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Wnt-regulated temporal control of BMP exposure directs the choice between neural plate border and epidermal fate

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The non-neural ectoderm is divided into neural plate border and epidermal cells. At early blastula stages, Wnt and BMP signals interact to induce epidermal fate, but when and how cells initially acquire neural plate border fate remains poorly defined. We now provide evidence in chick that the specification of neural plate border cells is initiated at the late blastula stage and requires both Wnt and BMP signals. Our results indicate, however, that at this stage BMP signals can induce neural plate border cells only when Wnt activity is blocked, and that the two signals in combination generate epidermal cells. We also provide evidence that Wnt signals do not play an instructive role in the generation of neural plate border cells, but promote their generation by inducing BMP gene expression, which avoids early simultaneous exposure to the two signals and generates neural plate border instead of epidermal cells. Thus, specification of neural plate border cells is mediated by a novel Wnt-regulated BMP-mediated temporal patterning mechanism.

KEY WORDS: BMP, Neural plate border, Wnt, Chick, Epidermal, Neural crest

INTRODUCTION

Early in development, the vertebrate ectoderm becomes subdivided into neural, neural plate border and epidermal cells. Neural plate border cells develop at the junction between the neural and epidermal ectoderm and give rise to placodal and neural crest cells, which are components of the peripheral nervous system. Previous results have provided evidence that at the early blastula stage, medial and lateral epiblast cells are specified as neural and epidermal cells, respectively (Wilson and Edlund, 2001). When and how epiblast cells initially acquire neural plate border character has, however, remained undefined.

At the early blastula stage, prospective epidermal cells are exposed to *Wnt3A* and *Wnt8C*, and to bone morphogenetic protein 4 (*Bmp4*) and *Bmp7*, and these two classes of signals interact to promote the generation of epidermal cells at the expense of neural cells (Wilson et al., 2000; Wilson et al., 2001). Later in development, at the late gastrula stage, Wnt and BMP signals are also involved in the generation of neural plate border cells. At rostral forebrain levels where cells evade Wnt signals, BMP signals promote the generation of neural plate border cells of rostral placodal character, and the time of exposure to BMP signals plays a key role in the differential specification of olfactory and lens placodal cells (Sjodal et al., 2007). At caudal levels of the neuraxis where neural plate border cells of caudal/neural crest character are generated (Patthey et al., 2008), BMP signals can induce neural plate border character in prospective neural plate cells, whereas Wnt signals are required to impose caudal character on neural plate border cells. However, Wnt signals can also induce neural plate border cells of caudal/neural crest character at rostral forebrain levels (Patthey et al., 2008). Moreover, both Wnt and BMP signals are required for the generation of neural crest in intact embryos (Abu-Elmagd et al., 2006; Endo et al., 2002; Lewis et al., 2004; Tucker et al., 2008; Wawersik et al., 2005), although the mechanism by which these two classes of signals interact to induce neural crest cells, and how they interact to differentially generate neural plate border and epidermal cells, have not been determined.

In this study, we have established chick explant assays of ectodermal cell differentiation to examine the roles of Wnt and BMP signals in the initial specification of neural plate border cells. We provide evidence that the specification of neural plate border cells is initiated at the late blastula stage and requires both Wnt and BMP signals, and that BMP, but not Wnt, signals play an instructive role in this process. Our results also indicate that at this stage, BMP signals induce neural plate border cells only when Wnt activity is blocked, and that simultaneous exposure to Wnt and BMP signals generates epidermal cells. Furthermore, we show that Wnt signals induce BMP gene expression in prospective neural plate border cells, which avoids early simultaneous exposure to the two signals and thereby generates neural plate border instead of epidermal cells. Thus, the early subdivision of the embryonic ectoderm is mediated by a novel Wnt-regulated BMP-mediated temporal patterning mechanism.

MATERIALS AND METHODS

Embryos

Fertilized White Leghorn chicken eggs were obtained from Agrisera AB, Umeå, Sweden. The use of chick embryos in this study was approved by the Ethical Committee on Animal Experiments for Northern Sweden. Chick embryos were staged according to published protocols (Eyal-Giladi and Kochav, 1976; Hamburger and Hamilton, 1951).

Isolation and culture of tissue explants

Ectodermal explants were removed from the underlying hypoblast and isolated using a tungsten needle. Medial, border and lateral explants were isolated from stage XI and stage 2 embryos, and rostral neural and neural plate border explants were isolated from stage 4 embryos. All explants were cultured in vitro in collagen (Invitrogen) in serum-free OPTI-MEM (Gibco) containing N2 supplement (Invitrogen) and fibronectin (Sigma). Wnt3A was used at an estimated concentration of 300 ng/ml, mouse (m) frizzled receptor 8 (mFrz8CRD) conditioned media (CM) were used at 150 μl/ml culture medium and Noggin at an estimated concentration of 25 ng/ml. Explants cultured in the presence of control CM generated the same combination of cells as explants cultured alone (data not shown). Human BMP4 (R&D Systems) was used at 5-10 ng/ml.

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Preparation of factors

Various assays have shown that Wnt3A, Wnt8C and Wnt11 have similar activities (http://www.stanford.edu/%7Ernusse/wntwindow.html). In the present study, the effects of Wnt signaling were examined using Wnt3A CM, previously shown to have reliable biological activity (Gunhaga et al., 2003; Nordstrom et al., 2002; Patthey et al., 2008; Wilson et al., 2001). Soluble Wnt3A and control CM were obtained from stably transfected or untransfected mouse L cells, respectively (Shibamoto et al., 1998), soluble mFrz8CRD and control CM from HEK-293 cells transfected with *mFrz8CRD* or *lacZ* reporter constructs (Hsieh et al., 1999) using GenePORTER 2 (GTS_{INC}, San Diego, CA), and soluble Noggin and control CM from stably transfected or untransfected CHO cells (Lamb et al., 1993).

Immunohistochemistry

For immunohistochemistry, embryos and explants were fixed as described (Gunhaga et al., 2003) and serially sectioned at 8-10 μm. Anti-Sox1, anti-Sox2, anti-Gbx2, anti-Otx2, wide spectrum screening anti-cytokeratin (DakoCytomation) and anti-BF1 (Watanabe et al., 2005) rabbit antibodies, and monoclonal anti-Slug (Snail2) (Liem et al., 1995), anti-HNK-1 (Liu and Jessell, 1998), anti-Pax6 (Ericson et al., 1997) and anti-Nkx2.1 (AbCam) mouse antibodies were used. Nuclei were stained with DAPI (Sigma). Rabbit anti-Otx2 antibodies were raised against the peptide CLDYKNQTSSWKFQVL at Agrisera, Umeå, Sweden.

Quantitative real-time PCR analysis

Total RNA was prepared from stage 2 and stage 4 explants (n=20) before culture or after 10 hours of culture as previously described (Patthey et al., 2008). The CT value for each gene was the average of a triplicate, and expression levels were normalized using the average of CT values for *Gapdh*, histone H4 and *S17*. Primer sequences for *Dlx5* were: 5′-TTTTT-CGGCTCAGCTGTGG-3′ and 5′-GGCACCATTGACAGTGTCCA-3′. Primer sequences for *Bmp4*, *Gapdh*, histone H4, *Sp5* and *S17* have been described previously (Patthey et al., 2008).

Western blot analysis

Medial explants (n=20) were isolated from stage 2 embryos and cultured for 1 hour on a Millicell membrane (PICM01250, Millipore) in serum-free OPTI-MEM. The explants were homogenized by a 10-minute incubation in lysis buffer at 100°C. Tissue extracts containing 3.5 μg total protein were applied to each well. Antibodies used were rabbit anti-phosphorylated Smad1/5/8 (Cell Signaling, 1:5000), anti-β-actin (Cell Signaling, 1:10,000) and HRP-conjugated anti-rabbit (Jackson Laboratories, 1:10,000).

Statistical analysis

Consecutive sections from the same explants were stained in multiple ways. The percentage of antigen-expressing cells was quantified by counting the number of stained cells in two to five sections per explant (n=8-17 explants). The total number of cells per section was determined by counting the number of DAPI-stained nuclei. For quantification of Sox1, Sox2, Snail2, HNK-1 and cytokeratin (Ker), the mean number of cells positively stained for these antigens was expressed as a percentage of total cell number.

RESULTS

Neural plate border cells are specified at the late blastula stage

At the early blastula stage, epiblast cells are subdivided along the mediolateral axis into prospective neural and epidermal cells (Wilson et al., 2000). The stage at which cells acquire neural plate border character remains, however, to be determined. To address this issue, we isolated medial (M), border (B) and lateral (L) epiblast explants of early to late blastula stage (stage EG XI to HH2) chick embryos and cultured the explants for 28 or 36 hours, corresponding in time to a stage ~10 embryo (Fig. 1A,C). After culture, we monitored the generation of neural, rostral placodal, neural crest and epidermal cells on consecutive sections of the explants. By stage 10, Sox1 is expressed exclusively in neural progenitor cells (Patthey et al., 2008; Wood and Episkopou, 1999), Snail2 (previously known as Slug) is preferentially expressed in premigratory and early migratory neural crest cells, HNK-1 is expressed in migratory neural crest (Del Barrio and Nieto, 2004), cytokeratins (Ker) and Sox2 are coexpressed in prospective cranial placodes, and Ker expression alone defines cells of epidermal character (Comte et al., 2004; Rex et al., 1997; Wood and Episkopou, 1999) (Fig. 1B). Early blastula stage (stage XI) B explants generated $Sox1^+$ $Sox2^+$ neural cells and Ker^+ epidermal cells, no $Sox2^+$ Ker^+ placodal cells, and, only occasionally, a small number of $Snail2^+$ $HNK\text{-}1^+$ neural crest cells (see Fig. S1A in the supplementary material). By contrast, stage 2 B explants generated a large number of $Snail2^+$ $HNK\text{-}1^+$ neural crest cells, but only a few $Sox1^+$ $Sox2^+$ neural cells or Ker^+ epidermal cells (Fig. 1F). Stage 2 M explants generated $Sox1^+$ $Sox2^+$ neural cells but no, or only a few, $Snail2^+$ $HNK\text{-}1^+$ and Ker^+ cells (Fig. 1D). Stage 2 L explants generated Ker^+ epidermal cells, but no $Snail2^+$ $HNK\text{-}1^+$ or $Sox1^+$ $Sox2^+$ cells (Fig. 1H). Thus, the specification of neural plate border cells is initiated at the late blastula stage, after the specification of epidermal cells.

Previous studies have provided evidence that at the late gastrula stage (stage 4), rostral neural plate border cells are specified as olfactory/lens placodal cells and caudal border cells as neural crest cells (Basch et al., 2006; Patthey et al., 2008; Sjodal et al., 2007). To determine whether rostrocaudal patterning of prospective neural plate border cells is initiated at stage 2, we isolated explants of the prospective neural plate border from the entire rostrocaudal extent of stage 2 embryos (Fig. 1C). Under these conditions, $Snail2^+$ $HNK\text{-}1^+$ neural crest cells were generated to the same extent at all rostrocaudal levels, but no $Sox2^+$ Ker^+ placodal cells, and only a few neural and epidermal cells appeared (Fig. 1E-G). Moreover, stage 2 rostral neural plate border (RB) explants cultured alone did not generate placodal cells even after prolonged (40 hours) culture (see Fig. S1B in the supplementary material). By contrast, and in agreement with previous studies (Basch et al., 2006; Patthey et al., 2008; Sjodal et al., 2007), stage 4 RB explants cultured for 22 hours, corresponding in time to a stage ~10 embryo, generated $Sox2^+$ Ker^+ placodal cells, but almost no $Sox1^+$, $Snail2^+$ or $HNK\text{-}1^+$ cells (see Fig. S1C in the supplementary material). Taken together, these results provide evidence that at the late blastula stage, and independent of rostrocaudal position, prospective neural plate border cells are initially specified as neural crest cells.

Wnt and BMP signals are required for the specification of neural plate border cells

At the late gastrula stage, Wnt and BMP signals promote the generation of neural plate border cells of caudal/neural crest character (Patthey et al., 2008), raising the possibility that prospective neural plate border cells are initially exposed to both BMP and Wnt signals. Consistent with this idea, in stage 2 B explants cultured in the presence of Noggin, which is a selective antagonist of BMP signals (Lamb et al., 1993), the generation of $Snail2^+$ and $HNK\text{-}1^+$ neural crest cells was blocked, and $Sox1^+$ and $Sox2^+$ neural cells were generated (Fig. 2B). In addition, the mRNA level of the BMP target gene *Dlx5* (Jadlowiec et al., 2006) was downregulated ~11-fold to the level observed in stage 4 rostral (R) neural explants, which are not influenced by BMP signals, verifying that BMP signaling is blocked by Noggin (Fig. 2D). By stage 4, Wnt signals impose caudal character on neural plate border cells, and when Wnt activity is blocked, prospective caudal neural plate border cells generate olfactory/lens placodal cells at the expense of neural crest cells (Patthey et al., 2008). Culturing stage 2 B explants in the presence of mouse (m) frizzled receptor 8 (mFrz8CRD), a soluble Wnt inhibitor (Gunhaga et al., 2003; Hsieh et al., 1999; Wilson et

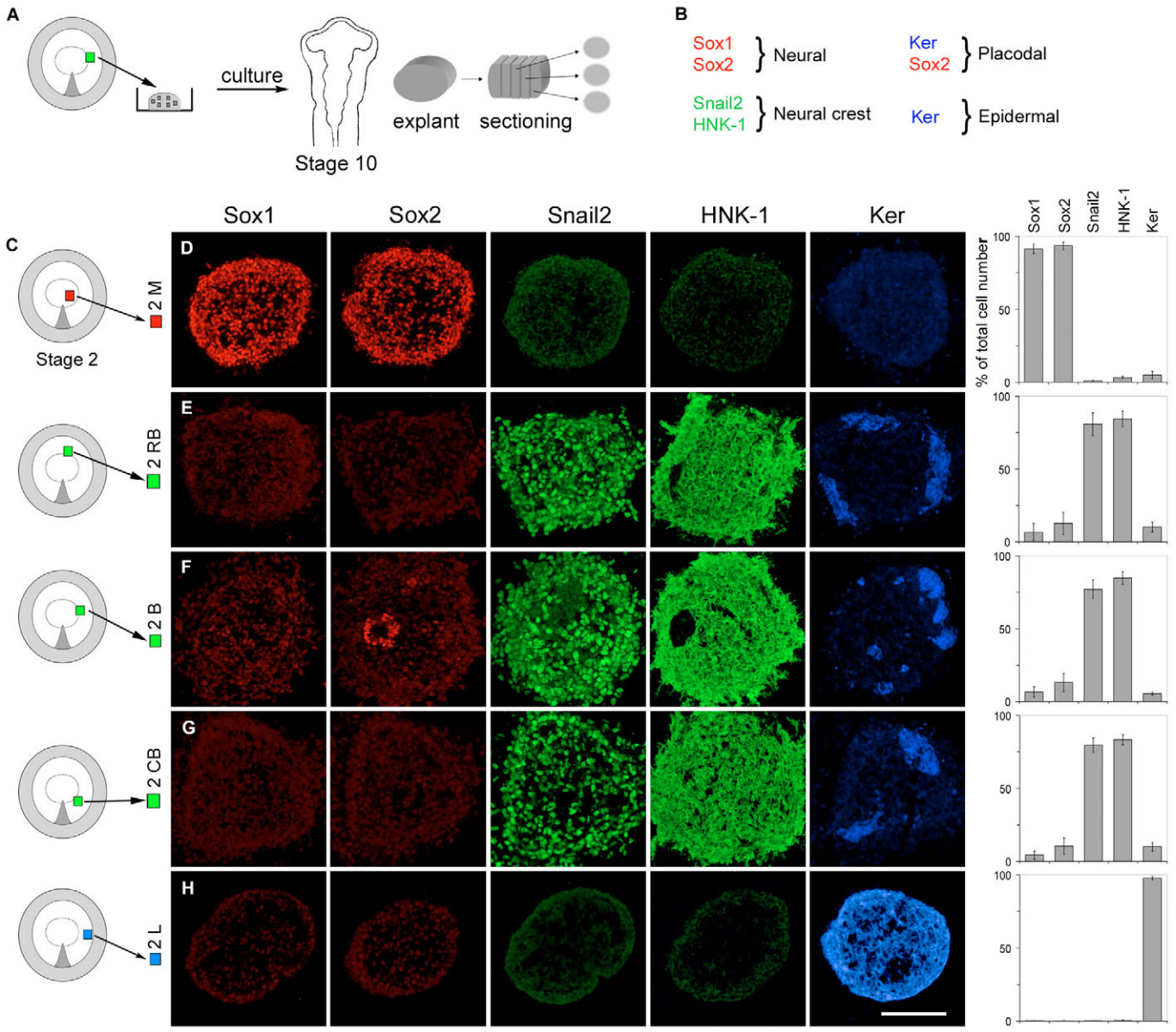

Fig. 1. Chick neural plate border cells are specified as neural crest cells at the late blastula stage. (**A**) Ectodermal explants were isolated, separated form the hypoblast/endoderm, and cultured in vitro to the developmental equivalent of stage 10 before fixation, freezing and sectioning. (**B**) Different combinations of molecular markers define specific ectodermal fates. (**C**) Schematic representations of stage 2 embryos indicating the position at which medial (M, red box), prospective border [RB (rostral neural plate border), B (border) and CB (caudal border), green boxes] and lateral (L, blue box) explants were isolated. (**D-H**) Consecutive sections showing expression of molecular markers in explants cultured for 28 hours. (D) Stage 2 M explants (n>30) generated Sox1$^+$ and Sox2$^+$ cells, but no Snail2$^+$, HNK-1$^+$ or Ker$^+$ cells. (E-G) Stage 2 B explants at different rostrocaudal positions (RB, n=23; B, n>30; CB, n=13) generated Snail2$^+$ and HNK-1$^+$ cells, but no, or only a few, Sox1$^+$, Sox2$^+$ or Ker$^+$ cells. (H) Stage 2 L explants (n>30) generated Ker$^+$ cells, but no Sox1$^+$, Sox2$^+$, Snail2$^+$ or HNK-1$^+$ cells. Data are represented as the mean ± s.e.m. Scale bar: 100 μm.

al., 2001), also inhibited the generation of Snail2$^+$ and HNK-1$^+$ neural crest cells, but unlike at stage 4, Sox1$^+$ and Sox2$^+$ neural cells were generated instead of rostral placodal cells (Fig. 2C). In addition, the mRNA level of *Sp5*, a Wnt target gene (Weidinger et al., 2005), was downregulated ~17-fold to the level observed in stage 4 R explants, which are not influenced by Wnt signals, verifying that Wnt signaling is blocked by mFrz8CRD (Fig. 2E). Thus, both BMP and Wnt signals are required for the initial specification of neural plate border cells and, in the absence of either signal, cells acquire neural character.

Both Wnt and BMP signals are required for the generation of epidermal cells

At the early blastula stage, Wnt and BMP signals interact to promote the generation of epidermal cells (Wilson and Edlund, 2001). To analyze whether both Wnt and BMP signals are also required for the generation of epidermal cells at the late gastrula stage, we cultured stage 2 L explants in the presence of Noggin and/or mFrz8CRD. In stage 2 L explants cultured in the presence of Noggin, the generation of Ker$^+$ epidermal cells was blocked and Sox1$^+$ Sox2$^+$ neural cells, but no Snail2$^+$ HNK-1$^+$ neural crest or Sox2$^+$ Ker$^+$ placodal cells, were

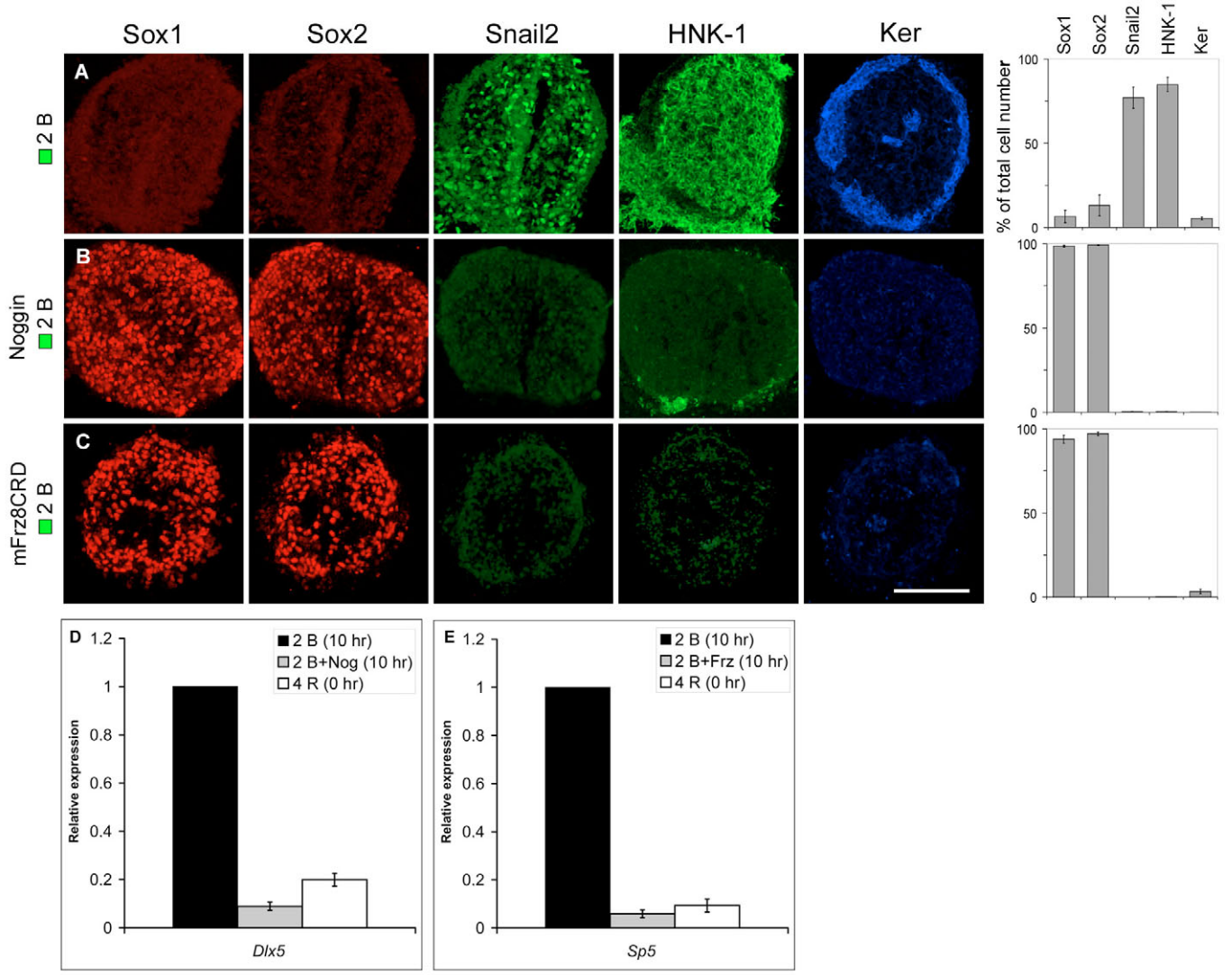

Fig. 2. BMP and Wnt signals are required for the specification of neural crest cells. (**A-C**) Consecutive sections showing expression of molecular markers in chick explants cultured for 28 hours. (A) Stage 2 B explants (*n*>30) generated Snail2$^+$ and HNK-1$^+$ cells, but no, or only a few Sox1$^+$, Sox2$^+$ or Ker$^+$ cells. (B) Stage 2 B explants cultured in the presence of Noggin (*n*=26) generated Sox1$^+$ and Sox2$^+$ cells, but no Snail2$^+$, HNK-1$^+$ or Ker$^+$ cells. (C) Stage 2 B explants cultured in the presence of mFrz8CRD (*n*>30) generated Sox1$^+$ and Sox2$^+$ cells, but no Snail2$^+$, HNK-1$^+$ or Ker$^+$ cells. Data are represented as mean ± s.e.m. Scale bar: 100 μm. (**D**,**E**) Relative *Dlx5* and *Sp5* mRNA levels measured by quantitative real-time PCR. mRNA expression of the BMP target gene *Dlx5* (D) and of the Wnt target gene *Sp5* (E) is inhibited in stage 2 B explants cultured in the presence of Noggin (D) or mFrz8CRD (E). Bars represent mean ± s.e.m. of four (D) and five (E) independent experiments.

generated (Fig. 3B). Thus, in the absence of BMP activity, prospective epidermal cells acquire neural character. Inhibition of Wnt activity in stage 2 L explants by exposure to mFrz8CRD also blocked the generation of Ker$^+$ epidermal cells, while Sox2$^+$ Ker$^+$ placodal cells, but no Snail2$^+$ HNK-1$^+$ neural crest cells, were generated (Fig. 3C). To elucidate the identity of the Sox2$^+$ and Ker$^+$ placodal cells, we monitored the expression of molecular markers characteristic of olfactory and lens cells after 48 hours of culture (corresponding to a stage ~17 embryo). Under these conditions, Ker$^+$, *Raldh3*$^+$ and HuCD$^+$ cells, which are characteristic of the olfactory placode, and Ker$^+$ δ-crystallin$^+$ cells characteristic of the lens placode, were generated (data not shown). In addition, in stage 2 L explants cultured in the presence of mFrz8CRD for 10 hours, the mRNA level of the Wnt target gene *Sp5* was downregulated 5.5-fold, whereas the mRNA level of the BMP target gene *Dlx5* was unaffected by mFrz8CRD (Fig. 3E). Thus, these results provide evidence that Wnt signals are required for the generation of epidermal cells, and suggest that in the absence of Wnt signals, endogenous BMP activity induces rostral placodal cells, but not neural crest cells. Consistently, stage 2 L explants cultured in the presence of both mFrz8CRD and Noggin generated Sox1$^+$ Sox2$^+$ neural cells, but no Ker$^+$ Snail2$^+$ or HNK-1$^+$ cells (Fig. 3D). Taken together, these results indicate that at the late blastula stage, both Wnt and BMP signals are required for the generation of epidermal character.

Wnt signals promote the generation of neural plate border cells by a BMP-dependent mechanism

The requirement for both BMP and Wnt signals for the generation of epidermal cells and for the initial specification of neural plate border cells raised the issue of how these two classes of signals interact to specify neural plate border cells instead of epidermal cells. To address

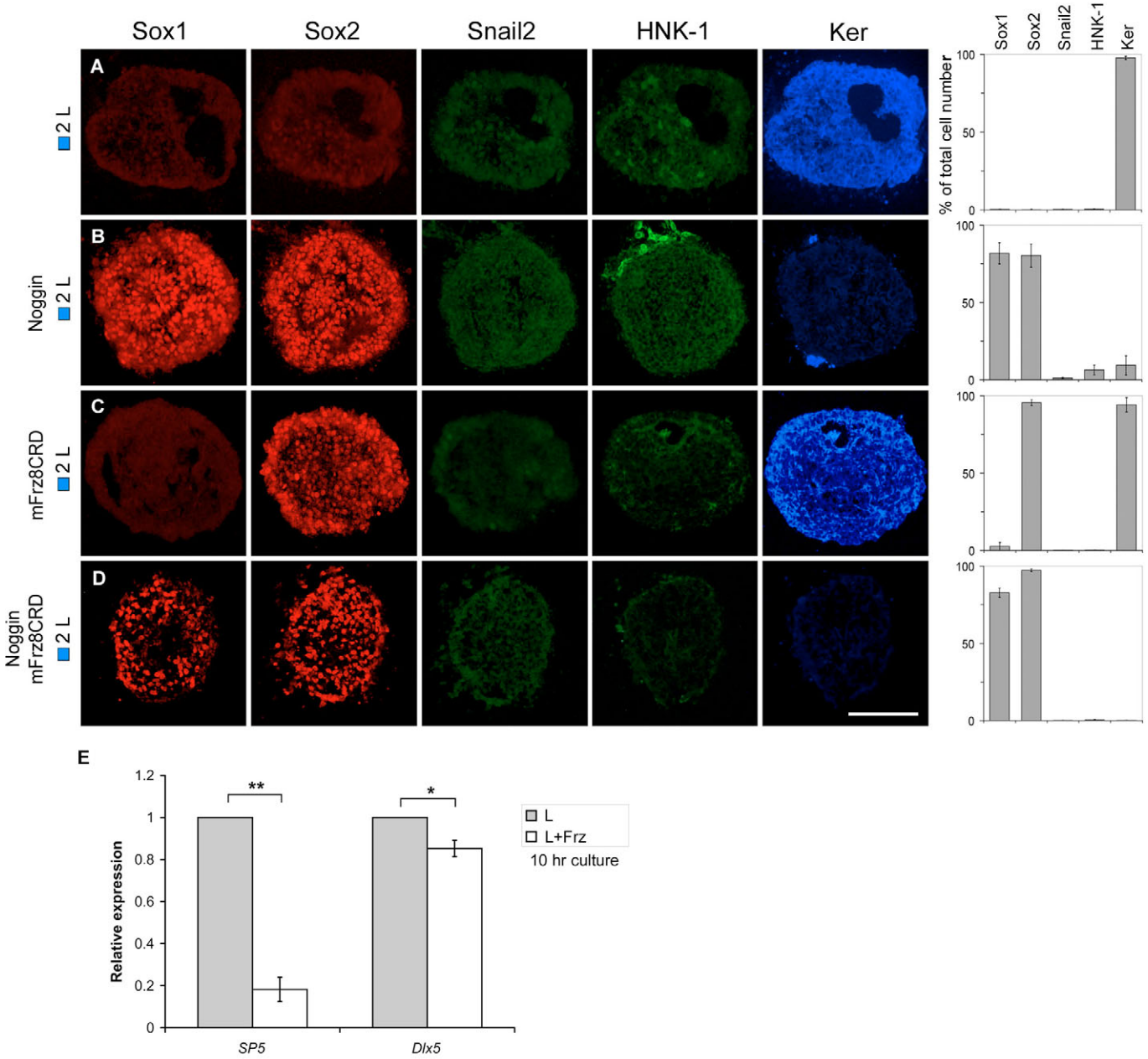

Fig. 3. BMP and Wnt signals are required for the generation of epidermal cells. (**A-C**) Consecutive sections showing expression of molecular markers in chick explants cultured for 28 hours. (A) Stage 2 L explants (*n*>30) generated Ker$^+$ cells, but no, or only a few, Sox1$^+$, Sox2$^+$, Snail2$^+$ or HNK-1$^+$ cells. (B) Stage 2 L explants cultured in the presence of Noggin (*n*>30) generated Sox1$^+$ and Sox2$^+$ cells, but no Snail2$^+$, HNK-1$^+$ or Ker$^+$ cells. (C) Stage 2 L explants cultured in the presence of mFrz8CRD (*n*>30) generated Sox2$^+$ and Ker$^+$ cells, but no Sox1$^+$, Snail2$^+$ or HNK-1$^+$ cells. (**D**) Stage 2 L explants cultured in the presence of both Noggin and mFrz8CRD (*n*=18) generated Sox1$^+$ and Sox2$^+$ cells, but no Snail2$^+$, HNK-1$^+$ or Ker$^+$ cells. Data are represented as the mean ± s.e.m. Scale bar: 100 µm. (**E**) Relative *Sp5* and *Dlx5* mRNA levels measured by quantitative real-time PCR. The mRNA expression of the Wnt target gene *Sp5*, but not of the BMP target gene *Dlx5*, is inhibited in stage 2 L explants cultured in the presence of mFrz8CRD. Bars represent mean ± s.e.m. of five independent experiments. **P*<0.05, ***P*<0.01; two-sided, one-sample *t*-test.

this, we first exposed stage 2 M explants to BMP and/or Wnt signals. BMP4 (5-10 ng/ml) blocked the generation of Sox1$^+$ Sox2$^+$ neural cells in stage 2 M explants and induced Ker$^+$ epidermal cells, but no Snail2$^+$ HNK-1$^+$ neural crest cells or Sox2$^+$ Ker$^+$ placodal cells were detected (Fig. 4B). By contrast, although Wnt3A also blocked the generation of Sox1$^+$ Sox2$^+$ neural cells, Snail2$^+$ HNK-1$^+$ neural crest cells were induced, but no or very few Ker$^+$ epidermal cells or Sox2$^+$ Ker$^+$ placodal cells were generated (Fig. 4C). BMP4 (5 ng/ml) and Wnt3A in combination also blocked the generation of Sox1$^+$ Sox2$^+$ neural cells, and, as with BMP4 alone, Ker$^+$ epidermal cells were induced but no, or very few, neural crest or placodal cells were generated (Fig. 4D). Taken together, these results provide evidence that at stage 2, Wnt signals induce neural plate border character in prospective neural cells, whereas BMP signals alone or in combination with Wnt signals impose epidermal character.

The finding that Wnt signals alone induce neural plate border cells, but that both Wnt and BMP signals are required for the specification of neural plate border cells, raised the possibility that

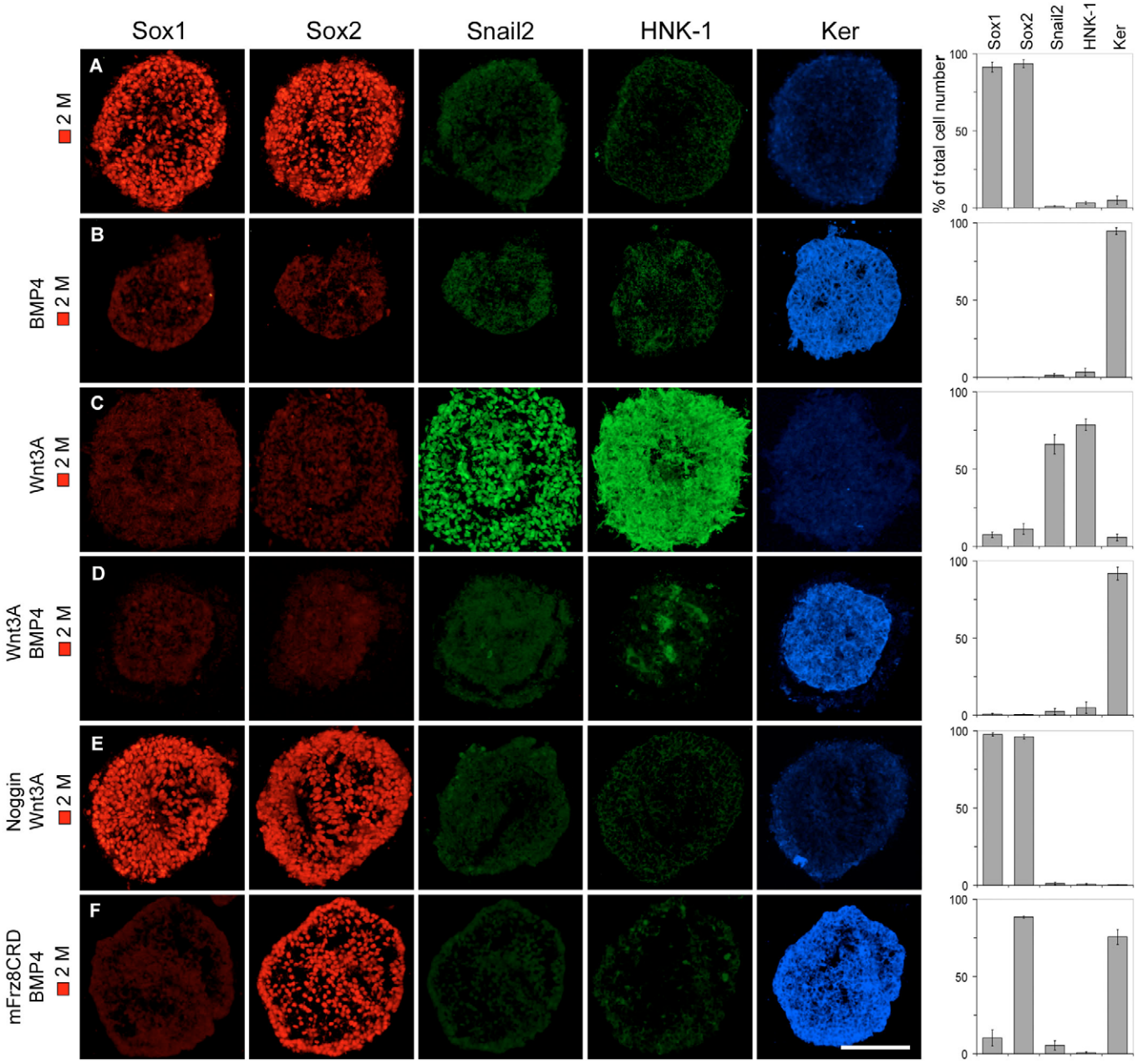

Fig. 4. BMP, but not Wnt, signals play an instructive role in the specification of neural plate border cells. Consecutive sections showing expression of molecular markers in chick explants cultured for 28 hours. (**A**) Stage 2 M explants (n>30) generated Sox1$^+$ and Sox2$^+$ cells, but no Snail2$^+$, HNK-1$^+$ or Ker$^+$ cells. (**B**) Stage 2 M explants cultured together with BMP4 (5 ng/ml) (n>30) generated Ker$^+$ cells, but no, or only a few, Sox1$^+$, Sox2$^+$, Snail2$^+$ or HNK-1$^+$ cells. (**C**) Stage 2 M explants cultured together with Wnt3A (n>30) generated Snail2$^+$ and HNK-1$^+$ cells, but no, or only a few, Sox1$^+$, Sox2$^+$ or Ker$^+$ cells. (**D**) Stage 2 M explants cultured together with Wnt3A and Noggin (n=23) generated Sox1$^+$ and Sox2$^+$ cells, but no Snail2$^+$, HNK-1$^+$ or Ker$^+$ cells. (**E**) Stage 2 M explants cultured together with Wnt3A and BMP4 (5 ng/ml) (n=25) generated Ker$^+$ cells, but no Sox1$^+$, Sox2$^+$, Snail2$^+$ or HNK-1$^+$ cells. (**F**) Stage 2 M explants cultured together with BMP4 (5 ng/ml) and mFrz8CRD (n=17) generated Sox2$^+$ and Ker$^+$ cells, but no Sox1$^+$, Snail2$^+$ or HNK-1$^+$ cells. Data are represented as the mean ± s.e.m. Scale bar: 100 μm.

the induction of neural plate border cells by Wnt signals requires BMP activity. To examine this, we exposed stage 2 M explants to Wnt3A and Noggin. Under these conditions, the generation of Snail2$^+$ and HNK-1$^+$ neural crest cells was blocked, and Sox1$^+$ Sox2$^+$ neural cells (Fig. 4E) of caudal character were generated (see Fig. S2A,B in the supplementary material), which is consistent with the previously described caudalizing activity of Wnt signals (Nordstrom et al., 2002; Patthey et al., 2008; Villanueva et al., 2002). Thus, the induction of neural plate border character in prospective neural cells by Wnt signals requires BMP activity, and, in the absence of BMP activity, Wnt signals induce caudal character in neural cells.

We next addressed whether Wnt signals induce BMP gene expression in late blastula stage prospective neural cells by culturing stage 2 M explants in the presence of Wnt3A for 10 hours and monitoring *Bmp4* mRNA levels by quantitative real-time PCR. Under

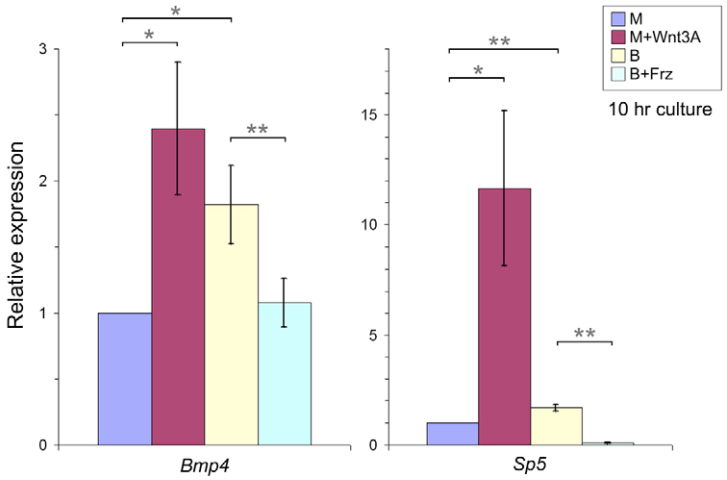

Fig. 5. Wnt signaling promotes the generation of neural crest cells by inducing *Bmp4* expression. Relative *Bmp4* and *Sp5* mRNA levels measured by quantitative real-time PCR in stage 2 M chick explants cultured in the absence (M) or presence (M+Wnt3A) of Wnt3A, or in stage 2 B explants cultured in the absence (B) or presence (B+Frz) of mFrz8CRD. Bars represent mean ± s.e.m. of five independent experiments. **P*<0.05, ***P*<0.01; two-sided, one-sample *t*-test.

these conditions, *Bmp4* mRNA levels were upregulated 2.4-fold (Fig. 5). A ~12-fold induction of *Sp5* mRNA confirmed the activation of the Wnt pathway (Fig. 5). Conversely, inhibition of Wnt activity in stage 2 B explants by exposure to mFrz8CRD downregulated *Bmp4* 1.7-fold (Fig. 5). In addition, after 10 hours of culture, *Bmp4* mRNA levels were 1.8-fold higher in stage 2 B explants than in stage 2 M explants (Fig. 5). Thus, at the late blastula stage, Wnt signals induce *Bmp4* expression in neural plate border cells. Taken together, these results indicate that Wnt signals induce BMP gene expression in prospective neural plate border cells, and that BMP activity is required for the induction of neural plate border cells.

Simultaneous exposure of epiblast cells to Wnt and BMP signals induces epidermal cells

Our results provide evidence that Wnt signals induce BMP activity that in turn induces neural plate border cells, whereas BMP signals either alone or in combination with Wnt signals induce epidermal cells. This raises the possibility that simultaneous early exposure of epiblast cells to Wnt and BMP signals generates epidermal instead of neural plate border cells, implicating in turn that at stage 2, prospective neural cells are exposed to Wnt activity. Previous results have provided evidence that Wnt signals impose dorsal character on prospective forebrain cells (Gunhaga et al., 2003). Consistent with this, stage 2 M explants cultured for 48 hours generated Sox1$^+$, BF1$^+$ and Pax6$^+$ cells (see Fig. S3A in the supplementary material), which are characteristic of dorsal forebrain cells, supporting the idea that stage 2 prospective neural cells are exposed to Wnt activity. Moreover, stage 2 M explants cultured in the presence of mFrz8CRD for 48 hours generated Sox1$^+$, BF1$^+$ and Nkx2.1$^+$ cells of ventral forebrain character at the expense of dorsal forebrain cells (see Fig. S3B in the supplementary material). Thus, at stage 2, prospective neural cells are exposed to Wnt activity, but at levels that are lower than those required to promote the generation of neural plate border cells. Collectively, these results support the idea that early simultaneous exposure of epiblast cells to Wnt and BMP signals generates epidermal instead of neural plate border cells.

To directly test whether the induction of epidermal cells requires simultaneous exposure to BMP and Wnt signals and whether, in the absence of Wnt activity, BMP signals induce neural plate border cells, we exposed stage 2 M explants to increasing concentrations of BMP4 (5-10 ng/ml) and mFrz8CRD. Under these conditions, the generation of Sox1$^+$ Sox2$^+$ neural cells was blocked, and Sox2$^+$ Ker$^+$ placodal cells were generated, but no Snail2$^+$ HNK-1$^+$ neural crest or Ker$^+$ epidermal cells appeared (Fig. 4F). After 48 hours of culture, Ker$^+$, *Raldh3*$^+$ and HuCD$^+$ cells, which are characteristic of the olfactory placode, and Ker$^+$ δ-crystallin$^+$ cells characteristic of the lens placode, were generated (data not shown). To confirm that increased levels of BMP4 correlated with increased BMP signaling, we monitored by western blotting the levels of phosphorylated (p) Smad1/5/8, indicative of active BMP signaling, in stage 2 M explants exposed to 5 or 10 ng/ml BMP4 in the presence of mFrz8CRD. Under these conditions, p-Smad1/5/8 was induced by 5 ng/ml BMP4, an effect that was blocked by Noggin (see Fig. S4 in the supplementary material), and a very large relative increase of p-Smad1/5/8 was observed using 10 ng/ml BMP4 (see Fig. S4 in the supplementary material). Thus, when Wnt activity is blocked, BMP signaling, even at high levels, induces neural plate border cells of rostral placodal character instead of epidermal cells, thereby providing evidence that simultaneous early exposure of epiblast cells to BMP and Wnt signals induces epidermal cells. These results also indicate that Wnt signals do not play an instructive role in the specification of neural plate border cells, raising the possibility that the role of Wnt signals in the initial specification of neural plate border cells is to provide temporal control of exposure to BMP signals so as to avoid simultaneous early exposure of epiblast cells to the two classes of signals.

Wnt-regulated temporal control of BMP exposure mediates the induction of neural plate border cells

To address whether the temporal order of exposure of epiblast cells to Wnt and BMP signals is important for the induction of neural plate border cells, we first compared the expression of *Bmp4* and of the BMP target gene *Dlx5* in stage 2 B and stage 4 caudal neural plate border (CB) explants. The levels of *Bmp4* and *Dlx5* mRNA were 6.0- and 14.7-fold higher, respectively, in stage 4 CB explants than in stage 2 B explants, suggesting that BMP activity is upregulated in prospective neural plate border cells around the late gastrula stage (see Fig. S5A in the supplementary material). Next, we compared the mRNA levels of the Wnt target gene *Sp5* in stage 2 B, stage 4 RB and stage 4 CB explants. Compared with stage 2 B explants, *Sp5* mRNA levels were 2.5-fold lower in stage 4 RB explants and 3.1-fold higher in stage 4 CB explants (see Fig. S5B in the supplementary material). Thus, these results provide evidence that at stage 2, prospective neural plate border cells are exposed to Wnt signals, and around the late gastrula stage caudal neural plate border cells are exposed to both Wnt and BMP signals, whereas rostral neural plate border cells are exposed to BMP signals in the absence of Wnt activity.

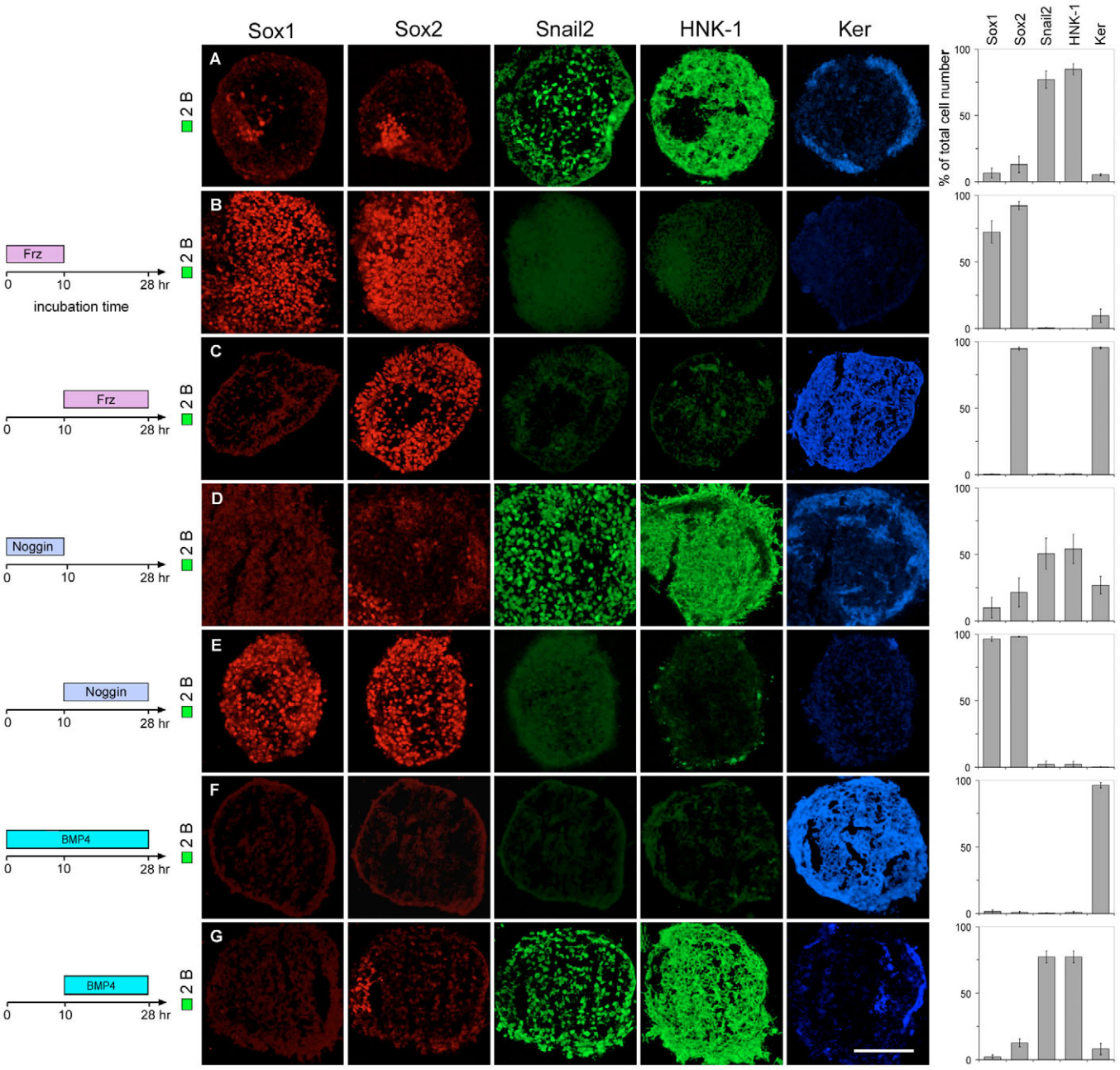

Fig. 6. Wnt-regulated temporal control of BMP exposure mediates the induction of neural plate border cells. Consecutive sections showing expression of molecular markers in chick explants cultured for 28 hours. The schematics to the left illustrate the periods of exposure to mFrz8CRD (Frz), Noggin and BMP4 during the culture. (**A**) Stage 2 B explants (*n*>30) generated Snail2$^+$ and HNK-1$^+$ cells, but no, or only a few Sox1$^+$, Sox2$^+$ or Ker$^+$ cells. (**B**) Stage 2 B explants cultured together with mFrz8CRD for the first 10 hours of culture (*n*=29) generated Sox1$^+$ and Sox2$^+$ cells, but no Snail2$^+$, HNK-1$^+$ or Ker$^+$ cells. (**C**) Stage 2 B explants cultured together with mFrz8CRD for the last 18 hours of culture (*n*>30) generated Sox2$^+$ and Ker$^+$ cells, but no Sox1$^+$, Snail2$^+$ or HNK-1$^+$ cells. (**D**) Stage 2 B explants cultured together with Noggin for the first 10 hours of culture (*n*=17) generated Snail2$^+$ and HNK-1$^+$ cells, and a few Sox2$^+$ and Ker$^+$ cells, but no Sox1$^+$ cells. (**E**) Stage 2 B explants cultured together with Noggin for the last 18 hours of culture (*n*=16) generated Sox1$^+$ and Sox2$^+$ cells, but no Snail2$^+$, HNK-1$^+$ or Ker$^+$ cells. (**F**) Stage 2 B explants cultured together with BMP4 (5 ng/ml) for the entire culture period (*n*=27) generated Ker$^+$ cells, but no Sox1$^+$, Sox2$^+$, Snail2$^+$ or HNK-1$^+$ cells. (**G**) Stage 2 B explants cultured together with BMP4 (5 ng/ml) for the last 18 hours of culture (*n*>30) generated Snail2$^+$ and HNK-1$^+$ cells, and a few Sox2$^+$ and Ker$^+$ cells, but no Sox1$^+$ cells. Data are represented as the mean ± s.e.m. Scale bar: 100 μm.

We next examined the temporal requirement for Wnt activity in the specification of neural plate border cells. We blocked Wnt activity in prospective neural plate border cells at different times in culture by exposing stage 2 B explants to mFrz8CRD for the first 10 hours, or the last 18 hours, of a total 28 hours of culture (10 hours of culture corresponds to a stage ~4 embryo). In stage 2 B explants cultured in the presence of mFrz8CRD for the first 10 hours of culture, the generation of Snail2$^+$ and HNK-1$^+$ neural crest cells was blocked and Sox1$^+$ Sox2$^+$ neural cells were generated, but no cells of epidermal or prospective placodal character appeared (Fig. 6B).

By contrast, in stage 2 B explants exposed to mFrz8CRD from 10 to 28 hours of culture, the generation of Snail2$^+$ and HNK-1$^+$ neural crest cells was blocked and instead Sox2$^+$ and Ker$^+$ placodal cells, but no Sox1$^+$ neural cells, were generated (Fig. 6C). After 48 hours of culture, Ker$^+$, *Raldh3*$^+$ and HuCD$^+$ cells, which are characteristic of the olfactory placode, and Ker$^+$ δ-crystallin$^+$ cells characteristic of the lens placode, were generated (data not shown). Thus, the initial specification of neural plate border cells requires an early phase (~10 hours) but not late phase of Wnt signaling, and, in agreement with previous results (Patthey et al., 2008), prolonged exposure of prospective neural plate border cells to Wnt signals induces neural crest at the expense of olfactory/lens placodal cells.

The early requirement for Wnt signals in the specification of neural plate border cells, and the finding that BMP but not Wnt signals block the generation of neural cells, suggest that neural plate border cells are derived from cells that are initially of neural character. In support of this, previous studies have shown that *Sox2* mRNA can be transiently detected in the prospective neural crest region but not in the prospective epidermal area in chick embryos (Wakamatsu et al., 2004). Consistently, after 8 hours of culture, cells in stage 2 B, or stage 2 M explants grown alone or together with Wnt3A expressed Sox2 and Otx2 (see Fig. S6A-C in the supplementary material), which is characteristic of neural cells (Bally-Cuif et al., 1995; Rex et al., 1997). By contrast, exposure of stage 2 B explants to BMP4 (5 ng/ml) for 8 hours inhibited the generation of Sox2$^+$ and Otx2$^+$ neural cells (see Fig. S6D in the supplementary material). Thus, under these explant conditions, neural plate border cells are derived from cells that initially exhibit neural character.

We next examined the temporal requirement of exposure to BMP signals for the generation of neural plate border cells. Stage 2 B explants were cultured in the presence of Noggin for the first 10 hours, or the last 18 hours, of a total 28 hours of culture. Stage 2 B explants exposed to Noggin for only the first 10 hours of culture still generated Snail2$^+$ and HNK-1$^+$ cells and a few Ker$^+$ cells, but no Sox1$^+$ or Sox2$^+$ cells (Fig. 6D). By contrast, when stage 2 B explants were exposed to Noggin between 10 and 28 hours of culture, the generation of Snail2$^+$ HNK-1$^+$ neural crest cells was blocked and Sox1$^+$ Sox2$^+$ neural cells, but no Ker$^+$ epidermal cells, were generated (Fig. 6E). Thus, the specification of neural plate border cells requires a late but not early phase of BMP activity.

To confirm that the absence of BMP signals is required during the early Wnt-dependent phase of neural plate border induction, we first compared the expression of *Bmp4* and of the BMP target gene *Dlx5* in stage 2 B and stage 2 L explants before culture. Consistently, the levels of *Bmp4* and *Dlx5* mRNA were 4.1-fold and 3.5-fold higher, respectively, in stage 2 L explants than in stage 2 B explants (see Fig. S7 in the supplementary material). Next, we cultured stage 2 B explants in the presence of BMP4 (5 ng/ml) for the first 10 hours of culture or for the entire culture period. Under either condition, the generation of Snail2$^+$ and HNK-1$^+$ neural crest cells was blocked, and Ker$^+$ epidermal cells were generated (Fig. 6F; see Fig. S8B in the supplementary material). By contrast, when BMP4 (5 ng/ml) was added after 10 hours of culture to stage 2 B explants, or when stage 2 M explants were exposed to Wnt3A for the entire culture period and to BMP4 after 10 hours culture, Snail2$^+$ and HNK1$^+$ neural crest cells were generated (Fig. 6G; see Fig. S8D in the supplementary material). Thus, the absence of BMP activity is required during the early Wnt-dependent phase of neural plate border induction. Taken together, these results indicate that the Wnt-mediated induction of BMP gene expression avoids the simultaneous exposure of prospective neural plate border cells to BMP and Wnt signals, thereby ensuring that neural plate border cells are generated instead of epidermal cells. Exposure of epiblast cells to BMP signals in the absence of Wnt signals generates rostral neural plate border cells.

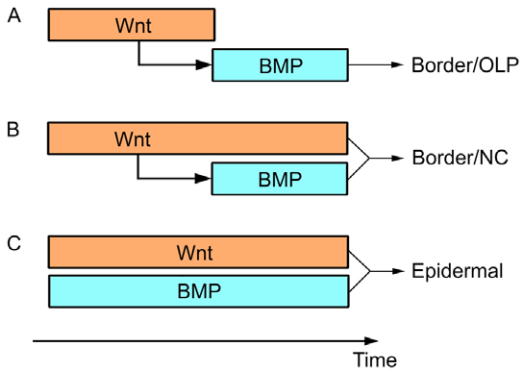

Fig. 7. Early patterning of the chick non-neural ectoderm is regulated by temporal exposure to Wnt and BMP signals. Proposed Wnt and BMP signaling events required for the specification of rostral and caudal neural plate border cells, and of epidermal cells. (**A**) An early phase of Wnt signals and a late phase of BMP signals specify rostral neural plate border cells of olfactory and lens placodal (OLP) character. (**B**) An early phase of Wnt signals and a late phase of both Wnt and BMP signals specify caudal neural plate border cells of neural crest (NC) character. (**C**) An early and a late phase of both Wnt and BMP signals specify cells of epidermal character.

DISCUSSION

Early in development, the embryonic ectoderm becomes subdivided along the mediolateral axis into neural, neural plate border and epidermal cells. At the early blastula stage, Wnt and BMP signals interact to block neural fate and to induce epidermal cells (Basch et al., 2006; Nordstrom et al., 2002; Patthey et al., 2008; Sjodal et al., 2007; Tucker et al., 2008; Villanueva et al., 2002; Wilson and Edlund, 2001). We now provide evidence that the specification of neural plate border cells is initiated ~8 hours later, at the late blastula stage, and also requires Wnt and BMP signals. We also conclude that Wnt signals do not act in an instructive manner during the specification of neural plate border cells, but act by inducing BMP gene expression, which avoids early simultaneous exposure to the two signals and thereby generates neural plate border instead of epidermal cells (Fig. 7). Thus, the specification of the neural plate border is mediated by a novel Wnt-regulated BMP-mediated temporal patterning mechanism.

Our results indicate that the induction of epidermal cells is mediated by early simultaneous exposure to Wnt and BMP signals, and that the induction of neural plate border cells requires early temporal separation of Wnt and BMP activity. The signaling mechanism whereby early convergent Wnt and BMP signals induce epidermal instead of neural plate border cells remains, however, to be established. A possible level of integration is at the convergence of Wnt/β-catenin and Smad signals on a common promoter, as is the case in *vent* and *vox* activation during mesoderm patterning in zebrafish (Ramel and Lekven, 2004). Similarly, it has been shown in vitro that Lef1/β-catenin and Smad4/Smad1 interact on the *Msx2* promoter, explaining the synergy between these two pathways in *Msx2* induction (Hussein et al., 2003). A recent study has also suggested that Wnt activity stabilizes Smad1, which results in prolonged Smad activity in response to BMP signals (Fuentealba et

al., 2007), providing a possible molecular explanation for the cooperation between Wnt and BMP signals. However, this mechanism seems unlikely because our results indicate that when Wnt signals are blocked in prospective epidermal cells, the BMP target gene *Dlx5* is still expressed at the same levels as in untreated cells, even after 10 hours of culture, and under these conditions prospective epidermal cells acquire a rostral neural plate border character.

Previous results in *Xenopus*, zebrafish and chick have suggested that the generation of neural plate border or epidermal cells is regulated by the level of BMP signals, such that epidermal cells are generated at higher levels of BMP activity (Raible, 2006; Schlosser, 2006). Our results provide evidence, however, that simultaneous early exposure of epiblast cells to Wnt and BMP signals induces epidermal cells, and that the induction of neural plate border cells requires an early phase of Wnt and a later phase of BMP exposure, thereby avoiding simultaneous early exposure to both signals. In support of our model, we show that both Wnt and BMP signals are required for the specification of epidermal cells. In the absence of BMP activity, prospective epidermal cells acquire neural character, whereas in the absence of Wnt signals cells acquire rostral placodal character in response to BMP activity. Furthermore, when Wnt activity is inhibited in prospective neural cells, BMP signals induce neural plate border cells of olfactory/lens placodal character instead of epidermal cells, even at high levels of BMP and p-Smad1/5/8 signals. Thus, the initial specification of epidermal or neural plate border cells at the late blastula stage does not appear to be mediated by the level of BMP signals; instead, simultaneous early exposure to Wnt and BMP signals induces epidermal fate. It remains possible, however, that the precise molecular mechanism underlying the initial differential specification of neural plate border and epidermal fate might differ between chick, *Xenopus* and zebrafish, and that different levels of BMP signals influence the maintenance of these fates at later stages of development.

At the late gastrula stage, neural plate border cells give rise to olfactory and lens placodal cells at rostral levels and to neural crest cells at caudal levels of the neuraxis (Couly et al., 1993; Couly and Le Douarin, 1985). We now provide evidence, however, that at the late blastula stage and independent of rostrocaudal position, prospective neural plate border cells are initially specified as neural crest cells in response to Wnt-regulated exposure to BMP signals. These results are in agreement with a recent study showing that *Snail1*, a marker of caudal neural plate border cells, is transiently expressed in rostral neural plate border cells in early gastrula stage *Xenopus* embryos (Carmona-Fontaine et al., 2007). Our results also indicate that when Wnt signals induce BMP signals in prospective neural plate border cells and Wnt activity is thereafter inhibited, cells acquire olfactory/lens placodal character (Fig. 7). Thus, these results suggest that the specification of rostral neural plate border cells at late gastrula stages in response to BMP signals requires the absence, or low levels, of Wnt signals, which is mediated, at least in part, by exposure to Wnt inhibitors. These results are in agreement with previous results showing that at the late gastrula stage, Wnt signals impose caudal character on neural plate border cells (Litsiou et al., 2005; Patthey et al., 2008; Villanueva et al., 2002), and that the most rostral part of the embryo is exposed to the Wnt antagonists dickkopf 1 and crescent (Carmona-Fontaine et al., 2007; Chapman et al., 2004). Similarly, previous studies in chick and zebrafish have provided evidence that around the gastrula stages, the specification of the most rostral part of the central nervous system, the telencephalon, requires the absence of Wnt signals (Houart et al., 2002; Kudoh et al., 2002; Nordstrom et al., 2002). Taken together, our results provide evidence that Wnt signals play distinct temporal roles during the generation of neural crest cells. An early phase of non-instructive Wnt activity at the late blastula stage mediates temporal exposure of epiblast cells to BMP signals that specify neural plate border cells, and a later phase of Wnt activity, corresponding to the late gastrula stage, induces caudal/neural crest character in prospective neural plate border cells.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/1/73/DC1

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