

Avian neural crest-derived neurogenic precursors undergo apoptosis on the lateral migration pathway

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SUMMARY

Neural crest cells of vertebrate embryos disperse on distinct pathways and produce different derivatives in specific embryonic locations. In the trunk of avian embryos, crest-derived cells that initially migrate on the lateral pathway, between epidermal ectoderm and somite, produce melanocytes but no neuronal derivatives. Although we found that melanocyte precursors are specified before they disperse on the lateral pathway, we also observed that a few crest-derived neuronal cells are briefly present on the same

pathway. Here, we show that neuronal cells are removed by an episode of apoptosis. These observations suggest that localized environmental factor(s) affect the distribution of fate-restricted crest derivatives and function as a 'proof-reading mechanism' to remove 'ectopic' crest-derived cells.

Key words: Neural crest, Migration, Hu, *mitf*, Melanocyte, Apoptosis, Chick, Quail

INTRODUCTION

Neural crest cells originate from the dorsal neural tube of vertebrate embryos and enter a space delimited by the dorsal neural tube and the dorsal somite. In this location, the migration staging area (MSA; Weston, 1991), crest-derived cells choose one of two distinct migration pathways and ultimately produce diverse derivatives in precise embryonic locations. In avian and mammalian embryos, the peripheral nervous system contains neurons and glia that arise from crest-derived cells that migrate early on a medial pathway (MP) between the neural tube and the rostral sclerotome of the somite. Other crest-derived cells disperse later on a lateral migration pathway (LP), which initially consists of the space between epidermal ectoderm and somitic dermamyotome, but at later stages, also includes epidermis, dermal mesenchyme and myotome. It is believed that these cells normally produce melanocytes in the epidermis and dermis. No crest-derived peripheral ganglia arise from crest cells on this pathway.

The spatially distinct distribution of melanocytes on the LP could be the result of two distinct mechanisms. First, developmentally labile crest cells might undergo progressive fate restrictions as they disperse on the LP, or after they colonize the skin, and differentiate into melanocytes in response to specific localized environmental cues. This alternative appears to gain support from studies in which crest cells taken from the LP were cultured at clonal density. In such cultures, crest-derived clonal progenitors present on the LP at early stages of development gave rise to colonies containing

cells that expressed neuronal traits. At later stages, neurogenic ability was absent in crest-derived clonal progenitors on the LP. These results were interpreted to indicate that multipotent crest cells became progressively restricted to melanogenic fate after the onset of dispersal on the LP (Sieber-Blum et al., 1992; Richardson and Sieber-Blum, 1993).

Alternatively, crest cells might become fate-restricted *before* they disperse from the MSA. If this were the case, environmental cues localized on the LP would promote selective migration and/or survival of fate-restricted melanocyte precursors. This alternative is supported by a number of recent reports. For example, we have recently shown by sequential clonal analysis that a significant fraction of crest cells newly emerged from avian neural tube explants give rise to distinct fate-restricted precursor populations in a culture environment that is permissive for the differentiation of all major crest-derived cell types, such as neuron, glia and melanocyte. This result indicates that fate-restricted progenitors segregate early in the crest-derived population (Henion and Weston, 1997). Moreover, Erickson and Goins (1995) have shown that cultured quail crest-derived melanogenic cells precociously and preferentially disperse on the LP when grafted into the MSA of host chicken embryos. These results are consistent with the idea that fate-restricted melanocyte precursors in the MSA selectively disperse on the LP.

The idea that fate-restricted melanocyte precursors selectively disperse on the LP appears inconsistent with the reported presence on the LP of crest cells with neurogenic ability (Richardson and Sieber-Blum, 1993). It is important

for understanding developmental mechanisms, therefore, to try to distinguish between these two distinct explanations of the precise pattern of localization of crest derivatives. To do so, and to identify the source and fate of neurogenic cells on the LP, we must be able to recognize and follow the fates of developmentally distinct crest-derived precursors. Accordingly, we have used early molecular markers to detect the existence of both melanocyte precursors and neurogenic/neuronal precursors on the LP. We find that melanocyte precursors represent a majority, and neurogenic cells a minority, of crest-derived cells on the LP. We have also shown that the LP is not capable of supporting the survival of neurogenic/neuronal cells that happen to be present there.

MATERIALS AND METHODS

Experimental animals

Fertilized eggs of chicken (*Gallus gallus*) and Japanese quail (*Coturnix coturnix japonica*) were obtained from Oregon State University, Corvallis, Oregon and incubated at 38°C. Embryos were staged according to Hamburger and Hamilton (1951).

Whole-mount in situ hybridization

Riboprobes were prepared from a *Bam*HI fragment of cDNA, consisting primarily of the 3' untranslated region of the chicken *Microphthalmia* gene product, *mitf* (Mochii et al., 1998a). To do so, the cDNA template was transcribed in vitro with T7 RNA polymerase and digoxigenin-11-UTP (Boehringer-Mannheim) according to the manufacturer's instructions. Whole-mount in situ hybridization was performed as described previously (Wakamatsu and Weston, 1997).

Immunological staining

Immunological staining on tissue sections and in cell cultures was done as described previously (Wakamatsu et al., 1993). 16A11 (anti-Hu) antibody (mouse IgG2b; Marusich et al., 1994) and HNK-1 antibody (mouse IgM; Vincent et al., 1983) were prepared from hybridomas and were provided by the Monoclonal Antibody Facility, Institute of Neuroscience, University of Oregon. The details of the anti-MITF rabbit antibody has been described previously (Mochii et al., 1998a). Quail cell-specific antibody against a perinuclear antigen (QCPN; mouse IgG1; Catala et al., 1996) was obtained from Developmental Studies Hybridoma Bank, National Institute of Child Health & Human Development, University of Iowa. Melanocyte-specific antibody, recognizing melanosome matrix protein 115 (MEBL-1; mouse IgG1; Kitamura et al., 1992), was kindly provided by Dr Kunio Kitamura. Rabbit polyclonal anti-chicken neurofilament 150 kDa antibody was purchased from Calbiochem. TRITC-conjugated goat anti-mouse IgM, TRITC-conjugated goat anti-mouse IgG1, FITC-conjugated goat anti-mouse IgG2b antibodies purchased from Southern Biotechnologies, and TRITC-conjugated donkey anti-mouse IgG, FITC-conjugated donkey anti-rabbit IgG antibodies purchased from Jackson ImmunoResearch were used for detection of primary antibodies. After staining with antibodies, nuclei were labeled with DAPI. Stained sections and cultures were mounted in Vectashield (Vector).

Whole-mount antibody staining was performed as described previously (Wakamatsu and Weston, 1997).

Transplantation

Preparation of CM-Dil-labeled crest cells

Neurogenic (24 hours) and non-neurogenic (48 hours) neural crest clusters were prepared as described previously (Glimelius and Weston, 1981; Vogel and Weston, 1988). Harvested crest clusters were labeled with culture medium containing 10 µM of Cell Tracker CM-Dil (Molecular Probes; Andrade et al., 1996) for 30 minutes at 38°C.

At the time that neural crest clusters were taken for transplantation, sister cell populations were placed in secondary culture to confirm their neurogenic and melanogenic ability (Fig. 1B,C).

Transplantation

Labeled cluster-derived cells were transplanted to the LP or MP of stage 13-14 chicken embryos as follows. The LP or MP of a host embryo was opened with a sharpened tungsten needle and approximately 50 CM-Dil-labeled crest cells were inserted with a blunt tungsten needle. These procedures are diagramed in Fig. 1A. Quail neural tubes were transplanted into stage 13-14 chicken host embryos as previously described (Wakamatsu et al., 1997).

Detection of cell death by TUNEL

Histological sections

Frozen sections of 4% paraformaldehyde (PFA)-fixed chimeric embryos were treated with 1 µg/ml of proteinaseK/PBS for 2 minutes at room temperature and then re-fixed in 4% PFA/PBS for 10 minutes. After preincubation with TdT buffer (Wakamatsu and Weston, 1997), sections were incubated with TdT buffer containing 120 units/ml of terminal deoxynucleotidyl transferase (TdT) (Boehringer) and 8 µM of biotin-14-dATP (Gibco) at 37°C for 30 minutes. After washing, labeled cells were visualized by avidin-FITC (Vector) followed by nuclear-staining with DAPI.

Whole mounts

PFA-fixed embryos were treated with 1 µg/ml of proteinaseK in PBST (PBS containing 0.2% Tween20) for 30 minutes, and then re-fixed with 4% PFA, 0.2% glutaraldehyde in PBST for 20 minutes. After washing with TdT buffer, embryos were incubated in TdT buffer containing 120 units/ml of TdT and 5 µM DIG-11-dUTP (Boehringer) at 4°C for 1 hour, then at 37°C for 1 hour. After washing in TBST (100 mM TrisHCl (pH 7.5), 150 mM NaCl, 0.1% Tween20), and blocking with TBST containing 10% heat-inactivated normal goat serum for 2 hours, embryos were incubated with alkaline phosphatase-conjugated anti-DIG antibody (Boehringer) at 4°C overnight. Embryos were then washed and developed with NBT/BCIP.

RESULTS

Melanocyte precursors preferentially migrate on the lateral pathway

The *Microphthalmia* (*mitf*) gene product is a transcription factor known to be essential for melanocyte differentiation. At the wing level of stage 19 avian embryos, cells expressing *mitf* mRNA were first detected in the crest cell population that remained in the MSA after many crest cells had already departed on the MP (Fig. 2A,C). Subsequently, *mitf*⁺ cells were observed only on the LP (Fig. 2B). Sections treated with both anti-MITF and a melanocyte-specific antibody (MEBL-1), revealed that MEBL-1 immunoreactivity (IR) begins to appear after the onset of dispersal on the LP only in MITF⁺ cells (Fig. 2D-F). Later in development, most MITF⁺ cells were MEBL-1⁺. No cells were observed to exhibit MEBL-1-IR without MITF-IR. Likewise, no *mitf*⁺ cells were ever seen to co-express neuronal markers (anti-Hu and anti-neurofilament antibodies, see Methods). Taken together with other reports on the role of *mitf* in melanogenesis (see Discussion), these results suggest that *mitf*⁺ cells are melanocyte precursors, which are specified prior to their dispersal on the LP.

Even after being placed on the MP, transplanted *mitf*⁺ melanocyte precursors are present primarily on the LP of host embryos. Thus, cultured crest cell populations, which are known to be primarily melanogenic, and which lack

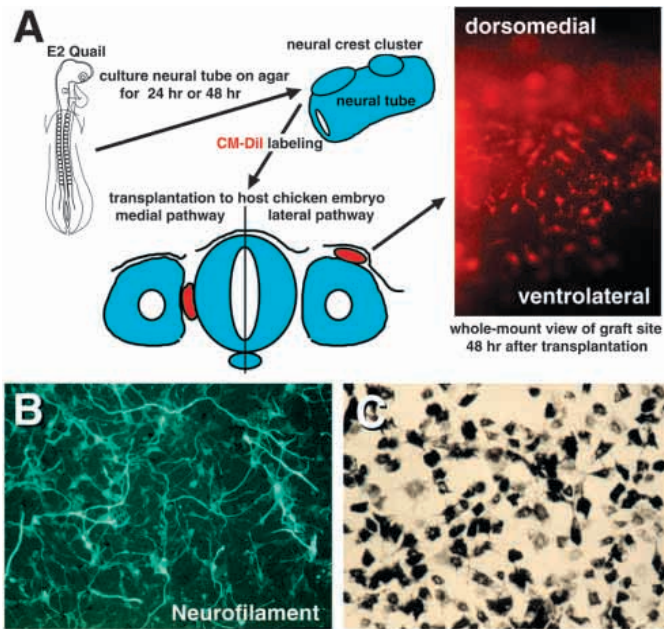


Fig. 1. Transplantation of cultured crest cells to the lateral and medial pathways. (A) Overview of procedure for grafting neurogenic and melanogenic crest cell populations to the LP and MP. Note that CM-Dil-labeled crest cells, detected in whole-mount preparations, are seen to disperse extensively on the LP. (B) Cultures of crest-derived neurogenic populations corresponding to cells grafted to LP. Note extensive appearance of cells expressing neurofilament-IR (green fluorescence). (C) Cultures of crest-derived melanogenic populations corresponding to cells transplanted both to LP and MP. Note that virtually all cells contain melanin granules. No neuronal cells were observed (not shown, but see Vogel et al., 1988).

neurogenic ability (Vogel and Weston, 1988), were obtained from cell clusters that form from crest cells that emerge when quail neural tubes are explanted on non-adhesive substrata and that are prevented from dispersing in a timely way (Glimelius and Weston, 1981). These cell populations were transplanted onto the MP of stage 13-14 chicken embryos at the level of wing bud (see Materials and Methods, Fig. 1A). Sister cell populations were cultured in permissive medium to verify their melanogenic fate (Fig. 1C). In hosts fixed 4 days after the transplantation (stage 29), virtually all the transplant-derived cells detected by anti-quail perinuclear antigen QCPN antibody were found in the epidermal and dermal skin, and expressed MITF-IR (Fig. 3B,C). Only a few cells were found to remain on the MP (data not shown), and none of these expressed neuron-specific Hu RNA-binding proteins characteristic of neuronal differentiation (mAb 16A11-IR; see Marusich et al., 1994; Wakamatsu and Weston, 1997).

Based on the staining and transplantation results, therefore, we conclude that crest-derived cells appear to be restricted to a melanogenic fate prior to the onset of dispersal from the MSA, and these specified melanocyte precursors preferentially migrate on the LP (see also Erickson and Goins, 1995).

Some crest-derived neuronal cells can be identified on the LP

After whole-mount in situ hybridization with *mitf* antisense probe, embryos were sectioned and examined for expression

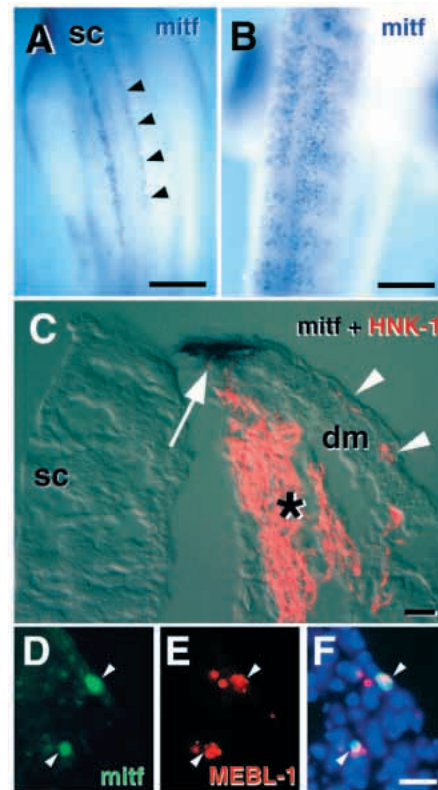


Fig. 2. *mitf* expression reveals melanocyte differentiation and migration on the LP. (A) Dorsal view of stage 19 chicken embryo, showing *mitf* mRNA expression in the MSA at the level of the wing buds (arrowheads; see text). (B) Dorsal view of stage 22 embryo, showing *mitf* mRNA expression in cells dispersed on the LP at wing level. (C) Transverse section of stage 21 embryo at hind limb level. HNK-1 antibody staining (red) reveals neural crest-derived cells. While *mitf*⁺ cells remain in MSA (dark purple, arrow), some HNK-1⁺ crest cells have dispersed on the LP (arrowheads). HNK-1 staining in *mitf*⁺ cells was masked by the *mitf* probe color reaction product. (D-F) Transverse section of stage 23 embryo, showing epidermis and dermal mesenchyme on the LP. Nuclear staining of anti-MITF antibody (green, D,F) coincides with MEBL-1 antibody staining (red, E,F; arrowheads). Blue staining in F shows DAPI nuclear staining. dm, dermomyotome; sc, spinal cord. *, crest cells migrating on the MP. Scale bars, (A) 250 μ m, (B) 500 μ m, (C, F) 20 μ m.

of the neural crest marker HNK-1. At the time and axial level where all *mitf*⁺ cells still remained in the MSA, (i.e. stage 19 at wing level, or stage 21 at hind limb level), some *mitf*⁺, HNK-1⁺ cells were also detected on the LP (Fig. 2C). This result suggested the possibility that some non-melanogenic crest-derived cells disperse on the LP. We also observed a few cells randomly distributed on the LP that expressed the neuron-specific Hu-IR (Fig. 4A). In transverse sections, these cells were found either in dermal mesenchyme (Fig. 4B) or within the myotome (not shown), well away from other nervous system structures such as dorsal root ganglia, sympathetic ganglia and neural tube. Since functional neurons expressing Hu-IR are confined to the central and peripheral nervous systems at late stages of development, we refer to the Hu⁺ cells outside of these structures as 'ectopic' neuronal cells. None of the ectopic Hu⁺ cells was *mitf*⁺ (data not shown). Ectopic Hu⁺ cells were present on the LP briefly and in small numbers

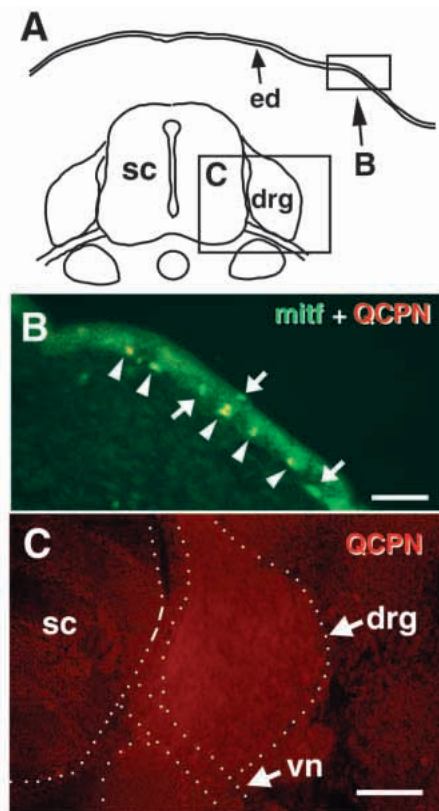


Fig. 3. Non-neurogenic quail crest cells transplanted on the MP of chicken embryo hosts are found primarily on the LP. (A) Schematic view of transverse section of stage 29 embryo (wing level) that received non-neurogenic crest cells on the MP. (B) Combined image of anti-MITF (green) and quail cell-specific (QCPN) antibody staining (red). Double-stained cells appear yellow. Transplanted cells (QCPN⁺; arrowheads) express MITF-IR in the skin (yellow), but host-derived melanocyte precursors (OCPN⁻; arrows) also express MITF-IR (green). (C) QCPN-staining of the same section as B, showing no transplant-derived cells remain in tissues derived from crest cells on the MP. drg, dorsal root ganglia; ed, epidermis; sc, spinal cord; vn, ventral nerve. Scale bars, (B) 40 μ m, (C) 200 μ m.

(Table 1). They were first seen at stage 20. At stage 22-23, more ectopic Hu⁺ cells were observed, after which the number appeared to decline. To determine whether these ectopic Hu⁺ cells were crest-derived, the neural tube of stage 13-14 chick embryo hosts was replaced at the wing level with a segment of quail neural tube before the onset of crest cell emigration. Hosts were then fixed at stage 22, and double-stained with the anti-Hu and quail cell-specific antibodies. All the Hu⁺ cells in dermis or myotome were derived from the quail donor (Fig. 4C-E). We conclude, therefore, that crest-derived neurogenic/neuronal cells, which were developmentally distinct from melanocyte precursors, reside transiently on the LP.

Crest-derived neurogenic/neuronal cells do not persist on the LP

Since crest-derived neuronal structures do not form on the LP, it is essential to learn the fate of the cells with neuronal phenotype. There are at least three possibilities: neurogenic cells on the LP could change their fate, they could re-localize

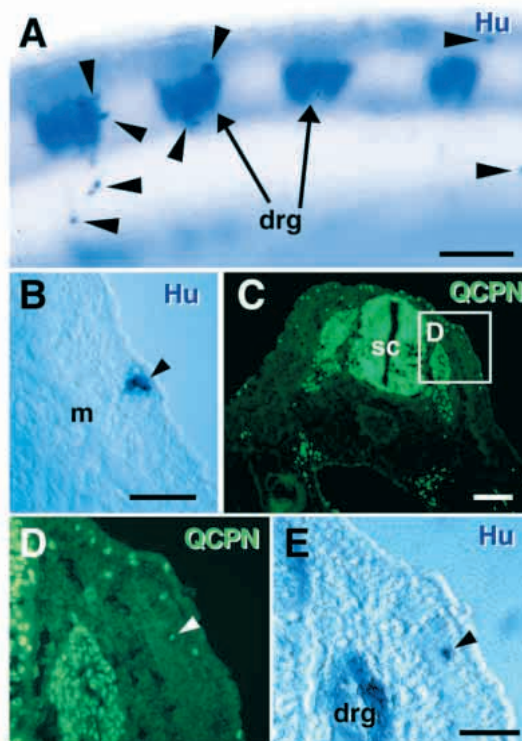


Fig. 4. Neural crest-derived Hu⁺ neuronal cells are present on the LP. (A) Side view of stage 22 chicken embryo stained with anti-Hu antibody (mAb 16A11) in whole-mount preparation. In addition to staining in the dorsal root ganglia (drg, arrows), randomly scattered superficial Hu⁺ cells are observed (arrowheads). (B) Transverse section of stage 22 embryo stained with 16A11 anti-Hu antibody in whole mount. Hu⁺ cells (arrowhead) are found in the dermal mesenchyme. (C) Transverse section of chicken embryo in which a segment of quail neural tube has replaced host neural tube. QCPN-IR marks graft-derived spinal cord (sc) cells and neural crest derivatives. (D) High magnification of the part of section indicated in (C). (E) Bright-field image of (D). Dark staining shows Hu⁺ cells in the drg and ectopically in the dermatome (arrowhead). m, myotome. Scale bars, (A) 200 μ m, (B, C) 100 μ m, (E) 50 μ m.

or they could die. Consistent with the last alternative, cell death on the LP has been inferred previously by Nile blue vital dye staining (Jeffs and Osmond, 1992). We extended these results by whole-mount TUNEL analysis, which reveals DNA fragmentation and/or degradation in dying cells. We found that

Table 1. The number of ectopic Hu⁺ cells on the LP during normal development

Stage	<i>n</i>	Whole trunk and tail Average (range)	Wing level* Average (range)
18, 19	5	0 (0)	0 (0)
20, 21	5	14.2 (9-17)	4.2 (2-7)
22, 23	7	34.4 (12-49)	16.3 (0-38)
24, 25	6	22.8 (1-37)	8.8 (0-15)
26, 27	6	1.8 [‡] (0-5)	0 (0)

All Hu⁺ cells localized superficially on the trunk and the tail were counted. *n*, the number of embryos examined.

*Axial level corresponding to somite 16-21.

[‡]Ectopic Hu⁺ cells were found only in the tail.

transient cell death occurred in the LP at the wing axial level of stage 21-24 embryos (Fig. 5A-D). This cell death was predominantly observed in the dermatome/dermal mesenchyme (Fig. 5E,F). In addition, we observed that some of the ectopic Hu^+ cells appeared to have fragmented nuclei (data not shown), one of the indicators of apoptotic cells. Taken together, these results suggested that neurogenic/neuronal crest cells were undergoing apoptosis on the LP. However, because there were few ectopic Hu^+ cells and the TUNEL method does not stain dying cells until the end of the apoptosis process when Hu expression can no longer be detected, these results do not indicate unequivocally that crest-derived neuronal cells on the LP were undergoing apoptosis.

To confirm that the neurogenic/neuronal cells on the LP were undergoing apoptosis and to achieve quantitative as well as qualitative results, we placed crest cells with known neurogenic ability onto the LP and observed the effect on the number of cells undergoing apoptosis. Specifically, we prepared quail crest cell populations from nascent crest cell clusters, which are known to contain both neurogenic and melanogenic precursors (Fig. 1B; Vogel and Weston, 1988). We labeled these cells with CM-DiI and transplanted them onto the LP of stage 13-14 chicken embryo hosts (see Methods; Fig. 1A). Neurofilament- and Hu -immunostaining of cultured sister populations confirmed the presence of abundant neurogenic cells in the transplanted populations (Fig. 1B). 48 hours after transplantation, graft-derived cells were dispersed on the LP about 3- to 5-somite length along the anteroposterior axis (Fig. 1A). Most of the CM-DiI-labeled cells present in the epidermis were $mitf^+$ melanocyte precursors, whereas the labeled cells in the dermis and myotome included both $mitf^+$ and $mitf^-$ cells (data not shown). Graft-derived melanocytes developed normally, and eventually contributed to donor-specific

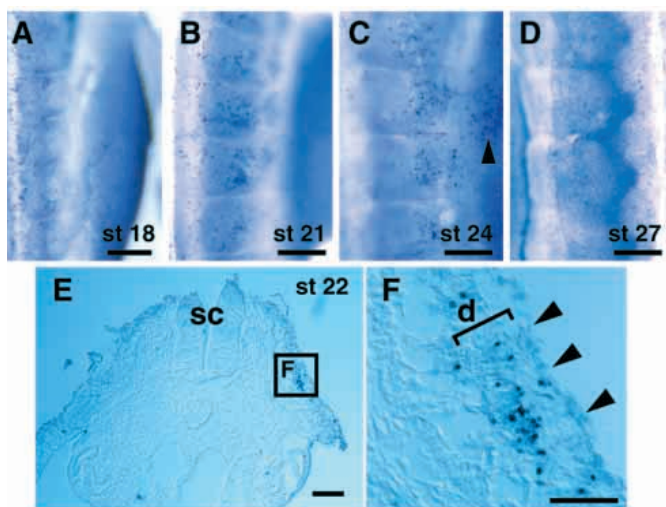


Fig. 5. Transient cell death normally occurs on the LP. (A-D) Flat mount preparation of TUNEL-stained embryos at wing level. Mid-line is on the left of each photograph. Increased cell death can be seen at stage 21 and 24. Arrowhead in C indicates cell death in the proximal part of the limb bud. (E) Transverse section of stage 22 embryo after whole-mount TUNEL staining. (F) Higher magnification of E. TUNEL-stained dying cells are found in dermatome (d), but not in epidermis (arrowheads). sc, spinal cord. Scale bars, (A-D) 200 μ m, (E) 100 μ m, (F) 50 μ m.

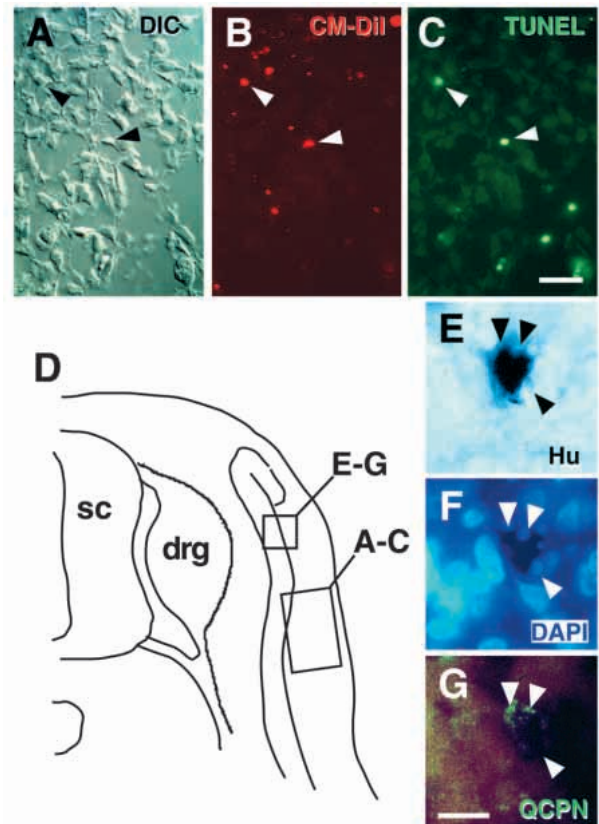


Fig. 6. Neurogenic/neuronal crest cells transplanted on the LP die in the dermis and myotome. (A-C) Transverse section of a chimeric embryo, 48 hours after transplantation of CM-DiI-labeled neurogenic crest cells on the LP, showing dermal mesenchyme. (A) DIC optics. (B) CM-DiI fluorescence represents transplant-derived cells. (C) Some of the TUNEL-stained dying cells (arrowheads) are transplant-derived. (D) Schematic view of transverse section, showing the location of A-C and E-G. (E-G) Transverse section of chimeric embryo, 36 hours after transplantation of neurogenic crest cells on the LP. Example of a Hu^+ cell on the LP (E, dark purple), indicating fragmented nucleus (F, arrowheads), typical of apoptosis with DAPI staining. (G) This dying Hu^+ cell still possesses residual QCPN-IR. Scale bars, (C) 20 μ m, (F) 10 μ m.

pigmentation of host feathers (data not shown). Similar chimeric embryos were examined for DNA fragmentation by the TUNEL method (Table 2, see also Fig. 6A-C). 48 hours after the transplantation (stage 23-24), approximately 5% of the CM-DiI-labeled cells located in the dermal mesenchyme and myotome exhibited the small, rounded morphology typical of apoptotic cells, and were also labeled by the TUNEL reaction. In contrast, virtually no TUNEL⁺ cells were found in the epidermis (Table 2, Fig. 6A-C). The observations that apoptosis and $mitf^-$ cells were predominant in the dermal mesenchyme and myotome coincided well with the data that the ectopic Hu^+ cells and transient apoptosis in normal development were found in the same area. When sections were double-labeled with anti- Hu and anti-quail (QCPN) antibodies, some Hu^+ cells were again stained with the quail-specific antibody, confirming their origin from graft cells. Moreover, some of the double-stained cells possessed fragmented nuclei consistent with apoptosis (Fig. 6E-G). However, none of the

Table 2. The number of graft-derived dying cells after neurogenic populations were transplanted onto the LP

	Epidermis*			Dermis and myotome		
	TUNEL ⁻	TUNEL ⁺	% TUNEL ⁺	TUNEL ⁻	TUNEL ⁺	% TUNEL ⁺
No. 1	102	1	1.0	267	12	4.3
No. 2	359	1	0.3	538	32	5.6
No. 3	169	0	0	302	12	5.3
Total	620	2	0.3	1107	62	5.3

*Labeled cells were either integrated within the epidermis or associated with epidermis.

transplanted Hu⁺ cells was labeled by the TUNEL method, probably because Hu proteins are degraded before nuclei become susceptible to TUNEL (data not shown).

To determine whether there was a critical period of apoptosis of neurogenic/neuronal crest cells on the LP, cell death was examined in chimeric embryos fixed 24, 36, 48 and 72 hours (stage 17-18, 21-22, 23-24 and 26-27 respectively) after receiving grafts of CM-DiI-labeled neurogenic populations, or an equivalent number of cells from melanogenic crest populations (Figs 7, 8). The location of transplant-derived cells was identified by CM-DiI fluorescence on whole mounts (Fig. 1A), and trunk segments containing transplant-derived cells (usually 3-5 somites length) were processed for TUNEL staining. Embryos examined 24 or 72 hours after transplantation of neurogenic crest populations showed no difference in the number of TUNEL⁺ cells on the operated and control sides (Fig. 7A,C). In contrast, at 36 and 48 hours after transplantation, the number of TUNEL⁺ cells on the side of the host that received neurogenic populations was markedly increased compared to the contralateral side (Fig. 7B). Importantly, this transient increase in the number of apoptotic cells was observed only when neurogenic crest-derived populations were transplanted. Thus, at all stages examined, no significant increase of cell death was observed when melanogenic crest-derived populations were transplanted on the LP (Fig. 8). The increase in the number of apoptotic cells observed when neurogenic populations were transplanted to the LP is not consistent either with the possibilities that transplanted neurogenic cells re-localized or changed their developmental fate. Accordingly, we conclude that neurogenic/neuronal crest-derived cells are subject to the transient wave of apoptosis on the LP.

DISCUSSION

Melanocyte specification and pathway choice

The *microphthalmia* (*mitf*) gene product is expressed in a subpopulation of neural crest-derived cells on the LP in mouse development (Hodgkinson et al., 1993; Hughes et al., 1993). *mitf* encodes a 'basic helix-loop-helix leucine zipper' transcription factor, which binds to a regulatory *cis*-element referred to as an M-box (Hemesath et al., 1994) and upregulates the expression of melanocyte-specific genes such as *tyrosinase*, *tyrosinase-related protein 1* (Bentley et al., 1994; Yasumoto et al., 1995), *c-kit* receptor tyrosine kinase (Tsujiyama et al., 1996) and *melanosome matrix protein 115* (MMP115; M. M. et al., unpublished data). *mitf* mutants have been found in both mouse and quail, and these mutants lack

pigmentation (Hodgkinson et al., 1993; Homma, 1971; Mochii et al., 1998b; see Moore, 1995 for a review). It is clear that gene expression cannot be used universally as an indicator of cell lineage specification. Nevertheless, based on its unique expression pattern and its role in regulating melanocyte-specific gene products, it seems reasonable to conclude that *mitf* expression is useful as an early marker for melanocyte precursors (see also Opdecamp et al., 1997).

In avian embryos, *mitf* expression is first detected in a subset of neural crest-derived cells residing in the MSA after many

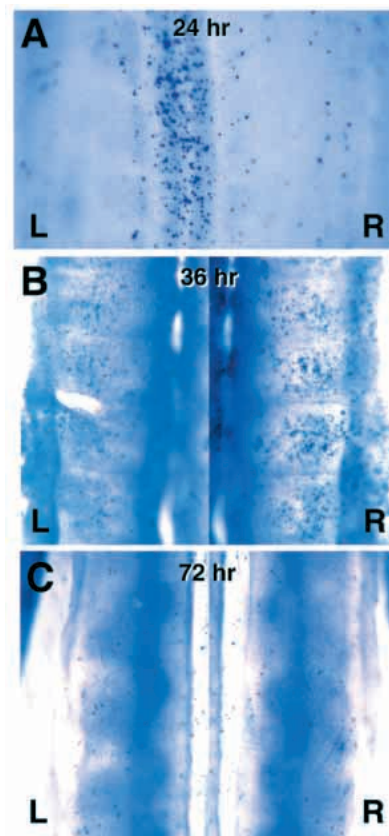


Fig. 7. Transplantation of neurogenic crest-derived population on the LP increases the number of cells undergoing apoptosis. Flat-mount preparations of TUNEL in embryos that received neurogenic crest-derived cells on the LP. (A) 24 hours after transplantation. No obvious difference is observed between transplanted side (right) and control (left). (B) 36 hours after transplantation. Note increase of TUNEL⁺ cells on the transplanted side (R, right). (C) 72 hours after transplantation. No obvious difference in the number of TUNEL⁺ cells is observed between transplanted side (R) and control side (L).

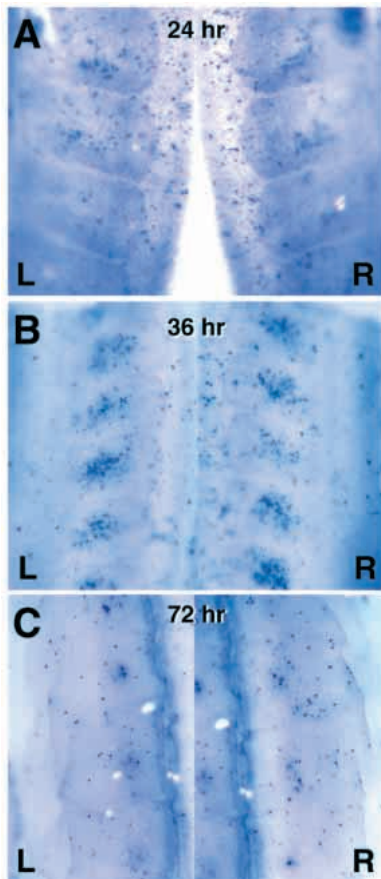


Fig. 8. Transplantation of non-neurogenic crest-derived population on the LP does not change the number of cells undergoing apoptosis. Flat-mount preparations of TUNEL in embryos that received non-neurogenic crest-derived cells on the LP. (A-C) 24, 36 and 72 hours after transplantation, respectively. Although more labeled cells are present at 36 hours, no obvious increase of cell death can be seen when transplanted (R) and control (L) side are compared.

crest-derived cells have already dispersed on the MP. At later stages, cells expressing *mitf* can be seen on the LP, at which time these cells co-express the MEBL-1 antigen MMP115. Previous lineage tracing studies have revealed that melanogenic/melanocyte precursors segregate from the dorsal neural tube and migrate later than neurogenic populations (Kitamura et al., 1992; Sharma et al., 1995; Henion and Weston, 1997). Our inference that melanocyte precursors are already specified prior to the onset of migration on the LP is consistent with these observations. Moreover, in mice, melanocyte precursors expressing tyrosinase related protein 2 are first observed in a subpopulation of crest cells in the MSA (Wehrle-Haller and Weston, 1995) similar to the *mitf*⁺ crest cells in the MSA of avian embryos.

Crest-derived melanogenic populations transplanted into the MSA have been shown to migrate preferentially on the LP (Erickson and Goins, 1995). In this study, we have confirmed that melanogenic crest populations migrate onto the LP, even when they were transplanted ventrally on the MP. Although the mechanism of pathway choice of avian melanocyte precursors is not yet clear, work in murine embryos suggests that diffusible Steel factor present on the LP may be sufficient to

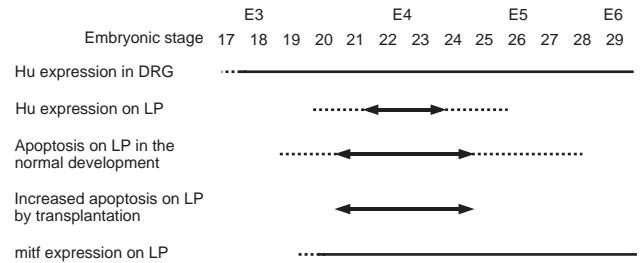


Fig. 9. Time course of marker expressions and cell death on the LP.

promote dispersal of melanocyte precursors, which express the receptor for Steel factor, c-kit (Wehrle-Haller and Weston, 1995, 1997). It seems very likely, therefore, that melanocytes are specified in the MSA prior to their migration and that they preferentially choose the LP in avian embryos.

Crest-derived neurogenic/neuronal cells are transiently present on the LP

In this study, we have shown that crest-derived cells that express neuronal traits are transiently present on the LP (see Fig. 9). Recent sequential clonal analyses have established that neurogenic and melanogenic lineages segregate early so that only a small proportion of crest-derived cells ever showed the potential to give rise to both neurons and melanocytes (Henion and Weston, 1997). Such cells, which also give rise to glial derivatives, soon disappeared altogether from crest-derived populations (Henion and Weston, 1997). It is possible, therefore, that these early 'unspecified' cells are the source of ectopic neurons on the LP. In any case, although the melanogenic population is likely to emigrate from the neural tube late (Kitamura et al., 1992; Sharma et al., 1995; Henion and Weston, 1997), it is yet not clear when the neurogenic/neuronal crest population that is found on the LP segregates from the neural tube. Since *mitf*⁻ crest-derived cells are found on the LP slightly earlier than *mitf*⁺ melanocyte precursors, the neurogenic/neuronal crest cells on the LP may leave the neural tube earlier than the melanogenic population, but later than the majority of neurogenic/neuronal population, which migrates on the MP.

Since some neurogenic crest cells are present on the LP, and eventually begin to differentiate as neurons, it was not clear why fully differentiated neuronal derivatives of the crest are not found on this pathway later in development. As we described above, however, apoptosis occurs transiently on the LP in normal development (see also Jeffs and Osmond, 1992) at the time that neuronal cells are present on the LP (Fig. 9). This suggests that a critical period of apoptosis on the LP selectively eliminates neurogenic crest cells that happen to reside there.

Because apoptosis occurs rapidly and leaves no traces, the observed number of neuronal cells on the LP is likely to be an underestimate of the number that might actually be present on that pathway at some time during the migration phase of crest development (see Raff et al., 1993). Since some Hu⁺ neuronal cells present on the LP possessed fragmented nuclei, it seemed somewhat puzzling that we were unable to detect Hu⁺ cells that were labeled with the TUNEL method. However, we believe the failure to detect such cells is due to the fact that DNA fragmentation is a late event in apoptosis, which is likely to be

preceded by loss of cell-type-specific proteins such as Hu. Nevertheless, transplantation analyses allowed us to infer that neuronal/neurogenic cells were undergoing apoptosis. Thus, when crest populations that retained both neurogenic and melanogenic ability were transplanted on the LP, *mitf*^{-/-} melanocyte precursors preferentially localized in or beneath the epidermis, whereas increased cell death was observed transiently at stage 21-24 (Fig. 9) in the dermis and myotome where ectopic Hu⁺ cells were also observed. In contrast, transplantation of non-neurogenic, primarily melanogenic, crest cells on the LP did not increase the proportion of TUNEL⁺ cells there. Taken together, we conclude apoptosis of neurogenic/neuronal crest-derived cells on the LP is the most parsimonious explanation for the lack of neuronal derivatives on this pathway.

Previous clonal analyses in vitro revealed the transient existence of crest cells on the LP that were able to produce mixed clones that contained cells expressing neuronal traits (Sieber-Blum et al., 1992; Richardson and Sieber-Blum, 1993). Although many progenitors gave rise to melanocyte clones at all stages, the neurogenic clonal progenitors were found predominantly at stage 21. Fewer neurogenic progenitors were detected at stage 26, and none was found at stages 29 and 34 (Sieber-Blum et al., 1992; see also Fig. 9). These results were considered to support the idea that multipotent crest cells underwent progressive fate restriction in response to the unknown environmental cues on the LP. It is important to emphasize, however, that the appearance of neurons in cultures of crest cells taken from the LP could also be explained by the rescue, in vitro, of neurogenic crest cells on the LP that would have undergone apoptosis if they had remained in situ.

Cell-type-specific death and survival may depend on localized environmental cues

Crest cells are clearly not the only cells undergoing apoptosis on the LP. Some of the dying cells in the dermal mesenchyme of normal embryos are likely to be somite-derived, since dying cells were still observed in the dermal mesenchyme even when a part of the neural tube was ablated before crest cell emigration (data not shown). Therefore, even though it is not known why somite cells die, it is tempting to speculate that some somite cells are sacrificed as crest-derived ectopic neurogenic cells/neurons (and possibly other ectopic cell types) are eliminated from the LP.

Presently, it is not clear what mechanism(s) are involved in the death of neurogenic/neuronal crest cells on the LP. Despite the lack of candidates, it is possible that some positive regulators of apoptosis of neurogenic/neuronal crest cells may exist on the LP. Involvement of extracellular factors in neural crest development has been extensively studied. Recently, the importance of localized extracellular factors for the survival of crest-derived cell types has been noted. For instance, murine melanocyte precursors, which differentially express the receptor tyrosine kinase, *c-kit*, are known to depend on its ligand, Steel factor, for survival (Steel et al., 1992; Morrison-Graham and Weston, 1993). This ligand, in turn, is localized on the LP at the time that murine melanocyte precursors migrate there (Wehrle-Haller and Weston, 1995). Likewise, Neurotrophin-3 (NT-3) activity, which is required by early neurogenic subpopulations as a survival factor, appears to be present on the MP and selectively depleted on

the LP (Henion et al., 1995). Neuregulin/glia growth factor has been reported as an instructive differentiation factor for the glial cell lineage (Shah et al., 1994), but is also known to be a survival factor for Schwann cell precursors (Dong et al., 1995), locally provided by neurons (Marchionni et al., 1993; Ho et al., 1995; Meyer et al., 1997). Therefore, many crest-derived cell types appear to require specific localized extracellular factor(s) for their survival (see also Wehrle-Haller and Weston, 1997).

Interestingly, *c-kit* is known to be positively regulated by *mitf*, along with other melanocyte-specific genes (Tsujimura et al., 1996). Therefore, *mitf* links melanocyte specification and Steel-factor dependence. Moreover, the HuD RNA-binding protein, which is recognized by mAb 16A11 and used as an early neuronal marker in this study, seems to promote both neuronal differentiation and neurotrophin-dependence of the crest-derived neuronal precursors (Wakamatsu and Weston, 1997). Therefore, the *mitf*⁺ and Hu⁺ cells identified in this study, seem to require localized cell-type-specific environmental factor(s) for their survival.

It should be emphasized that ectopically located crest-derived cells were also observed in the MP. Thus, Hu⁺ cells were occasionally found along the spinal nerve as well as on the LP. Like Hu⁺ cells on the LP, these ectopic neurons also appeared randomly and transiently. Neural tube replacement experiments proved that these Hu⁺ cells originated from donor quail neural tube (data not shown). Anti-MITF antibody staining also revealed the existence of some melanocyte precursors on the MP (data not shown). Those *mitf*⁺ cells were found around the nascent dorsal root ganglia, where melanocytes do not normally differentiate.

Taken together, it seems likely that crest-derived subpopulations are specified early, prior to the onset of migration, and that specific localized factor(s) are required by such subpopulations for survival and subsequent differentiation. Further, it seems likely that inappropriately located cells, which are deprived of these factors, are eliminated by apoptosis as a 'proof-reading mechanism'.

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