Neural crest emigration from the neural tube depends on regulated cadherin expression

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SUMMARY

During the emergence of neural crest cells from the neural tube, the expression of cadherins dynamically changes. In the chicken embryo, the early neural tube expresses two cadherins, N-cadherin and cadherin-6B (cad6B), in the dorsal-most region where neural crest cells are generated. The expression of these two cadherins is, however, downregulated in the neural crest cells migrating from the neural tube; they instead begin expressing cadherin-7 (cad7). As an attempt to investigate the role of these changes in cadherin expression, we overexpressed various cadherin constructs, including N-cadherin, cad7, and a dominant negative N-cadherin (cN390Δ), in neural crest-generating cells. This was achieved by injecting adenoviral expression vectors encoding these molecules into the lumen of the closing neural tube of chicken embryos at stage 14. In neural tubes injected with the viruses, efficient infection was observed at the neural crest-forming area, resulting in the ectopic cadherin expression also in migrating neural crest cells. Notably, the distribution of neural crest cells with the ectopic cadherins changed depending on which constructs were expressed. Many crest cells failed to escape from the neural tube when N-cadherin or cad7 was overexpressed. Moreover, none of the cells with these ectopic cadherins migrated along the dorsolateral (melanocyte) pathway. When these samples were stained for Mitf, an early melanocyte marker, positive cells were found accumulated within the neural tube, suggesting that the failure of their migration was not due to differentiation defects. In contrast to these phenomena, cells expressing non-functional cadherins exhibited a normal migration pattern. Thus, the overexpression of a neuroepithelial cadherin (N-cadherin) and a crest cadherin (cad7) resulted in the same blocking effect on neural crest segregation from neuroepithelial cells, especially for melanocyte precursors. These findings suggest that the regulation of cadherin expression or its activity at the neural crest-forming area plays a critical role in neural crest emigration from the neural tube.

Key words: Adenoviral vector, Cadherin, Melanocyte precursor, Neural crest, Cell adhesion, Cell migration, Mouse
cadherins preferentially binds like molecules. However, N-cadherin binds neither cadB nor cad7 (Nakagawa and Takeichi, 1995). CadB shows a partial affinity to cad7, but cells expressing these cadherins segregate from one another within their chimeric aggregates, suggesting that their heterophilic interaction is less stable than the homophilic interaction of each subtype (Nakagawa and Takeichi, 1995).

Based on these observations, we have performed experiments that in cadherin expression during neural crest development play a role in the segregation of the neural crest cells from the neural tube. For example, downregulation of N-cadherin and cad6B in the crest cells may be a prerequisite for their detachment from neural tube cells.

To test the above idea, we sought to perturb the cadherin expression in neural crest cells by overexpressing various cadherin constructs in the chicken embryonic neural tube by use of adenoviral expression vectors. This approach was technically successful; we could efficiently label not only dorsal neural tube cells but also crest cells with ectopic cadherin molecules. Our results show that the overexpression of N-cadherin or cad7 suppressed the emergence of neural crest cells from the neural tube, but the migration of crest cells that had already detached from the neural tube was not affected by the ectopic cadherin expression. These findings support the idea that the regulated cadherin expression, occurring during neural crest emigration, is a necessary step for this morphogenetic process.

**MATERIALS AND METHODS**

**Antibody production**

To raise antibodies against the extracellular domain of chicken cad6B or cad7, we prepared chimeric proteins consisting of this domain and the human immunoglobulin Fc region: A cDNA fragment encoding the N-terminal 605 amino acids of cad6B or the N-terminal 597 amino acids of cad7 was amplified by PCR, and cloned into pEF-fc (Suda and Nagata, 1994). These plasmids were introduced into COS-7 cells by electroporation, and the cells were incubated for 24 hours in a HBSS, and further incubated in a fetal calf serum. They were then washed twice with a Hepes-buffered balanced salt solution (HBSS), and permeabilized by incubation in −20°C methanol for 5 minutes. Non-specific binding of antibodies was blocked by immersing the sections in TBS containing 5% skim milk (Difco). When using HNK1 as the primary antibody, TBS containing 2% BSA (Sigma) was used as a blocking solution instead. For double-label immunostaining, sections were first incubated with a primary antibody for 1 hour, followed by incubation with an appropriate secondary fluorescence-labeled antibody for 30 minutes, and finally with a second set of primary and secondary antibodies.

For staining with HNK1, the incubation time was extended to 16 hours. Fluorescence was visualized with an epifluorescence microscope (Zeiss) or a laser-scanning confocal microscope (Bio-Rad).

For whole-mount immunostaining, embryos were fixed in 4% paraformaldehyde/HBSS for 2 hours. Fixed embryos were washed twice with HBSS, and incubated for 6 hours in TBS containing 0.2% Triton X-100, 5% heat-inactivated FBS, and 0.5% peroxide. Embryos were then incubated overnight with an appropriately diluted primary antibody, and washed six times (1 hour each time) with TBS containing 0.2% Triton X-100. HRP-conjugated antibodies were used as secondary antibodies, and the antibody incubation and washing procedures were the same as for the primary antibodies. After having been visualized with DAB and photographed under a binocular microscope (Nikon SMZ-U), the samples were sectioned with a microtome (D. S. K. DTK-1000, Kyoto) at a thickness of 100 μm, cleared with 75% glycerol, and mounted on a glass slide. These sections were examined under a Zeiss microscope using Nomarski optics.

For immunoblotting, embryos at stage 14 were lysed with Laemmli’s sample buffer and boiled for 5 minutes. The samples were run in a 7.5% SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane. The blots incubated with appropriate antibodies were processed for chemiluminescence with the ECL detection system (Amersham).

**Plasmid construction**

The adenoviral shuttle vectors encoding various cadherin cDNAs were constructed as follows: The whole coding regions of N-cadherin, cad6B, and cad7 were amplified by PCR using Z10T6, cN390 (Hatta and Takeichi, 1986), mouse monoclonal antibody HNK1 (Becton Dickinson 347990), mouse monoclonal antibody M2 (Kodak IB13010), rabbit polyclonal antibody against Mif (Mochii et al., 1998; Cy3-conjugated anti-mouse, -rat, and -rabbit IgGs (Chemicon AP12AC, AP136C, AP132C); FITC-conjugated anti-mouse IgM (ZYMED, 62-6811); HRP-conjugated anti-mouse and -rabbit IgGs (Amersham RPN1001, RPN1004); streptavidin-labeled FITC (Amersham RPN1232); Texas Red-conjugated anti-rabbit IgG (CAPPEL 55675). Embryos were fixed with 4% paraformaldehyde in HBSS at 4°C for 2 hours.

For cryosections, embryos were immersed in a graded series of sucrose solutions (12%, 15%, 18% sucrose in HBSS), embedded in Tissue Tek (Miles), and frozen in liquid nitrogen. Cryostat sections of 10 μm thickness were collected, and dried on silane-coated glass slides. Sections were rehydrated in TBS (50 mM Tris, 150 mM NaCl, 1 mM CaCl2), and permeabilized by incubation in −20°C methanol for 5 minutes. Non-specific binding of antibodies was blocked by immersing the sections in TBS containing 5% skim milk (Difco). When using HNK1 as the primary antibody, TBS containing 2% BSA (Sigma) was used as a blocking solution instead. For double-label immunostaining, sections were first incubated with a primary antibody for 1 hour, followed by incubation with an appropriate secondary fluorescence-labeled antibody for 30 minutes, and finally with a second set of primary and secondary antibodies. For staining with HNK1, the incubation time was extended to 16 hours. Fluorescence was visualized with an epifluorescence microscope (Zeiss) or a laser-scanning confocal microscope (Bio-Rad).

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were amplified, and purified by CsCl2 step-gradient centrifugation of the CAG promoter (Niwa et al., 1991). The recombinant adenoviruses AdV-cN/CBR(-), expressing these cadherins under the control of the HpaI site. L-cells were then transiently transfected with these pCMV constructs, and the cells were then transfected with the anti-FLAG antibody M2 to ensure proper protein expression. An adenovirus shuttle vector plasmid pAdV-CA-pA was made by inserting the CA promoter sequence (Niwa et al., 1991), followed by insertion of a poly(A) signal into the EcoRV site of pAdV (Moriyoshi et al., 1996). The whole coding sequences including the FLAG epitope were cut from the pCMV constructs with SpIi and NotI, blunted using the Klenow fragment, and cloned into the Hpal site of pAdV-CA-pA to yield pAdV-CA-cN/FLAG-pA, pAdV-CA-cN390Δ/FLAG-pA, pAdV-CA-c7/FLAG-pA, pAdV-CA-cN/CBR(-)/FLAG-pA.

**Construction of recombinant adenoviruses**

AdV-CA-lacZ, adenovirus expressing β-galactosidase driven by a CA promoter, was a kind gift from K. Moriyoshi at Kyoto University. Construction of recombinant viruses was performed according to the methods described previously (Moriyoshi et al., 1996). Recombinant adenoviruses were obtained by homologous recombination in HEK 293 cells (ATCC CRL 1573), which were maintained in DMEM supplemented with 10% FBS. HEK 293 cells cultured in 6-well plates (Nunc) were co-transfected with viral genome fragments (0.2 µg) and linearized adenoviral shuttle vector plasmids (1 µg) using LipofectAMINE (GIBCO). On the next day, the cells were divided and placed into collagen-coated 24-well plates (IWAKI, Japan). Ten days later, some wells were full of dead cells, caused by viral propagation, of which debris was screened for proper construction of recombinant viruses. Partially infected cells were discarded. The injected embryos were further incubated at 38°C for 24 hours to allow embryos to develop to stage 14 (Hamburger and Hamilton, 1951). The posterior neuropore of the chicken embryos from this farm closed earlier than that of White Leghorn embryos, which was an essential requirement to obtain higher infection efficiency. The tip of pulled glass micropipettes was broken by forceps and front-filled with adenovirus solution. Fast Green (Sigma) was added to the virus solution at a final concentration of 0.2% to help visualization of the injected solution. The injection pipette was inserted obliquely into the neural tube at the caudal-most level by means of a micromanipulator (Narishige). The virus solution up to 100 nl was very slowly injected by air pressure until the solution filled the entire lumen of the neural tube. In most cases, diffused virus solution was seen to reach the level of the midbrain. Occasionally the virus solution leaked from the neural tube. In such cases, the embryos were discarded. The injected embryos were further incubated at 38°C until they were fixed for immunostaining.

**RESULTS**

**Changes in cadherin expression during neural crest emigration**

We determined the protein expression patterns for three cadherins, N-cadherin, cad6B, and cad7, during neural crest development in the chicken embryos, since previous information was limited to mRNA expression for the latter two. Two mouse monoclonal antibodies, CCD6-1 and CCD7-1, which specifically recognize chicken cad6B and cad7, respectively, were prepared; and a rabbit antisera specific for cad6B, designated as anti-6B, was also generated. In western blotting, CCD6B-1/anti-6B and CCD7-1 specifically detected a major 120 kDa protein and 105 kDa protein, respectively (Fig. 1). These antibodies as well as NCD-2, specific for chicken N-cadherin (Hatta and Takeichi, 1986), were used for subsequent immunostaining studies. HNK1 antibody was also used to identify migrating neural crest cells (Tucker et al., 1984; Bronner-Fraser, 1986). In the trunk of embryos at stage 16, N-cadherin was expressed throughout the neural tube, although its expression was reduced at the dorsal-most region where neural crest cells were being generated (Fig. 2A) (Hatta and Takeichi, 1986; Akita and Bronner-Fraser, 1992). In this dorsal-most portion, cad6B was strongly expressed (Fig. 2B), overlapping the weak N-cadherin expression. When neural crest cells had left the neural tube, both of these cadherins were downregulated. They instead expressed cad7 (Fig. 2C). Double-staining for cad7 and the HNK1 antigen showed that most of HNK1-positive cells were also cad7 positive (Fig. 2C,D), suggesting that the majority of migrating crest cells expressed this cadherin. At subsequent stages, e.g., at stage 22, cad7 and the HNK1 antigen became co-expressed by cells migrating towards the space between the dermamyotome and overlying ectoderm (Fig. 2E,F arrows), which were likely melanocyte precursors entering the dorsolateral pathway (Erickson et al., 1992). The cad7 was also expressed in the neural tube at the boundary zone between the alar and basal plates. These expression patterns of cadherin proteins were basically identical to those of their mRNAs previously reported (Nakagawa and Takeichi, 1995), although the immunostaining turned out to be more sensitive than the mRNA in situ hybridization, resulting in the detection of more positive cells.
For closer observations of the changing process of the cadherin expression pattern, we employed confocal microscopy (Fig. 2G-I). Staining for N-cadherin and cad6B indicated that both of them were distributed throughout the lateral contact sites between dorsal neural tube cells, although more cadherin proteins were accumulated at the luminal side of the neural tube, where the adherens junctions are localized between neuroepithelial cells. Major cad7 signals were detected only in cells that had detached from the neural tube (Fig. 2H,I, arrows), although some faint signals were also detectable in the adherens junction area (Fig. 2H,I, arrowheads). To confirm this feature of cad7 expression, we examined thick sections at the dorsal-most region of the neural tube stained for cad7 by conventional immunofluorescence microscopy at higher magnification (Fig. 2J,K). Cad7 signals were indeed already present in the neural tube, but these were confined to the luminal junctional sites, never extending to the main body of neuroepithelial cells. Strong cad7 signals covering the entire cell body appeared only after crest cells had become isolated from the neuroepithelium.

**Ectopic β-galactosidase or cadherin expression induced by adenoviral expression vectors**

To investigate the role of the above changes in cadherin expression during neural crest development, we designed experiments to induce over or ectopic expression of various cadherin constructs in neural crest precursors as well as in surrounding neural tube cells by use of adenoviral expression vectors. To test adenoviral vectors for usefulness in our system, we injected those carrying the β-galactosidase gene (provided by K. Moriyoshi, Kyoto University), whose expression was under a control of the CAG promoter (Niwa et al., 1991), into the lumen of the neural tube of stage 14 embryos. The injected embryos were fixed at various times, and subjected to β-gal staining as whole-mount samples. In embryos fixed at 4 hours, no ectopic expression of β-gal was observed (Fig. 3A). At 10 hours, however, enzymatic activity of β-gal was detected in cells scattered around the dorsal portion of the embryos as well as in those aligned along their dorsal midline (Fig. 3B). A similar pattern of positive cells was observed at 16 hours also, although they were more intensely labeled (Fig. 3C). At 24 hours, dorsal root ganglia (DRG) became positive (Fig. 3D). These results indicate that the virus-derived protein expression was initiated at a time between 4 and 10 hours after viral injection and that its level increased up to 24 hours of incubation. Thereafter, the expression level gradually decreased presumably due to a dilution of this replication-incompetent virus by cell proliferation (data not shown).

For more precise localization of β-gal-positive cells, embryos at 24 hours after viral injection, which had reached stage 20, were sectioned and immunostained for β-gal. In the neural tube, infection was observed in various regions of the tube; however, the most reproducible, intense infection occurred at the dorsal-most portion where premigratory neural crest cells reside (Fig. 3E). This infection resulted in β-gal labeling of migrating neural crest cells also, as confirmed by double-staining for β-gal and the HNK1 epitope: for example, at the hindlimb level, a cluster of β-gal-positive cells was found migrating down the typical ventral pathway for neural crest migration (Fig. 3E); and these cells were also HNK1 positive (Fig. 3F). Some of the myotome cells occasionally expressed β-gal, possibly due to some leakage of viruses from the injected sites. In these experiments, more than half of the HNK1-positive neural crest cells expressed the lacZ gene, although the infection efficiency varied with the embryos and experiments.
Cadherin-dependent neural crest emigration

Fig. 3. Adenovirus-mediated exogenous protein expression in neural crest cells. (A–D) Dorsal view at the trunk region of embryos injected with β-gal-expressing adenoviruses, and fixed after 4 hours (A), 10 hours (B), 16 hours (C), and 24 hours (D). The samples were processed for X-gal staining. (E,F) Double-staining for β-gal (E) and HNK1 (F) in a section at the hindlimb level of an embryo incubated for 24 hours after viral injection. Virus infection is observed in premigratory and migrating neural crest cells that are immunoreactive for HNK1 as well as in the neural tube. (G,H) Embryos were injected with AdV-Ncad (G) or AdV-cad7 (H), and stained for FLAG epitope 24 hours after the injection. Note that cells strongly expressing exogenous cadherins are migrating along the ventral pathway (arrowheads).

Next, we generated recombinant viruses expressing N-cadherin (AdV-Ncad) and cad7 (AdV-cad7), whose carboxy terminus was attached to the FLAG tag (Fig. 4), and injected them into stage 14 embryos, as described above. At 24 hours after the injection, the embryos were stained for the tag epitope. As expected from the above experiments, migrating neural crest cells as well as cells localized in the roof plate of the neural tube were intensely labeled with the anti-FLAG antibody (Fig. 3G,H). Strong expression of the ectopic cadherins was observed not only in the roof plate but also in migrating crest cells (Fig. 3G,H arrows).

Alteration of neural crest behavior by ectopic cadherin expression

In the above experiments, we did not notice significant effects of the ectopic cadherin expression on neural crest behavior. However, when the injected embryos were incubated longer, a dramatic difference in neural crest patterning became evident between the embryos expressing β-gal and the full-length cadherin constructs. In embryos incubated for 30 hours after the injection of viruses carrying the β-gal gene, cells expressing this gene were detected not only in DRG but also along the dorsolateral pathway, as revealed by whole-mount staining of the embryos (Fig. 5A) as well as by sectioning of them (Fig. 5B, arrows). However, in embryos expressing ectopic N-cadherin or cad7, no infected cells were found migrating along the dorsolateral pathway (Fig. 5C–F), as judged by staining with anti-FLAG antibody. Notably, a number of infected cells had accumulated in the midline of the neural tube, some of which had invaded the lumen of the neural tube (Fig. 5D,F arrowheads). In these embryos, the number of FLAG-positive cells that had been incorporated into the DRG also tended to be slightly reduced.

As controls for the above experiments, we constructed recombinant viruses expressing cN390Δ (AdV-cN390Δ), and cN/CBR(–) (AdV-cN/CBR(–)). cN390Δ is a mutant N-cadherin with a deletion at the extracellular domain, but having the normal intracellular domain; it exhibits a dominant negative effect on the activity of endogenous cadherins (Kintner 1992; Fujimori and Takeichi, 1993). cN/CBR(–) is another mutant form of N-cadherin lacking only the catenin-binding region. When these constructs were injected, cN390Δ expression did not induce any abnormal clustering of infected cells in the dorsal neural tube, allowing many of them to migrate along the dorsolateral pathway (Fig. 5G,H). Cells expressing cN/CBR(–) showed an intermediate phenotype; some of them migrated along the dorsolateral pathway, but a considerable number of them remained localized in the midline of the neural tube (Fig. 5I,J).

To confirm the ectopic protein expression for N-cadherin and cad7 in the above experiments, we immunostained embryos incubated for 30 hours after viral injection for these proteins. As expected, both proteins were overexpressed in the roof plate region of the neural tube (Fig. 6). As mentioned above, in these neural tubes, some roof plate cells invaded their lumen (Fig. 6A,B arrows). These findings demonstrate that overexpressed N-cadherin or cad7 inhibited the escape of a particular population of neural crest precursors from the neural tube. This population likely includes melanocyte precursors, as only these crest derivatives are capable of migrating in the dorsolateral path (Erickson and Goins, 1995).

Differentiation is not inhibited by ectopic cadherin expression

The above inhibition of neural crest emigration along the dorsolateral pathway could have been brought about by a suppression of their differentiation specific for this particular pathway, that is, melanocyte differentiation (LeDouarin, 1982). To test this possibility, we examined the expression of Mitf, an early melanocyte marker (Opdecamp et al., 1997; Y. Wakamatsu, personal communication), in embryos expressing ectopic N-cadherin or cad7. Embryos were incubated for 48 hours after viral injection to allow a sufficient expression of Mitf. In control embryos injected with the β-gal vector, Mitf-positive future melanocytes were detected not only in DRG but also in other parts of the trunk and hindlimb (Fig. 7A,B). In embryos injected with N-cadherin- or cad7-expressing viruses, cells co-expressing Mitf and FLAG-positive future melanocytes were detected not only in DRG but also in other parts of the trunk and hindlimb (Fig. 7C,D). To confirm the ectopic protein expression for N-cadherin and cad7 in the above experiments, we immunostained embryos incubated for 30 hours after viral injection for these proteins. As expected, both proteins were overexpressed in the roof plate region of the neural tube (Fig. 8). As mentioned above, in these neural tubes, some roof plate cells invaded their lumen (Fig. 8A,B arrows). These findings demonstrate that overexpressed N-cadherin or cad7 inhibited the escape of a particular population of neural crest precursors from the neural tube. This population likely includes melanocyte precursors, as only these crest derivatives are capable of migrating in the dorsolateral path (Erickson and Goins, 1995).

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had accumulated in the dorsal lumen of the neural tube (Fig. 7C,E arrows), although some of the Mitf-positive cells were FLAG negative (Fig. 7C-F, arrowheads). Some Mitf-positive cells were also scattered dorsal to the neural tube as in control embryos (Fig. 7C,E), but none of them expressed exogenous cadherins (Fig. 7D,F). In embryos injected with cN390Δ-expressing viruses, Mitf-positive cells behaved like those in the control embryos (Fig. 7G,H). These results suggest that melanocyte differentiation took place in cells with ectopic N-cadherin or cad7 but that these cells were unable to escape from the neural tube and instead invaded the lumen of the tube.

DISCUSSION

Neural crest emigration from the neural tube involves multiple processes (Tosney, 1978; Erickson and Weston, 1983; Erickson and Perris, 1993). Disruption of the attachment of premigratory crest cells to neuroepithelial cells is one of these processes. The neural tube cells are connected to one another along their lateral surfaces. At their luminal side which corresponds to the apical side of simple epithelia, the adherens junction (AJ) develops, which probably represents the major sites for the interconnection of neuroepithelial cells (Duband et al., 1988). Cadherin adhesion molecules are localized throughout the lateral contact sites between these cells, being highly enriched in the AJs. For neural crest cells to escape from the tube, these cell-cell contacts must be disrupted. We can imagine at least two possible mechanisms for the disruption of neuroepithelial junctions. One is a physiological mechanism to down-regulate the AJ function. Cadherin-based junctions are believed to be a machinery whose activity can be regulated (Takeichi et al., 1993). Cadherins are associated with catenins, and this association is essential for the normal activity of the former (Aberle et al., 1996; Barth et al., 1997). Physiological modification of catenins could de-stabilize cadherin-mediated cell-cell adhesion (Takeichi, 1993). It is of note that cells expressing chimeric proteins of E-cadherin and α-catenin, to which β-catenin is unable to bind, are more stably connected with one another than those expressing the normal cadherin/catenin complex, suggesting that β-catenin is a regulator for cadherin activity (Nagafuchi et al., 1994). Such mechanisms could serve to enhance neural crest detachment from neuroepithelial cells.

![Fig. 4. Schematic representation of full-length or mutant cadherin constructs of recombinant adenovirus. All constructs have the FLAG epitope at their carboxyl terminus. TM, transmembrane domain.](image)

![Fig. 5. Behavior of neural crest cells ectopically expressing various cadherin constructs. Embryos were injected with adenoviruses expressing β-gal (A,B), N-cadherin (C,D), cad7 (E,F), cN390Δ (G,H), or cN/CBR(−) (I,J), and fixed 30 hours after the injection. The specimens were stained with anti-β-gal (A,B) or anti-FLAG M2 antibody (C-J). (A,C,E,G,I) Dorsal view of whole-mount embryos. (B,D,F,H,J) The whole-mount samples were sectioned at a thickness of 100 μm, and photographed under Nomarsky optics. Arrows indicate cells migrating along the dorsolateral pathway; note the absence of labeled cells on this pathway in C-F. Arrowheads point to infected cells localized in the roof plate, some of which are invading the neural tube lumen.](image)
The second mechanism concerns cadherin type switching, which occurs during neural crest development. Dorsal-most neuroepithelial cells express N-cadherin and cad6B, while migrating crest cells express cad7. The expression of cad7 by neural crest cells seems to begin in advance of their escape from the tube, but its substantial expression appeared only after they had left the tube. In these cells, N-cadherin and cad6B were no longer detected, indicating that N-cadherin and cad6B are replaced with cad7, when crest cells are leaving the neural tube. Previous observations showed that heterophilic cad7-cad6B binding is less stable than homophilic cad6B-cad6B or cad7-cad7 binding and also that cad7 has little affinity to N-cadherin (Nakagawa and Takeichi, 1995). Therefore, the observed replacement of cadherins during neural crest emergence is expected to facilitate their detachment from neuroepithelial cells. It should be stressed that the loss of N-cadherin and cad6B from premigratory crest cells is, in itself, theoretically sufficient to facilitate this detachment process.

Our results of N-cadherin or cad7 overexpression support the idea that the control of cadherin activity or expression is essential for neural crest emigration. When these cadherins were overexpressed in the dorsal neural tube, emigration of melanocyte precursors was prohibited. In considering the mechanism of this phenomenon, however, we were concerned that the cadherin overexpression might have affected melanocyte differentiation, but not adhesion processes. A melanocyte marker, Mitf, was found to be expressed by the neural crest cells stuck in the neural tube, suggesting that the melanocyte differentiation normally proceeded during the observed events. Another concern along a related line would be a possible involvement of Wnt signaling. Two Wnt genes, Wnt-1 and Wnt-3a, are expressed in the midline of the mouse neural tube; and double mutation for these genes causes inhibition of neural crest generation and migration (Ikeya et al., 1997). It is known that cadherin overexpression depletes β-catenin, a component essential for Wnt signaling, and therefore blocks this signaling system (Heaseman et al., 1994; Fagotto et al., 1996; Sanson et al., 1996). However, cN390Δ, which can bind β-catenin as well as intact cadherins, did not suppress the dorsolateral migration of the crest cells. Moreover, the expression of cN/ΔBR(−), which lacks the β-catenin binding domain, exhibited a partial suppression of crest cell migration. These findings suggest that the failure of neural crest migration did not depend on inhibition of the β-catenin-associated Wnt signaling. The fact that the effect of cadherin overexpression mimicked that of the Wnt mutation is interesting, as it raises the possibility that a role of Wnt signals in neural crest development is to antagonize cadherin function. In this context, it should be interesting to note that E-cadherin expression is upregulated in the roof plate of the midbrain in Wnt-1 mutant mice (Shimamura et al., 1994).

Thus, a likely mechanism underlying the above phenomenon is that the overexpressed cadherins directly modulated the neural crest adhesion properties. Although the endogenous N-cadherin and cad7 were expressed in different patterns by the...
original neural crest, the ectopic expression of them produced a novel profile of cadherin expression; i.e., a single type of cadherin, either N-cadherin or cad7, was expressed throughout from the neural tube to migrating crest cells, abolishing the cadherin type-switching pattern. This altered cadherin expression likely caused a persistent sticking of neural crest precursors to the neuroepithelial layer, interfering with either or both of the above-proposed mechanisms for neural crest detachment from the neural tube: that is, the ectopic cadherins expressed by both neuroepithelial cells and crest precursors might have prevented them from separating, or an excess amount of cadherins accumulated at the dorsal neural tube might have competed with a mechanism to down-regulate cadherin-mediated intercellular adhesion. One could propose a third mechanism, i.e., that the overexpression of cadherins affected cell motility, thereby inhibiting crest emigration. This is unlikely, because crest cells with a high level of ectopic cadherins still normally migrated (Fig. 3G, H arrows). In these cells, the cadherin overexpression was probably induced after they had escaped from the neural tube (see below).

It remains unsolved as to why crest cells that had failed to escape from the neural tube eventually accumulated in the lumen of the tube. Essentially all Mitf-positive cells expressing ectopic cadherins were excluded from the neuroepithelial layer into its lumen. Interestingly, the excluded cell clusters contained a population of cells not expressing the ectopic cadherins. This finding implies that the exclusion was not induced by cadherin overexpression in neural crest precursors, but rather by that in the neuroepithelial cells. A possible mechanism underlying this phenomenon is the following. The regulated expression of cadherins in the neuroepithelium may be required for maintaining its integrity, such as apicobasal polarity, and this could be essential for the neuroepithelium to send out neural crest cells in the right direction. Cadherin overexpression might disorganize the polarized neuroepithelial structures, leading some of crest cells to migrate in a wrong direction. If this is the case, this possibility also must be considered as one of the mechanisms by which melanocyte precursor emigration was inhibited. It should also be pointed out that a similar luminal accumulation of crest cells was observed when their migration onto the extracellular matrix had been blocked (Bilozur and Hay, 1988). In this case, we should assume that crest cells were rather passively brought down into the lumen. For the presence case, such passive mechanism also should be considered.

Why was behavior of melanocyte precursors selectively affected in our experiments? The crest cell migration along the ventral pathway was not dramatically inhibited by the cadherin overexpression, although some small effects were detectable. In our experimental protocol, stage 14 embryos were injected. During the initial 20 hours following the injection, only cells that would migrate through the ventral pathway are generated; and after about 24 hours (stage 21), melanocyte precursors first appear in the trunk areas (Erickson et al., 1992), therefore leaving the neural tube relatively late (Henion and Weston, 1997). As the virus-derived cadherin expression gradually increased during 24 hours, the maximum overexpression of these cadherins happened to coincide with the onset of the generation of melanocyte precursors; i.e., among various neural crest precursors being generated, the future melanocytes and surrounding neuroepithelial cells must have undergone the strongest exogenous cadherin expression while the former were still localized in the neural tube. These considerations explain why the melanocyte pathway was most severely affected. In many of the crest cells that migrated along the ventral pathway, ectopic cadherin expression would have been induced only after their escape from the neural tube, as there was a lag period in the viral vector expression.

In the present studies, we used two control N-cadherin constructs, cN390Δ and cN/CBR(−). Although the former showed no effect on neural crest emigration processes, the latter one exhibited a partial effect. The adhesive function of this cadherin construct was not extensively analyzed. Since this construct lacks the catenin-binding region, its activity as a cadherin adhesion molecule should be not as good as that of the intact N-cadherin. Nevertheless, the juxtanuclear region of the cadherin cytoplasmic domain, left intact in the cN/CBR(−), was shown to have some biological activity (Kintner, 1992; Riehl et al., 1996), implying that this construct still possesses certain functions to regulate cell adhesion. The effect of its expression on neural crest emigration is possibly based on such partial adhesion-related functions. However, cN390Δ did not show any inhibition of neural crest emigration. This result is consistent with the fact that cN390Δ cannot function as an adhesion molecule, because of the deletion in the extracellular domain. cN390Δ is known to exhibit a dominant negative effect on endogenous cadherin-mediated adhesion in certain epithelial cell lines (Fujimori and Takeichi, 1993). Such inhibitory effect was not clearly detected in the present system. Perhaps, the expression level of cN390Δ was not sufficient to inhibit endogenous cadherin activity, or the neural tube system was not sensitive enough to detect adhesion defects.

The present study did not focus on the role of cadherins in the later migration processes of crest cells including homing, mainly because of the technical reason that the virus-derived cadherin expression was transient, diminishing during the migration. We previously hypothesized that cadherins expressed by migrating crest cells may play roles in the regulation of their homing behavior (Nakagawa and Takeichi, 1995), but this idea remains to be tested in future studies.

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