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Melanocyte development in vivo and in neural crest cell cultures: crucial dependence on the Mitf basic-helix-loop-helix-zipper transcription factor

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SUMMARY

The more than 20 different *Mitf* mutations in the mouse are all associated with deficiencies in neural crest-derived melanocytes that range from minor functional disturbances with some alleles to complete absence of mature melanocytes with others. In the trunk region of wild-type embryos, *Mitf*-expressing cells that coexpressed the melanoblast marker *Dct* and the tyrosine kinase receptor *Kit* were found in the dorsolateral neural crest migration pathway. In contrast, in embryos homozygous for an *Mitf* allele encoding a non-functional Mitf protein, *Mitf*-expressing cells were extremely rare, no *Dct* expression was ever found, and the number of *Kit*-expressing cells was much reduced. Wild-type neural crest cell cultures rapidly gave rise to cells that expressed *Mitf* and coexpressed *Kit* and *Dct*. With time in culture, *Kit* expression was increased, and pigmented, dendritic cells developed. Addition of the Kit ligand Mgf or endothelin 3 or a combination of these factors all rapidly increased the number of *Dct*-positive cells. Cultures from *Mitf* mutant embryos initially displayed *Mitf*-positive cells similar in numbers and *Kit*-expression as did wild-type cultures. However, *Kit* expression did not increase with time in culture and the mutant cells never responded to Mgf or endothelin 3, did not express *Dct*, and never showed pigment. In fact, even *Mitf* expression was rapidly lost. The results suggest that *Mitf* first plays a role in promoting the transition of precursor cells to melanoblasts and subsequently, by influencing Kit expression, melanoblast survival.

Key words: DOPAchrome tautomerase, tyrosinase-related protein 2, Trp2, Kit, mast cell growth factor, Steel factor, stem cell factor, endothelin B receptor, endothelin 3

INTRODUCTION

The neural crest of vertebrates is a transient population of cells localized in the dorsal portion of the closing neural tube. The cells are morphologically indistinguishable from each other and remarkably pluripotent, capable of giving rise, at least initially, to derivatives belonging to multiple lineages that include neurons and glia of the peripheral nervous system, smooth muscle cells, bone and cartilage cells, and all melanocytes of the skin, inner ear, choroid and part of the iris (Hörstadius, 1950; Le Douarin, 1982; review, Bronner-Fraser, 1995). The cells proliferate extensively and, to reach their final destinations, migrate along characteristic pathways; this process is exhaustive such that upon completion, the neural crest is gone.

Some of the factors that influence the development of neural crest derivatives have been identified by analyzing genetic mutations, most notably those that affect melanocyte formation in the laboratory mouse. Such mutations are abundant and their molecular analysis has revealed that both cell intrinsic and extrinsic factors are important for melanocyte development. Among the latter are mast cell growth factor (Mgf), a polypeptide growth factor whose gene is mutated, for instance, in Mgf^{Sl} (*Steel*) mice (Copeland et al., 1990). Another cell extrinsic factor is endothelin 3 (Edn3), a peptide ligand whose gene is mutated in $Edn3^{ls}$ (*lethal spotting*) and *Edn3* gene-targeted mice (Baynash et al., 1994). Mutations in either of these genes interfere with normal generation of melanocytes and lead to pigment dilution, white spotting, or total lack of coat pigmentation (Steel et al., 1992; Murphy et al., 1992; Baynash et al., 1994; Reid et al., 1995; Barsh, 1996). Not surprisingly, the corresponding cellular receptors are equally important for melanocyte development. Thus, mutations in *Kit*, the gene encoding the tyrosine kinase receptor for Mgf, affect melanocytes, as seen for instance in Kit^{W} (*dominant spotting*) mice (Geissler et al., 1988). Similarly, mutations in *Ednrb*, the gene encoding the G-coupled receptor for Edn3, also lead to defects in melanocytes, as seen in mice carrying the $Ednrb^{s}$ (*piebald*), $Ednrb^{s-l}$ (*piebald-lethal*) or an *Ednrb* gene-targeted allele (Hosoda et al., 1994).

The first cells that can be assigned to the neural crest-derived melanocyte lineage and that are subject to regulation by these factors are called melanoblasts. They can be defined as cells that express *Kit* (likely also *Ednrb*) and *Dct*, the gene that encodes the melanin-synthetic enzyme DOPAchrome tautomerase or tyrosinase-related protein-2, and are capable of giving rise to differentiated melanocytes (Steel et al., 1992; Pavan and Tilghman, 1994; Reid et al., 1995). Interestingly, even a complete lack of function in the above mentioned signaling pathways still allows for the initial generation of at least some *Dct*-positive cells; in $Ednrb^{s-l}$ or *Ednrb* gene-targeted mice, they are considerably reduced in number yet may develop into mature coat melanocytes that can be seen as pigmented spots around the ears and eyes, and in *Edn3* mutant mice, such spots are even larger (Pavan and Tilghman, 1994; Hosoda et al., 1994; Baynash et al., 1994). In Mgf^{Sl} and several *Kit* mutant mice, the numbers of *Dct*-positive cells may initially be similar to wild type, but the cells are lost soon thereafter (Cable et al., 1995; Wehrle-Haller and Weston, 1995; Klüppel et al., 1997). These observations suggest that the respective signaling pathways are not required for melanoblast determination but rather for melanoblast proliferation and/or survival.

Like mice with defects in the above mentioned ligand/receptor systems, mice with mutations at the *microphthalmia* (*Mitf*) locus also have defects in coat pigmentation. Depending on the mutant allele, such mice may in addition have an abnormal retinal pigment epithelium, small eyes, sensorineural deafness and osteopetrosis (Green, 1989; Mouse Genome Database, 1997). The gene whose mutations are responsible for these defects has recently been identified and is now known as *Mitf* (Hodgkinson et al., 1993; Hughes et al., 1993; Steingrímsson et al., 1994; Hemesath et al., 1994). It encodes a transcription factor of the basic-helix-loop-helix-zipper class and thus shares structural and biochemical features with basic-helix-loop-helix proteins such as Mash1, NeuroD or those belonging to the MyoD family which are known for their role as potent regulators of cell fate (Jan and Jan, 1993; Sommer et al., 1995; Lee et al., 1995). The gene has undergone multiple independent mutations in the mouse so that to date, at least 21 different *Mitf* alleles have been observed (Mouse Genome Database, 1997; Steingrímsson, personal communication). In fact, in the mouse alone, *Mitf* mutations represent one of the largest and phenotypically best characterized collections of mutations in any member of this class of transcription factors. In addition, the homologous gene is mutated in some families with Waardenburg type IIa syndrome, a particular form of congenital hearing impairment with associated pigment alterations in eye, hair and skin (Tassabehji et al., 1994).

In this paper, we present evidence that in embryos homozygous for severe *Mitf* mutations, neural crest-derived cells do not reach the *Dct*-positive stage. In addition, in the neural crest of wild-type embryos, *Mitf* expression is found just in a few cells before expression of *Dct*, thus rendering an in vivo analysis of the early fate of *Mitf* mutant cells extremely difficult. Hence, we reasoned that culturing embryonic neural tubes might allow us to study these cells more easily and explore their characteristics and response to growth factors. In fact, neural crest cell cultures, whether derived from wild-type embryos or embryos carrying a severe *Mitf* mutation, were equally capable of producing a small number of *Mitf*-expressing cells. However, in contrast to wild-type cells, the *Mitf* mutant cells did not go on to express *Dct*, never became pigmented, and were rapidly lost. Also, they could not be maintained by addition of Mgf although their initial *Kit* expression was unaffected. Likewise, Edn3 was without effect for mutant cells even though it potently increased the number of wild-type *Dct*-positive cells and the melanocytes derived from them. These observations establish that Mitf is a crucial transcription factor required early on to allow neural crest cells to enter the melanocyte differentiation pathway.

MATERIALS AND METHODS

Mice

Embryos expressing wild-type *Mitf* were derived from the albino strain A2G or the pigmented strains C57BL/6 or they were of a mixed C57BL/6/C3H background. *Mitf* mutant embryos were either homozygous for the $Mitf^{vga-9}$ allele that resulted from a transgenic insertion in the putative *Mitf* promoter region (mixed C57BL/6/C3H background, Tachibana et al., 1992; Hodgkinson et al., 1993), or homozygous for the $Mitf^{mi-ew}$ (eye-less white) allele that encodes a non-functional protein (background strain Naw) (Miner, 1968; Wood and Minor, 1969; Steingrímsson et al., 1994). The homozygous mutant embryos were obtained by mating homozygous parents. Timed pregnancies were obtained by checking mating plugs, and the morning a plug was detected was defined as E0.5.

Neural crest cell preparations

Neural tube explants were obtained from embryos at E9.5 (15-25 pairs of somites). Embryos were removed from the uterus and placed in a 3.5 cm Petri dish containing equal volumes of DMEM and PBS. Using fine forceps under a dissecting microscope, embryos were cleaned of membranes and soft tissues and a portion of the neural tube corresponding to the 10 posterior-most somites was cut and digested on ice for 3 minutes in 1% trypsin in PBS. The trypsin was neutralized by transferring the neural tube segment to a new 3.5 cm Petri dish containing DMEM supplemented with 10% fetal calf serum. Using forceps, the neural tube was then cleaned from the surrounding somites and transferred to the culture dishes.

Culture conditions, media and gowth factors

Neural crest cells were cultured on 3.5 cm primaria dishes (Falcon) or on chamber glass slides, both coated with 10 μg/ml of human plasma fibronectin (GIBCO BRL) diluted in PBS. The basal culture medium (2 ml per dish or chamber) consisted of F10 medium supplemented with 10% fetal calf serum, 16 nM TPA (Sigma), 20 nM cholera toxin (GIBCO BRL) and 10 μg/ml [Nle^4, D-Phe^7]-α-MSH (Sigma) and was changed every 2nd to 3rd day. The cultures were incubated in 5% CO_2/95% air at 37°C. Mgf (recombinant murine c-kit ligand, R&D System) or Edn3 (Sigma) were added at a final concentration of 0.5 nM or 1 nM, respectively.

Riboprobes

The Mitf sense and antisense riboprobes were prepared as described previously (Hodgkinson et al., 1993). The template for the mouse Dct probe was described previously (Steel et al., 1992) and corresponded to a 1200 bp Dct cDNA kindly provided by Dr I. J. Jackson. Linearization with either *Hin*dIII or *Bam*HI yielded antisense and sense probes, respectively. A Kit riboprobe was prepared using a plasmid containing a mouse Kit cDNA that was linearized with *Bam*HI to generate the 2.5 kb antisense transcript. In vitro transcriptions were performed using either the T3 or T7 polymerase and a digoxigenin RNA labeling kit (Boehringer). For double-labeling experiments, ^{35}S-

UTP-labeled riboprobes were mixed with digoxigenin-labeled probes. Sense probes were used for control purposes.

In situ hybridizations

Non-radioactive in situ hybridization of paraformaldehyde fixed, sucrose embedded cryostat sections were performed according to standard procedures essentially as described by Hodgkinson et al. (1993). For in situ hybridizations of cultured cells, cultures were washed with PBS and fixed for 30 minutes at room temperature in 4% paraformaldehyde in PBS, pH 7.2, incubated in 0.05% Triton-X-100 in PBS for 5 minutes, 0.2 N HCl in water for 5 minutes, proteinase K (1 μg/ml in 20 mM Tris pH 7.5) for 5 minutes, and fixed again for 5 minutes in 4% paraformaldehyde. In situ hybridizations were done according to standard procedures. The probes were revealed with an anti-digoxigenin-alkaline-phosphatase-conjugated antibody followed by incubation with the color substrate solution (Boehringer). For double-labeling experiments, cells were dehydrated with 50% and 95% ethanol after the completion of the alkaline phosphatase staining. Culture dishes were then coated with an LM emulsion (Amersham). Exposure times varied from 10 to 15 days prior to development with D19 developer.

Antibodies and immunostaining

For immunization of rabbits, recombinant Mitf protein containing an aminoterminal stretch of six histidines was expressed in *E. coli* and purified by affinity chromatography on Ni-NTA-columns under native conditions followed by FPLC ion exchange chromatography on a Mono-Q column. The specificity of the polyclonal sera for immunostaining puposes was tested in two ways. First, no staining was found in sections derived from *Mitf* null mutant mice. Second, affinity fractionation of the IgG fraction using an aminoterminal Mitf fragment lacking the conserved bHLH-Zip domain yielded an antibody fraction with specific staining similar to the full serum. For Kit labeling, the rat monoclonal antibody ACK2 (Gibco) was used.

For double indirect immunolabeling, fresh frozen tissue sections were fixed with methanol (100%, 15 minutes, –20°C) followed by 2% paraformaldehyde (2 minutes, room temperature) and exposed to a mixture of the two antibodies. Cultured cells were fixed with 2% paraformaldehyde (30 minutes), permeabilized with Triton-X-100 (0.1%, 5 minutes) and exposed to anti-Mitf serum (30 minutes, room temperature) and subsequently to ACK2 (60 minutes, room temperature). The primary antibodies were revealed with RITC-coupled goat anti-rabbit (Fab)2 and FITC-coupled goat anti-rat (Fab)2, using a Reichert-Jung Polyvar microscope equipped for epifluorescence and differential interference contrast.

RESULTS

Analysis of *Mitf* expression in vivo – wild-type embryos

Previous in situ hybridization results suggested that in E13.5-16.5 wild-type embryos, *Mitf* is expressed in neural crest-derived cells around the otic vesicles and in hair follicles; coexpression of *Dct*, the melanin synthetic enzyme DOPAchrome tautomerase, suggested that they were indeed melanoblasts (Hodgkinson et al., 1993). We now extended these observations to the trunk region and analyzed wild-type and *Mitf* mutant embryos containing 25-45 pairs of somites (E10.5-E11.5). The first *Mitf*-positive cells were observed at the 27-28 somite stage close to the dorsal midline of the neural tube. Thereafter, the number of *Mitf*-positive cells progressively increased; in embryos with 40 pairs of somites, numerous dispersed cells were found underneath the surface ectoderm as shown for the area of the hind limb bud (Fig. 1A), suggesting that they were part of the characteristic dorsolateral migration pathway of trunk neural crest cells. Cells expressing the melanoblast marker *Dct*, labeled on adjacent sections, were not seen in early embryos at time points when *Mitf* expression first appeared. Soon thereafter, however, they were present in areas largely overlapping with those of *Mitf*-positive cells but rarely close to the dorsal midline (Fig. 1B). Double label in situ hybridization experiments showed that many cells in the areas lateral to the neural tube coexpressed *Mitf* and *Dct* (Fig. 1B, inset). The results suggested that *Mitf*-positive cells originate in the neural crest and develop into *Dct*-positive melanoblasts soon after their emigration from the neural tube.

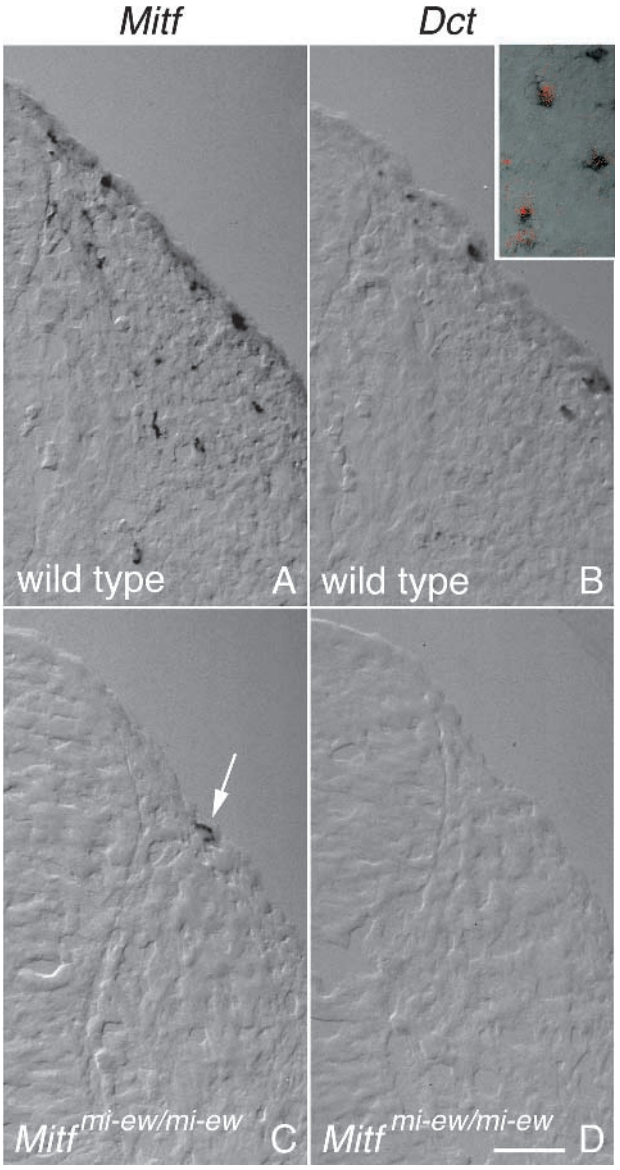

Fig. 1. Expression of *Mitf* and *Dct* in vivo. Shown are sections through the hind limb bud area of wild-type (A,B) and *Mitf*$^{mi\text{-}ew}$ homozygous (C,D) embryos at E11.5. (A,C) In situ hybridization for *Mitf*. (B,D) In situ hybridization for *Dct* in adjacent sections. (Inset) Double label in situ hybridization using a digoxigenin-labeled Mitf riboprobe (black) and an ^{35}S-labeled Dct riboprobe (dark field image converted to red dots) shows cells co-expressing these two genes. Bar, 50 μm.

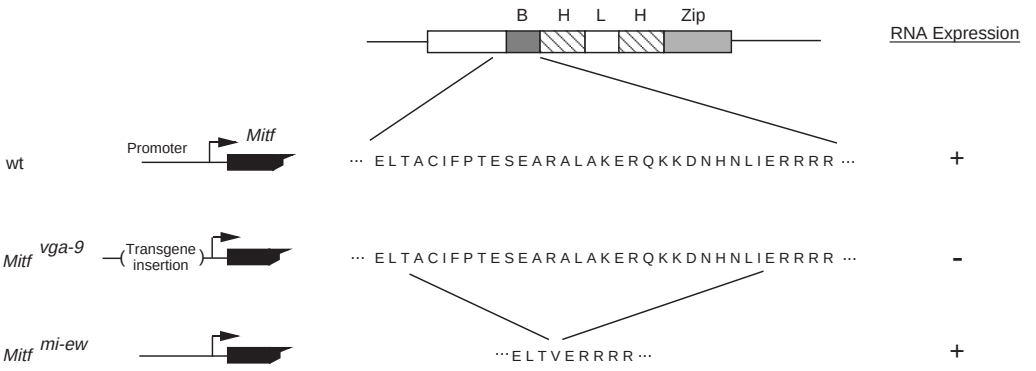

Fig. 2. Molecular characteristics of the *Mitf* mutant alleles used in this study. The left part of the figure schematically depicts the upstream regulatory region of the *Mitf* gene. In the $Mitf^{vga\text{-}9}$ allele, this region is interrupted by insertion of a transgene (Hodgkinson et al., 1993). The middle part of the figure depicts the amino acid sequence (single letter code) of the DNA-binding basic domain and adjacent amino-terminal flanking region of Mitf. The $Mitf^{mi\text{-}ew}$ encodes a protein characterized by replacement of 25 residues from the basic and adjacent amino-terminal region by a single valine. The right part of the figure lists the detectability of Mitf mRNA based on in vivo and in vitro observations.

Analysis of Mitf expression in vivo – mutant embryos

In previous experiments, we were unable to identify neural crest-derived, *Dct*-positive melanoblasts in *Mitf* mutant embryos, and adult mutant mice lack the neural crest-derived, melanocytic intermediate cells in the stria vascularis of the inner ear (Hodgkinson et al., 1993; Tachibana et al., 1992; Motohashi et al., 1994; unpublished observations). To determine when and where these cells were lost during development, we now used embryos homozygous for either one of two *Mitf* alleles, $Mitf^{vga\text{-}9}$ and $Mitf^{mi\text{-}ew}$, whose molecular characteristics are shown in Fig. 2. $Mitf^{vga\text{-}9}$ is an apparent null allele that resulted from the insertion of multiple copies of an unrelated transgene in the upstream regulatory region of *Mitf* without affecting the *Mitf* coding region (Hodgkinson et al., 1993; unpublished observations). $Mitf^{mi\text{-}ew}$ arose spontaneously and is characterized by expression of a mRNA with a 75 base deletion, thereby encoding a protein with a replacement, by a single valine, of 25 residues including all but 5 residues of the DNA-binding basic domain (Steingrímsson et al., 1994). This protein, when translated in vitro, forms homo- and heterodimers but does not bind DNA in gel shift assays (Hemesath et al., 1994). Unlike the nuclear located wild-type protein, $Mitf^{mi\text{-}ew}$ protein accumulates in both nucleus and cytoplasm when experimentally expressed in fibroblasts (Takebayashi et al., 1996).

In $Mitf^{vga\text{-}9}$ embryos, *Mitf* signals were never observed, consistent with the molecular nature of this null allele. In $Mitf^{mi\text{-}ew}$ embryos, however, *Mitf*-positive cells could occasionally be detected underneath the surface ectoderm in areas corresponding to those where *Mitf*-positive cells were found in wild type (Fig. 1C). However, they were extremely rare, necessitating complete sectioning of several embryos, and no positive cells were ever found beyond E12.5. Staining of adjacent sections for *Dct* never showed a positive cell, either in $Mitf^{vga\text{-}9}$ (not shown) or $Mitf^{mi\text{-}ew}$ embryos (Fig. 1D). Thus, in *Mitf* mutant embryos, authentic melanoblasts, as defined by their marker gene *Dct*, could not be found even though some rare *Mitf*-positive cells were generated. Because of the paucity of these *Mitf*-positive cells, we reasoned that further in vivo experiments would be difficult but that culturing these cells in vitro might give us easier access to study their fate and response to growth factors.

Mitf expression in wild-type neural crest cell cultures

To analyze the role of *Mitf* in more detail, we established neural crest cell cultures under conditions that favor generation of differentiated melanocytes. The cultures were prepared from trunk neural tubes harvested just prior to the onset of *Mitf* expression in vivo, i.e. from embryos with 15-25 pairs of somites (E9.5).

Upon explantation, neural tubes attached readily to culture dishes and cells were seen to migrate out 6 hours later. Ten days thereafter, the cultures showed a number of dispersed, melanin-positive cells with extended processes. To determine at which stage presumptive melanoblasts would appear, we performed a series of in situ hybridization tests, using digoxigenin-labeled or ^{35}S-labeled riboprobes for *Mitf* and *Dct*. The results are shown in Fig. 3. At day 1 of culture, a small subset of the cells surrounding the neural tube expressed *Mitf* (Fig. 3A), and a similarly small subset expressed *Dct* (Fig. 3B). The number of these cells was variable, usually ranging from 20-50 cells per tube. With time in culture, their numbers increased but remained variable from tube to tube, most likely because of embryo-to-embryo variation of the number of precursor cells present in the tube at time of harvest. Double label experiments, performed at day 2 of culture (Fig. 3C,D), showed overlap of *Mitf* and *Dct* expression in the majority of cells. At 2 days of culture, a typical experiment showed a total of 103 labeled cells, 71 (69%) of which were double positive, 26 (25%) positive only for *Mitf*, and 5 (5%) only for *Dct*. At 4 days, there were a total of 208 labeled cells, 193 (93%) of which were double-positive, 9 (4%) *Mitf* single-positive, and 6 (3%) *Dct* single-positive. These results indicated that *Dct*-positive cells appeared in culture at a time point consistent with the appearance of *Dct*-positive cells in vivo (E 10.5-11.5, Steel et al., 1992; Pavan and Tilghman, 1994; Wehrle-Haller and Weston, 1995; Fig. 1) and that the majority of *Mitf*-expressing cells were melanoblasts as defined by their *Dct*-positivity. They further suggested that cells expressed *Mitf* before they expressed *Dct*. The appearance of a small number of *Dct* single-positive cells may have reflected dynamic changes in the levels of the respective mRNAs.

At 10 days of culture, with the appearance of melanin-positive cells, the number of *Mitf* and *Dct*-positive cells was further increased. However, cells that displayed dendritic

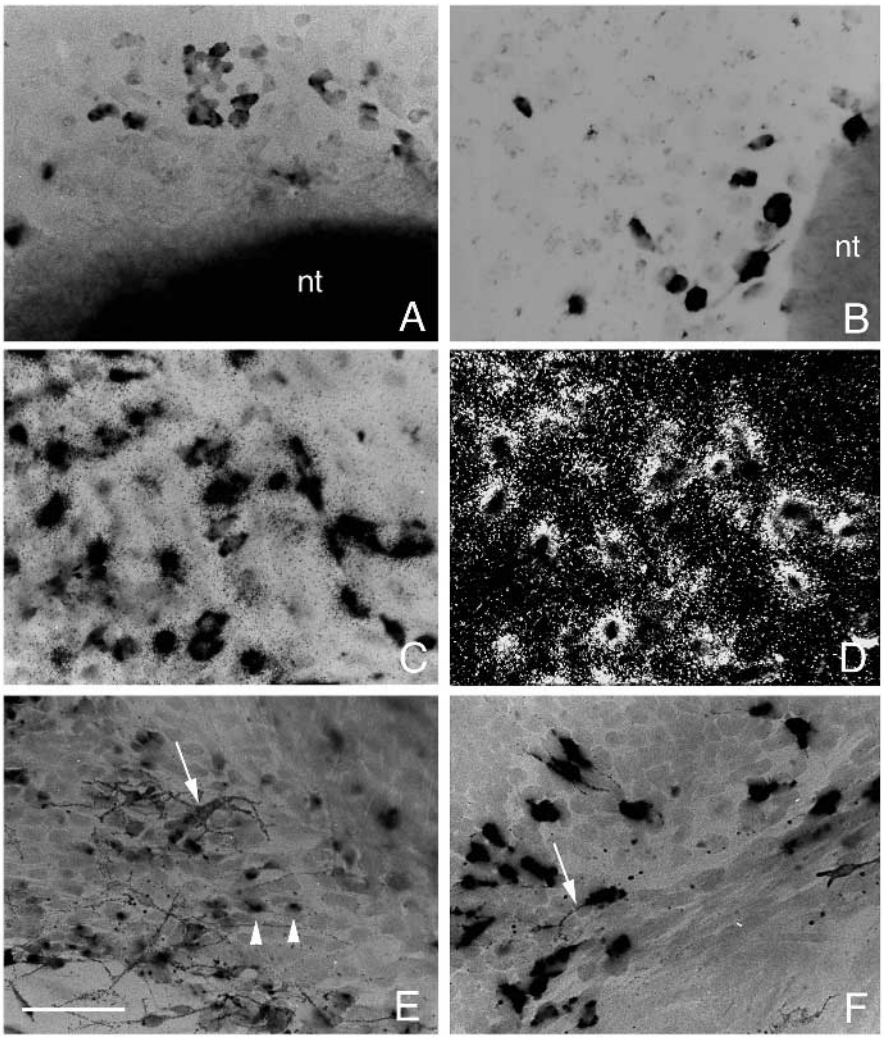

Fig. 3. *Mitf* and *Dct* expression in wild-type neural crest cell cultures. Cultures were processed for single label in situ hybridization, using digoxigenin-labeled Mitf or Dct riboprobes, or for double-label in situ hybridization, using a combination of the digoxigenin-labeled Mitf and a ^{35}S-labeled Dct-specific riboprobe. (A) *Mitf* expression 1 day after explantation. (B) *Dct* expression 1 day after explantation. (C) *Mitf/Dct* double label 2 days after explantation, bright-field image showing *Mitf* signal. (D) Same field, dark-field image showing grains of *Dct* signal. Note that a majority of cells are double labeled. (E) *Mitf* expression 10 days after explantation. Note that at this stage, there are cells that display melanin pigment but are low or negative for *Mitf* (arrow) while others with high *Mitf* signal do not display melanin (arrowheads). (F) *Dct* expression 10 days after explantation. Note that melanin-positive cells still express high levels of *Dct* (arrow). nt, neural tube. Bar, 100 μm.

extensions and were strongly positive for melanin (arrow in Fig. 3E) were negative for *Mitf*. Such melanin-positive cells (arrow in Fig. 3F) remained positive for *Dct*; cells that still expressed high levels of *Mitf* at this stage did not display pigment (arrowheads, Fig. 3E). It is conceivable that high levels of *Mitf* might be required for differentiation into melanocytes but not for maintenance of the differentiated state, or that high levels might interfere with pigment synthesis. Down regulation of developmentally important transcription factors upon differentiation is not without precedent. For instance, Mash1, a mammalian homolog of the achaete-scute family of proteins that determine neuronal fate in *Drosophila*, is expressed in neural crest-derived precursors of sympathetic and enteric neurons, but becomes undetectable upon neuronal differentiation (Li-Ching et al., 1991); similar transient expression modes apply to its *Drosophila* homologs (Cabrera et al., 1987; Cubas et al., 1991).

Mitf expression in *Mitf* mutant neural crest cell cultures

Neural tubes from either *Mitf*$^{vga\text{-}9}$ or *Mitf*$^{mi\text{-}ew}$ embryos gave rise to explant cultures as readily as wild-type neural tubes. However, no melanin-positive cells were ever found, even

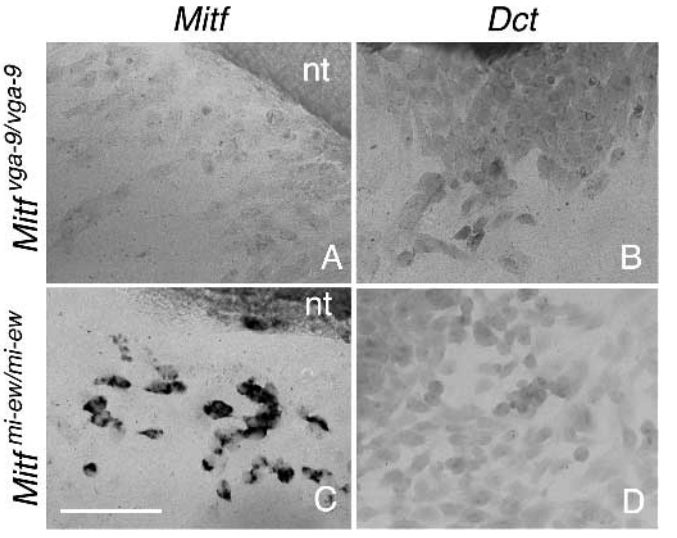

Fig. 4. In situ hybridization for *Mitf* and *Dct* in *Mitf*$^{vga\text{-}9}$ and *Mitf*$^{mi\text{-}ew}$ neural crest cell cultures 1 day after explantation. Note that *Mitf*$^{vga\text{-}9}$ cultures express neither *Mitf* (A) nor *Dct* (B), while *Mitf*$^{mi\text{-}ew}$ cultures contain cells expressing *Mitf* (C) but not *Dct* (D). nt, neural tube. Bar, 100 μm.

after prolonged periods in culture. In situ hybridization results of these cultures are shown in Fig. 4. As was expected from the molecular nature of the *Mitf*$^{vga-9}$ allele, no *Mitf* signal was detected in *Mitf*$^{vga-9}$ cultures at 1 or 4 days (Fig. 4A for 1 day), and the cultures also remained negative for *Dct* (Fig. 4B). These results, while demonstrating the specificity of the two probes, did not allow us to follow the respective cells. In *Mitf*$^{mi-ew}$ cultures, Mitf mRNA-positive cells appeared at day 1 in numbers similar to those in wild-type cultures (Fig. 4C), but they became undetectable beyond day 3. As with *Mitf*$^{vga-9}$ cultures, however, there were at no stages any *Dct*-positive cells (Fig. 4D), except in a single culture (of 10 analyzed) in which five cells expressed *Dct* at day 1. These results suggested that functional Mitf protein was necessary for the persistence of Mitf mRNA-positive cells beyond day 4, and for *Dct* expression.

Expression of Kit in wild-type and mutant neural crest cell cultures

The above experiments showed that *Mitf*$^{mi-ew}$-expressing cells are transiently present but then become undetectable. As mentioned, *Kit* mutant embryos also display melanocyte precursors only transiently although these cells may reach the *Dct*-positive stage (Cable et al., 1995; Wehrle-Haller and Weston, 1995; Klüppel et al., 1997). Hence, it was conceivable that *Mitf* mutations would lead to reduced levels of *Kit* expression, thereby lowering the rate of proliferation and/or survival of *Mitf*-positive cells, and that the lack of *Dct*-expression in these cells was yet another consequence of the absence of functional *Mitf*. Thus, we tested by in situ hybridization whether *Mitf*-positive cells from wild-type or *Mitf*$^{mi-ew}$ mutant embryos would coexpress *Kit* or not. Fig. 5A shows

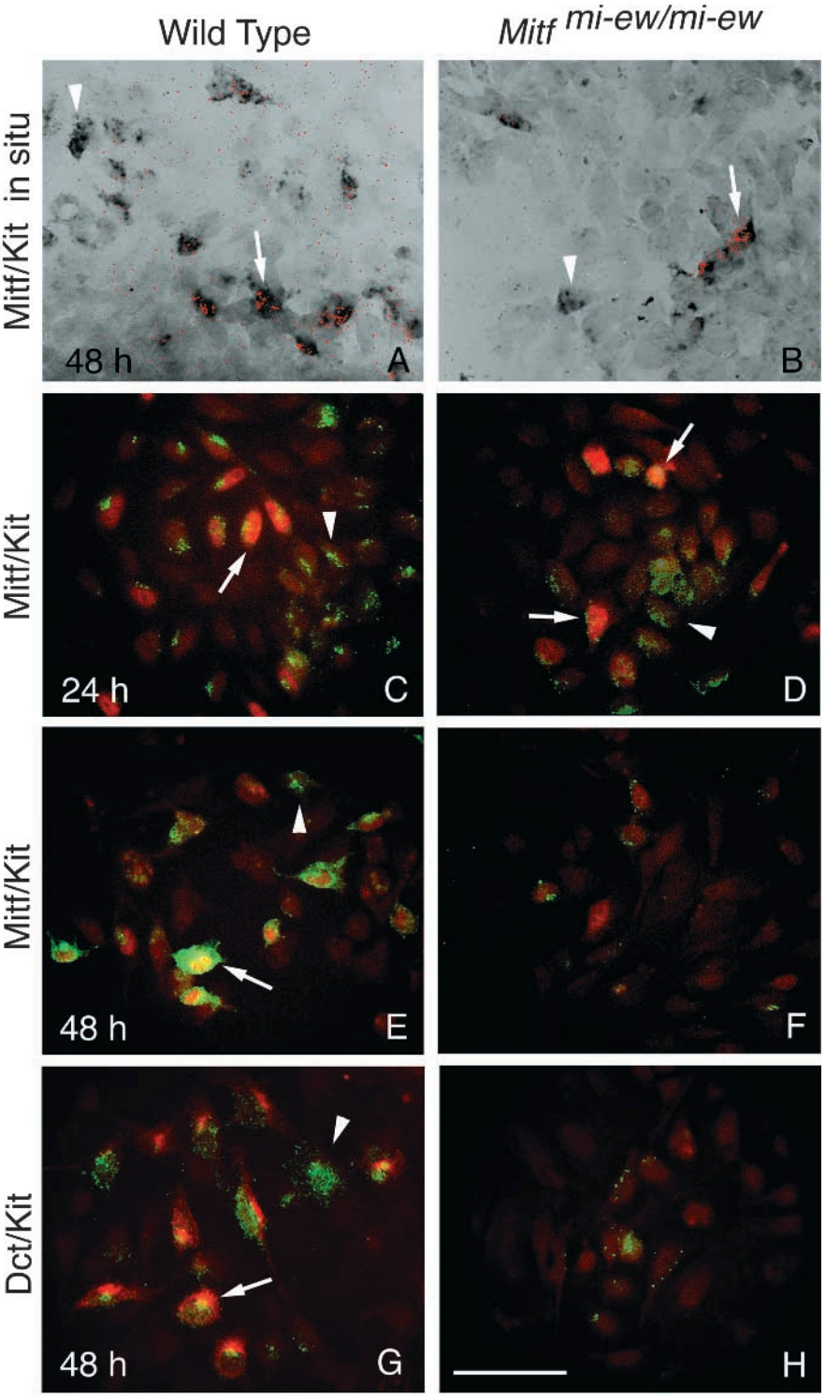

Fig. 5. *Kit* expression in wild-type (A,C,E,G) and *Mitf*$^{mi-ew}$ (B,D,F,H) neural crest cell cultures. (A,B) Double-label in situ hybridization of 2-day-old cultures using an ^{35}S-labeled Mitf riboprobe (red) and a digoxigenin-labeled Kit riboprobe (black). The image of the silver grains in the emulsion was converted to the red color and overlayed (slightly offset) on the image of the digoxigenin label. Note double-positive cells (arrows) and *Kit* single-positive cells (arrowheads) in either culture, suggesting that the onset of *Kit* expression does not depend on *Mitf*, either in *Mitf*-expressing cells or cells lacking *Mitf* expression. (C-F) Double immunolabeling for Mitf protein (red-yellow) and Kit protein (green). Note Kit expression in Mitf$^+$ (arrows) and in Mitf$^-$ (arrowheads) cells at 24 hours of culture (C,D), independent of the Mitf genotype. At 48 hours (E,F), Kit expression is increased in wild-type cells but not in *Mitf* mutant cells. Compared with the in situ hybridizations depicted in A and B, antibody labeling shows a more prominent difference between the two types of cultures. This is most likely due to the lower sensitivity of the immunofluorescent assay. (G,H) Double immunolabeling for Dct protein (red-yellow) and Kit protein (green). Note double-labeled (arrows) and Kit single-labeled (arrow head) cells in wild-type (G) but only Kit single-labeled cells in *Mitf*$^{mi-ew}$ cultures (H). For G, the superimposed images were slightly shifted to highlight presence of the individual signals. Bar, 50 μm.

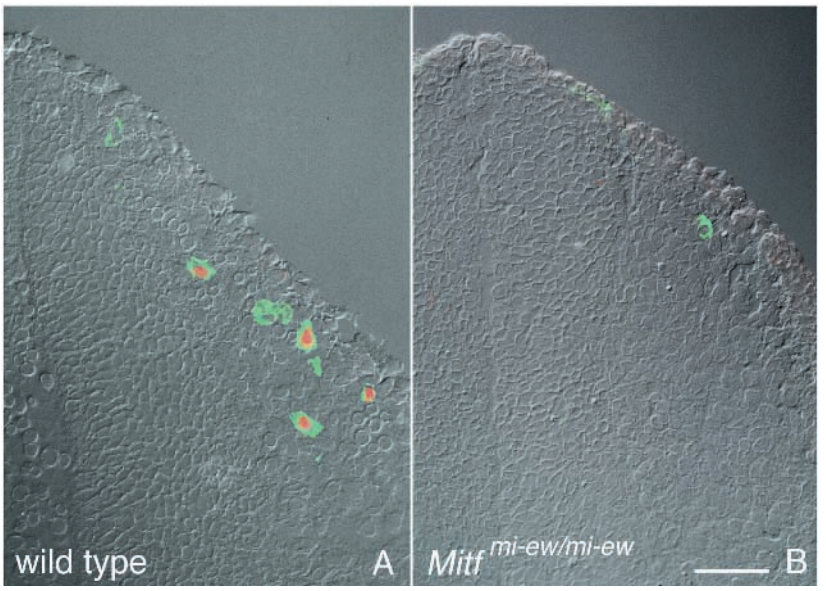

Fig. 6. Double immunolabeling of tissue sections of embryos of 34-38 pairs of somites. (A) wild type. Nuclear Mitf label is in red and cytoplasmic Kit label is in green. Note double-labeled cells in dorsolateral pathway. (B) *Mitf*$^{mi\text{-}ew}$ embryo. Note Kit-positive cell while Mitf label is absent. Bar, 50 μm.

that in 2-day-old wild-type cultures, *Mitf*-positive cells indeed coexpressed *Kit*. In addition to the double-positive cells, there were similar numbers of *Kit* single-positive cells. These cells may already have downregulated *Mitf* expression, may go on to express *Mitf* subsequently, or may belong to a different, non-melanocytic lineage; their precise identification would require lineage tracing experiments. More importantly, however, in *Mitf*$^{mi\text{-}ew}$ cultures (Fig. 5B), *Mitf*-expressing cells likewise coexpressed *Kit*, suggesting that a functional Mitf protein was not required for the onset of *Kit* expression in these cells.

To test whether this *Mitf*-independent onset of *Kit* expression was also reflected at the protein level, we used double indirect immunofluorescent labeling techniques. As shown in Fig. 5C and D, at 24 hours after explantation, both wild-type and *Mitf*$^{mi\text{-}ew}$ cultures showed Mitf/Kit double-labeled cells (arrows) as well as Kit single-labeled cells (arrowheads). However, Kit staining was still weak at this early stage and concentrated mostly in the perinuclear area. Over the following days in culture, both Kit and Mitf expression in the two types of cultures differed. In wild-type cultures, Mitf staining persisted in many cells, and Kit expression became more prominent and was now distributed throughout the cytoplasm, particularly in double-positive cells (arrow). In some Kit single-positive cells, expression stayed low (arrowhead, Fig. 5E) while in others, it increased (not shown). In *Mitf*$^{mi\text{-}ew}$ cultures, Mitf soon became difficult to visualize, and Kit staining never increased even though Kit-positive cells could still be identified at day 2 (Fig. 3F) as suggested by the more sensitive in situ hybridization assays. At day 3, Mitf staining was absent and only a few Kit-labeled cells were seen (not shown). The results confirmed the rapid disappearance of *Mitf*-positive cells in mutant cultures as seen by in situ hybridization (see above) and suggested that while onset of Kit expression was independent of Mitf, functional Mitf was necessary for increasing its expression. Furthermore, in early wild-type cultures, a subset of Kit-expressing cells was positive for Dct (Fig. 5G) while no Dct-positive cells were found in *Mitf*$^{mi\text{-}ew}$ cultures (Fig. 5H).

The results obtained in wild-type cultures were confirmed through in vivo observations. Double immunolabeling of the dorsolateral trunk region of embryos with 34-38 pairs of somites showed both Kit single-positive and Mitf/Kit double-positive cells (Fig. 6A). In contrast, in *Mitf*$^{mi\text{-}ew}$ embryos, Kit-positive cells, though identifiable in the dorsolateral pathway, were considerably reduced in number, but Mitf protein-expressing cells (and hence double-positive cells), were not found (Fig. 6B), perhaps because the period of Mitf positivity in vivo was even shorter or the expression levels lower than in vitro. Nevertheless, the in vivo results confirm that Kit single-positive cells are being generated both in wild-type and *Mitf*$^{mi\text{-}ew}$ embryos, thus consistent with the interpretation that at least for some cells, functional Mitf is not required for the onset of Kit expression but is needed for development of melanocytes.

Effect of Mgf and Edn3 on wild-type and mutant neural crest cell cultures

Since these cells initially expressed *Kit* independent of their *Mitf* genotype, we could test whether deliberate addition of soluble Mgf (above the level presumably present in these mixed-cell cultures) might rescue *Mitf* mutant cells. For these experiments, Mgf was added to the cultures at 0.5 nM at day 0 and replenished at day 2 or 3, and the cells were assayed for *Dct* or *Mitf* expression at day 4 of culture. The dose corresponded to the minimum saturating concentration as determined in dose/response curves with wild-type cultures. As mentioned above, at this time point, untreated wild-type cultures still contained cells expressing *Mitf* and *Dct* while *Mitf* mutant cultures were negative for both markers. As shown in Fig. 7, Mgf addition increased the median number of *Dct*-positive cells by 1.7-fold in wild-type cultures but did not lead to the appearance of any *Dct*-positive cells in mutant cultures. This was expected if *Mitf* was necessary for *Dct* expression. Notably, however, addition of Mgf to *Mitf*$^{mi\text{-}ew}$ cultures did also not lead to the persistence of *Mitf*-expressing cells. This result suggested that even though such *Mitf*$^{mi\text{-}ew}$ cultures initially displayed *Kit*-positive, *Mitf*-expressing cells, addition of the corresponding bioactive ligand could not maintain them.

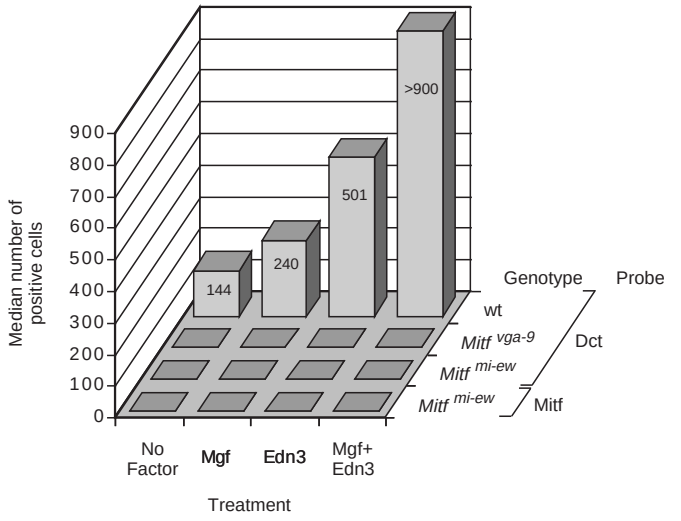

Fig. 7. Mgf and Edn3 do not rescue *Mitf* mutant neural crest-derived melanoblast precursors to the *Dct*-positive stage and do not promote maintenance of *Mitf*-positive cells. Control cultures received medium without added growth factors. Mgf was added at 0.5 nM, and Edn3 at 1 nM. In situ hybridization was performed 4 days after explantation and the positive cells were counted. The values correspond to the median number of cells derived from individual embryos with the following absolute values: for wild type: control, 122, 144, 160; Mgf, 180, 240, 367; Edn3, 329, 500, 502, 570; and Mgf/Edn3, 637, >900, >900, >900. The median number was chosen because the combined treatment produced numbers so high (>900) that in some areas of the culture, individual positive cells could no longer be clearly distinguished from each other. The numbers for triplicate or quadruplicate $Mitf^{mi\text{-}ew}$ cultures was 0 in all conditions. Thus, at 4 days, *Mitf*- or *Dct*-positive cells can only be detected in wild-type but not in *Mitf* mutant cultures.

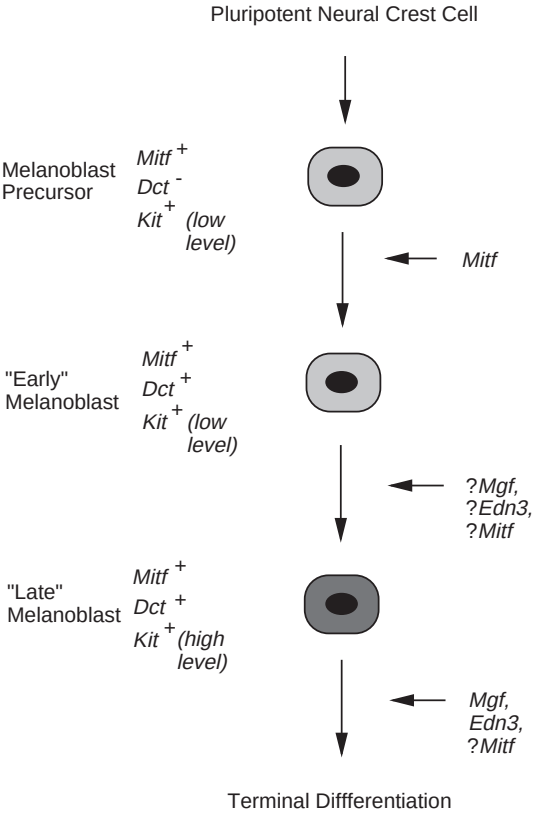

Fig. 8. Diagram illustrating the role(s) of *Mitf* in melanocyte development. A pluripotent neural crest cell gives rise to a melanoblast precursor that may be defined as a cell expressing *Mitf* and *Kit* but not *Dct*. This cell then develops into an 'early' melanoblast that is $Mitf^{+}$, Kit^{+} (low level) and Dct^{+}. Be it under further regulation by *Mitf*, or be it a late and indirect consequence of earlier functions of *Mitf*, the cell then upregulates *Kit* and becomes a 'late' melanoblast. In *Mitf* mutant cells, *Dct* is not turned on and *Kit* is not upregulated. This blocks the development of the lineage either at the melanoblast precursor stage or at an aberrant, Dct^{-} 'early' melanoblast stage. The model is consistent with the possibility that *Mitf* might be important for the specification of an uncommitted neural crest cells to enter the melanocyte differentiation pathway.

Edn3, the ligand for Ednrb, has recently been found to enhance the number of melanocytes in avian or murine neural crest cultures (Lahav et al., 1996; Reid et al., 1996). We, therefore, also tested whether addition of Edn3 might overcome the lack of functional *Mitf*. As shown in Fig. 7, addition of Edn3 at 1 nM (a dose chosen after establishing a dose/response curve as mentioned for Mgf) to wild-type cultures between day 0-4 increased the median number of *Dct*-positive cells 3.5-fold, and the combination of Edn3 with Mgf increased this number further to >6.25-fold. Nevertheless, at day 4, no *Dct*- or *Mitf*-positive cells were ever found in similarly treated $Mitf^{mi\text{-}ew}$ cultures. Thus, the block in melanocyte development as observed in *Mitf* mutant cultures could not be overcome by addition of two potent growth factors that regulate melanocyte development both in vivo and in vitro.

DISCUSSION

We have demonstrated that the basic-helix-loop-helix-zipper transcription factor gene *Mitf* is expressed in isolated cells that are located in the characteristic dorsolateral migration pathway of the trunk neural crest which normally gives rise to melanocytes. Identification of these cells as part of the melanocyte lineage rests on the facts that they coexpress *Kit* and the melanoblast marker *Dct* and that in embryos with mutations in *Mitf*, Dct^{+} melanoblasts and mature melanocytes are absent. Nevertheless, in mutant embryos, *Mitf*-expressing cells may appear, but their numbers are extremely small and their presence is extremely short-lived. These latter observations necessitated an analysis of these cells in an appropriate neural crest cell culture system. In fact, the cultures faithfully reproduced the in vivo development and allowed us to follow the cells more easily. Wild type cells started to express *Mitf* at day 1 in culture, corresponding to E10.5, the time point at which *Mitf*-positive cells first appeared in the trunk area in vivo. These cells coexpressed *Kit* and *Dct*. They also showed increased proliferation and/or survival in response to Mgf and Edn3 whose absence severely impairs melanocyte development in vivo. Finally, terminal differentiation into pigmented cells was also observed. Cultures prepared from $Mitf^{mi\text{-}ew}$ embryos likewise generated *Mitf*-positive and *Mitf/Kit* double-positive cells, but these cells were not maintained, did not respond to growth factors, did not express *Dct* (except for a small number once observed in a single dish), and pigmented

cells were never generated. The results suggested that the cells in mutant cultures, much as the corresponding cells in vivo, were blocked in their development at an early stage. We do not know at present, however, whether they died out or assumed (or have the potential to assume) alternative differentiation pathways.

The results can be interpreted as schematically depicted in Fig. 8. In wild type, a pluripotent neural crest cell gives rise to a melanoblast precursor that expresses *Mitf* and *Kit*. Soon thereafter, while continuing to express these markers, the cell also starts to express *Dct* and thus displays the hallmarks of a melanoblast. As time progresses, *Kit* expression increases, prompting us to name the melanoblast that expresses Kit at a low level an 'early' melanoblast, and the one that expresses Kit at a higher level a 'late' melanoblast. This interpretation makes no a priori assumptions as to the lineage relationships of these cells and whether the phenotypic transitions require cell division or the action of external factors; it predicts, however, that the late melanoblast would be the one responding best to Mgf. In the *Mitf* mutant, the developmentally arrested cell shares the phenotype of the above melanoblast precursor – *Mitf/Kit* double positivity and *Dct* negativity – and it remains Kit^{low}. However, the limited set of available markers do not allow us to determine whether this cell truely represents a melanoblast precursor or, alternatively, has proceeded to a stage more akin to an early melanoblast, albeit an aberrant one that is negative for *Dct*. Nevertheless, the phenotype of this cell suggests that *Mitf* plays a dual role, inducing *Dct* expression and upregulating *Kit*, and that the ultimate demise of the mutant cell is a consequence of insufficient levels of *Kit*. This view is supported by the following three observations. (1) The human *Dct* promoter contains two E-box elements that are necessary for transcriptional activity and are potential binding sites for *Mitf* (Yokoyama et al., 1994). (2) The *Kit* promoter also contains an E-box element that interacts with Mitf (Tsujimura et al., 1996). (3) The lack of Kit or its neutralization with antibodies interferes with proper melanocyte development (Reid et al., 1995; Yoshida et al., 1996). However, whether *Dct* and *Kit* are direct target genes of *Mitf* is not known at present. In fact, the following considerations might suggest that they are not, at least not in all stages of development.

First, while in neural crest cells *Dct* expression critically depends on functional *Mitf*, it apparently does not in retinal pigment cells (unpublished observation). Thus, it is conceivable that the lack of *Dct* expression in the mutant neural crest is but an additional, indirect consequence of the stage at which the cells become arrested.

Second, while upregulation of *Kit* did not occur in mutant cells, the onset of its expression, as shown by in situ hybridization and immunolabeling, was independent of *Mitf*, both in cells that at a given time express *Mitf*, and those that do not. Thus, in these cells, and at these stages of development, *Kit* does not appear to be a crucial target gene of *Mitf*. It is noteworthy to compare such an interpretation with one prompted by the study of *Mitf* mutant mast cells (Tsujimura et al., 1996). Adult mutant mast cells are low in *Kit* expression and do not respond to Mgf, but both *Kit* expression and response to Mgf can be rescued by over-expression of wild-type *Mitf*, suggesting that *Mitf* plays a role in the regulation of *Kit* (Ebi et al., 1992; Tsujimura et al., 1996). However, in adult wild-type mast cells, *Mitf* expression may be barely detectable while *Kit* expression may be high (Tsujimura et al., 1996). Thus, in wild-type cells, *Mitf* may not be critical for *Kit* expression and, hence, experimental over-expression in mutant cells may not probe the normal function of *Mitf*. Rather, wild-type and mutant mast cells may represent two different developmental stages, each one operating under its own transcriptional requirements for *Kit* expression. Also, in earlier experiments, expression of *Fms*, a tyrosine kinase receptor that rescues *Kit* mutant mast cells, could not rescue *Mitf* mutant mast cells when exposed to the corresponding ligand (Dubreuil et al., 1991). This observation suggests that dysregulation of *Kit* is not the only defect in the *Mitf* mutant mast cells. Thus, it is likely that experimentally expressing *Kit* at higher levels might not rescue *Mitf* mutant neural crest cells. Similar reasoning may apply to *Ednrb*. This interpretation is consistent with the observation that exposing the mutant cells to Mgf and Edn3 did not rescue them. Even though these growth factors potently increased the number of Dct^+ cells beyond day 4 in wild-type cultures, in mutant cultures not a single Dct^+ cell was ever found beyond day 4, and not a single $Mitf^+$ cell ever persisted beyond day 4.

Since the onset of *Kit* expression did evidently not depend on *Mitf*, yet the two genes were coexpressed in melanoblasts, it is conceivable that *Mitf*, rather than regulating *Kit*, is regulated by *Kit* in these cells. However, this possibility is unlikely for the following reason. Embryos with mutations which severely hamper *Kit* signaling may still generate *Dct*-positive neural crest cells (Cable et al., 1995; Wehrle-Haller and Weston, 1995; Klüppel et al., 1997). Since our results, which are based on genetic evidence, indicate that *Mitf* is a prerequisite for cells to develop to the *Dct*-positive stage, it is unlikely that *Kit* mutations would enable cells to bypass this requirement. In similar ways, malfunctioning of the *Ednrb*-mediated signaling also still allows for generation of some *Dct*-positive cells, in fact even some differentiated melanocytes. Thus, although there is coexpression of *Kit* and *Mitf* and although both gene products are needed for proper development of melanocytes, the two proteins may initially be expressed independently of each other. This interpretation does not preclude, however, that Kit or Ednrb signaling may modulate the transcriptional activity of Mitf.

Our results are consistent with previous observations that *Kit* and *Ednrb*-mediated signaling are necessary for proliferation and/or survival of the lineage. As argued above, *Kit* is expressed early on in $Mitf^+$ cells but is not required for cells to develop to the Dct^+ stage, in contrast to *Mitf* which is required for this transition. Because *Mitf* is a member of a family of transcription factors known to control cell fate decisions, and because it acts early in development, it may in fact be involved in lineage specification and thus commit a pluripotent precursor cell to the melanocyte pathway. Alternatively, *Mitf* could play a role analogous to that of *Mash1* which promotes the differentiation of a committed neuronal precursor but does not commit a multipotent cell to the neuronal fate (Sommer et al., 1995).

In sum, if functional *Mitf* is lacking early on, the melanocyte lineage cannot be rescued and its development is aborted. This establishes that *Mitf* is a crucial factor required early on in the generation of neural crest-derived melanoblasts.

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