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Bimodal functions of Notch-mediated signaling are involved in neural crest formation during avian ectoderm development

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SUMMARY

Neural crest is induced at the junction of epidermal ectoderm and neural plate by the mutual interaction of these tissues. In previous studies, BMP4 has been shown to pattern the ectodermal tissues, and BMP4 can induce neural crest cells from the neural plate. In this study, we show that epidermally expressed *Delta1*, which encodes a Notch ligand, is required for the activation and/or maintenance of *Bmp4* expression in this tissue, and is thus indirectly required for neural crest induction by BMP4 at the epidermis-neural plate boundary. Notch activation in the epidermis additionally inhibits neural crest formation in this tissue, so that neural crest generation by BMP4 is restricted to the junction.

Key words: Neural crest, Notch signaling, Slug, BMP4, Ectoderm, Neural plate, Quail

INTRODUCTION

The neural crest is a vertebrate-specific group of cells formed at the boundary of neural and epidermal ectoderm (Le Douarin and Kalcheim, 1999). Neural crest cells undergo epithelial-mesenchymal transition, delaminate from the epithelial ectoderm, and subsequently migrate on distinct pathways to give rise to various tissues including neurons, glial cells and melanocytes, as well as cranial skeletogenic mesenchyme. Neural crest cells can be identified in the neural fold of avian and *Xenopus* embryos by the expression of a zinc-finger transcription factor, *Slug* (Nieto et al., 1994; Linker et al., 2000), before the epithelial-mesenchymal transition. *Slug* has been shown to regulate neural crest formation and subsequent delamination (Nieto et al., 1994; LaBonne and Bronner-Fraser, 2000).

How are neural crest cells specified at the junction of the prospective neural ectoderm and the prospective epidermis? In vertebrates, bone morphogenetic protein (BMP)-mediated signaling is important for the specification of epidermal ectoderm. In *Xenopus*, for example, *Bmp4* is initially expressed throughout the ectoderm, but BMP antagonists such as *chordin*, *noggin* and *follistatin* cause neural ectoderm to be specified in the vicinity of the dorsal midline (reviewed by Sasai and De Robertis, 1997). As neural ectoderm is specified, *Bmp4* expression becomes restricted to the epidermal region. In avian embryos, it has been shown that BMP and fibroblast growth factor (FGF) signaling define the boundary of these ectodermal derivatives (Streit and Stern, 1999), and neural and epidermal fate is regulated by the state of Wnt signaling (Wilson et al., 2001). It has been proposed in *Xenopus* that neural ectoderm, neural crest and epidermis are specified by different concentrations of BMP, with an intermediate concentration of BMP-inducing neural crest (Marchant et al., 1998). It has been shown both in avian and amphibian embryos that neural crest is generated when neural plate and epidermal ectoderm are juxtaposed. For example, neural crest is induced when a medial fragment of neural plate, which normally does not generate neural crest, is transplanted into the prospective epidermal region, where the BMP concentration is high (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Dickinson et al., 1995; Mancilla and Mayor, 1996). Furthermore, when neural plate and epidermal ectoderm explants are co-cultured, expression of neural crest markers becomes detectable (Selleck and Bronner-Fraser, 1995; Selleck and Bronner-Fraser, 2000). Thus, these observations suggest that neural crest induction may be a secondary event after the specification of neural and epidermal ectoderm. As neural crest is generated from both tissues when transplanted, the interaction of these tissues seems to be reciprocal. In this model, BMP4 is one of the important molecules for neural crest formation. Consistently, BMP4 can induce *Slug* expression and subsequent neural crest segregation from medial neural plate explants taken from avian embryos (Liem et al., 1995). In *Bmp2* knockout mouse embryos, moreover, generation of neural crest cells in the cranial region is severely reduced, implicating BMP-mediated signaling in this process (Kanzler et al., 2000).

In addition to BMP signaling, the involvement of Wnt, FGF and Notch signaling in neural crest induction has been suggested (Coffman et al., 1993; LaBonne and Bronner-Fraser, 1998; Cornell and Eisen, 2000). Notch is a membrane-bound receptor that has been shown to regulate many developmental processes in both vertebrates and invertebrates (reviewed by Artavanis-Tsakonas et al., 1999). Unlike BMP, Wnt and FGF

signaling, all of which are mediated by secreted ligands, the ligands for Notch receptors are predominantly membrane bound, so that Notch signaling occurs between immediate neighbors. Injection of mRNA encoding a constitutively activated form of Notch into early *Xenopus* embryos inhibits cranial neural crest formation, as monitored by *twist* expression, while the neural plate expands and the epidermis regresses (Coffman et al., 1993). It is not clear, however, whether the inhibitory effect of Notch activation on neural crest formation is direct, or a secondary consequence of the expansion of the neural plate.

We show that the Notch signaling pathway has dual functions for neural crest formation: (1) maintenance of *Bmp4* expression to promote neural crest specification; and (2) inhibition of *Slug* expression and subsequent delamination of crest cells from the ectoderm. We propose that bimodal functions of Notch signaling restrict neural crest formation at the neural and epidermal ectoderm boundary.

MATERIALS AND METHODS

Experimental animals

Japanese quail (*Coturnix coturnix japonica*) eggs were obtained from Sendai Jun-ran, Sendai. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Antibodies and immunological staining

62.1E6 anti-Slug (mouse IgG1; a kind gift from Dr Jessell) (Liem et al., 1995) and HNK-1 (mouse IgM) (Tucker et al., 1988) antibodies were used as described previously. M2 anti-Flag (mouse IgG1, Sigma), anti-Flag (rabbit polyclonal, Zymed) and anti-GFP (mouse IgG, Clontech) antibodies were purchased from commercial suppliers. Fluorochrome or enzyme-conjugated secondary antibodies were purchased from Southern Biotechnologies (anti-mouse IgM-TRITC), and Jackson (anti-rabbit IgG-FITC, anti-mouse IgG-cy3 and anti-mouse IgM-HRP).

Immunological staining on sections was performed as described previously (Wakamatsu et al., 1993). Cryosections (8 μm) were prepared on VectaBond coated slides (Vector). Sections treated with antibodies were also exposed to DAPI (Sigma) to visualize nuclei, and subsequently mounted with VectaShield mounting medium (Vector). Fluorescent images were captured by a cooled CCD camera (COOL SNAP, Roper) on Zeiss AxioplanII microscope, and processed using Adobe Photoshop (version 5) software.

Whole-mount immunostaining using HNK-1 antibody was performed essentially as described (Wakamatsu and Weston, 1997) with a few modifications. Briefly, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 3 hours. The fixed embryos were bleached in H_2O_2/methanol for several hours. The embryos were then rehydrated in PBS, and heated for 30 minutes at 63°C. Blocking was performed in TTBST (150 mM NaCl, 100 mM TirsHCl (pH 7.5), 0.1% Tween 20, 0.5% Triton X-100) containing 10% heat-inactivated goat serum for 1.5 hours, followed by overnight incubation with HNK-1-containing hybridoma supernatant in the blocking solution at 4°C. After extensive washing in TTBST, embryos were incubated with a secondary antibody (anti-mouse IgM-HRP) diluted in the blocking solution at 4°C overnight. After washing in TTBST, the localization of HNK-1 immunoreactivity was visualized by the DAB color reaction.

In situ hybridization

In situ hybridization on sections and in wholemounts was performed as described previously (Wakamatsu and Weston, 1997). Detailed protocols are available upon request. The use of quail *Slug*, *Sox2*, *Notch1*, *Notch2*, *Delta1*, *Serrate1* probes have been described previously (Wakamatsu et al., 2000). A fragment of chicken *Bmp4* probe was prepared from E2 embryos with SuperscriptII (Gibco) as a template, according to the previously described sequence (Francis et al., 1994). Chicken *Hairy1*, *Hairy2*, *Pdgfrα*, *Serrate2* and *Keratin19* cDNAs were generous gifts from Drs Pourquie, Richardson, Henrique and Yasugi, respectively (Palmeirim et al., 1997; Jouve et al., 2000; Ataliotis, 2000; Laufer et al., 1997; Sato and Yasugi, 1997).

Expression vectors

Flag epitope-tagged expression vectors of jellyfish green fluorescent protein (*GFP*), *CNIC* (constitutively active form of chick *Notch1*) and *CNIC*$^{\Delta C89}$ (*CNIC* that lacks C-terminal end) have been described previously (Wakamatsu and Weston, 1997; Wakamatsu et al., 1999; Wakamatsu et al., 2000). pEGFP-N1 was purchased from Clontech. RCAS (B)-chicken *Delta1*^{stu} was kindly provided by Dr Henrique (Henrique et al., 1997). Quail *Delta1* in pcDNA3.1 (Maynard et al., 2000) was digested with *Xba*I and *Hin*dIII, and subcloned into an expression vector, pmiwSV (Wakamatsu et al., 1997). Subsequently, the intracellular domain of quail *Delta1* was removed by *Stu*I and *Xba*I digestion to make the pmiw-quail *Delta1*^{stu} construct. RCAS (B)-chicken *Delta1*^{stu} and pmiw-quail *Delta1*^{stu} gave identical results in the misexpression experiments when transfected as naked DNAs (and thus were used interchangeably). Expression vectors of *Xenopus Noggin* and mouse *Bmp4* were provided by Dr Takahashi with permission from Drs Sasai and Ueno (Tonegawa et al., 1997; Tonegawa and Takahashi, 1998). An expression vector of *X-Su(H)*^{DBM} was kindly provided by Dr Kintner (Wettstein et al., 1997), and the insert was subcloned into pmiwSV for transfection studies.

Whole embryo culture and electroporation

Fertilized quail eggs were incubated at 38°C in a humidified atmosphere for 20 hours to obtain stage 5-6 embryos. Embryos were cut from the yolk, transferred with the vitelline membrane to Hanks BSS (137 mM NaCl, 5.4 mM KCl, 5.6 mM glucose, 0.34 mM NaH_2PO_4, 10 mM Hepes), and the excess yolk was carefully removed. The embryos were then carefully detached from the vitelline membrane and transferred to fresh Hanks BSS. Collagen-coated filter membranes were prepared in advance by soaking Millipore filter membranes (JHWP01300, Millipore) in 0.17% acetic acid containing 0.25 mg/ml collagen (C-7661, Sigma) at 4°C overnight, and then washing twice in sterilized water. A hole was made in the center of the membranes, and several radial cuts were made around the hole, so that embryos would adhere easily to the membranes (Fig. 1B). Petri dishes (35 mm) were filled with a pre-culture medium (Hanks BSS diluted with an equal volume of the thin albumen), and the vitelline membranes, as isolated above, were transferred to the dishes. The embryos were kept at 20°C for a few hours to allow them to attach to the membrane.

Before the electroporation, a chamber with a 2 mm^2 positive electrode (Unique Medical Imada) was filled with Hanks BSS (Fig. 1A, Fig. 2). The embryo on the filter membrane was then set on the platform of the chamber with its dorsal side upwards (Fig. 1A,B). A tungsten needle was used as the negative electrode (Unique Medical Imada, Fig. 1B). DNA solution (1.2 μl of 5 μg/μl in PBS containing 0.025% Fast Green) was carefully placed on the right prospective neural fold of the embryo (Fig. 1A, part 1). In most cases, the electroporator (CUY21, Tokiwa Science) applied three rectangular pulses (7 V, 25 msecond duration) at 200 mseconds. To achieve lower transfection efficiency, 5 V was used. Silpot culture wells were prepared, in advance, by punching a 10 mm diameter hole in 1.5 mm thick and 1.5 cm square silpot (SILPOT 184 w/c, Dow Corning) and were washed in sterilized water and subsequently in 70% ethanol (Marusich and Weston, 1992). The well was placed in the center of a 35 mm Petri dish and the vitelline membrane was transferred to the dish (Fig. 1A, part 3). After electroporation, the embryo was placed

on the vitelline membrane with the ventral side up and the dish was filled with culture medium (F12 containing 5% fetal bovine serum diluted with three volumes of thin albumen) (Fig. 1A, part 3). The embryo with the vitelline membrane was placed over the hole of the silpot culture well in the dish, and was incubated in a moist box at 38°C (Fig. 1A, part 3). The efficiency of the electroporation was monitored by GFP expression (Fig. 1D) under a fluorescence dissection microscope (MZFLIII, Leica). Under this condition, we did not detect significantly increased cell death on the transfected side of the embryo, except when high concentration of *Bmp4* expression vector was used. For misexpression studies, pEGFP-N1 and the other expression vector were usually mixed at 1:1. When the *Bmp4* expression vector was co-transfected with pEGFP-N1, the ratio was 1:19 to minimize BMP4-induced apoptosis (see Results). When the *Bmp4* expression vector was co-transfected with pEGFP-N1 and a third expression vector, the ratio was 1:9:10.

RESULTS

Expression of Notch1 and a Notch ligand, Delta1 during neural crest formation

To correlate the expression of *Notch* receptors and ligands with neural crest formation, the expression of *Notch1*, *Notch2*, *Delta1*, *Serrate1*, and *Serrate2* were examined in whole-mount preparations of at the 1-3 somite stage (Hamburger and Hamilton stage 6-8) quail embryos, and compared with the expression of the neural crest marker *Slug* (Nieto et al., 1994) and that of *Pdgfrα*, which is expressed in the head fold (Takakura et al., 1997; Le Douarin and Kalcheim, 1999) and in the skeletogenic head mesenchyme (Schatteman et al., 1992; Morrison-Graham et al., 1992). *Slug* expression was initially observed as faint, broad staining around the prospective head fold region at the one- to two-somite stage (Fig. 2A,B). As development proceeded, *Slug* expression became restricted to the head-fold region and was upregulated in the region of premigratory neural crest (Fig. 2C). Expression of *Pdgfrα* began slightly later (Fig. 2E), but the expression domain overlapped well with that of *Slug* (Fig. 2E,F). Among the genes encoding Notch receptors and ligands, only *Notch1* and *Delta1* mRNAs were detected in the head fold region (Fig. 2G-L), so we focused on these genes for the rest of our analysis. *Notch1* is expressed broadly in the ectoderm, with higher levels in the neural plate region (Fig. 2J). At later stages, *Notch1* expression was less clear in the epidermal region, and high levels of *Notch1* expression were observed both in the neural plate and the head fold (Fig. 2K,L). Although *Delta1* expression was observed in the neural plate (Fig. 2I, asterisk) and paraxial mesoderm (Fig. 2G, arrow), it was also detected in the epidermal ectoderm, with higher levels in the head fold (Fig. 2G-I).

To correlate the expression domains of *Notch1*, *Delta1* and *Slug* in the ectoderm, we examined the expression of these genes together with a neural plate marker *Sox2* on neighboring sections. At the one-somite stage, when *Slug* expression was faintly detected (Fig. 3E), *Notch1* expression was observed throughout the ectoderm (Fig. 3A), and *Delta1* expression was restricted to the epidermal region (Fig. 3C). *Sox2* was clearly expressed in the neural plate as described previously (Streit and Stern, 1999), but at the boundary of the neural plate and epidermis, the expression was only weakly detectable (Fig. 3G). At the five-somite stage, *Slug* expression could be detected clearly in presumptive neural crest cells in the neural fold (Fig. 3F). The *Delta1* expression domain was distinct from the *Slug* domain and was restricted to epidermal ectoderm (Fig. 3D), while *Notch1* expression was detected weakly in the epidermal ectoderm and strongly in the neural plate (Fig. 3B). Strong *Sox2* expression was observed in the neural plate, but

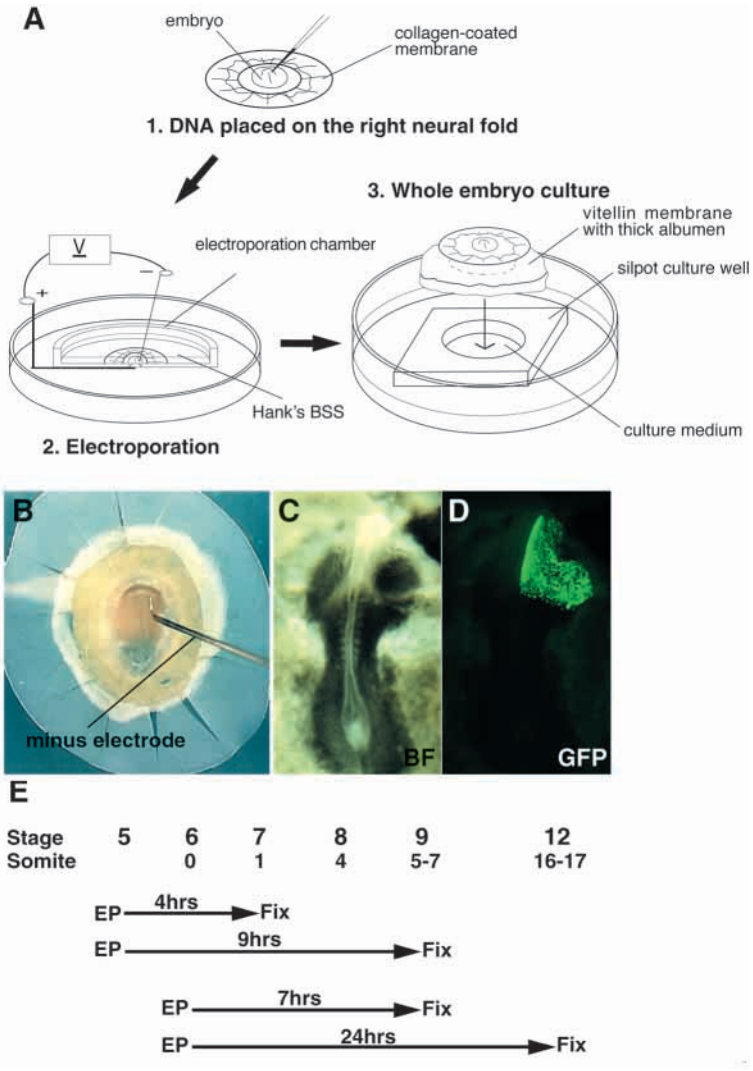

Fig. 1. Electroporation of expression plasmids into ectoderm of cultured quail embryos. (A) Electroporation and whole embryo culture. Plasmid DNA is placed on the dorsal surface of the stage 5-6 quail embryo adhering to the collagen-coated membrane (1). Electroporation is performed in the chamber filled with Hanks BSS (2). The embryo is cultured on vitelline membrane in culture medium (3). (B) A demonstrative picture showing how electroporation is performed. As a negative electrode, L-shaped tungsten needle is used. The tip of the electrode is placed over the right side of the ectoderm. The square-shaped positive electrode is fixed underneath the embryo. (C,D) An example of cultured embryo. Electroporation was performed at stage 6, and cultured until stage 10 (approximately 12 hours). Expression of GFP is visible in the right side of the embryo. BF, bright field. (E) Correlation of schedule of electroporation (EP), culture length, Hamburger and Hamilton stages and somite number.

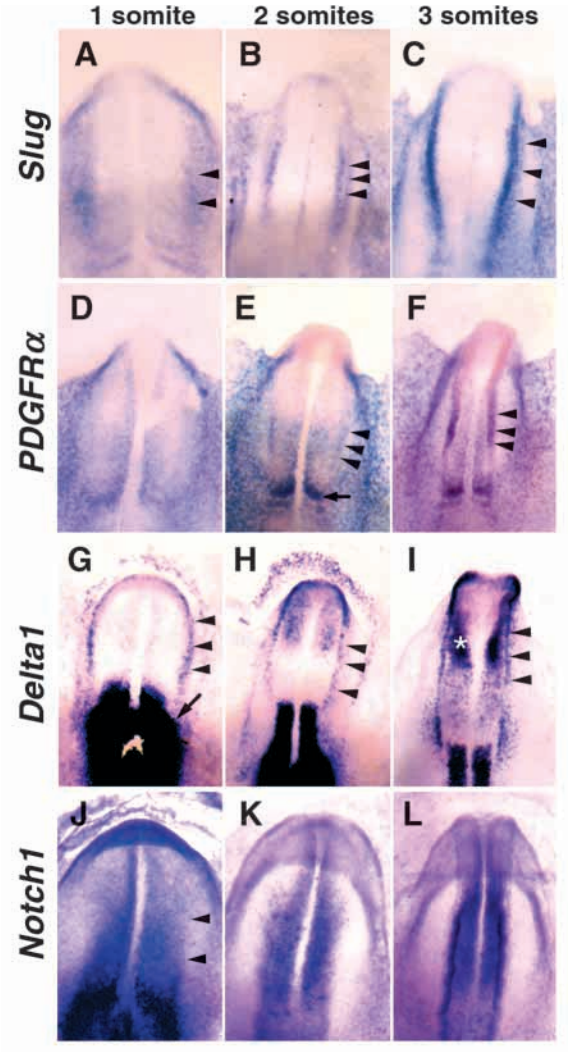

Fig. 2. *Notch1* and *Delta1* are expressed in the neural fold of quail embryos during neural crest formation. (A-C) *Slug* expression in the premigratory neural crest cells (arrowheads). At the one-somite stage, *Slug* expression is barely detectable in whole-mount preparation (but see Fig. 3E). (D-F) *Pdgfrα* expression in the premigratory cranial crest cells (arrowheads). An arrow in E indicates somite expression. (G-I) *Delta1* expression in the epidermal ectoderm (arrowheads). Arrow in G indicates strong expression in presomitic mesoderm. Asterisk in I indicates expression in the neural plate. (J-L) Broad expression of *Notch1*. Arrowheads in J indicate the boundary of the neural plate and the epidermal ectoderm.

expression was barely detectable in the *Slug*-positive cells (Fig. 3H). Taken together, *Delta1* expression is restricted to the epidermal ectoderm together with *Notch1* during neural crest formation, so that Notch activation by Delta1 is likely to occur only in the epidermal ectoderm.

Notch signaling regulates neural crest formation without affecting neural plate and epidermal ectoderm development

Electroporation of expression constructs into the ectoderm of cultured quail embryos

Recently, electroporation has become a powerful tool to introduce DNA into embryonic tissue, particularly in the avian system (reviewed by Yasugi and Nakamura, 2000). Thus, to study the function of Notch signaling in neural crest formation, we adopted the electroporation method for use on whole avian embryos cultured in vitro (see Fig. 1 and Materials and Methods section for details). Stage 5-6 embryos were cultured on a membrane, and the cultured embryos successfully developed to stage 10-12 (Fig. 1C). This method allowed easy access to the ectoderm, from which neural crest cells segregate. Expression vector DNAs were locally applied onto the right side of the embryo, and after electroporation, the expression of exogenous reporter genes such as GFP could be detected in the right neural fold, as well as in the neural plate and the epidermal ectoderm (Fig. 1D). The conditions for the electroporation were carefully determined to allow normal development of cultured quail embryos. The timetable of electroporation and embryo fixation is indicated in Fig. 1E.

Notch activation in the neural fold inhibits neural crest formation

To elucidate the function of Notch signaling in neural crest formation, an expression vector of the constitutively active forms of chicken *Notch1*, Flag-epitope-tagged cytoplasmic domain was electroporated with a GFP expression vector into the ectoderm of stage 6 embryos. In most cases, transfection

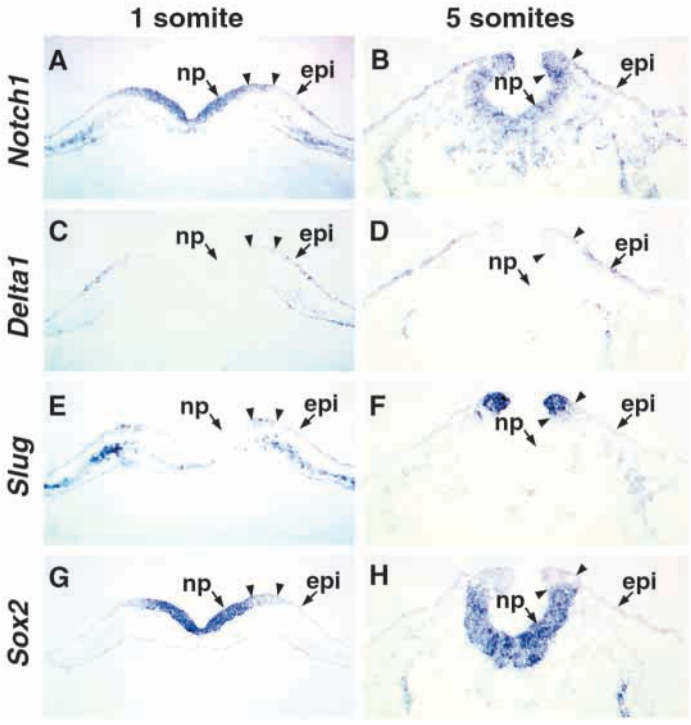

Fig. 3. *Delta1* expression is restricted to the epidermal side of the ectoderm. Neighboring sections of stage 7 (one-somite stage) and stage 9 (five-somite stage) were hybridized with *Notch1*, *Delta1*, *Slug* and *Sox2* probes. *Notch1* is expressed in broadly in the ectoderm (A,B). *Delta1* mRNA expression (C,D) is highly localized in the epidermal ectoderm, and shows a sharp boundary juxtaposed to the neural crest domain, marked by *Slug* expression (E,F). *Sox2* is expressed in the neural plate, and downregulated in the neural crest domain (G,H).

of the full-length cytoplasmic domain of chicken *Notch1* (*CNIC*) caused severe reduction of endogenous *Slug* expression in the transfected neural fold 7 hours after electroporation (four out of six cases; Fig. 4C,D). Transfection of the cytoplasmic domain without the PEST sequence at the C-terminal end (*CNIC*$^{\Delta C89}$) also caused clear reduction or loss of expression of both *Slug* and *Pdgfrα* (*Slug*, nine out of nine cases, Fig. 4E,F; *Pdgfrα*, seven out of eight cases, Fig. 4I,J). No significant effect was observed on *Slug* (none out of five cases) or *Pdgfrα* expression (none out of nine cases) when only *GFP* was transfected (Fig. 4A,B,G,H).

To examine the downregulation of *Slug* in more detail, sections of *CNIC*$^{\Delta C89}$-transfected embryos were double-stained with anti-Slug and anti-Flag antibodies (Fig. 5A-F). No obvious increase in cell death was observed, based on the morphology of the nuclei (Fig. 5D). In most cases, *CNIC*$^{\Delta C89}$-transfected Flag-positive cells appeared to have lost their endogenous Slug protein expression, suggesting that *CNIC*$^{\Delta C89}$-transfected neural fold cells failed to differentiate into neural crest (Fig. 5D-F). The loss of Slug expression caused by *CNIC*$^{\Delta C89}$-transfection later coincided with a decrease in migrating neural crest cells (Fig. 5G,H,M,N). Endogenous expression of *Slug* diminished during migration, but with some overlap; migrating crest cells and cells colonizing the brachial arches expressed the HNK-1 epitope [data not shown, but see Nieto et al. (Nieto et al., 1994)]. When *CNIC*$^{\Delta C89}$ was transfected at stage 6 and embryos were allowed to develop for 24 hours, the number of migrating neural crest cells possessing HNK-1 immunoreactivity was severely reduced on the transfected side (ipsilateral side, Fig. 5H), compared with the untransfected side (contralateral, Fig. 5G), in whole-mount preparations. To study the effect of *CNIC*$^{\Delta C89}$ in detail, individual *CNIC*$^{\Delta C89}$-transfected cells were identified by their anti-Flag immunoreactivities, and their location and HNK-1 expression were examined in sections. *CNIC*$^{\Delta C89}$-transfected cells often remained epithelial in the ectoderm (Fig. 5M), and very few of them emigrated from the neural tube, while in the same embryo, HNK-1-positive, untransfected neural crest cells colonized the branchial arches of the contralateral side normally (Fig. 5P). When GFP was transfected, many HNK-1-positive transfected cells were observed migrating dorsolaterally (Fig. 5I,J), or colonizing the branchial arches (Fig. 5K,L), similar to the contralateral side in *CNIC*$^{\Delta C89}$-transfected embryos (Fig. 5P). Because in the experiments above the transfection efficiency was high, it was not clear whether the observed reduction of neural crest by *CNIC*$^{\Delta C89}$ misexpression was a cell-autonomous effect or a community effect caused by changes in environmental factors (such as BMP4). Thus, to minimize possible community effect(s) on transfected cells, the efficiency of gene transfer was reduced (see Materials and Methods), and the number of transfected, HNK1-positive migrating cells was counted (Table 1). In this analysis, only a small number of *CNIC*$^{\Delta C89}$-transfected cells appeared to delaminate and express HNK-1. Therefore, strong activation of Notch signaling with *CNIC*$^{\Delta C89}$ in the neural fold cell-autonomously repressed Slug expression, and subsequently inhibited delamination and migration.

Table 1. *CNIC*$^{\Delta C89}$-transfected ectoderm cells fail to delaminate and do not express HNK-1

Expression vector	Number of cells*
GFP	1237±231 (3)
CNIC$^{\Delta C89}$	57±31 (4)†

~80-100 sections were examined in each embryo. Values are mean±s.d. (*n*).
*Counts of HNK-1^{+}/FLAG^{+} or HNK-1^{+}/GFP^{+} cells in the head mesenchyme of transfected side.
$^{\dagger}P<0.0002$ (*t*-test).

Delta1-mediated signals are required for neural crest formation

As forced activation of Notch signaling inhibited neural crest formation in the head fold, an expansion of neural crest would be expected from inhibition of Notch signaling. Therefore, we transfected the ectoderm with expression vectors of chicken or quail *Delta1*^{stu}, which lack the intracellular domain and have been reported to act as a dominant-negative in various situations (Henrique et al., 1997). Unexpectedly, however, *Slug* expression was clearly reduced in the cranial neural fold, when embryos were electroporated at stage 5 and cultured for 9 hours until stage 9 (Fig. 6C,D, 14/18 cases). GFP transfection did not have a significant effect on *Slug* expression (one out of seven cases, Fig. 6A,B). Interestingly, the effect of *Delta1*^{stu} was

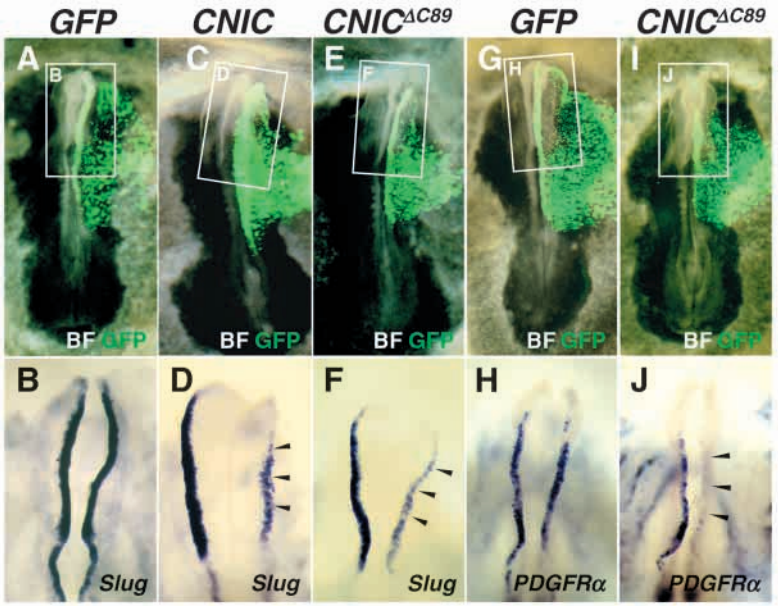

Fig. 4. Forced activation of Notch signaling in the ectoderm represses expression of head-fold markers. Electroporation of GFP (A,B,G,H), GFP+*CNIC* (C,D) and GFP+*CNIC*$^{\Delta C89}$ (E,F,I,J) was performed on stage 6 embryos, and the embryos were cultured for 7 hours until stage 9. Transfected areas are visualized by the fluorescence of GFP overlaid on bright field image of electroporated live embryos (A,C,E,G,I). *Slug* and *Pdgfrα* expression in the head fold (B,D,F,H,J) are shown at higher magnification, corresponding to the boxed areas of A,C,E,G,I, respectively. Forced activation of Notch signaling by *CNIC* and *CNIC*$^{\Delta C89}$ (cytoplasmic domain of chicken *Notch1*) reduced the expression of *Slug* and *Pdgfrα* in the head fold (arrowheads in D,F,H).

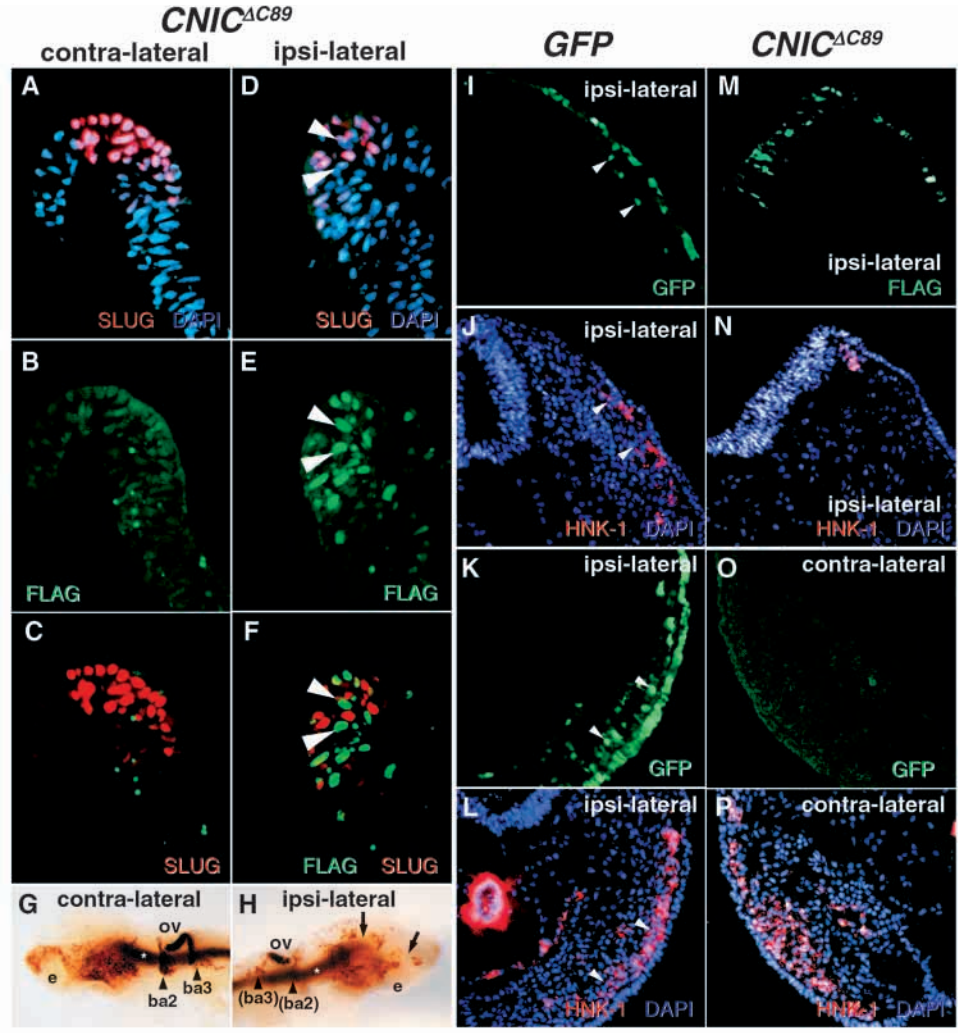

Fig. 5. *CNIC*$^{\Delta C89}$-transfected ectoderm cells fail to express Slug and HNK-1, and do not delaminate from the ectoderm. Electroporation of *GFP* (I-L) or *CNIC*$^{\Delta C89}$ (A-H and M-P) was performed on stage 6 embryo, and the embryos were cultured for 7 hours (A-F), or 24 hours (G-P). (A-F) A *CNIC*$^{\Delta C89}$-transfected neural fold (ipsilateral, D-F) and an untransfected neural fold (contralateral, A-C) of the same embryo, stained with anti-Flag (FITC, green), anti-Slug (cy3, red) and DAPI (blue). In the contralateral neural fold, many Slug-positive premigratory neural crest nuclei are observed (A,C). By contrast, in the ipsilateral side, the number of Slug-positive nuclei is reduced (D). Many Flag-positive nuclei of *CNIC*$^{\Delta C89}$-transfected cells can be identified (E), and most of the Flag-positive nuclei do not possess Slug-immunoreactivity (D-F, arrowheads). (G,H) Whole-mount HNK-1 staining of a *CNIC*$^{\Delta C89}$-transfected embryo. Compared with the contralateral side (G), migrating neural crest cells are severely reduced in the *CNIC*$^{\Delta C89}$-transfected side (H, black arrows in midbrain-forebrain level and black arrowheads in branchial arches). Strong HNK-1 staining indicated by white asterisks is in the axial mesoderm. ba2, second branchial arch; ba3, third branchial arch; e, eye; op, otic placode. (I-L) GFP-transfected HNK-1-positive neural crest cells migrate laterally underneath the epidermis (I,J, arrowheads), and colonize the branchial arch (K,L, arrowheads). (M,N) *CNIC*$^{\Delta C89}$-transfected cells failed to delaminate from the ectoderm. Note that *CNIC*$^{\Delta C89}$-transfected neural fold sometimes fails to close. (O,P) In the contralateral side of the *CNIC*$^{\Delta C89}$-transfected embryo, HNK-1-positive neural crest cells normally colonize the branchial arch (P).

stage-sensitive, and no change in *Slug* expression was observed in embryos transfected with *Delta1*^{stu} at stage 6 and cultured for 7 hours until stage 9 (none out of seven cases). Transfection of an expression vector of chicken *Numb*, a Notch antagonist (Wakamatsu et al., 1999; Wakamatsu et al., 2000), at stage 5 also reduced *Slug* expression in the head fold (Fig. 6E,F, seven out of 19 cases). Again, *Numb* misexpression had no effect on *Slug* expression if transfection was performed at stage 6 (none out of 10 cases). These results indicate that Notch activation by *Delta1* is required for neural crest formation in the ectoderm, but such a requirement is only transient.

Epidermis and neural ectoderm development is not affected by Notch signaling

As endogenous *Delta1* is expressed in the epidermal ectoderm region, and because electroporation of exogenous genes could not be precisely targeted to the presumptive neural folds, the observed reduction of neural crest by manipulating Notch signaling might be a secondary effect of perturbed differentiation of the epidermis or the neural plate. We reasoned, therefore, that it would be necessary to examine the differentiation of the epidermal ectoderm and the neural plate with molecular markers such as *Keratin19* (Sato and Yasugi, 1997) and *Sox2* (Uwanogho et al., 1997; Streit and Stern, 1999), respectively (Fig. 7). Embryos were electroporated at stage 5 and cultured for 4 hours until stage 7. *GFP*-transfection had no effect on the expression of *Keratin19* and *Sox2* (none out of seven and none out of eight cases, respectively; Fig. 7A-C,J-L). In no case did misexpression of *CNIC*$^{\Delta C89}$ change the distribution of *Keratin19* and *Sox2* (none out of 18 and none out of 12 cases, respectively; Fig. 7D-F,M-O). Nor did *Delta1*^{stu} affect the expression of *Keratin19* and *Sox2* (one out of 33 and none out of 16 cases, respectively; Fig. 7G-I,P-R). These results suggest that changes in the differentiation of these non-crest ectodermal tissues could not account for the effect of the manipulation of Notch signaling on *Slug* expression and neural crest formation.

Moderate levels of Notch signaling are required for the maintenance of *Bmp4* expression

Given that it has been shown that exogenous BMP4 can induce neural crest from neural plate explant (Liem et al., 1995), we realized that the effect of manipulating Notch signaling could be indirect. Accordingly, we examined the expression of *Bmp4*

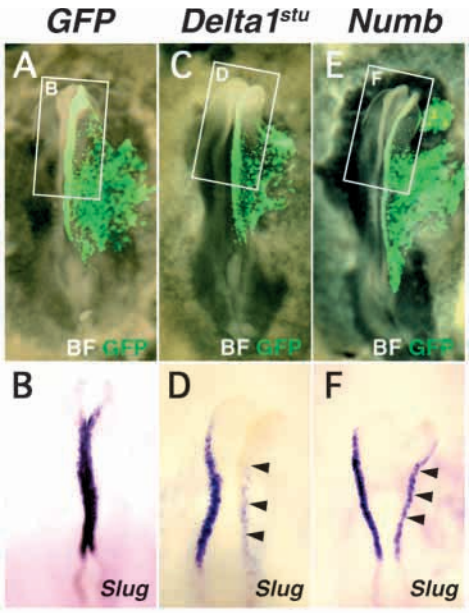

Fig. 6. Reduced Notch signaling results in the decrease of *Slug* expression. Electroporation of GFP (A,B), GFP+*Delta1*^{stu} (dominant-negative *Delta1*, C,D), GFP+chick *Numb* (E,F) was performed on stage 5 embryo, and the embryos were cultured for 9 hours until stage 9. Transfected areas are visualized by the fluorescence of GFP overlaid on bright field image of electroporated live embryos (A,C,E). *Slug* expression in the head fold (B,D,F) is shown at higher magnification, corresponding to the boxed areas of A,C,E, respectively. Reduction of Notch signaling results in the decrease of the *Slug* expression in the head fold (arrowheads in D,F).

at stage 5-9. As described previously (Liem et al., 1995), we found that *Bmp4* was expressed in the presumptive epidermal ectoderm prior to crest formation and later in the neural crest itself (data not shown). GFP-transfection had no effect on *Bmp4* expression (none out of 8 cases; Fig. 8A,B). *CNIC*$^{\Delta C89}$ transfection at stage 5 caused a severe reduction of *Bmp4* expression in the epidermis (4 hours after electroporation, eight out of 10 cases; Fig. 8C,D), and in the neural fold (9 hours after electroporation, eight out of 12 cases). Considering the endogenous *Delta1* expression in the epidermis is likely to activate Notch signaling in this region, transfection of *CNIC*$^{\Delta C89}$ might over-activate Notch signaling. *Delta1*^{stu} transfection at stage 5 also caused a reduction of *Bmp4* expression in the epidermal region (4 hours after transfection, ten out of 12 cases; Fig. 8E,F), suggesting the requirement of modest activation of Notch signaling for *Bmp4* expression.

To test whether BMP signaling is required for formation of neural crest, an expression vector of *Xenopus Noggin*, a BMP-antagonist, was transfected at stage 5 and embryos were cultured for 9 hours until stage 9 (Fig. 9C,D). This resulted in a clear decrease in *Slug*-positive cells (12/16 cases), consistent with previous observations in transgenic mouse embryos (Kanzler et al., 2000) (see Discussion).

To determine the role of Notch signaling on *Bmp4* expression and function, co-transfection of expression vectors of *Bmp4* and either *CNIC*$^{\Delta C89}$ or *Delta1*^{stu} was performed at stage 5 and embryos were cultured for 9 hours until stage 9. First, *Bmp4* alone was transfected into the ectoderm. Various concentrations of *Bmp4* expression vector were tested, because the concentration used for other constructs in this study appeared to induce significant apoptosis, as judged by the presence of many pyknotic nuclei stained with DAPI (data not shown). At one concentration of exogenous *Bmp4* (see Materials and Methods), however, the *Slug* expression domain often expanded (eight out of 13 cases; Fig. 9E,F). *Delta1*^{stu} -induced loss of *Slug* expression in the neural fold (see above, Fig. 6C,D) was rescued by the co-transfection of the *Bmp4* expression vector (13/15 cases; Fig. 9I,J), suggesting that Notch signaling acts upstream of *Bmp4* to promote neural crest formation. By contrast, co-transfection of *Bmp4* failed to rescue the loss of *Slug* expression by *CNIC*$^{\Delta C89}$ (seven out of eight cases; Fig. 9G,H). Thus, the inhibition of neural crest specification by strong Notch activation (Fig. 5) is likely to be independent of BMP4-mediated signaling.

A possible signal transduction pathway following Notch activation in epidermal ectoderm

A major signal transduction of the Notch signal is mediated by

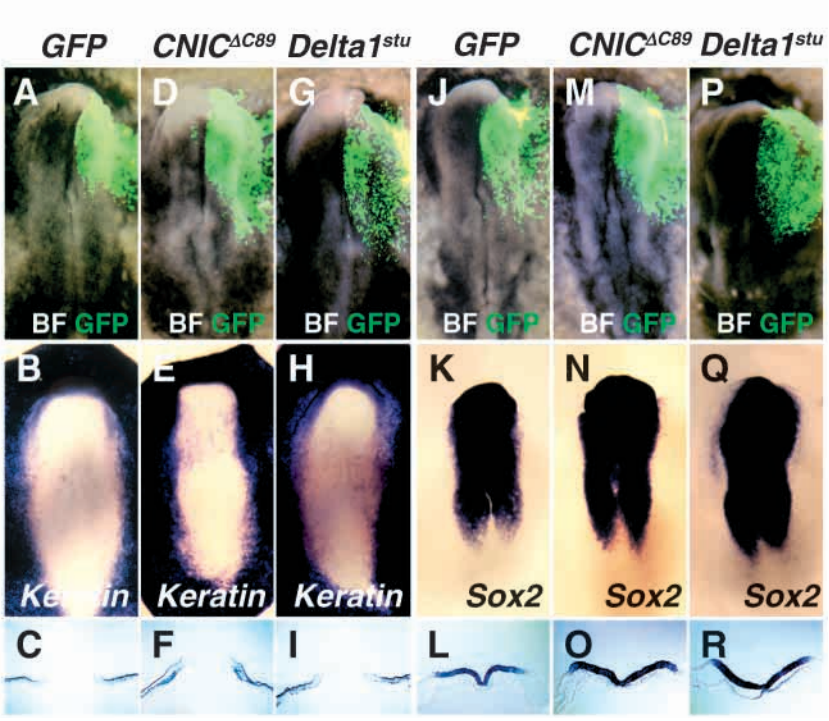

Fig. 7. Manipulation of Notch signaling does not affect the differentiation of the neural plate or the epidermis. Electroporation of GFP (A-C,J-L), GFP+*CNIC*$^{\Delta C89}$ (D-F,M-O), GFP+*Delta1*^{stu} (G-I,P-R), was performed on stage 5 embryo, and the embryos were cultured for 4 hours until stage 7. Transfected areas are visualized by the fluorescence of GFP overlaid on bright field image of electroporated live embryos (A,D,G,J,M,P). Expression of an epidermis marker *Keratin19* and a neural plate marker *Sox2* in these embryos are shown as whole-mount preparations (B,E,H,K,N,Q) and as sections (C,F,I,L,O,R).

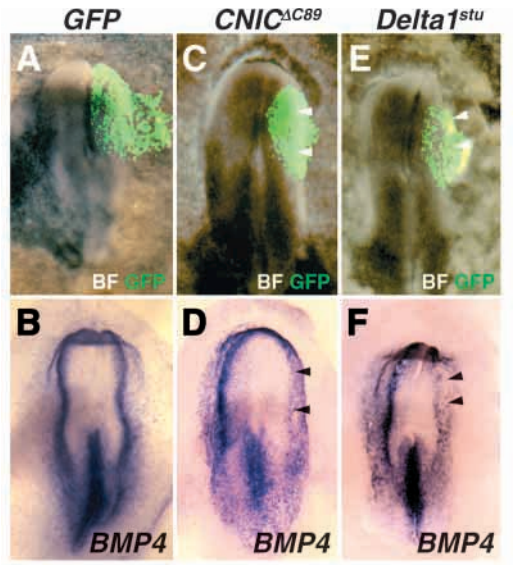

Fig. 8. Manipulation of Notch signaling in the ectoderm downregulates the epidermal expression of *Bmp4*. Electroporation of GFP (A,B), GFP+*CNIC*$^{\Delta C89}$ (C,D) and *GFP+Delta1*^{stu} (E,F) was performed on stage 5 embryos, and the embryos were cultured for 4 hours until stage 7. Transfected areas are visualized by the fluorescence of GFP overlaid on bright field image of electroporated live embryos (A,C,E). Both forced activation and inactivation of Notch signaling reduce *Bmp4* expression in the epidermal ectoderm (arrowheads in D,F).

a transcriptional complex formed by the Notch intracellular domain and Suppressor of Hairless [Su(H)/RBP-J (Oka et al., 1995; Kato et al., 1997)]. Thus, we tested if a dominant-negative form of Su(H) could interfere with neural crest formation. An expression vector of *X-Su(H)*^{DBM} (Wettstein et al., 1997) was transfected into the ectoderm of stage 5 embryo with the GFP vector, but *Slug* expression was unaffected (20/23 cases; Fig. 10A,B). This result suggested that *Su(H)/RBP-J*-independent pathway(s) was/were used in neural crest formation.

Expression of Hes family transcription factor(s) is often upregulated upon Notch activation, and mediates Notch signaling (Kageyama and Ohtsuka, 1999). We thus examined expression of avian homologs of Hes genes, *Hairy1* (Palmeirim et al., 1997) and *Hairy2* (Jouve et al., 2000). *Hairy2* expression was detected in the epidermal ectoderm at stage 5-8 (data not shown), indicating a response to the activation of Notch signaling in this tissue. To test this possibility, expression vectors of *Delta1*^{stu} or *CNIC*$^{\Delta C89}$ were transfected to stage 5 embryos, and *Hairy2* expression was examined after 4 hours of culture. Forced Notch activation with *CNIC*$^{\Delta C89}$ had no effect on *Hairy2* expression in the epidermis (nine out of 11 cases; Fig. 10C,D), but ectopic expression of *Hairy2* was often observed in the neural plate area (eight out of 11 cases; Fig. 10D, inset). This observation suggests that Notch signaling is activated in the epidermal ectoderm by *Delta1* in normal development. Consistently, most of the *Delta1*^{stu}-transfected embryos showed a significant reduction of *Hairy2* expression (11/15 cases, Fig. 10E,F). Electroporation of GFP had no effect on *Hairy2* expression (none out of three cases).

DISCUSSION

In this study, temporally controlled gene transfer into the avian embryonic ectoderm, including the prospective neural fold, revealed involvement of Notch signaling in neural crest formation. Endogenous *Delta1* is expressed in the epidermal ectoderm, and misexpression of dominant-negative *Delta1* (*Delta1*^{stu}) leads to the loss of a neural crest marker, *Slug*. Given that *Bmp4* expression in the epidermal ectoderm is decreased by the misexpression of *Delta1*^{stu}, and as co-transfection of *Bmp4* with *Delta1*^{stu} effectively restores *Slug* expression, Delta1-mediated Notch activation in the epidermis regulates *Slug* expression, probably through *Bmp4* function. A role for BMPs in neural crest induction has been suggested in avian system, as exogenous *Bmp4* can induce neural crest in explanted medial neural plate (Liem et al., 1995). Consistent with this, misexpression of exogenous *Noggin*, a BMP antagonist, in the ectoderm leads to a decrease in *Slug*-

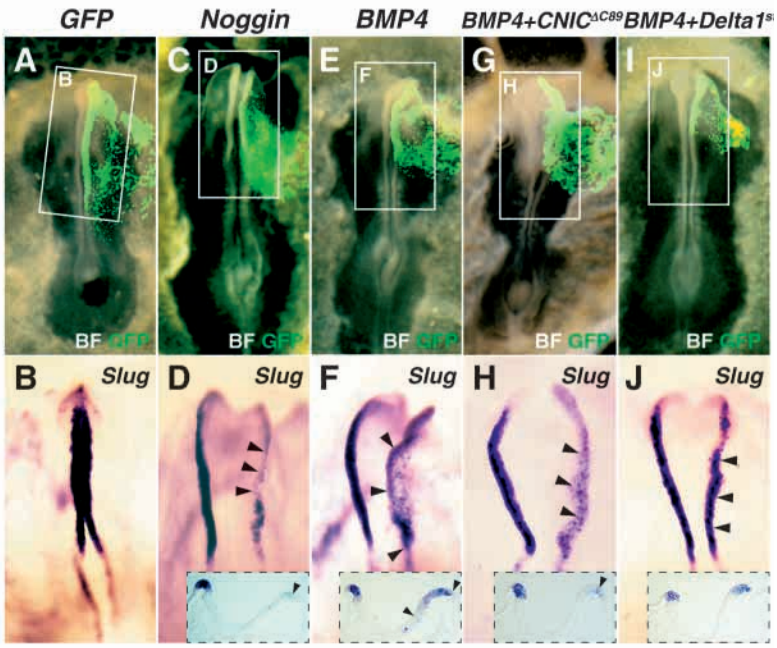

Fig. 9. Exogenous *Bmp4* rescues the reduction of *Slug* expression by *Delta1*^{stu}, but not the reduction by *CNIC*$^{\Delta C89}$. Electroporation of *GFP* (A,B), GFP+*Noggin* (C,D), GFP+*Bmp4* (E,F), GFP+*Bmp4* +*CNIC*$^{\Delta C89}$ (G,H), GFP+*Bmp4*+*Delta1*^{stu} (I,J), was performed on stage 5 embryo, and the embryos were cultured for 9 hours until stage 9. Transfected areas are visualized by the fluorescence of GFP overlaid on bright field image of electroporated live embryos (A,C,E,G,I). Misexpression of *Noggin* reduces *Slug* expression (D, see also the section in the inset, arrowhead), while overexpression of *Bmp4* results in the expansion of *Slug* domain (F, see also the section in the inset, arrowheads). The loss of *Slug* expression by *CNIC*$^{\Delta C89}$ cannot be rescued by the co-expression of *Bmp4* (H, see also the section in the inset, arrowhead). The decrease of *Slug* expression by *Delta1*^{stu} (see Fig. 6D) is rescued by the co-expression of *Bmp4* (J, see also the section in the inset).

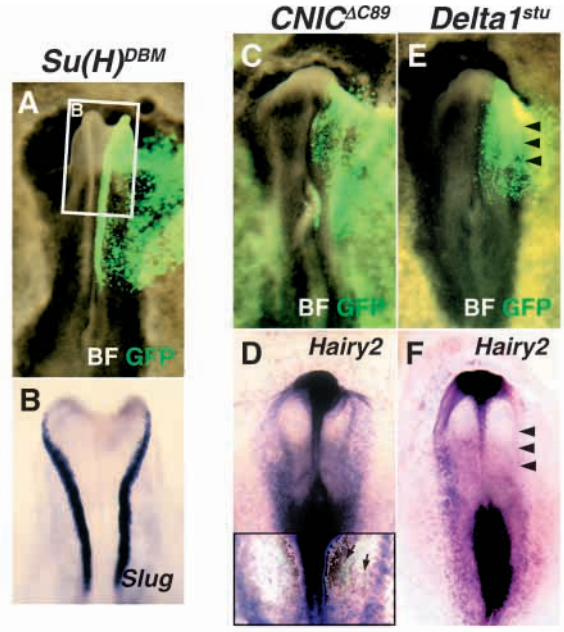

Fig. 10. Notch activation is necessary and sufficient for *Hairy2* expression in the ectoderm, but *Su(H)/RBP-J* activity is not required. Electroporation of *GFP* + *Su(H)*^{DBM} was performed on stage 5 embryos, and the embryos were cultured for 9 hours until stage 9 (A,B). *Slug* expression is not affected (B). Electroporation of GFP+*CNIC*$^{\Delta C89}$ (C,D) or GFP+*Delta1*^{stu} (E,F) was performed on stage 5 embryo, and the embryos were cultured for 4 hours until stage 7. Transfection of *CNIC*$^{\Delta C89}$ has no effect on *Hairy2* expression in the epidermis (D), while ectopic *Hairy2* expression in the neural plate is observed (D, arrows in inset). Transfection of *Delta1*^{stu} reduced the level of *Hairy2* expression in the epidermis (F, arrowheads)

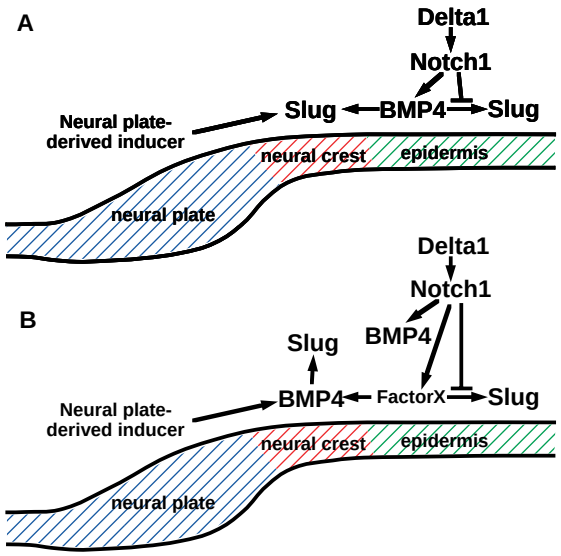

Fig. 11. Alternative models of regulatory relationship of BMP signaling, Notch signaling and *Slug* expression.

expressing presumptive neural crest (Kanzler et al., 2000), and exogenous *Bmp4* induces expansion of *Slug* expression in the head fold. Thus, we speculate that Delta1-mediated Notch activation promotes *Bmp4* expression in the epidermal ectoderm, and that the epidermal BMP4 subsequently induces neural crest at the junction of the neural plate and epidermal ectoderm (Fig. 11A). However, the timing of the requirement for BMP in neural crest formation has been argued. Because of the epidermal expression of BMPs before crest formation, it has been suggested that epidermal BMPs promote neural crest formation (Liem et al., 1995). More recently, however, Selleck et al. (Selleck et al., 1998) showed that, at least in the trunk, *Bmp4* expressed in the neural crest itself is important. If this is also the case at the cranial level, because *Bmp4* has been shown to promote epidermis differentiation both in *Xenopus* and in avian embryos (Wilson and Hemmati-Brivanlou, 1995; Pera et al., 1999), *Bmp4* may induce another yet-to-be identified epidermal factor (factor X) that may in turn induce *Bmp4* expression in neural crest. In this model, Notch activation in the epidermis may induce factor X, and such a factor might induce *Bmp4* expression in the head fold (Fig. 11B). Whichever is the case, epidermal Notch signaling appears to be required for neural crest formation at the cranial level after the neural plate-epidermis boundary is set.

Because activated *Notch1* (*CNIC*$^{\Delta C89}$) downregulates *Bmp4* expression, the apparent loss of neural crest by strong and continuous Notch activation might be explained by the reduction of the inductive signal. However, *CNIC*$^{\Delta C89}$ misexpression in the ectoderm at late stages of development, when *Delta1*^{stu} misexpression no longer affects neural crest formation, still inhibits *Slug* expression and subsequent epithelial-mesenchymal transition, even when the transfection efficiency is low. Because such inhibition of neural crest formation by *CNIC*$^{\Delta C89}$ misexpression appears to be cell-autonomous, Notch signaling may inhibit neural crest specification directly. Thus, in normal development epidermally expressed *Delta1* may moderately activate Notch signaling to inhibit *Slug* expression in this tissue (Fig. 11).

In an earlier report (Coffman et al., 1993), the loss of neural crest in *Xenopus* embryo injected with mRNA of activated *Notch1* was accompanied by the expansion of the neural plate. This could be interpreted as a transformation of the neural crest to neural plate. By contrast, the expansion of the neural plate in *Xenopus* embryos injected with *Sox2* mRNA increased *Slug*-positive presumptive crest cells, together with the neural plate expansion (Mizuseki et al., 1998). Thus, neural plate expansion and neural crest formation are not necessarily be correlated. We thus propose that the loss of neural crest in *Xenopus* embryos induced by the injection of activated *Notch1* is a direct effect, and the increase of neural crest by the *Sox2* mRNA injection is a secondary effect of the observed neural plate expansion, perhaps through an increase in a putative neural plate-derived neural crest inducing factor. In fact, upon *Slug* expression, *Sox2* expression is downregulated in the neural crest of avian embryo, and misexpression of *Sox2* in the neural fold decreases the number of *Slug*-positive neural crest cells (Y. E. and Y. W., unpublished observations). Taken together, we propose that *Delta1*-mediated Notch activation in the epidermis promotes and/or maintains *Bmp4* expression in this region, while it inhibits *Slug* expression in the epidermis (Fig. 11). Then, either *Bmp4* or unidentified factor X in the epidermis may be able to induce *Slug* expression and neural crest formation only in the neural fold, in cells where Notch

signaling is not activated, and neural plate-derived inductive signal(s) is/are available (Fig. 11).

In the epidermal ectoderm, expression of *Hairy2*, which encodes a Hes family transcription factor, is positively regulated by Notch signaling. This is consistent with the previous reports that expression of Hes genes are activated by Notch signaling, in multiple developing tissues such as neural epithelium of the central nervous system and segmental plate of the paraxial mesoderm (Kageyama and Ohtsuka, 1999; Jouve et al., 2000). Thus, *Hairy2* may mediate Notch signaling to induce neural crest. Unlike other systems, however, *Su(H)*/*RBP-J* does not appear to be required for neural crest formation, as dominant-negative *Su(H)* transfection had no obvious effect on crest formation. Unexpected mutation(s) in the plasmid used in this study cannot account for this observation, because the same construct has been successfully used in other studies (Wettstein et al., 1997; Cornell and Eisen, 2000). Furthermore, transfection of this construct into the chick neural tube caused increased neuronal differentiation, probably as a result of reduced Notch signaling (Y. W., unpublished). Although the nature of the *RBP-J*-independent signal transduction pathway in the epidermis remains to be determined, deltex/DTX family proteins have been shown to mediate Notch signaling (Matsuno et al., 1995; Matsuno et al., 1998). It has not been studied whether Dtx genes are involved in neural crest formation, but injection of mouse *Dtx2* mRNA into *Xenopus* embryo caused an expansion of the neural plate (Kishi et al., 2000), similar to injection of activated *Notch* mRNA (Coffman et al., 1993). Because a chick homolog of Dtx is expressed in the epidermis (Frolova and Beebe, 2000), it will be interesting to study the function of Dtx genes in neural crest formation.

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