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Scatter factor/hepatocyte growth factor as a regulator of skeletal muscle and neural crest development

(melanocytes/Met/migration/transgenic mice/TRP-2)

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ABSTRACT **Factors that regulate cellular migration during embryonic development are essential for tissue and organ morphogenesis. Scatter factor/hepatocyte growth factor (SF/HGF) can stimulate motogenic and morphogenetic activities in cultured epithelial cells expressing the Met tyrosine kinase receptor and is essential for development; however, the precise physiological role of SF/HGF is incompletely understood. Here we provide functional evidence that inappropriate expression of SF/HGF in transgenic mice influences the development of two distinct migratory cell lineages, resulting in ectopic skeletal muscle formation and melanosis in the central nervous system, and patterned hyperpigmentation of the skin. Committed TRP-2 positive melanoblasts were found to be situated aberrantly within defined regions of the transgenic embryo, including the neural tube, which overproduced SF/HGF. Our data strongly suggest that SF/HGF possesses physiologically relevant scatter activity, and functions as a true morphogenetic factor by regulating migration and/or differentiation of select populations of premyogenic and neural crest cells during normal mammalian embryogenesis.**

Scatter factor/hepatocyte growth factor (SF/HGF) stimulates proliferation and/or movement in a variety of cultured epithelial cells (1–3). SF/HGF is produced in mesenchymal embryonic tissues lying in close proximity to epithelial cells expressing its tyrosine kinase receptor Met, suggesting that this paracrine signaling pathway plays an important role in development (4). In fact, mouse embryos carrying a null mutation in genes encoding either SF/HGF or Met die *in utero*, and show aberrant development of placenta, liver, and muscle (5–7). SF/HGF also mediates the morphogenetic organization of cultured epithelial cells into ordered tubular structures and has been implicated in epithelial/mesenchymal conversion (8–12). However, despite extensive study of *in vitro* biological activities, the precise *in vivo* role of SF/HGF is still poorly understood. To elaborate physiologically relevant functions of SF/HGF, transgenic mice were generated in which a mouse SF/HGF cDNA was overexpressed in a wide variety of fetal and adult tissues. Here we show that inappropriate expression of SF/HGF induces the ectopic development of skeletal muscle and melanocytes in the central nervous system (CNS), indicating that SF/HGF possesses *in vivo* morphogenetic and scatter activity.

MATERIALS AND METHODS

Transgenic Mice. SF/HGF transgenic mice were generated as described (13). A 2.2-kbp mouse SF/HGF cDNA was cloned from a library constructed in the phagemid λpCEV27 from NIH 3T3 fibroblast mRNA. The library was screened using the coding region of human SF/HGF cDNA as a probe (14). Full-length mouse SF/HGF clones (728 amino acids) were identified by PCR and DNA sequence analysis. The 20.7-kbp *SalI–SalI* microinjection fragment included the complete mouse SF/HGF cDNA coding region, the mouse metallothionein (MT) I promoter, the human growth hormone polyadenylylation site, and the mouse MT gene 5′ and 3′ flanking regions, which served as locus control regions to confer copy number-dependent and position-independent expression to the transgene (15). All mouse work was performed in accordance with regulations established by the National Institutes of Health on the care and maintenance of laboratory animals.

Analysis of RNA. Total RNA was prepared from tissues or whole embryos using guanidine thiocyanate, as described (13). For Northern blot hybridization total RNA was loaded at 20 μg per lane and transferred to nylon membranes. SF/HGF transcripts were detected with a ^{32}P-labeled 2.2-kbp mSF/HGF cDNA probe synthesized by PCR using the transgene as template and the following primer set: 5′-ATTATAGGATCCATGATGTGGGGGACCAAACTTCTGCCG-3′ and 5′-ATTATAGGATCCTTACAACTTGTATGTCAAAATTAC-3′. To control for RNA loading and transfer variation, filters were rehybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Localization of committed melanoblasts in midgestation embryos was by *in situ* hybridization, using the method described by Fox and Cottler-Fox (16) and a ^{33}P-labeled 1.2-kb mouse TRP-2 antisense riboprobe. A control nonsense riboprobe demonstrated no specific signal (data not shown).

Microscopy and Analysis of Protein. All mouse tissues were fixed in 10% buffered formalin except when used for electron microscopy, where Karnofsky's fixative was used. Analysis of tissue by electron microscopy was as described (17). All other tissues were stained with hematoxylin and eosin, or with phosphotungstic acid-hematoxylin (PTAH) to visualize muscle striations. For immunohistochemistry, SF/HGF was detected using a goat anti-human SF/HGF polyclonal antibody (R&D Systems) at a 1:20 dilution, and a goat Vectastain Elite ABC kit (Vector Laboratories). Muscle tissue was identified using a mouse α-actin monoclonal antibody (Dako) at a 1:100 dilution. For quantification of mouse SF/HGF in serum and urine, a two-site ELISA kit using a cross-reacting anti-rat SF/HGF monoclonal antibody was employed according to instructions from the manufacturer (Institute of Immunology, Tokyo).

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Abbreviations: CNS, central nervous system; MT, metallothionein; PTAH, phosphotungstic acid-hematoxylin; SF/HGF, scatter factor/hepatocyte growth factor; E, embryonic day.

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RESULTS

Generation of Transgenic Mice Expressing SF/HGF. Transgenic mice were generated carrying a mouse SF/HGF cDNA under the control of the mouse MT-I promoter and 5′/3′ MT gene flanking sequences (15) (Fig. 1*A*). Nine transgenic founder mice were generated harboring between 7 and 27 transgene copies. The transgene expressed a characteristic 2.4-kb RNA in virtually all adult tissues, at a level between 3- and 50-fold higher than the major 6-kb endogenous SF/HGF transcript (Fig. 1*B*). An ELISA assay using an anti-rat HGF monoclonal antibody was used to show that, relative to controls, the average level of immunodetectable SF/HGF in all transgenic lines was significantly higher in both serum (16.4 ± 2.5 versus 3.9 ± 0.6 ng/ml) and urine (21.4 ± 2.2 versus 0.2 ± 0.1 ng/ml). Both transgenic serum and conditioned media from cultured transgenic hepatocytes possessed strong biological activity based on a scatter assay using MDCK cells (H. Sakata, W. Taylor, and J. Rubin, unpublished observations). Transgenic transcripts were detectable in embryos on embryonic day (E) 9.5, and were approximately 4- and 10-fold higher than endogenous RNAs on E11.5 and E14.5, respectively (Fig. 1*C*). Analysis of protein by immunohistochemistry demonstrated that SF/HGF was abundant on E11.5 throughout the transgenic embryo, including the neural tube and skin (Fig. 2*B*).

Ectopic Skeletal Muscle Development in the Transgenic CNS. Based on transgene expression two lines, MH19 and MH37, were selected for detailed analysis. Nearly 80% of MH19 and 65% of MH37 mice died by 6 months of age, typically from either sporadic intestinal obstruction or progressive renal failure. Approximately 5% of MH19 mice (nine animals to date) developed hind limb paralysis between 4.5 and 6.5 weeks of age. Histopathologic evaluation of paralyzed adults revealed ectopic development of striated muscle in the CNS, revealed by staining with PTAH (Fig. 2 *D* and *G*). The spinal cords of paralyzed mice often exhibited cytoarchitectural asymmetry in which dorsal and ventral horns were distorted (Fig. 2*D*). Although more severe in paralyzed mice, ectopic muscle development was observed in the cord of most (14 of 20) MH19 mice examined, and less frequently in the brain (5 of 28). Ectopic muscle, which showed immunohistochemical positivity for α-actin, was most prominent in the leptomeninges of the ventrolateral thoracic cord (Fig. 2 *D* and *E*), but appeared occasionally in cervical and lumbar cords and on the ventral surface of the medulla (Fig. 2*F*). Smaller fibers were detected within the adult cord by blood vessels, and within ventral nerve roots (Fig. 2*G*).

Interestingly, the development of ectopic muscle in the CNS was delayed relative to native muscle. Muscle tissue was detectable in neonatal and juvenile MH19 cords, but only as immature cells and small fibers (Fig. 2 *H* and *I*). In contrast, adult cords contained mature muscle. Striated muscle in the CNS was characterized as skeletal based on electron microscopic examination; fibers had peripheral nuclei, did not demonstrate branching and rejoining, and contained t-tubules at the junction of the A and I bands (Fig. 2*J*).

Aberrant Development of Melanocytes in SF/HGF Transgenic Mice. Based on the apparent effect of SF/HGF on myogenic cell migration, we next examined neural crest-derived melanocytes, another major migratory cell lineage. Albino MH19 and MH37 mice were crossbred with pigmented C57BL/6 mice. Eight-day-old transgenic F_1 mice from both crosses exhibited patterned hyperpigmentation in skin of the extremities, and on dorsal and ventral surfaces (Fig. 3*A*). The latter appeared as black abdominal spots. Microscopically, melanocytes in adult transgenic skin appeared not only in hair shafts, their normal location, but in great excess throughout the dermis and epidermis (Fig. 3*G*).

Significantly, in adult transgenic mice, melanosis was extensive in and around the meninges throughout the CNS, including the brain (Fig. 3 *B* and *C*), and in lymph nodes (Fig. 3*I*). Ectopic melanocytes and muscle occasionally developed together on the lateral surface of the cord, suggesting a common mechanism in pathogenesis (Fig. 3*D*). Pigmentation was much more limited in younger mice. Melanocytes in 8-day-old transgenics were found only in the dorsolateral meninges of

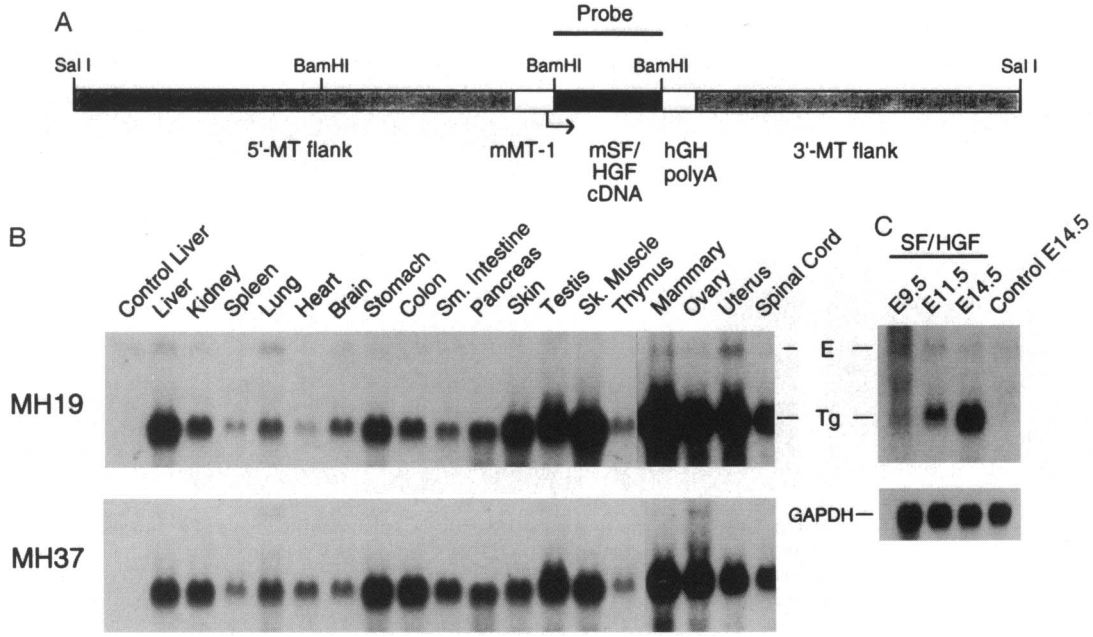

FIG. 1. Structure and transcriptional activity of the MT-SF/HGF transgene. (*A*) The 20.7-kbp *SalI–SalI* microinjection fragment includes 2.2 kbp of cDNA encoding the entire mouse SF/HGF (black-shaded box), the mouse MT-1 promoter, the human growth hormone polyadenylylation site (open boxes), and mouse MT gene 5′ and 3′ flanking regions (grey-shaded boxes), which contain locus control regions. Region used as Northern hybridization probe is shown at top. (*B*) Analysis of SF/HGF RNA in adult transgenic tissues by Northern hybridization. Shown is expression of 2.4-kb transgenic (Tg) and 6.0-kb endogenous (E) SF/HGF transcripts from two independent lines (MH19 and MH37 indicated at left). (*C*) Analysis by Northern hybridization of SF/HGF RNA in E9.5, E11.5, and E14.5 total embryo tissue. Filter was rehybridized with a GAPDH probe to assess loading and transfer of embryonic RNAs.

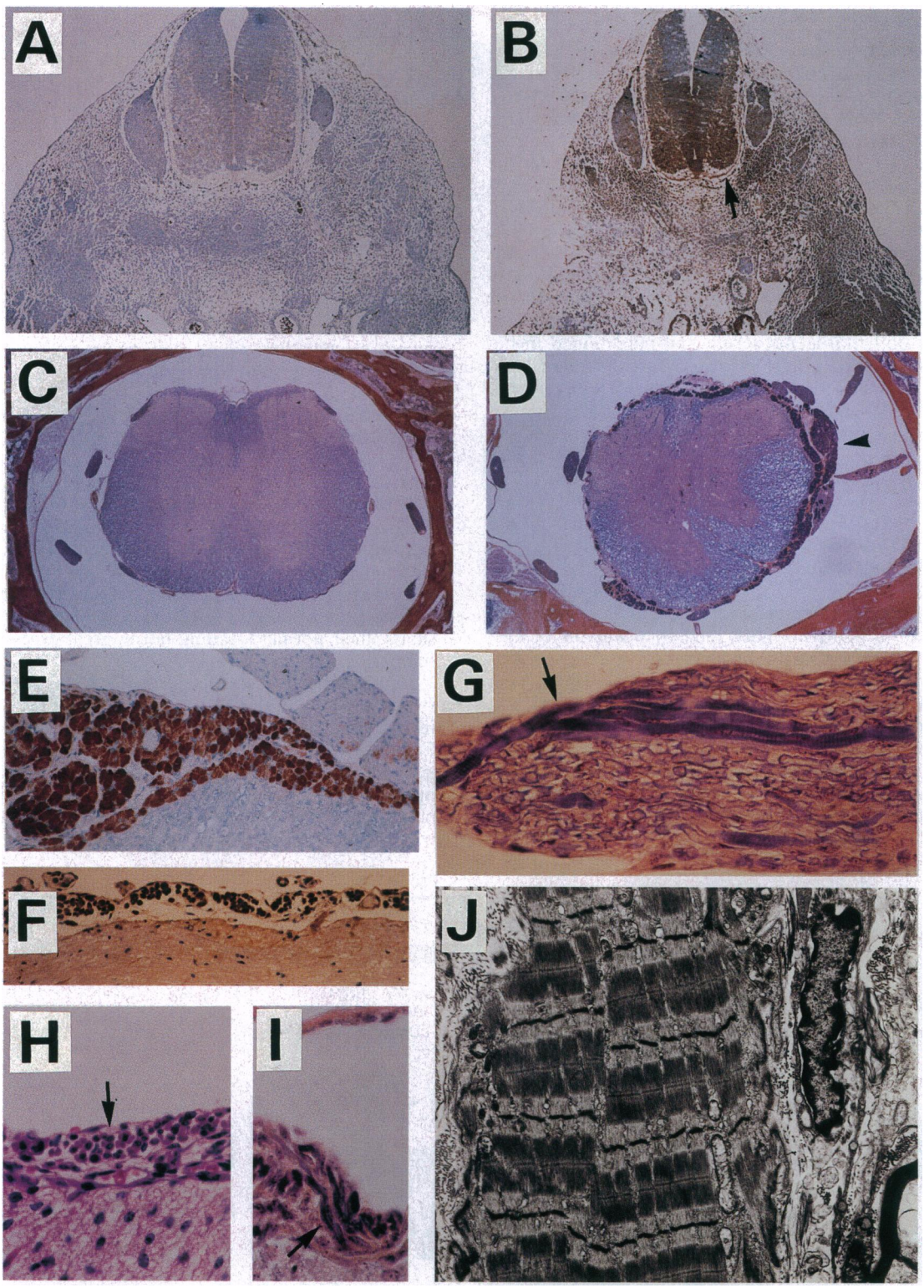

FIG. 2. (*A* and *B*) Expression of SF/HGF protein in the transgenic mouse embryo. Immunohistochemical localization of mouse SF/HGF in (*A*) control FVB/N and (*B*) transgenic MH19 E11.5 embryos (magnification ×55). Note heavy staining in neural tube (arrow). (*C–J*) Ectopic development of skeletal muscle in the transgenic CNS. Low magnification of adult thoracic spinal cord from (*C*) control and (*D*) paralyzed transgenic mice (×55); purple PTAH stain suggests development of striated muscle in transgenic cord (arrowhead). Antibody to α-actin positively identifies muscle in adult (*E*) cord (red chromagen, ×225) and (*F*) brain (brown chromagen, ×200). (*G*) PTAH-positive mature striated muscle (arrow) along adult transgenic ventral root (×630). (*H*) Immature muscle cells (arrow) in 4.5-day-old hematoxylin and eosin-stained and (*I*) small muscle fibers (arrow) in 8-day-old PTAH-stained male MH19 spinal cords (×630). (*J*) Electron micrograph of ectopic muscle around cord of paralyzed adult MH19 male (×3600).

the upper spinal cord and cerebellum. At 4 days of age, a smaller number of newly differentiated melanocytes was detected only on the dorsal midline of the thoracic cord (Fig. 3*E*), corresponding to the region from which trunk neural crest-derived melanocytes arise (18, 19). Clearly, local constitutive SF/HGF overexpression greatly stimulated the proliferation

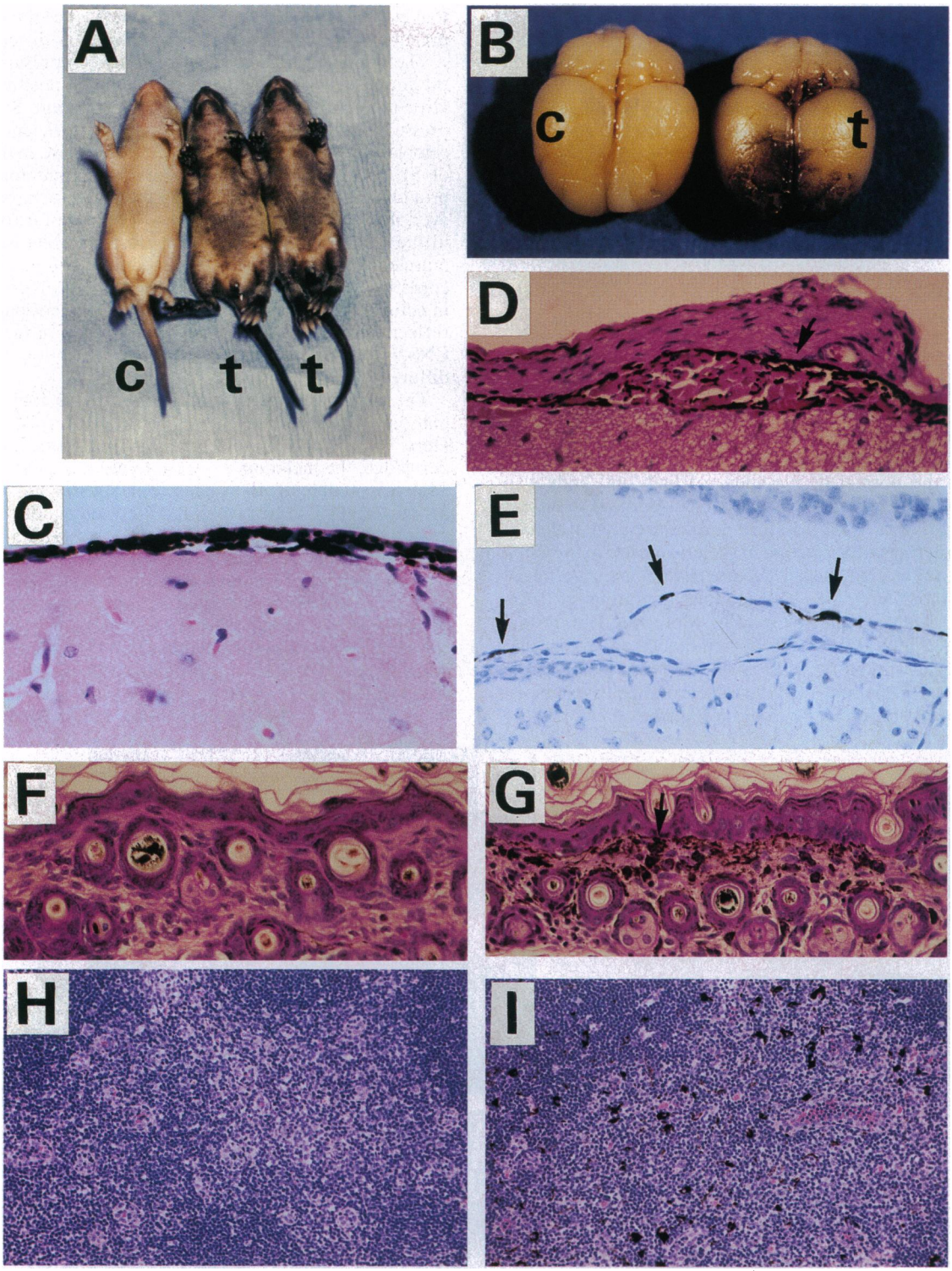

FIG. 3. Aberrant development of melanocytes in SF/HGF transgenic mice. (*A*) Hyperpigmentation in skin of extremities and abdomen in anesthetized 10-day-old (t) MH19 × C57BL/6 F_1 transgenic and (c) FVB/N × C57BL/6 F_1 control mice. Note black spots on abdomen. (*B*) Brains from 5-week-old F_1 (c) control and (t) transgenic mice (magnification ×3.6). (*C*) Microscopic detection of black pigment cells on brain of 4-week-old F_1 transgenic female (×700). (*D*) Simultaneous ectopic development of muscle and pigment cells (arrow) on lateral surface of thoracic spinal cord from 4-week-old F_1 transgenic female (×400). (*E*) Detection of differentiated melanocytes (arrows) exclusively at dorsal midline of cord from 4-day-old F_1 transgenic male (×450). (*F* and *G*) Enhanced melanocyte development in epidermis and dermis of dorsal skin from 8-day-old F_1 (*G*, arrow) transgenic relative to (*F*) control females (×400). (*H* and *I*) Lymph nodes of inguinal mammary gland from 5-week-old F_1 (*H*) control and (*I*) transgenic males (×225). Pigment cells are never found in the brain, cord, or lymph nodes of control F_1 mice.

and/or differentiation of melanocytes in the adult CNS; however, this does not adequately explain the presence of melanocytes in the spinal cords of transgenic neonates.

At emigration many neural crest cells are multipotent, but become sequentially restricted in developmental potency en route through interations with environmental factors (20–23). One of the earliest markers for commitment to the melanocyte lineage is TRP-2, which identifies melanoblasts after emigration from the neural tube (24, 25). We used *in situ* hybridization with a TRP-2 riboprobe to visualize melanoblast migra-

tion in transgenic embryos. In control E12.5 embryos TRP-2 positive cells were observed migrating away from, but not dorsal to, the neural tube (Fig. 4 *C* and *E*), consistent with a previous report (25). In contrast, transgenic TRP-2 positive cells were inappropriately detected near their site of origin in the dorsal marginal area and roof plate of SF/HGF-expressing neural tubes (Fig. 4 *B* and *D*), in presumptive muscle and by dorsal root ganglia, suggesting that migration of committed melanoblasts had been perturbed.

DISCUSSION

Skeletal muscle development is a multistep process involving commitment of somitic mesodermal precursor cells to two myogenic lineages (26–28). Axial muscle is derived from myotomal cells, whereas Met-expressing ventrolateral cells of the dermamyotome migrate at midgestation (E10–E10.5) and differentiate into limb bud and body wall muscle (4, 29, 30). Recently, Bladt *et al.* (7) demonstrated that in mice carrying *met* null mutations myogenic precursor cells fail to migrate, preventing the normal development of limb and body wall muscle, whereas axial muscle is unaffected. Here we show that ectopic expression of SF/HGF in the adjacent neural tube induces inappropriate formation of skeletal muscle in the CNS, supporting the notion that in normal development SF/HGF plays an important role in regulating migration of Met-containing myogenic precursor cells. Moreover, our results suggest that SF/HGF serves as a chemotactic agent to guide migratory Met-containing cells to the neural tube in transgenic embryos, and to the limbs (7) during normal embryogenesis. Although less compelling, we cannot yet dismiss the possibility that ectopic SF/HGF expression can influence the behavior of precursor populations by perturbing cellular differentiation.

The developmental relationship between presumptive neural and muscle tissue is complex, involving positive and negative regulatory factors (31–33). Inappropriate SF/HGF expression is necessary, but may not be sufficient to induce ectopic muscle development. The fetal CNS may, in the presence of SF/HGF, be a preferred site for muscle formation by providing other factors necessary for myoblast survival. However, it is noteworthy that the development of ectopic muscle in the CNS was also significantly retarded relative to native muscle. Muscle development in neonatal and juvenile MH19 cords was limited to immature cells and small fibers. The delay in ectopic muscle development and subsequent paralysis may reflect the absence of optimal differentiation factors in the CNS, or the presence of a neural factor inhibitory to myoblast differentiation.

The vertebrate neural crest is another migratory cell population, able to give rise to a wide variety of derivatives (18, 19). Here we show that cells from at least one neural crest derivative, the melanocytes, were found within the transgenic adult in a number of abnormal ectopic sites, including the CNS. Moreover, TRP-2-positive committed melanoblasts were aberrantly localized to inappropriate sites within the E12.5 transgenic embryo, including the neural tube. Our data suggest that, as in muscle development, distinct subsets of neural crest cells become Met-positive just before or at emigration, rendering them responsive to SF/HGF. It is known that SF/HGF can stimulate melanin production and movement in cultured melanocytes (34). We propose that along with c-Kit, the receptor for Steel, Met is required for and helps define determination in the melanocyte lineage of the neural crest

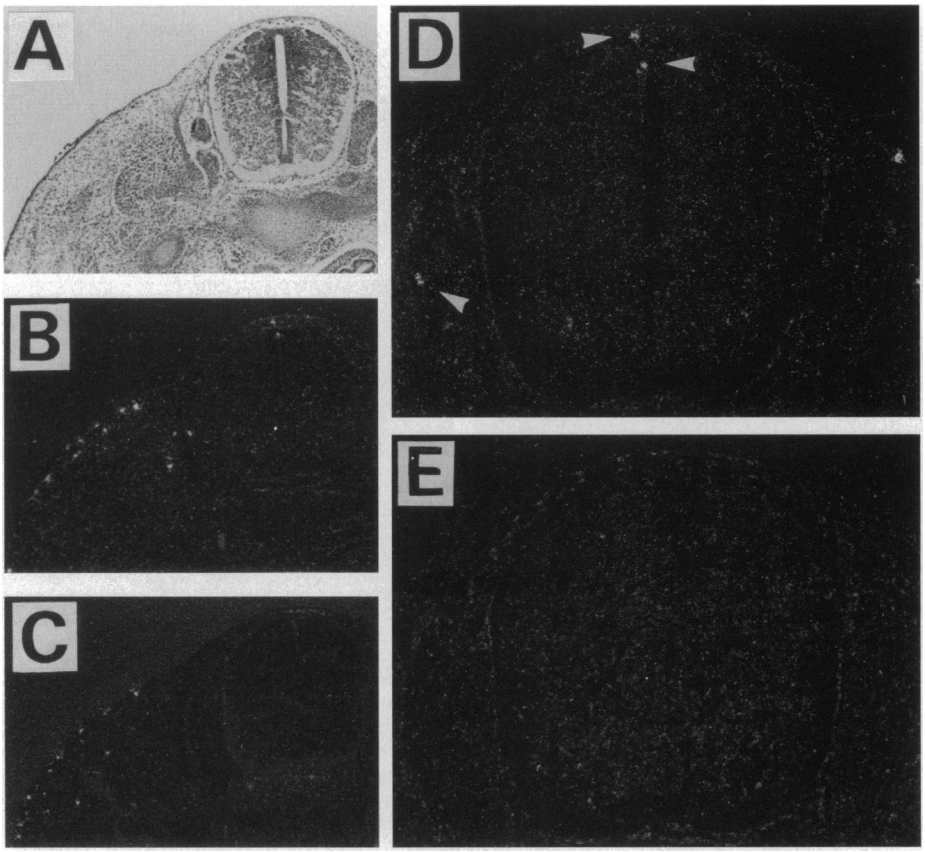

FIG. 4. Aberrant localization of TRP-2 positive melanoblasts in SF/HGF transgenic midgestation embryos. TRP-2 transcripts were identified in melanoblasts by *in situ* hybridization. Dorsal region of transverse sections through forelimb of (*A*, *B*, and *D*) SF/HGF transgenic and (*C* and *E*) control E12.5 embryos. (*A–C*) Magnification (×50) showing dorsolateral pathway through which melanoblasts migrate. (*D* and *E*) Magnification (×100) of neural tube region. Note aberrant localization of melanoblasts in transgenic neural tube and near dorsal root ganglion (*D*, arrowheads). Transgenic E13.5 embryos had the same general phenotype (data not shown). (*A*) Brightfield. (*B–E*) Darkfield.

during normal embryogenesis. While the Steel factor is needed mostly for survival of predetermined cells (24, 35), SF/HGF is likely to influence migration of Met-expressing melanoblasts. This is supported not only by aberrant migration of melanoblasts in transgenic embryos, but by inappropriate localization of melanocytes in lymph nodes and throughout the epidermis and dermis of the skin, which overexpresses SF/HGF in both transgenic fetus and adult.

The influence of SF/HGF on the developing neural crest may not be limited to the melanocyte lineage. SF/HGF transgenic mice have an unusually high incidence of gastrointestinal obstruction, which may be caused by aberrant development of enteric ganglia, another neural crest derivative, as has been found in a variety of spontaneous and gene-targeted mouse mutants and in Hirschprung disease in humans (36–38). In addition, we have noted that SF/HGF transgenic mice also demonstrate abnormal development of the olfactory mucosa and the mammary gland; a variety of tumors then develop at a relatively low incidence in these and other transgenic tissue types (unpublished results).

Our results have important implications concerning mechanisms regulating cellular migration. The *in vitro* scatter activity of SF/HGF, in which epithelial sheets dissociate into mobile spindle shaped cells, appears analogous to the *in vivo* process whereby epithelial cells acquire mesenchymal properties and migrate. The ectopic imposition in the transgenic CNS of skeletal muscle and melanocytes, both requiring cellular epithelial/mesenchymal conversion and relocation for normal development, strongly suggests that select Met-containing myogenic and melanocytic precursor cells require SF/HGF for appropriate migration during embryogenesis. Evidence is provided for the first time implicating SF/HGF as an important regulator of neural crest lineage determination and diversification. The MT-SF/HGF transgenic mouse represents an ideal model for analysis of morphogenetic events associated with neural crest and muscle development, and for dissection of mechanisms underlying regulation of cell migration and differentiation during mammalian embryogenesis.

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