The Delayed Entry of Thoracic Neural Crest Cells into the Dorsolateral Path Is a Consequence of the Late Emigration of Melanogenic Neural Crest Cells from the Neural Tube

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Neural crest cells migrate along two pathways in the trunk: the ventral path, between the neural tube and somite, and the dorsolateral path, between the somite and overlying ectoderm. In avian embryos, ventral migration precedes dorsolateral migration by nearly 24 h, and the onset of dorsolateral migration coincides with the cessation of ventral migration. Neural crest cells in the ventral path differentiate predominantly as neurons and glial cells of the peripheral nervous system, whereas those in the dorsolateral path give rise to the melanocytes of the skin. Thus, early- and late-migrating neural crest cells exhibit unique morphogenetic behaviors and give rise to different subsets of neural crest derivatives. Here we present evidence that these differences reflect the appearance of specified melanocyte precursors, or melanoblasts, from late- but not early-migrating neural crest cells. We demonstrate that serum from Smyth line (SL) chickens specifically immunolabels melanocyte precursors, or melanoblasts. Using SL serum as a marker, we first detect melanoblasts immediately dorsal and lateral to the neural tube beginning at stage 18, which is prior to the onset of dorsolateral migration. At later stages every neural crest cell in the dorsolateral path is SL-positive, demonstrating that only melanoblasts migrate dorsolaterally. Thus, melanoblast specification precedes dorsolateral migration, and only melanoblasts migrate dorsolaterally at the thoracic level. Together with previous work (Erickson, C. A., and Goins, T. L., *Development* 121, 915–924, 1995), these data argue that specification as a melanoblast is a prerequisite for dorsolateral migration. This conclusion suggested that the delay in dorsolateral migration (relative to ventral migration) may reflect a delay in the emigration of melanogenic neural crest cells from the neural tube. Several experiments support this hypothesis. There are no melanoblasts in the ventral path, as revealed by the absence of SL-positive cells in the ventral path, and neural crest cells isolated from the ventral path do not give rise to melanocytes when explanted in culture, suggesting that early, ventrally migrating neural crest cells are limited in their ability to differentiate as melanocytes. Similarly, neural crest cells that emigrate from the neural tube *in vitro* during the first 6 h fail to give rise to any melanocytes or SL-positive melanoblasts, whereas neural crest cells that emigrate at progressively later times show a dramatic increase in melanogenesis under identical culture conditions. Thus, the timing of dorsolateral migration at the thoracic level is ultimately controlled by the late emigration of melanogenic neural crest cells from the neural tube.   © 1998 Academic Press

INTRODUCTION

Neural crest cells detach from the dorsal surface of the neural tube and migrate throughout the embryo along stereotyped pathways (Le Douarin, 1982; Erickson and Perris, 1993). In avian embryos, trunk neural crest cells take

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one of two routes (Loring and Erickson, 1987; Serbedzija *et al.,* 1989). The first neural crest cells to detach from the neural tube migrate ventrally, between the neural tube and somite, and then through the sclerotome. These cells differentiate as neurons or glial cells of the peripheral nervous system. Later-emigrating neural crest cells take a dorsolateral path between the somite and overlying ectoderm, where they differentiate as melanocytes (Teillet, 1971; Hulley *et al.,* 1991).

What accounts for the final distribution of neural crest derivatives? The predominant model of neural crest patterning postulates that when neural crest cells leave the neural tube, they are multipotent and migrate haphazardly according to the environmental conditions they encounter at the time that they emigrate. Once in a particular path, these developmentally naive neural crest cells are exposed to cues unique to that path that direct them to differentiate as a derivative appropriate for that locale. Thus, this model argues that the final pattern of neural crest derivatives reflects the interaction between mutipotent neural crest cells and unique, instructive environments. For simplicity, we refer to this model as the environment-directed model of neural crest patterning. Support for this model comes from numerous studies (reviewed in Le Douarin *et al.,* 1993; Selleck *et al.,* 1993; Stemple and Anderson, 1993) showing that some neural crest cells are multipotent *in vitro* and *in vivo,* and that backgrafted neural crest derivatives (e.g., sensory or enteric ganglia) often differentiate according to their new environment rather than their site of origin.

However, the developmental plasticity of neural crest cells under these experimental conditions does not rule out the possibility that subpopulations of neural crest cells might be specified early, prior to entering a migratory path. Here we define a specified neural crest cell as one that is not fully differentiated but is expressing biochemical or molecular characteristics unique to a particular neural crest lineage. Assuming that the specification event is reversible, meaning that the cells are differentiating but not yet irreparably committed to a particular fate (Slack, 1991), then they could still generate multiple derivatives when cultured *in vitro* or grafted to another region of the embryo (e.g., Loring *et al.,* 1982; Coulombe and Bronner-Fraser, 1986). If specification dictates which migratory route a neural crest cell will follow, then the final distribution of neural crest derivatives will reflect the stereotyped morphogenetic behaviors of fate-specified neural crest cells. We refer to this as the phenotype-directed model of neural crest patterning.

The best evidence that trunk neural crest cells are specified prior to migration, and that specified neural crest cells exhibit stereotyped migratory behaviors, comes from *in vivo* lineage analysis and direct time-lapse observations of individual zebrafish trunk neural crest cells by Raible and Eisen (1994). Unfortunately, these sorts of experiments are not possible in amniotes, since the cells are smaller and the embryos are not translucent. Nevertheless, there is some support for the phenotype-directed model in avian embryos, in particular as it applies to the dorsal distribution of melanocytes. Kitamura and co-workers (1992) demonstrated that at least some neural crest cells express the melanogenic epitope MEBL-1 prior to entering the dorsolateral path. In addition, grafting studies by Erickson and Goins (1995) showed that neural crest cells presumed to be melanoblasts, but not other neural crest cells, invade the dorsolateral path when grafted into younger embryos whose own neural crest cells are only migrating ventrally, suggesting that melanoblasts have unique migratory properties. In this study we provide definitive evidence that the phenotype-directed model of neural crest patterning explains the final distribution of melanocytes.

First, we show that serum from Smyth line (SL)[2] chickens (Smyth, 1989), who produce an autoantibody against the melanogenic enzyme tyrosinase-related protein-1 (TRP-1; Austin *et al.,* 1995), specifically immunolabels embryonic melanoblasts at very early stages of melanocyte differentiation. Using SL serum as a marker, we demonstrate that a subpopulation of neural crest cells is specified as melanoblasts early, prior to entering the dorsolateral path, and that every HNK-1-positive neural crest cell in the dorsolateral path at later stages is also SL-positive. Taken together with previous grafting studies (Erickson and Goins, 1995), these results argue that utilization of the dorsolateral path (at the thoracic level) requires prior specification as a melanoblast.

Why is dorsolateral migration delayed with respect to ventral migration? SL immunoreactivity at the wing bud level first appears in a subset of neural crest cells at stage 18, approximately 18 h after the onset of neural crest emigration and ventral migration. This observation suggested that the delayed appearance of SL-positive cells (and therefore the delay in dorsolateral migration) may reflect the late emigration of melanogenic neural crest cells from the neural tube. Several experiments support this conclusion. Neural crest cells isolated from the ventral path fail to differentiate as melanocytes when cultured under conditions permissive for melanogenesis. Similarly, melanocytes do not arise in cultures of neural crest cells that emigrate from the neural tube during the first 6 h *in vitro,* whereas there is a significant increase in the percentage of melanocytes arising in cultures of later-emigrating neural crest cells under identical culture conditions. These latter data demonstrate that there is heterogeneity in the phenotypic biases of neural crest cells that detach from the neural tube at different times, and suggest that these differences account for the pathway choices at different stages in neural crest morphogenesis.

MATERIALS AND METHODS

Quail Neural Crest Cultures

Fertilized Japanese quail eggs (*Coturnix japonica japonica*) from the Avian Sciences Department (University of California–Davis) were maintained in a humidified 37°C incubator until they reached stages 13–15 (Hamburger and Hamilton, 1951). Neural tubes at the thoracic level were excised from the embryo and separated from surrounding tissues (somites, notochord, and ectoderm) after digestion with Pancreatin (Gibco) as described previously (Erickson and Goins, 1995). Neural tubes were then transferred to 35-mm tissue culture dishes (Corning) and maintained in Ham's F-12 medium

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[2] Abbreviations used: Smyth line, SL; migration staging area, MSA; phosphate-buffered saline, PBS; bovine serum albumin, BSA; Light Brown leghorn, LBL.

(Gibco) supplemented with 10% fetal bovine serum (Gibco), 3% 10-day chick embryo extract, and 100 units/ml penicillin/ streptomycin (Gibco). The medium was changed every 48 h. The same lot of fetal bovine serum and the same preparation of embryo extract were used throughout this study. Cultures were incubated at 37°C in a humidified, 10% CO_2 incubator. Neural crest cells begin to migrate onto the surface of the culture dish within a few hours, and by 20 h of incubation clusters of neural crest cells also form on top of the neural tube (Loring *et al.,* 1981). Under these conditions, pigment granules routinely appear in a subset of neural crest cells by 96 h.

Generating Neural Crest Cultures with Different Melanogenic Potential

We used three different subpopulations of neural crest cells with varying melanogenic characteristics. All of these cultures were maintained as described above. (1) Cultures containing essentially 100% melanoblasts were generated by removing neural crest clusters from 36- or 48 h-old cultures of neural tubes (Loring *et al.,* 1981; Vogel and Weston, 1988) and allowing the cells in these clusters to disperse for an additional 36 h in a fresh dish. (2) Cultures entirely devoid of melanoblasts (Ciment and Weston, 1983, 1985; Ito and Sieber-Blum, 1993) were obtained by dissecting posterior branchial arches from 3.5-day-old quail embryos, cutting these into small pieces, and culturing the arch fragments (which contain neural crest cells from the vagal axial level) for 3 days. (3) Cultures containing no melanoblasts are also generated by explanting neural tubes for 6 h, at which point the neural tubes were removed. Neural crest cells that had emigrated from the neural tube during this 6 hour period were cultured for an additional 4 days before immunolabeling. No melanocytes differentiate in these cultures (see Results).

SL Immunocytochemistry on Cultured Neural Crest Cells

Cells were fixed for 10 min at room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS), washed 3× for 5 min each in PBS, and blocked for 15 min in PBS containing 0.5% bovine serum albumin (BSA; Sigma). After blocking, the cells were incubated in serum from depigmented SL chickens (diluted 1:800 in 0.5% BSA/PBS; a generous gift of R. Boissy) for 3 h at room temperature. Serum from age-matched Light Brown leghorn (LBL) chickens was used as a negative control. The cultures were washed in 0.5% BSA/PBS and incubated in a 1:100 solution of a rhodamine-conjugated secondary antibody (rabbit-anti-chicken IgG; Chemicon) for 2 h. Cultures were then washed in PBS and mounted in 70% glycerol with 0.1 M $NaHCO_3$ (pH 7.8) and 2% *n*-propyl gallate. Immunolabeled cells were viewed with a Lietz Dialux 20 fluorescence microscope.

Immunolabeling of Frozen Sections with SL Serum and HNK-1

Fertile White leghorn chicken eggs from Western Scientific (Sacramento, CA) were incubated at 37°C until they reached the appropriate stage. Embryos were fixed in 4% paraformaldehyde in PBS for 1–3 h at room temperature and washed in PBS, and the wing bud region of the trunk was excised by cutting anterior to somite 10 and posterior to somite 17. The tissue was incubated in 5% sucrose/PBS for 1 h and transferred to 15% sucrose/PBS overnight at 4°C. The next morning the pieces were infiltrated for 4–6 h at 37°C in 7.5% gelatin/15% sucrose/PBS, transferred to small plastic cryomolds, and snap-frozen in liquid nitrogen. Ten-micrometer sections were cut on a Bright cryostat and air dried onto Superfrost Plus slides (Fisher).

Slides were washed twice for 5 min each in 0.1 M glycine/PBS, rinsed for 5 min in PBS, blocked for 15 min in 1% BSA in PBS, and incubated overnight at 4°C in blocking solution with SL serum (diluted 1:500). The following morning slides were washed three times (5, 10, and 15 min) in PBS, blocked as above, and incubated with 1:100 diluted secondary antibody (either goat anti-chicken IgG fluorescein-conjugated from Southern Biotechnologies or rabbit anti-chicken IgG rhodamine-conjugated from Chemicon) for 90 min at room temperature. After washing in PBS, the slides were incubated in HNK-1 (undiluted hybridoma supernatant) at room temperature for 2 h, washed, and blocked as above, and incubated in secondary antibody (1:100; rhodamine- or fluorescein-conjugated rabbit-anti-mouse from Cappell) for 90 min. Slides were washed in PBS, coverslipped, and viewed under epifluorescence on a Leitz Dialux 420 microscope. The emission spectra for the FITC-conjugated secondary antibodies used in these experiments peaks at 520 nm and extends to ca. 560 nm. To lessen the likelihood of FITC fluorescence bleed-through in the double-label experiments, we used a rhodamine filter set with a longpass suppression filter (Leica) that excludes wavelengths under 580 nm. Using this filter set, we did not observe any significant fluorescence bleed-through in control slides stained with SL serum and a FITC-conjugated secondary antibody.

Mapping the Distribution of SL-Positive Cells in Vivo

Serial cryosections through the wing bud level of stage 16 through 26 White leghorn chicken embryos were immunolabeled with SL serum and HNK-1 as described above. We report here our analysis of stages 18, 20, and 23 only, since after stage 23 neural crest cells in the dorsolateral path begin to lose HNK-1 immunoreactivity (Erickson *et al.,* 1992; our unpublished observations). Forty serial sections, each 10 μm in thickness, were examined for each embryo. For each section, the position of every SL-positive cell was plotted onto a schematic diagram of an embryo cross section. These plots were then combined on a single schematic to generate a composite map of all 40 sections. Thus, the composite maps shown in Fig. 4 depict the dorsal–ventral and medial–lateral position of every SL-positive cell in the 400-μm range examined. We analyzed at least 6 embryos for each stage and did not detect any obvious variability in the distribution of melanoblasts within a stage.

Isolation and Culture of Neural Crest Cells from the Ventral Migratory Pathway

Neural tubes and associated somites from the level of the last 12 somites (somites I–XII; see Christ and Ordahl, 1995, for nomenclature) of a stage 16 quail embryo were removed from the embryo with tungsten needles, digested with Pancreatin for 1 min, and transferred to cold Hanks' saline, and somites VII–XII were carefully separated from the neural tube. Like somites were cultured together (12 somites from 6 embryos per experiment; 6 separate experiments) for 6 days. The cultures were fixed as above and immunolabeled with HNK-1 (1:25 diluted hybridoma supernatant), to identify neural crest cells that had invaded the somites (Loring

and Erickson, 1987), and SL serum (see above), to check for latent melanoblasts. All cultures were examined for the presence of melanocytes under brightfield illumination.

Determining the Percentage of Melanocytes in Cultures of Early- and Late-Migrating Neural Crest Cells

Neural tubes from stage 16 quail embryos were excised from the thoracic region at the level of the last 6 somites, as described above. Six neural tubes were plated per culture dish. After 12 h the neural tubes were gently peeled from the plastic surface with a tungsten needle and transferred to a fresh dish, leaving behind neural crest cells that emigrated from the neural tubes during the first 12 h of culture. This process was repeated at 24 and 48 h (time after dissection) to generate a set of neural crest cultures from serially replated neural tubes. Six days after the dissection, neural crest cells were removed from the plastic culture surface by gentle enzymatic digestion with Pancreatin, transferred to cold F-12 medium, and centrifuged for 5 min at 5000 rpm to pellet the cells. After resuspension of the pelleted cells in Locke's saline solution, the total number of cells and the number of pigmented melanocytes were determined for each culture using a hemocytometer. The experiment was performed 4 times (18 neural tubes/experiment) with virtually identical results.

RESULTS

Cellular Patterns of SL Immunoreactivity

To verify that SL serum immunolabels pigmented melanocytes (Searle *et al.*, 1993), we stained pigmented 96-h-old neural crest clusters with SL serum (Figs. 1A and 1B). All of the cells are SL-positive, although the fluorescence signal is partially masked by the melanin in some cells. We also compared SL staining within a cell to that of HNK-1 (Figs. 1C and 1D). It is apparent that the HNK-1 and SL epitopes have different distributions in cells in which they colocalize, in all focal planes observed. Whereas HNK-1 labels neural crest cells diffusely around the cell surface (Fig. 1C), SL serum labels cells in a more punctate pattern along (or immediately under) the cell membrane (Fig. 1D). Searle *et al.* (1993) suggest that the punctate nature of SL staining reflects the fact that SL serum immunolabels premelanosomes distributed just under the cell membrane. The unique patterns of SL and HNK-1 staining cells are important because they allowed us to eliminate concerns about bleed through in double-label experiments (below).

Smyth Line Serum Is a Specific Marker for Melanoblasts both in Vitro and in Vivo

To determine whether SL serum recognizes embryonic melanoblasts, we immunolabeled cells from 48-h-old quail neural crest clusters. Previous experiments (Loring *et al.,* 1981; Vogel and Weston, 1988) have suggested that these clusters contain almost 100% melanoblasts based on the
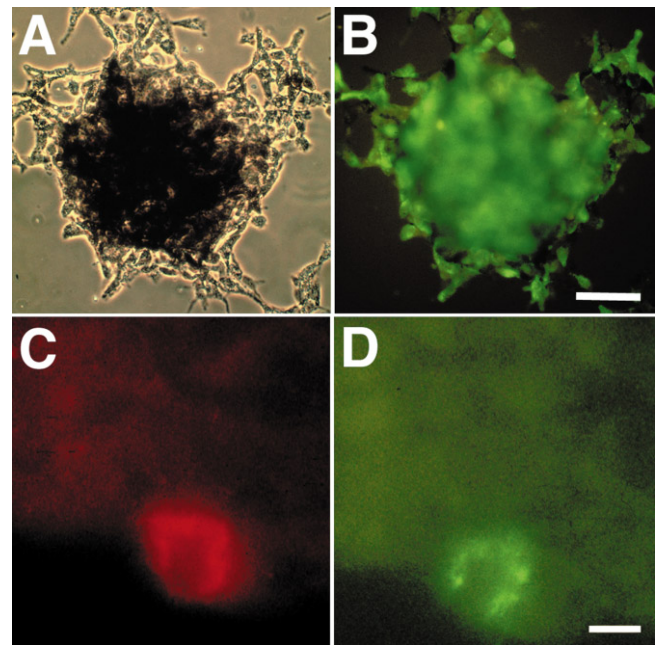

FIG. 1. Patterns of SL immunostaining. (A, B) A partially pigmented 96-h-old neural crest cluster immunolabeled with SL serum and photographed in phase (A) and under fluorescence (B). The pigmented cells (A) are SL-positive (B). (C, D) 10-μm cryosection of a stage 23 chick embryo showing a single neural crest cell in the ectoderm. The section was double-labeled with HNK-1 (C) and SL serum (D) and photographed under 100× magnification. Note the difference in staining patterns between HNK-1 and SL serum. SL serum labels cells in a punctate fashion along (or just under) the cell membrane (see also Searle *et al.*, 1993), whereas HNK-1 exhibits a much more diffuse pattern of staining Scale bar: 500 μm for A and B; 75 μm for C and D.

fact that melanocytes are the primary derivative that arise from explanted 48-h-old neural crest clusters *in vitro*. All of the cells derived from 48-h-old neural crest clusters are SL-positive (Figs. 2A and 2B; some cells appear to be unlabeled in Fig. 2B because we could not photograph the entire fluorescence field in the same focal plane). As a negative control, we immunolabeled 36-h-old neural crest clusters with either SL serum or serum from age-matched LBL chickens. LBL chickens have normal pigmentation, and do not produce detectable anti-melanocyte autoantibodies (Austin *et al.,* 1992; Searle *et al.,* 1993). Only the SL serum immunolabels 36-h-old clusters (Figs. 2C and 2D). To determine if SL serum specifically labels melanoblasts and not other neural crest cells, we immunolabeled neural crest cells isolated from the branchial arches of 3.5-day-old quail embryos. This subpopulation of neural crest cells is devoid of melanoblasts (Ciment and Weston, 1983, 1985; Ito and Sieber-Blum, 1993) and is SL-negative (data not shown). Thus, SL serum is a specific marker for melanoblasts *in vitro*.
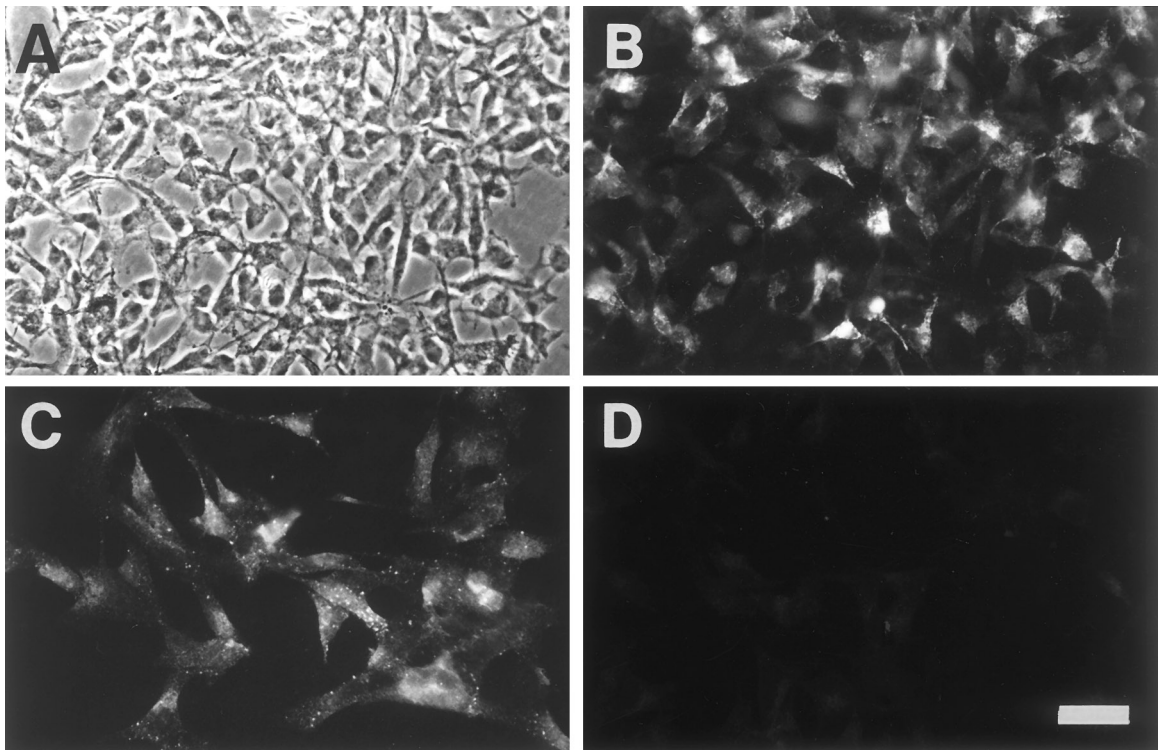
FIG. 2. SL serum immunolabels melanoblasts *in vitro.* Photomicrographs of cells derived from 48-h-old (A, B) or 36-h-old (C, D) neural crest clusters. Comparison of phase (A) and immunofluorescence (B) images shows that neural crest cells from 48-h-old clusters, which are highly enriched for melanoblasts, immunolabel with SL serum. Although it appears that some cells are not labeled, this simply reflects our inability to resolve all of the fluorescence in the same focal plane at this magnification. As a control, 36-h-old neural crest clusters were immunolabeled with either SL serum (C) or serum from age-matched Light Brown leghorn (LBL) chickens (D). Note the punctate pattern of staining in C. No immunoreactivity is observed with the LBL serum (D). Scale bar: 36 μm for A and B; 31 μm for C and D.

To determine whether SL serum can be used to identify melanoblasts *in vivo,* we double-labeled cross sections of stage 16 and 23 chicken embryos (wing bud level) with HNK-1 and SL serum. HNK-1 is a monoclonal antibody that recognizes neural crest cells in both the dorsolateral and ventral pathways (Tucker *et al.,* 1988; Erickson *et al.,* 1992). There is abundant neural crest cell migration in the ventral path at stage 16 (Fig. 3A), but none of these cells is SL-positive (Fig. 3B). By stage 23 there is extensive dorsolateral migration (Fig. 3C), and in each section (12 embryos; 40–60 serial sections/embryo; ca. 6000 cells), every HNK-1-positive cell in the dorsolateral path is also SL-positive (Figs. 3C and 3D). We do not detect SL-positive neural crest cells in the ventral path at stage 23 except for occasional SL-positive cells associated with the dorsal aspect of the developing sensory ganglia (arrowhead, Fig. 3D). Because pigment cells in most chickens are not found in ventral tissues (e.g., Teillet, 1971; Hulley *et al.,* 1991), the distribution of SL-positive neural crest cells *in vivo* strongly suggests that these cells are melanoblasts.

Patterns of Melanoblast Specification and Migration

Previous models of neural crest morphogenesis have proposed that melanoblasts are specified just prior to entering the dorsolateral path from a pool of pluri- or multipotent neural crest cells residing in the area between the neural tube and dermamyotome. This region has been referred to as the "migration staging area" (MSA; Weston, 1991; Erickson *et al.,* 1992). We detect SL-positive neural crest cells as early as stage 18 at the level of the wing bud (Fig. 4A). The majority of these cells are located immediately dorsal to the neural tube, suggesting that they have recently delaminated from the neuroepithelium (Fig. 4B). At stage 18 there are only a few SL-positive neural crest cells as far lateral as the MSA (Fig. 4B), even though there are numerous HNK-1-positive neural crest cells filling the MSA at this time (not shown, but see Erickson *et al.,* 1992). These observations demonstrate that neural crest cells are specified as melanoblasts soon after they leave the neural tube, and before reaching the MSA.
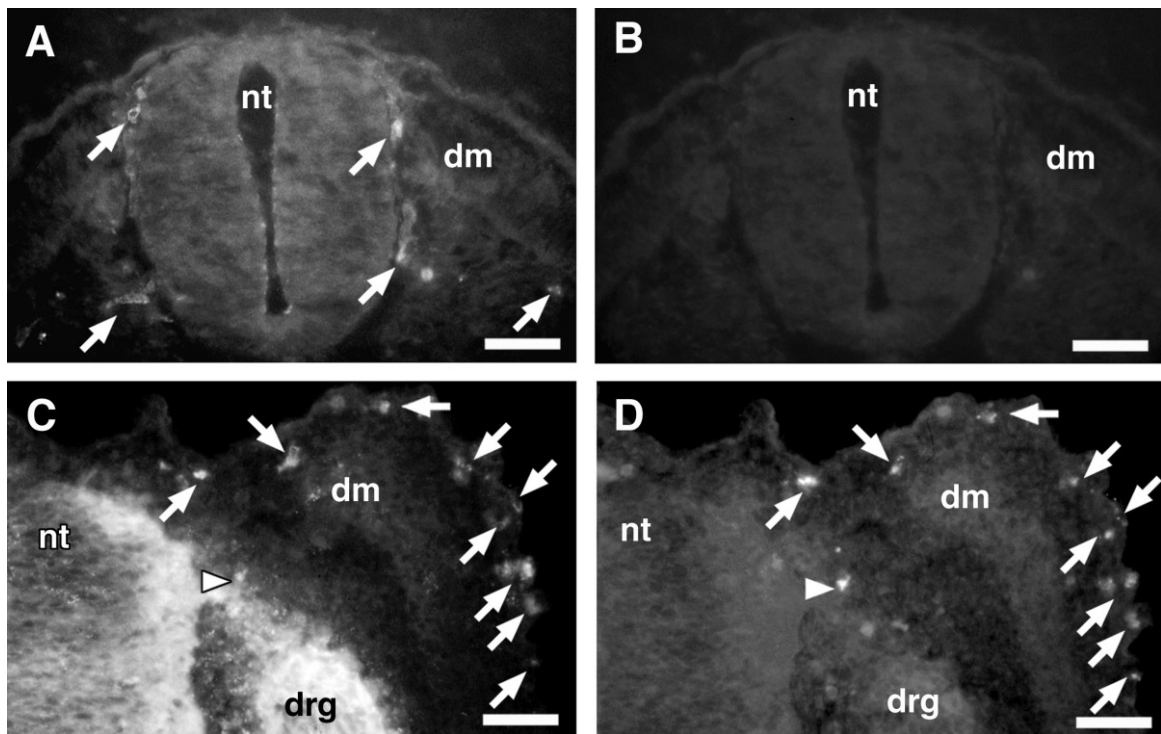
FIG. 3. SL serum is a specific marker for melanoblasts *in vivo.* Transverse cryosections through the forelimb level of stage 16 (A, B) and stage 23 (C, D) chicken embryos double-labeled with HNK-1 (A, C) and SL serum (B, D). At stage 16, no ventrally migrating neural crest cells (arrows in A) are SL-positive (B). At stage 23, the only HNK-1-positive cells that are also SL-positive are in the dorsolateral path (arrows in C, D). Occasionally we find a rare SL-positive cell as far ventral as the dorsal root ganglion (arrowhead in C, D). Abbreviations: nt, neural tube; dm, dermamyotome; drg, dorsal root ganglia. Scale bar = 45 μm.

By stage 20 there are many more SL-positive cells than at stage 18, and some have begun to enter the dorsolateral path (Figs. 4C and 4D). For some of these embryos we double-labeled the sections with HNK-1 (data not shown). As was true for stage 23 embryos (Fig. 3), each HNK-1-positive cell in the dorsolateral path at stage 20 was also SL-positive (40–50 sections/embryo; 5 embryos; ca. 300 cells total). These observations demonstrate that melanoblasts are the only neural crest cells that enter the dorsolateral path.

The Earliest Migrating Neural Crest Cells Fail to Differentiate as Melanocytes

The absence of melanocytes from ventral sites in most adult birds, as well as the sudden appearance of SL-positive cells at stages 18–20, suggests the hypothesis that, as a population, early migrating neural crest cells are nonmelanogenic. To test this idea, we obtained neural crest cells that had migrated ventrally by explanting somites VII–XII (see Christ and Ordahl, 1995, for nomenclature), which contain invading neural crest cells (Loring and Erickson, 1987), and cultured them under conditions permissive for melanogenesis. The explanted somites disperse on the plastic substratum and form a monolayer of cells. All

cultures were examined after 6 days to identify pigmented melanocytes.

Figure 5 shows representative photomicrographs of somite IX-derived cultures (Fig. 5A) and somite XII-derived cultures (Fig. 5B). Of 36 cultures (representing 432 somites and 6 separate experiments), we observed only 4 melanocytes. In addition, immunolabeling with SL serum did not reveal any latent melanoblasts in these cultures (Fig. 5C). The absence of melanocytes and melanoblasts is not because neural crest cells fail to survive, as immunolabeling with HNK-1 reveals many neural crest cells in these cultures (Fig. 5D). In addition, the somite cells do not inhibit pigmentation under our culture conditions because 24-h-old neural crest clusters (Loring *et al.,* 1981) cocultured with explanted somites readily differentiate into melanocytes (data not shown). Thus, neural crest cells isolated from the ventral path are restricted in their ability to become melanocytes.

This experiment could not distinguish between an intrinsic restriction in the melanogenic capability of the earliest migrating neural crest cells and a change induced in these cells as they pass through the ventral path and into the somite. To address this question, we cultured neural tubes for 6 h, removed the neural tubes, and cultured the neural
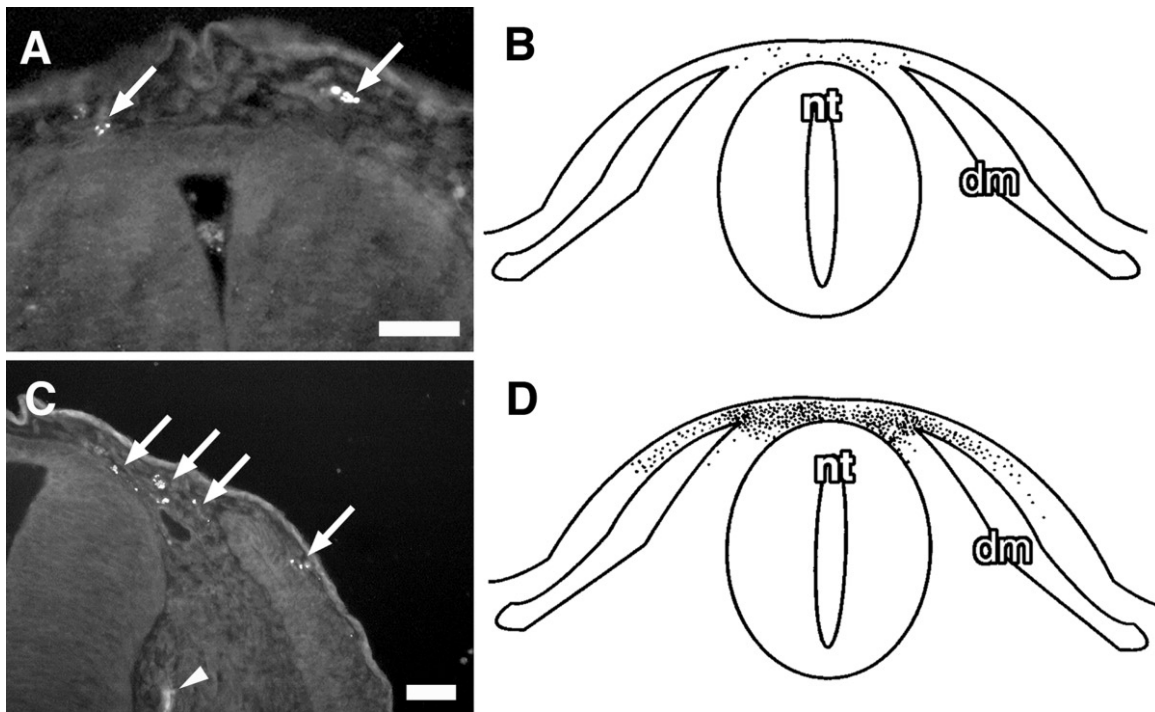
FIG. 4. Distribution of SL-positive cells at the early stages of melanoblast migration. (A, C) Photomicrographs of SL-immunolabeled cryosections through the forelimb level of stage 18 (A) and stage 20 (C) chick embryos. Arrows indicate SL-positive cells. The arrowhead in C indicates autofluorescing erythrocytes. (B, D) Maps representing the dorsal–ventral and medial–lateral position of every SL-positive cell in a 400-μm-long segment at the forelimb level of a representative stage 18 (B) and stage 20 (D) chick embryo. At stage 18, the earliest stage at which we detect SL-positive cells in the trunk, most of the SL-positive cells are immediately dorsal to the neural tube. By stage 20, SL-positive cells have migrated laterally and entered the dorsolateral path. Abbreviations: nt, neural tube; dm, dermamyotome. Scale bars, 45 μm.

crest cells that had emigrated from them for an additional 96 h. No pigmented melanocytes differentiated in these cultures (Fig 6A), and SL-immunolabeling did not reveal any latent melanoblasts (Fig. 6B). We conclude that, as a population, the earliest migrating neural crest cells lack the ability to differentiate as melanocytes under permissive culture conditions.

Later Migrating Neural Crest Cells Have an Inherent Melanogenic Bias

We proposed two different mechanisms to account for the delayed appearance of melanoblasts *in vivo* and the failure of early migrating neural crest cells to generate melanocytes *in vitro.* One possibility is that environmental factors external to the neural epithelium, for example, from the overlying ectoderm or nearby dermamyotome, direct later migrating neural crest cells to undergo melanogenesis. Alternatively, perhaps late-emigrating neural crest cells are predisposed to differentiate as melanocytes independent of signals from adjacent tissues. To distinguish between these two possibilities, we isolated trunk neural tubes from the axial level of the last 6 somites of stage 16 quail embryos

and serially replated the tubes 12, 24, and 48 h after the initial plating. Cultures were examined for melanocytes 6 days after the initial dissection.

There is a progressive increase in the number of melanocytes that arise in cultures of later migrating neural crest cells (Fig. 7), even though all cultures were maintained under identical conditions. To determine whether this qualitative increase in melanocytes was statistically significant, we dissociated the cells from the culture dish and counted the total numbers of cells and melanocytes. There is a significant increase in the percentage of melanocytes that differentiate in cultures of progressively later emigrating neural crest cells (Fig. 8). The frequency of melanogenesis is most pronounced in cultures derived from cells that emigrated from the neural tube between 48 and 144 h after the initial dissection. Nearly 70% of the cells in these cultures are melanocytes (Fig. 8). Unfortunately, we could not further refine this last time point because neural tubes replated after 48 h usually failed to readhere to the plastic culture substratum. However, virtually 100% of the cells immediately surrounding the neural tube are pigmented in the 144-h-old cultures (not shown). Assuming that these melanocytes are derived from the most recently emigrated
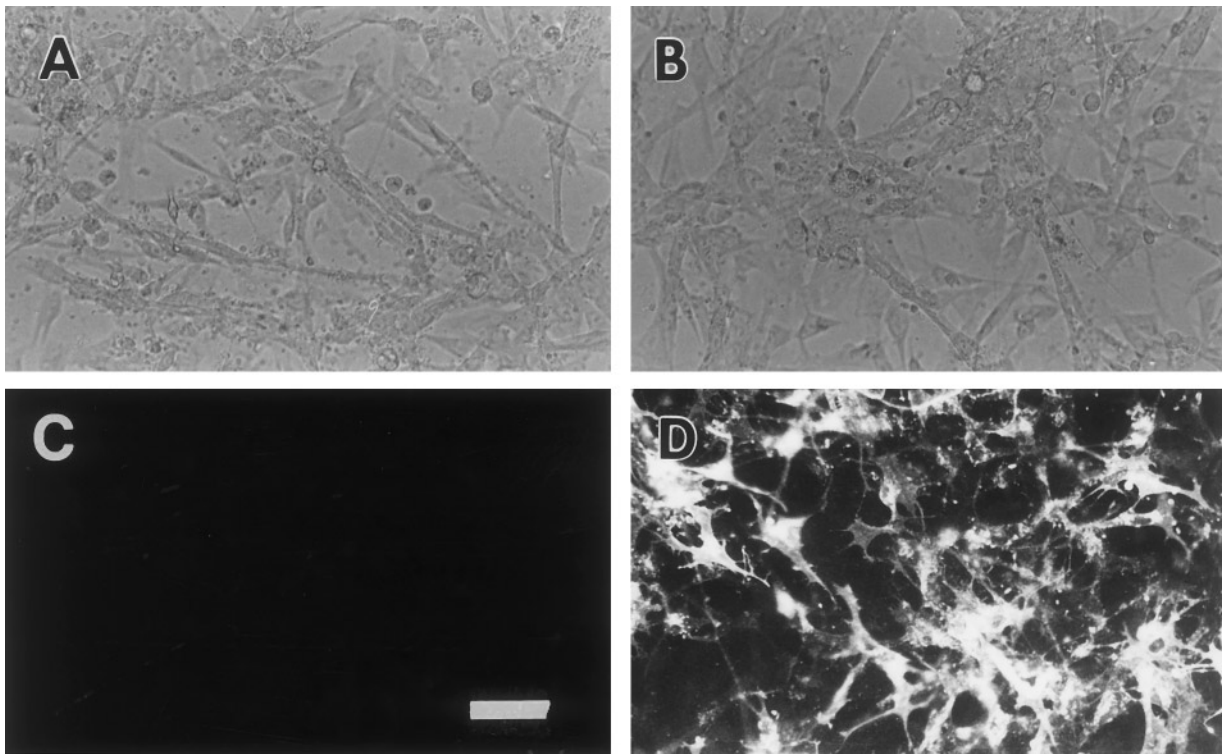
FIG. 5. Ventrally migrating neural crest cells fail to form melanocytes under permissive culture conditions. Thoracic-level somites with their associated neural crest cells were explanted and cultured for 6 days in medium permissive for melanogenesis. Brightfield photomicrographs of somite IX-derived (A) and somite XII-derived cultures (B) show the lack of differentiated melanocytes in these explants. (C, D) The same field of a somite XII-derived culture double-labeled with SL serum (C) and HNK-1 (D). There are no melanoblasts in the culture (C), even though numerous HNK-1-positive neural crest cells are present (D). Identical results were obtained with explants of somites VII–XI (data not shown). Scale bar, 75 μm.

neural crest cells, we believe that 70% is an underestimate of the percentage of melanocytes that arise from the latest-migrating neural crest cells.

In this experiment the increase in melanogenesis over time appears to be gradual. This is in contrast to our Smyth line staining data (above) and the observations of Kitamura *et al.,* (1992), which suggest a more abrupt transition in the emigration of melanogenic neural crest cells. The most likely explanation for this apparent discrepancy is that in the serial replating experiments each culture contains neural crest cells of different relative ages, since the explanted neural tubes encompass 6 somite lengths. For example, at 24 h, neural crest cells leaving the anterior-most level of the explanted neural tube will be 20–24 h old (relative to the time when neural crest migration began at that axial level), whereas at the posterior-most level the neural crest cells will be approximately 10–14 h old (again, relative to the time when migration was initiated at that level). Consequently the transition from nonmelanogenic to melanogenic neural crest cells may be quite distinct at any given axial level, but we cannot resolve this transition very

sharply in our cultures since they include cells from multiple axial levels.

DISCUSSION

Using SL serum as a marker for embryonic melanoblasts, we have demonstrated that a subset of neural crest cells are specified as melanoblasts prior to the onset of dorsolateral migration and that at later stages melanoblasts are the only neural crest cells that migrate dorsolaterally. These observations are significant because they show that melanocyte differentiation is initiated before neural crest cells are exposed to the dorsolateral environment, and that melanoblasts exhibit a pattern of migration that differs from nonmelanoblasts. We further show that neural crest cells isolated from the ventral path, as well as very early migrating neural crest cells *in vitro,* fail to generate melanocytes under culture conditions where later migrating neural crest cells preferentially differentiate as melanocytes. Thus, the delay in dorsolateral migration reflects the delayed emergence of melanogenic neural crest cells from the neural
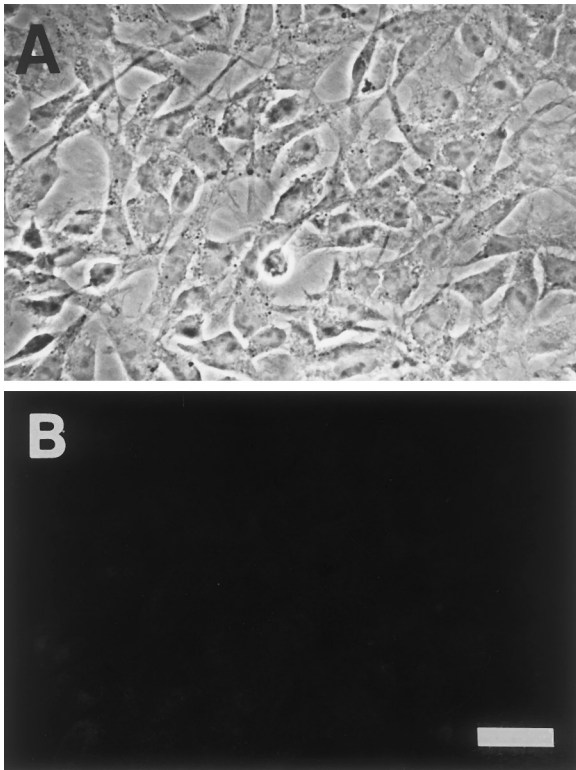
FIG. 6. Cultures derived from the earliest migrating neural crest cells fail to generate melanocytes or melanoblasts. Quail neural tubes from the thoracic level were explanted into culture medium permissive for melanogenesis. After 6 h the neural tubes were removed, leaving behind only those neural crest cells that had migrated away from the neural tube during those 6 h. Cells were then cultured for an additional 4 days. Phase (A) and SL-immunolabeling (B) reveals no differentiated melanocytes or latent SL-positive melanoblasts in these cultures. Scale bar, 31 μm.

tube. This study now proves what we inferred from previous experiments (Erickson and Goins, 1995), which is that the final distribution of at least one neural crest derivative, melanocytes, is established because specified neural crest cells follow a unique migratory pathway.

Smyth Line Serum Is a Useful Reagent for Studying Melanoblast Morphogenesis and Differentiation

SL serum immunolabels neural crest cells from 36- and 48-h-old neural crest clusters (Fig. 2), which are already committed to melanogenesis (Loring *et al.,* 1981), but not neural crest cells derived from branchial arch mesenchyme (Ciment and Weston, 1983, 1985; Ito and Sieber-Blum, 1993) or 6-h-old neural crest outgrowths (Fig. 6), which do not differentiate as melanocytes. The pattern of SL immunoreactivity in embryos (Figs. 3 and 4) is consistent with the dorsal distribution of melanocytes in older embryos and adult birds (e.g., Teillet, 1971; Hulley *et al.,* 1991). Although

SL serum specifically labels melanoblasts, it does not necessarily follow that SL serum recognizes all neural crest cells with melanogenic potential. The primary target of the autoantibodies in SL serum is the enzyme TRP-1 (Austin and Boissy, 1995). Given the role of TRP-1 in melanin production (Hearing and Tsukamoto, 1991), it is likely that cells expressing TRP-1 have already begun to differentiate as melanocytes. Thus, SL serum is a useful marker for melanoblasts in the early stages of melanogenesis, but may not recognize cells with melanogenic potential that have not yet begun to differentiate.

Environment-Directed versus Phenotype-Directed Models of Neural Crest Patterning

The environment-directed model of neural crest patterning predicts that developmentally naive neural crest cells are directed into either the ventral or dorsolateral paths by environmental conditions they encounter at the time of their emigration. Once in a particular pathway, they are exposed to specific cues that cause them to assume a final phenotype appropriate for that location. In contrast, the phenotype-directed model of neural crest patterning proposes that neural crest cells are specified (or conditionally specified) prior to entering a particular migratory path, and specification dictates which path they will enter. Obviously, these two models represent extremes. Nevertheless, they provide a useful framework for addressing the relationship between migratory behavior and differentiation in the neural crest.

The phenotype-directed model makes several testable predictions. First, it predicts that neural crest cells will be specified as melanoblasts before entering the dorsolateral path. The first evidence for this was provided by Kitamura and co-workers (1992), who described a novel melanocyte marker, MEBL-1, and showed that some neural crest cells are MEBL-1-positive prior to entering the dorsolateral path. Using a different melanoblast marker, SL serum, we have confirmed that a subset of neural crest cells is specified as melanoblasts at stage 18, prior to entering the dorsolateral path. With the MEBL-1 results, we can now conclude that neural crest cells express two distinct melanocyte-specific antigens before they initiate dorsolateral migration. Thus, neural crest cells initiate melanogenesis independent of exposure to cues from the dorsolateral environment.

The second prediction of the phenotype-directed model is that melanoblasts migrate preferentially in the dorsolateral path. New observations from this study support this conclusion. First, the vast majority of SL-positive cells migrate dorsolaterally (Figs. 3 and 4), although we do see a few SL-positive cells (5–7 cells/embryo) located near the dorsal-most aspect of the developing sensory ganglia at stage 23. The ultimate fate of these cells is unknown (i.e., do they continue ventrally and undergo melanogenesis?), but our observations suggest that melanoblasts are not absolutely prohibited from entering the ventral path. Even so, the dorsolateral path clearly is the preferred migratory route for
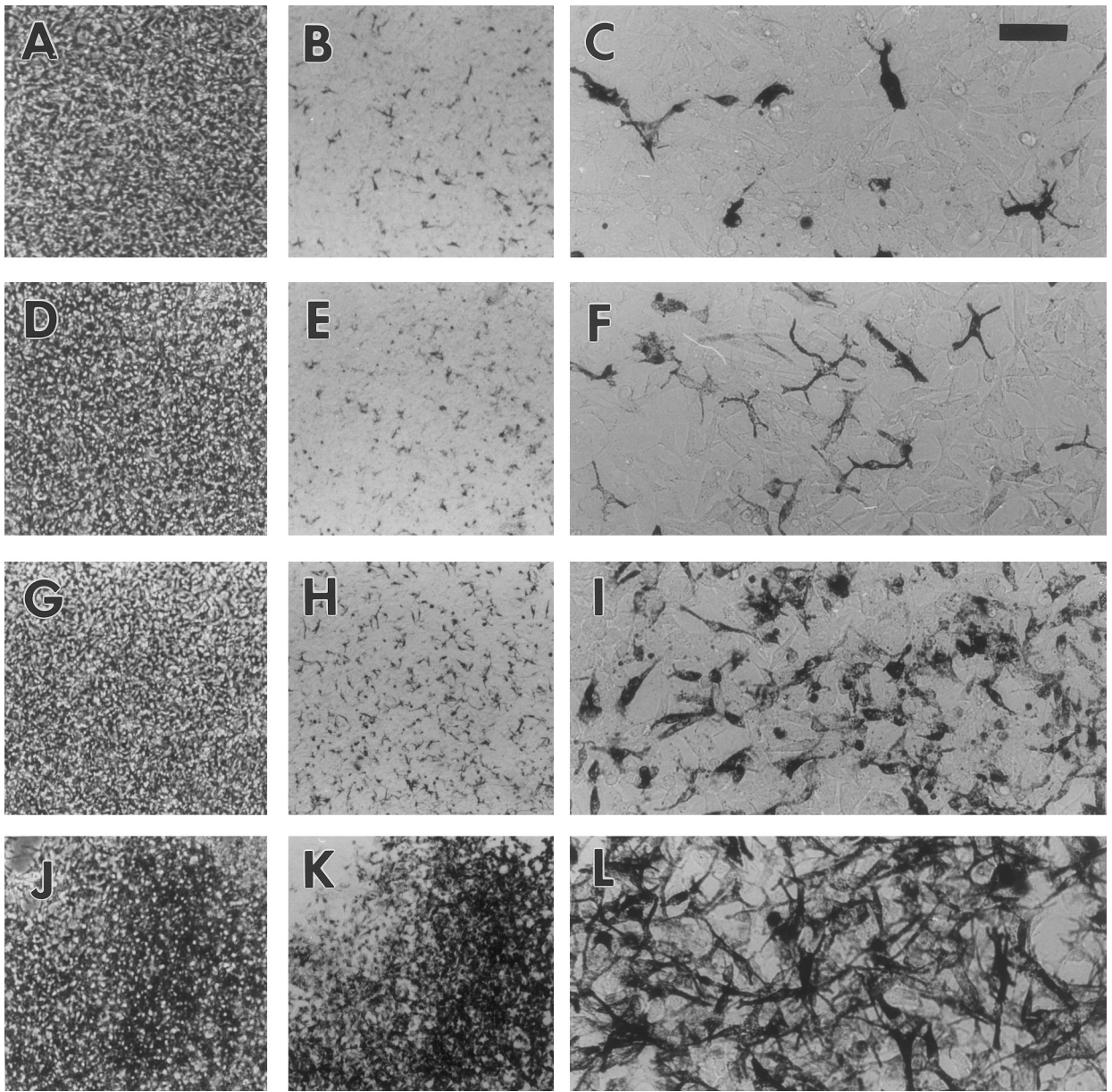
FIG. 7. There is a significant increase in melanogenesis of neural crest cells that emigrate from the neural tube at progressively later times *in vitro.* Quail neural tubes from the thoracic level were explanted into culture and serially transferred into new dishes after 12, 24, and 48 h to generate a set of cultures of neural crest cells that emigrated from the neural tube at 0–12 h (A–C), 12–24 h (D–F), 24–48 h (G–I), or after 48 h (J–L). All cultures were examined for the presence of melanocytes 144 h after the initial dissection. Photomicrographs of these cultures viewed under low magnification with phase illumination (A, D, G, J) and brightfield illumination (B, E, H, K) show more melanocytes present in cultures derived from later migrating neural crest cells. C, F, I, and L show the same cultures viewed in brightfield under higher magnification. Scale bar: 240 μm for A, B, D, E, G, H, J, and K; 100 μm for C, F, I, and L.

melanoblasts. Second, at the level of the wing bud, we first detect SL-positive cells immediately dorsal to the neural tube at stage 18 (Fig. 4). This is precisely when the first neural crest cells destined for the dorsolateral path emigrate from the neural tube, as revealed by vital dye labeling studies (Serbedzija *et al.,* 1989; Kitamura *et al.,* 1992; Oakley *et al.,* 1994). Thus, neural crest cells are specified as melanoblasts prior to entering the dorsolateral path or even reaching the MSA, and the timing of melanoblast appearance correlates with the switch from ventral to dorsolateral
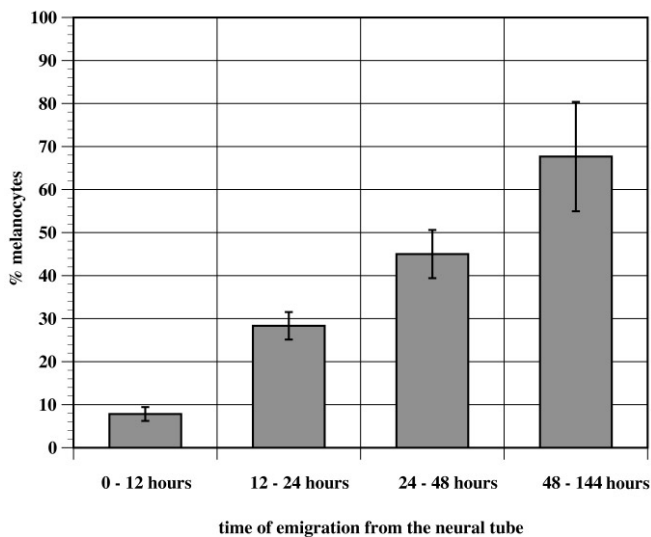
FIG. 8. The mean percentage of pigment cells for each time point in the serial replating experiment was determined 144 h after the initial dissection. Data from 3 separate experiments (18 neural tubes per experiment) were combined for this analysis. The percentage of melanocytes at each time point is significantly greater than that at the preceding time point (Mann–Whitney nonparametric test; $P < 0.05$). Error bars represent standard deviations from the mean over the 3 experiments.

migration. Third, by double-labeling tissue with HNK-1 and SL serum and carefully mapping the distribution of SL-positive cells at different stages, we have demonstrated that, in every embryo at each stage we examined, each HNK-1-positive neural crest cell in the dorsolateral path is also SL-positive (e.g., Fig. 3). It is possible that there are HNK-1-negative, SL-negative neural crest cells in the dorsolateral path. Nevertheless, our data strongly suggest that, at the thoracic level, melanoblasts are the only neural crest cells that migrate dorsolaterally.

A third prediction of the phenotype-directed model is that only melanoblasts have the ability to enter the dorsolateral path. This conclusion was inferred from earlier grafting studies (Erickson and Goins, 1995), which showed that the ability of backgrafted neural crest cells to invade the dorsolateral path depended not on the age of the host embryo, but instead on the source of the backgrafted cells. Specifically, cells from subpopulations of the crest that generate few if any melanocytes *in vitro* (e.g., 12-h outgrowths or branchial arch-derived crest mesenchyme) fail to migrate dorsolaterally even when grafted into later staged embryos whose own crest are so doing. In contrast, cells from 24- and 48-h-old neural crest clusters, which differentiate almost exclusively as melanocytes (Loring *et al.,* 1981; Vogel and Weston, 1988), rapidly enter the dorsolateral path when grafted into young embryos whose own crest are only migrating ventrally. By demonstrating that neural crest cells in 36- and 48-h-old clusters are SL-positive (Fig. 2), we

can say definitively that these cells are melanoblasts, and therefore can conclude that melanoblasts, but not other neural crest cells, have the ability to invade the dorsolateral path.

Why do only melanoblasts migrate dorsolaterally (at least at the thoracic level)? Perhaps melanoblasts are uniquely sensitive to guidance cues from the dermamyotome. For example, in murine embryos, Steel factor produced by the dermamyotome influences the dispersion of melanoblasts, which express the receptor for Steel factor, c-kit (Wehrle-Haller and Weston, 1995; Wehrle-Haller *et al.,* 1996). The relatively late onset of c-kit expression in chick melanoblasts *in vivo* (Lecoin *et al.,* 1995; our unpublished data) argues that Steel factor does not play a similar role in avian melanoblast morphogenesis, although it is certainly possible that the dermamyotome produces other positive guidance cues. Another possibility is that melanoblasts are insensitive to migration-inhibiting molecules present in the dorsolateral path (e.g., Oakley *et al.,* 1994; Krull *et al.,* 1997; Wang and Anderson, 1997) that prevent other neural crest cells from entering that space. We are attempting to distinguish between these possibilities.

Temporal Differences in the Developmental Biases of Neural Crest Cells

Neural crest cells isolated from the ventral path are SL-negative and do not differentiate as melanocytes when cultured under conditions permissive for melanogenesis (Fig. 5). Similarly, neural crest cells that emigrate from the neural tube during the first 6 h of culture do not generate any melanocytes or SL-positive melanoblasts (Fig. 6). Also, sympathetic ganglia, which are derived at least in part from early, ventrally migrating neural crest cells (Serbedzija *et al.,* 1989), do not give rise to pigmented melanocytes when backgrafted between the neural tube and somite of younger embryos (Le Lievre *et al.,* 1980). Together, these observations demonstrate that, as a population, trunk neural crest cells that emigrate early from the neural tube are strongly biased against the melanocyte phenotype. Whether these cells absolutely lack melanogenic potential remains unknown, and would be difficult to demonstrate conclusively. At a minimum it would require challenging the cells with multiple *in vitro* and *in vivo* environments, but even then one could not be certain that all possibilities had been tested.

Nevertheless, it is clear that, as populations, early- and late-migrating neural crest cells differ in their ability to differentiate as melanocytes. Whereas the earliest migrating neural crest cells fail to generate melanocytes, serially replating neural tubes reveals a significant melanogenic bias in later emigrating neural crest cells under identical culture conditions (Figs. 7 and 8). The reasons for the melanogenic bias of late-migrating neural crest cells are unknown. One interesting possibility is that prolonged exposure to the neural tube stimulates neural crest progeni-

tors to adopt a melanogenic fate, as suggested by Derby and Newgreen (1982).

Other studies have also documented heterogeneities in the phenotypic biases of early- and late-emigrating avian trunk neural crest cells. Artinger and Bronner-Fraser (1992) report differences in the classes of neurons that arise from cultures of early- and late-emigrating neural crest cells. *In vitro* lineage analysis by Henion and Weston (1997) also reveals differences in the timing of appearance of fate-restricted precursors for many neural crest derivatives. The Henion and Weston study is of particular interest to our work, since it reports at the single-cell level what we see at the population level: the failure of early-migrating neural crest cells to differentiate as melanocytes under culture conditions where later migrating neural crest cells primarily differentiate as melanocytes. These studies, and the data presented here, demonstrate unexpected differences in the phenotypic biases of neural crest cells that emigrate from the neural tube at different times.

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