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Ectopic c-kit Expression Affects the Fate of Melanocyte Precursors in *Patch* Mutant Embryos

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The *Patch* (*Ph*) mutation in the mouse, a deletion that includes the gene for PDGFRα, is a recessive lethal that exhibits a dominant pigment phenotype in heterozygotes. To assess whether the *Ph* mutation acts cell-autonomously or non-autonomously on melanocyte development, we have examined the melanogenic potential of neural crest populations from normal and mutant crest cells *in vitro* and the pattern of dispersal and survival of melanocyte precursors (MPs) *in vivo*. We report that trunk neural crest cells from homozygous *Ph* embryos give rise to pigmented melanocytes *in vitro* in response to Steel factor (SlF). *In vivo*, homozygous *Ph* embryos contain a subpopulation of crest-derived cells that express c-kit and tyrosinase-related protein-2 characteristic of MPs. These cells begin to migrate normally on the lateral crest migration pathway, but then fail to disperse in the dermal mesenchyme and subsequently disappear. Although dermal mesenchyme is adversely affected in *Ph* homozygotes, SlF mRNA expression by the cells of the dermatome is normal in *Ph* embryos when neural crest-derived MPs start to migrate on the lateral pathway. In contrast, mRNA for the SlF receptor, c-kit, was observed to be ectopically expressed in somites and lateral mesenchyme in embryos carrying the *Ph* mutation. Based on this ectopic expression of c-kit in *Ph* mutant embryos, and the observed distribution of SlF protein in normal and mutant embryos, we suggest that competition for limited amounts of SlF localized on the lateral neural crest migration pathway alters melanocyte dispersal and survival.

INTRODUCTION

Several mouse pigment pattern mutants have been described and their defects analyzed at the molecular level. Two of the best characterized are the *Dominant spotting* (*W*) and the *Steel* (*Sl*) mutations, which encode the c-kit receptor tyrosine kinase (Geissler *et al.*, 1988) and its ligand, Steel factor (SlF, also known as stem cell factor, mast cell growth factor, or kit ligand), respectively. Both mutations affect development of pigment cells as well as hematopoietic and germ cells (Anderson *et al.*, 1990; Copeland *et al.*, 1990; Huang *et al.*, 1990; Williams *et al.*, 1990).

Another pigment pattern mutant, *Patch* (*Ph*), is a recessive lethal that shows a dominant pigment phenotype in heterozygotes. Homozygous *Ph* embryos die between e8-9 and e16-17 depending on the genetic background (Morrison-Graham *et al.*, 1992; Orr-Urtreger *et al.*, 1992). In *Ph* homozygotes, characteristic defects in mesenchymal tissues are accompanied by cleft palate, epidermal blisters, kinky neural tube, and failure of heart septation (Grüneberg and Truslove, 1960; Erickson and Weston, 1983; Morrison-Graham *et al.*, 1992; Orr-Urtreger *et al.*, 1992; Schatteman *et al.*, 1992). The *Ph* locus is located on chromosome five in the mouse, adjacent to the *W* locus that encodes c-kit, and has been shown to be a deletion of about 200 kb spanning the entire coding region of PDGFRα (Smith *et al.*, 1991; Stephenson *et al.*, 1991, 1994; Nagle *et al.*, 1994). Although much is known about the role of the c-kit gene for melanocyte development and survival (Nishikawa *et al.*, 1991; Steel *et al.*, 1992; Morrison-Graham and Weston, 1993; Wehrle-Haller and Weston, 1995; Reid *et al.*, 1995), it is not yet known how the lack of the PDGFRα gene results in the pigment pattern phenotype seen in *Patch* heterozygotes.

Since PDGFRα transcripts are normally expressed by cranial ectomesenchyme in branchial arches and the cardiac outflow tract, but not by neurons of the central nervous system or the crest-derived peripheral nervous system (Morrison-Graham *et al.*, 1992; Schatteman *et al.*, 1992), the defects in *Ph* homozygous embryos, like cleft palate and the absence of conotruncal septation, may be directly caused by the failure of nonneurogenic neural crest-derived cells to survive or proliferate in these locations. Similarly, since a

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subset of nonneurogenic crest-derived cells normally undergoes melanogenesis, the pigment phenotype observed in *Patch* heterozygotes might result cell-autonomously from a lack of PDGFRα expression in mutant melanocyte precursors (MPs). However, since melanocytes disperse and proliferate in the dermal mesenchyme (Wehrle-Haller and Weston, 1995), which is known to require PDGFRα activity (Morrison-Graham *et al.*, 1992; Schatteman *et al.*, 1992), the *Patch* mutation might alter the pigment pattern noncell-autonomously by adversely affecting the environment in which melanocytes develop.

Thus, various alternative hypotheses can explain the pigment phenotype in *Patch* mutants. First, as is the case for c-kit, PDGFRα might act cell-autonomously to promote MP survival and development. Second, loss of PDGFRα activity might indirectly affect the production or localization of SlF or other growth factors (Murphy *et al.*, 1994) required for MP migration, proliferation, and/or survival. Alternatively, the genomic deletion in *Patch* mentioned above might disrupt adjacent genes, such as c-kit (Nagle *et al.*, 1994; Duttlinger *et al.*, 1995; Bucan *et al.*, 1995), directly or indirectly causing the pigment phenotype.

In order to distinguish among these possibilities, we used a variety of techniques to examine melanogenesis *in vivo* and *in vitro*. We show that melanocyte precursors from *Patch* homozygotes can survive, express c-kit, and undergo melanogenesis in a supportive environment *in vitro*. We further demonstrate that the initial appearance of melanocyte precursors *in vivo* is normal, but their ultimate distribution and survival is altered in *Ph* mutant embryos. These results are not consistent with the hypothesis that PDGFRα acts cell-autonomously on crest-derived melanocyte precursors. We also show that expression of SlF mRNA and protein by the dermatome is not perturbed in *Patch* homozygotes. Finally, we show that c-kit is ectopically expressed in *Patch* embryos, which, taken together with our observations on the pattern of distribution of SlF protein in these embryos, suggests that melanocyte precursors might compete with mesenchyme cells that ectopically express c-kit for a limiting amount of ligand. We suggest that such a competition for SlF might explain the observed *Patch* pigment phenotype.

MATERIALS AND METHODS

Embryos

The *Patch* mutation has been carried on a C57Bl6 inbred background for many generations. In order to promote fecundity of the highly inbred strain we backcrossed it once with our C57Bl6 line and subsequently maintained it by brother sister mating. The *Patch* mutation within this background does not yield homozygous embryos older than e11.5. In order to obtain *Ph*/*Ph* homozygous embryos with a cleft palate phenotype (viable embryos older than e11.5), we mated F1 animals from a *Ph* $\times$ Balb/C cross (Morrison-Graham *et al.*, 1992). All embryos used for *in situ* hybridization and antibody staining were derived from such matings. E9.5 homozygous *Ph* embryos were identified by their kinky neural tube and occasional blisters in the trunk. Alternatively, a 500-bp band could be amplified as previously described (Wehrle-Haller and Weston, 1995) by PCR from genomic DNA of wildtype and heterozygous littermates, but not from homozygous embryos, using a forward primer ACCTCCTTTCGGACGATGAC from the interkinase domain of PDGFRα (bases 2417–2436; Wang *et al.*, 1990, Accession No. M57683) and a reverse primer from within the flanking intron ATCACTTCAGAATGGCTCCA (Dr. Peter Lonai; personal communication). A control PCR band, obtained in the same reaction with primers specific for the *Sl* gene (forward CCATGGCATTGCCGGCTCTC; bases 665–684; and reverse CTGCCCTTGTAAGACTTGACTG; complement of bases 757–736; Huang *et al.*, 1990; Steel *et al.*, 1992), verified the quality of the DNA (Wehrle-Haller and Weston, 1995). Homozygous embryos identified by PCR also exhibit kinky neural tube, blisters, and cleft palate when e11.5 or older. Heterozygous embryos were identified either by their melanocyte phenotype or by PCR analysis of genomic DNA obtained from a limb bud or tail by digestion in 50 μl of 10 m*M* Tris, pH 8, 2 m*M* EDTA, 0.2% Triton X-100, 200 mg/ml proteinase K for 3 hr at 55°C. Samples were boiled for 5 min and 10 μl was amplified with PCR using primer pairs for the *D5MIT135* locus (Research Genetics). This primer pair maps just outside of the *Ph* deletion and produces a 241-bp band from the *Ph* chromosome which originated in a C57Bl6 background and a 217-bp band from the Balb/C-derived chromosome (mouse genome database (MGD); Whitehead Institute/MIT Center for Genome Research (WI/MIT CGR); Dietrich *et al.*, 1994; Copeland *et al.*, 1993).

Neural Tube Cultures

C57Bl6 embryos were used to determine SlF-dependent survival of c-kit expressing neural crest cells. To determine the number of c-kit expressing cells in *Ph* neural tube cultures, we used homozygous *Patch* and heterozygous or wildtype littermates as controls from a F1 *Ph* $\times$ Balb/C mating (see above). All *Patch* crest cell cultures used to assess the ability to form pigment were derived from embryos produced by *Ph* $\times$ C57Bl6 mating. e9.5 embryos were dissected and cultured as described (Morrison-Graham and Weston, 1993). Recombinant SlF (obtained from Dr. D. Williams) was added at 100 ng/ml when indicated. c-kit expressing cells (putative melanocytes) were visualized as follows: Live cells were exposed to rat monoclonal antibody against mouse c-kit (ACK-2 conditioned medium; generously supplied by Dr. S. Nishikawa) for 45 min at 37°C, washed, and fixed in 4% paraformaldehyde. After blocking with 5% goat serum, biotinylated goat anti-rat antibody followed by avidin–Texas red was used to detect bound ACK-2 antibody. Neuronal cells were identified by staining with an anti-Hu monoclonal antibody (Marusich and Weston, 1992) as described (Morrison-Graham and Weston, 1993). In order to visualize melanocytes in culture prior to overt pigmentation, we fixed and stained cultures with D,L-DOPA (Sigma) as described (Morrison-Graham and Weston 1993).

RNA Probes and Whole Mount in Situ Hybridization

Digoxigenin-labeled riboprobes were synthesized according to standard protocols (Boehringer-Mannheim) from linearized cDNAs coding for mouse SlF (KL-M1; kindly provided by Dr. John Flanagan, Boston); mouse c-kit (bp 745–2380; obtained from Dr. Robert J.

Arceci, Boston), mouse PDGFRα (bp 952–1837; Wang *et al.,* 1990; obtained from Dr. Dan Bowen-Pope, Seattle) and mouse tyrosinase-related protein-2 (TRP-2; obtained from Dr. Ian Jackson, London, UK). Whole mount *in situ* hybridization was performed according to Conlon and Rossant (1992) with the following minor modifications: Proteinase K treatment was prolonged for older embryos, hybridization was performed at 68°C, and some embryos were developed in the presence of 5% polyvinyl alcohol (low molecular weight, Aldrich) to enhance color development. Detailed protocols will be provided upon request. Color photographs were taken on a Wild stereo microscope, scanned, and converted to grayscale in Photoshop 3.0 (Adobe). In all cases figures shown were representative of the typical appearance of at least three embryos per stage, genotype, and riboprobe.

Antibody Production and Immunostaining

Affinity-purified mouse SlF antiserum was prepared from rabbit immunized against a fusion protein of SlF with bacterial glutathione S-transferase (GST-SlF). Mouse SlF DNA was PCR amplified from KL-M1 plasmid (kindly provided by Dr. J. Flanagan; Flanagan *et al.,* 1991) using primers TATGGATCCAAGGAGATCTGCGGG and TATGGATCCTTATGCAACAGGGGGTAACAT. The obtained fragment was cleaved and inserted at the *Bam*HI site of pGEX-2T expression vector (Pharmacia). GST-SlF present in inclusion bodies was denatured with 8 *M* urea and 10 m*M* DTT, renatured in 50 m*M* Tris, pH 8, and 0.5 *M* NaCl, and purified with glutathione Sepharose 4B (Pharmacia). In order to enhance the specificity of the GST-SlF antiserum we affinity purified SlF-specific antibodies with double-tagged recombinant SlF lacking GST sequences. Two complementary oligonucleotides (GTCCCGAGCAGAAGCTTATCTCC-GAGGAGGACCTCG and GACCGAGGTCCTCCTCGGAGATAAGCTTCTGCTCGG) coding for an epitope recognized by the c-myc antibody (9E-10; Evans *et al.,* 1985) were annealed and cloned into the *Ppu*MI site in KL-M1 (Flanagan *et al.,* 1991) located in the extracellular domain of SlF (KL-M1/myc). From KL-M1/myc a PCR fragment was amplified with the same primers used to construct the GST-SlF fusion protein and cloned into pTrcHisA (Invitrogen) at the *Bam*HI site, which resulted in the addition of six N-terminal histidines and a linker to the myc-tagged SlF. His/myc-tagged SlF was affinity purified from bacterial lysates with Ni^{2+}-agarose (Quiagen) and coupled to Cn-activated Sepharose 4B (Pharmacia). Anti-SlF-specific antibodies were affinity purified from GST-SlF antiserum and eluted with 0.1 *M* glycine, pH 2.5, and immediately neutralized with 1 *M* Tris, pH 8.0. Specificity of the affinity-purified antiserum was tested on Western blots with crude bacterial extracts from induced bacteria transformed with His/myc-tagged SlF in pTrcHisA.

For whole mount immunostaining, embryos were fixed for 2–5 hr in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C, washed in PBS, and transferred and stored in methanol at −20°C. Embryos were rehydrated in Tris-buffered saline, 0.1% Tween 20 (TBST), heat inactivated for 30 min at 70°C, and then blocked in 10% heat-inactivated normal goat serum (hiNGS) in TBST, 0.5% Triton X-100 (TBSTT). Embryos were incubated in 1:200 affinity-purified anti-SlF antiserum in 5% hiNGS in TBSTT at 4°C overnight. Primary antibody was absorbed with embryo acetone powder from e15.5 *Sl*/*Sl* embryos, which lack SlF protein. Embryos were extensively washed in TBSTT and incubated at 4°C in affinity-purified goat anti-rabbit alkaline phosphatase (Bio-Rad) diluted 1:2000 in 5% hiNGS in TBSTT, previously absorbed with e13.5 mouse acetone powder. After washing in TBSTT embryos were equilibrated in 100 m*M* Tris, pH 9.5, 25 m*M* $MgCl_2$, 100 m*M* NaCl, 0.1% Tween 20, 0.5% Triton X-100, and 5% polyvinyl alcohol. Embryos were stained in the same buffer in the presence of NBT and BCIP, washed in TBST, dehydrated through methanol, rehydrated in TBST, and equilibrated in 30% sucrose in PBS or 80% glycerol in PBS. Embryos in sucrose were embedded in Tissue-Tek OCT (Miles) and cryosectioned at 16 μm. In order to enhance morphological structures, sections were counterstained with 1:200 rabbit anti-laminin serum (Collaborative Research) and 1:200 Texas red-conjugated goat anti-rabbit antiserum as described (Wehrle-Haller and Weston, 1995). Color photographs of whole mounts and sections were taken on a Wild stereo microscope and Zeiss Axioplan, respectively. Photographic transparencies were scanned and converted to grayscale in Photoshop 3.0 (Adobe). Contrast and brightness was adjusted to improve presentation of visual appearance of stained tissue. All changes were made identically in wild-type and mutant material so that no artifactual differences were introduced.

RESULTS

Cultures of Crest Cells from Ph Homozygotes Contain c-kit Expressing Cells, Which Undergo Melanogenesis in the Presence of SlF

The melanogenic subpopulation of neural crest cells transiently depends on SlF for its survival *in vivo* and *in vitro* (Morrison-Graham and Weston, 1993) and hence must express functional c-kit. To confirm that c-kit staining specifically marks melanocytes *in vitro,* we correlated c-kit antibody staining with SlF-dependent survival in cultures of normal mouse neural crest cells. Cells that express c-kit are first detected in a subpopulation of crest-derived cells by Day 2 of culture. Initially, neither the intensity of the staining nor the number of positive cells are affected by the addition of SlF. However, the persistence of c-kit expressing cells depended upon SlF (Fig. 1). In cultures containing exogenous SlF (100 ng/ml), the numbers of c-kit-positive cells increased over the next few days (Days 2–5). By Day 6, both c-kit expression (ACK-2 staining) and melanin granules could be detected in melanocytes. In contrast, following the initial appearance of ACK-2-immunoreactive cells at Day 2, the numbers of c-kit-positive cells in cultures deprived of SlF (for example, in the presence of antibody to SlF in the culture medium) decreased progressively between Days 2 and 5, and by Day 6, no positive cells were detectable (Fig. 1). Since no neurogenic (anti-Hu-immunoreactive) neural crest-derived cell ever expressed c-kit immunoreactivity (c-kit-IR) (Figs. 2A and 2B), we conclude that the c-kit expression reliably marks melanocyte precursors in our cultures and that the loss of c-kit immunoreactive cells in the absence of SlF represents a loss of melanocyte precursors (see Morrison-Graham and Weston, 1993; Reid *et al.,* 1995).

Using c-kit-IR as a marker for melanocytes and their precursors, we then cultured *Ph*/*Ph* neural tubes in the presence of exogenous SlF and examined them for c-kit expres-

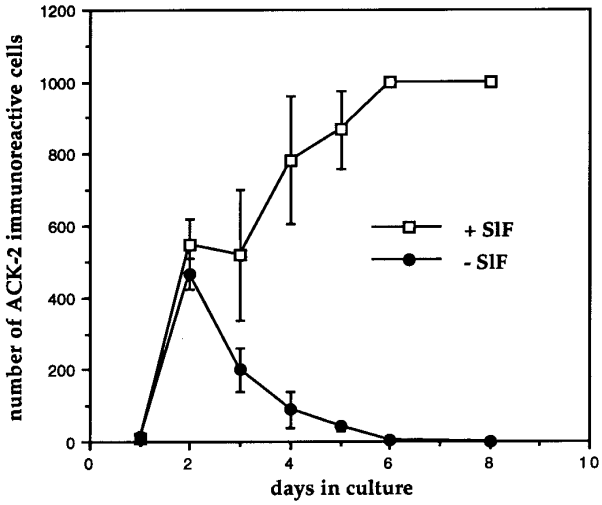

FIG. 1. The number of c-kit expressing cells decreases in the absence of SlF in mouse neural crest cultures. The number of ACK-2-immunoreactive cells (c-kit expressing cells) per neural tube culture was plotted against the time in culture in the presence of SlF (100 ng/ml) (squares) and in the absence of SlF (anti-SlF antibody) (filled circles). The standard deviation is given for Days 1 and 2 (n = 2); for all other data points the standard error is given (n = 3). In the presence of SlF at Days 6 and 8 the number of ACK-2-IR cells exceeded 1000 per culture and has not been determined accurately.

sion after 4–5 days. Although crest cell outgrowth from explanted neural tubes of *Ph* homozygotes was smaller than outgrowth from neural tubes of wildtype or heterozygous littermates, c-kit expressing cells were observed in all cultures. (*Ph*/*Ph:* 279 ± 192; n = 3; +/+ or heterozygotes: 754 ± 204; n = 3). Thus, the *Patch* mutation does not prevent expression of c-kit by a subset of crest-derived cells (presumed melanocyte precursors). To determine if such cells could undergo melanogenesis, some cultures were left for a 10-day culture period. Pigmentation in such cultures was diminished, but not absent. Pigmented cells were observed in approximately half of the cultures (5/12), suggesting that PDGFRα is not required for the initial differentiation of melanocytes.

The Patch Mutation Alters Dispersal of MPs in the Dermis, but Not Their Initial Migration on the Lateral Pathway

To follow the initial migration and subsequent dispersal of MPs *in vivo,* we used whole mount *in situ* hybridization to detect melanocyte-specific mRNA for TRP-2 (Steel *et al.*, 1992; Wehrle-Haller and Weston, 1995) in e11.5–e13.5 wildtype, *Ph*/+, and *Ph*/*Ph* embryos. At e11.5, MPs have already reached lateral positions in the head and anterior trunk but have just begun to migrate onto the lateral crest migration pathway in the posterior trunk. In the posterior trunk, TRP-2 mRNA expressing cells are initially distributed within the dorsal aspect of the lateral crest migration pathway extending from the migration staging area (MSA: Wehrle-Haller and Weston, 1995) to the dorsal aspect of the dermatome. In contrast to wildtype, MPs of *Ph*/*Ph* embryos did not reach the base of the hind limb bud (Fig. 3A; compare with Figs. 2A and 2E in Wehrle-Haller *et al.*, 1995). At developmentally more advanced (more rostral) axial levels, TRP-2 mRNA-positive cells were clustered in a segmental pattern dorsolaterally in mutant embryos (Fig. 3B, arrow), compared to the more uniform distribution of cells observed in wildtype embryos (Fig. 3C).

The patterns of MP distribution were qualitatively simi-

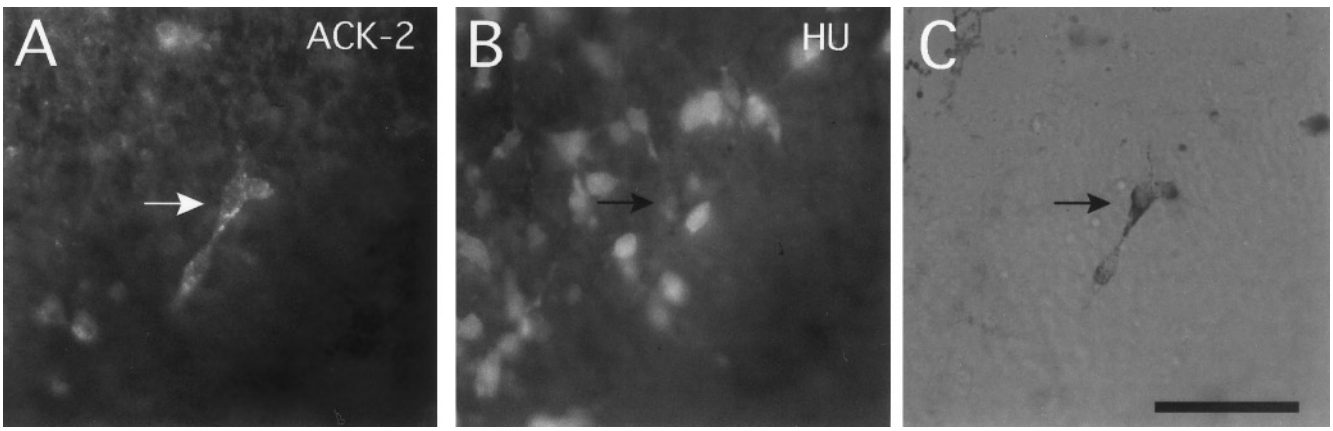

FIG. 2. Melanocyte precursors (c-kit$^+$) and neurogenic cells (Hu$^+$) represent distinct neural crest subpopulations. Neural tube explants cultured in the presence of SlF (100 ng/ml) were cultured for 8 days and stained with ACK-2 rat monoclonal antibody (A) and with anti-Hu mouse monoclonal antibody (B) as described under Materials and Methods. (C) Bright-field photo reveals pigmented melanocytes. Arrows point to the same ACK-2-reactive, Hu-negative, and pigmented melanocyte. Bar, 66 μm.

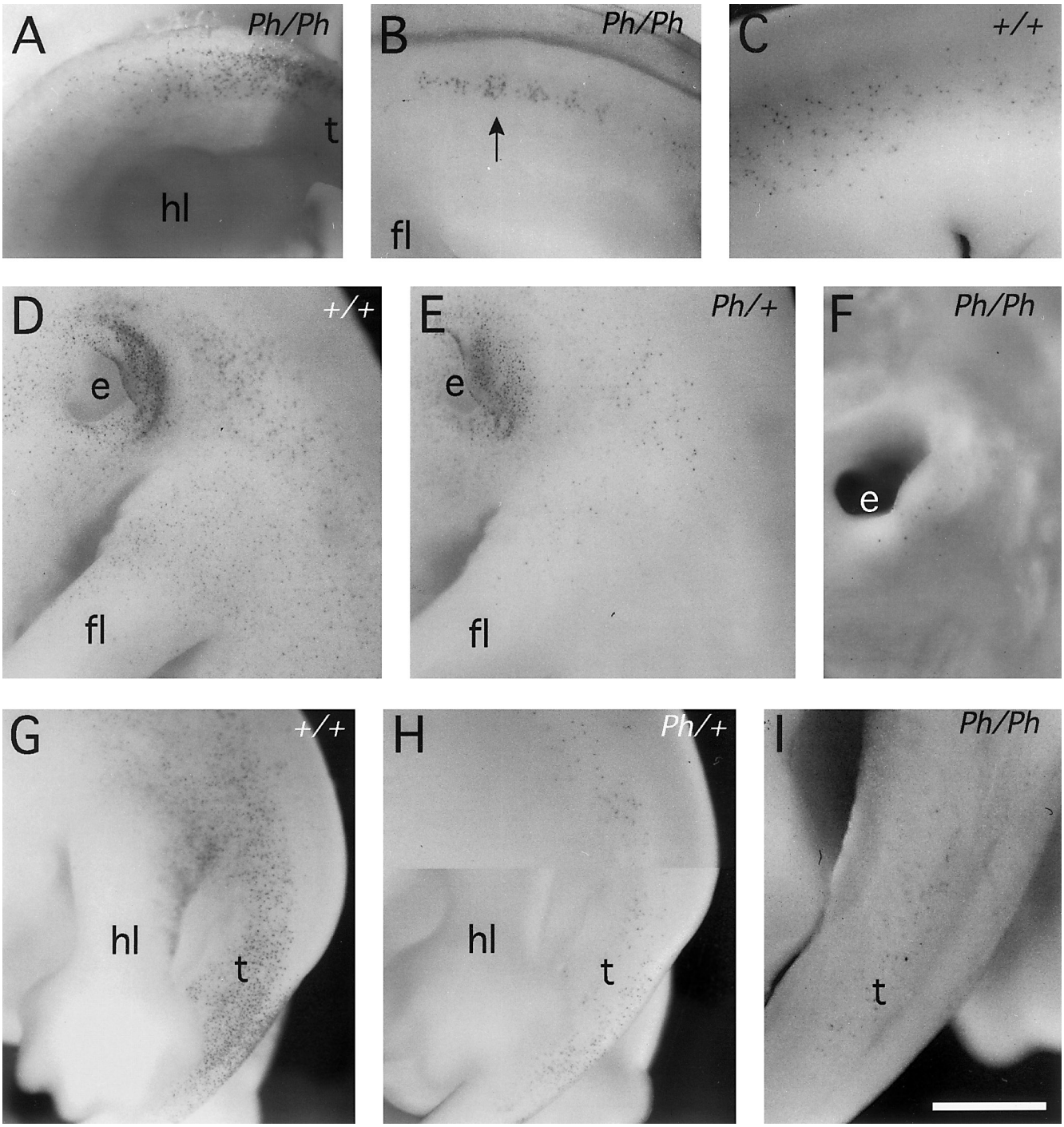

FIG. 3. Melanocyte phenotype of wildtype and Ph mutant embryos. TRP-2 mRNA *in situ* hybridization of e11.5 (A–C) and e13.5 (D–I) +/+ and *Ph* mutant embryos. (A) Lateral view of hind limb bud and tail of a *Ph*/*Ph* embryo. (B) Lateral view of the mid-trunk of a *Ph*/*Ph* embryo. (C) Lateral view of the mid-trunk region of a +/+ embryo. (D, E) Lateral view of the shoulder and ear of +/+ and *Ph*/+ embryos, respectively. (F) Magnified view of the ear from a *Ph*/*Ph* embryo. (G, H) Hind limb bud and tail region of a +/+ and *Ph*/+ embryo respectively. (I) Magnified view of the tail of a *Ph*/*Ph* embryo. Arrow points to clusters of MPs in e11.5 *Ph*/*Ph* embryos. Note the presence of only a few MPs in the ear (F) and tail (I) of e13.5 *Ph*/*Ph* embryos. In H, two pictures taken at different focal levels of the tail region are combined into a montage. e, ear; fl, fore limb bud; hl, hind limb bud; t, tail. Each photograph is representative of the reproducible staining pattern seen in multiple embryos, as follows: A, B, $n = 3$; C, $n = 6$; D, $n = 3$; E, F, $n = 4$; G, $n = 3$; H, I, $n = 4$. Bar corresponds to 0.6 mm in A, B, C, F; to 0.93 mm in D, E; to 1.08 mm in G, H; and to 0.46 mm in I.

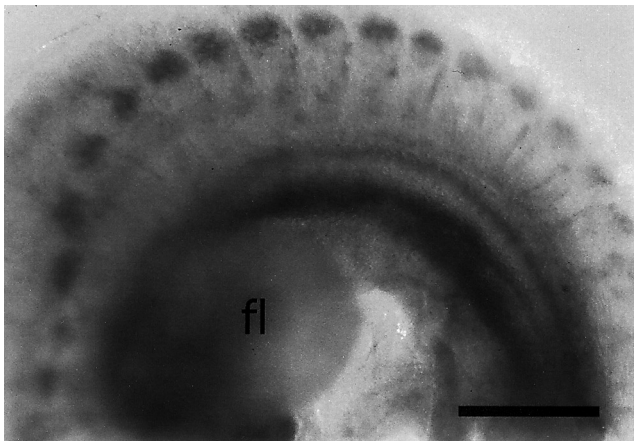

FIG. 4. SlF mRNA expression in *Ph*/*Ph* e10.5 embryo. Lateral view of the trunk of a whole mount *in situ* hybridization with the antisense SlF riboprobe. The photograph represents the reproducible staining pattern seen in three different mutant embryos. fl, fore limb bud. Bar, 360 μm.

lar in e12.5 and e13.5 embryos. Generally, the number of MPs was reduced in heterozygotes and was almost absent from homozygotes compared to wildtype (Figs. 3D–3I). In the trunk of heterozygotes, MPs were present at the shoulder, absent in the mid-trunk region, reduced at the base of the hind limb, and present in the tail (Fig. 3H; compare to wildtype, Fig. 3G). In *Patch* homozygotes, a few cells could be found at shoulder level at e12.5, but were no longer present at e13.5. MPs were not detected in the trunk of e12.5 and e13.5 embryos, but remain in small numbers in the ear (Fig. 3F) and in dorsolateral locations in the tail (Fig. 3I).

SlF mRNA Expression Pattern Is Not Affected in Ph/Ph Mutant Embryos

Melanocytes start to disappear in *Ph* embryos when SlF is known to be required for survival of melanocytes *in vivo* (Nishikawa *et al.*, 1991; Wehrle-Haller and Weston, 1995) and *in vitro* (Morrison-Graham and Weston, 1993). To determine whether expression patterns of SlF by somite-derived mesenchymal cells was affected in mutant embryos, we used whole mount *in situ* hybridization to examine the distribution of SlF mRNA in *Ph*/*Ph* embryos. At e10.5, when SlF mRNA is known to be expressed at sites which are later populated by melanocytes in wildtypes (Wehrle-Haller and Weston, 1995), the expression pattern of SlF mRNA in *Ph*/*Ph* was comparable to those of wildtype or heterozygous littermates (Fig. 4; compare with Figs. 5A and 5B in Wehrle-Haller and Weston, 1995).

SlF Protein, Initially Localized to the Epithelial Dermatome, Is Subsequently Found at Low Levels in the Dermal Mesenchyme

In order to test for possible changes in SlF protein distribution in *Patch* mutant tissue, we used immunohistochemistry to determine SlF protein distribution along the lateral crest migration pathway of mutant and wildtype embryos. At e10.5 (35–36 somites), SlF protein pattern in both mutant and wildtype embryos was similar to the localization of its mRNA. In the trunk, SlF protein was detected in the dorsal aspect of the epithelial dermatome (Figs. 5A and 5F).

At e11 (39–40 somites), in wildtype and *Patch* embryos, SlF protein is expressed in dorsal aspects of the dermatome at hind limb levels (not shown), whereas at more anterior axial levels, SlF immunoreactivity (SlF-IR) associated with the epithelial dermatome decreases as the epithelial dermatome transforms into mesenchymal dermis (Figs. 5B and 5G). When these whole mounts were sectioned and SlF-IR was compared between wildtype and *Ph*/*Ph* embryos, weak SlF-IR was consistently detected in the newly transformed dermal mesenchyme of wildtype embryos (Figs. 5C and 5E), whereas under identical staining conditions, SlF-IR could not be detected in corresponding dermal mesenchyme of *Patch* homozygotes (Figs. 5H and 5J).

c-kit mRNA Expression Is Altered in Ph/Ph and Ph/+ Embryos Compared to Wildtype

In the course of studying c-kit expression by MPs *in vivo*, we observed altered c-kit mRNA expression patterns in *Ph* mutant embryos. Specifically, at e10.5 in *Ph* mutant embryos, c-kit mRNA expression can be detected in MPs in the head similar to that of wildtype embryos (Steel *et al.*, 1992; Wehrle-Haller and Weston, 1995). However, in mutant embryos, c-kit mRNA expression was strongly enhanced in the dorsal aspect of the spinal cord compared to wildtype embryos (Figs. 6A and 6B). Likewise, mesenchymal c-kit expression in the first and second branchial arches (not shown), the lateral mesenchyme, and the limb buds was dramatically enhanced in *Ph* mutant embryos (compare Fig. 6A with 6B). In addition, in mutant embryos c-kit mRNA was expressed ectopically in the newly transformed dermis at mid-trunk levels (arrow in Fig. 6B) and the limb bud apical ectodermal ridge (AER) (Fig. 6B). Interestingly, the ectopic c-kit expression pattern in *Patch* mutant embryos was similar to the expression pattern of PDGFRα mRNA in wildtype embryos (Fig. 6C).

By e11.5, the c-kit mRNA staining initially detected in wildtype mesenchyme had disappeared, whereas it was still strongly expressed in *Ph* mutant embryos, especially within the developing ear pinna, the limb buds, and the lateral and dermal mesenchyme. The enhanced expression of c-kit in the neural tube as well as ectopic expression in the AER seen at e10.5 was still evident in e11.5 mutant embryos (Fig. 6D).

In e12.5 and e13.5 *Ph* mutant embryos, in contrast to wildtype, ectopic mesenchymal c-kit expression persisted in the ear pinna, the limb buds, the dermal mesenchyme, and the neural tube (Fig. 7). These results suggest that embryos carrying the *Ph* mutation express c-kit mRNA not only in MPs but also ectopically in tissues adjacent to the lateral neural crest migration pathway.

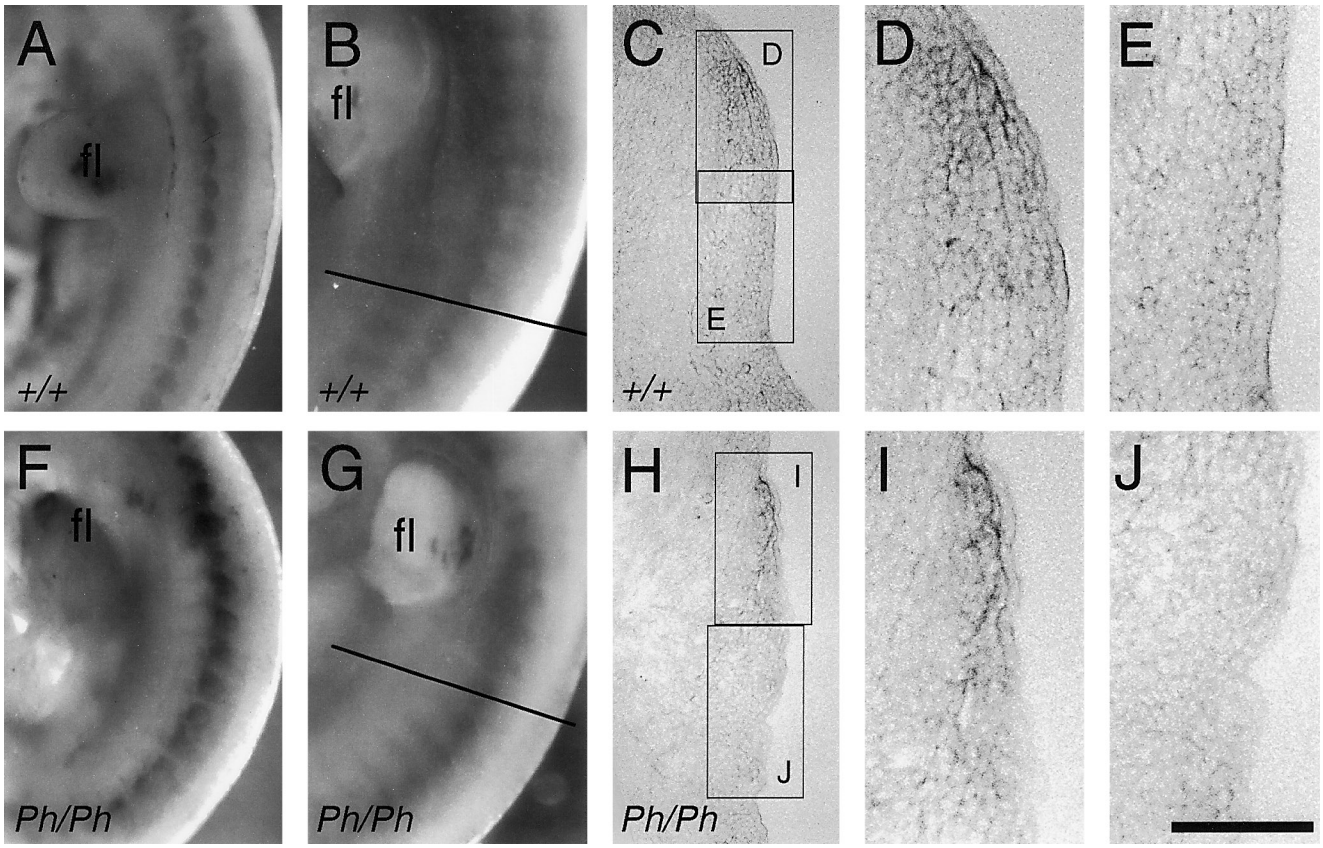

FIG. 5. SlF protein distribution in e10.5 and e11 wildtype and *Ph*/*Ph* mutant embryos. Lateral view of e10.5 +/+ (A) and *Ph*/*Ph* (F) embryos processed for whole mount immunohistochemistry with anti-SlF antiserum. Lateral view at the fore limb level of e11 +/+ (B) and *Ph*/*Ph* (G) embryos processed for anti-SlF antiserum whole mount immunohistochemistry. Control embryos treated with secondary antibodies, but not anti-SlF antiserum, were unstained (not shown). Cross section of whole mount immunostained e11 embryos. 16-μm cryostat sections of +/+ (C) and *Ph*/*Ph* (H). Boxed areas in C and H are magnified in D,E and I,J respectively. Boxed areas D, I correspond to the epithelial dorsal aspect of the dermatome. Boxed areas E, J correspond to the mesenchymal mediolateral aspect of the dermis. Approximate plane of sections shown in C and H are indicated in B and G, respectively. Each photograph is representative of the reproducible staining pattern seen in three different embryos. Bar corresponds to 0.8 mm in A, F; to 0.69 mm in B, C; to 0.16 mm in C, H; and to 0.06 mm in D, E, I, J.

DISCUSSION

We and others have shown that MPs transiently require SlF signaling via c-kit for survival *in vivo* and *in vitro* (Nishikawa *et al.*, 1991; Steel *et al.*, 1992; Morrison-Graham and Weston, 1993; Lahav *et al.*, 1994; Reid *et al.*, 1995). Although *Patch* homozygotes do not survive long enough to observe pigmentation, a function for PDGFRα in MP development has been suggested since altered pigment patterns are observed in *Patch* heterozygotes. As discussed below, we have now elucidated how the PDGFRα deletion in *Patch* mutants might affect pigment pattern in the trunk.

The Patch Mutation Acts Non-Cell-Autonomously with Respect to Melanogenesis

In order to understand whether the PDGFRα signal-transduction mechanism acts cell-autonomously in MPs, we determined the melanogenic ability of neural crest populations from mouse embryos homozygous for the *Ph* mutation. We have shown that c-kit-positive MPs disappear within 3 days when normal neural crest cells are cultured in the absence of the c-kit ligand SlF. In cultures of both *Ph*/*Ph* and wildtype neural crest cells, however, melanocytes survive in the presence of SlF well beyond this critical period and terminally differentiate. This suggests that PDGFRα, which is absent in *Ph* homozygotes, is not required for the initial expression and maintenance of c-kit in MPs, nor is it absolutely required for terminal differentiation. It is possible that PDGFRα activity is required for some aspect of early neural tube development or later in melanocyte differentiation or survival. However, since pigment cells do differentiate in cultures of *Ph*/*Ph* neural crest cells, it seems likely that the *Ph* pigment pattern results from an initially non-cell-autonomous defect. This conclu-

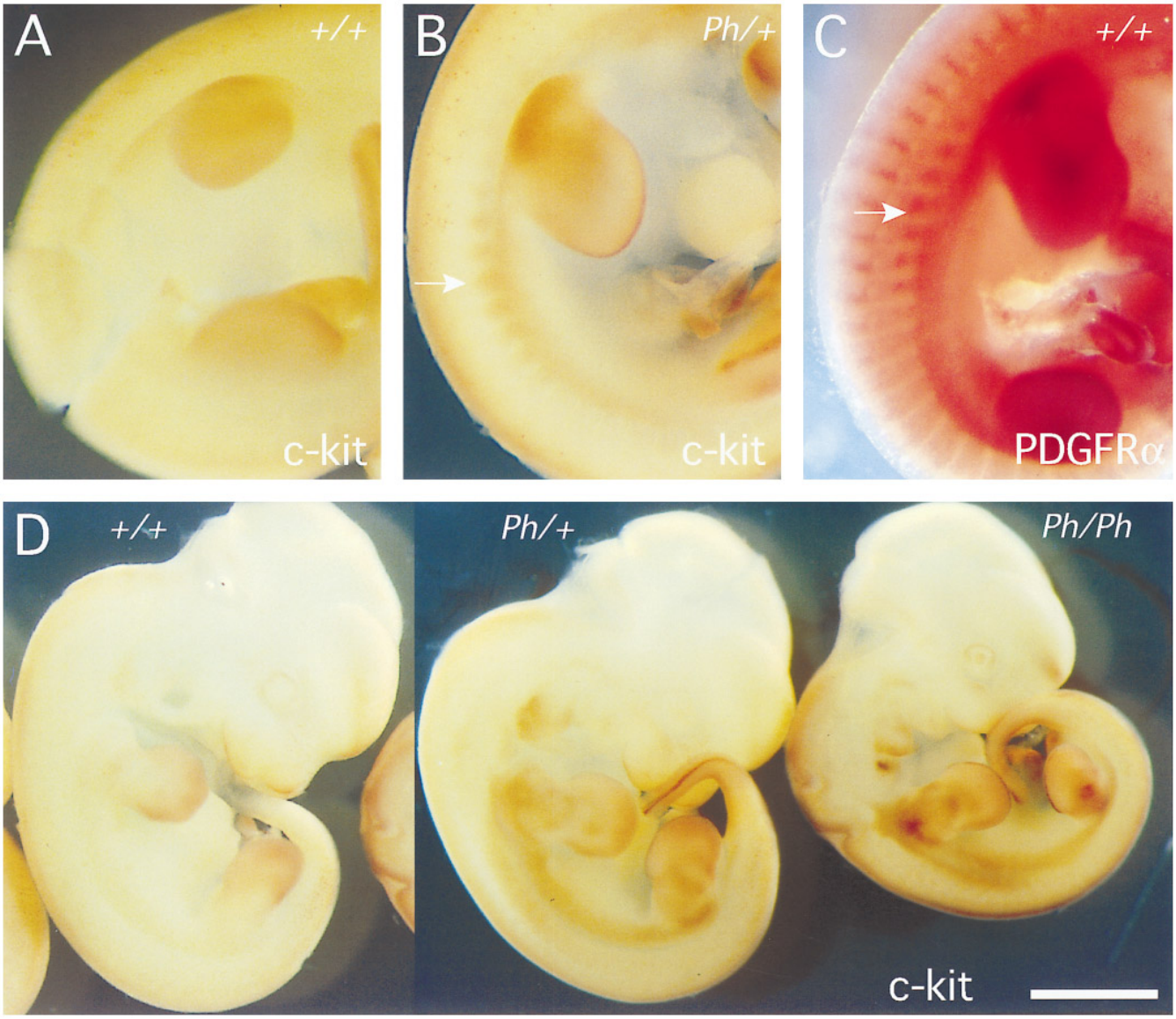

FIG. 6. c-kit mRNA expression pattern is altered in *Ph* mutant embryos. Lateral views of whole mount *in situ* hybridizations with c-kit and PDGFRα antisense riboprobe. Trunk of e10.5 embryos of +/+ (A) and *Ph*/+ (B) genotypes stained with c-kit riboprobe. Trunk of an e10.5 wildtype embryo stained with PDGFRα riboprobe (C). (D) c-kit mRNA localization in embryos of an e11.5 *Ph* litter, showing +/+ (left), *Ph*/+ (middle), and *Ph*/*Ph* (right). Arrows in B and C point to the newly transformed mesenchymal dermis expressing ectopic c-kit in *Ph* mutant and PDGFRα in wildtype embryos. All embryos stained for c-kit mRNA were treated identically to reveal differences in the staining pattern and intensities. Each photograph is representative of the reproducible staining pattern seen in multiple embryos, as follows: A, $n = 5$; B, $n = 7$; C, $n = 4$; D, (+/+) $n = 4$, (*Ph*/+) $n = 7$, (*Ph*/*Ph*) $n = 7$. Bar corresponds to 0.75 mm in A, B; to 0.8 mm in C; and to 2.2 mm in D.

sion is consistent with our inability to detect PDGFRα transcripts in migrating melanocytes *in vivo* and *in vitro* and the lack of PDGFRα expression by various melanoma cell lines (Halaban, 1994).

Since PDGFRα function is thought to be required in glial cells of the central and peripheral nervous systems (Barres *et al.*, 1992; Morrison-Graham *et al.*, 1992), it is interesting to note that PDGFRα appears to be absent in one of the lineages believed to segregate from a crest-derived glial/melanocyte precursor (Weston, 1991; Stocker *et al.*, 1991). This would suggest that the putative glial/melanocyte precursor lacks functional PDGFRα, but that this receptor and the

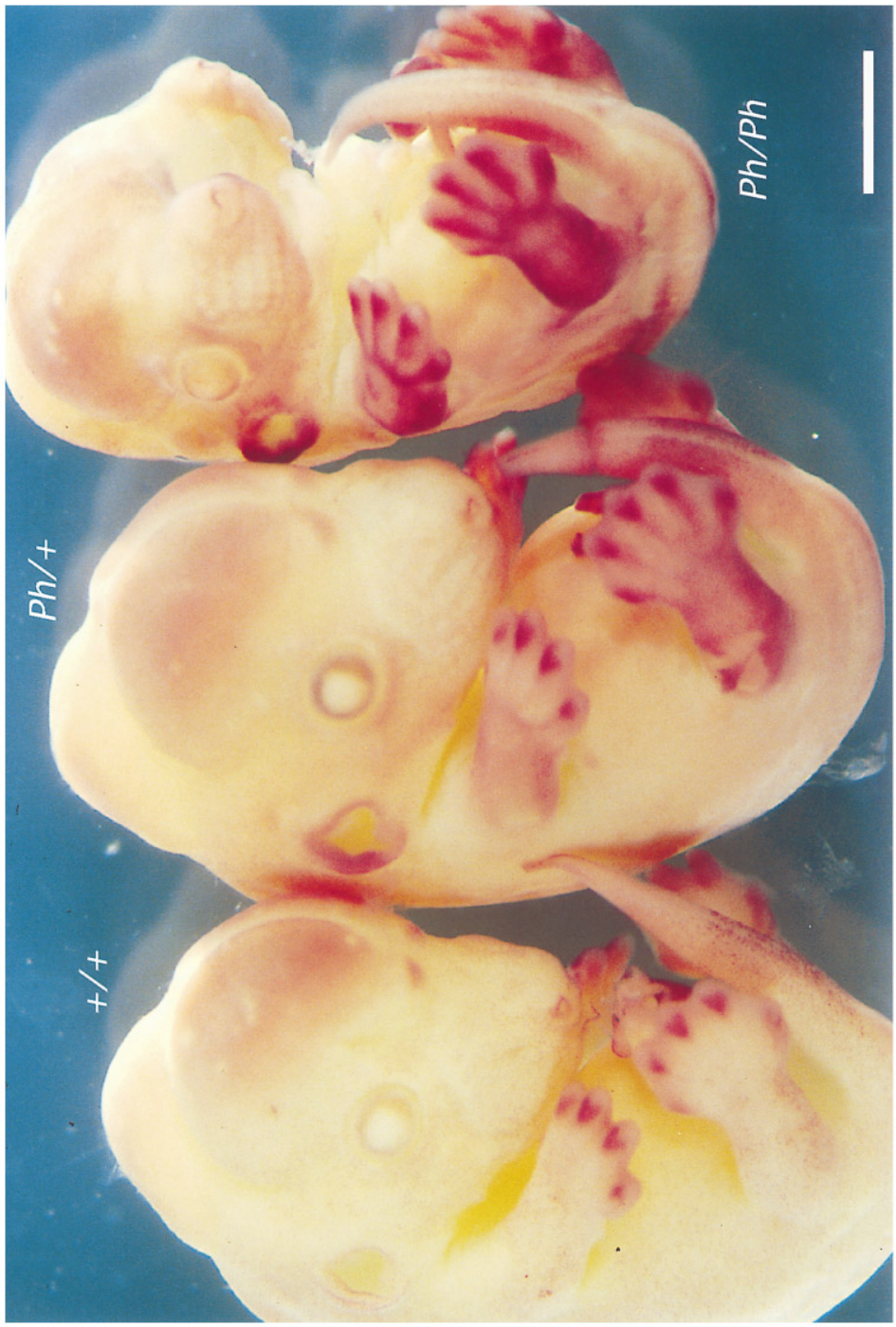

FIG. 7. c-kit mRNA expression pattern is also altered in older *Ph* mutant embryos. Lateral views of whole mount *in situ* hybridizations with c-kit antisense riboprobe of e13.5 +/+ (left), *Ph*/+ (middle), and *Ph*/*Ph* (right) embryos. All embryos were treated identically to reveal differences in the staining pattern and intensities. The photograph is representative of the reproducible staining pattern seen in multiple embryos, as follows: (+/+) $n = 3$, (*Ph*/+) $n = 5$, (*Ph*/*Ph*) $n = 3$. Bar, 2.4 mm.

requirement for its ligand arises later in the glial cell lineage, but not in the melanocyte. This notion remains to be tested using appropriate markers for the crest-derived glial lineage in older embryos.

SlF Localization, but Not Production, Is Altered in Patch Mutant Mice

Initial dispersal of MPs onto the lateral migration pathway and subsequent survival of melanocytes in the dermis both depend on SlF activity (Steel *et al.,* 1992; Wehrle-Haller and Weston, 1995). In the *Ph* mutant embryos, the initial SlF-dependent melanocyte precursor migration appears to be the same as that in wildtype embryos (see Wehrle-Haller and Weston, 1995). This supports our conclusion (above) that c-kit expression in melanocytes and SlF expression by target tissues are initially not affected in embryos homozygous for the *Ph* mutation. However, by e11.5, subsequent dispersal of MPs within the newly formed mesenchymal dermis of *Ph* embryos is dramatically different from that in wildtype embryos. This corresponds to a time when SlF is still required by migrating MPs (Nishikawa *et al.,* 1991; Morrison-Graham and Weston, 1993), but before SlF is expressed by the overlying epidermis, which is reported to occur at e12.5–e13.5 (Motro *et al.,* 1991; Bedell *et al.,* 1995). Interestingly, beginning at e10.5, wildtype embryos begin to express PDGFRα in the lateral dermatome as it starts to transform into dermal mesenchyme (Fig. 6C). In the absence of PDGFRα the newly transformed dermal fibroblasts fail to thrive (Morrison-Graham *et al.,* 1992; Schatteman *et al.,* 1992), which could possibly affect localization of SlF or other growth factors required for melanocyte development. It should be emphasized, however, that possible changes in the structure of the dermis are not sufficient to explain changes in pigment pattern since melanocytes are absent in the mid-trunk level of *Patch* heterozygotes where dermal mesenchyme appears to be normal.

Whether the absence of PDGFRα activity has an effect on the localization or presentation of SlF by the dermal mesenchyme can best be tested by immunohistochemical detection of SlF protein. In wildtype embryos, SlF-IR is detected in the dorsal aspect of the dermatome and ventrolaterally in the newly formed mesenchymal dermis. In contrast, SlF-IR in *Ph*/*Ph* embryos can be detected in the dorsal epithelial dermatome, but is absent from the newly formed ventrolateral mesenchymal dermis.

The loss of SlF-IR in the ventrolateral mesenchymal dermis might be related to our observation that c-kit is ectopically expressed by these cells in embryos carrying the *Patch* deletion. Thus, based on the appearance of the SlF-immunostained epithelial dermatome and the ventrolateral mesenchymal dermis (see Fig. 5), we suggest that some cell-bound SlF is retained by the dermatome and some is normally released by the epithelial dermatome and diffuses ventrolaterally. We further suggest that c-kit, which is ectopically expressed by dermal mesenchyme in *Patch* embryos, mediates internalization and degradation of the diffusible SlF initially expressed and released by the dermatome as it transforms into mesenchyme. As a consequence of ectopic c-kit expression, therefore, the limiting amounts of SlF activity provided by diffusible SlF protein normally present in the dermal mesenchyme would be reduced in the mutant tissue. The fate of MPs in mutant embryos is consistent with this interpretation, since they are seen to cluster (Fig. 3B) in the area of the dorsal epithelial dermatome, where SlF remains (see Fig. 5I), but do not disperse as in wildtype embryos (Fig. 3C) in ventrolateral mesenchyme, which lacks the SlF-IR that is present in wildtype embryos (compare Figs. 5E and 5J). It is presently not clear why the MPs also eventually disappear from these dorsal locations. We postulate, however, that after complete transformation of the epithelial dermatome into mesenchyme, the levels of SlF activity also decline in dorsal locations because of ectopic c-kit expression in mesenchymal cells (Fig. 7).

The Ph Deletion Might Interfere with Regulatory Elements of the c-kit Gene

Recently, two dominant pigmentation mutations, mapping to the *Patch* locus have been molecularly characterized. *Rump-white,* (*Rw*) and W^{sash}, characterized by a white sash/belt at mid-trunk level, have been shown to be large chromosomal inversions (Nagle *et al.,* 1994; Stephenson *et al.,* 1994; Bucan *et al.,* 1995; Duttlinger *et al.,* 1995). In both of these inversions, one of the breakpoints is located between the PDGFRα and the c-kit gene, apparently in the same genomic region that contains one end of the deletion in *Patch*. An analysis of the c-kit expression pattern has not been performed in the *Rw* embryo. However, in W^{sash}, c-kit mRNA and protein are ectopically expressed in somitic mesenchymal derivatives in a pattern that resembles that of *Patch* (Duttlinger *et al.,* 1993, 1995; Besmer *et al.,* 1993). In W^{sash}, where the heterozygous adult pigment pattern is similar to that of *Patch* heterozygotes, the phenotype might also be caused by a disruption of MP migration onto the lateral pathway as observed in *Patch*. Interestingly, in *Patch* as well as in W^{sash}, c-kit is ectopically expressed in locations that normally express PDGFRα (Duttlinger *et al.,* 1993, 1995; Besmer *et al.,* 1993).

Since *Ph* and W^{sash} pigment pattern phenotypes are almost identical *in vivo,* the behavior of MPs might also be similar *in vitro.* Indeed, it has been shown that neural crest explants from W^{sash} homozygous embryos can undergo pigmentation *in vitro* similar to that of *Ph*-derived neural crest cells (Huszar *et al.,* 1991). However, a possible non-cell-autonomous defect in W^{sash} is apparently not supported by a mosaic analysis of melanocyte development (Huszar *et al.,* 1991).

Taken together, these results suggest that the pigment defect in *Patch* mutants is likely to be caused by a competition for limited amounts of SlF on the lateral crest migration

pathway. This competition results from ectopic expression of c-kit by dermal mesenchyme, possibly caused by the removal of c-kit regulatory elements by the *Patch* genomic deletion. This inference is supported by the phenotype of mice carrying a targeted mutation in the PDGFRα gene, which, unlike the *Patch* deletion, do not exhibit a pigmentation phenotype as heterozygotes (P. Soriano, personal communication). This result suggests that the *Patch* mutation acts non-cell-autonomously to affect the development of MPs and further emphasizes the importance of characterizing the role of c-kit regulatory elements for c-kit expression *in vivo*.

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