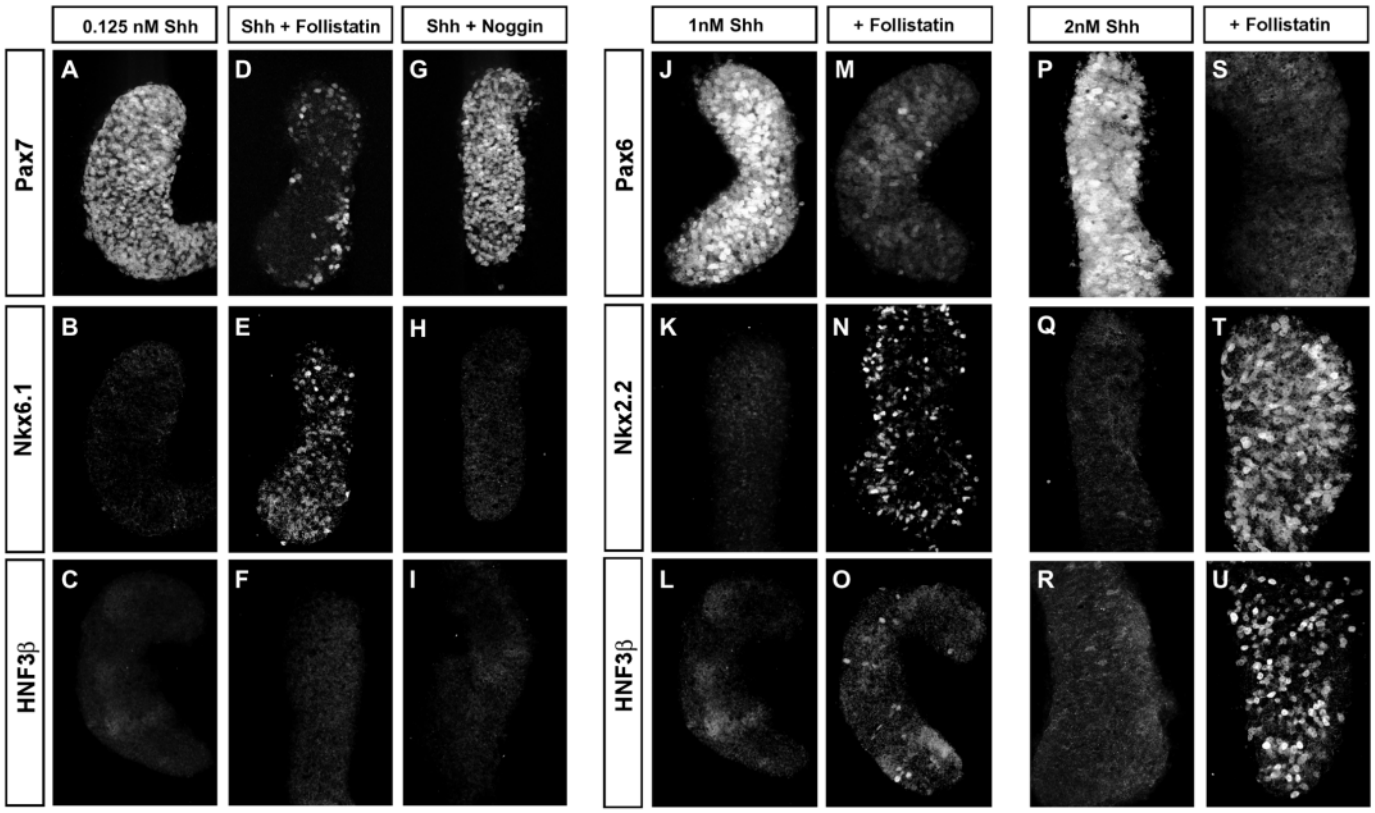

Fig. 6. Follistatin regulates neural progenitor cell responses to Shh. (A-C) Stage 10 [i] explants cultured for 24 hours in the presence of 0.125 nM Shh-N give rise to Pax7$^+$ cells (>500 cells, *n*=4 explants) (A), but not to Nkx6.1$^+$ cells (0 cells; *n*=4 explants) (B), nor to HNF3β$^+$ cells (0 cells, *n*=4 explants) (C). (D-F) Stage 10 [i] explants cultured for 24 hours in the presence of 0.125 nM Shh-N and 80 pM follistatin give rise to many fewer Pax7$^+$ cells (48±11 cells, *n*=4 explants) (D), many Nkx6.1$^+$ cells (55±11 cells, *n*=4 explants) (E) but to no HNF3β$^+$ cells (0 cells, *n*=4 explants) (F). (G-I) Stage 10 [i] explants grown for 24 hours in the presence of 0.125 nM Shh-N and 20 nM noggin give rise to many Pax7$^+$ cells (>500 cells, *n*=3 explants) (G), but not to Nkx6.1$^+$ cells (0 cells, *n*=3 explants) (H), nor to HNF3β$^+$ cells (0 cells, *n*=3 explants) (I). Similar findings were made with addition of 100 nM noggin. (J-L) Stage 10 [i] explants grown for 24 hours in the presence of 1 nM Shh-N give rise to many Pax6$^+$ cells (>500 cells, *n*=4 explants) (J) but not to Nkx2.2$^+$ cells (0 cells, *n*=4 explants) (K), nor to HNF3β$^+$ cells (0 cells, *n*=4 explants) (L). (M-O) Stage 10 [i] explants grown for 24 hours in the presence of 1 nM Shh-N and 80 pM follistatin give rise to a few cells that express high levels of Pax6 (27±10, *n*=7) (M), to many Nkx2.2$^+$ cells (233±44, *n*=4 explants) (N), and to few HNF3β$^+$ cells (<5 cells, *n*=4 explants) (O). (P-R) Stage 10 [i] explants grown for 24 hours in the presence of 2 nM Shh-N still give rise to many Pax6$^+$ cells (>500 cells, *n*=4 explants) (P), but few Nkx2.2$^+$ cells (2±1 cells, *n*=5 explants) (Q) and no HNF3β$^+$ cells (<5 cells, *n*=4 explants) (R). (S-U) Stage 10 [i] explants grown for 24 hours in the presence of 2 nM Shh-N and 80 pM follistatin give rise to few Pax6$^+$ cells (0 cells, *n*=4) (S), many Nkx2.2$^+$ cells (>500 cells, *n*=4 explants) (T), and to many HNF3β$^+$ cells (115±28 cells, *n*=4 explants) (U).

BMP inhibitors with distinct binding specificities, regulate the pattern of cell differentiation in [i] explants. We first examined whether the action of these BMP inhibitors alone, in the absence of Shh, was sufficient to induce the differentiation of ventral neural cell types. Addition of follistatin and noggin, alone, or in combination, to [i] explants did not suppress the expression of Pax7 and did not result in the induction of Nkx6.1, Nkx2.2 or HNF3β expression (data not shown; see also Pierani et al., 1999). Thus, these two BMP inhibitors do not mimic the ability of Shh to induce ventral progenitor cell types in vitro.

We next examined the effects of follistatin and noggin on responses to Shh signaling in [i] explants. Cells in [i] explants were exposed to a concentration of Shh-N (125 pM), that alone, was below the threshold for repression of Pax7 expression (Fig. 6A), and was insufficient to induce Nkx6.1, Nkx2.2 or HNF3β expression (Fig. 6B,C; data not shown). In contrast, addition of 125 pM Shh-N in the presence of 80 pM follistatin decreased by approx. ten fold the number of cells that expressed Pax7 (Fig. 6D) and induced the expression of Nkx6.1 in approx. 20% of neural cells (Fig. 6E). No induction of Nkx2.2 or HNF3β expression was detected (Fig. 6F; data not shown). This result shows that follistatin is able to modulate the sensitivity of progenitor cells to Shh signaling, converting dorsal to ventral progenitors. However, addition of Shh-N (125 pM) in the presence of noggin (80 pM: a concentration sufficient to block both BMP4 and epidermal ectoderm-derived signals, Liem et al., 1997), failed to repress Pax7 and did not induce the expression of Nkx6.1, Nkx2.2 or HNF3β (Fig. 6G-I; data not shown).

We next examined whether follistatin can alter the subtype identity of ventral progenitor cells generated in response to Shh signaling. To generate ventral progenitors we exposed [i] explants to 1 nM Shh-N, a concentration at which Pax7 was completely repressed (data not shown), at which Pax6 expression persisted at high levels (Fig. 6J), and at which

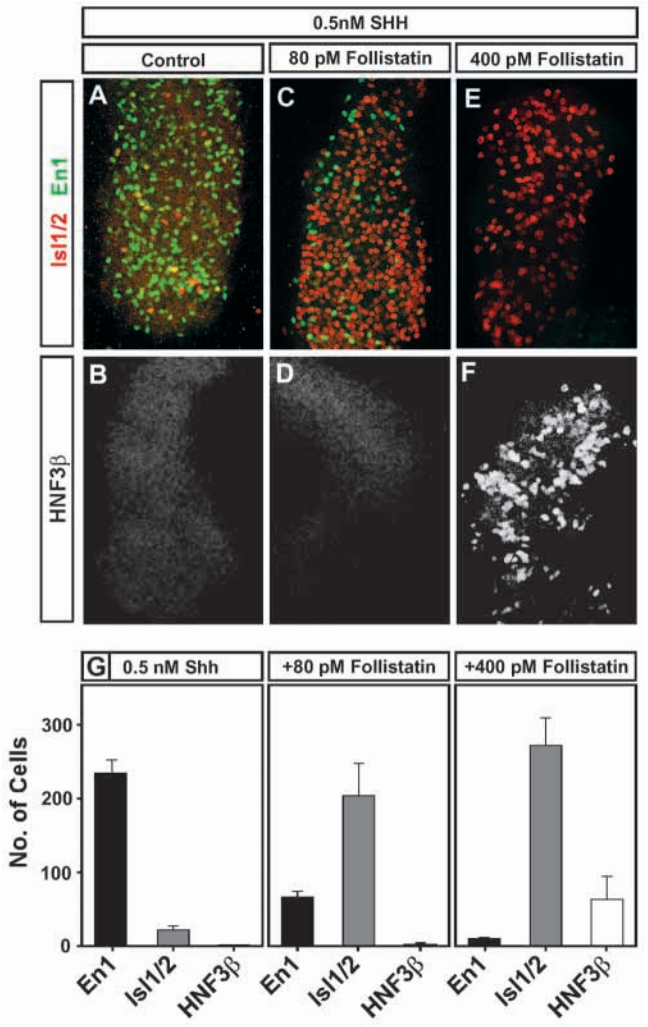

Fig. 7. Follistatin regulates the Shh-mediated assignment of ventral neuronal fate. (A-B) Stage 10 [i] explants cultured for 48 hours in the presence of 0. 5 nM Shh-N give rise to many En1$^+$ V1 interneurons (green; 234±18 cells, *n*=6 explants), to few Isl1/2$^+$ motor neurons (red; 22±5 cells, *n*=5 explants) (A), but to no HNF3β$^+$ floor plate cells (0 cells, *n*=4 explants) (B). (C-D) Stage 10 [i] explants cultured for 48 hours in the presence of 0. 5 nM Shh-N and 80 pM follistatin give rise to few En1$^+$ V1 interneurons (green; 67±8, *n*=5 explants) and many Isl1/2$^+$ motor neurons (red; 204±43 cells, *n*=8 explants), (C) and to few HNF3β$^+$ cells (3±2 cells, *n*=11 explants) (D). (E-F) Stage 10 [i] explants cultured for 48 hours in the presence of 0.5 nM Shh-N and 400 pM follistatin give rise to few En1$^+$ V1 neurons (green; 10±3 cells, *n*=5 explants), to many Isl1/2$^+$ motor neurons (red; 272±37 cells, *n*=5 explants) (E) and to HNF3β$^+$ cells (63±31 cells, *n*=6 explants) (F). (G) Quantitation of En1$^+$, Isl1/2$^+$, and HNF3β expression in [i] explants exposed to Shh-N (0.5 nM) alone or in the presence of 80 pM or 400 pM follistatin. (Histogram indicates mean ± s.e.m., *n*=4 explants).

neither Nkx2.2 nor HNF3β were induced (Fig. 6K,L). Addition of Shh-N (1 nM) in the presence of follistatin (80 pM) resulted in an approx. 20-fold decrease in the number of Pax6 cells (Fig. 6M), and led to the expression of Nkx2.2 by approx. 30% of cells (Fig. 6N). At these factor concentrations, only very few cells expressed HNF3β (Fig. 6O). In a separate set of experiments we exposed [i] explants to 2 nM Shh-N; a concentration that permitted expression of Pax6 (Fig. 6P; Ericson et al., 1997), but again was below the threshold for induction of Nkx2.2 or HNF3β (Fig. 6Q,R). Addition of 2 nM Shh-N in the presence of follistatin (80 pM) resulted in a virtually complete repression of Pax6 (Fig. 6S), and in the induction of Nkx2.2 expression by approx. 75% of neural cells (Fig. 6T). At these factor concentrations many cells were also induced to express HNF3β (Fig. 6U). Thus, follistatin can markedly enhance the response of neural cells to a set level of

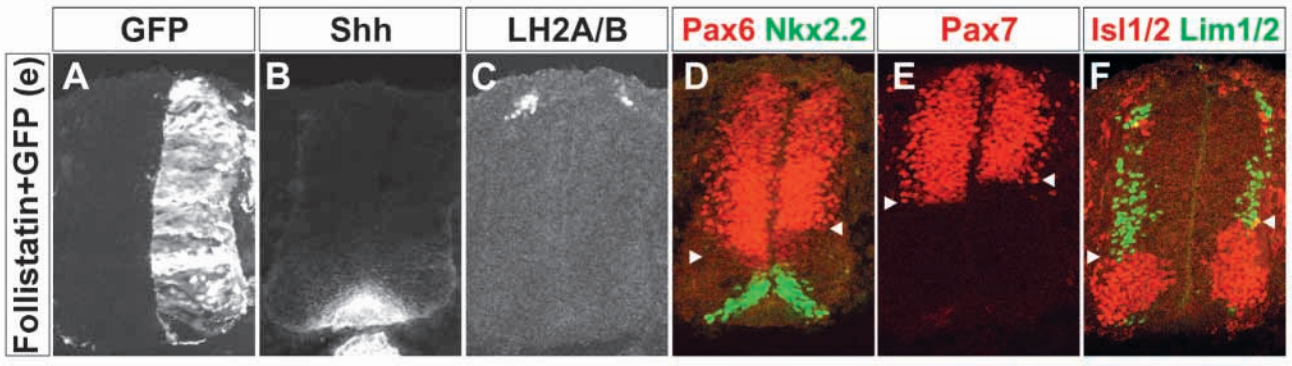

Fig. 8. Follistatin expression changes neural progenitor cell identity and neuronal fate in vivo. Ectopic expression of follistatin in chick neural tube. Vectors encoding follistatin and GFP were introduced into chick neural tube by in ovo electroporation and the effect on expression of marker proteins was assayed 48 hours later. All images are orientated with the electroporated side to the right. The non-electroporated (left) side serves as a control. (A) Broad ectopic expression of electroporated constructs is detected, as assessed by GFP expression. (B) Expression of follistatin does not affect the domain or level of Shh expression. (C) A significant decrease in the number of LH2A/B$^+$ (D1) dorsal interneurons is observed on the electroporated side of the neural tube. (D) Ectopic expression of follistatin results in a dorsal shift in the domain of progenitor cells that express low levels of Pax6 (arrowheads mark ventral limit of high level Pax6 progenitors). (E) Ectopic expression of follistatin results in a dorsal shift in the ventral boundary of Pax7 expression (arrowheads mark ventral limit of Pax7 expression). (F) Ectopic expression of follistatin results in a dorsal expansion in the domain of generation of Isl1/2$^+$ motor neurons and a dorsal restriction in the domain of expression of Lim1/2$^+$ (V0 and V1) ventral neurons (boundary marked by arrowheads). Similar findings were obtained in 7 electroporated embryos.

Shh signaling, as assessed by dorsal-to-ventral switch in the subtype identity of ventral progenitor cells.

In this latter set of experiments, the detection of HNF3β$^+$ cells raised the issue of whether the secondary induction of floor plate cells, and accompanying *Shh* expression, could contribute to this change in ventral progenitor cell identity. However, the present experiments were analyzed at 24 hours and the induction of *Shh* expression in vitro is not detected before this time (Tanabe et al., 1995; Roelink et al., 1995; Pons and Marti, 2000). Thus, it is unlikely that induction of floor plate cells and the secondary neural expression of *Shh* contributes significantly to the observed changes in progenitor cell fate detected at 24h. Addition of noggin (80 pM) did not affect the response of cells to either 1 nM or 2 nM Shh-N (data not shown; see below).

Regulation of ventral neural fate by follistatin in vitro

We next determined whether follistatin exposure alters the subtype identity of ventral neurons generated in response to a set level of Shh signaling. To test this possibility, [i] explants were exposed to 0.5 nM Shh-N, in the absence or presence of follistatin, for 48h. Exposure of [i] explants to Shh-N alone generated approx. 250 En1$^+$ V1 neurons, but only approx. 25 Isl1/2$^+$ motor neurons, and no HNF3β$^+$ floor plate cells (Fig. 7A,B,G). In contrast, [i] explants grown in the presence of 0.5 nM Shh-N and 80 pM follistatin generated approx. three-fold fewer En1$^+$ V1 neurons and an eight-fold greater number of Isl1/2$^+$ motor neurons (Fig. 7C,G). At this follistatin concentration, no HNF3β$^+$ cells were induced (Fig. 7D,G), indicating that the change in subtype identity of ventral neurons is independent of floor plate induction. Addition of Shh-N (0.5 nM) in the presence of a higher concentration of follistatin, (400 pM), resulted in an almost complete suppression in the generation En1$^+$ V1 neurons (Fig. 7E,G), and was associated with a further increase in the number of Isl1/2$^+$ motor neurons (Fig. 7E,F,G). Moreover, at this concentration of follistatin, many HNF3β$^+$ cells were induced (Fig. 7F,G). Thus coincident exposure of cells to Shh and follistatin can promote the generation of ventral cell types induced by an approx. ten-fold higher concentration of Shh alone (see Ericson et al., 1997b). Together, these findings provide evidence that modulation of endogenous BMP signaling within neural tissue by follistatin in vitro dramatically alters the identity of ventral progenitor cells and postmitotic neurons generated in response to a set level of Shh protein.

Regulation of ventral neural fate by follistatin in vivo

To examine whether the modulation of BMP signaling also affects ventral neural cell fates in vivo, we ectopically expressed follistatin in the chick neural tube. Vectors directing expression of follistatin and GFP were co-electroporated into one side of HH stage 10-12 chick neural tube, and embryos were permitted to develop in ovo until stages 20-22. Regions of ectopic follistatin expression were marked by expression of GFP (Fig. 8A and data not shown). The expression of Shh, the progenitor cell markers Pax7, Pax6, Nkx2.2, and the neuronal subtype markers Isl1/2, Lim1/2 and LH2A/B were examined.

We detected an approx. 70% decrease in the number of LH2A/B$^+$ (D1) interneurons in the dorsal neural tube (Fig. 8C). This result provides evidence that dorsal neural expression of follistatin attenuates BMP signaling from the roof plate that is necessary for the induction of LH2A/B$^+$ D1 neurons (Liem et al., 1997; Lee et al., 2000). The expression of Shh by floor plate cells was unaffected by unilateral follistatin expression (Fig. 8B). In regions of the ventral neural tube within which follistatin was ectopically expressed, we detected a marked dorsal-to-ventral shift in the identity of ventral neural progenitor cells and in neuronal fate (Fig. 8). On the electroporated side of the neural tube, the domain occupied by progenitor cells expressing low levels of Pax6 was shifted dorsally (Fig. 8D). In addition, the ventral boundary of Pax7 expression was shifted dorsally by three to five cell diameters (Fig. 8E). The ventralization of progenitor cell identity was accompanied by a similar ventralization in neuronal fate. We detected a dorsal expansion in the position of MN generation and a concomitant dorsal restriction in the position of generation of ventral Lim1/2$^+$ (V0 and V1) interneurons (Fig. 8F; see Pierani et al., 1999). Together these findings provide evidence that follistatin expression in vivo results in a marked ventralization of ventral neural cell fates, in a manner independent of any change in Shh expression. These results support the idea that the modulation of endogenous BMP signaling alters the response of ventral progenitor cells to Shh signaling in vivo.

DISCUSSION

During the patterning of the neural tube, the level of Shh signaling activity appears to have a critical role in determining the identity of ventral progenitor cells and the fate of postmitotic neurons (Ericson et al., 1997b; Briscoe et al., 1999, 2000). The mechanisms that determine how neural cells respond to specific levels of Shh activity, however, remain poorly defined. The response of neural cells might be a direct reflection of the local concentration of active Shh protein within a specific domain of the ventral neural epithelium. Alternatively, cellular responses could depend on the actions of factors that activate distinct signaling pathways that modulate the Shh-signaling pathway within responsive cells. In this study we provide evidence that BMP signaling plays such a role. Our results lead to three main conclusions: (1) that prospective ventral neural plate cells are exposed to ongoing BMP activity, (2) that BMP signaling attenuates Shh signaling, and (3) that follistatin modulates BMP activity and sensitizes the response of neural cells to Shh. We discuss these findings in the context of the role of regulated BMP signaling in ventral neural patterning.

BMPs modulate the response of neural cells to Shh

One conclusion that emerges from our studies is that the response of ventral neural progenitors to a specific level of Shh signaling activity is dependent on ambient BMP signaling. The exposure of neural progenitor cells to a fixed Shh concentration in the presence of BMPs causes a marked ventral-to-dorsal shift in the identity of neural progenitor cells and postmitotic neurons. Exposure of neural cells to BMPs has been shown previously to block the differentiation of motor neurons in vitro and floor plate cells in vivo (Basler et al., 1993; Liem et al., 1995; Arkell and Beddington, 1997), but these studies did not

examine the identity of the cell types generated in response to BMP exposure.

The patterning roles of BMPs have been analyzed in most detail in the dorsal half of the neural tube (Lee and Jessell, 1999). Nevertheless, it seems likely that prospective ventral neural plate cells are also exposed to BMPs. Two lines of evidence support this idea. First, cells in neural plate explants initially express BMPs at very low levels (Liem et al., 1995; Streit et al., 1998), yet there is a marked elevation in *BMP* expression when neural explants are grown alone in vitro (Liem et al., 1995, 1997; our unpublished data). Since BMPs markedly induce *BMP* gene expression in neural cells (Liem et al., 1995), the elevation in *BMP* expression is likely to be initiated by BMP protein present in neural plate tissue at the time of its isolation. Second, and more persuasively, follistatin changes the response of neural cells to Shh signaling both in vitro and in vivo, eliciting a pronounced dorsal-to-ventral shift in progenitor cell identity and neuronal fate. The analysis of *BMP* mutant phenotypes in zebrafish embryos has also revealed an expansion in the domain of expression of ventral neural markers (Barth et al., 1999; Nguyen et al., 2000), consistent with the idea that the fate of cells in prospective ventral regions of the neural plate is regulated BMP signaling at neurula stages.

What might be the source of BMP protein in early neural plate tissue? Several *BMP* genes are expressed by ectodermal cells immediately preceding their differentiation into neural tissue (Fainsod et al., 1994; Streit et al., 1998; Streit and Stern, 1999). Thus, BMP protein is likely to persist in newly induced neural cells at levels sufficient to influence the patterning of early neural cells. In addition, certain *BMP* family members are expressed at later stages by ventral neural tube cells themselves (Bruneau and Rosa, 1997 Jones et al., 1994). Such ventral neural sources of BMPs could have a role in attenuating neural responses to Shh signaling after neural tube closure. *BMP*s are also expressed by roof plate cells at the dorsal midline of the neural tube (see Lee and Jessell, 1999). In mouse, genetic ablation of the roof plate eliminates its resident *BMP*s and causes a minor expansion in the domains of expression of ventral neural markers (Lee et al., 2000). Thus, BMPs derived from dorsal midline cells may have a minor influence on ventral patterning at stages after neural tube closure.

The mechanism by which BMPs regulate neural cell responses to Shh signaling is not known. Our findings indicate that BMPs act on target cells to modify their responses to Shh signaling, rather than regulating the availability or presentation of Shh protein itself. In addition, exposure of neural cells to BMPs blocks the Shh-mediated induction of *HNF3β* and *Ptc*, two genes that are thought to be induced as a direct response to hedgehog signaling (Ruiz i Altaba 1995; Sasaki et al., 1997; Ingham, 1998). Thus, BMPs are likely to act at a proximal step in the transduction of Shh signals within neural cells. BMP signal transduction typically involves the phosphorylation and nuclear translocation of Smad effector proteins (Whitman, 1998). Recent evidence suggests that Smads are sequestered in a protein complex that contains Gli proteins, a class of zinc finger transcription factors that transduce many Shh signals (Liu et al., 1999). Thus, Shh and BMP signaling may converge in neural cells at the level of a transcriptional regulatory complex that contains both Smad and Gli proteins.

Secreted BMP inhibitors and the regulation of neural cell sensitivity to Shh

The ability of BMPs to regulate the response of neural progenitor cells to Shh signaling raises the issue of how the net level of BMP activity is set within neural tissue. One prominent method for regulating BMP signaling is through the secretion of proteins that bind directly to BMPs or to BMP-receptor complexes, thus attenuating downstream transduction events (Piccolo et al., 1996; Zimmerman et al., 1996; Iemura et al., 1998).

Follistatin produces a dorsal-to-ventral shift in the response of neural plate cells to Shh signaling. In contrast, the exposure of neural plate cells to noggin, a BMP inhibitor with differing target specificity, did not change the response of neural progenitors to Shh. Follistatin inhibits activin and the BMP5-BMP7 subgroup, whereas noggin preferentially blocks signaling by the BMP2/4 and GDF5-GDF7 subgroups (Nakamura et al., 1990; Yamashita et al., 1995; Lee et al., 1998). Thus, our results suggest that in chick, neural plate cells are exposed primarily to follistatin-sensitive members of the TGFβ superfamily.

Genetic studies in mice have provided support for the idea that BMP antagonists play a role in ventral patterning in vivo. In *noggin* null mice there is a marked defect in ventral patterning, most notably, the absence of floor plate cells and motor neurons at caudal axial levels (McMahon et al., 1998). This defect in ventral neural differentiation is associated with the ectopic expression of *Bmp4* within the ventral neural tube (McMahon et al., 1998). Thus, in mouse, noggin appears to function normally to prevent ventral neural expression of *Bmps*, in effect sensitizing neural cells to Shh signals. In chick neural tissue grown in vitro, however, noggin failed to modify the sensitivity of neural cells to Shh. The differing activities of noggin suggested by studies in chick and mouse could be related to the expression in these two species of a different spectrum of BMPs in and around the caudal neural tube (Liem et al., 1995, 1997; Lee et al., 1998).

Although ectopic expression of follistatin in chick neural tube cells in vivo markedly changes ventral neural pattern, the analysis of *follistatin* null mice has not revealed developmental defects indicative of a profound alteration in early neural patterning (Matzuk et al., 1995). One possible explanation for the lack of a developmental phenotype in *follistatin* mutants is that noggin serves as the predominant BMP inhibitor in mice, at least at this developmental stage. An alternative explanation is that *flik*, a follistatin-related gene expressed by the notochord and by midline neural cells (Patel et al., 1996), may have activities redundant with those of follistatin. Chick embryos treated in ovo with *flik* antisense oligonucleotides have been reported to lack a floor plate (Towers et al., 1999), a patterning defect that is consistent with a role for *flik* in promoting ventral cell type differentiation in the chick embryo.

Our results do not exclude that factors other than BMPs also contribute to the control of neural cell responses to Shh activity. Shh signaling has been shown to induce the ventral neural expression of *HIP*, a gene encoding a surface membrane protein that binds to Shh and appears to attenuate its signaling activity in non-neural tissues (Chuang and McMahon, 1999). Vitronectin, an extracellular matrix protein is also induced by Shh in ventral neural cells (Martinez-Morales et al., 1997), and has been reported to bind to Shh and regulate its activity (Pons

and Marti, 2000). Neurally expressed peptides have also been suggested to regulate Shh signaling, through their ability to elevate protein kinase A activity (Hynes et al., 1995; Epstein et al., 1996; Fan et al., 1995; Waschek et al., 1998).

Finally, our findings may be relevant to the general issue of how graded Shh signaling activity within the ventral neural tube controls the specification of neural cell fate. In particular they raise the possibility that regulated BMP signaling has a role in establishing a ventral-to-dorsal gradient of Shh activity within the neural tube. Several BMP inhibitors are expressed by cells in or around the ventral neural tube (Amthor et al., 1996; Connolly et al., 1997; Dale et al., 1999). The ventrally restricted expression of these BMP inhibitors would be expected to reduce the net level of BMP signaling in more ventral regions of the neural tube. Such ventral sources of BMP inhibitors may serve to sharpen a ventral-to-dorsal gradient of Shh signaling activity within the ventral neural tube. The establishment of a steep gradient of Shh activity is likely to be a necessary step in ventral patterning, given the evidence that five distinct neuronal subtypes are generated in the ventral neural tube in response to progressive two- to three-fold changes in the level of Shh activity (Ericson et al., 1997b). Thus, the interplay between Shh, BMPs and BMP inhibitors may be crucial in establishing a dorsoventral gradient of Shh signaling activity that underlies the generation of neuronal diversity within the ventral neural tube.

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REFERENCES

Acampora, D., Gulisano, M. and Simone, A. (1999). Otx genes and the genetic control of brain morphogenesis. *Mol. Cell Neurosci.* **13**, 1-8.

Alder, J., Lee, K. J., Jessell, T. M. and Hatten, M. E. (1999). Generation of cerebellar granule neurons in vivo by transplantation of BMP-treated neural progenitor cells. *Nature Neurosci.* **2**, 533-540.

Amthor, H., Connolly, D., Patel., K., Brand-Saberi, B., Wilkinson, D., Cooke, J. and Christ., B. (1996). The Expression and regulation of follistatin and a follistatin-like gene during avian somite compartmentalization and myogenesis. *Dev. Biol.* **178**, 343-362.

Arkell, R. and Beddington, R. S. P. (1997). BMP7 influences pattern and growth of the developing hindbrain of mouse embryos. *Development* **124**, 1-12.

Barth, K. A., Kishimoto, Y., Rohr, K. B., Seydler, C., Schulte-Merker, S. and Wilson, S. W. (1999). BMP activity establishes a gradient of positional information throughout the entire neural plate. *Development* **126**, 4977-4987.

Basler, K., Edlund, T., Jessell, T. M. and Yamada, T. (1993). Control of cell pattern in the Neural tube: Regulation of cell differentiation by *dorsalin-1*, a novel TGFβ family member. *Cell* **73**, 687-702.

Briscoe, J., Sussel, L., Serup, D., Hartigan-O'Connor, D., Jessell, T. M., Rubenstein, J. L. R. and Ericson, J. (1999). Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic Hedgehog signaling. *Nature* **398**, 622-627.

Briscoe, J., Pierani, A., Jessell, T. M. and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**, 435-445.

Bruneau, S. and Rosa, F. M. (1997). Dynamo, a new zebrafish DVR member of the TGFβ superfamily is expressed in the posterior neural tube and is up-regulated by Sonic Hedgehog. *Mech. Dev*. **61**, 199-212.

Capdevila, J. and Johnson, R. L. (1998). Endogenous and ectopic expression of noggin suggests a conserved mechanism for regulation of BMP function during limb and somite patterning. *Dev. Biol.* **197**, 205-217.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. T., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic Hedgehog gene function. *Nature* 3**83**, 407-413.

Chuang, P. -T. and McMahon, A. P. (1999). Vertebrate hedgehog signaling modulated by induction of a Hedgehog-binding protein. *Nature* **397**, 617-621.

Connolly, D. J., Patel, K. and Cooke, J. (1997). Chick noggin is expressed in the organizer and neural plate during axial development, but offers no evidence of involvement in primary axis formation. *Int. J. Dev. Biol.* **41**, 389-396.

Dale, J. K., Vesque, C., Lints, T., Sampath, T. K., Furley, A., Dodd, J. and Placzek, M. (1997). Cooperation of BMP7 and Shh in the induction of forebrain ventral midline cells by prechordal mesoderm. *Cell* **90**, 257-269.

Dale, K., Sattar, N., Heemskerk, J., Clarke, J. D., Placzek, M. and Dodd, J. (1999). Differential patterning of ventral midline cells by axial mesoderm is regulated by BMP7 and chordin. *Development* **26**, 397-408.

Epstein, D. J., Marti, E., Scott, M. P. and McMahon, A. P. (1996). Antagonizing camp-dependent protein kinase A in the dorsal CNS activates a conserved Sonic hedgehog signaling pathway. *Development* **122**, 2885-2894.

Ericson, J., Thor, S., Edlund, T., Jessell, T. M. and Yamada, T. (1992). Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1 *Science* **256**, 1555-1560.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-673.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J., (1997a). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.

Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V. and Jessell, T. M. (1997b). Graded Sonic Hedgehog signaling and the specification of cell fate in the ventral neural tube. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 451-466.

Fainsod, A., Steinbeisser, H. and De Robertis, E. M. (1994). On the function of BMP-4 in patterning the marginal zone of the Xenopus embryo. *EMBO J.* **13**, 5015-5025.

Fan, C. M., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A. and Tessier-Lavigne, M. (1995). Long-range sclerotome induction by sonic hedgehog: direct role of the amino-terminal cleavage product and modulation by the cyclic AMP signaling pathway. *Cell* **81**, 457-465.

Fuse, N., Maiti, T., Wang, B., Porter, J. A., Hall, T. M., Leahy, D. J. and Beachy, P. A. (1999). Sonic hedgehog protein signals not as a hydrolytic enzyme but as an apparent ligand for patched. *Proc. Natl. Acad. Sci. USA* **96**, 10992-10999.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A. and Scott, M. P. (1996). Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev.* **10**, 301-312.

Graham, A. and Lumsden, A. (1996). Interaction between rhombomeres modulate krox-20 and follistatin expression in the chick embryo hindbrain. *Development* **122**, 473-480.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of chick embryos. *J. Morph.* **88**, 49-92.

Hammerschmidt, M., Brook, A. and McMahon, A. P. (1997). The world according to hedgehog. *Trends Genet*. **13**, 14-21.

Hynes, M., Porter, J. A., Chiang, C., Chang D., Tessier-Lavigne, M., Beachy, P. A. and Rosenthal, A., (1995). Induction of midbrain dopaminergic neurons by Sonic hedgehog. *Neuron* **15**, 35-44.

Hynes, M., Weilan, Y., Wang, K., Stone, D., Murone, M., de Sauvage, F and Rosenthal, A. (2000). The seven-transmembrane receptor Smoothened cell-autonomously induces multiple ventral cell types. *Nature Neurosci.* **3**, 41-46.

Iemura, S., Yammamoto, T. S., Takagi, C., Uchiyama, H., Natsume, T., Shimasaki, S., Sugino, H. and Ueno, N. (1998). Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *Proc. Natl. Acad. Sci. USA* **95**, 9337-9342.

Ingham, P. W. (1998). Transducing hedgehog: the story so far. *EMBO J.* **17**, 3505-3511.

Jones, C. M., Lyons, K. M. and Hogan, B. L. (1991). Involvement of Bone

Morphogenetic Protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. *Development* **111**, 531-542.

Lee, K. J., Mendelsohn, M. and Jessell, T. M. (1998). Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. *Genes Dev*. **12**, 3394-3407.

Lee, K. J. and Jessell, T. M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. *Annu. Rev. Neurosci.* **22**, 261-294.

Lee, K. J., Dietrich, P. and Jessell, T. M. (2000). Genetic abalation reveals that the roof plate is essential for dorsal interneuron specification. *Nature* **403**, 734-740.

Liem, K. F. Jr., Tremml, G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.

Liem, K. F. Jr., Tremml, G. and Jessell, T. M. (1997). A role for the roof plate and its resident TGFβ-related proteins in neuronal patterning in the dorsal spinal cord. *Cell* **91**, 127-138.

Liu, F., Massague, J. and Ruiz i Altaba, A. (1998). Carboxy-terminally truncated gli3 proteins associate with smads. *Nat. Genet.* **20**, 325-326.

Lumsden, A. and Krumlauf, R. (1996). Pattering the vertebrate neuraxis. *Science* **274**, 1109-1115.

Marigo, V., Scott, M. P., Johnson, R. L., Goodrich, L. V. and Tabin, C. J. (1996) Conservation in hedgehog signaling: induction of a chicken patched homologue by Sonic Hedgehog in the developing limb. *Development* **122**, 1225-1233.

Marigo, V. and Tabin C. J. (1996). Regulation of patched by Sonic hedgehog in the developing neural tube. *Proc. Nat. Acad. Sci. USA* **93**, 9346-9351.

Marti, E., Bumcrot, D. A., Takada, R. and McMahon, A. P. (1995). Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375**, 322-325.

Martinez-Morales, J. R., Barbas, J. A., Marti, E., Bovalenta, P., Edgar, D., Rodriguez-Tebar, A. (1997). Vitronectin is expressed in the ventral region of the neural tube and promotes the differentiation of motor neurons. *Development* **124**, 139-147.

Matzuk, M. M., Lu, N., Vogel, H., Sellheyer, K., Roop, D. R. and Bradley, A. (1995). Multiple defects and perinatal death in mice deficient in follistatin. *Nature* **374**, 360-363.

McMahon, J. A., Takada, S., Zimmerman, L. B., Fan, C. M., Harland, R. M. and McMahon, A. P. (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* **12**, 1438-52.

Mullen R. J., Buck, C. R. and Smith, A. M. (1992). NeuN, a neuronal specific nuclear protein in vertebrates. *Development* **116**, 201-211.

Murone, M., Rosenthal, A. and de Sauvage, F. J. (1999). Hedgehog signal transduction: from flies to vertebrates. *Exp. Cell Res.* **253**, 25-33.

Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K. and Sugino, H. (1990). Activin-binding protein from rat ovary is follistatin. *Science* **247**, 836-838.

Nothias, F., Fishell, G. and Ruiz i Altaba, A. (1998). Cooperation of intrinsic and extrinsic signals in the elaboration of regional identity in the posterior cerebral cortex. *Curr. Biol.* **8**, 459-462.

Nguyen, V. H., Trout, J., Connors, S. A. Andermann, P., Weinberg, E. and Mullins, M. C. (2000). Dorsal and intermediate neuronal cell types of the spinal cord are established by a BMP signaling pathway. *Development* **127**, 1209-1220.

Patel, K., Connolly, D. J., Amthor, H., Nose, K. and Cooke, J. (1996). Cloning and early dorsal axial expression of Flik, and chick Follistatin-related gene: evidence for involvement in dorsalization/neural induction. *Dev. Biol.* **178**, 327-342.

Pepinsky, R. B., Rayhorn, P., Day, E. S., Dergay, A., Williams, K. P., Galdes, A., Taylor, F. R., Boriack-Sjodin, P. A. and Garber, E. A. (2000). Mapping sonic hedgehog-receptor interactions by steric interference. *J. Biol. Chem*. **275**, 10995-11001.

Piccolo S., Sasai Y., Lu B. and De Robertis E. M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86**, 589-598.

Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A Sonic Hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 1-20.

Pons, S. and Marti, E. (2000). Sonic Hedgehog synergizes with the extracellular matrix protein Vitronectin to induce spinal motor neuron differentiation. *Development* **127**, 333-342.

Porter, J. A., von Kessler, D. P., Ekker, S. C., Young, K. E., Lee, J. J., Moses, K. and Beachy, P. A. (1995). The product of hedgehog autoproteolytic cleavage active in local and long-range signaling. *Nature* **374**, 363-366.

Quirk, J., van den Heuvel, M. Henrique, D., Marigo, V., Jones, T. A., Tabin, C. and Ingham, P. W. (1997). The smoothened gene and hedgehog signal transduction in Drosophila and vertebrate development. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 217-226.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.

Ruiz i Altaba, A., Placzek, M., Baldassare, M., Dodd, J. and Jessell, T. M. (1995). Early stages of notochord and floor plate development in the chick embryo defined by normal and induced expression of HNF3β. *Dev. Biol.* **170**, 299-313.

Sasaki, H., Hui, C., Nakafuku, M. and Kondoh, H. (1997). A binding site for gli proteins is essential for HNF3β floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313-1322.

Smith, W. C. (2000). TGF beta inhibitors. New and unexpected requirements in vertebrate development. *Trends Genet.* **15**, 3-5.

Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q, Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J. E., de Sauvage, F. and Rosenthal, A. (1996). The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* **384**, 129-134.

Streit, A., Lee, K. J., Woo, I., Roberts, C., Jessell, T. M. and Stern, C. (1998) Chordin regulates primitive streak development and the stability of induced neural cells, but is not sufficient for neural induction in the chick embryo. *Development* **125**, 507-519.

Streit, A. and Stern, C. D. (1999). Establishment and maintenance of the border of the neural plate in the chick: involvement of FGF and BMP activity. *Mech Dev*. **82**, 51-66.

Sussel, L., Marin, O., Kimura, S. and Rubenstein, J. L. (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. *Development* **126**, 3359-3370.

Tanabe, Y. and Jessell, T. M. (1996). Diversity and pattern in the developing spinal cord. *Science* **274**, 1115-1123.

Tanabe, Y., Roelink, H. and Jessell, T. M. (1995). Induction of motor neurons by Sonic hedgehog is independent of floor plate differentiation. *Curr. Biol.* **5**, 651-658.

Thery, C., Sharpe, M. J., Batley, S. J., Stern, C. D. and Gherardi, E. (1995). Expression of HGF/SF, HGF1/MSP, and c-met suggests new functions during early chick development. *Dev. Genet.* **17**, 90-101.

Towers, P., Patel, K., Withington, S., Isaac, A. and Cooke, J. (1999). Flik, a chick follistatin-related gene, functions in gastrula dorsalisation/neural induction and in subsequent maintenance of midline sonic hedgehog signaling. *Dev. Biol.* **214**, 298-317.

Tsuchida,T., Ensini, M., Morton, S., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79**, 957-970.

Varela-Echavarria, A., Pfaff, S. L. and Guthrie, S. (1996). Differential expression of LIM homeobox genes among motor neuron subpopulations in the developing chick brain stem. *Mol. Cell Neurosci.* **8**, 242-257.

Waschek, J. A., Castillas, R. A., Nguyen, T. T., DiCicco-Bloom, E. M., Carpenter, E. M. and Rodriguez, W. I. (1998). Neural tube expression of pituitary adenylate cyclase-activating peptide (PACAP) and receptor: potential role in patterning and neurogenesis. *Proc. Natl. Acad. Sci. USA* **95**, 9602-9607.

Whitman, M. (1998). Smads and early developmental signaling by the TGFβ superfamily. *Genes Dev.* **12**, 2445-2462.

Yamada, T., Placzek, M. Tanaka, H., Dodd, J. and Jessell, T. M. (1991). Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. *Cell* **64**, 635-647.

Yamada, T., Pfaff, S. L., Edlund, T. and Jessell, T. M. (1993). Control of cell pattern in the neural tube: Motor neuron induction by diffusible factors from notochord and floor plate. *Cell* **73**, 673-686.

Yamashita, H., ten Dijke, P., Huylebroeck, D., Sampath, T. K. andries, M., Smith, J. C., Heldin, C. and Miyazono, K. (1995). Osteogenic protein-1 binds to activin type II receptors and induces certain Activin-like effects. *J. Cell Biol.* **130**, 217-226.

Zimmerman, L., De Jesus-Escobar, J. and Harland, R. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein-4. *Cell* **86**, 599-606.