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

6.1E8 anti-Slug (mouse IgG1; a kind gift from Dr Jessell) (Li 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 IgG1, 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 AxiosplanII 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 H2O2/methanol for several hours.

Expression vectors

Flag epitope-tagged expression vectors of jellyfish green fluorescent protein (GFP), CNIC (constitutively active form of chick Notch1) and CNICAC89 (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 Delta1m was kindly provided by Dr Henrique (Henrique et al., 1997). Quail Delta1 in pcDNA3.1 (Maynard et al., 2000) was digested with XbaI and HindIII, and subcloned into an expression vector, pmiwSV (Wakamatsu et al., 1997). Subsequently, the intracellular domain of quail Delta1 was removed by Stul and XbaI digestion to make the pmiw-quail Delta1m construct. RCAS (B)-chicken Delta1m and pmiw-quail Delta1m 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 (Tonge et al., 1997; Tonge and Takahashi, 1998). An expression vector of X-SufHDBM 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 NaH2PO4, 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 mm2 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 ms, 200 ms) at 200 ms. To achieve lower transfection efficiency, 5 V was used. Silpolt 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 (Mausch 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). The Delta1 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. 2L, 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 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). The Delta1 expression vector was co-transfected with pEGFP-N1 and a third expression vector, the ratio was 1:9:10.

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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. 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.
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 (CNICAC89) also caused clear reduction or loss of expression of both Slug and Pdgfra (Slug, nine out of nine cases, Fig. 4E,F; Pdgfra, seven out of eight cases, Fig. 4I,J). No significant effect was observed on Slug (none out of five cases) or Pdgfra 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 CNICAC89-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, CNICAC89-transfected Flag-positive cells appeared to have lost their endogenous Slug protein expression, suggesting that CNICAC89-transfected neural fold cells failed to differentiate into neural crest (Fig. 5D-F). The loss of Slug expression caused by CNICAC89-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 CNICAC89 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, Fig. 5G, in whole-mount preparations. In this analysis, only a small number of CNICAC89-transfected cells appeared to delaminate and express HNK-1. Therefore, strong activation of Notch signaling with CNICAC89 in the neural fold cell-autonomously repressed Slug expression, and subsequently inhibited delamination and migration.

**Table 1. CNICAC89-transfected ectoderm cells fail to delaminate and do not express HNK-1**

<table>
<thead>
<tr>
<th>Expression vector</th>
<th>Number of cells*</th>
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<tbody>
<tr>
<td>GFP</td>
<td>1237±231 (3)</td>
</tr>
<tr>
<td>CNICAC89</td>
<td>57±31 (4)†</td>
</tr>
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~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.

†P<0.0002 (t-test).

**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+CNICAC89 (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 Pdgfra expression in the head fold (B,D,F,H) 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 CNICAC89 (cytoplasmic domain of chicken Notch1) reduced the expression of Slug and Pdgfra in the head fold (arrowheads in D,F,H).
stage-sensitive, and no change in Slug expression was observed in embryos transfected with Delta1 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 CNICDC89 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 Delta1stu 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

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**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. Then they 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).
Notch in neural crest development

Fig. 6. Reduced Notch signaling results in the decrease of Slug expression. Electroporation of GFP (A,B), GFP+Delta1stu (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).

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+CNICAc89 (D-F,M-O), GFP+Delta1stu (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).

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 CNICAc89 or Delta1stu 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). Delta1stu - 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,L), 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 CNICAc89 (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

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). CNICAc89 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 CNICAc89 might over-activate Notch signaling. Delta1stu transfection at stage 5 also caused a reduction of Bmp4 expression in the epidermal
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 Delta1ΔN or CNICΔC89 were transfected to stage 5 embryos, and Hairy2 expression was examined after 4 hours of culture. Forced Notch activation with CNICΔC89 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 Delta1ΔN-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 (Delta1ΔN) leads to the loss of a neural crest marker, Slug. Given that Bmp4 expression in the epidermal ectoderm is decreased by the misexpression of Delta1ΔN, and as co-transfection of Bmp4 with Delta1ΔN effectively restores Slug expression, Delta1-mediated Notch activation in the epidermis regulates Bmp4 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). Consistently, misexpression of exogenous Noggin, a BMP antagonist, in the ectoderm leads to a decrease in Slug-expression.

**Fig. 8.** Manipulation of Notch signaling in the ectoderm downregulates the epidermal expression of Bmp4. Electroporation of GFP (A,B), GFP+CNICΔC89 (C,D) and GFP+Delta1ΔN (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).

**Fig. 9.** Exogenous Bmp4 rescues the reduction of Slug expression by Delta1ΔN, but not the reduction by CNICΔC89. Electroporation of GFP (A,B), GFP+Noggin (C,D), GFP+Bmp4 (E,F), GFP+Bmp4 +CNICΔC89 (G,H), GFP+Bmp4+Delta1ΔN (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 CNICΔC89 cannot be rescued by the co-expression of Bmp4 (H, see also the section in the inset, arrowhead). The decrease of Slug expression by Delta1ΔN (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)\textsubscript{RBP-J} activity is not required. Electroporation of GFP + Su(H)\textsubscript{RBP-J} 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\textsuperscript{ΔC89} (C,D) or GFP+Delta1\textsubscript{stu} (E,F) was performed on stage 5 embryo, and the embryos were cultured for 4 hours until stage 7. Transfection of CNIC\textsuperscript{ΔC89} 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\textsubscript{stu} reduced the level of Hairy2 expression in the epidermis (F, arrowheads).

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 plate 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\textsuperscript{ΔC89}) 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\textsuperscript{ΔC89} misexpression in the ectoderm at late stages of development, when Delta1\textsubscript{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\textsuperscript{ΔC89} 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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