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Wnt/BMP signal integration regulates the balance between proliferation and differentiation of neuroepithelial cells in the dorsal spinal cord

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Abstract

Multiple signaling pathways regulate proliferation and differentiation of neural progenitor cells during early development of the central nervous system (CNS). In the spinal cord, dorsal signaling by bone morphogenic protein (BMP) acts primarily as a patterning signal, while canonical Wnt signaling promotes cell cycle progression in stem and progenitor cells. However, overexpression of Wnt factors or, as shown here, stabilization of the Wnt signaling component β-catenin has a more prominent effect in the ventral than in the dorsal spinal cord, revealing local differences in signal interpretation. Intriguingly, Wnt signaling is associated with BMP signal activation in the dorsal spinal cord. This points to a spatially restricted interaction between these pathways. Indeed, BMP counteracts proliferation promoted by Wnt in spinal cord neuroepithelial cells. Conversely, Wnt antagonizes BMP-dependent neuronal differentiation. Thus, a mutually inhibitory crosstalk between Wnt and BMP signaling controls the balance between proliferation and differentiation. A model emerges in which dorsal Wnt/BMP signal integration links growth and patterning, thereby maintaining undifferentiated and slow-cycling neural progenitors that form the dorsal confines of the developing spinal cord.

Keywords: Spinal cord development; Neuroepithelial stem cell; Proliferation; Differentiation; Wnt; β-catenin; BMP

Introduction

The formation of the vertebrate nervous system involves a complex process of cell division, cellular rearrangement, and differentiation, that has to be tightly coordinated in space and time. In the spinal cord early patterning is established by BMP and sonic hedgehog (Shh) counter gradients (Liem et al., 1997, 2000; Marcelle et al., 1997). While BMPs are expressed in the dorsal-most portion of the neural tube, the roof plate, Shh is secreted from the ventral-most portion, the floorplate. Dorsal progenitor populations are specified by BMP signaling, which also influences domains of Wnt ligand, receptor and antagonist expression (Panchision et al., 2001; Parr et al., 1993). The resulting mitogenic Wnt gradient from the dorsal midline of the neural tube is responsible for progenitor expansion via cell cycle regulation (Chenn and Walsh, 2002; Dickinson et al., 1994; Megason and McMahon, 2002; Zechner et al., 2003). As the cells exit the cell cycle, they migrate laterally and adopt specific neuronal identities. The establishment of dorsal identity involves the transcription factor Pax7 at early stages, and transcription factors like Math1, Ngn1 and Mash1 at later stages (Helms and Johnson, 2003). Whether Wnt signaling is involved in patterning processes, in addition to its role in promoting cell cycle progression, is still debated. Ngn1 and Mash1 expression was downregulated upon ablation of *Wnt1* and *Wnt3a* (Muroyama et al., 2002), but this effect might be due to altered selective proliferation in interneuron progenitor domains (Chesnutt et al., 2004). According to this hypothesis, patterning is primarily controlled by BMP, linking patterning and neuronal differentiation to Wnt-mediated proliferation. Similarly, Wnt acts

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downstream of BMP to coordinate G1/S cell cycle transition in neural crest cells with their delamination from the dorsal neural tube (Burstyn-Cohen et al., 2004).

Although Wnt signaling via the signaling component β-catenin plays an essential role in proliferation, cell-intrinsic and environmental properties might change over time during development, thereby modulating the response of the cells (Kleber and Sommer, 2004). The presence of a mitogenic Wnt gradient along the dorsoventral axis theoretically predicts continuous growth of the dorsal spinal cord. In order to allow neural tube closure and spinal cord formation, however, neuroepithelial expansion has to be terminated in the dorsal-most spinal cord. Intrinsic properties such as rate limiting steps in the signaling cascade have been proposed to regulate this process (Megason and McMahon, 2002). Alternatively, modulation of Wnt signal activity by the local spatiotemporal context might contribute to the controlled proliferation of neuroepithelial cells in the dorsal spinal cord. This might conceivably involve dorsally expressed BMPs, which modulate the biological activity of Wnt in other systems (Kleber et al., 2005). In this study we investigated the response of spinal cord neuroepithelial cells to Wnt/β-catenin signaling and found that Wnt-mediated proliferation can be counteracted by BMP signaling. Moreover, BMP-dependent differentiation is antagonized by Wnt, indicating that proliferation and differentiation in the dorsal spinal cord are regulated by cross-inhibitory interactions between Wnt and BMP signaling.

Materials and methods

Mating scheme and genotyping

Wnt1-Cre (Danielian et al., 1998) and *Brn4-Cre* (Ahn et al., 2001) mice were mated with *β-catenin*$^{flox(ex3)/+}$ animals (Harada et al., 1999). Embryos with a *Cre* and a *β-catenin*$^{flox(ex3)}$ allele were referred to as mutant embryos. The fate of neuroepithelial cells was followed in compound transgenic animals expressing *Cre* and bred to the ROSA26 mouse reporter *(R26R)* line (Soriano, 1999).

Staining of tissue sections

Non-radioactive in situ hybridization with digoxigenin-labeled riboprobes was performed on cryosections as previously described (Paratore et al., 1999). For immunohistochemistry, sections were fixed for 15 min in 4% paraformaldehyde (PFA)/PBS at room temperature (RT) and treated with 10% goat serum, 0.3% Triton-X-100 and 0.1% BSA in PBS for 30–60 min before incubation with rat anti-Ki67 antibody (1:20; DAKO), monoclonal mouse anti-βIIITubulin (1:250; Sigma), polyclonal rabbit anti-CyclinD1 (1:100 Santa Cruz, CA, No. SC-717), polyclonal rabbit anti-pHH3 (ser10) (1:200; Upstate), polyclonal rabbit anti-Smad1/5/8 (1:200; Cell Signaling), polyclonal rabbit anti-Pax2 (1:50; Zymed San Francisco, No. 71–6000), monoclonal anti-Islet1 (1:50; DSHB), monoclonal anti-Mash1 (1:100; BD Biosciences) or anti-β-catenin (1:300, Sigma) for 1 h at RT or overnight at 4 °C. Ki67, Smad1/5/8, Pax2 and Mash1 antigen presentation was performed in citrate buffer. Smad1/5/8, Pax2 and Mash1 stainings were visualized by tyramide signal amplification (TSA) Cyanine 3 System (Perkin Elmer) as described by the manufacturer. Other immunostainings were visualized by incubation for 1 h at RT using goat anti-rat Texas red antibody (1:200), goat anti-mouse FITC antibody (1:200), goat anti-mouse Cy3 antibody (1:200), or goat anti-rabbit FITC antibody (1:200) (Jackson ImmunoResearch Laboratories). Nuclei were visualized by DAPI staining (1:1000; Sigma). Confocal analysis was done using a TCS SP2 AOBS confocal microscope (DMIRBE, Leica). X-Gal staining on cryosections was performed as previously described (Paratore et al., 2002). All stainings have been performed on at least 3 embryos of each relevant genotype.

Quantification of proliferation and differentiation in vivo

An average of ten Ki67/βIIITubulin immunofluorescence images were taken from 10 μm forelimb level sections of 4 control, 3 *Brn4-Cre/β-catenin*$^{\Delta ex}$ and 2 *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ embryos. From each image the neural tube was graphically cropped in Photoshop CS2 (Adobe Systems Inc.). The cropped neural tubes were horizontally segmented in ImageMagick (http://www.imagemagick.org) into four equal stripes, resulting in a dorsal fragment (dorsal), two medial fragments (medial 1 and medial 2) and a ventral fragment (ventral). Regions of interest were defined for the proliferative (Ki67-positive) and the differentiated (βIIITubulin-positive) areas and quantified in ImageJ (http://rsb.info.nih.gov/ij/).

pHH3 quantification

Serial sections from two E11.5 controls were stained for pHH3 and the nuclei were visualized with DAPI. The roof plate was morphologically determined and graphically confined. Consecutive sectors of 5 cells along the ventricle were determined adjacent to the roof plate. pHH3-positive nuclei per sector were counted. For each sector the values were averaged over 10 sections.

Neuroepithelial cell cultures

Trunk neural tubes from E10.5 rat embryos were prepared by digestion in 0.4 mg/ml Dispase I (Roche) at 4 °C for 5 min. Cleaned tubes were dissociated in 0.025% trypsin/EDTA. Selection for Sox2-positive progenitors was achieved by a 2-day incubation on poly-D-lysine (pDL)/laminin-coated tissue culture dishes (Corning) under non-differentiating conditions (Kalyani et al., 1997). The medium was supplemented with BrdU (Roche) to label the cell population. Four conditions were used in the clonal and the cohort analysis: monolayers of control fibroblasts were used for control and BMP conditions (BMP2, Peprotec), while Wnt-1-producing fibroblasts were used for Wnt and Wnt/BMP conditions (Lee et al., 2004). Neuroepithelial basal medium (Kalyani et al., 1997) was used without FGF and chicken embryo extract, but containing 100 ng/ml EGF (Peprotec), 50 ng/ml NGF (R&D) and BrdU.

To assess BrdU incorporation and differentiation of neuroepithelial cells in cohort cultures, 1000 cells/condition were plated for each replicate and condition (3 independent experiments). After 2 days, BrdU-labeled cells were fixed in 4% PFA for 10 min at RT and stained with anti-BrdU Labeling Kit I antibody (Roche). Differentiation was assayed with an anti-βIIITubulin primary antibody (1:200 Sigma) followed by an alkaline phosphatase-coupled secondary antibody (1:1000 Southern Biotechnology Associates). Detection was performed with a 4-Nitrophenyl phosphate (Fluka) solution at RT for 5 min. The OD was measured at 405 nm (SpectraMAX 190). MTT-Assay for cell proliferation was performed on independent quadruplicates. BMP gradients (0, 5, 10, 15, 20 and 100 ng/ml BMP) were generated on Wnt1-producing fibroblast layers and control fibroblast layers. 3000 neuroepithelial cells/condition were plated for each replicate and condition. After 2 days, the cells were incubated with 0.5 mg/ml MTT (Sigma-M5655) in DMEM for 2 h and fixed in 4% PFA for 10 min at RT. The converted dye in each well was solubilized with 0.5 ml acidic isopropanol (0.04 M HCl in absolute isopropanol) for 10 min at RT. Detection was performed by OD measurement at 570 nm with background subtraction at 650 nm (SpectraMAX 190).

To prospectively identify neuroepithelial cells on fibroblast monolayers for clonal analysis, cells were stained with PKH26 (Sigma). For each condition 50 cells/well were plated in 12-well dishes on Wnt1-producing or control fibroblast monolayers. Upon attachment, clone founder cells were mapped, fixed after 48 h and stained for BrdU. Differentiation was assessed by immunofluorescence for βIIITubulin.

Neurosphere assays

Neuroepithelial cells were prepared from three E10.5 rat embryos. For each embryo 100 cells were plated on 12-well Nunclon dishes coated with poly-HEMA (Poly-[2-hydroxyethylmethacrylate]) (Sigma). The medium was

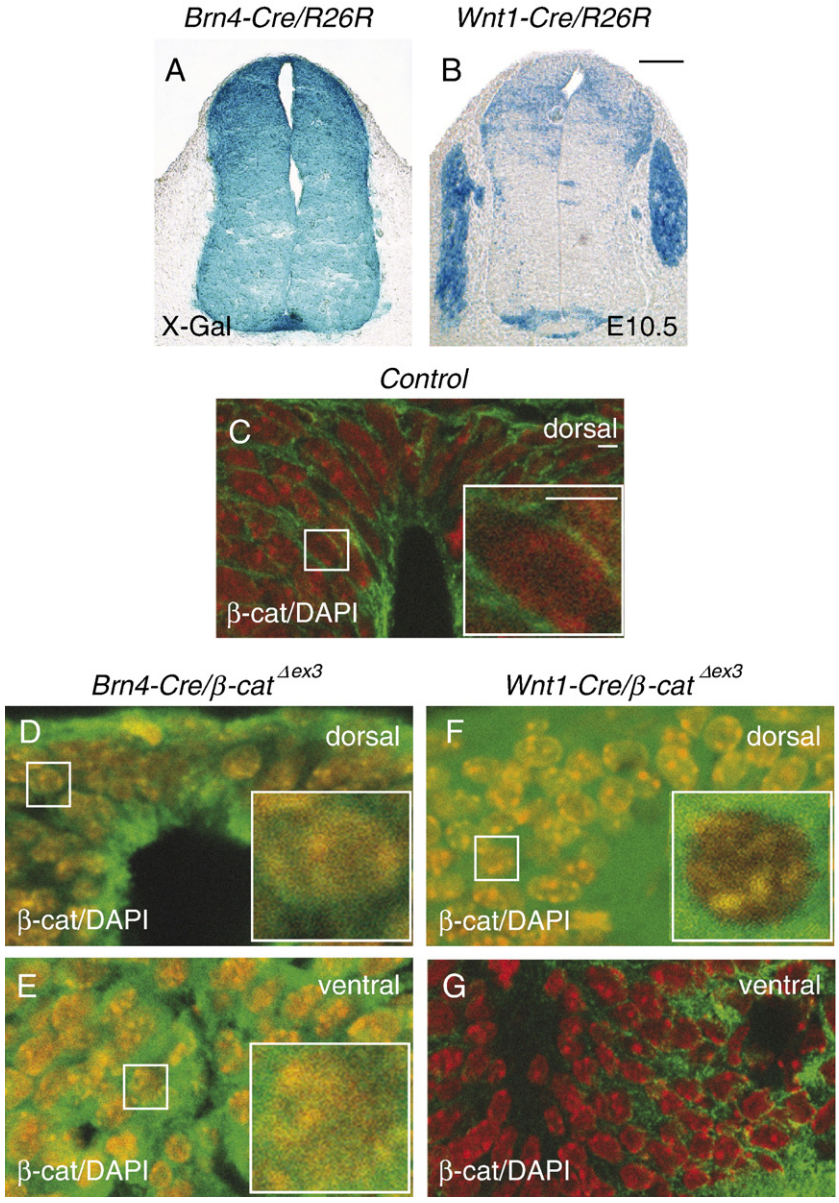


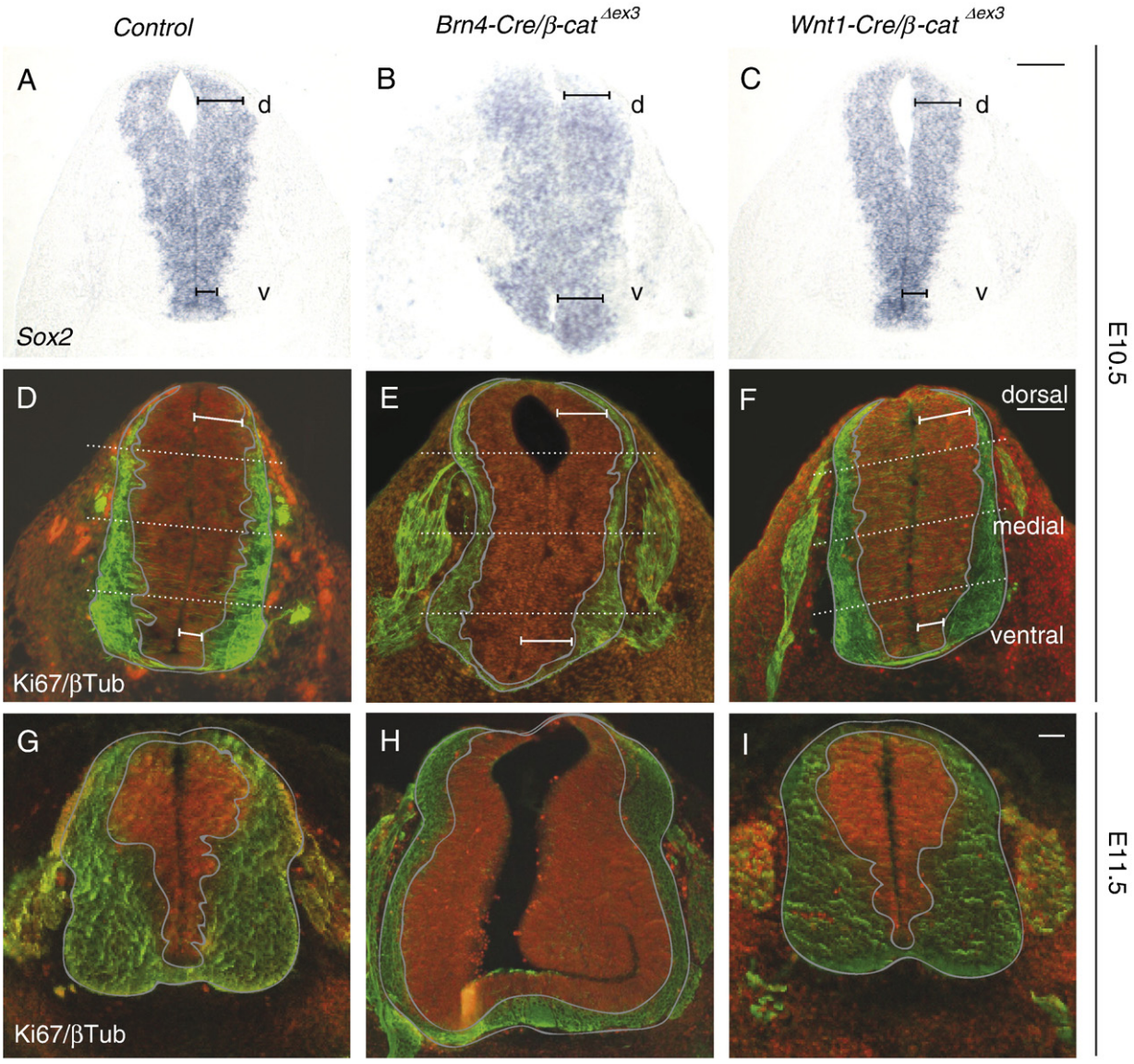
Fig. 1. Conditional expression of constitutively active β-catenin in the developing spinal cord. X-gal staining visualizing Cre-mediated expression of β-galactosidase at E10.5 in the entire spinal cord of *Brn4-Cre/R26R* embryos (A) and in the dorsal spinal cord of *Wnt1-Cre/R26R* embryos (B). Confocal microscopy fails to reveal β-catenin protein (β-cat; green) in DAPI-stained nuclei (red) in the dorsal spinal cord of control animals (C). Upon *Brn4-Cre*-mediated exon3 deletion (Δex3) of *β-catenin*, the protein accumulates throughout the spinal cord, including in the cell nuclei (yellow) of dorsal (D) and ventral (E) areas. In *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ mutants, increased nuclear β-catenin (yellow) is detectable on the same tissue section in the dorsal (F) but not ventral (G) spinal cord. Insets, magnifications of cell nuclei marked by white boxes. Scale bars, (B) 50 μm and (C) 5 μm.

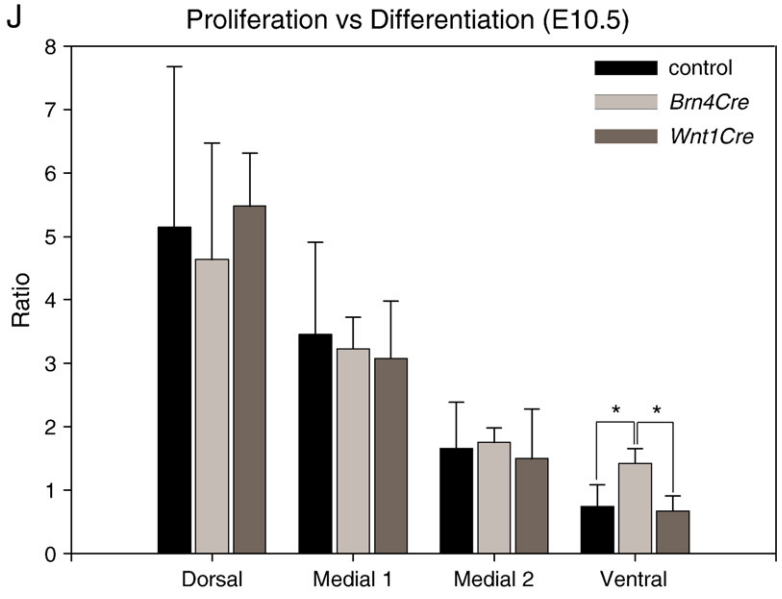
as for the clonal analysis but complemented with 50 ng/ml soluble human Wnt1 (Peprotec) and/or 20 ng/ml BMP2. Spheres were dissociated in trypsin/EDTA (0.025%) for passaging, and 100 cells/well were replated. The number of spheres for each condition and replicate was averaged for each passage.

Western blot analysis

Proteins from primary sphere cells were prepared as described (Atanasoski et al., 2001) and immunoblotted with antibodies against CyclinD1 (1:100

Fig. 2. Differential effects of β-catenin signaling on proliferation and differentiation along the dorsoventral spinal cord axis. (A) In situ hybridization detecting *Sox2* mRNA at E10.5 reveals that the lateral extent of the dorsal ventricular zone (bar labeled by 'd') is comparable in control (A), *Brn4-Cre/β-catenin*$^{\Delta ex3}$ (B) and *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ embryos (C). In contrast, the ventral ventricular zone (bar marked by 'v') is laterally expanded in *Brn4-Cre/β-catenin*$^{\Delta ex3}$ as compared to control and *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ embryos. Zones of Ki67-positive proliferating cells (Ki67; red) and of neuronal cells stained with anti-βIIITubulin antibodies (βTub; green) are comparable in dorsal and medial portions of the spinal cord at E10.5 in control, *Brn4-Cre/β-catenin*$^{\Delta ex3}$ and *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ mutants, while the proliferative zone is expanded in the ventral spinal cord of *Brn4-Cre/β-catenin*$^{\Delta ex3}$ mutants (white bars; D–F). At E11.5, Ki67 and βTub expression domains reveal a similar spinal cord morphology of control and *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ embryos (G, I). (H) The spinal cord of *Brn4-Cre/β-catenin*$^{\Delta ex3}$ embryos is ventrally distorted due to overproliferation, indicating the responsiveness of the ventral neuroepithelium to β-catenin signaling. (J) Ratios of proliferation vs. differentiation were quantified for each segment dorsal, medial 1, medial 2 and ventral (depicted by the dotted lines in (D–F)) at E10.5 (see Materials and methods). The ventral-most segment of *Brn4-Cre/β-catenin*$^{\Delta ex3}$ embryos exhibits significantly enhanced proliferation and reduced differentiation. No significant differences were detected in the medial and dorsal spinal cord of *Brn4-Cre/β-catenin*$^{\Delta ex3}$ as compared to control and *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ embryos. (*), $p<0.05$. Scale bars, 50 μm.


Control
Brn4-Cre/β-cat Δex3
Wnt1-Cre/β-cat Δex3
A
B
C
d
v
Sox2
D
E
F
dorsal
medial
ventral
Ki67/βTub
G
H
I
Ki67/βTub
E10.5
E11.5


J
Proliferation vs Differentiation (E10.5)
control
Brn4Cre
Wnt1Cre
Ratio
0
1
2
3
4
5
6
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8
Dorsal
Medial 1
Medial 2
Ventral
*
*

Santa Cruz, CA, No. SC-717) and β-actin (Sigma; 1:1000). After incubation with goat anti-rabbit horseradish peroxidase-conjugated (Santa Cruz Biotechnology, Inc.) or goat anti-mouse κ-chain alkaline phosphatase-conjugated secondary antibodies (Southern Biotechnology Associates), immunoreactive bands were visualized by Immobilon (Millipore Corporation) or CDP-Star (Roche).

Statistical analysis

Statistical analysis was performed in Systat (SYSTAT Software Inc.). All the plots were prepared with SigmaPlot (SYSTAT Software Inc.). The unpaired *t*-test was used to statistically analyze the data in Figs. 2J, 5, 6, 7, 8E.

Results

Wnt/β-catenin signal activation in the developing spinal cord causes ventral but not dorsal neuroepithelial expansion

In mice carrying an allele of the *β-catenin* gene in which exon 3 is flanked by LoxP sites (*β-catenin*$^{flox(ex3)}$ allele) Cre recombinase-mediated exon 3 excision (*β-catenin*$^{\Delta ex3}$) leads to expression of a stabilized, constitutively active form of β-catenin (Harada et al., 1999). When expressed under the control of the *Brn4* promoter, Cre recombinase was active in the entire neural tube as early as at E9.5, as assessed by mating of *Brn4-Cre* mice (Ahn et al., 2001) to *Rosa26* Cre reporter *(R26R)* mice (Soriano, 1999) that express β-galactosidase upon Cre-mediated recombination (Fig. 1A). However, Cre recombinase expression under the control of the *Wnt1* promoter leads to recombination only in the dorsal part of the neural tube (Fig. 1B) (Brault et al., 2001). Similar to *Brn4-Cre*, *Wnt1-Cre* is active in the neural tube from E9.5 onwards (data not shown). Stabilization of β-catenin results in its nuclear accumulation (Huber et al., 1996; Tolwinski and Wieschaus, 2004). While there was no detectable nuclear β-catenin protein in control neural tube at E10.5 (Fig. 1C), *Brn4-Cre/β-catenin*$^{\Delta ex3}$ mice displayed a drastic increase in β-catenin levels, including nuclear protein, throughout the neural tube (Figs. 1D and E). In contrast, nuclear β-catenin accumulation was restricted to the dorsal neural tube of *Wnt1-Cre/β-catenin-* embryos (Fig. 1F), while β-catenin expression and localization in the ventral neural tube of these mutants were comparable to that in controls (Fig. 1G).

Expression of a constitutive active form of β-catenin has been reported to induce expansion of the neural progenitor population in the developing CNS (Chenn and Walsh, 2002; Zechner et al., 2003). In agreement with these earlier findings, the ventricular zone containing *Sox2*-positive neural progenitors was enlarged in the spinal cord of *Brn4-Cre/β-catenin*$^{\Delta ex3}$ embryos at E10.5 relative to control embryos (Figs. 2A and B) (Zechner et al., 2003). However, this enlargement was confined to medial and ventral levels along the dorsoventral axis and not apparent in the most dorsal aspects of the developing spinal cord. These results were unlikely due to differential β-catenin activation along the dorsoventral axis because Cre recombinase expression in *Brn4-Cre* mice can be detected both in the dorsal and ventral neural tube already at E9.5 (data not shown). Rather, the data suggest that dorsal neuroepithelial cells might be less sensitive to increased β-catenin levels than more ventral cells. To further address this issue, we generated *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ mice to activate β-catenin exclusively in the dorsal neural tube. Strikingly, the developing spinal cord in these animals did not display morphological alterations as compared to control embryos. Moreover, we did not observe an expansion of the *Sox2*-positive neuroepithelium in *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ embryos at E10.5 (Fig. 2C), despite increased nuclear β-catenin (Fig. 1F) and recombination as early as at E9.5 (data not shown). Co-staining of spinal cord sections at E10.5 for the proliferation marker Ki67 and the neuronal differentiation marker βIIITubulin revealed expansion of the ventricular zone in the ventral spinal cord of *Brn4-Cre/β-catenin*$^{\Delta ex3}$ mutant embryos (Figs. 2D–F). However, the extent of the proliferative and differentiated areas was comparable in the dorsal spinal cord of control, *Brn4-Cre/β-catenin*$^{\Delta ex3}$ and *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ mutants. The differential effect of constitutively active β-catenin on ventral vs. dorsal spinal cord portions was even more pronounced at E11.5, when the ventral spinal cord of *Brn4-Cre/β-catenin*$^{\Delta ex3}$ mutants was often distorted due to a massively expanded proliferative zone (Fig. 2H). Thereby, the increase in proliferation correlated with a decrease in neuronal differentiation in the mutant ventral spinal cord. In contrast, the spinal cord was morphologically normal in *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ mutant embryos at E11.5, and proliferation and neuronal differentiation appeared not to be affected in these mutants (Fig. 2I).

To quantify the effects of β-catenin activation along the dorsoventral axis, we measured the relative areas occupied by proliferating vs. neuronally differentiated cells. To this end, spinal cord sections of control, *Brn4-Cre/β-catenin*$^{\Delta ex3}$ and *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ mutant embryos at E10.5 were double labeled for Ki67/βIIITubulin and virtually segmented in four portions of equal dorsoventral extension (dotted lines in Figs. 2D–F). Area measurements revealed that the ratio between proliferation and differentiation was unaltered in dorsal and medial sections of controls and both mutants (Fig. 2J). In contrast, this ratio was significantly increased in ventral spinal cord sections of *Brn4-Cre/β-catenin*$^{\Delta ex3}$ mutant embryos as compared to corresponding sections of control and *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ embryos. Thus, activation of Wnt/β-catenin signaling influences the balance between proliferation and differentiation in ventral but not dorsal portions of the developing spinal cord.

Modulation of proneural gene expression upon Wnt/β-catenin signal activation in the dorsal spinal cord accompanied by changes in BMP signaling

Apart from its mitogenic effect, canonical Wnt signaling has been associated with patterning in the developing spinal cord. Discrete dorsal interneuron (dI) populations dI1, dI2 and dI3 are defined by the proneural transcription factors Math1, Ngn1 and Mash1, respectively (Helms and Johnson, 2003). Loss of dorsally expressed *Wnt1* and *Wnt3a* resulted in loss of *Math1* and *Ngn1* transcripts, with a subsequent loss of the dorsal interneuron subclass dI1, marked by Lhx2 (Muroyama et al., 2002). However, it has been suggested that these results might be secondary to altered proliferation (Chesnutt et al., 2004). The

availability of the *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ model system allowed us to revisit neural tube patterning upon Wnt/β-catenin signal activation specifically in the dorsal spinal cord, in the absence of apparent changes in proliferation. In situ hybridization revealed an enlargement of the *Math1* expression domain in mutant as compared to control embryos at E10.5 (Figs. 3A and B), concomitant with an increase in the *Lhx2*-positive dI1 population at E11.5 (Figs. 3K and L). Intriguingly, dorsal activation of β-catenin led to a loss of *Ngn1* expression and, accordingly, to a reduction of the dI2 population marked by *Lhx1* (Figs. 3C, D, M, N). Expression of *Mash1* was unaltered, as assessed both by in situ hybridization analysis and immunostaining (Figs. 3G–J). In contrast, expression of *Ngn2*, which controls number but not identity of interneuron subtypes (Helms et al., 2005), was increased in the mutant (Figs. 3E and F). Overexpression of *Ngn2* in the chicken neural tube leads to reduction of dI3 and an increase in dI4 populations (Helms et al., 2005). In agreement with these data, increased *Ngn2* expression in the spinal cord of *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ mice was accompanied by a decrease in Isl1-positive dI3 and an increase in Pax2-expressing dI4 neuron numbers (Figs. 3O and P). Thus, β-catenin signal activation affected patterning processes in the dorsal spinal cord.

The differential changes in proneural gene expression cannot simply be explained by expansion of progenitor domains throughout the dorsal neural tube. Moreover, loss of *Ngn1* was not due to impaired cell survival in the *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ mutant because cell death was minimal both in control and mutant neural tube at E10.5 (data not shown). Hence, our findings point to an involvement of Wnt/β-catenin signaling in neural tube patterning. This is in apparent contrast to recent studies, in which BMP signaling has been identified as the primary effector in neural tube patterning, coordinating patterning and growth by regulating Wnt ligand expression (Chesnutt et al., 2004). However, the effect of β-catenin stabilization on proneural gene expression in the dorsal neural tube might be indirect and possibly explained by modulated BMP signaling. *Msx1* is a target gene of both Wnt and BMP signaling, but its

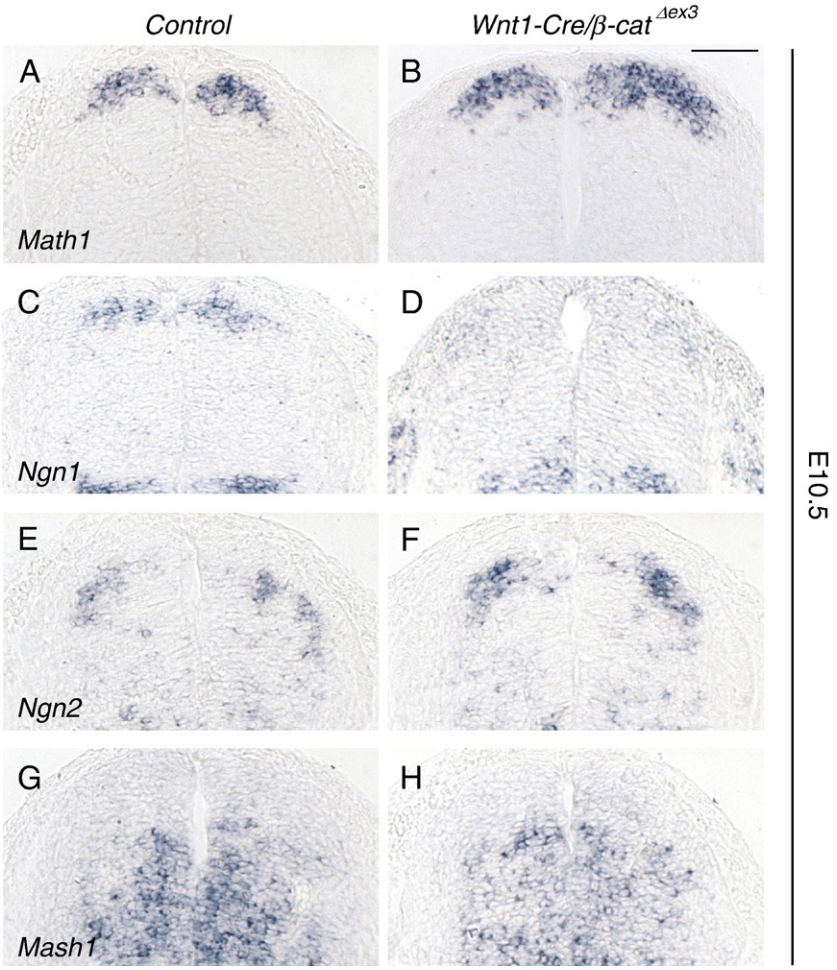

Fig. 3. Dorsal spinal cord patterning defects in *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ embryos. (A, B) In situ hybridization experiments show that at E10.5, the expression domain of the dorsal proneural gene *Math1* is expanded upon β-catenin signal activation in *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ mutants. (C, D) Conversely, *Ngn1* is downregulated in the mutant. (E, F) *Ngn2* is considerably upregulated in the mutant. The expression domain of *Mash1* is not detectably altered in the mutant (H) when compared to control embryos (G). (I, J) Similarly, at E11.5 Mash1 immunoreactivity is comparable in control and mutant. At E11.5, the dI1 interneuron population marked by in situ hybridization for *Lhx2* mRNA is increased in the mutant (L) when compared to the control (K). Conversely, the adjacent *Lhx1*-positive dI2 domain (marked by horizontal brackets) is reduced in the mutant (N) as compared to the control (M). (O, P) Immunostaining for Isl1 (green) and Pax2 (red) reveals a decrease in the dI3 and an increase in the dI4 interneuron population, respectively. Scale bars, 25 μm.

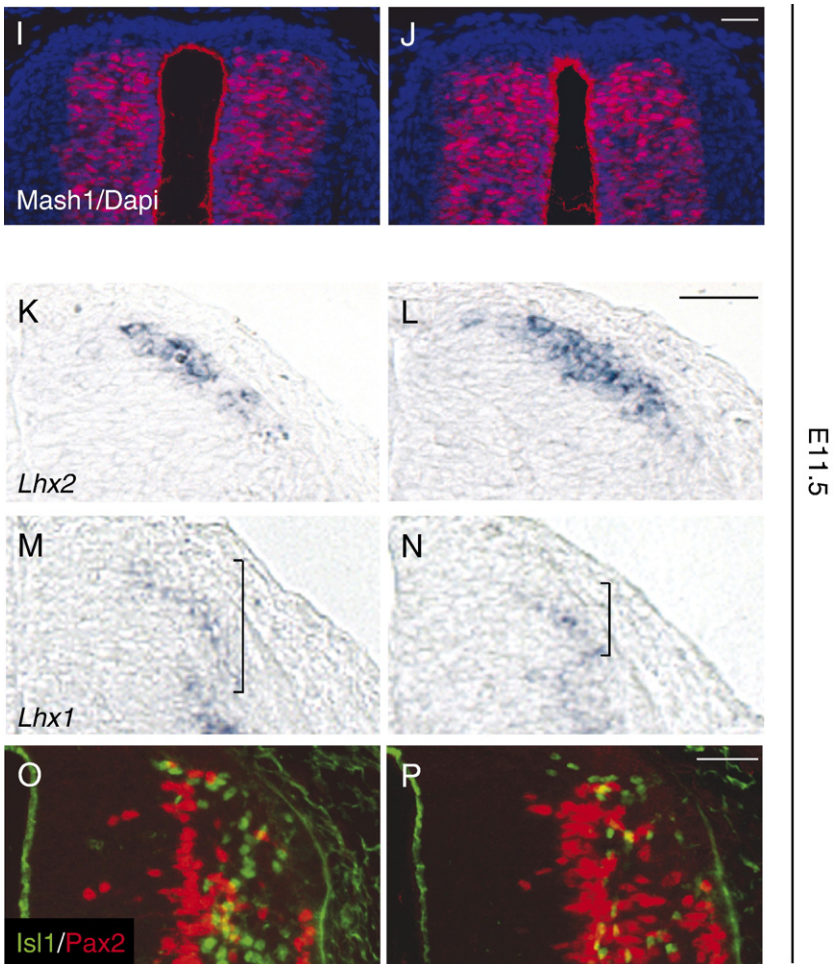

Fig. 3 (*continued*).

expression is greatly enhanced by the combination of both factors (Timmer et al., 2002; Willert et al., 2002). While in the control *Msx1* mRNA expression was restricted to the most dorsal aspects of the spinal cord at E11.5 (Fig. 4A), *Msx1* expression was strongly expanded in *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ mutant embryos (Fig. 4B). Similarly, *Msx1* expression was upregulated in the dorsal but not ventral spinal cord of *Brn4-Cre/β-catenin*$^{\Delta ex3}$ mutants (data not shown). Importantly, β-

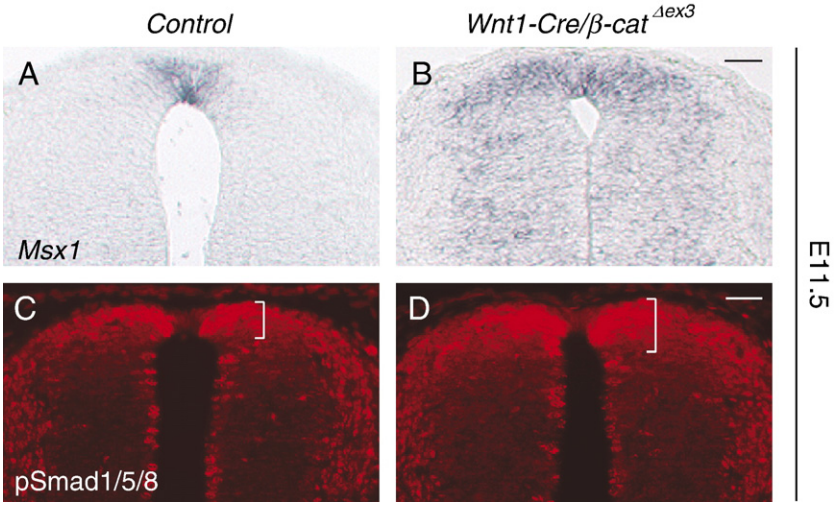

Fig. 4. Activation of β-catenin signaling induces BMP signaling in the dorsal spinal cord. At E11.5, expression of the Wnt/BMP target *Msx1* is restricted to the roof plate in the control (A), while it is expanded in the spinal cord of *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ mutant embryos (B), as analyzed by in situ hybridization. (C, D) Likewise, increased immunostaining of phosphorylated (p)Smad1/5/8 (white brackets) illustrates BMP signal activation upon β-catenin stabilization in the dorsal spinal cord. Scale bars, 25 μm.

catenin activation also led to drastically enhanced phosphorylation of the BMP signaling components Smad1/5/8 in the dorsal spinal cord (Figs. 4C and D). These findings demonstrate that signaling by activated β-catenin upregulates BMP signaling. Thus, at least some of the changes in proneural gene expression observed in mouse mutants expressing constitutively active β-catenin are conceivably due to Wnt/β-catenin-dependent BMP signaling.

Crosstalk between Wnt and BMP signaling regulating proliferation and differentiation of neuroepithelial cells

Increased BMP signaling might also explain the limited proliferation in the dorsal spinal cord of *Brn4-Cre/β-catenin*$^{\Delta ex3}$ and *Wnt1-Cre/β-catenin*$^{\Delta ex3}$ mutants, given that BMP can promote mitotic arrest in CNS progenitor cells (Mabie et al., 1999; Panchision et al., 2001). To directly assess whether and how BMP signaling might modulate Wnt-dependent proliferation on the cellular level, we used an adhesive culture system of neuroepithelial cells obtained from E10.5 rat spinal cords (Kalyani et al., 1997). Culturing of the cells for 1 to 2 days in a medium containing chicken embryo extract allowed the selection of undifferentiated proliferative neuroepithelial progenitor cells (Kalyani et al., 1997), 99.9±0.1% of which expressed the neural stem and progenitor marker Sox2 (data not shown). In a first set of experiments, these neuroepithelial progenitor cells were exposed in high density cultures to either control conditions, Wnt1 alone (provided by feeder cell layers), BMP2 alone (20 ng/ml), or a combination of Wnt1 and BMP2. In all cases, FGF2 was removed from the culture medium because FGF2 may lead to ventralization of dorsal progenitor cells, which would possibly alter the behavior of neuroepithelial progenitor cells in response to the administered factors (Gabay et al., 2003). Proliferation on the population level was assayed by cumulative BrdU incorporation and subsequent colorimetric analysis. As compared to control conditions in the absence of Wnt1 and BMP2, exposure of neuroepithelial progenitor cells to Wnt1 alone promoted proliferation 3.13±0.2-fold, while BrdU incorporation was at approximately 70% of control levels in the presence of BMP2 only (Fig. 5A). Intriguingly, upon treatment with both Wnt1 and BMP2, BrdU incorporation was intermediate to that obtained with Wnt1 alone and BMP2 alone (Fig. 5A), indicating an inhibitory role of BMP signaling on Wnt-induced mitosis.

BMP2 elicits a dose-dependent control of midbrain precursor cell numbers (Panchision et al., 2001). Similarly, low doses of BMP2 (5 ng/ml) promoted proliferation of spinal cord neuroepithelial cells by approximately 2-fold, whereas BMP2 concentrations above 20 ng/ml slowed down proliferation as compared to control conditions (Fig. 5B). To investigate whether BMP influences Wnt activity in a dose-dependent manner, spinal cord neuroepithelial cells exposed to Wnt1 were treated with varying doses of BMP2 (Fig. 5B). Intriguingly, although high doses of BMP2 had the strongest effect, Wnt-dependent proliferation was also decreased at low BMP2 concentrations at which BMP2 alone is mitogenic.

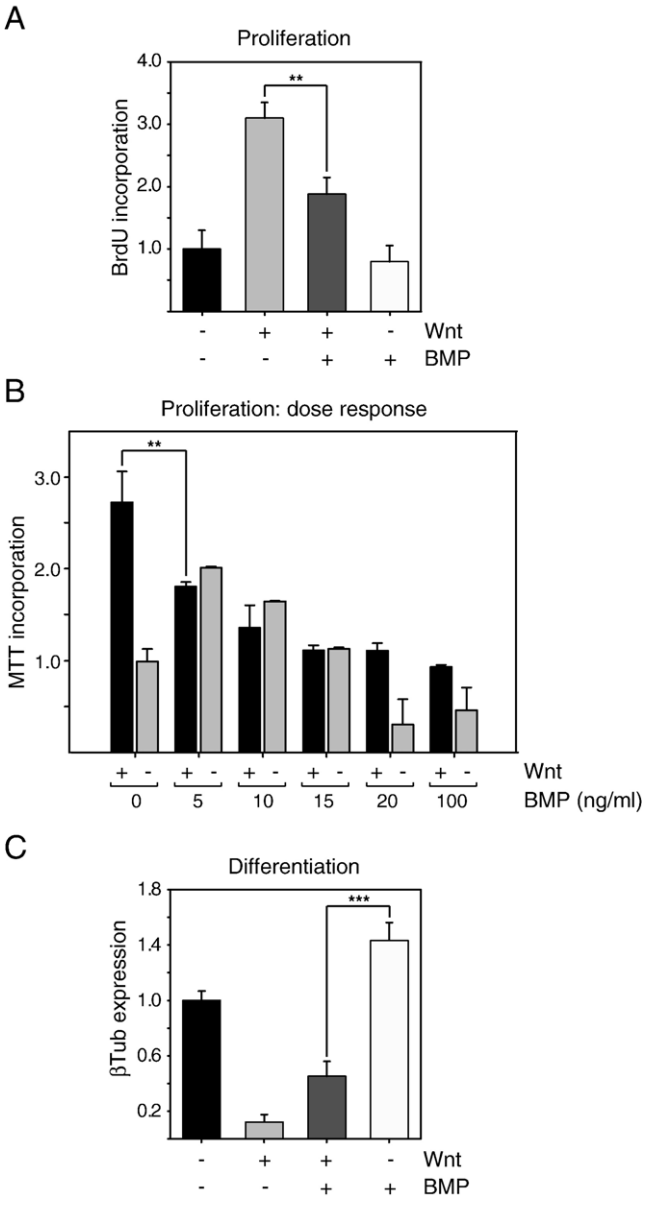

Fig. 5. Proliferation and differentiation of spinal cord neuroepithelial cells treated with Wnt and BMP. Proliferation and differentiation were assessed in neuroepithelial cell cohorts isolated from E10.5 rat spinal cords. (A) BrdU incorporation demonstrates an increase in the relative proliferation of cells cultured on Wnt1-expressing feeder layers as compared to cells in control conditions. In the presence of BMP2 alone (20 ng/ml), BrdU incorporation was reduced. Upon combinatorial Wnt/BMP signaling, proliferation was significantly reduced in comparison to Wnt only conditions. (B) A dose response to increasing amounts of BMP2 indicates that BMP alone promotes proliferation at low concentrations and suppresses proliferation at high concentrations, as measured by an MTT incorporation assay. In contrast, at all BMP2 doses tested, Wnt-induced proliferation was reduced by BMP signaling. (C) The relative rates of differentiation appear reciprocal to the proliferation values. Both Wnt and Wnt plus BMP reduced differentiation as compared to control conditions (Wnt1 provided by feeder cell layers; BMP2 used at 20 ng/ml). Importantly, Wnt also significantly reduced BMP-promoted differentiation. (**), $p<0.005$; (***), $p<0.0005$.

In vivo, Wnt/β-catenin-dependent proliferation in the ventral neural tube was accompanied by reduced neuronal differentiation, while in the dorsal spinal cord both

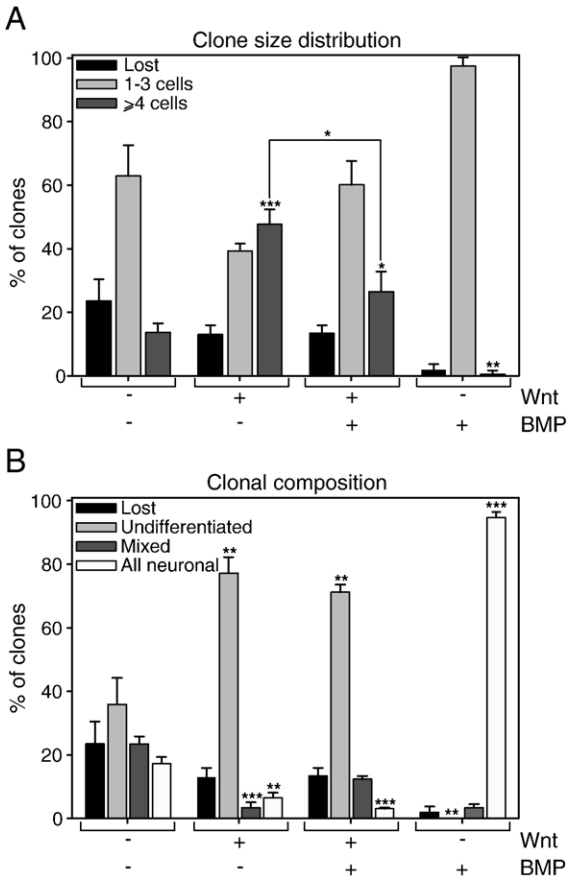

Fig. 6. Antagonistic effects of Wnt and BMP on neuroepithelial cells at clonal density. (A) Size distribution of clones generated from neuroepithelial clone founder cells treated with the factors indicated. In control conditions, most clones consisted of 1–3 cells, while the clone size was considerably shifted to large clones in the presence of Wnt1 (provided by feeder cell layers). Treatment with Wnt1 and BMP2 (20 ng/ml) significantly reduced the number of clones containing 4 or more cells. In the presence of BMP alone, virtually all clones contained less than three cells. (B) Evaluation of the clone composition. Few clones contained βIIITubulin-positive neuronal cells upon treatment with Wnt alone, whereas with BMP alone most clones consisted exclusively of differentiated neurons. Upon combinatorial Wnt/BMP signaling, most neuroepithelial cells failed to generate clones containing neurons. (*), $p<0.05$; (**); $p<0.005$; (***); $p<0.0005$; (control vs. condition).

proliferation and differentiation were unaffected upon Wnt/β-catenin signal activation and concomitant increase of BMP signaling (Figs. 2 and 4). Therefore, in addition to proliferation, we examined the effect of Wnt and BMP signaling on neuronal differentiation of neuroepithelial progenitor cells by colorimetric analysis of βIIITubulin expression. While BMP2-only conditions (20 ng BMP2/ml) reduced proliferation the most, they induced the highest degree of neuronal differentiation (Fig. 5C). In contrast, incubation of the cells with mitogenic Wnt1 drastically reduced neuronal differentiation to 11.6±5% of that of control conditions. Combinatorial Wnt1/BMP2 signaling displayed intermediate differentiation levels. However, these were yet significantly lower than the degree of differentiation observed in control and BMP2-only conditions (Fig. 5C). Thus, Wnt signaling counteracts BMP-induced differentiation.

The effect of Wnt1 and BMP2 on proliferation and neurogenesis of neuroepithelial progenitor cells could indicate selective mechanisms, in that Wnt and/or BMP signaling would promote proliferation, survival or differentiation of subpopulations of spinal cord cells. Alternatively, neuroepithelial progenitor cells might represent a relative homogeneous cell population in terms of their potential, irrespective of their origin within the developing spinal cord; in this case, combinatorial Wnt and BMP might act instructively on individual cells. To distinguish between these two possibilities, neuroepithelial cells were plated at clonal density, individual cells were marked, and their developmental potential was assessed by exposing them to either Wnt1, BMP2 or to a combination of both. Under the chosen conditions, many founder cells in the control generated clones of one to three cells, while only 13.6±2.9% of the cells produced clones larger than four cells (Fig. 6A). 23.5±6.8% of all clones in control conditions were lost. Addition of Wnt1 produced a considerable shift towards clones larger than 4 cells (47.6±4.6% of all clones) at the expense of smaller sized clones, indicating that Wnt signaling induced proliferation in our culture system. In the presence of BMP2 alone, virtually all clones survived but these were small (Fig. 6A), and many clones consisted of a single cell (data not shown). In agreement with experiments performed at the level of cell populations (Fig. 5), the number of clones larger than four cells was significantly reduced in Wnt1/BMP2 conditions as compared to Wnt1 alone, and almost as many clones as in the control (61±6.3%) consisted of one to three cells only. In addition, survival of founder cells was increased in Wnt1/BMP2 conditions relative to control cultures. Thus, our data reveal that BMP2 antagonizes the proliferative effect of Wnt1 on spinal cord-derived neuroepithelial progenitor cells at clonal density.

To investigate the neuronal differentiation potential of individual neuroepithelial cells, we analyzed the cellular composition of clones generated in control conditions or in the presence of Wnt1, BMP2 or both factors. In the control, 35.8±8.3% of all clones did not contain a single βIIITubulin-positive neuronal cell ("undifferentiated clones"), while 17.2±

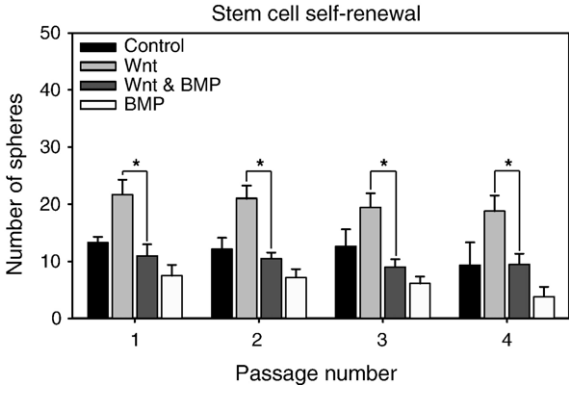

Fig. 7. BMP counteracts neural stem cell self-renewal promoted by Wnt. Neurospheres treated with the factors indicated were passaged 4 times and the sphere numbers were counted 5 days after each passage. The sphere number (mean±SD per 2400 cells plated) was maximal in the presence of soluble Wnt1, while upon addition of Wnt1 plus BMP2 the sphere numbers were significantly reduced as compared to Wnt1 alone. (*), $p<0.005$.

2% of all clones consisted exclusively of βIIITubulin-positive neurons ("all neuronal clones") (Fig. 6B). 23.3±2.4% of all clones were "mixed" and contained at least one neuron and one non-neuronal cell. Under Wnt1 conditions, 77.2±5% of the clones were undifferentiated, with very few mixed clones (3.3±1.7%) and strongly reduced numbers of all-neuronal clones (6.4±1.6%). In contrast, BMP2 alone promoted maximal differentiation, with 94.7±1.6% of all clones consisting of neurons only. Interestingly, however, the number of all-neuronal clones was drastically reduced to only 3.0±0.4% of all clones in cultures containing Wnt1 plus BMP2, and the cellular composition of Wnt1/BMP2-treated clones was similar to that of clones exposed to Wnt1 alone, with 71.2±2.3% of all clones remaining undifferentiated in Wnt1 plus BMP2 conditions (Fig. 6B). Hence, while BMP signaling counteracted Wnt-induced proliferation, Wnt signaling antagonized BMP-dependent differentiation. Thereby, loss of clones was minimal in all conditions (Figs. 6A and B), virtually all cells turned out to be responsive to BMP signaling, and a large fraction of the cells displayed responsiveness to Wnt or Wnt plus BMP. Thus, neuroepithelial progenitor cells prepared from rat spinal cord at E10.5 are fairly homogeneous with respect to their ability to respond to Wnt and BMP signaling.

Simultaneous repression of proliferation and differentiation by combinatorial Wnt/BMP signaling has also been observed in neural crest stem cells emigrating from the dorsal neural tube (Kleber et al., 2005). Therefore, we investigated whether Wnt and BMP influence the behavior of spinal cord-derived neuroepithelial cells with stem cell properties. To this end, we made use of the neurosphere culture assay (Reynolds and

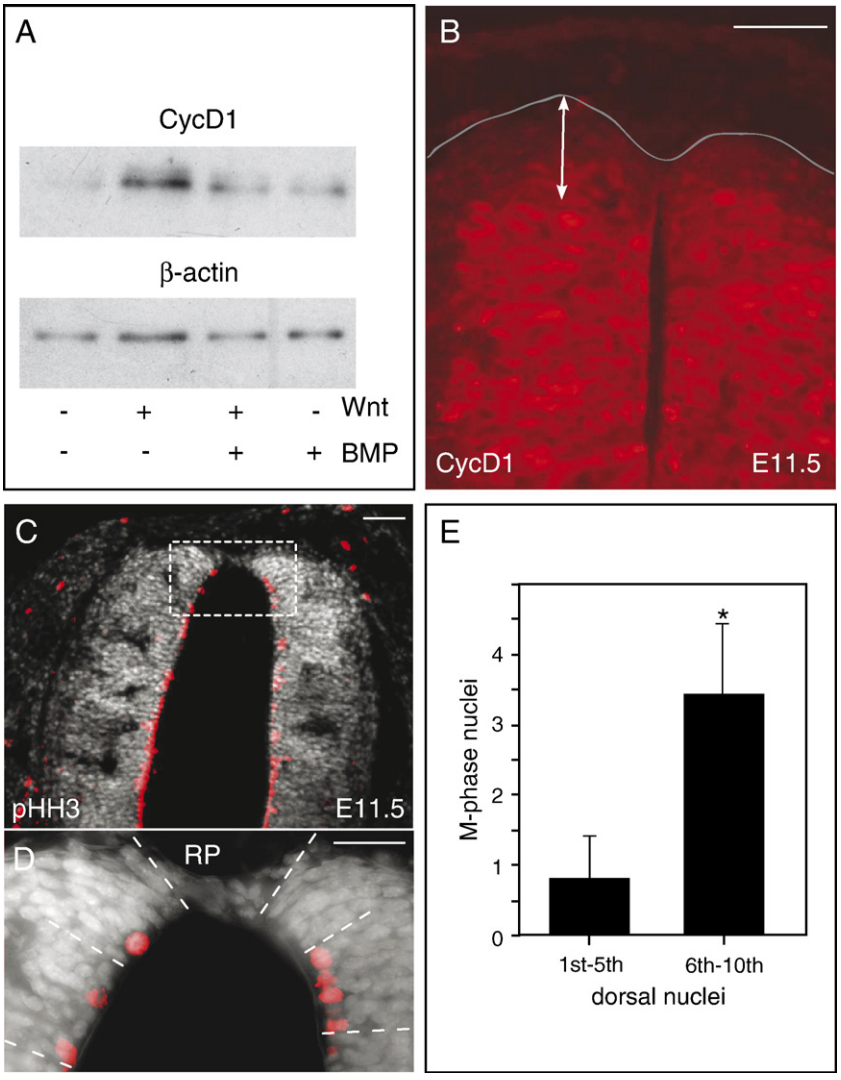

Fig. 8. Cyclin D1 expression and number of mitotic cells are low in the dorsal-most spinal cord. (A) Western blot analysis of primary neurospheres treated with Wnt and/or BMP. Cyclin D1 levels (CycD1) were highest in the presence of Wnt. Addition of Wnt/BMP and of BMP alone resulted in reduced CycD1 levels. (B) Although highly expressed in the medial and dorsal spinal cord, CycD1 protein expression was low in the dorsal-most spinal cord (double arrow) of wild-type mice at E11.5, in agreement with Wnt/BMP being expressed in the dorsal spinal cord. (C) Likewise, phospho-histone H3 (pHH3) staining revealed reduced mitotic cell numbers in the dorsal-most spinal cord. (D) High magnification of area indicated by box in panel C. (E) Quantification of apical M-phase nuclei in the spinal cord at E11.5. Proceeding from the roof plate (RP) margin in a ventral direction, the number of pHH3-positive nuclei among the 1st to 5th and the 6th to 10th apical nuclei was quantified. Segments taken into account are illustrated by dotted lines in D. 0.8±0.6 (mean±SD) nuclei were pHH3 positive within a distance of 1–5 nuclei from the RP. However, 3.45±1 M-phase nuclei were detected within a distance of 6–10 nuclei from the RP. (*), $p<0.05$. Scale bar, 50 μm.

Weiss, 1996), in which self-renewal of neural stem cells can be operationally addressed. As with the above-described adhesive cell cultures, ventralizing FGF (Gabay et al., 2003) was avoided in the neurosphere cultures. In the presence of soluble Wnt, the number of primary spheres generated from rat spinal cord at E10.5 was increased as compared to control cultures (Fig. 7). Moreover, incubation with Wnt increased the self-renewal activity of neural stem cells, as assessed by dissociation of primary neurospheres and serial propagation at clonal density for up to four passages in the presence of the growth factor. In contrast, addition of BMP2 to Wnt-treated cultures significantly reduced primary sphere formation and stem cell self-renewal to the numbers of control conditions (Fig. 7).

Regulation of CyclinD1 levels by combinatorial Wnt and BMP signaling

In various cell types, Wnt-dependent proliferation is mediated by upregulation of the Wnt target gene *CyclinD1* (Nusse, 1999; Shtutman et al., 1999; Tetsu and McCormick, 1999). Therefore, the capacity of BMP to inhibit Wnt-induced neuroepithelial cell proliferation and stem cell self-renewal might be explained by repression of *CyclinD1* expression. To address this issue, we measured CyclinD1 protein levels in primary neurospheres derived from rat spinal cord at E10.5 and incubated in control cultures or in cultures treated with either soluble Wnt, BMP2 or a combination of both. As expected, exposure of cells to soluble Wnt increased CyclinD1 levels relative to control levels (Fig. 8A), consistent with the increase in proliferation observed in Wnt-treated neuroepithelial cells (Figs. 5 and 6). However, when Wnt was combined with BMP2, CyclinD1 levels were strongly reduced in comparison to conditions with Wnt alone. These data suggest that BMP signaling prevents Wnt from promoting proliferation in neuroepithelial cells by suppressing Wnt-dependent activation of CyclinD1 expression.

Our data predict that, in wild-type spinal cord, CyclinD1 expression would be reduced in most dorsal regions because of dorsal Wnt/BMP signaling. Analysis of CyclinD1 protein expression at E11.5 revealed that the mantle zone of differentiating cells in the developing spinal cord was CyclinD1-negative, while there was generally strong expression in the proliferative ventricular zone, in particular at medial–dorsal levels (Fig. 8B) (Megason and McMahon, 2002). However, the most dorsal cell layers of the spinal cord neuroepithelium expressed lower levels of CyclinD1 than seen in adjoining more ventral domains (Fig. 8B). To investigate whether decreased CyclinD1 expression correlates with decreased cell cycle progression, we counted the number of nuclei expressing phosphorylated histone H3 (pHH3), a marker for late G2 and M-phase progression, in the dorsal neuroepithelium of spinal cord at E11.5. In general, pHH3 staining was confined to the apical side of the developing spinal cord (Fig. 8C). Strikingly, however, the number of nuclei in M-phase of the cell cycle was more than four fold reduced in dorsal-most ventricular cells as compared to ventrally adjoining cells (Figs. 8D and E). These data are consistent with the model that BMP modulates Wnt-dependent CyclinD1 expression and cell cycle progression in vivo.

Discussion

In this study we show that the balance between proliferation and differentiation of neuroepithelial cells in the developing dorsal spinal cord is finely tuned by the crossregulatory activities of Wnt and BMP signaling. Overexpression of the canonical Wnt signaling component β-catenin leads to ventral but not dorsal expansion of the spinal cord neuroepithelium, along with dorsal upregulation of BMP signaling. Furthermore, both in mass and clonal cultures of neuroepithelial cells, BMP antagonizes Wnt-induced CyclinD1 expression and cell proliferation, while Wnt counteracts neuronal differentiation promoted by BMP. These findings plus the observation that cell cycle progression and differentiation are low in the dorsal-most portions of the developing spinal cord indicate that the dorsal-to-ventral Wnt-dependent mitogen gradient and the ventral-to-dorsal differentiation gradient (Megason and McMahon, 2002) are shaped by combinatorial Wnt/BMP signaling. This allows maintenance of slow-cycling, undifferentiated progenitor cells defining the dorsal limits of the spinal cord neuroepithelium (Fig. 9).

Patterning effects by Wnt/BMP signaling

Wnt/β-catenin signaling has multiple functions in the developing nervous system, regulating – apart from progenitor cell proliferation – cell fate specification and differentiation in a context-dependent manner (Ille and Sommer, 2005). In the developing telencephalon, ablation and activation of *β-catenin*

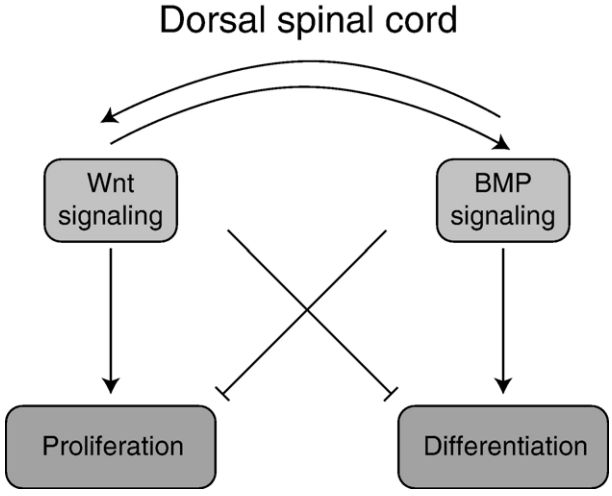

Fig. 9. Model for inhibitory Wnt/BMP crossregulation in the dorsal spinal cord. Crossinhibition of proliferation-inducing Wnt signaling and differentiation-inducing BMP signaling results in maintenance of slow-cycling, undifferentiated neuroepithelial cells during early developmental stages of the dorsal spinal cord. Mutual regulation (curved arrows) of the expression of Wnt and BMP signaling components by BMP and Wnt, respectively, further contributes to the finely tuned balance between proliferation and differentiation. Combinatorial Wnt/BMP signaling thus helps to coordinate the process of neuronal differentiation with growth of the dorsal spinal cord.

altered expression of genes involved in dorsoventral specification (Backman et al., 2005). Similarly, simultaneous loss of *Wnt1* and *Wnt3a* in the spinal cord (Muroyama et al., 2002) resulted in reduced expression of both *Math1* and *Ngn1*, which specify dI1 and dI2 populations, respectively (Helms and Johnson, 2003), indicating a role of Wnt signaling in dorsal spinal cord patterning. Complementary to these data, mutants expressing a stabilized form of β-catenin in the dorsal spinal cord (Fig. 1) displayed increased *Math1* expression and an expanded dI1 domain (Fig. 3). However, as in mice lacking *Wnt1/Wnt3a* (Muroyama et al., 2002), *Ngn1* expression was abolished upon β-catenin gain-of-function in the dorsal spinal cord. Thus, modulation of proneural gene expression by canonical Wnt signaling is complex and might involve the interplay with further cues. In particular, β-catenin signal activation led to increased levels of the BMP signal effectors phospho-Smad1/5/8 and to increased expression of *Msx1* (Fig. 4), which is also activated by BMP and synergistic Wnt/BMP signaling (Timmer et al., 2002; Willert et al., 2002). This points to a possible involvement of BMP signaling in mediating Wnt/β-catenin-modulated proneural gene expression. Indeed, several studies have implicated signaling by roof plate-derived BMP factors in dorsal spinal cord patterning (Liu and Niswander, 2005). In spinal cord explant cultures and in vivo, BMP signal activation promoted the formation of dorsal interneurons, while BMP signal inactivation resulted in loss of dI1 interneurons (Chesnutt et al., 2004; Liem et al., 1997; Timmer et al., 2002; Wine-Lee et al., 2004). Intriguingly, dependent on the method of signal manipulation (or possibly the species), reducing BMP signal activity produced either elevated or decreased numbers of dI2 interneurons (Chesnutt et al., 2004; Wine-Lee et al., 2004). This discrepancy can likely be explained by the finding that the generation of Ngn1-dependent dI2 interneurons requires a specific dose of BMP (Timmer et al., 2002). Accordingly, loss of *Ngn1* expression upon activation of β-catenin signaling in the dorsal spinal cord (Fig. 3) is conceivably due to alteration of BMP signal activity to levels inappropriate for *Ngn1* expression. Alternatively, downregulation of *Ngn1* might be secondary to the elevated levels of *Math1* or upregulation of *Math1* might be secondary to reduced *Ngn1* expression, given that these proneural genes are engaged in crossinhibitory activities (Gowan et al., 2001).

Our findings are consistent with the notion that neural tube patterning effects of Wnt/β-catenin signaling might be indirect and that BMP signaling constitutes the main patterning activity in the dorsal neural spinal cord (Caspary and Anderson, 2003; Chesnutt et al., 2004). This, however, does not exclude roles of Wnt/β-catenin signaling in regulating proneural gene expression independently of BMP signaling. *Ngn2* was upregulated upon β-catenin signal activation in the dorsal spinal cord, while *Mash1* expression remained unchanged (Fig. 3). Consistent with the described role of *Ngn2* downstream of *Mash1* to control dI3 vs. dI4 interneuron numbers in the spinal cord (Helms et al., 2005), increased *Ngn2* expression was associated with a decrease in dI3 and a concomitant increase in dI4 neurons (Fig. 3). As in the spinal cord, *Ngn2* expression is also activated by canonical Wnt signaling in the developing telencephalon (Backman et al., 2005) and in the neural crest (Lee et al., 2004). At least in the neural crest, this process appears to be independent of BMP signaling (Kleber et al., 2005). Moreover, in a reporter assay, *Ngn2* has been suggested to be a direct target of canonical Wnt signaling (Israsena et al., 2004). Whether in the dorsal spinal cord the regulation of *Ngn2* by Wnt is indeed BMP-independent has to be addressed, however, by Wnt/β-catenin signal activation in the absence of any BMP signaling. In any case, though, it is unlikely that altered proneural gene expression upon β-catenin signal activation is simply due to a mitogenic activity of canonical Wnt signaling in dorsal interneuron progenitors because we did not observe enhanced proliferation in the dorsal spinal cord of mutant mice (Fig. 2). In summary, we propose that, although BMP is a major patterning factor (Liu and Niswander, 2005), Wnt signaling participates in neural specification in the spinal cord, both by modulating BMP signaling and possibly by directly activating the expression of specific proneural genes.

Combinatorial Wnt and BMP signaling regulating growth and differentiation in the dorsal spinal cord

A main function of Wnt/β-catenin signaling in the CNS is the regulation of cell cycle progression of neural stem and progenitor cells (Kleber and Sommer, 2004). Accordingly, overexpression of Wnt ligands or β-catenin in different regions of the early CNS resulted in enlarged brain tissue (Chenn and Walsh, 2002; Chesnutt et al., 2004; Dickinson et al., 1994; Megason and McMahon, 2002; Zechner et al., 2003). However, when we expressed an activated form of β-catenin in the entire developing spinal cord, only ventral but not dorsal portions underwent expansion, although nuclear β-catenin was present throughout the neural tube (Figs. 1 and 2). Moreover, restricted expression of constitutively active β-catenin exclusively in the dorsal neural tube had no effect on spinal cord morphology and neuroepithelial cell proliferation. Similarly, overexpression of Wnt1 or Wnt3a in the chicken spinal cord predominantly affected ventral rather than dorsal proliferation (Megason and McMahon, 2002). These findings could be explained by two alternative models: either there exist intrinsic differences among neuroepithelial cells along the dorsoventral axis of the spinal cord with respect to their sensitivity to Wnt/β-catenin signaling, or the response to Wnt/β-catenin is differentially modulated in dorsal vs. more ventral neuroepithelial cells. The findings presented in this study rather support the latter model: Wnt promotes proliferation in high density neuroepithelial cell cultures, increases the size of clones derived from single neuroepithelial cells and enhances neurosphere formation and self-renewal (Figs. 5–7). The neuroepithelial cells used in these experiments were derived from whole developing spinal cords, comprising ventral and dorsal portions. Importantly, clonal assays (Fig. 6) demonstrated that the sensitivity to Wnt is not confined to a small subpopulation of neuroepithelial cells. Instead, the majority of neuroepithelial cells isolated from the developing spinal cord are receptive to mitogenic Wnt, demonstrating that the local environment rather than specific

intrinsic properties determines the cellular behavior of dorsal vs. ventral neuroepithelial cells in response to Wnt/β-catenin.

BMP factors expressed in the dorsal spinal cord are likely candidates to influence dorsal Wnt signaling, given that synergistic and antagonistic Wnt/BMP signaling have previously been shown in other systems to control cell fate decisions, patterning or tumorigenesis (Ellies et al., 2000; Jin et al., 2001; Kaphingst and Kunes, 1994; Soshnikova et al., 2003; Takaku et al., 1998). Indeed, all effects of Wnts on spinal cord-derived neuroepithelial cells assessed in this study were counteracted by the addition of BMP (Figs. 5–7), indicating that BMP signaling can antagonize Wnt-induced proliferation and stem cell self-renewal. Intriguingly, even low concentrations of BMP – which on their own act mitogenically – reduced Wnt-dependent proliferation (Fig. 5). This further emphasizes the modulation of Wnt signaling by BMP and strengthens the notion that, similar to their role on neural crest stem cells (Kleber et al., 2005), combinatorial Wnt and BMP do not just have additive effects on neuroepithelial cells, but rather elicit responses not seen with the individual signals alone. Furthermore, clonal analysis demonstrated that the antiproliferative activity of high BMP concentrations on neuroepithelial cells was not due to selective processes. Rather, virtually all clone founder cells treated with BMP, and the majority of cells treated with Wnt and BMP, underwent maximally one cell cycle during the duration of the experiment, revealing a general responsiveness of spinal cord-derived neuroepithelial cells to Wnt/BMP.

Intriguingly, we not only found Wnt activity to be suppressed by BMP, but also BMP activity itself to be antagonized by Wnt. While BMP alone promoted robust neuronal differentiation in neuroepithelial cells of the developing spinal cord, simultaneous exposure to Wnt efficiently prevented differentiation (Figs. 5 and 6). As for the mitogenic effect of Wnt and the antimitogenic effect of BMP, clonal assays demonstrated the instructive rather than selective effect of all three, Wnt, BMP, and Wnt plus BMP on neuronal differentiation of neuroepithelial cells. While small BMP-treated clones consisted almost exclusively of neurons, both large Wnt-treated clones and smaller Wnt/BMP-treated clones were composed primarily of undifferentiated cells. Thus, the net outcome of combinatorial Wnt/BMP treatment is maintenance of an undifferentiated, slow-cycling neuroepithelial cell (Fig. 9). In principle, it would be valuable to further test this model in vivo by using spinal cord-specific *β-catenin* loss-of-function mutants. Such an analysis was, however, precluded by the cell adhesion defects observed upon ablation of *β-catenin* in the neural tube, which led to partial disintegration of the mutant spinal cord (Ille F., Kemler R., and Sommer L., unpublished) (Junghans et al., 2005). Nonetheless, in vivo the rate of differentiation (Megason and McMahon, 2002) as well as CyclinD1 expression and mitosis (Fig. 8) is low in the dorsal-most spinal cord, in accordance with dorsally expressed Wnt/BMP suppressing both differentiation and proliferation during normal development of the spinal cord. Likewise, Wnt together with BMP suppresses differentiation and fast cell cycle progression of early neural crest stem cells, thereby maintaining stem cell properties in these cells (Kleber et al., 2005).

It remains to be shown how the crosstalk between Wnt and BMP signaling is regulated in the dorsal spinal cord, but it might involve direct physical interaction of β-catenin with SMAD proteins that mediate BMP signaling (Fischer et al., 2002; Hu et al., 2003; Hussein et al., 2003; Labbe et al., 2000; Nishita et al., 2000; Riese et al., 1997; Szeto and Kimelman, 2004; Theil et al., 2002; Willert et al., 2002). Moreover, it is unclear to what extent changes in Wnt/BMP signaling strength that presumably occur along the dorsoventral axis involve changing levels of activated signaling components, such as of phosphorylated Smads and nuclear β-catenin, versus qualitative changes in their interactions. In addition, canonical Wnt might switch usage of BMP receptors that mediate distinct effects of BMP factor signaling (Panchision et al., 2001). Signaling cues such as PTEN and Akt kinase might also play a role in antagonistic Wnt/BMP signaling (He et al., 2004). In the spinal cord, the interaction between Wnt/β-catenin and BMP signaling is not only evident by the result of their combined activity, but is also reinforced by the mutual upregulation of signaling components, with Wnt activating expression of BMP signaling molecules and BMP regulating Wnt expression (Marcelle et al., 1997; Panchision et al., 2001; Timmer et al., 2002; Wine-Lee et al., 2004; this study). Accordingly, we hypothesize that the reason that BMP signal inactivation does not lead to a Wnt-dependent increase in proliferation and expansion of the dorsal spinal cord is because of the concomitant downregulation of Wnt signaling components (Chesnutt et al., 2004; Wine-Lee et al., 2004). Furthermore, given this signal crossregulation, we cannot formally exclude that the proliferative effect attributed to canonical Wnt might involve the participation of BMP signaling components, although in culture even low doses of exogenous BMP antagonized mitogenic Wnt signaling. Interestingly, upregulation of the BMP downstream components pSmad1/5/8 and Msx1 is restricted to dorsal portions of the spinal cord, even when β-catenin is activated throughout the dorsoventral axis of the spinal cord (Fig. 4; data not shown). Therefore, additional factors must exist that confine combinatorial Wnt/BMP signaling to the appropriate location within the developing CNS. Ventral Shh might be involved in this process as it can inhibit *Msx* gene expression (Watanabe et al., 1998). Moreover, specific Wnt pathway inhibitors have recently been implicated in regulating development of the ventral neural tube (Lei et al., 2006). Thus, we propose that a network of signals shapes and modulates the inverse gradients of mitogenesis and differentiation present in the developing spinal cord (Megason and McMahon, 2002). According to this model, dorsal Wnt/BMP signaling ensures that proliferation and differentiation rates are leveling off in the dorsal spinal cord and hence that patterning processes are coordinated with growth during spinal cord development.

Acknowledgments

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