Cell-autonomous and cell non-autonomous signaling through endothelin receptor B during melanocyte development

Ling Hou[1,*], William J. Pavan[1], Myung K. Shin[2] and Heinz Arnheiter[3]

[1]Genetic Disease Research Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892-4472, USA
[2]Cell and Developmental Biology Program, Fox Chase Cancer Center, Philadelphia, PA 19111, USA
[3]Laboratory of Developmental Neurogenetics, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA
*Author for correspondence (e-mail: lhou@mail.nih.gov)

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Summary

The endothelin receptor B gene (*Ednrb*) encodes a G-protein-coupled receptor that is expressed in a variety of cell types and is specifically required for the development of neural crest-derived melanocytes and enteric ganglia. In humans, mutations in this gene are associated with Waardenburg-Shah syndrome, a disorder characterized by pigmentation defects, deafness and megacolon. To address the question of whether melanocyte development depends entirely on a cell-autonomous action of *Ednrb*, we performed a series of tissue recombination experiments in vitro, using neural crest cell cultures from mouse embryos carrying a novel *Ednrb*-null allele characterized by the insertion of a *lacZ* marker gene. The results show that *Ednrb* is not required for the generation of early neural crest-derived melanoblasts but is required for the expression of the differentiation marker tyrosinase. Tyrosinase expression can be rescued, however, by the addition of *Ednrb* wild-type neural tubes. These *Ednrb* wild-type neural tubes need not be capable of generating melanocytes themselves, but must be capable of providing KIT ligand, the cognate ligand for the tyrosine kinase receptor KIT. In fact, soluble KIT ligand is sufficient to induce tyrosinase expression in *Ednrb*-deficient cultures. Nevertheless, these tyrosinase-expressing, *Ednrb*-deficient cells do not develop to terminally differentiated, pigmented melanocytes. Pigmentation can be induced, however, by treatment with tetradecanoyl phorbol acetate, which mimics EDNRB signaling, but not by treatment with endothelin 1, which stimulates the paralogous receptor EDNRA. The results suggest that *Ednrb* plays a significant role during melanocyte differentiation and effects melanocyte development by both cell non-autonomous and cell-autonomous signaling mechanisms.

Key words: G-protein-coupled receptor, Endothelin, Kit ligand, Mitf, Phorbol ester, Neurocristopathy, Neural crest cell culture, Tyrosinase, Melanogenesis

Introduction

The neural crest is a pluripotent population of cells that originates along the dorsal line of the developing neural tube and gives rise to a variety of cell types, including pigment cells of the skin, inner ear and choroid, peripheral nervous system cells, enteric ganglia, endocrine cells, most of the craniofacial skeletal and connective tissues, and parts of the heart outflow tracts (Le Douarin and Kalcheim, 1999). The generation of each of these cell types from neural crest stem cells depends on a complex interplay between cell-extrinsic and cell-intrinsic factors (Dorsky et al., 2000). Genetic evidence indicates that pigment cell development requires endothelin 3 (EDN3), a peptide ligand that along with two related ligands, endothelin 1 (EDN1) and endothelin 2 (EDN2), acts through the G-protein-coupled receptor endothelin receptor B (EDNRB) (Pla and Larue, 2003). It also requires KIT-ligand (KITL) (formerly known as Steel factor, stem cell factor or mast cell growth factor) that acts through the tyrosine kinase receptor KIT (Copeland et al., 1990; Geissler et al., 1988). Pigment cell development further depends on a set of transcription factors that include the SRY-like high mobility group protein SOX10 and the basic helix-loop-helix-leucine zipper protein MITF (Hodgkinson et al., 1993; Southard-Smith et al., 1998). Although interactions between signaling pathways and transcription regulation have to some extent been investigated in vitro (Goding, 2000), much needs to be learned about the interactions between distinct signaling pathways. The fact that melanocytes develop in an environment that is shared with many other cell lineages requires us to focus not only on signaling crosstalks within one cell type but also on crosstalks between different cell types. We address the question of whether EDNRB acts exclusively in melanoblasts themselves or effects melanocyte development also indirectly, by acting in other cell types.

It is well known that *Ednrb* is expressed in mouse melanoblasts (Lee et al., 2003; Opdecamp et al., 1998) and hence thought to act cell-autonomously (Hosoda et al., 1994), in contrast to its ligand EDN3 which is secreted and clearly works in a paracrine fashion during melanocyte development. Nevertheless, a study in chimeric mice composed of wild-type

and *Ednrb* null mutant cells (*Ednrb*$^{s\text{-}l}$, *piebald-lethal*) has suggested that *Ednrb* can act cell non-autonomously during enteric neuroblast development (Kapur et al., 1995). Because of a lack of suitable markers for mutant melanoblasts, however, this chimeric study did not allow conclusions as to the fate of the mutant pigment lineage in the wild type environment, and so the question of whether melanocyte development is likewise subject to cell non-autonomous actions of *Ednrb* remained unanswered.

In order to be able to track *Ednrb*-expressing pigment cells even when they are deficient in functional *Ednrb*, we here used a novel null allele, *Ednrb*^{lacZ}, that was obtained by targeted insertion of the bacterial *lacZ* marker gene into the first exon of mouse *Ednrb* (Lee et al., 2003). Although chimeric mice composed of wild-type and mutant cells, as mentioned above, usually provide a straightforward approach to test for cell non-autonomous gene actions, they may offer limited possibilities to further characterize the cell types and growth factors involved. This limitation prompted us to perform tissue recombination experiments in vitro, using neural crest cell cultures established from explanted mid-gestation neural tubes (NTs).

The experiments show that in the absence of functional *Ednrb*, the development of the melanocyte lineage is initiated but no tyrosinase-positive or mature melanocytes are being generated. The mutant cells can be rescued, however, by a two-step procedure whereby a first step, involving cell non-autonomous EDNRB signaling in cells other than melanoblasts, leads to tyrosinase expression but not pigmentation, and a second step, mimicking cell autonomous EDNRB signaling in melanoblasts, leads to pigmentation. The results imply that *Ednrb* plays a significant role in melanocyte differentiation at time points beyond the narrow developmental period between E10.5 and E12.5 through which it has been shown to be required in vivo (Shin et al., 1999), and they suggest that this later role is mediated by both cell-autonomous and cell non-autonomous signaling pathways.

Materials and methods

Mice and genotyping

The line of *Ednrb*^{lacZ} mice, in which the *lacZ* reporter gene is inserted into the *Ednrb* locus thereby creating a null allele, has been described (Lee et al., 2003). It was kept on a mixed C57BL/6//C3H/HeJ background. As *Ednrb*^{lacZ}/*Ednrb*^{lacZ} homozygous mice do not usually survive beyond weaning because of megacolon, matings were set up between *Ednrb*^{lacZ}/+ heterozygotes. To generate *Ednrb*^{lacZ} homozygotes that were also heterozygous for *Kit*^{lacZ}, we first crossed *Ednrb*^{lacZ}/+ mice with *Kit*^{lacZ}/+ mice (Bernex et al., 1996) and then intercrossed the double heterozygotes. Mice carrying the *Kitl*^{Sl} allele on a WC/ReJ background were obtained from Jackson Laboratories and bred with C57BL/6xC3H/HeJ F1 mice. Mice carrying the allele *Mitf*$^{mi\text{–}ew}$ on a Naw background were originally obtained form N. Jenkins/N. Copeland.

Noon on the day a vaginal plug was found was defined as embryonic day 0.5 (E0.5). Embryos were harvested at E9.5. The polymerase chain reaction (PCR) was used to genotype embryos, using the head portion as source of genomic DNA. Three oligonucleotides were used: Ednrb1, 5′-CCAGACTGAAAA-CAGCAGAGCGGC-3′ (forward); Ednrb2, 5′-GGTCTCCC-AGAGCCAGACTGGCGATA-3′ (reverse); and Ednrb-*lacZ*, 5′-CTGTTGGGAAGGGCGATCGGTGC-3′ (reverse). Because of a deletion associated with the targeted insertion, *Ednrb*^{lacZ}/*Ednrb*^{lacZ} embryos could be detected by the presence of a 270 bp band and the absence of a 495 bp band, wild-type embryos by the presence of the 495 bp band and the absence of the 270 bp band, and heterozygotes by the presence of both bands. To genotype embryos heterozygous or homozygous for *Kitl*^{Sl}, the oligonucleotides used were those described previously (Ono et al., 1998). For genotyping, genomic DNA was denatured (94°C for 3 minutes) in the presence of primers, and subjected to PCR in a 50 μl reaction for 30 cycles (94°C for 40 seconds, 55°C for 1 minute and 72°C for 1 minute) using Taq polymerase (Promega) and the manufacturer's buffer in the presence of 1.5 mM $MgCl_2$.

Neural tube explant cultures, in vitro reconstitutions and growth factor treatments

Embryos at E9.5 were obtained from the matings described above. Pure NT explants containing neural crest cells were isolated and cultured as described previously (Ito and Takeuchi, 1984). For most experiments, culture medium consisted of 90% DMEM, 1 mM L-glutamine, 1 mM penicillin-streptomycin, 10% FBS and EDN3 (Sigma) at 10 nM. When mentioned, KITL (R&D Systems) was added at 5 nM, 12-O-Tetradecanoylphorbol 13-acetate (TPA) at 40 nM and endothelin 1 (EDN1, Sigma) at 40 nM. For in vitro reconstitution experiments, embryonic NT explants were isolated at E9.5 from the following matings: +/+ × +/+; *Ednrb*^{lacZ}/+ × *Ednrb*^{lacZ}/+; *Mitf*$^{mi\text{–}ew}$/*Mitf*$^{mi\text{–}ew}$ × *Mitf*$^{mi\text{–}ew}$/*Mitf*$^{mi\text{–}ew}$; or *Kitl*^{Sl}/+ × *Kitl*^{Sl}/+. Neural crest cell cultures were established separately for each embryo and the embryos were genotyped. Twenty-four hours later, NTs from *Ednrb*^{lacZ}/*Ednrb*^{lacZ} cultures were removed and replaced with NTs from either wild type, *Ednrb*^{lacZ}/*Ednrb*^{lacZ}, *Mitf*$^{mi\text{–}ew}$/*Mitf*$^{mi\text{–}ew}$ or *Kitl*^{Sl}/*Kitl*^{Sl} cultures. For each reconstitution experiment, the medium contained 10 nM EDN3.

Antibodies and immunostaining

At various days in culture, neural crest cells were fixed in 4% formaldehyde in PBS (pH 7.5) for 25 minutes at room temperature, and then permeabilized with 0.1% Triton-X-100 for 5 minutes. For double indirect immunolabeling, the cells were incubated with rabbit anti-Mitf (Opdecamp et al., 1997) or anti-tyrosinase antisera (Vincent Hearing) for 60 minutes, respectively, and then with anti-β-galactosidase (β-gal) monoclonal antibody (Promega) for 30 minutes. These antibodies were revealed with RITC-coupled goat anti-rabbit $(Fab)_2$ and FITC-coupled goat anti-mouse $(Fab)_2$.

Identification of β-gal-positive cells and pigmented melanocytes

The cultured neural crest cells were fixed at 14 days in 4% formaldehyde in PBS. X-gal staining was performed in X-gal buffer (2 mM $MgCl_2$, 0.02% NP-40, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, 0.05% X-gal in PBS, pH 7.5), and postfixed in 4% formaldehyde. The appearance of melanocytes was examined at various time points in live cultures. Their differentiation from neural crest cells was judged by the presence of melanin granules (one of the markers of mature melanocytes) and the characteristic dendritic morphology under phase-contrast and bright-field microscopy.

Results

Melanocyte differentiation depends on *Ednrb*

To be able to experimentally manipulate the development of *Ednrb* mutant melanocytes, we first established an in vitro culture system using murine trunk neural tubes (NTs) harvested from embryos at E9.5, a stage at which melanoblasts have not yet emigrated from the neural crest. As shown in Fig. 1A, EDN3 promoted the generation of pigmented cells from *Ednrb*^{lacZ}/+ NTs, as found previously with wild-type NTs (Hou et al., 2000; Reid et al., 1996). When stained for the expression

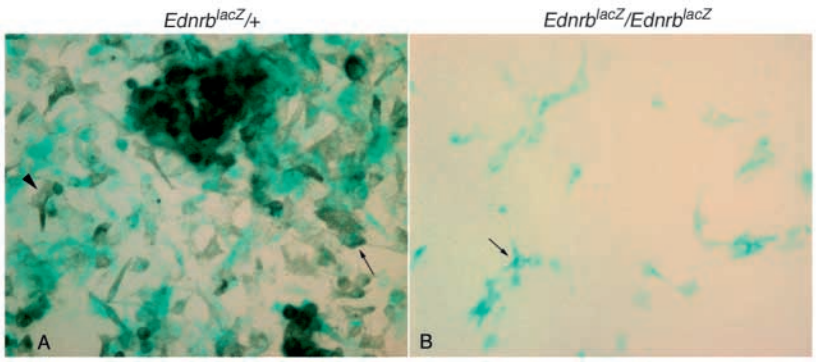

Fig. 1. Lack of melanocyte differentiation in *Ednrb*^{lacZ}/*Ednrb*^{lacZ} neural crest cells in culture. Primary cultures from embryos of the indicated genotypes were established and kept in the presence of EDN3 for 2 weeks before fixation. X-gal staining was then performed and the cultures were photographed in bright field to show melanin and β-gal staining. (A) *Ednrb*^{lacZ}/+ cultures show melanin-positive cells, many of which co-express β-gal (arrow) although some lack clear β-gal staining (arrowhead). (B) *Ednrb*^{lacZ}/*Ednrb*^{lacZ} cultures lack melanin-positive cells, although β-gal-positive cells are present (arrow).

of β-gal, 14-day-old *Ednrb*^{lacZ}/+ cultures showed β-gal-positive cells that were pigmented (Fig. 1A, arrow), along with some that lacked pigmentation and some that were pigmented but lacked clear β-gal staining (Fig. 1A, arrowhead). These latter cells may reflect a progressive downregulation of *Ednrb* in mature melanocytes. By contrast, in cultures established from *Ednrb*^{lacZ}/*Ednrb*^{lacZ} NTs, no pigmented cells were seen, although β-gal-positive cells survived (Fig. 1B). These β-gal-positive cells lacked characteristic melanoblast markers, including MITF (see below), and probably represent cells of other lineages. The results indicate that in vitro, *Ednrb* is required for the generation of melanocytes and suggest that *Ednrb* is expressed in the melanocyte lineage beyond E12.5, i.e. beyond the developmental period through which it has been shown to be important in melanocyte development in vivo (Shin et al., 1999).

The onset of expression of early melanoblast markers does not depend on *Ednrb*

To determine up to which stage *Ednrb*^{lacZ}/*Ednrb*^{lacZ} cells progress during in vitro development, we stained NT explant cultures for markers that are sequentially expressed in the melanocyte lineage (Hou et al., 2000). The transcription factor MITF, for example, normally begins to be expressed in melanoblasts at E10.5 and is required for the development of the melanocyte lineage (Hodgkinson et al., 1993; Hou et al., 2000; Nakayama et al., 1998; Opdecamp et al., 1997). As shown in Fig. 2A,B, after 2 days of in vitro culture in the absence of EDN3, MITF protein is expressed in the nuclei of β-gal-positive cells, regardless of whether they were derived from *Ednrb*^{lacZ}/+ or *Ednrb*^{lacZ}/*Ednrb*^{lacZ} embryos. Not surprisingly, the transcription factor SOX10, which is needed for *Mitf* expression and plays crucial roles in the development of neural crest derivatives (Potterf et al., 2000; Potterf et al., 2001; Southard-Smith et al., 1998; Verastegui et al., 2000), is also normally expressed in both β-gal-positive *Ednrb*^{lacZ}/+ and *Ednrb*^{lacZ}/*Ednrb*^{lacZ} cells (not shown). These results are consistent with the previous observation by whole-mount in situ hybridization that the early melanoblast marker dopachrome tautomerase (*Dct*) is expressed in *Ednrb*^{lacZ}/*Ednrb*^{lacZ} embryos (Lee et al., 2003) and suggest that *Ednrb* is not required for the initiation of melanoblast development.

We then tested for the expression of the tyrosine kinase receptor KIT, an additional marker characteristic of early melanoblasts. As direct antibody labeling of KIT may not give high level signals and requires fixation criteria that are not easily compatible with double-labeling for other antigens (Opdecamp et al., 1997), we decided to test for KIT expression in an indirect way, using *Kit*^{lacZ} mice in which sequences encoding a nuclear β-gal have been inserted into the *Kit* gene (Bernex et al., 1996). By crossing *Ednrb*^{lacZ} and *Kit*^{lacZ} mice, we obtained doubly marked mice in which the two signals are distinct in that the *Ednrb*-derived β-gal is cytoplasmic and the *Kit*-derived β-gal is nuclear. As shown in Fig. 2C,D, double nuclear/cytoplasmic β-gal is visible independent of whether the cells were derived from NTs that were heterozygous or homozygous at *Ednrb*^{lacZ}. Thus, as for the onset of MITF or SOX10 expression, *Ednrb* is also not required for the onset of KIT expression.

Despite the fact that lineage development is initiated in the absence of functional *Ednrb*, the cultures do not generate pigmented cells. As the rate-limiting step in pigmentation is tyrosinase activity, we reasoned that cells devoid of functional *Ednrb* might not reach the tyrosinase-positive stage. Indeed, double-labeling for β-gal and tyrosinase showed that even after 14 days in culture, i.e. 8 days after the usual onset of tyrosinase expression (Hou et al., 2000), *Ednrb*^{lacZ}/*Ednrb*^{lacZ} NTs did not generate tyrosinase-positive cells, while *Ednrb*^{lacZ}/+ NTs did (Fig. 2E,F). This result suggested that in vitro, *Ednrb* signaling is required for the development of cells to the differentiated, tyrosinase-positive stage and hence explains why *Ednrb*^{lacZ}/*Ednrb*^{lacZ} cells never become pigmented.

Ednrb$^{+/+}$ NTs induce melanoblast differentiation in *Ednrb*^{lacZ}/*Ednrb*^{lacZ} neural crest cells

To test whether *Ednrb*-deficient melanoblasts may be rescued in the presence of wild-type NT or neural crest cells, we performed in vitro recombination experiments in which the *Ednrb*^{lacZ}/*Ednrb*^{lacZ} NTs, after a 24 hour culture period during which the neural crest cells emigrated, were removed and replaced with *Ednrb*$^{+/+}$ NTs. For these experiments, we used *Ednrb*$^{+/+}$ NTs from embryos homozygous for the *Mitf* allele, *Mitf*$^{mi–ew}$, which cannot generate melanoblasts that survive for more than 24-48 hours and hence neither contain tyrosinase-positive nor pigmented cells (Opdecamp et al., 1997). In this way, we could avoid the potential complication that a transfer of melanosomes from pigmented *Ednrb*$^{+/+}$ cells to the non-pigmented *Ednrb*^{lacZ}/*Ednrb*^{lacZ} cells might have created. As a control, *Ednrb*^{lacZ}/*Ednrb*^{lacZ} NTs were exchanged for *Ednrb*^{lacZ}/*Ednrb*^{lacZ} NTs obtained from parallel cultures, and, as expected under these conditions, no tyrosinase-positive cells were seen (Fig. 3A). The reconstitution with

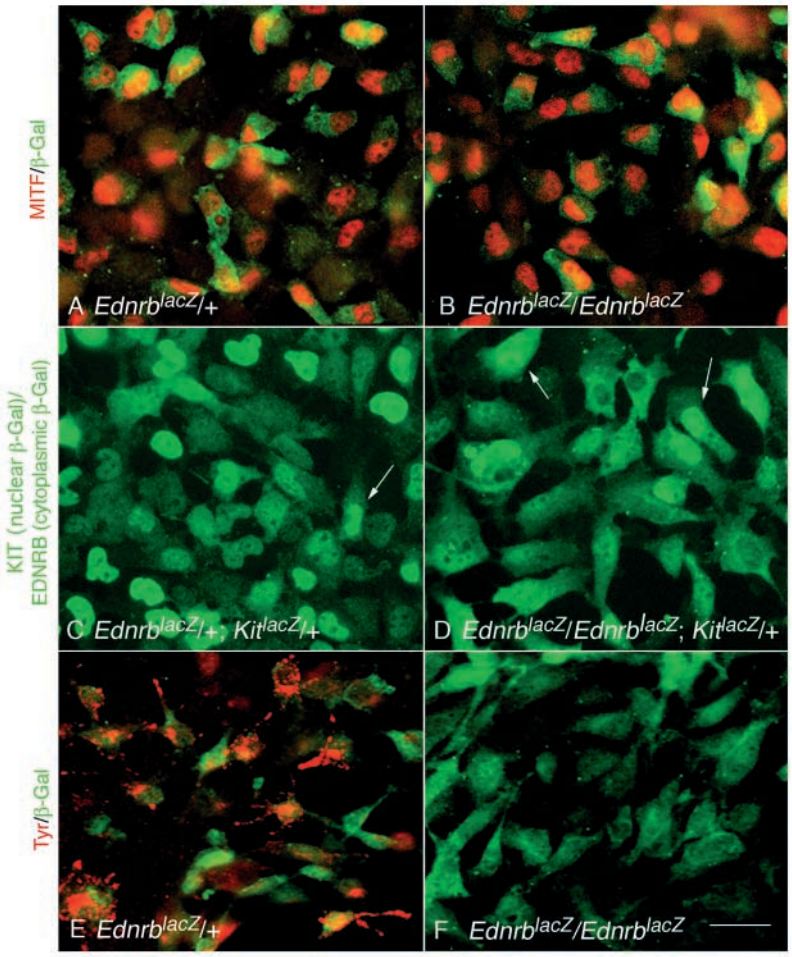

Fig. 2. Expression of MITF and KIT does not depend on *Ednrb*, whereas that of tyrosinase does. Neural crest cultures were established from embryos of the indicated genotypes and kept in the absence of EDN3 for 2 days (A-D) or in the presence of EDN3 for 2 weeks (E,F). They were then subjected to double indirect immunofluorescent labeling using MITF- and β-gal-specific antibodies (A,B), β-gal-specific antibody alone (C,D), or tyrosinase- and β-gal-specific antibodies (E,F). (A,B) Merged images show that regardless of the *Ednrb* genotype, MITF/β-gal double-positive cells were generated. (C,D) Regardless of the *Ednrb* genotype, cells double-positive for cytoplasmic β-gal (marking *Ednrb* expression) and nuclear β-gal (marking *Kit* expression) were generated (arrows). Note that in cultures from Kit^{lacZ}/+ embryos, β-gal staining is confined to the nucleus (Hou et al., 2000) (data not shown), and in cultures from $Ednrb^{lacZ}$/+ embryos, to the cytoplasm. (E,F) Merged images show Tyr/β-gal double-positive cells along with β-gal single-positive cells in $Ednrb^{lacZ}$/+ cultures (E) but only β-gal single-positive cells in $Ednrb^{lacZ}/Ednrb^{lacZ}$ cultures (H). Scale bar: 25 μm for A,B,E,F; 20 μm for C,D.

$Mitf^{mi-ew}/Mitf^{mi-ew}$ NTs, however, led to the generation of β-gal/tyrosinase double-positive cells in $Ednrb^{lacZ}/Ednrb^{lacZ}$ cultures (Fig. 3B). This result suggested that the *Ednrb*-positive neural crest cells provided an exogenous signal capable of rescuing tyrosinase expression in the co-cultured *Ednrb*-negative cells, and that melanocytes were not required for this rescue. Nevertheless, the possibility, however remote, that at least some of the double-positive cells might have been the product of cell fusions cannot formally be excluded with this experiment.

It has been shown previously that KIT signaling, much as EDNRB signaling, is a crucial regulator of melanocyte development (Copeland et al., 1990; Geissler et al., 1988). In vitro, *Kitl*-deficient ($Kitl^{Sl}/Kitl^{Sl}$) NT explants do not support the generation of melanocytes (Ono et al., 1998), and *Kit*-deficient (Kit^{lacZ}/Kit^{lacZ}) explants do not give rise to tyrosinase-expressing melanoblasts and do not generate pigmented cells (Hou et al., 2000). These observations prompted us to test whether $Kitl^{Sl}/Kitl^{Sl}$ NTs, which carry a wild-type *Ednrb* locus but lack KITL, would rescue $Ednrb^{lacZ}/Ednrb^{lacZ}$ melanocyte precursors. Consistent with previous results (Ono et al., 1998), NT explants from E9.5 $Kitl^{Sl}/Kitl^{Sl}$ embryos neither generated tyrosinase-positive nor pigmented cells, even after prolonged periods in culture and in the presence of EDN3 (not shown). The addition of $Kitl^{Sl}/Kitl^{Sl}$ NTs to $Ednrb^{lacZ}/Ednrb^{lacZ}$ cultures indeed showed that in contrast to *Kitl*-positive $Mitf^{mi-ew}/Mitf^{mi-ew}$ NTs, *Kitl*-deficient NTs do not allow for *Ednrb*-deficient cells to become tyrosinase positive (Fig. 3C). As there is no reason to assume that the conditions for potential cell fusion in these co-cultures was any different from those in co-cultures between $Mitf^{mi-ew}/Mitf^{mi-ew}$ + $Ednrb^{lacZ}/Ednrb^{lacZ}$ cells, cell fusion can safely be excluded as an explanation for the rescue in the latter cultures. Furthermore, we can conclude that even though the *Ednrb*-deficient cultures carried a wild-type *Kitl* locus, their production of KITL was apparently neither sufficient to rescue their own melanocyte precursors nor, conversely, those derived from the *Kitl*-deficient cultures. Hence, *Ednrb* signaling is required for appropriate KITL expression in these cultures, and KITL is required for tyrosinase expression in *Ednrb*-deficient melanoblasts.

KITL is both required and sufficient to rescue $Ednrb^{lacZ}/Ednrb^{lacZ}$ melanocyte precursors to the tyrosinase-positive stage

As the above reconstitution experiments suggested that KITL is required to allow tyrosinase expression in *Ednrb*-negative melanocyte precursors, we then asked whether it was sufficient. In fact, we found that the treatment of $Ednrb^{lacZ}/Ednrb^{lacZ}$ cultures with soluble KITL from the day of NT explantation led to the generation of tyrosinase-expressing cells (Fig. 4). Thus, KITL was both required and sufficient to promote the differentiation of *Ednrb*-deficient melanocyte precursors.

Rescue of pigmentation

Although the above results demonstrated a cell non-autonomous rescue of *Ednrb*-negative melanocyte precursors to the tyrosinase-positive stage, differentiation to mature, pigmented melanocytes was not observed, either in co-cultures or after the addition of KITL. This is probably not due to the possibility that KITL or other rescue factors were not present

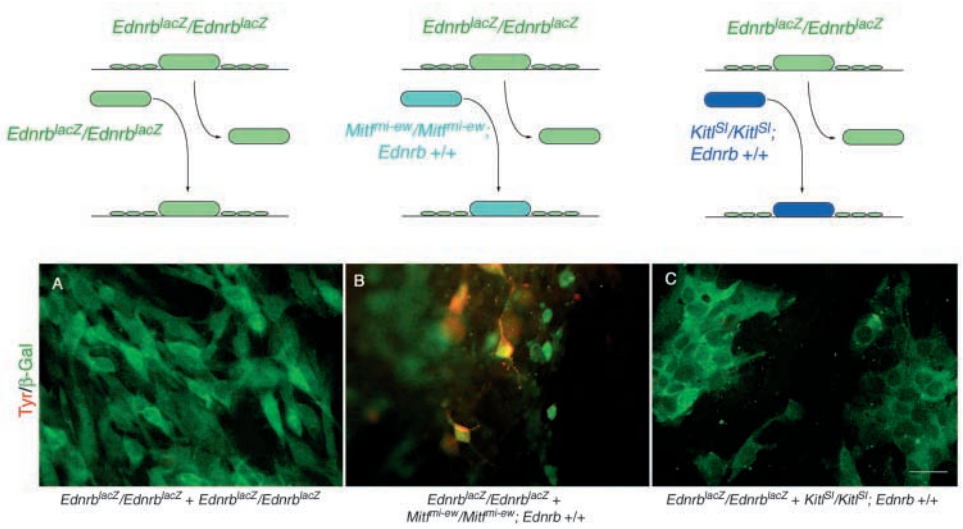

Fig. 3. *Ednrb* wild-type neural tubes (NTs) induce tyrosinase expression in *Ednrb*-deficient cultures. (Top) Tissue recombination experiments. In a first step, E9.5 NT explants were isolated from ($Ednrb^{lacZ}$/+ × $Ednrb^{lacZ}$/+) matings as well as from [($Mitf^{mi-ew}$/$Mitf^{mi-ew}$; $Ednrb^{+/+}$) × ($Mitf^{mi-ew}$/$Mitf^{mi-ew}$; $Ednrb^{+/+}$)] or [($Kitl^{Sl}$/+; $Ednrb^{+/+}$) × ($Kitl^{Sl}$/+; $Ednrb^{+/+}$)] matings. The NTs were placed separately into individual dishes and the corresponding embryos were genotyped. On the following day, the NTs from identified $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ cultures were removed and replaced with NTs from the respective $Ednrb^{+/+}$ cultures. As a control, NTs from $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ cultures were given to other $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ cultures whose own NTs had been removed. The cultures were kept for 2 weeks and then stained with β-gal and tyrosinase antibodies as described in the legend for Fig. 2 and the Materials and methods section. (Bottom panel) (A) $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ culture control-reconstituted with $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ NT. (B) $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ culture reconstituted with ($Mitf^{mi-ew}$/$Mitf^{mi-ew}$; $Ednrb^{+/+}$) NT. (C) $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ culture reconstituted with ($Kitl^{Sl}$/$Kitl^{Sl}$; $Ednrb^{+/+}$) NT. $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ cultures always generated β-gal-positive cells. However, only ($Mitf^{mi-ew}$/$Mitf^{mi-ew}$; $Ednrb^{+/+}$) NTs (B), but not $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ NTs (A) or ($Kitl^{Sl}$/$Kitl^{Sl}$; $Ednrb^{+/+}$) NTs (C), led to the generation of tyrosinase-positive cells. Neither ($Mitf^{mi-ew}$/$Mitf^{mi-ew}$; $Ednrb^{+/+}$) nor ($Kitl^{Sl}$/$Kitl^{Sl}$; $Ednrb^{+/+}$) cultures carry the *lacZ* marker, nor are they capable of generating tyrosinase-positive cells on their own (Hou et al., 2000) (this paper), indicating that Tyr/β-gal double-positive cells in B are derived from the $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ culture. Scale bar: 25 μm.

at high enough concentrations. Rather, it suggests that EDNRB signaling has an additional, obligatory cell-autonomous role in terminal cell differentiation. EDNRB signaling is known to activate PKC, which in turn activates tyrosinase (Park et al., 1999). As TPA activates PKC (Oka et al., 1996), as does EDNRB signaling, we tested whether TPA might rescue pigmentation in KITL-treated, *Ednrb*-deficient cultures. As shown in Fig. 5A, after 6 days in the presence of KITL and another 8 days in the presence of both KITL and TPA, $Ednrb^{lacZ}$/+ control cultures generated pigmented cells, consistent with previous results with wild-type cells (Murphy et al., 1992). In *Ednrb*-deficient cultures, despite the presence of KITL, no pigmented cells appeared if TPA was omitted (Fig. 5B). By contrast, when TPA was added at 6 days, mature melanocytes were generated during the following 8 days (Fig. 5C). Thus, TPA can rescue pigmentation in KITL-treated, *Ednrb*-deficient cells.

It has been suggested earlier that signaling through the paralogous G-coupled receptor EDNRA, which responds preferentially to endothelin 1 (EDN1) and endothelin 2, but has no evident role in early melanoblast development (Opdecamp et al., 1998), could possibly account for pigmentation at later stages (Reid et al., 1996). We, therefore, also tested whether EDN1 might induce pigmented cells in KITL-treated, $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ cultures. Whereas 40 nM of EDN1 (which can interact equally well with EDNRA and EDNRB) was perfectly able to induce pigmented cells in $Ednrb^{lacZ}$/+ cultures, it was unable to do so in $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ cultures (Fig. 5D,E). This suggests that in these cells, signaling through EDNRA is either quantitatively insufficient or qualitatively distinct from signaling through EDNRB. Taken together, the above experiments suggest that while tyrosinase-positive melanoblasts can be generated through cell non-autonomous signaling pathways, terminal cell differentiation still seems to require *Ednrb* signaling or a drug like TPA that mimics EDNRB signaling.

Discussion

The analysis of the in vitro development of *lacZ*-marked, *Ednrb*-deficient melanocyte precursors allowed us to reach four main conclusions. First, regardless of the presence or

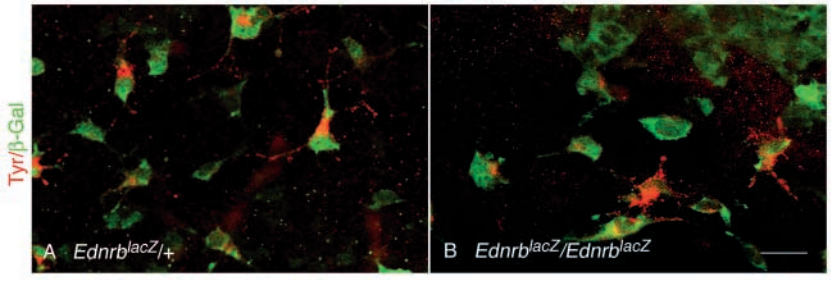

Fig. 4. KITL rescues melanoblast differentiation in $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ neural crest cells. Primary cultures from embryos of the indicated genotypes were established and kept in the presence of KITL for 2 weeks before fixation. The cultures were then double-labeled for β-gal and tyrosinase as described in Fig. 2. Note the presence of Tyr/β-gal double-positive cells in KITL-treated cultures, regardless of whether they were derived from $Ednrb^{lacZ}$/+ (A) or $Ednrb^{lacZ}$/$Ednrb^{lacZ}$ embryos (B). Scale bar: 25 μm.

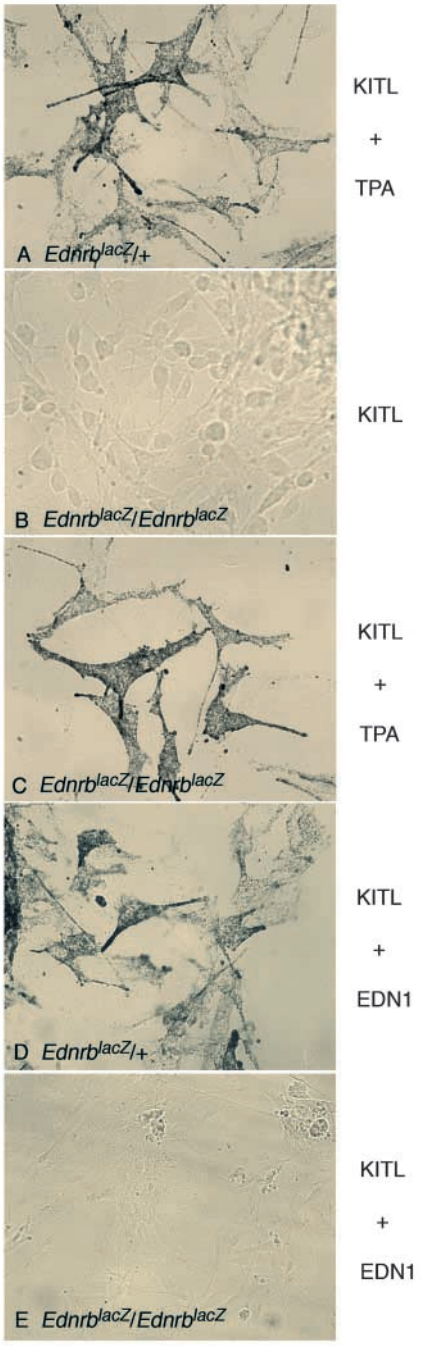

Fig. 5. TPA but not EDN1 rescues pigmentation in KITL-treated, tyrosinase-positive *Ednrb*-deficient melanoblasts. (A-C) Cultures from embryos with the indicated genotypes were kept in the presence of KITL for 6 days, and then in the presence of KITL and TPA (A,C) or KITL alone (B) for another 8 days. Mature melanocytes are present in TPA-treated $Ednrb^{lacZ}/+$ and $Ednrb^{lacZ}/Ednrb^{lacZ}$ cultures (A,C) but absent in melanocytes in $Ednrb^{lacZ}/Ednrb^{lacZ}$ cultures when TPA was omitted (B). (D,E) Cultures of the indicated genotypes were treated with KITL and EDN1 for 14 days. Pigmented cells are not present in $Ednrb^{lacZ}/Ednrb^{lacZ}$ cultures (E).

absence of functional *Ednrb*, cells expressing early lineage markers such as SOX10, MITF and KIT are generated normally, indicating that EDNRB signaling has no instructive role in melanocyte development. Second, the *Ednrb* mutant cells do not express the melanogenic enzyme tyrosinase and hence are blocked in cell differentiation. Third, the mutant cells can be differentiated to express tyrosinase, if a source of KITL is provided. KITL can be added directly to the medium or can come from co-cultured neural tubes or neural crest cells that are wild type at *Ednrb*. This suggests that *Ednrb* signaling stimulates the synthesis and/or secretion of KITL and hence is capable of inducing melanocyte differentiation indirectly. Fourth, the presence of *Ednrb* wild-type cells, or of KITL, does not allow the tyrosinase-expressing, *Ednrb*-deficient cells to become pigmented. Pigmentation can be induced, however, by stimulating PKC, a downstream signal of *Ednrb*, suggesting that the activation of tyrosinase is a cell-autonomous function of *Ednrb*.

At first sight, a role for *Ednrb* in melanocyte differentiation would seem to contradict previous in vivo findings that showed, based on conditional downregulation, that *Ednrb* is no longer needed for melanocyte development after E12.5 (Shin et al., 1999); at this developmental time point, tyrosinase is not yet expressed, at least not in the trunk area, and pigmentation also is not seen until several days later. We have to consider, though, that our in vitro results are based on analyzing isolated NT and neural crest cells, not intact embryos. Conceivably, the role of *Ednrb* in melanocyte differentiation may not be revealed in vivo because of compensatory mechanisms that operate efficiently only in the embryo. Thus, parallel signaling pathways, whether acting cell-autonomously or not, might substitute for the lack of *Ednrb* beyond E12.5 in vivo. In fact, *Ednrb*-deficient mice can have pigmented spots on the head and at the base of the tail, indicating that in vivo, melanocytes can be generated, albeit rarely, in the complete absence of *Ednrb*. The fact that, in vitro, conditions can be found that rescue *Ednrb*-negative cells to the fully mature, pigmented stage is entirely consistent with the appearance of these pigmented spots in vivo, and suggests that they are derived from a few somatically variant precursor cells that have crossed a threshold of sensitivity to rescuing pathways that are similar, or identical, to those we have identified in vitro. Although it was believed that signaling through EDNRA might be responsible for these pigment spots (Reid et al., 1996), this is unlikely, because our in vitro studies with EDN1 provide no evidence for a role for EDNRA in pigmentation.

There are several alternative explanations for the seeming discrepancy between a role for EDNRB beyond the equivalent of E12.5 in vitro and not in vivo. It is possible, for example, that the conditional downregulation of the *Ednrb* mRNA in vivo was not followed by an equally rapid downregulation of EDNRB protein or of its activated downstream targets. In addition, the conditional downregulation of *Ednrb*, even if complete in melanoblasts, may not have been complete in other cell types, and incomplete downregulation in other cell types may then have added indirectly to the rescue of melanocytes as suggested by our in vitro analysis.

The cell non-autonomous action of *Ednrb* during melanocyte development lends support to previous observations in chimeric mice in which a wild-type environment helped *Ednrb* mutant enteric neuroblasts to colonize the large intestine (Kapur et al., 1995). The details of the underlying mechanisms may differ, though, as it has been suggested that EDNRB signaling may normally delay

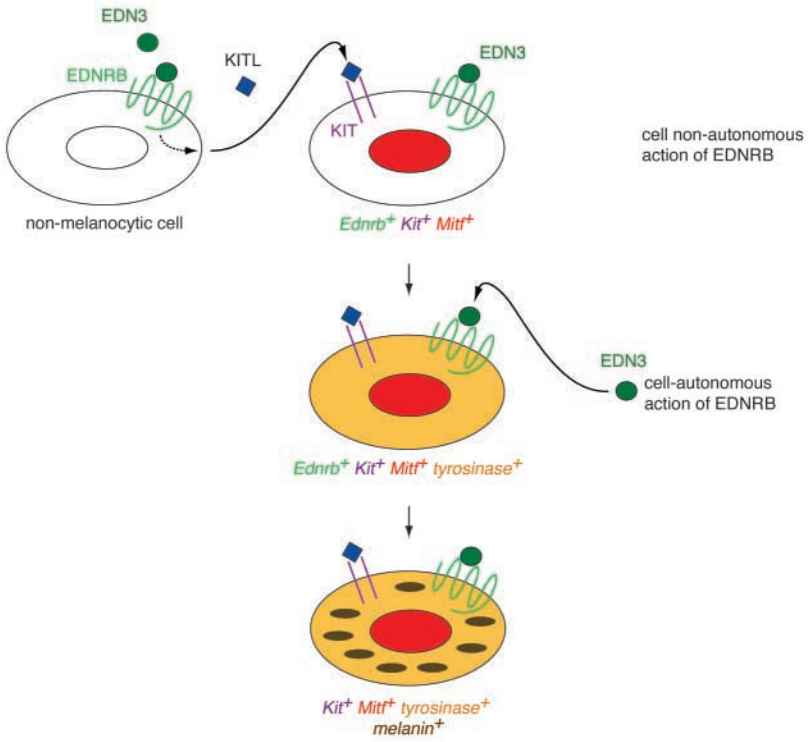

Fig. 6. The effect of EDNRB signaling on melanocyte development both cell-autonomously and cell non-autonomously. Both melanoblasts and non-melanogenic NT or neural crest cells express *Ednrb*. As the addition of *Ednrb*$^{+/+}$ NTs can induce tyrosinase expression in *Ednrb*-deficient melanoblasts, we postulate that in response to EDN3, these *Ednrb*$^{+/+}$ NTs or their neural crest derivatives produce one or several factors that indirectly help melanoblasts to differentiate. As these rescuing *Ednrb*$^{+/+}$ NTs need not be capable of generating melanocytes themselves but must be capable of providing KITL, the helper cell type(s) can be non-melanocytic but must provide KITL as a major helper factor. It is likely that KITL acts directly on melanoblasts even though an indirect action involving yet other cell types cannot be excluded. Nevertheless, even though the cell non-autonomous action of *Ednrb* can rescue *Ednrb* mutant cells to the tyrosinase-positive stage, it is insufficient to induce pigmentation. Pigmentation can be seen, however, if the rescued cells are given TPA which induces PKC as does EDNRB signaling. Thus, terminal differentiation to mature, pigmented melanocytes probably depends on an additional, cell-autonomous EDNRB signaling step. Note that the above scenario does not preclude the possibility that in wild-type melanoblasts, *Ednrb* can also act cell-autonomously throughout development.

neuroblast differentiation (Wu et al., 1999), allowing for prolonged cell proliferation and migration, and not promote cell differentiation, as described here for melanoblasts. In any event, cell non-autonomous actions of growth factor receptors are not without precedents. A telling example are embryos deficient in a tyrosine kinase receptor, fibroblast growth factor receptor 1 (*Fgfr1*), which is expressed in several cell types, including presomitic mesoderm precursors. In such embryos, somites fail to form, suggesting *Fgfr1* plays a cell-autonomous role in somite specification, but a chimeric analysis showed that upon development in a wild-type environment, the mutant cells can contribute to somites (Rossant and Spence, 1998).

The existence of cell non-autonomous mechanisms would predict that genetic modifiers causing phenotypic variabilities in receptor mutants, including *Ednrb* mutants of mice (Pavan et al., 1995) and humans, need not necessarily influence the expression or activity of the corresponding signaling pathways in the very cell types that cause disease. It is often unknown, however, what cell types might be responsible for such indirect rescue. In the case of *Ednrb*, we have only demonstrated that the melanocyte lineage, at least its later tyrosinase-positive cell population, is not involved, and we can but speculate as to the cell type that is involved (see below). In addition, we do not know whether in the *Ednrb*-deficient NTs and their derivatives, the respective 'helper' cell type(s) are missing altogether, or are present but simply unable to respond to EDN3. Regardless of the nature of the responsible cell type, our results clearly indicate that the helper cells need to provide at least one factor – KITL.

The rational for testing KITL as the prime rescue factor for *Ednrb*-deficient cells was based on a number of previous studies that showed that *Ednrb* and *Kit* are both required for melanocyte development (Baynash et al., 1994; Geissler et al., 1988) and that EDN3 and KITL cooperate to effect melanogenesis (Hou et al., 2000; Reid et al., 1996). Moreover, we have previously found that although *Kit*-deficient neural crest cells respond to EDN3 with enhanced survival, they do not develop to the tyrosinase-positive state (Hou et al., 2000), and we report here that *Ednrb* deficiency does not interfere with *Kit* expression. Last, variant alleles of the *Kitl* locus on mouse chromosome 10 serve to phenotypically modify the hypomorphic, non-lethal *Ednrb*^{s} (*piebald*) allele (Pavan et al., 1995; Rhim et al., 2000). Taken together, these findings provided strong indications that it was the lack of sufficient amounts of KITL that was responsible for the absence of tyrosinase expression in *Ednrb*-deficient melanocyte precursors. Indeed, KITL was able to induce tyrosinase expression in *Ednrb*-deficient cells, as was the addition of *Ednrb*$^{+/+}$ NTs, even *Ednrb*$^{+/+}$ NTs that were incapable of generating melanocytes of their own as long as they could synthesize KITL. It appears, then, that EDNRB, by still unknown mechanisms, stimulates KITL synthesis and/or secretion. A likely source for this KITL is the neural tube or its derivatives where both *Ednrb* (Lee et al., 2003) and *Kitl* (Guo et al., 1997; Wehrle-Haller and Weston, 1999) are expressed.

Although the above results showed a role for KITL in rescuing the *Ednrb*-deficient cells to the tyrosinase-positive stage, KITL was not sufficient to render them pigmented. This was unlikely to be because of insufficient expression of tyrosinase; the intensity of its immunofluorescent signal in KITL-treated *Ednrb*^{lacZ}/*Ednrb*^{lacZ} cells was similar to that observed in equally treated *Ednrb*^{lacZ}/+ cells. Rather, it appears that tyrosinase is not properly activated in the rescued cells. In fact, even in *Ednrb*-wild type cultures, KITL is insufficient to stimulate melanogenesis (Morrison-Graham and Weston, 1993; Murphy et al., 1992; Reid et al., 1996), but the addition

of TPA (Murphy et al., 1992) or EDN3 (Reid et al., 1996) induces pigmentation. Similarly, the addition of TPA induces pigmentation in our rescued cells. A major site of action of both EDNRB signaling and TPA is the activation of protein kinase C (PKC), which has been shown to activate tyrosinase by serine phosphorylation (Park et al., 1999). If indeed melanogenesis in *Ednrb*-deficient cells requires the stimulation of PKC, then our results strongly suggest that for melanin synthesis to occur, the cells also depend on cell-autonomous EDNRB signaling pathways. In fact, during terminal differentiation, the KITL-mediated cell non-autonomous action of *Ednrb* may be replaced by its cell-autonomous signaling. This view is consistent with the observation, based on the in vivo use of neutralizing KIT antibodies, that KIT signaling is no longer needed for melanogenesis after ~E14.0 when melanocytes begin their terminal differentiation in the epidermis (Nishikawa et al., 1991).

Although our observations do not assess the relative importance of cell non-autonomous *Ednrb* signaling during melanocyte development in wild-type embryos in vivo, they clearly show that such mechanisms exist. This fact suggests, therefore, that the well-known dual dependency of the melanocyte lineage on two major signaling pathways, G-protein coupled signaling through EDNRB and tyrosine kinase receptor signaling through KIT, may actually result from complex interplays between cell-autonomous and cell non-autonomous actions. As schematically shown in Fig. 6, these different modes of action probably operate at distinct stages in lineage development, allowing for an intricate mechanism to fine-tune the proper deposition of melanocytes in the different bodily compartments.

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