

Long-Distance Cue From Emerging Dermis Stimulates Neural Crest Melanoblast Migration

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Neural crest melanoblasts display unique navigational abilities enabling them to colonize the dorsal path between ectoderm and somite. One signal shown here to elicit melanoblast migration is a chemotactic cue supplied by the emerging dermis. Until dermis emerges, melanoblasts fail to enter the dorsal path. The dermis emerges from a site that is too distant to stimulate migration by cell contact. Instead, surgeries show that dermis elicits migration from a distance. When dermis is grafted distally, neural crest cells enter the path precociously. Moreover, large grafts recruit melanoblasts from the control sides (without increasing crest cell numbers) as well as a few crest cells from ventral somite. Because other grafted tissues fail to stimulate migration, the dermis stimulus is specific. This report is the first documentation that trunk neural crest cells can be guided chemotactically. It also extends evidence that migration is exquisitely sensitive to temporal–spatial patterns of somite morphogenesis. *Developmental Dynamics* 229:99–108, 2004. © 2003 Wiley-Liss, Inc.

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INTRODUCTION

Neural crest cells are renowned for their ability to navigate along complex paths. Until recently, all neural crest paths were considered as general freeways able to guide any neural crest cells, rather than as private-access roads accessible only to neural crest subsets. This generality was supported by the early findings that crest cells transplanted to foreign axial levels can colonize local neural crest paths promiscuously, regardless of their original destinations (Weston, 1970; Noden, 1975).

However, in the trunk, the neural crest melanoblasts diverge from this rule. Melanoblasts colonize a path that other crest cells cannot, i.e., the

dorsal migration path between the somite and the ectoderm, which inhibits entry by other neural crest cells (Santiago and Erickson, 2002). Moreover, the melanoblasts are temporally segregated, emerging relatively late into the “migration staging area” circumscribed by the neural tube, ectoderm, and somite. They enter the dorsal path only well after other neural crest cells have finished migrating ventrally into the somite (Weston and Butler, 1966; Serbedzija et al., 1990; Erickson et al., 1992; review: Erickson, 1993). During neural crest cell emergence or during their sojourn in the staging area, they become committed to the melanocyte fate (Erickson and Goins, 1995;

Reedy et al., 1998a; Kos et al., 2001; Faraco et al., 2001; Luo et al., 2003) and acquire the special properties that allow them to colonize the dorsal path (Santiago and Erickson, 2002).

Guidance cues in the dorsal path are generally thought to be short range, requiring direct cell contact. Inhibitory elements within the dorsal path are supplied by the epithelial dermatome (Erickson et al., 1992; Oakley et al., 1994) and include ephrin-B ligands (Koblar et al., 2000; Santiago and Erickson, 2002), chondroitin sulfate, and an uncharacterized substance detected by peanut agglutinin lectin binding (Oakley et al., 1994). Permissive extracellular

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matrix components such as hyaluronic acid, thrombospondin, collagen, laminin, fibronectin, and basal lamina are also found in trunk paths (reviews, Erickson, 1988; Peris and Perissinotto, 2000) but a diffusible cue has yet to be directly implicated.

This work tests the hypothesis that emerging dermis supplies a diffusible attractant that stimulates melanoblasts to leave the staging area and enter the dorsal path. If emerging dermis does supply a long-range directional cue, then dermis should emerge shortly before melanoblasts enter the dorsal path, it should be too distant to directly contact entering melanoblasts, and it should be able to stimulate directed migration into the path from a distance, even when transplanted before melanoblasts normally enter the path and are still residing in the staging area. These predictions were tested using anatomic and surgical techniques and were validated. The findings show that dermis does emerge at a distance shortly before melanoblasts enter the dorsal path. Moreover, grafts that form dermis do elicit a precocious and directed neural crest cell migration into the dorsal path. Dermal grafts stimulate migration from a distance, in accord with a chemotactic response to a diffusible cue.

RESULTS

Temporal-Spatial Relations Between Neural Crest Entry and Dermis Emergence

As revealed by serial section analysis, dermis emerges at a time and place that make it a viable candidate as a source of a diffusible attractant (Fig. 1A). Dermis emerges shortly before neural crest cells enter the dorsal path. At chick and quail hindlimb levels, the epithelial dermatome initiates an epithelial-mesenchymal transition and the first dermal cells begin to disperse between stages 19 and 20, just before neural crest cells enter the path at stage 20–21, 6–12 hr later (see also Erickson et al., 1992). Most important, these first dermal cells arise far from the migration staging area contain-

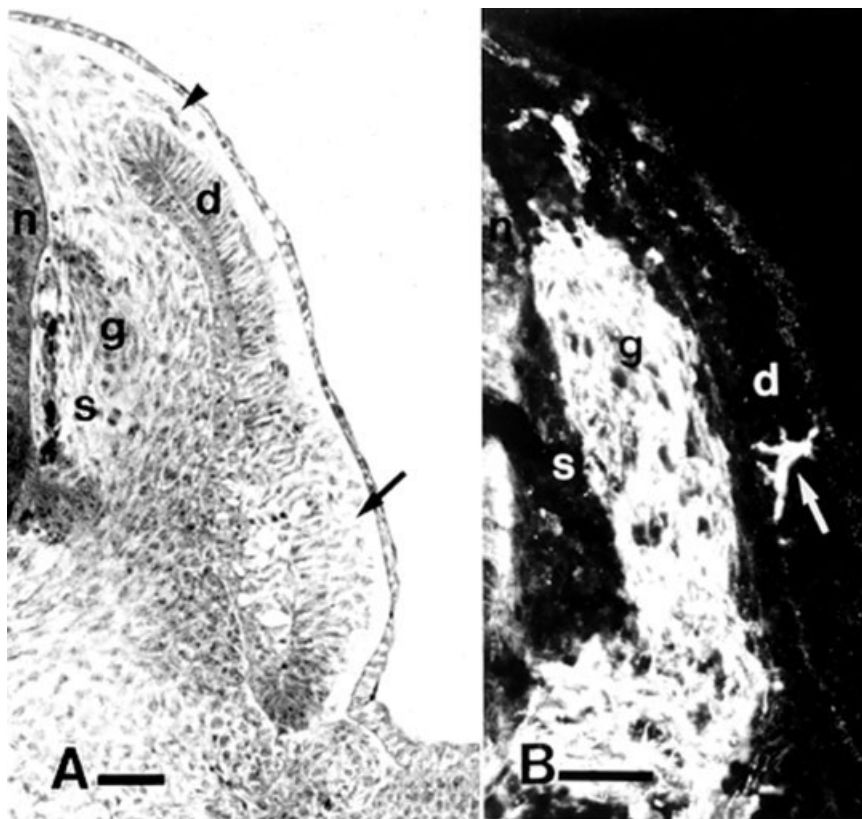


Fig. 1. The time and position in which dermis emerges correlates with neural crest cell behavior. **A:** Shortly before neural crest cells (arrowhead) enter the dorsal path, dermis (arrow) begins to disperse from the dermatome at a site 300–500 μm from the path entryway. Dermis was identified by its distinctive mesenchymal morphology. A 25- μm plastic cross-section. **B:** An HNK1-positive neural crest cell (arrow) has intercalated within the dermatome (d) as dermis begins to form. A 12- μm frozen cross-section. d, dermatome; s, sclerotome; n, neural tube; g, sensory ganglion. Stage 19.5 chick embryos. Dorsal toward top. Scale bars = 50 μm in A,B.

ing the melanoblasts. They emerge from central dermatome, 300–500 μm from the path entryway. The entryway remains free of dermal cells until a couple of days after colonization commences, because the medial dermatome generates dermis only after stage 23. Thus, dermis emerges at the right time to stimulate entry but emerges at a distance that precludes stimulation by direct cell contact. It could potentially stimulate by means of a long-range mechanism.

A curious spatial-temporal correspondence during normal development is also consistent with a long-range stimulation. As dermis begins to develop, some neural crest cells intercalate within the dermatome and/or myotome (see also Oakley et al., 1994). In medial dermatome next to the staging area, the intercalated cells lie in anterior as well as in

posterior somite positions and are detected throughout migration. In contrast, neural crest intercalation into the central dermatome (Fig. 1B; also see Fig. 4) is stringently restricted in space and time. Three features of this restriction suggest that the intercalated cells originate from crest cells that had migrated into sclerotome, rather than from melanoblasts that had already colonized the dorsal path. First, like the ventrally migrating neural crest cells, they are situated only in the anterior half of segment. Second, they are unlikely to have entered the dermatome from a dorsal location, because they invade central dermatome even before melanoblasts have invaded the dorsal path. Of most interest, cells intercalate centrally only at stages and levels where dermis has begun to emerge, either within the same or adjacent sec-

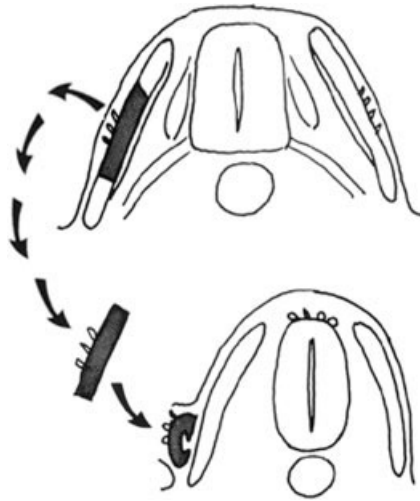


Fig. 2. Schematic diagram shows the surgical strategy. Central dermatome was grafted from a quail embryo (top) to the distal dorsal path of a stage 16–17 chick embryo. This strategy leaves the proximal path unaltered while providing a distant source of dermis.

tions. These relationships suggest that emerging dermis may provide such a compelling stimulus that some neural crest cells will breach intervening myotome and dermatome to reach it.

Grafted Older Dermis Stimulates Precocious Migration

To test whether neural crest cells can respond to distant dermis, older central dermatome that was on the verge of producing dermis was grafted from stage 20–21 quail embryos to stage 16–17 chick embryos. The graft was positioned between the ectoderm and the somite at the distal edge of the dorsal path (Fig. 2). Because such dermatome is still epithelial, it is easy to isolate cleanly and transplant. However, it begins to generate dermis within a few hours after grafting, providing a premature source of dermis. This strategy leaves the proximal portion of the dorsal path intact while offering neural crest cells an early and distant source of dermis. Embryos were fixed before and during the stages when melanoblasts normally enter the dorsal path at the operated levels. In normal and sham-operated embryos, the pattern of migration is stereotyped and is uniform across the

midline. At stage 19, crest cells have yet to enter this path and remain in the staging areas; at stage 20, the first cells are in the path entryway, dorsal to the medial lip of the dermatome; at stage 21, crest cells have entered but still lie in the most proximal portion of the path (also see Erickson et al., 1992, and reconstructed sham operated embryos in Fig. 4). These stages offer the greatest sensitivity for detecting altered melanoblast migration. Grafted quail cells (QCPN-positive) and neural crest cells (HNK1-positive) are easily identified, and precocious entry is easily detected by comparison to the control side.

When older dermatome was grafted, it formed dermis and neural crest cells entered the path precociously, despite the distant source of the dermis. For instance, in Figure 3A, three crest cells lie in the staging area on both control and operated sides. No crest cells have entered on the control side. In striking contrast, on the side with the graft, the neural crest cells have traversed the path to its midpoint. Likewise, in Figure 3B, one crest cell has entered the path on the control side and lies just dorsal to the medial lip of the dermatome. On the grafted side, crest cells have moved farther distally along the path. This stimulation of migration cannot be mediated by cell contact, because grafted cells remained in a lateral position. Dermal cells failed to spread to medial sites where crest cells could contact them directly. Instead, the dermis stimulated migration from graft sites lying up to 600 μm from the entry to the path.

The degree of stimulation depended on the size of the graft (Table 1). Small grafts did not stimulate migration detectably, whereas larger grafts stimulated migration proportionally. Indeed, the larger grafts recruited crest cells from the control side. At graft levels, a larger proportion of crest cells lay within the dorsal path on the operated side. A shift in population was obvious as early as stage 20 (Fig. 4D) and was consistently seen at stage 21 (Fig. 4E). In the stage 21 embryos with large grafts, 71 \pm 4.5% of the population lay on the graft side, strikingly differ-

ent from the uniform 50 \pm 4% distribution seen in controls ($n = 12$; $P < 0.05$). The nonuniform distribution is not explained by increased cell numbers in the population. At stage 21, cells sampled per 100 μm averaged 26 \pm 5 in sham grafts ($n = 4$), 31 \pm 5 in age-matched dermatome controls ($n = 4$), and 30 \pm 6 in embryos with large grafts. The graft, therefore, did not stimulate neural crest cell division to a degree large enough to account for enhanced migration. Instead, it evidently attracted melanoblasts from the contralateral side, where they were lying in the staging area between the neural tube and adjacent dermatomes.

Large dermal grafts also attract crest cells from the ventral path. When grafts were large, crest cells were invariably seen inside the grafts, and/or in the dermatome or myotome between the graft and the sclerotome (Figs. 3B,C, 4D–F; $n = 20$ grafts). There are three possible sources of these cells: they could come from the ventral path, from the dorsal path, or from quail crest cells included in the graft. The dorsal path is the least likely alternative, because several crest cells lie within grafts even when the front of crest cell migration is up to 300 microns from the graft (Fig. 4D,E). The cells are not neural crest cells inadvertently included in the graft, as they consistently failed to label with the quail-specific antibody QCPN (Fig. 3C). These cells are thus recruited from crest cells that have already migrated ventrally. The recruitment was not dependent on local damage to the host dermatome. In a few cases, the host dermatome was cut during surgery and the graft partially fused with host dermatome (e.g., Fig. 3B). However, such damage was not required for crest cells to intercalate in dermatome or enter the graft (e.g., Fig. 3C). Moreover, crest cells never entered control grafts, or intercalated in the dermatome adjacent to such grafts, even when the host dermatome had been injured.

Stimulation was specific to dermis. Grafts of other embryonic tissues failed to stimulate migration, even when these grafts were large (Fig. 5;

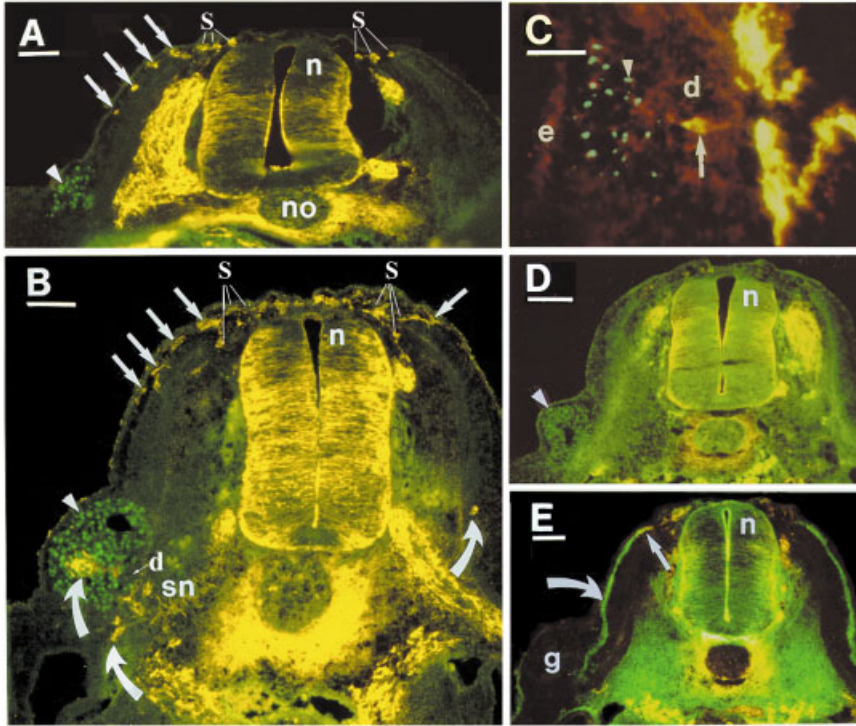


Fig. 3.

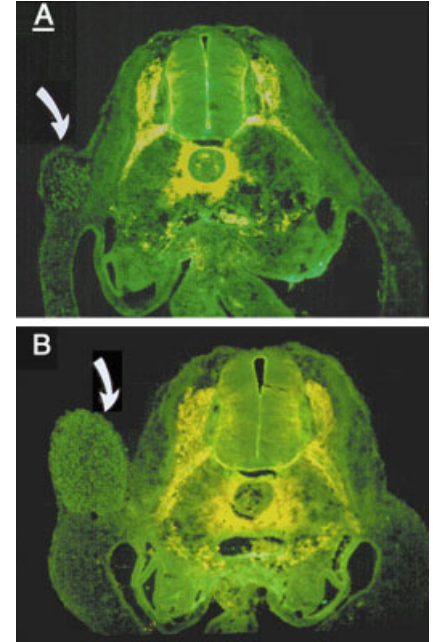


Fig. 5.

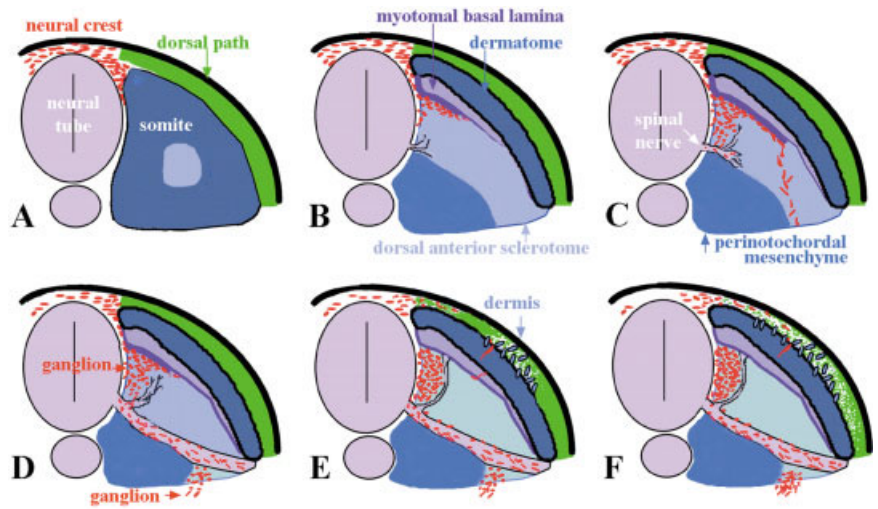


Fig. 6.

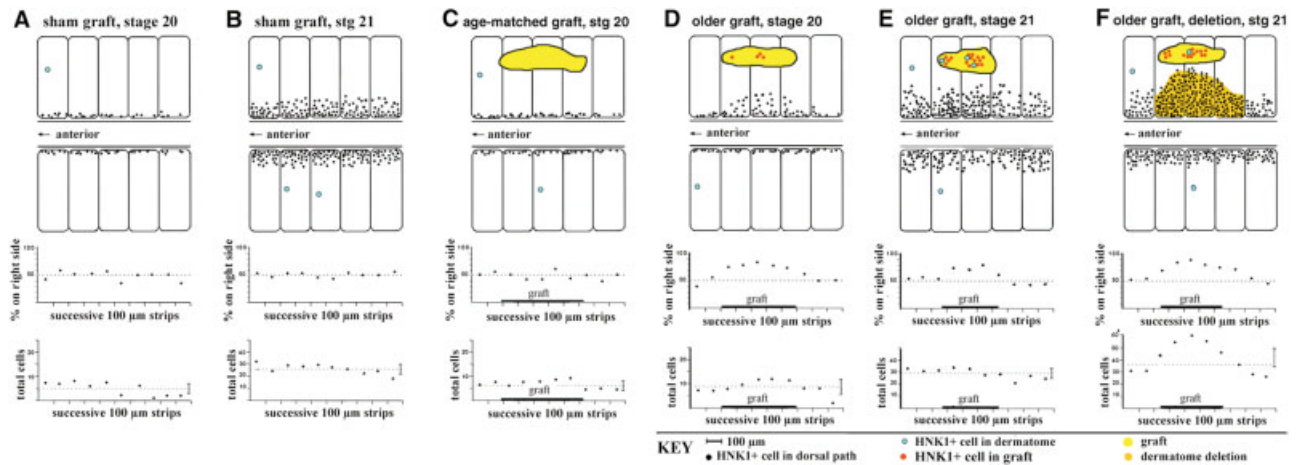


Fig. 4.

Table 1). Moreover, the epithelial precursor of central dermis also failed to stimulate migration. Crest cells entered on schedule after younger or age-matched dermatome was grafted (Figs. 3D, 4C). Medial dermatome also failed to stimulate migration in this assay, even when it was taken from older embryos just before crest cells begin to colonize the path ($n = 8$). All such grafts retained an epithelial morphology and failed to generate dermis before fixation. The stimulation is not an artifact of surgical trauma, because migration was unaltered by sham grafts ($n = 25$; Fig. 4A,B). The stimulation is likewise not an artifact of using quail tissue as a donor. The same stimulation ensued when older dermatome is transplanted from a chick donor to a chick host and the neural crest cells are visualized with a nontoxic vital dye ($n = 11$). Control surgeries ruled out the possibilities that migration was stimulated by some diffusible substance

produced by medial epithelial dermatome at the path entryway or that migration is stimulated merely by the increased mass of grafted dermatome, rather than by the dermis itself.

Relation Between Attractive Dermal Grafts and Inhibition

The results above show that grafted dermis can entice neural crest cells into the dorsal path, even though the graft lies distally where it is unlikely to have directly altered inhibitory properties at the path entryway. The likelihood that inhibitory qualities are retained is supported by dual surgeries designed to supply long-range stimulation and to eradicate the local inhibition simultaneously, by combining grafts with medial dermatome deletions. If dermis acts by removing the inhibition, grafts alone and combined grafts/deletions should generate a similar stimulation. In contrast, if dermal and in-

hibitory cues are separable, then the stimulation should be synergistic: the dermal cue would attract melanoblasts, whereas the loss of inhibition would foster invasion by neural crest cells that normally migrate ventrally. In accord with a synergism, crest cell invasion was enhanced. After dual surgeries, more crest cells entered the path and these often reached farther distally than they did after grafts or deletions alone ($n = 7$; Fig. 4F). At stage 21, 50 ± 4.3 sampled cells are seen per $100 \mu\text{m}$, contrasting with the average of 30 ± 4.5 cells that typify both controls and older dermal grafts ($P < 0.05$). These experiments thus provide evidence that the dermal cue acts independently, rather than by directly removing an inhibitory influence.

A diffusible cue from dermis could directly attract cells (chemotaxis) or could alter the extracellular matrix within the path and make it more suitable for migration (haptotaxis). Although these alternatives are diffi-

Fig. 3. Grafts with dermis (but not with dermatome alone) stimulate precocious migration into the dorsal path and recruit ventral crest cells, but fail to alter an inhibitory marker distribution. Arrows, yellow HNK1-positive neural crest cells; arrowheads, grafted QCPN-positive quail nuclei. HNK1 also labels mesoderm surrounding the notochord (no). n, neural tube. Operated sides on the left. **A:** Grafted dermis stimulates precocious migration. Stage 21 quail dermatome, grafted at stage 17, has formed dermis. Crest cells on both sides lie in the staging area, proximal to the dorsal path (S). On the control side, no crest cells have entered the path. On the operated side, crest cells have migrated halfway through the path. Stimulation by direct contact is ruled out, as quail nuclei remain distal. **B:** A larger graft also recruits ventral crest cells. Crest cells have just entered the control side path from the medial edge and by traversing dermatome (right curved arrow). On the operated side, a large graft of stage 19 quail dermatome has formed dermis and an epithelial vesicle that has partially integrated with host dermatome (d). Crest cells have precociously entered this path. Moreover, several HNK1-positive cells (curved arrows) have diverged from sclerotome toward the graft. sn, spinal nerve. **C:** QCPN-positive nuclei indicate grafted cells between ectoderm (e) and intact dermatome (d). An HNK1-positive cell within dermatome is QCPN-negative. **D:** Age-matched dermatome graft remains epithelial and fails to stimulate migration. **E:** PNA binding activity (green, curved arrow) is retained proximally, despite grafted dermis (g) and precocious neural crest cell entry. Stage 20 embryos. All are $12\text{-}\mu\text{m}$ frozen sections. Scale bars = 20 m in C, $100 \mu\text{m}$ in A,B,D,E.

Fig. 4. Grafts with dermis promote early migration and recruit neural crest cells from the control sides without detectably increasing population sizes, whereas grafts combined with dermatome deletions both stimulate migration and increase population size. The top of each panel shows reconstructions from alternate serial sections, as viewed from the dorsal surface, with the operated side toward the top. Lines indicate the somite boundaries. Yellow indicates grafts. Crest cells are color coded by location. Note that cells in dermatome are restricted to the anterior halves of a segment, even in regions with grafts. Crest cells in the dorsal path on each side were tabulated in $100\text{-}\mu\text{m}$ strips. Graphs in register with the reconstructions display the percentage of the population that lies on the graft side and the population numbers. **A–C:** Sham grafts and age-matched dermatome grafts do not detectably alter migration. **D,E:** In grafts with dermis, up to 80% of the population lies on the operated side, but population number is not detectably larger at graft levels. **F:** When grafts with dermis are combined with medial dermatome deletions (gold), the population size increased, as though crest cells that normally migrate ventrally were recruited.

Fig. 5. Grafts of other tissues fail to promote migration into the dorsal path. Grafts of sclerotome (**A**, arrow) and distal limb bud (**B**, arrow) failed to stimulate crest cells to enter the path early. Both images are $12\text{-}\mu\text{m}$ frozen sections. Scale bar = $100 \mu\text{m}$.

Fig. 6. Relations between crest migration and somite morphogenesis. **A:** Crest cells emigrate but wait in the staging area while the somite is epithelial and both paths are inhibitory. **B:** As ventral somite forms mesenchyme, crest cells enter dorsal anterior sclerotome (a permissive but not preferred substrate) by traveling laterally on myotomal basal lamina. The first motor axons likewise emerge. **C:** Once available basal lamina is coated with crest cells, remaining cells move ventrally in two streams: one medial and one lateral. Medially, crest cells are contained by the spinal nerve and dorsally advancing epaxial motor nerve. Both axons and crest cells avoid traversing the inhibitory perinotochordal mesenchyme. **D:** Some crest cells are captured by the spinal nerve, which spreads across the boundary between dorsal-anterior sclerotome and perinotochordal mesenchyme. Others bypass the advancing nerve tip and lodge in the sympathetic ganglion site. The sensory ganglion is cupped between the neural tube, myotome, and spinal and epaxial nerves. **E:** Ventral migration is complete, and dorsal anterior sclerotome becomes more inhibitory. As dermis emerges, melanoblasts leave the staging area and enter the dorsal path. A few ventral cells home toward dermis through intervening tissues. **F:** Crest cells continue to enter the dorsal path, which loses inhibitory character as more dermis emerges.

TABLE 1. Precocious Neural Crest Migration Is Regularly Elicited by Medium to Large Grafts of Older Dermatome, but Not by Age-Matched Dermatome, Younger Dermatome, or Other Tissue^a

	Small graft	Medium graft	Large graft
Obviously precocious			
Older dermatome	0	9	20
Detectably precocious			
Older dermatome	3	5	0
Not precocious			
Older dermatome	8	0	0
Age-matched dermatome	1	2	5
Younger dermatome	0	1	6
Medial older dermatome	1	2	5
Distal limb bud	1	2	7
Gut epithelium	0	1	1
Mesonephros	0	0	2
Neural crest/dorsal neural tube	0	1	3
Notochord	0	1	1
Perinotochordal mesenchyme	0	1	2
Posterior sclerotome	0	0	4
Anterior sclerotome	0	0	2

^aOlder dermatome grafts that were small only sometimes (3/11) elicited precocious migration. Small grafts had a maximum density per 10- μ m section of less than 10 cells, and extended less than 50 μ m along the anterior-posterior axis. Medium grafts reached maximum densities of 10 to 20 cells per section and extended 90–200 μ m. Larger grafts reached maximum densities of 20 to 50 cells (a maximum of 100 cells in two cases) and extended 120–400 μ m.

cult to distinguish, it is notable that the distribution of a marker for inhibitory function in the proximal path is unchanged by the dermal grafts. Peanut agglutinin lectin, an inhibitory marker (Oakley and Tosney, 1991; Oakley et al., 1994), is retained within the proximal path even in the presence of a large graft (Fig. 3E; $n = 12$). Therefore, grafts fail to obviously alter a characteristic molecular profile of extracellular matrix. In particular, the entry region retains the inhibitory marker. Although subtle but significant changes are not ruled out, the dermal stimulation is thus more likely to be explained by a diffusible cue that by a wholesale remodeling of extracellular matrix.

DISCUSSION

This work establishes a mechanism for directed melanoblast migration into the dorsal path. It identifies dermis as the source of a cue that can elicit melanoblast migration from the distant staging area. During normal development, a directing gradient is established due to the spatial-tem-

poral pattern of dermis emergence. Dermis arises from central dermatome, at a distance from the staging area. The absence of dermis along the medial portion of the path, both during normal development and after grafts, shows that dermis cannot stimulate migration directly, by means of cell contact. At the medial site, dermis forms only well after crest cells have colonized the entire path. Indeed, medial dermatome generates a molecularly as well as spatially distinct population of dermis (Houzelstein et al., 2000) and when epithelial it displays no activity in the graft assay. By virtue of its initial emergence from the distant central dermatome, dermis supplies a distant source of attractant. The cue that dermis supplies must thus be diffusible. It is unlikely to work by altering inhibitory features at the path's entryway.

Within the dorsal path, both the stimulus and the target are specific. Only dermis stimulates directed migration. Other tissues tested failed to attract crest cells into the dorsal

path, and even grafts of dermatome that remained epithelial are unable to stimulate migration from a distance, regardless of the graft size. The target within the dorsal path is clearly the melanoblast population, which has developed special properties while still in the staging area that allow it to access the dorsal path (Santiago and Erickson, 2002). As convincingly shown by Erickson and her associates, crest cells that enter dorsal path are specified as melanoblasts and would comprise the crest cell population in the staging area during stages when dermatome grafts generated dermis (Erickson and Goins, 1995; Reedy et al., 1998a; Kos et al., 2001; Faraco et al., 2001). The dermal grafts do not alter the size of this population. Instead, the population shifted toward the graft, as though only one population was capable of being recruited by this stimulus. Specificity was confirmed by combining grafts with medial dermatome deletions, which would be expected to entice a second population into the dorsal path, ventral crest cells that are normally inhibited from entering by inhibition supplied by medial dermatome (Erickson et al., 1992; Oakley et al., 1994; Santiago and Erickson, 2002). Such dual surgeries increased the size of the population in the dorsal path, as predicted.

An extraordinary finding was that a few neural crest cells that had already migrated ventrally can be attracted to dermis, both during normal development and in response to dermal grafts. These results further establish that the dermal cue cannot act by direct contact. They also show that a few cells on the ventral path can detect and respond to this diffusible cue. These cells may or may not be a part of the melanoblast lineage. During normal development, bipotential glial/melanocyte progenitors are found on ventral paths (Nichols and Weston, 1977; Nichols et al., 1977; Hallett and Ferrand, 1984; Reedy et al., 1998b) and, in some cases melanocytes, erroneously migrate ventrally (Faraco et al., 2001). However, the responding cells may have other fates and may simply be misdirected. For instance, a few cells with neurogenic

potential mistakenly enter the dorsal path, but these errors are removed by apoptosis (Wakamatsu et al., 1998).

Long-range cues may characterize other neural crest paths as well. The otic placode may attract hind-brain neural crest cells (Sechrist et al., 1994); FGF-2 may be chemotactic in the mouse mesencephalon (Kubota and Ito, 2000); GDNF is chemotactic to the enteric neural crest (Young et al., 2001). In the somite, both crest cells and axons preferentially enter the anterior sclerotome (Keynes and Stern, 1984; Rickmann et al., 1985), which can attract sensory and motor axons from a distance (Hotary and Tosney, 1996), suggesting that the anterior sclerotome may attract crest cells as well. If it attracts ventral crest cells but not melanoblasts, it would help to explain why melanoblasts in the staging area fail to migrate ventrally. Melanoblasts and ventral cells may each have a specific attractant. Alternatively, the ventral path may specifically prohibit melanoblast migration ventrally or, as suggested by Reedy et al. (1998a), timing may suffice to dictate specificity: melanoblasts may begin migration only after ventral somite becomes inhibitory to any crest cell movement.

The attractive substance released by the dermis is uncharacterized, but two candidates are of interest. First, a soluble form of Steel factor has been suggested to aid the dispersion or directed migration of melanoblasts. Steel factor (aka mast cell factor or stem cell factor; see Witte, 1990) is required for the maintenance but not the differentiation of melanoblasts, and crest cells express its receptor, c-kit (Murphy et al., 1992; Steel et al., 1992). In mouse, experiments that uncouple melanoblast survival and dispersal suggest that the soluble version supplies a migration signal (Wehrle-Haller and Weston, 1995; Wehrle-Haller et al., 2001). However, its temporal-spatial distribution argues against it being the dermal directional signal. Before melanoblasts enter the dorsal path, Steel factor is initially expressed medially in the epithelial dermatome (Wehrle-Haller and Weston, 1995; Guo et al., 1997), but the medial der-

matome fails to generate dermis early enough to be the dermal stimulant. Finally, Steel factor is also produced and released by the neural crest cells themselves while melanoblasts are still in the staging area, where it may act as an autocrine factor to induce melanogenesis in the nearby crest cells (Guo et al., 1997). Steel production in the staging area would likely preclude the development of an effective Steel factor gradient from emerging dermis.

Instead of providing a directional signal, Steel factor may be a 'scatter factor,' promoting dispersion in both the neural crest and the dermatome. Homozygous Patch mutants support this possibility (Wehrle-Haller et al., 1996). They express Steel normally but also express c-kit ectopically in the distal somite, which could increase the competition for Steel in the dorsal path. Dermis development is defective, and crest cells initiate migration but fail to disperse normally or survive, suggesting that reducing Steel reduces both survival and dispersal.

A second candidate is soluble ephrin-B1, which has been shown to attract melanoblasts in a chemotaxis assay (Santiago and Erickson, 2002), just as ephrin A-1 is chemotactic to endothelial cells (Pandey et al., 1995). Although ephrins are predominantly cell-surface molecules, the ADAM-10 metalloprotease can cleave ephrin-A2 (Hattori et al., 2000), potentially releasing a soluble fraction suitable to establish a chemotactic gradient, as proposed by Santiago and Erickson (2002). ADAM-10 is expressed in the dispersing dermis during the dermatome-dermis transition (Hall and Erickson, 2003), which is accompanied by the local remodeling of matrix and the local reduction of an inhibitory marker (Oakley et al., 1994). It could thus generate a local source of soluble ephrin. Alternatively, it could facilitate changes that allow the dermis to produce some other attractive substance.

The evidence that the dorsal path is accessible only to melanoblasts raises the possibility that other paths may also be population-specific. For the ventral path, this possibility is commonly disregarded, based on

an assumption that the ventral somite offers just one route and that differences in destination are dictated only by how far crest cells can travel before the somite matures and grows too inhibitory to support migration (Weston and Butler, 1966). However, ventral migration is more intricately patterned than is generally thought, and the patterns accord with quite detailed spatial-temporal patterns of somite maturation (Fig. 6 summarizes results from Rickmann et al., 1985; Bronner-Fraser, 1986; Loring and Erickson, 1987; Teillet et al., 1987; Tosney, 1987; Serbedzija et al., 1990; Tosney and Oakley, 1990; Bronner-Fraser and Stern, 1991; Oakley et al., 1994; Tosney et al., 1994; Hotary and Tosney, 1996; Sela-Donenfeld and Kalcheim, 2000). It remains unclear whether different ventral routes provide population-specific cues. If these routes lack specific cues, then the intricate migration could result simply from transient accessibility of different substrates as somitic tissues develop. For instance, the basal lamina substrate that crest cells initially use to enter the somite would be transiently available, so that cells that enter later would use different substrates to colonize the sensory ganglion destination (see Tosney et al., 1994). However, if each route does provide some specific information, then migration patterns could result from preference: sensory cells could ignore the basal lamina, whereas sympathetic ganglion precursors colonize it avidly. If specific cues also help to specify cell identity en route, then the full repertoire of a population's possible phenotypes is a function of its migratory options (c.f., Lumsden, 1988; Noden, 1993; McGonnel and Graham, 2002). Specific cues may also select against survival of phenotypes that colonize an incorrect path (e.g., Wakamatsu et al., 1998; Maynard et al., 2000). Even if routes lack population-specific cues, they could guide specified populations if paths and cells mature in synchrony. Coordinated timing could be a consequence of the early induction of neural crest emigration by developing somite (Sela-Donenfeld and Kalcheim, 2000). These unresolved issues must

be studied by investigating the qualities of the distinctive paths directly.

In summary, long-range as well as short-range cues guide the melanoblasts into the dorsal path. The dermatome supplies both these cues in temporal sequence. It initially provides a transient, short range inhibition that prevents the entry of ventrally migrating cells (Erickson et al., 1992; Oakley et al., 1994) but supports the entry of specified melanoblasts (Santiago and Erickson, 2002). As it matures, it generates dermis distally that stimulates migration from a distance. Neural crest migration, thus, is subject to precise control that accords temporally and spatially with the detailed developmental program of somite morphogenesis.

EXPERIMENTAL PROCEDURES

Histology

To examine the temporal-spatial pattern of dermis emergence, embryos were examined in thick plastic sections that preserve excellent cellular detail, prepared as in Tosney and Landmesser (1986). Briefly, stage 16–24 embryos were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 12–18 hr, post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer overnight at 4°C, dehydrated in graded alcohols, and embedded in Epon-Araldite through propylene oxide. The polymerized block was trimmed, and the embryos were serially sectioned at 12.5 or 25 μm with a heated steel knife on a Leitz rotary microtome. Sections were mounted in 10% glycerin. The time table of neural crest migration was established by using immunocytochemistry (below), and the same database as that used in Erickson et al. (1992) and Tosney et al. (1994).

To examine neural crest entry into the dorsal path after surgeries, embryos were examined by using immunocytochemistry. Operated embryos were fixed at stages 19.5 through stage 21 in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2–4 hr at room temperature, washed three times for 10 min each in PBS, infiltrated at 4°C in 5% sucrose for 1–24 hr, in 15% sucrose for 18–24 hr, and in solution of 7.5% gelatin and 15% sucrose in PBS for 4–6

hr at 37°C, in which they were then embedded. After 24 hr at 4°C, blocks were rapidly frozen in isopentane cooled on dry ice. Serial 10- μm frozen sections were produced by using a Reichert cryostat. Selected sections were labeled by using PNA (Vector) to detect putative inhibitory domains, using QCPN (kind gift of B. Carlson) to detect transplanted quail cells, and using HNK1 (kind gift of K. Barald) to detect neural crest cells. No melanoblast-specific marker was used, as it was important to monitor all putative crest cells. Without this strategy, the crest cells invading the dermatome from the ventral path might have been missed. PNA binding was detected by using goat anti-PNA (Vector) as in Tosney and Oakley (1990). HNK1 and QCPN were detected by using rabbit anti-goat fluorescein isothiocyanate or rabbit anti-mouse tetra-rhodamine isothiocyanate as in Erickson et al. (1992). Fluorescent and phase images were recorded separately by using an intensified CCD video camera (Pulnix model TM-74, Motion Analysis, Eugene, OR) on optical disc (Panasonic). Black and white images of each photofluor were pseudocolored and combined by using Metamorph (Universal Imaging).

Reconstructions of each embryo that had a graft and of six sham-operated embryos were reconstructed from every other transverse serial section. Crest cell numbers reported, therefore, represent ca 50% of the crest cells actually present. The position of each labeled HNK1-positive cell in the dorsal path was measured from the midline and transferred to a planar representation that also recorded the somite borders and the graft position and size. Likewise, the numbers and position of crest cells in epithelial dermatome and within quail grafts was also recorded. All such HNK1-positive cells were QCPN-negative. Reconstructions covered operated segments, plus at least one segment anterior and posterior to the operation site. Statistical significance was tested using unpaired Student's *t*-test.

Surgeries

Quail tissues were implanted into the lateral edge of the chick dorsal path (Fig. 1). Eggs were obtained from David Bilbe (Ann Arbor, MI). In brief, 1–2 cc of albumin was removed through a hole in the blunt end of chick eggs so that the embryo would fall away from the shell, the shells were windowed, India ink was injected beneath the embryo to enhance visibility, the vitelline membrane was removed, and the amnion was opened. The ectoderm overlying one to four somites was slit laterally at lower thoracic and/or lumbar levels of stage 16–17 chick embryos (Hamburger and Hamilton, 1951). Stage 15–23 quail embryos (staged by using the same criteria) were removed from the egg to Sylgard-coated dishes. Ectoderm overlying somites was removed. The epithelial dermatome was harvested by using tungsten needles from the central region of somites, as this region normally generates dermis first. Excised dermatome was rinsed to remove any adherent sclerotome or myotome, moved using a wire loop to the chick embryo, and nudged by blunt needles into a gap resulting from slitting the host ectoderm. "Old dermatome" grafts were from stage 20–21 embryos. Control dermatomal grafts include younger central dermatome (stage 15–16), stage-matched dermatome, and medial older dermatome (stage 20). Sclerotome and myotome contamination was minimal, as these tissues fail to adhere to central dermatome at these stages (Tosney, unpublished data). Additional control tissues were harvested from stage 17 (neural crest, sclerotome) or stage 20–23 embryos by using needles (see Table 1). Generally, the host ectoderm enclosed and healed over the graft, but in some cases, the ectoderm healed under the graft, lifting it and excluding it from the embryo ($n = 25$); these were considered to be sham grafts. In no cases did sham grafts show precocious neural crest migration. In seven embryos, in addition to grafting older dermatome laterally, the medial half of one to four dermatomes was deleted as in Erickson et al. (1992). Embryos were

fixed at stages 19.5, before neural crest cells enter the dorsal path at the operated levels, at stage 20, as the first cells are commonly seen in the path entryway, dorsal to the dorsal lip of the dermatome, and at stage 21, after crest cells have begun to enter the dorsal path but when all still lie proximally. See Erickson et al. (1992) for confirmation of migration events relative to stages at these levels.

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