Canonical Wnt activity regulates trunk neural crest delamination linking BMP/noggin signaling with G1/S transition

Tal Burstyn-Cohen, Jonathan Stanleigh, Dalit Sela-Donenfeld* and Chaya Kalcheim†

Department of Anatomy and Cell Biology, Hebrew University-Hadassah Medical School, PO Box 12272, Jerusalem 91120, Israel
*Present address: Division of Developmental Neurobiology, The NIMR, The Ridgeway, Mill Hill, NW7 1AA, London, UK
†Author for correspondence (e-mail: kalcheim@nn-shum.cc.huji.ac.il)

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Summary

Delamination of premigratory neural crest cells depends on a balance between BMP/noggin and on successful G1/S transition. Here, we report that BMP regulates G1/S transition and consequent crest delamination through canonical Wnt signaling. Noggin overexpression inhibits G1/S transition and blocking G1/S abrogates BMP-induced delamination; moreover, transcription of Wnt1 is stimulated by BMP and by the developing somites, which concomitantly inhibit noggin production. Interfering with β-catenin and LEF/TCF inhibits G1/S transition, neural crest delamination and transcription of various BMP-dependent genes, which include Cad6B, Pax3 and Msx1, but not that of Slug, Sox9 or FoxD3. Hence, we propose that developing somites inhibit noggin transcription in the dorsal tube, resulting in activation of BMP and consequent Wnt1 production. Canonical Wnt signaling in turn stimulates G1/S transition and generation of neural crest cell motility independently of its proposed role in earlier neural crest specification.

Key words: Avian embryo, Cell cycle, Cyclin D1, Epithelial to mesenchymal transition, Neural tube, Somite

Introduction

The neural crest (NC) is a unique population of embryonic progenitors that generates a rich variety of derivatives (Le Douarin and Kalcheim, 1999; Kalcheim, 2000; Knecht and Bronner-Fraser, 2002). Born as epithelial cells in the dorsal neural folds, NC cells must convert into mesenchyme and engage in migration throughout the embryo in order to reach their homing sites and differentiate. Epithelio-mesenchymal transition (EMT) is, therefore, an essential stage in NC ontogeny. It is also a widespread mechanism during development of various systems other than the NC and is a crucial step in carcinoma metastasis (Savagner, 2001).

EMT of NC cells requires the coordinated action of transcription factors, extracellular matrix, cytoskeletal and cell-adhesion proteins (Nieto, 2001; Halloran and Berndt, 2003). The identity and mechanism of action of upstream factors that initiate the process remained, however, elusive. In previous studies, we have shown that a high rostral to low caudal gradient of BMP4 activity is established along the dorsal neural tube by a reciprocal gradient of expression of its inhibitor noggin. BMP then triggers emigration of NC progenitors in a manner that is independent of initial specification events. *Noggin* downregulation is in turn carried out by the developing somites, which consequently determine the timing of NC delamination (Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld and Kalcheim, 2000; Sela-Donenfeld and Kalcheim, 2002). More recently, the cell cycle was found to play a pivotal role in EMT of NC cells. We showed that trunk-level avian NC cells synchronously emigrate in the S-phase of the cell cycle. Next, we reported that the transition from G1 to S is a necessary event for NC delamination because specific inhibition of the transition from G1 to S blocked NC emigration, whereas arrest at S or G2 phases had no immediate effect (Burstyn-Cohen and Kalcheim, 2002).

These findings raise the question of whether BMP signaling and G1/S transition independently regulate EMT of NC cells or, alternatively, whether these processes interact with each other. In the present study, we report that noggin overexpression prevents the entry of neuroepithelial cells into the S phase of the cycle at axial levels corresponding to NC emigration (epithelial and dissociated somites) but not at levels opposite the segmental plate where NC cells have not yet initiated delamination. Moreover, mimosine, which specifically blocks G1/S transition, inhibits BMP-induced NC cell delamination. Hence, BMP regulates NC delamination in a cell cycle-dependent manner. We then examined the hypothesis that Wnt signaling, which controls G1/S transition by regulating *cyclin D1* transcription (Tetsu and McCormick, 1999) mediates BMP activity on both G1/S transition and NC delamination. We find that expression of *Wnt1* along the dorsal tube, but not that of *Wnt3a*, is reciprocal to the pattern of *noggin* transcription and depends upon BMP activity. In addition, grafting dissociating somites in place of segmental plate mesoderm, a procedure that results in premature downregulation of *noggin* mRNA, upregulates *Wnt1* transcription and leads to precocious NC emigration from the caudal tube. Furthermore, abrogating canonical Wnt signaling downregulates transcription of various BMP-dependent genes in the dorsal tube. Thus, in the context of NC delamination, BMP acts upstream of Wnt1. Consistent with this notion, inhibition of the canonical branch of Wnt activity, but not of the non-canonical pathway, prevents both G1/S transition and

NC delamination and overexpression of β-catenin rescues NC delamination in noggin-inhibited neural primordia. Thus, BMP-dependent Wnt signaling is necessary for NC delamination.

Materials and methods

Embryos

Chick (*Gallus gallus*) and quail (*Coturnix coturnix* Japonica) eggs were from commercial sources.

In ovo grafting of noggin-secreting cells

CHO cells producing *Xenopus* noggin and dhfr-CHO control cells were grown as previously described (Lamb et al., 1993; Sela-Donenfeld and Kalcheim, 2002). To establish confluent monolayers, cells were replated on eight-well chamber slides (Lab-Tek), grown in serum-containing medium for 2 days and then transferred to serum-free medium until explantation of neural primordia. For grafting purposes, confluent cultures were harvested and pelleted. The vitelline membrane of 16- to 20-somite stage chick embryos was removed. A slit was performed along the dorsal edge of the neural tube at levels corresponding either to the rostral segmental plate and two last formed epithelial somites, or along the caudal half of the segmental plate. Concentrated cell suspensions were applied on top of the neural tube with a micropipette. Cell implants were performed under an Olympus dissecting microscope with a ×40 total magnification. Embryos were further incubated for 8-10 or 20-24 hours and then fixed for immunocytochemistry and/or in situ hybridization.

Grafting of BMP-4-coated beads

Heparin-acrylic beads (Sigma) were immersed in a solution of BMP4 [R&D, 50 ng/ml in phosphate buffered saline (PBS)/1% fetal calf serum (FCS)] for 1.5 hours followed by repeated washings in PBS. To graft the beads, a slit was made along the dorsal aspect of the neural tube at the segmental plate level of the axis. A single BMP-coated bead per embryo was then inserted being held between the neural folds. Embryos were further incubated for 6-8 hours.

Explants of neural primordia

Neural tubes containing premigratory NC were excised from levels corresponding to the segmental plate and three most recently formed somites of 16-20 somite stage-quail embryos, and then explanted onto eight-well chamber slides (Lab-Tek) pre-coated with fibronectin (Sigma, 50 μg/ml), as described (Burstyn-Cohen and Kalcheim, 2002). Culture medium consisted of CHO-S-SFM II medium (Gibco-BRL) to which the following factors were added: L-mimosine (Calbiochem) and BMP4 (R&D). Mimosine was dissolved to a concentration of 0.05 M in 0.12 M HCl; BMP4 was reconstituted in PBS/1% FCS to a stock solution of 100 ng/μl. Further dilutions to 600 μM and 100 ng/ml, respectively, were performed in culture medium, and controls consisted of the appropriate diluents. Explants were cultured for a total of 10 hours and a pulse of BrdU (10 μg/ml, Sigma) was administered 1 hour before fixation.

In another experimental series, hemi-neural tubes were co-electroporated with Wnt1/GFP or β-catenin/GFP encoding DNAs (see below). Two hours later, the neural primordia were enzymatically isolated and plated either on fibronectin-coated wells or onto monolayers of control CHO or CHO-noggin cells previously grown for 17 hours in serum-free medium. Cultures were incubated for 14 hours and co-cultures for 16-20 hours and pulsed with BrdU for 1 hour before fixation.

In ovo manipulations

Grafting of dissociating somites

Host embryos aged 16-18 somites were windowed and their vitelline membrane was removed. A fragment of caudal segmental plate corresponding to four or five prospective somites was removed unilaterally as previously described (Kalcheim and Teillet, 1989). Donor embryos aged 25-27 somites were pinned ventral side down on Sylgard-coated dishes. The fourth to ninth to last formed somites, corresponding to the onset of dissociation into dermomyotome and sclerotome, were excised in one piece along with a narrow strip of intermediate and lateral plate mesoderm. Donor somites were then grafted in the gap left after removal of the unsegmented mesoderm of the younger hosts. Special care was taken in keeping the correct dorsoventral and mediolateral orientations. In control experiments, sclerotomal fragments devoid of dermomyotome were excised from more rostral levels and similarly grafted. Embryos were incubated for additional 10 hours, fixed in 4% formaldehyde and processed for in situ hybridization.

In ovo electroporation

DNA (3-4 μg/μl) was microinjected into the lumen of the neural tube of 18- to 23-somite stage chick embryos with micropipettes, and electrodes were placed on either side of the embryos at the level of the segmental plate and recently formed somites. A square wave electroporator (BTX, San Diego, CA) was used to deliver four pulses of current at 25 volts, 10 mseconds each. Embryos were reincubated for 9 hours or 14-16 hours followed by a 1 hour pulse of BrdU (10 mM) or by processing for in situ hybridization. Another series was reincubated for 2 hours followed by explantation of isolated tubes on fibronectin, CHO or CHO-noggin monolayers. DNA expression vectors employed were: pCAGGS-AFP (Momose et al., 1999); pCAdelLEF-1 (Kubo et al., 2003); β-catenin fused to the engrailed repressor domain (β-Eng/pcDNA3.1+MT) (Montross et al., 2000); pEFBOSS/xNoggin (Endo et al., 2002), full-length mouse β-catenin/pCAGGS and Wnt1/pCAGGS (Nishihara et al., 2003). Truncated forms of Xdishevelled (from S. Sokol) were pCS2-Xdd1, pCS2-DEP+ and pCS2-D2-GFP (Sokol, 1996; Rothbacher et al., 2000; Tada and Smith, 2000). pCS2-Xdd1 and pCS2-DEP+ were fused to YFP (from eYFP-N1, Clontech) and subcloned into the pCAGGS electroporation vector.

Tissue processing and immunocytochemistry

Explants and embryos were fixed in 4% formaldehyde/PBS. Embryo fragments were embedded in paraffin wax and sectioned at 5 or 10 μm. Immunostaining for BrdU was as described (Burstyn-Cohen and Kalcheim, 2002). Rabbit anti-GFP antibodies (Molecular Probes) were used at 1:200 in combination with monoclonal anti-BrdU antibodies. Nuclei were visualized with Hoechst (Sigma).

In situ hybridization

In-situ hybridization was performed as previously described (Burstyn-Cohen and Kalcheim, 2002) with the following chick-specific probes: Wnt1 and Wnt3a (from A. P. McMahon), Cyclin D1 (from F. Pituello), RhoB (Liu and Jessell, 1998), Cad 6B (Nakagawa and Takeichi, 1995), Msx1 and Pax3 (Monsoro-Burq et al., 1996), FoxD3 (Dottori et al., 2001; Kos et al., 2001), Slug (Nieto et al., 1994), and Sox9 (Cheung and Briscoe, 2003).

Measurements of cell proliferation and NC delamination

Neural tube explants

The number of emigrating NC cells was counted using phase-contrast optics along a 900 μm length of tube explant. Cells (0-300/900 μm length of tube) were scored as +1; 300-600 cells/900μm length were scored as +2; more than 600 cells/900 μm length were scored as +3. The average scores were normalized to control values. In electroporated hemi-tubes, the total number of GFP+ NC cells was similarly counted along a length of 500 μm of tube explants. Five to eight explants were assayed per treatment. Results represent the average fold increase in NC delamination or the mean number of emigrated GFP+ NC cells (±s.d. of three or four similar experiments). Significance was examined using one way analysis of variance

(ANOVA). When significant differences were indicated in the F ratio test ($P<0.005$), the significance of differences between means of any two of these groups was determined using the modified Tukey method for multiple comparisons with an α of 0.05. Incorporation of BrdU into neural tube cells was taken as a general measure of drug efficacy.

Embryo sections

In electroporated embryos that received Xdd1-GFP or DEP+-GFP, the number of GFP+ and BrdU+ cells was monitored in 10-30 alternate sections. Between 100 and 130 GFP+ cells/hemi-tube were scored for BrdU incorporation. The proportion of BrdU-incorporating nuclei in all treatments described was found to be similarly affected in the dorsal region of the tube (when considering only eight or nine nuclei from the dorsal midline) as well as at more ventral areas. Therefore, to increase the sample size per embryo, counts included the entire dorsoventral extent of the epithelium. Results are expressed as percentage±s.d. of BrdU+ nuclei out of the GFP+ population. When electroporating the other constructs, assessment of cell proliferation was monitored as the number of total nuclei (Hoechst+) that incorporated BrdU. Between 250 and 300 nuclei were counted per embryo. Results represent the percentage±s.d. of BrdU+ cells from 4-14 embryos/treatment. NC delamination was similarly monitored in alternating sections. Results are expressed either as percentage of GFP+/total emigrating nuclei in embryos that received GFP alone versus Xdd1-GFP or DEP+-GFP or as number of Hoechst+ nuclei located up to the migration staging area (Weston, 1991) in intact versus transfected sides of co-electroporated embryos.

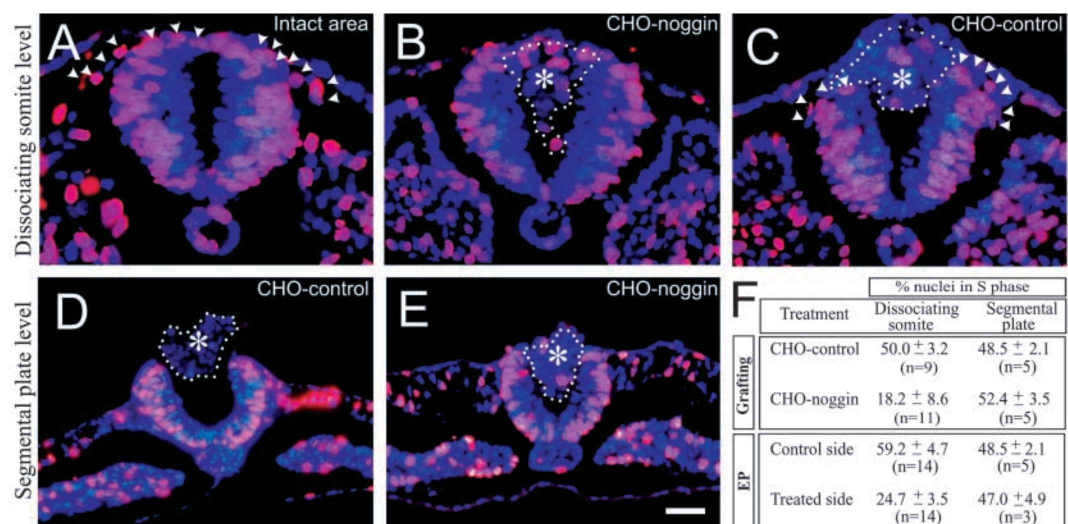
Results

BMP signaling controls G1/S transition in the neural tube

Noggin overexpression inhibits incorporation of BrdU in an axial level-dependent manner

To evaluate the possible relationship between BMP activity and G1/S transition, the BMP inhibitor noggin was overexpressed in the neural tube either by grafting noggin-expressing CHO cells or by unilateral electroporation of a noggin-encoding DNA. Embryos were reincubated for 10 hours, the approximate length of one cell cycle. When treatments concerned the level of rostral segmental plate, fixation corresponded to the time of somite dissociation. The percentage of total nuclei that incorporated BrdU was reduced by 64% in embryos grafted with noggin-expressing cells when compared with CHO controls (Fig. 1B,C,F; also compare A and B, which represent the same embryo immediately caudal to the graft and at the grafted level, respectively). A 58% reduction in BrdU incorporation was also measured in electroporated hemi-tubes compared with contralateral sides (Fig. 1F) or to embryos that received GFP alone (not shown) (see Burstyn-Cohen and Kalcheim, 2002). Hence, cell proliferation at epithelial to dissociated levels of the axis depends on BMP signaling. Consistent with previous findings (Sela-Donenfeld and Kalcheim, 1999), noggin overexpression also prevented the onset of NC delamination (Fig. 1B,C). Notably, although BMP is specifically expressed in the dorsal neural tube, its inhibition caused reduced proliferation throughout the dorsoventral extent of the neuroepithelium, suggesting a long-range activity of the factor.

In the caudal neural tube opposite the segmental plate mesoderm, the activity of BMP is relatively low because of high levels of noggin (Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld and Kalcheim, 2002). Nevertheless, the proportion of BrdU-positive nuclei reaches 48.5% (Fig. 1F), suggesting that cell proliferation in this area might not depend on BMP. To test this possibility, residual BMP activity was inhibited by overexpressing noggin in the tube at the level of the caudal segmental plate, which developed to be the level of the rostral segmental plate by the time of fixation. No effect on BrdU incorporation was measured when compared with controls (Fig. 1D-F). Hence, cell proliferation at caudal levels of the axis is BMP-independent. BMP signaling therefore controls G1/S transition in an axial level-dependent manner (see Discussion).



	Treatment	% nuclei in S phase: Dissociating somite	Segmental plate
Grafting	CHO-control	50.0 ± 3.2 (n=9)	48.5 ± 2.1 (n=5)
	CHO-noggin	18.2 ± 8.6 (n=11)	52.4 ± 3.5 (n=5)
EP	Control side	59.2 ± 4.7 (n=14)	48.5 ± 2.1 (n=5)
	Treated side	24.7 ± 3.5 (n=14)	47.0 ± 4.9 (n=3)

Fig. 1. BMP signaling controls G1/S transition in the neural tube in an axial-specific manner. Neural tube sections opposite dissociating somites (A-C) or segmental plate mesoderm (D,E). Hoechst+ nuclei are blue and BrdU+ nuclei are pink. CHO pellets are delineated by white dots and asterisks (*). In A, there is an intact area showing the neural tube with emigrating NC cells (arrowheads); B represents a nearby section from the same embryo at a level where CHO-noggin cells were implanted (*). No NC cells emigrate and BrdU incorporation is partially inhibited. (C) Implanted CHO-control cells (*) have no effect on NC delamination (arrowheads) and BrdU incorporation. (D,E) Noggin overexpression (E) has no effect on neuroepithelial cell proliferation at segmental plate levels of the axis when compared with control CHO (D). (F) Percentage of BrdU+/total nuclei (±s.d) at dissociating and segmental plate levels of the axis in embryos grafted with CHO-noggin cells or electroporated (EP) with a noggin-expressing vector. Numbers in parenthesis represent the embryos quantified. Scale bar: 25 μm in A-C; 40 μm in D,E.

Inhibition of G1/S transition with mimosine prevents BMP-induced BrdU incorporation and NC delamination

To further examine the relationship between BMP activity and the cell cycle, G1/S transition was specifically blocked with mimosine. This inhibitor was previously shown to upregulate levels of the cdk inhibitor p27 (Wang et al., 2000), causing its translocation into the nuclei of cultured NC cells and inhibiting NC delamination (Burstyn-Cohen and Kalcheim, 2002). Neural primordia containing premigratory NC were explanted onto fibronectin. Ten hours later, the first NC cells were present on the substrate of control cultures and treatment with BMP enhanced the number of emigrating mesenchymal cells by 72% (Fig. 2A,C,E). Consistent with previous results, mimosine alone inhibited the onset of NC delamination by 62% compared with controls and blocked DNA synthesis (Fig. 2B,E). The combination of mimosine and BMP resembled the effect of mimosine alone, with significant reduction of NC delamination to 50% of control values and to 29% of BMP-treated values (Fig. 2D,E). Furthermore, BMP co-treatment did not overcome the inhibition of G1/S transition caused by mimosine (Fig. 2D). These results suggest that BMP signaling is upstream of events controlling G1/S transition. As both BMP activity and G1/S transition are required for NC delamination (Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld and Kalcheim, 2000; Burstyn-Cohen and Kalcheim, 2002), our results suggest that these processes are epistatically related.

Wnt1 is a downstream target of BMP signaling in the dorsal neural tube

Next, we asked what factor/s mediate the BMP-dependent effect on cell entry into the S-phase and on NC delamination. Wnt proteins are likely candidates, as Wnt1 and Wnt 3a are expressed in the dorsal neural tube (Marcelle et al., 1997; Megason and McMahon, 2002; Sela-Donenfeld and Kalcheim, 2002; Parr et al., 1993), are required for expansion of NC progenitors (Ikeya et al., 1997) and have been shown to stimulate G1/S transition via transcriptional regulation of *cyclin D1* expression (Tetsu and McCormick, 1999; Willert et al., 2002).

Transcription of Wnt1, but not of Wnt 3a, is inhibited by short-term noggin treatment

Wnt1 mRNA is apparent along the rostral dorsal neural tube of avian embryos roughly to the level of the last formed somite pair/rostralmost portion of the segmental plate (Fig. 3A, Fig. 4B). This pattern is positively correlated with both the rostrocaudal gradient of NC delamination and with previously reported levels of BMP activity along the tube, and is reciprocal to that of *noggin* mRNA, which is high in the dorsal tube opposite segmental plate levels and decreases rostralwards (Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld and Kalcheim, 2000; Sela-Donenfeld and Kalcheim, 2002). Localized grafts of CHO-noggin cells completely abolished transcription of *Wnt1* as early as 6-10 hours following implantation (Fig. 3B,C; *n*=20), while control cells had no effect (*n*=9, not shown). Likewise, electroporation of a noggin-encoding vector, but not of GFP-encoding DNA, downregulated *Wnt1* along transfected hemi-tubes (*n*=9 for each construct, Fig. 3E and data not shown). Conversely, grafts of a BMP4-coated bead on the dorsal tube at the level of the segmental plate resulted in premature upregulation of *Wnt1*

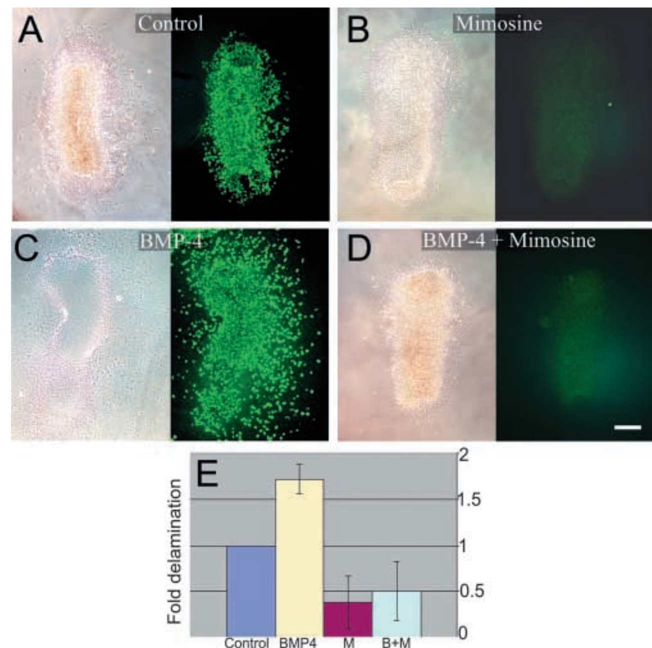


Fig. 2. BMP-dependent NC delamination from explanted neural primordia is inhibited by the G1/S transition blocker mimosine. (A) Control, and (B) 600 μm mimosine, (C) 100 ng/ml BMP4 and (D) mimosine+BMP4 treatment. Left panels are phase-contrast images and right panels depict BrdU immunoreactivity. (E) Data quantification. NC delamination is significantly stimulated by BMP4 ($P<0.005$) and inhibited by mimosine ($P<0.005$) relative to controls. Mimosine prevented BMP4-induced NC delamination ($P<0.002$ relative to BMP4 alone) and BrdU incorporation. Scale bar: 75 μm.

mRNA (arrow in Fig. 3F, *n*=5). Hence, BMP regulates transcription of *Wnt1* in the dorsal tube.

In contrast to the stable graded expression of *Wnt1*, normal expression of *Wnt3a* is temporally dynamic. Graded transcription of Wnt3a along the tube is apparent until the 20th somite stage (Fig. 3G) (see also Marcelle et al., 1997). However, from this stage onwards, *Wnt3a* mRNA is uniformly distributed along the dorsal tube reaching the Hensen's node (Fig. 3H) and noggin overexpression either as CHO-expressing cells or by electroporation, had no effect on transcription of *Wnt3a* at any axial level examined when monitored 10 hours after grafting (Fig. 3I, *n*=10, and data not shown). Downregulation of *Wnt3a* was, however, apparent following 20 hours of exposure to noggin (data not shown, *n*=4), consistent with previous observations (Marcelle et al., 1997). The differential transcriptional behavior of *Wnt1* versus *Wnt3a* under normal and experimental conditions suggests that only the former gene is likely to be a direct target of BMP signaling.

Wnt1 transcription in the dorsal tube is modulated by the developing somites

We have previously shown that the dorsomedial region of developing somites inhibits *noggin* transcription in the dorsal neural tube. Loss of noggin activity releases BMP4 from inhibition, resulting in NC emigration (Sela-Donenfeld and Kalcheim, 2000). We reasoned that if Wnt1 is part of the BMP/noggin-dependent signaling cascade, then experimentally induced downregulation of *noggin* should result in a

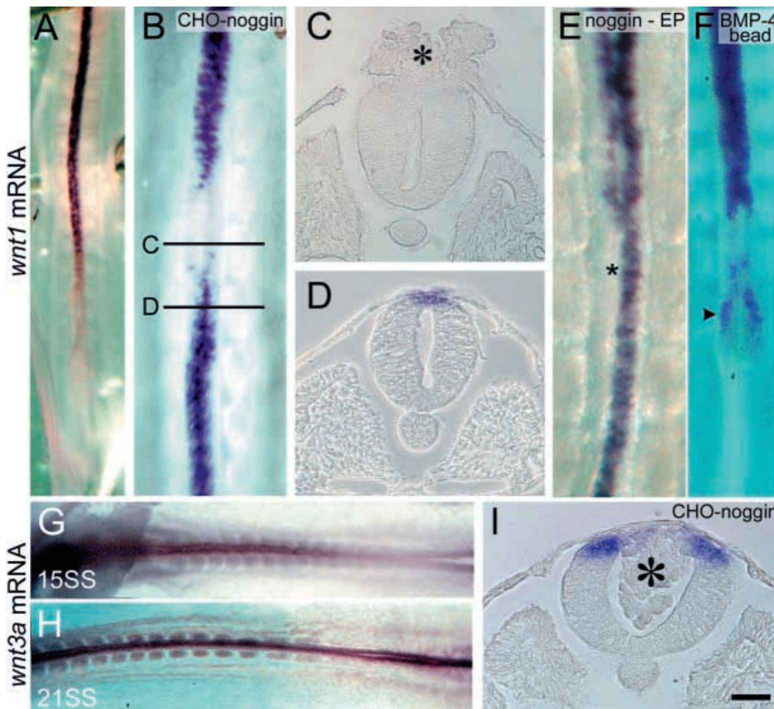


Fig. 3. Wnt1, but not Wnt3a, is an immediate downstream target of BMP signaling in the dorsal neural tube. (A-F) Expression of *Wnt1* mRNA. (A) An embryo aged 20 somites with decreasing to no signal opposite the segmental plate mesoderm. Rostral is towards the top. (B) Graft of CHO-noggin cells inhibits *Wnt1* transcription 10 hours later; (C,D) cross-sections of marked regions in B at grafted and intact levels, respectively. Asterisk (*) marks grafted cells. (E) Unilateral noggin electroporation (EP, *electroporated hemi tube) inhibits *Wnt1* transcription 10 hours later. (F) A BMP4-coated bead implanted on the neural tube at the segmental plate level induces ectopic and premature *Wnt1* expression (arrowhead). (G-I) Expression of *Wnt3a* mRNA. (G,H) Whole-mount in situ hybridization of 15 and 21 somite stage (SS) embryos, respectively. Rostral is towards the left. There is a rostrocaudal gradient of expression along the neural tube in the younger embryo with no signal adjacent to the segmental plate mesoderm, but uniform rostrocaudal signal at 21 somites and onwards (not shown). (I) Noggin overexpression (*) has no effect on *Wnt3a* mRNA following 10 hours incubation. Scale bar: 40 μm in C,D,I.

corresponding and premature upregulation of *Wnt1*. Dissociating somites (opposite which the tube is *noggin–/Wnt1+*) were unilaterally grafted in the place of the unsegmented mesoderm (opposite which the tube is *noggin+/Wnt1–*). This procedure precociously upregulated expression of *Wnt1* in the caudal hemi-tube adjacent to the graft (Fig. 4A, *n*=8). At a slightly more anterior level, opposite the rostral segmental plate where early *Wnt1* transcription is already apparent in the intact side, grafting of dissociating somites resulted in expansion of the domain of *Wnt1* expression. This was associated with premature emigration of NC cells, which retained *Wnt1* expression following delamination (Fig. 4B). By contrast, grafting of mesenchymal sclerotomal fragments devoid of dermomyotome, which has previously been shown to have no effect on *noggin* transcription (Sela-Donenfeld and Kalcheim, 2000), had no effect either on expression of *Wnt1* mRNA (Fig. 4C, *n*=5). Thus, similar to BMP/noggin, graded production of *Wnt1* along the dorsal tube is regulated by the dorsomedial region of developing somites. Moreover, *Wnt1* expression is directly correlated with BMP activity and reciprocally related to *noggin* production further supporting the notion that Wnt1 is a downstream effector of BMP signaling.

Wnt signaling mediates BMP-dependent G1/S transition and NC delamination

Inhibition of the canonical pathway of Wnt signaling prevents G1/S transition and NC delamination

To examine whether Wnt activity is necessary for the above processes, we overexpressed in the neural tube a truncated form of XDsh, a key component of the Wnt signaling pathway (Veeman et al., 2003; Wharton, 2003) that harbors a partial deletion in the PDZ domain (Xdd1-GFP). Previous studies have shown that Xdd1 acts in a dominant-negative form to abolish both canonical as well as non-canonical Wnt signaling (Sokol et al., 1995; Sokol, 1996; Tada and Smith, 2000; Rothbacher et al., 1995; Rothbacher et al., 2000). Electroporations were performed at segmental plate levels and embryos were further incubated for 16 hours corresponding to the onset of NC delamination at the treated levels of the tube. Hence, considering it takes 4-6 hours for the transgene to be expressed, the net effect was measured for the length of one

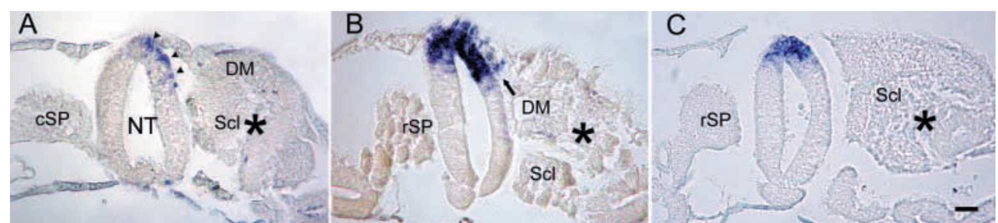


Fig. 4. *Wnt1* transcription in the neural tube is modulated by the developing somites. Dissociating somites were unilaterally grafted in the place of the unsegmented mesoderm. (A) There is premature expression of *Wnt1* mRNA (arrowheads) in the caudal hemi-tube adjacent to the graft (*) at the level of the caudal segmental plate (cSP). (B) Opposite the rostral segmental plate (rSP), *Wnt1* transcription is apparent in the intact hemi-tube of this embryo at the age of 23 somites. At this level, the graft (*) resulted in expansion of the *Wnt1* expression domain with associated emigration of NC cells that retained *Wnt1* expression (arrow). (C) Grafting of a fragment of sclerotomal tissue (Scl) had no effect on Wnt1 transcription or on NC delamination. Scale bar: 25 μm.

cell cycle approximately. This limited incubation time allows us to assay the direct effect of G1/S transition on NC behavior, as previously shown (Burstyn-Cohen and Kalcheim, 2002). Expression of a control GFP vector had no effect on DNA synthesis (Fig. 5A-C) as 50.2±7.4% of GFP+ cells in transfected hemi tubes incorporated BrdU into their nuclei, a similar value measured in control sides (data not shown) (Burstyn-Cohen and Kalcheim, 2002) (*n*=4 counted out of 10 similar embryos). By contrast, only 4.2±2.6% of GFP+ cells were BrdU+ in Xdd1-treated tubes (Fig. 5D-F, *n*=6 counted embryos of 12 embryos showing similar qualitative results). Thus, Xdd1 causes a 91.6% inhibition in DNA synthesis in neuroepithelial cells. This striking inhibition is only mildly reflected 16 hours following electroporation in the size of the neural tube; the amount of total nuclei in the treated sides was 91.2±3% of that in contralateral sides of Xdd1-treated embryos when compared with 100.2±4%, respectively, in embryos that received control GFP.

We next measured the effect of Xdd1 on NC delamination. In control GFP-expressing tubes, the percentage of emigrating Hoechst+ NC cell nuclei that co-expressed GFP attained 52.3±7.7%. By contrast, only 9.6±4.1% of emigrating NC cells co-expressed GFP in Xdd1-treated tubes. Thus, Xdd1 causes a 81.6% inhibition in NC cell delamination (Fig. 5). Notably, in all Xdd1+ embryos, GFP-negative NC cells normally exited the tube, implying that the effect of Xdd1 is cell autonomous (Fig. 5, compare F with D).

To determine whether the canonical pathway accounts for the effects of Xdd1, dominant-negative (dN)LEF1 [deleted in the β-catenin-binding domain (Kengaku et al., 1998; Kubo et al., 2003)] was electroporated. The LEF/T-cell transcription factor (TCF) family members act in conjunction with β-catenin to influence transcription of Wnt-responsive genes (Behrens et al., 1996; Molenaar et al., 1996; Cong et al., 2003). Overexpression of dNLEF1 resulted in 54% of total nuclei that incorporated BrdU when compared with the contralateral side (24.8±3.4% and 45.8±4.3%, respectively; *n*=5 embryos counted out of nine showing a similar phenotype). A corresponding 60% inhibition in NC delamination was measured (2.8±0.8 and 7.0±1.2 Hoechst+ NC cells/section, respectively). Likewise, inhibition of β-catenin activity by electroporation of β-catenin fused to the engrailed repressor domain reduced BrdU incorporation to 55% of control levels (27.0±3.5% versus 49.1±6.2% in treated versus control sides, respectively) and inhibited NC emigration by 56% (3.8±0.6 compared with 8.6±1.8 emigrating Hoechst+ NC cells/section) in four counted out of nine embryos with a similar effect. Furthermore, both dNLEF1 and β-catenin-engrailed downregulated transcription of *cyclin D1* in the transfected hemi-tubes (see Fig. 7C,D and data not shown), confirming their effect through the canonical pathway of Wnt signaling.

To examine whether the non-canonical branch also contributes to the effects of Xdd1, a truncated form of Dsh lacking the first 336 amino acids of the molecule (dsh-DEP+-GFP) was electroporated. Dsh-DEP+ was shown to block activin-induced axis elongation in *Xenopus* animal caps without affecting the canonical Wnt pathway (Tada and Smith, 2000). In contrast to Xdd1, dNLEF1 and β-catenin-engrailed, dsh-DEP+ had only a minor effect on DNA synthesis (39.6±3.6% compared with 50.2±7.4% of GFP+ nuclei incorporating BrdU) and on NC delamination (43.7±8.5% compared with 52.3±7.7% of delaminating NC cells that were

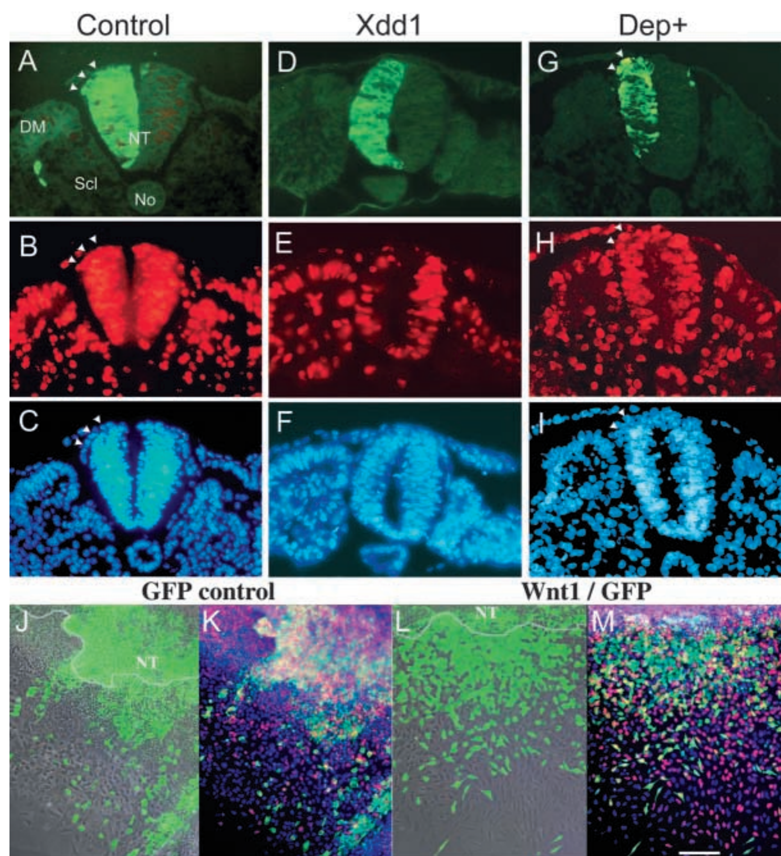


Fig. 5. The effects of loss and gain of function of Wnt signaling on BrdU incorporation and NC delamination. (A-I) Transverse sections of embryos that received control GFP (A-C), Xdd1 (D-F) or Dep+ (G-I). Upper panels represent GFP immunostaining, central panels are BrdU immunoreactivity, and lower panels depict Hoechst-stained nuclei. (A-C) Electroporation with a control GFP-encoding vector (green) into the hemi neural tube reveals no change in BrdU incorporation (red) when compared with contralateral side. Emigration of GFP+/BrdU+ NC cells is shown (arrowheads). (D-F) Transfection with Xdd1-GFP (green) caused a reduction in number of BrdU+ nuclei (red) and no GFP+ crest cells exiting the treated side of the tube. In D,F, GFP-negative NC cells emigrate from the transfected side. Emigration is normal from the contralateral hemi-tube. (G-I) Dep+ had no effect on either BrdU incorporation or NC emigration. Dep+/GFP+/BrdU+ delaminating cells (arrowheads). NC delamination is also normal from the control side, albeit not seen in this particular section. (J-M) Neural tube explants that received control GFP (J,K) or Wnt1-GFP at 3 μg/μl (L,M). (J,L) An overlay of GFP immunofluorescence (green) onto the phase-contrast images. The white lines depict the border of the tube explants. A larger number of Wnt1/GFP+ NC cells delaminate from the tube and migrate on the substrate when compared with GFP controls. (K,M) GFP in green, BrdU immunolabeling in red and Hoechst nuclear staining in blue. Yellow cells co-express GFP and BrdU. DM, dermomyotome; NT, neural tube; No, notochord; Scl, sclerotome. Scale bar: 35 μm in A-I; 200 μm in J-M.

GFP+) in DEP+ and controls, respectively (n=4 counted embryos of eight with similar phenotype, Fig. 5G-I). Notably, hemi-neural tubes expressing Dsh-DEP+ were somewhat narrower than the contralateral sides and their epithelial integrity was compromised (Fig. 5G-I), as also revealed by n-cadherin immunostaining (data not shown). Consistent with these results, Dsh-D2, which inhibited convergent extension movements (Wallingford et al., 2000; Wallingford and Harland, 2001) but did not affect formation of a secondary axis in *Xenopus* embryos (Rothbacher et al., 2000), had no detectable effect either on NC delamination or DNA synthesis (data not shown). Like Dsh-DEP+, Dsh-D2-treated embryos also displayed a somewhat disordered hemi-tube. Taken together, these results suggest that the non-canonical pathway is involved in maintaining epithelial integrity but not in EMT of NC cells.

We next examined whether lack of NC emigration in Xdd1 and dNLef1-treated embryos could be explained by enhanced cell death. In control sides of Xdd1, dNLef1 and DEP+-electroporated embryos, the proportion of pyknotic/total Hoechst+ nuclei was 5.9±0.9%, 5.9±1.2% and 4.7±1.7%, respectively (n=3 embryos/treatment). In treated sides, it slightly increased to 10.8±1.7% and 10.1±4% in Xdd1 and dNLef1 embryos, respectively, but remained unchanged upon treatment with DEP+ (4.9±2.0%). In the latter embryos, however, numerous cells were present in the lumen of the neural tube and were therefore not considered in the quantifications. Taken together, we conclude that enhanced cell death cannot account for the inhibition in NC delamination measured upon treatment with Xdd1 and dNLef1. Hence, the failure of NC cells to delaminate is likely to result from the lack of G1/S transition in the premigratory progenitors that is specifically mediated by canonical Wnt signaling.

The above loss-of-function experiments revealed that Wnt activity is necessary for NC delamination. Next, we examined whether specific overexpression of Wnt1 stimulates the process. To this end, hemi-neural tubes were co-electroporated with full-length mouse Wnt1 and GFP-encoding DNAs or with GFP alone. Two hours later, the transfected neural primordia were isolated and seeded onto fibronectin-coated wells and grown for 14 hours in serum-free medium. Assuming it takes ~4-6 hours for the transgenes to be significantly expressed, the net effect was measured over the length of one cell cycle. Three different concentrations of the Wnt1 DNA were tested (0.2, 0.8 and 3.0 μg/μl) and the number of labeled cells that emigrated from the hemi-tubes was counted as depicted in the Materials and methods. The average number of GFP+ NC cells that exited a given length of control hemi-tubes was 39.1±18 (about 20% of total NC cells that emigrated from both sides of the tube at this time). Electroporation with 0.2 μg/μl of Wnt1 DNA showed no significantly different emigration (43.7±19 GFP+ cells), but hemi-tubes that received 0.8 and 3.0 μg/μl of Wnt1 DNA revealed a 1.66 and 2.6 fold stimulation in delamination of labeled cells when compared with tubes that received GFP alone (64.7±8 and 101.8±23 GFP+ cells, $P<0.005$ and $P<0.001$, respectively, Fig. 5J,L). Consistent with previous findings (Burstyn-Cohen and Kalcheim, 2002), the delamination of transfected and untransfected NC cells in both control and experimental treatments, was associated with cells undergoing G1/S transition, as reflected by incorporation of BrdU into their nuclei (Fig. 5K,M; data not shown). Taken together, loss- and gain-of-function analysis substantiates a role for Wnt signaling in regulating NC delamination and further suggests that this effect may be exerted by Wnt1.

Inhibition of Wnt activity downregulates transcription of several BMP-dependent genes in the dorsal neural tube but has no effect on early genes associated with NC induction

Transcription of various dorsal neural tube-specific genes,

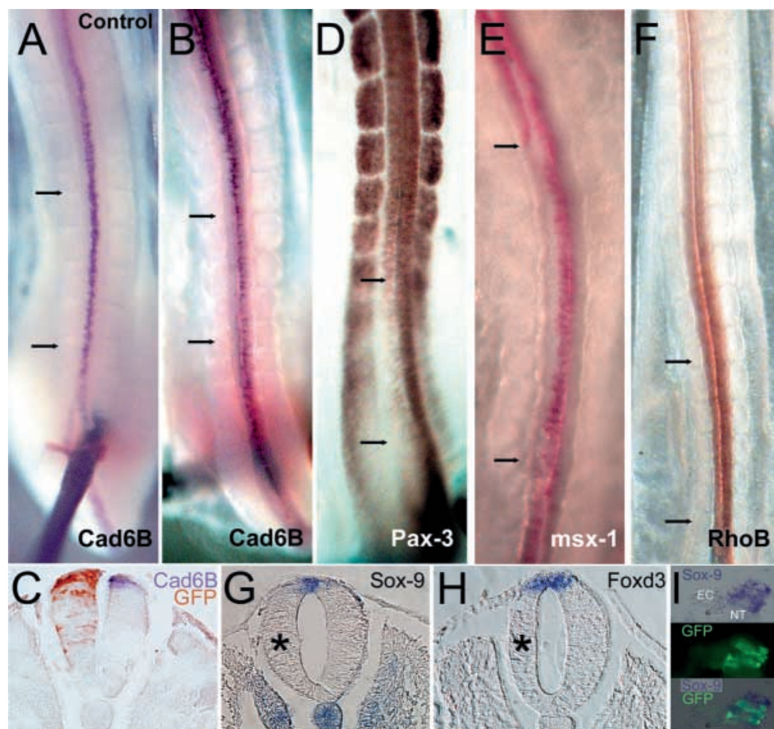


Fig. 6. The effects of inhibiting Wnt signaling on expression of dorsal tube-specific genes. (A,B,D-F) Whole-mount in situ hybridization following electroporation with (A) control GFP or (B,D-F) dNLEF1 to the left hemi-tubes and 10 hours reincubation. While the control vector had no effect on bilateral transcription of *Cad6B* (A), *Pax3*, *Msx1* or *RhoB* (not shown), there was unilateral downregulation of *Cad6B, Pax3* and *Msx1,* but not of *RhoB* in dNLEF1-treated hemi-tubes (transfected areas depicted between arrows). Results were confirmed by transverse section analysis.
(C) Transverse section of an embryo that received Xdd1-GFP and was analyzed for expression of *Cad6B* in combination with GFP immunostaining. Downregulation of *Cad6B* (blue) in dorsal hemi-tube expressing Xdd1-GFP (brown). NC cells emigrating from the treated side are devoid of Xdd1-GFP. (G,H) Transverse sections following electroporation with dNLEF1 to show bilaterally symmetrical expression of *Sox9* and *Foxd3.* The hemi-tubes in the transfected sides are slightly narrower (*). Similar results were obtained for all tested genes (RhoB, Sox9, Cad6B, Msx1, Pax3 and Foxd3) upon transfection with β-catenin/engrailed or Xdd1 (not shown). (I) High magnification of the dorsal hemi-tube of an embryo electroporated with Xdd1-GFP to show that individual cells expressing Sox9mRNA (upper panel) co-express GFP (middle panel). The lower panel is an overlay of the upper two. EC, ectoderm; NT, neural tube. Scale bar: 40 μm in C,G,H; 20 μm in I.

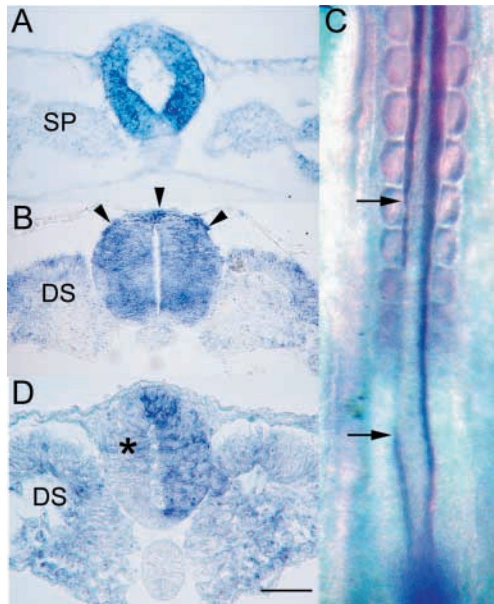


Fig. 7. Expression of *cyclin D1* under normal conditions and upon inhibition of Wnt signaling. (A,B) Normal expression patterns of *cyclin D1* in the neural tube at (A) a segmental plate (SP) level and (B) opposite dissociating somites (DS). In A, *cyclin D1* mRNA is expressed throughout the neural tube, except for the floor plate and dorsal midline. (B) At more rostral regions, *cyclin D1* signal becomes apparent in premigratory and early emigrating NC cells (arrowheads). (C,D) Electroporation of β-catenin fused to the engrailed repressor domain downregulates unilaterally expression of *cyclin D1* as shown in whole-mount (C, electroporation between arrows) and (D) transverse section (*treated hemi-tube). Scale bar: 35 μm in A,B,D.

including *Cad6B, Pax3, Msx1* and *RhoB* was found to depend upon BMP activity from the dorsal tube (Sela-Donenfeld and Kalcheim, 1999) (data not shown). If Wnt1 operates downstream of BMP, we reasoned that inhibiting Wnt activity will similarly affect their transcription. Unilateral electroporation of Xdd1, dNLEF1 or β-catenin-engrailed completely abolished expression of *Cad6B, Pax3 and Msx1* along the electroporated region (Fig. 6B-E and not shown), whereas control GFP had no effect (Fig. 6A and not shown). Expression of *cyclin D1*, a direct target of canonical Wnt signaling, reveals a dynamic pattern along the tube. Opposite the segmental plate mesoderm, *cyclin D1* mRNA is distributed throughout the tube except for the floor plate and dorsal midline regions, which reveal only low levels of transcription (Fig. 7A). At more rostral levels of the axis, concomitant with BMP and Wnt activation, *cyclin D1* expression also becomes apparent in the dorsal midline containing premigratory NC and in emigrating NC cells (Fig. 7B, arrowheads). Similar to *Cad6B, Pax3 and Msx1*, transcription of *Cyclin D1* was also downregulated both by noggin overexpression, as well as by the Wnt-inhibitory treatments Xdd1, dNLEF1 or β-catenin-engrailed (Fig. 7C,D; data not shown), further stressing the epistatic link between the BMP and Wnt pathways. By contrast, Dsh-DEP+ had no effect on transcription of either gene (data not shown). Hence, only canonical Wnt signaling plays a role in transcriptional activation of the above dorsal tube genes. Surprisingly, *RhoB* mRNA levels were unaffected upon treatment with either Xdd1, dNLEF1 or β-catenin-engrailed (Fig. 6F), suggesting that RhoB acts either upstream of Wnt, affects cyclin D1 production independently of Wnt activity, or is part of a separate BMP-dependent pathway (see Discussion).

In contrast to the above mentioned genes, transcription of *Slug, Foxd3* and *Sox9* is graded along the axis, resembling the pattern of *noggin* expression (not shown) (Sela-Donenfeld and Kalcheim, 1999). Co-expression of these genes under normal conditions with high levels of the BMP inhibitor suggests that their transcription is independent of tube-derived BMP signaling. This was confirmed for *Slug* the activity of which seems not to be required for either specification or delamination of trunk-level NC (Sela-Donenfeld and Kalcheim, 1999; del Barrio and Nieto, 2002). By contrast, *Foxd3* and *Sox9* were implicated in NC specification (Dottori et al., 2001; Kos et al., 2001; Cheung and Briscoe, 2003). To further examine whether tube-derived Wnt signaling is required for their synthesis, hemi-tubes were electroporated with Xdd1, dnLEF1 or β-catenin-engrailed and reincubated for 10 hours. Transcription of either *Foxd3*, *Sox9* or *Slug* was not affected in the electroporated hemi-tubes, which nonetheless were slightly narrower than the control sides and co-expressed GFP and either *Sox9* or *FoXd3* in individual cells (Fig. 6G-I; data not shown). Thus, at the stages considered here, Wnt activity mediates NC delamination without affecting maintenance of its specification state.

β-Catenin overexpression rescues NC delamination in noggin-treated neural tubes

We showed that *noggin* transcription along the tube is reciprocal to that of *Wnt1* (Fig. 3). In addition, noggin overexpression inhibits *Wnt1* transcription (Fig. 3), G1/S transition (Fig. 1) and NC delamination (Fig. 1) (Sela-Donenfeld and Kalcheim, 1999). Furthermore, inhibiting canonical Wnt signaling had a similar effect on cell cycle progression and NC emigration (Fig. 5). Altogether, these data support the notion that Wnt signaling acts downstream of BMP. In such a case, forced expression of full-length β-catenin is expected to rescue NC delamination in neural primordia treated with noggin. To test this prediction, hemi-neural tubes were co-electroporated in ovo with β-catenin/GFP and 2 hours later the transfected neural primordia were isolated and explanted on monolayers of control CHO or CHO-noggin cells.

When placed onto control CHO cells, GFP-positive NC cells exited the neural primordia that received either GFP alone or β-catenin/GFP and the emigrating cells stained positive for BrdU which was delivered to the co-cultures 1 hour prior to fixation (Fig. 8A,B, n=7 out of eight and six out of seven explants, respectively). By contrast, when placed onto noggin/CHO monolayers, no NC emigration was observed from any of the tubes that received a GFP-encoding plasmid (Fig. 8C, n=7). This inhibition was reversed upon transfection with β-catenin/GFP as both NC delamination and BrdU incorporation were observed in seven out of seven explants examined (Fig. 8D). Hence, canonical Wnt signaling acts downstream of BMP to stimulate NC delamination.

Discussion

We demonstrate that the canonical Wnt signaling pathway regulates delamination of trunk-level NC cells in the avian

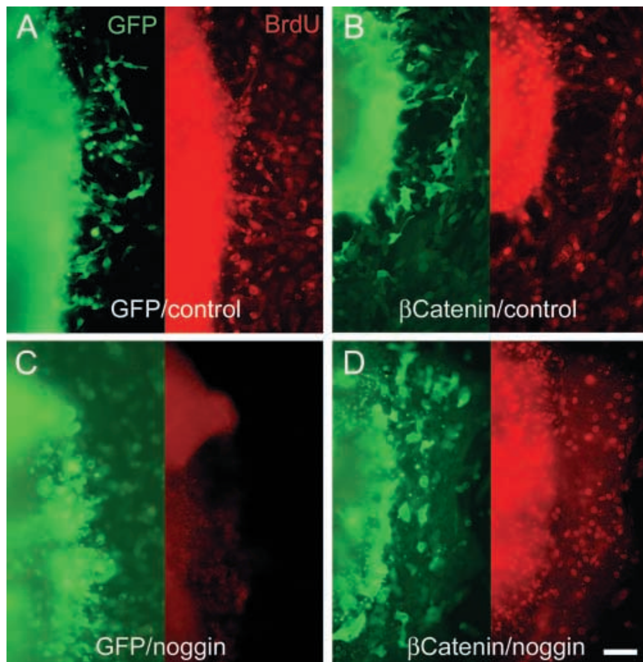


Fig. 8. β-Catenin overexpression rescues NC delamination in noggin-treated neural tubes. (A,B) Neural tubes that received (A) GFP only or (B) β-catenin/GFP were placed onto monolayers of control CHO cells (control). (C,D) Neural tubes that received (C) GFP only or (D) β-catenin/GFP were placed onto monolayers of noggin-producing CHO cells. GFP+NC cells (green) that incorporated BrdU (red) emigrated from the neural primordia onto the substrates in A,B,D, but not in C. CHO cells on the substrate have background fluorescence that is clearly distinct from the vibrant green fluorescence of the electroporated progenitors. Scale bar: 20 μm.

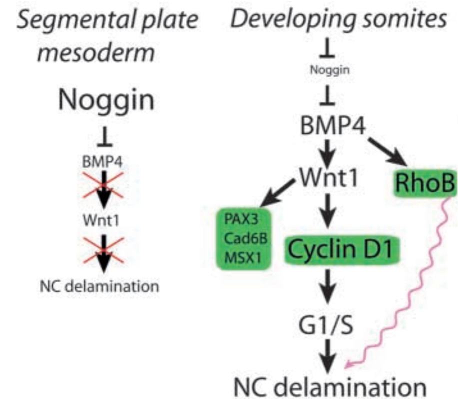


Fig. 9. A model of NC delamination that integrates data from the present and previous studies (see Discussion). Opposite the segmental plate mesoderm, high levels of noggin result in low BMP activity, no Wnt1 transcription, low Cyclin D1 and no NC cells emigrating from the caudal tube. G1/S transition and cell proliferation at this level are independent of BMP/Wnt1. With ongoing development, opposite mature epithelial and dissociating somites, a factor emitted by the dorsomedial region of the paraxial mesoderm inhibits *noggin* transcription in the dorsal tube, thereby relieving BMP activity from inhibition. BMP4 in turn positively regulates *Wnt1* transcription. Wnt signaling, via the canonical pathway, positively modulates transcription of *cyclin D1*, G1/S transition and NC cell delamination. Maintenance of *Pax3, Cad6B* and *Msx1* transcription in the dorsal tube is also regulated by the BMP/Wnt signaling pathway but their possible involvement in NC delamination awaits further testing. RhoB, at variance, is downstream of BMP but not of Wnt activities. The possible role of RhoB in NC delamination in vivo is still unknown. If it promotes delamination, it might act either via a parallel pathway (pink arrow), be upstream of Wnt1 or of Cyclin D1, or interact at a post-transcriptional level with molecules along the Wnt pathway.

embryo, an activity that is separable from earlier NC specification events. We also find that in the dorsal neural tube, Wnt1 behaves as a BMP-dependent gene, being likely to mediate the previously reported effect of BMP on NC delamination (Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld and Kalcheim, 2000). Furthermore, BMP and canonical Wnt pathways regulate G1/S transition in the dorsal tube. As most NC cells delaminate during S and successful G1/S transition of premigratory NC progenitors was found to be necessary for EMT (Burstyn-Cohen and Kalcheim, 2002), the present data provide a link between the effects of BMP/noggin, the transition between G1 and S phases of the cell cycle and the generation of cell movement.

BMP signaling differentially regulates G1/S transition along the axis

We show that inhibition of BMP activity affects neuroepithelial cell proliferation at somitic levels of the axis but not opposite the segmental plate. These results suggest that G1/S transition depends upon BMP signaling from segmented areas of the axis rostralwards, but not at caudal regions of the tube. The latter is consistent with the presence of high levels of noggin in the caudal dorsal tube which co-exist with normal proliferation rates (Burstyn-Cohen and Kalcheim, 2002). By contrast, inhibition of canonical Wnt signaling blocked cell cycle genes and G1/S transition along the entire length of the tube (T.B.-C., J.S., D.S.-D. and C.K., unpublished). Hence, at somitic levels of the axis, BMP-dependent Wnt signaling controls cell proliferation, whereas opposite unsegmented regions Wnt activity is independent of BMP. This differential regulation along the embryonic axis is, however, likely to be exerted via distinct Wnt proteins and cyclins. For example, proliferation in the caudal tube cannot depend upon Wnt1, because the latter is absent along this area and so is transcription of Wnt3a until the 18th-20th somite stage, when its expression becomes rostrocaudally homogeneous. Thus, Wnts other than Wnt1 and Wnt3a (temporarily) are likely to regulate the progression of the cell cycle in the caudal region of the dorsal tube. In addition, noggin overexpression inhibited BrdU incorporation and *Wnt1* but not *Wnt3a* transcription following 10-12 hours of treatment. Hence, residual endogenous Wnt3a does not appear to be sufficient for maintaining the progression of the cell cycle. The possibility remains to be tested that at segmented levels of the axis, the primary mechanism responsible for cell cycle progression is Wnt1/cyclin D1 dependent (see below).

A second axial difference resides in the dynamic pattern of cyclin D1 expression, which is very low in the dorsal neural tube at segmental plate areas and significantly increases from epithelial levels in the dorsal midline and early emigrating NC cells onward concomitant with the onset of Wnt1 transcription. By contrast, cyclin D2 reveals a reciprocal axial pattern (T.B.-C., J.S., D.S.-D. and C.K., unpublished). Given that somitic

signals downregulate transcription of *noggin* followed by BMP activation, synthesis of Wnt1 and NC delamination (Fig. 4) (Sela-Donenfeld and Kalcheim, 2000), we propose that the switch between differential mechanisms of cell cycle regulation along the axis also depends upon dynamic interactions with the paraxial mesoderm. Consistent with this notion, FGF8, the levels of which are high along the segmental plate mesoderm but decrease upon somitogenesis (Dubrulle et al., 2001; Dubrulle and Pourquie, 2004) controls levels of cyclin D2 expression in the caudal neural tube (F. Pituello, personal communication).

Wnt1 acts downstream of BMP in the dorsal neural tube

We have previously found that a balance between the activities of dorsal tube-derived BMP and its inhibitor noggin regulates NC delamination. Here, we report that BMP signaling also controls proliferation of neuroepithelial cells at axial levels corresponding to NC delamination. As the transition between G1 and S phases of the cell cycle is necessary for initiating NC migration, we hypothesized that BMP affects cell emigration by regulating the cell cycle. The Wnt proteins present in the dorsal tube, Wnt1 and Wnt3a, are likely candidates to mediate such an effect. This is because Wnt signaling directly targets cyclin D1 production (Tetsu and McCormick, 1999) and has been implicated in the control of cell proliferation in the CNS (Dickinson et al., 1994; Megason and McMahon, 2003), including NC precursors (Ikeya et al., 1997). In the present study, we find that Wnt1 acts downstream of BMP based on the following results. First, expression of *Wnt1* along the neural tube is reciprocal to that of *noggin*, is downregulated shortly after exposure to noggin and is prematurely upregulated by BMP overexpression. Likewise, in rodents, a constitutively active form of BMP receptor I induced ectopic expression of *Wnt1* in the neural tube (Panchinsion et al., 2001) and overexpression of constitutive active BMP receptor I in avian embryos led to increased NC emigration (Liu et al., 2004) in agreement with our results (Sela-Donenfeld and Kalcheim, 1999).

It is worth mentioning that the expression of *Wnt3a* is more dynamic, being absent from segmental plate levels of early embryos but present homogeneously along the entire axis of embryos aged 20 somites and older. Together with the finding that *Wnt3a* expression is downregulated following overnight exposure to noggin (Marcelle et al., 1997) (T.B.-C., J.S., D.S.-D. and C.K., unpublished) but not shortly afterwards, we infer that, unlike *Wnt1*, *Wnt3a* is not a direct target of BMP. However, our functional interference assays do not discriminate between different Wnt proteins and therefore do not rule out the possibility that Wnt3a may also be involved in some aspects of the NC delamination process.

Second, we show that somitic signals that inhibit *noggin* transcription in the dorsal tube resulting in activation of BMP (Sela-Donenfeld and Kalcheim, 2001), also stimulate initial transcription of *Wnt1* and altogether cause premature emigration of NC cells from the caudal tube. Third, inhibiting canonical Wnt signaling abrogates transcription of several BMP-dependent genes in the dorsal tube, except for early specification markers (see below). Fourth, overexpressing Wnt1 stimulates NC delamination. Fifth, activating the canonical pathway under conditions in which BMP signaling is inhibited by noggin overexpression, rescues both NC delamination and G1/S transition. Thus, BMP is upstream of Wnt signaling in the dorsal neural tube within the context leading to NC delamination. As BMPs and Wnts continue to be expressed after NC cells accomplished emigration, this hierarchical relationship between the two signaling systems may also remain for later events, such as the specification of dorsal interneurons in the spinal cord (Muroyama et al., 2002; Liu et al., 2004).

As discussed above, BMP signaling regulates Wnt at the transcriptional level. Whether Wnt protein activity in the dorsal neural tube is also modulated by its known antagonists, such as BMP, is regulated by noggin, remains to be clarified. In the trunk of avian embryos, Sfrp-1 and Sfrp-2 are expressed in the neural tube but are excluded from the dorsal midline, where the premigratory NC resides (Terry et al., 2000) (see also Esteve et al., 2000). The expression of another inhibitor, frzb-1 (Sfrp-3), is graded along the tube resembling that of noggin mRNA, with high levels being transcribed in the neural folds opposite the segmental plate and turning to low up to undetectable when advancing rostralward along the tube (Baranski et al., 2000; Duprez et al., 1999; Jin et al., 2001; Ladher et al., 2000). This expression pattern is largely reciprocal to that of Wnt1, which is already active at the level of epithelial somites based on the observed dorsalization of one of its direct target genes, cyclin D1, at this axial level (Fig. 7), and on the corresponding onset of detectable NC delamination, a function here reported to depend upon Wnt activity. Hence, it is possible that the low signal of frzb-1 mRNA still detected in 19-somite stage embryos at epithelial somite levels by Jin et al. (Jin et al., 2001) is compatible with a degree of Wnt activity that permits initial NC delamination to take place. Full activity of the protein is then enabled upon complete frzb-1 downregulation during the progression of the emigration process. Notably, in contrast to noggin, which titrates BMP activity at segmental plate levels of the axis, the intense expression of frzb-1 along this caudal region is likely to inhibit Wnt members other than Wnt1 or Wnt3a (till the 18th-20th somite stage) because these are absent from the caudal neuraxis.

Wnt signaling in initial NC specification versus subsequent delamination

Expression of *Slug, FoxD3* and *Sox9* is coincident with the induction of NC and is upregulated de novo by upstream signals that induce NC, including secreted molecules of the BMP and Wnt families that derive from the ectoderm (Garcia-Castro et al., 2002) (reviewed by Gammill and Bronner-Fraser, 2003). Here, we show that inhibiting canonical Wnt signaling in the neural tube at later stages resulted in decreased G1/S transition and impaired NC delamination but had no effect on transcription of genes involved in earlier NC specification, such as *Sox9, Foxd3* or *Slug*. These results suggest that the latter gene products are not involved in NC delamination mediated by Wnt. This notion is supported by results of overexpressing *FoxD3* and *Sox9*, which led to the formation of ectopic NC but not to their delamination (Chung and Briscoe, 2003; Kos et al., 2001) (but see Dottori et al., 2001). We note that our experiments temporally and spatially separate between these events because by the time of electroporation, the above transcripts are already apparent at segmental plate levels of the

tube and their subsequent expression is independent of dorsal tube-derived Wnt activity. Likewise, maintenance of Slug mRNA/protein was reported to be independent of BMP (Sela-Donenfeld and Kalcheim, 1999). In further support of this conclusion, a lack of correspondence can be observed at post-specification stages between *Wnt1* expression along the tube and that of the early genes. For example, *Wnt1* is still absent from the caudal tube, which already expresses highest levels of *Slug, FoxD3* and *Sox9*. Conversely, at more rostral levels, these genes are downregulated yet BMP/Wnt genes remain expressed and NC delamination is still under way.

Canonical Wnt signaling and EMT

Treatment of neural primordia with Xdd1 which blocks both canonical and non-canonical branches efficiently inhibited both BrdU incorporation and NC delamination. This was mimicked by treatment with dNLEF1 and β-catenin-engrailed, which specifically abrogate the canonical pathway. By contrast, dishevelled mutants selectively interfering with non-canonical Wnt signaling (DEP+ or D2) had no effect. Altogether, these data demonstrate that the canonical pathway of Wnt signaling, which involves β-catenin, LEF/TCF and transcriptional activation of genes acting on G1/S transition and execution of EMT, regulates the generation of NC cell migration. However, in mice, conditional ablation of β-catenin in Wnt1-expressing cells had no apparent effect on either cell proliferation or NC emigration (Hari et al., 2002), in contrast to our results and to data from previous studies (Ikeya et al., 1997; Megason and McMahon, 2002). Assuming that in mice, as in avian embryos, NC emigration begins immediately following the onset of *Wnt1* expression in the dorsal tube, it is possible that β-catenin protein persisted after the gene had been ablated and its residual activity still accounted for the observed emigration. To ensure early gene downregulation, our electroporations were performed at segmental plate levels of the axis, ~10 hours prior to initial Wnt1 expression and onset of NC delamination.

In line with our findings, de Melker et al. (Melker et al., 2004) have reported that β-catenin and Lef-1 proteins translocate to the nucleus of NC cells precisely during their delamination from explanted neural primordia. This nuclear localization is only transient, as it was found to disappear from cells undergoing advanced migration onto fibronectin substrates. These results are indicative of a signaling activity of the canonical Wnt pathway that is restricted to the delamination phase. As NC cells become synchronized to the S phase of the cell cycle during delamination, but lose synchrony during subsequent migration (Burstyn-Cohen and Kalcheim, 2002) and, moreover, upregulate cyclin D1 in the dorsal tube during this process (this paper), it follows that under normal conditions, the nuclear localization of β-catenin and Lef-1 proteins observed by de Melker et al. is positively associated with both G1/S transition and NC delamination. This observation is consistent both with our gain- and loss-of-function results. Surprisingly, this transient nuclear translocation of β-catenin and Lef-1 proteins reported by de Melker et al. (de Melker et al., 2004) to occur during NC delamination contrasts with the authors own experimental data showing that a pharmacological excess of Wnt1, achieved either by LiCl treatment or by co-culturing neural primordia with the Wnt1-producing 2.69.23 cell line, inhibited both BrdU incorporation as well as NC emigration, perhaps by reducing the ability of the cell to adhere to the culture substrate. These data are unlikely to be of physiological significance because in the embryo, as well as in explants, endogenous levels of Wnt activity in the dorsal tube are compatible with NC proliferation and delamination. These data also differ from our gain-of-function results (electroporation of full-length Wnt1 or β-catenin), which revealed a stimulation in cell delamination associated with BrdU incorporation (this paper), as well as from those of others (e.g. Megason and McMahon, 2002; Nishihara et al., 2003), which showed enhanced cyclin D1 expression followed by a stimulation of cell proliferation upon Wnt overexpression. Although the exact reason for this discrepancy remains unclear, it is possible that the paradigms used in de Melker's study led to a particularly robust overexpression of the protein, up to levels not attained upon Wnt1 electroporation, or, in the case of LiCl treatment, to the activation of additional non-specific processes. These, in turn, might have resulted in an adverse phenotype because they triggered inhibitory feedback mechanisms or receptors (e.g. Golan et al., 2004). Even under these conditions, the association between G1/S transition and NC delamination that we have previously found (Burstyn-Cohen and Kalcheim, 2002) was maintained. Altogether, it is possible that the dose and/or mode of presentation of Wnt proteins required for these processes have to be tightly regulated.

The notion we put forward in this study that the β-catenin/LEF transcription factor complex is associated with EMT is supported by results of several in vitro studies (Novak et al., 1998; Eger et al., 2000). In addition, IGFII induced rapid β-catenin translocation to the nucleus of cultured cells during EMT as well as transcription of target genes (Morali et al., 2001). Furthermore, activation of the β-catenin pathway directly induced EMT in normal corneal epithelium and DLD1 colon carcinoma cells (Kim et al., 2002). Hepatocyte growth factor and epidermal growth factor induced β-catenin signaling, whereas stimulating cell motility and ectopic expression of various forms of β-catenin mimicked the process (Muller et al., 2002).

In addition to its role in canonical Wnt signaling, β-catenin is also a member of adherens junctions, where it links cadherins to the cytoskeleton, thereby controlling intercellular interactions that characterize epithelial adhesions (Ozawa et al., 1989; Savagner, 2001). We observed that treatment with DEP+ and D2 perturbed epithelial integrity of the transfected hemi-tubes, as monitored by the loss of the pseudostratified structure, disorganized membrane n-cadherin and β-catenin immunostaining (T.B.-C., J.S., D.S.-D. and C.K., unpublished), but had no significant effects on gene transcription, G1/S transition or NC delamination. Hence, the non-canonical pathway mediated by specific domains of dishevelled (reviewed by Veeman et al., 2003) plays a role in stabilizing the neuroepithelium. This pathway has also been implicated in convergent extension, a type of cell movement that involves coordinated migration of cohesive cell sheets (reviewed by Locascio and Nieto, 2001). Instead, EMT of NC cells reflects the behavior of individual progenitors, further suggesting that these two types of cell movement are differentially regulated. Although results stemming mainly from in vitro studies suggest that Wnt-dependent β-catenin signaling and cadherin-β-catenin-mediated cell adhesion are possibly interrelated pathways (reviewed by Nelson and Nusse,

2004), our results, performed in a physiological context, suggest that emigration of NC cells is feasible as long as the β-catenin transcriptional activation pathway remains intact.

A model for NC delamination

The mechanisms underlying EMT differ among cell types and developmental contexts, emphasizing the complexity of the pathways. Our studies, which are focused on EMT of trunk-level NC cells stress the importance of two basic signaling systems, BMP and Wnt, which co-exist in the dorsal neural tube following initial NC specification. Previous results have shown that BMP4 is required both for de novo transcription as well as for later maintenance of *RhoB* (Liu and Jessell, 1998; Sela-Donenfeld and Kalcheim, 1999). In vitro inhibition also suggested that RhoB activity is required for NC delamination (Liu and Jessell, 1998); however, in vivo evidence is still missing. If found to be involved in NC emigration in the embryo, our finding that transcription of *RhoB* is under control of BMP but independent of canonical Wnt signaling (Fig. 9) would suggest either that RhoB activity is upstream of Wnt, that RhoB acts via a parallel pathway independent of Wnt and/or that Wnt and RhoB pathways, though genetically separate, interact at various levels, as shown for other systems (e.g. Gumbiner, 2000; Roovers et al., 2003).

By integrating previous and present results, we propose that the developing somitic mesoderm, which provides the substrate for NC migration and segmentation, acts upstream in the process of EMT of NC progenitors by producing an as yet unidentified cue/s that results in downregulation of *noggin* transcription in the dorsal tube (Sela-Donenfeld and Kalcheim, 2000) (Fig. 9). This event leads to activation of BMP signaling followed by upregulation of *Wnt1*. Wnt1 in turn activates the canonical pathway that includes β-catenin and LEF/TCF to regulate transcription of target genes such as *cyclin D1, Msx1, cadherin 6B* and *Pax3*. These proteins, and/or additional as yet unidentified genes, might play a role in the execution of the program of EMT; however, their involvement in the process and precise gene interactions await direct testing. Although found to be a necessary event for NC delamination, the precise mechanism by which Wnt-dependent G1/S transition is translated into generation of NC cell movement remains to be elucidated.

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