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Multiple roles for endothelin in melanocyte development: regulation of progenitor number and stimulation of differentiation

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SUMMARY

Melanocytes in the skin are derived from the embryonic neural crest. Recently, mutations in endothelin 3 and the endothelin receptor B genes have been shown to result in gross pigment defects, indicating that this signalling pathway is required for melanocyte development. We have examined the effects of endothelins on melanocyte progenitors in cultures of mouse neural crest. Firstly, they stimulate an increase in progenitor number and act synergistically with another factor, Steel factor, in the survival and proliferation of the progenitors. These findings are consistent with findings from mice with natural mutations in the endothelin receptor B gene, which show an early loss of melanocyte progenitors. Secondly, endothelins induce differentiation of the progenitors into fully mature pigmented melanocytes. This finding is consistent with the expression of endothelins in the skin of mice at the initiation of pigmentation. The melanocytes generated in endothelin-treated cultures also become responsive to α melanocyte-stimulating hormone, which then acts to regulate the activity of the pigmentation pathway. These findings indicate two key roles for endothelin in melanocyte development: regulation of expansion of the progenitor pool and differentiation of progenitors into mature melanocytes.

Key words: endothelin, Steel factor, melanocyte-stimulating hormone, neural crest, melanocyte development, mouse

INTRODUCTION

One of the major issues in development is how multipotential stem cells give rise to their differentiated progeny. The embryonic neural crest (NC) is an ideal system in which to study this question, since its potentiality extends to most of the cells of the peripheral nervous system, a multitude of mesenchymal cell types in the craniofacial region and skin melanocytes (Le Douarin and Smith, 1988). Genetic analysis of coat colour mutants of mice has revealed that several growth factors are involved in the development of melanocytes (Bennett, 1991; Jackson, 1992; Halaban and Moellmann, 1993). For example, two coat colour mutants, dominant white spotting (*W*) and steel (*Sl*) contain mutations in the genes for a receptor tyrosine kinase, c-kit (Chabot et al., 1988; Geissler et al., 1988) and its cognate ligand, Steel factor (SLF; Copeland et al., 1990; Huang et al., 1990; Williams et al., 1990; Zsebo et al., 1990). This establishes a critical role for this receptor-ligand relationship in melanocyte development. Further studies of the mode of action of SLF in mice have shown that it regulates melanocyte development through selective survival and proliferation of melanocyte progenitor cells, which exclusively express c-kit (Reid et al., 1995). In Aves, it appears to have similar, but perhaps broader activities on NC cells (Lahav et al., 1994).

Another receptor-ligand interaction important for melanocyte development has been revealed by genetic analysis of the lethal spotting (*ls*) and piebald-lethal (s^l) mutations. These mice carry mutations in a member of the endothelin family, endothelin 3 (ET3) and the endothelin receptor B (ETB), respectively (Greenstein Baynash et al., 1994; Hosoda et al., 1994). In addition to their pigment defects, these mice have aganglionic megacolon, which indicates that another NC lineage, enteric neurons, is regulated by ET3.

In the mouse, NC migration begins from as early as embryonic day 8 (E8) and c-kit mRNA, probably produced by melanocyte progenitors, can be detected along migration pathways from E10 (Keshet et al., 1991; Matsui et al., 1990). The progenitors invade the epidermis between E11 and E12 (Mayer, 1973), begin the production of unmelanised melanosomes at E14 and begin to differentiate into mature melanocytes at E16 (Hirobe, 1984). These mature melanocytes initially contain tyrosinase activity, but do not have visible pigment and can be detected with the dopa reaction (Hirobe, 1984). Pigmented melanocytes are first detected in the skin at E16 and increase dramatically after birth (Hirobe and Takeuchi, 1977).

We have examined the effects of two members of the ET family, ET1 and ET3, on melanocyte development using

primary cultures of mouse NC. We find firstly that they act in conjunction with SLF in the regulation of survival and proliferation of melanocyte progenitor cells. Secondly, and strikingly, they induce pigmentation in the NC cultures, most likely by directly stimulating the differentiation of the progenitor cells into fully mature melanocytes. Since targeted and natural mutations in the ET3 gene result in major coat colour defects, and targeted mutations in the ET1 gene do not, we conclude that ET3 is essential for melanocyte development during embryogenesis, whereas ET1 is not. For similar reasons, we conclude that signalling occurs through the ETB receptor.

MATERIALS AND METHODS

Cell culture

NC cultures were prepared from E9 CBA mouse embryos as previously described (Murphy et al., 1994), and cultured in Monomed medium (Commonwealth Serum Laboratories, Australia), with 10% fetal bovine serum added (control medium). Growth factors were added as indicated, at the following concentrations: SLF (R & D Systems) 100 ng/ml; ET1 or ET3 (Sigma) 1-100 nM; a melanocyte-stimulating hormone, (αMSH, Sigma) 1 μM. In all experiments, fresh growth factors were added every second day, and the cultures were refed with fresh control medium and growth factors once weekly.

Immunohistochemistry

NC cultures were fixed in methanol at the specified time and stained for c-kit using the ACK2 antibody, as previously described (Nishikawa et al., 1991; Ogawa et al., 1991). Briefly, cultures were blocked for 1 hour in a solution containing phosphate-buffered saline, 2% FBS and 0.5% rabbit serum, followed by incubation in ACK2 (1:100 for 1 hour). Cultures were treated with biotinylated anti-rat IgG (1:200, Vector Laboratories, CA), then StreptABComplex (DAKO, CA) and visualised using a metal-enhanced DAB substrate kit (Pierce, IL, USA).

For tyrosinase-related protein 2 (TRP-2) staining, cultures were fixed in 4% paraformaldehyde for 10-30 minutes, then incubated with an anti-TRP-2 antibody (1:100; Tsukamoto et al., 1992). Antibody binding was detected by incubation in biotinylated anti-rabbit IgG (1:200, Vector, CA), ABC-alkaline phosphatase complex (Vector, CA) and finally alkaline phosphatase substrate solution as previously described (Reid et al., 1995).

Determination of c-kit$^+$ cell number and proliferation assay

NC cultures were incubated in control medium, SLF, ETs or SLF+ETs for 7 days, then fixed and stained for c-kit. In order to determine total c-kit$^+$ cell number, an estimate was made by counting a representative sample in a known area and multiplying this by the total area of the NC culture as previously described (Murphy et al., 1994).

For proliferation experiments, NC cultures were incubated in the presence of either control medium or with growth factors as indicated, except that, for experiments with ET3, both control medium and cultures with added ET3 also contained ACK2 antibody (100 ng/ml) to block endogenous SLF (Reid et al., 1995). Cultures were incubated for 3 days, [^{3}H]thymidine (1.5 μCi/ml) was added for 1 hour, after which the cultures were fixed and stained for c-kit as described above. Following airdrying, autoradiography was performed as previously described (Reid et al., 1995) and the percentage of c-kit$^+$ cells that had incorporated [^{3}H]thymidine was determined for each of 5 wells per condition. Values are the means±s.d. of a representative experiment.

Pigmentation assay and dopa/melanin analysis

NC cultures were incubated in SLF with or without ET1 or ET3, as indicated, and observed daily. After 11 days, cultures were fixed and photographed, and the total number of melanocytes per NC culture were counted. Values are the means±s.d. from four separate cultures per condition.

For switching experiments, NC cultures were incubated in SLF for 11 days, then washed and switched to either control medium, SLF, ET-1 (100 nM) or SLF + ET-1, and the number of melanocytes that arose was counted after a further 7 days. In some experiments, ET3 was substituted for ET1.

For dopa/melanin analysis, NC cultures were incubated in SLF+ET3 with or without αMSH. After 11 days, each NC culture was harvested individually and cytospun onto 2 glass microscope slides. One of each pair was treated with 0.1% dihydroxyphenylalanine (dopa) for 3 hours to reveal mature but unpigmented melanocytes. Both samples were then counterstained with haematoxylin and coverslipped. The number of melanocytes was then counted for each sample and the result expressed as the percentage of total melanocytes (from the dopa-treated samples) that have visible pigment (from the untreated samples). Numbers given are the means±s.d. from 5 separate NC cultures per condition.

^{125}I-ET binding

NC were cultured for 6 days in the presence of SLF, then washed once in Ca^{2+}/Mg^{2+}-free Hank's Balanced salt solution with 0.05% bovine serum albumin (HBSS/BSA). Cultures were incubated in ^{125}I-ET1 (50 pM, Amersham, UK) in HBSS/BSA for 1 hour at 37°C in the presence and absence of 100 nM ET1, then washed twice in HBSS/BSA and fixed in 4% paraformaldehyde for 10-30 minutes. Cultures were then stained with an antibody to TRP-2 (as described above) and air dried, and a thin, even layer of photographic emulsion was applied, as previously described (Reid et al., 1995), and exposed for 4-14 days. The number of grains over approximately 70 TRP-2$^+$ cells was counted. A background count was made by counting an equivalent area adjacent to each TRP-2$^+$ cell and this was subtracted from the TRP-2$^+$ grain count to give a final grain count for each TRP-2$^+$ cell.

In situ hybridisation and northern blot analysis

For in situ hybridisation analysis, mouse ETA and ETB receptor genes were cloned by PCR using oligonucleotide primers deduced from their rat homologues (Sakurai et al., 1990; Lin et al., 1991). Probes for ETA and ETB receptors were obtained from plasmids containing 355 bp of the mouse ETA receptor cDNA or 537 bp of the mouse ETB receptor cDNA, respectively. Nonradioactive antisense RNA probes were synthesised with digoxigenin-11-UTP (Boehringer-Mannheim). Whole-mount fixation and in situ hybridization were performed in mice embryos at E9.5 as described (Wilkinson, 1992). Hybridized embryos were washed at high stringency, incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody and stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate.

For northern blots, skin samples from CBA mouse embryos ranging from E14 to postnatal day 2 (P2) were dissected and snap frozen. Total RNA was extracted from all samples and purified on a CsCl gradient (Sambrook et al, 1989). mRNA was then purified using a PolyATract mRNA isolation kit (Promega, Madison, WI). The *ET1* probe was as previously described (Kurihara et al, 1994) and the *ET3* probe was made by reverse transcriptase PCR of brain RNA, using oligonucleotides to the 5′ and 3′ end of the gene (Greenstein Baynash et al., 1994). Relative levels of RNA were assessed using a *GAPDH* probe (Fort et al., 1985) and RNA from adult brain was shown as a positive control (Greenstein Baynash et al., 1994).

ET1$^{-/-}$ skin analysis

The *ET1*$^-$ mutation was transferred onto a C57/Bl6 background and *ET1*$^{-/-}$, *ET1*$^{-/+}$ and *ET1*$^{+/+}$ were generated and killed immediately

after birth. Sections of skin from the dorsal midline around the thoracic level, and from the cranium were dissected from formalin-fixed specimens that had been previously genotyped (Kurihara et al., 1994). Excess surface keratin was then scraped away and the samples were dehydrated in ethanol and cleared in xylene before mounting on glass microscope slides in DePeX.

RESULTS

ETs and SLF regulate melanocyte progenitor number

Mice carrying mutations in either ET3 or ETB receptor genes show gross pigment defects. In addition, piebald-lethal mice, which lack functional ETB receptors (Hosoda et al., 1994), show a marked reduction in the number of melanocyte progenitors (Pavan and Tilghman, 1994). This indicates that ET signalling is involved in regulation of melanocyte progenitor number. We investigated this by incubating NC cultures with ET3 and identifying melanocyte progenitors by staining the cultures with c-kit, an early marker for melanocyte progenitors (Reid et al., 1995). We compared the effects of ET3 with SLF, which is the cognate ligand for c-kit and a key regulator of melanocyte progenitors (Reid et al., 1995). After 7 days of culture, very few progenitors were present in cultures with no added factors (Fig. 1). Treatment with either ET3 or SLF caused an increase in the number of melanocyte progenitors (Fig. 1), and treatment with both factors resulted in a further significant increase in progenitor number compared to either factor alone (P<0.0005; Fig. 1). Although ET3 is most strongly implicated in melanocyte development, we conducted similar experiments with another member of the endothelin family, ET1, and found essentially the same results as those observed with ET3 (Fig. 1).

To examine whether ET3 was proliferative for the melanocyte progenitors, we incubated NC cultures for 3 days in the presence of ET3, then pulsed them for 1 hour with [^{3}H]thymidine. ET3 stimulated an approximate 2-fold increase in the percentage of melanocyte progenitors that incorporated [^{3}H]thymidine (Fig. 2) compared to control cultures. In comparison, SLF stimulated a 3-fold increase in proliferation over controls. The stimulatory effects of ET3 on proliferation were additive to those of SLF (Fig. 2), suggesting that these factors act in concert but via independent signalling mechanisms to regulate proliferation of melanocyte progenitors. Similar results were obtained with ET1 (Fig. 2).

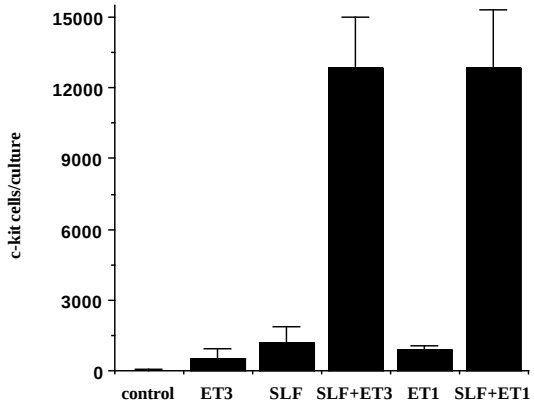

Fig. 1. ET and SLF cooperate to stimulate an increase in melanocyte progenitor numbers in NC cultures. NC cultures were incubated with or without ET1, ET3 and SLF for 7 days, fixed and stained with an anti-c-kit antibody, and numbers of c-kit$^+$ cells determined as described in Materials and methods.

ETs induce pigmentation in NC cultures

Our previous studies show that melanocyte progenitors require a discrete signal, not provided by SLF, in order for them to differentiate into mature pigmented melanocytes. We previously used the phorbol ester, TPA, to induce this step. However, addition of ETs not only stimulated melanocyte progenitor division but also induced pigmentation in NC cultures. In cultures incubated with SLF and either ET3 or ET1, pigmented melanocytes began to appear after 6-7 days, comparable to the time pigmentation begins in vivo, at E16 (Hirobe, 1984). These

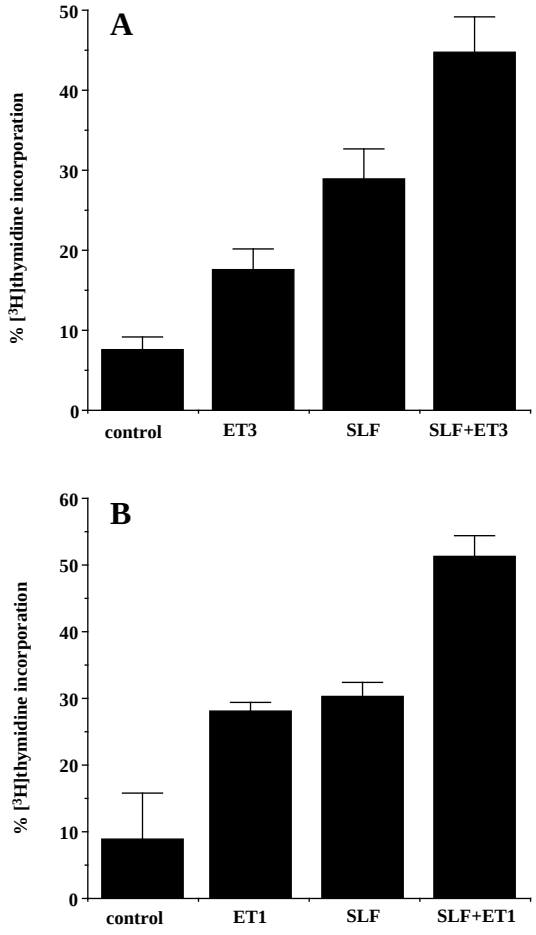

Fig. 2. (A) ET3 and SLF co-regulate melanocyte progenitor proliferation. NC cultures were incubated with 100 ng/ml ACK2 antibody (control), SLF, ET3+ACK2, or ET3+SLF as indicated, for 3 days, then with [^{3}H]thymidine for 1 hour, fixed, stained for c-kit, autoradiographed and the percentage of c-kit$^+$ cells which had incorporated [^{3}H]thymidine was determined. Values are means±s.d. of a representative experiment (n=6), and the experiment was performed three times. (B) ET1 has similar activity to ET3. In these experiments, controls and ET1 cultures did not contain ACK2 antibody.

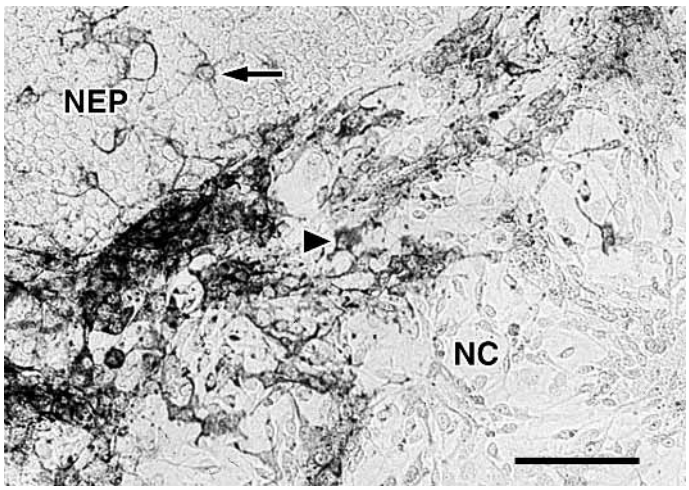

Fig. 3. ETs induce pigmentation in cultures of mouse NC. NC cultures incubated with 100 ng/ml SLF never gave rise to pigmented cells, but in the presence of 100 nM ET1 and SLF many pigmented melanocytes were generated. The same effect was observed when 100 nM ET3 was substituted for ET1 (data not shown). Pigmented melanocytes associated with the neuroepithelial sheet (NEP) had a highly dendritic morphology (arrow), while others associated with the NC population (NC) had a more polygonal appearance (arrowhead). Bar, 100 μm.

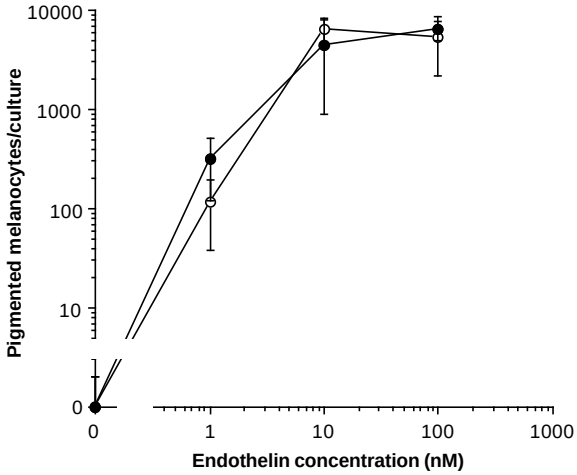

Fig. 4. Titration of ET1 (open circles) and ET3 (closed circles) in the presence of SLF shows that similar concentrations of either factor give rise to similar numbers of pigmented melanocytes. Cultures were fixed after 11 days and the total number of melanocytes per NC culture was counted. Values are means±s.d. (*n*=4).

pigmented cells were either dendritic or NC-like in appearance (Fig. 3), identical to pigmented melanocytes previously observed in NC cultures incubated in the presence of TPA (Murphy et al., 1992). By 11 days of culture, numerous melanocytes were present in ET-treated cultures (Figs 3, 4), and their number continued to increase over time. Both ET3 and ET1 gave the same dose response for pigmentation (Fig. 4) suggesting signalling through the ETB receptor, which binds ET3 and ET1 with equal affinity (Sakurai et al., 1990).

Further experiments involved incubating NC cultures in SLF alone to generate progenitor cells without stimulating pigment production (Reid et al., 1995), and switching these cultures to medium containing SLF+ET3, or SLF+ET1. Pigmented melanocytes began to appear as early as 16 hours after switching, which is consistent with ETs acting directly on the progenitors to induce pigmentation. Cultures that were switched from SLF to ETs alone gave rise to a similar number of pigmented melanocytes 7 days after switching (1070±370) as those switched to ET+SLF (1410±530; *n*=4), indicating that SLF is not required for pigment induction.

ET binds to melanocyte progenitors

To further assess whether endothelins could be acting directly on melanocyte progenitors, NC cultures were incubated in SLF alone to generate melanocyte progenitor cells (Reid et al., 1995). These cultures were incubated with ^{125}I-ET1 and bound ^{125}I-ET1 was cross linked to the cells by fixation in paraformaldehyde. Cultures were then stained for TRP-2 to identify the progenitors (Reid et al., 1995; Tsukamoto et al., 1992). TRP-2 was used in these experiments to mark melanocyte progenitors because fixation procedures required to cross link ^{125}I-ET1 to the cells could not be found that were compatible with c-kit staining. TRP-2$^+$ cells bound ^{125}I-ET1

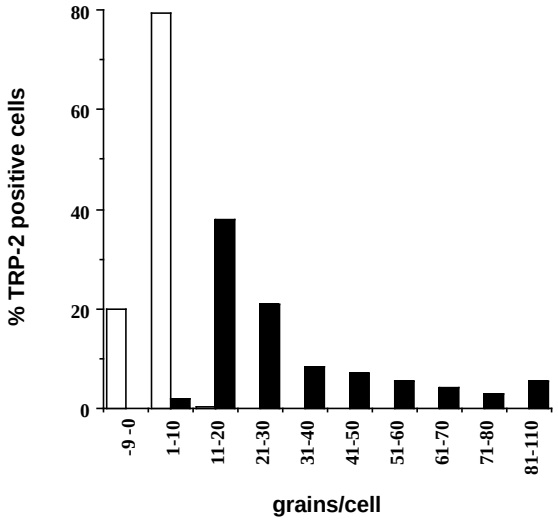

Fig. 5. Expression of ET receptors on melanocyte progenitors. Binding of ^{125}I-ET1 to NC cultures and localisation of binding to TRP-2$^+$ melanocyte progenitors was undertaken as described in materials and methods. Shown is the distribution of autoradiographic grain counts over TRP-2$^+$ cells in NC cultures, expressed as the percentage of cells binding the specified range of counts in cultures incubated either with ^{125}I-ET1 alone (filled bars) or with the addition of excess unlabelled ET1 (clear bars).

and binding could be inhibited by excess unlabelled ET1 (Fig. 5), indicating that melanocyte progenitors express ET receptors and thus can respond directly to ETs.

ETB receptor expression occurs in trunk regions of the embryo whereas ETA receptor expression is restricted to rostral region

Our NC cultures are derived from trunk regions of murine neural tube and the majority of melanocytes are derived from this region in vivo (Le Douarin and Dupin, 1993). To examine which of the ET receptors might be expressed in these regions,

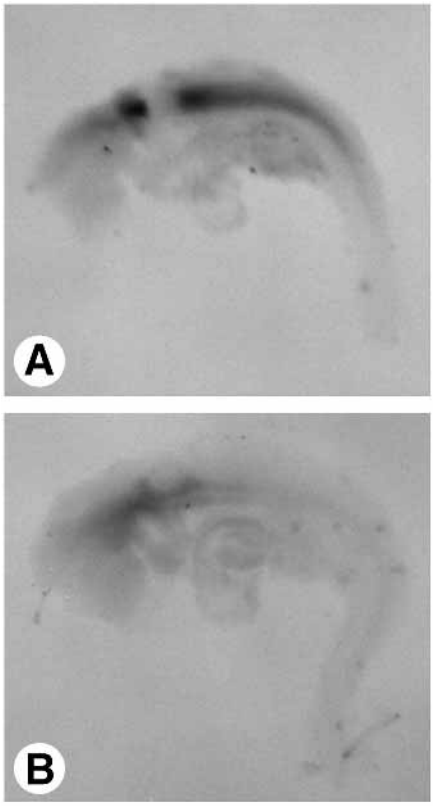

Fig. 6. In situ hybridisation of E9.5 embryos with ETB (A) and ETA (B) receptor probes. Hybridisation was conducted with digoxigenin-labelled probes as described in methods.

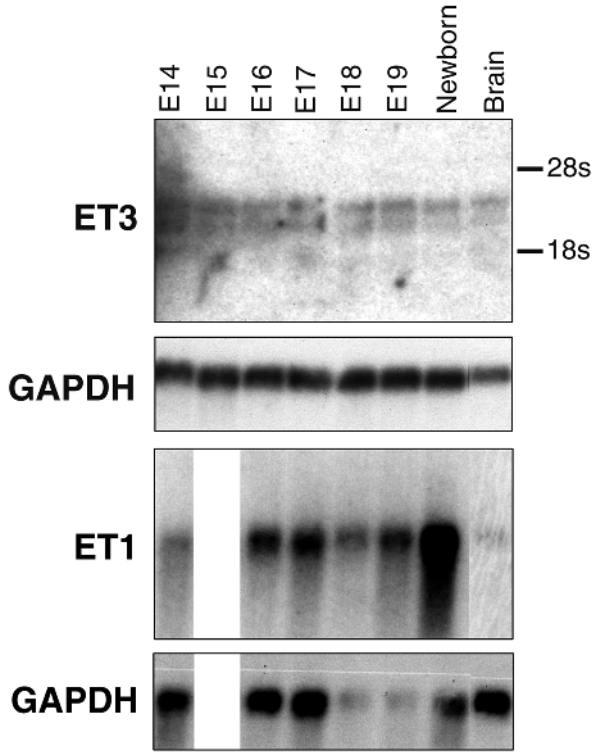

Fig. 7. Northern analysis of ET3 and ET1 expression in skin from E14-P2 mice. mRNA was isolated from skin, separated on a formaldehyde-agarose gel, blotted and hybridised with an ET3 or an ET1 probe as described in methods. Relative levels of RNA were assessed using a GAPDH probe and RNA from adult brain was shown as a positive control. E15 skin mRNA was not present in the ET1 northern analysis.

and thus can be implicated in signalling in melanocyte development, we undertook whole-mount in situ hybridisation of E9.5 embryos with ETB and ETA receptor gene probes. Expression of ETB mRNA extends extensively along the neural tube and includes the trunk regions as well as cranial areas (Fig. 6A). However, ETA receptor mRNA expression is localised to a rostral region of the embryo, around the pharyngeal arches, and there is no detectable signal in the trunk (Fig. 6B).

ET3 and *ET1* are expressed in embryonic skin at the time pigmentation begins in vivo

If ETs were responsible for induction of pigmentation, they should be expressed in skin when pigmentation is occurring. Melanin first appears in skin at E16 (Hirobe, 1984) and increases throughout the remainder of gestation and postnatally. To investigate whether ETs were expressed at this time in skin, we isolated mRNA from embryonic skin from as early as E14 and subjected it to northern blot analysis. Both *ET1* and *ET3* mRNAs are expressed throughout this period and in comparable levels to that seen in adult brain (Fig. 7).

ET1$^{-/-}$ mice contain pigment at birth

Mice with mutations in either ET3 or the ETB receptor genes are predominantly white (Hosoda et al., 1994; Greenstein Baynash et al., 1994), demonstrating the importance of these genes in melanocyte development. Since ET1 could also induce pigmentation in our cultures, we investigated whether *ET1*$^{-/-}$ mice (Kurihara et al., 1994) had defects in coat colour. Since these mice die at birth (Kurihara et al., 1994), before the coat is formed, skin samples were taken from these mice immediately after birth and examined microscopically. Examination of skin from *ET1*$^{-/-}$ animals bred onto a C57/Bl6 background revealed that these mice did contain pigmented hair follicles, and there was no apparent difference in the amount of pigmentation compared to *ET1*$^{+/+}$ littermates (Fig. 8). These data indicate that ET1 is not essential for melanocyte development during embryogenesis.

α-Melanocyte-stimulating hormone alone has no effect on pigmentation, but enhances ET induced pigmentation

We also studied the effects of αMSH in NC cultures. Addition of αMSH to NC cultures in the presence of SLF resulted in the generation of extremely few pigmented cells (Fig. 9A; Table 1), even after 3 weeks of culture, probably because the melanocyte progenitors in these cultures were incapable of synthesising pigment. Addition of ET in the absence of αMSH resulted in thousands of pigmented cells per culture (Figs 4, 9B). However, cultures treated with both αMSH and ET resulted in a marked increase in pigmented cells (Fig. 9C), compared to cultures incubated with ETs alone (Fig. 9B; Table 1).

αMSH probably increases pigment synthesis in cells once

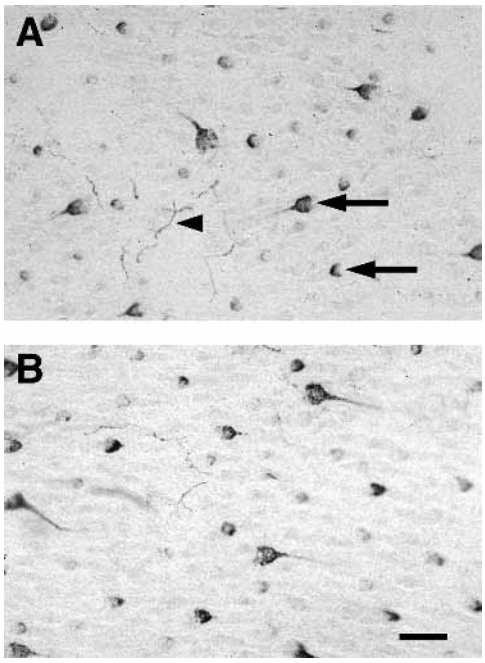

Fig. 8. *ET1*$^{-/-}$ mice have pigmented hair follicles and dermal melanocytes. Skin was taken from *ET1*$^{+/+}$ (A) and *ET1*$^{-/-}$ (B) mice immediately after birth, fixed, cleared, mounted and photographed. Pigmented hair follicles (arrow) and dermal melanocytes (arrowhead) are indicated. Bar, 100 μm.

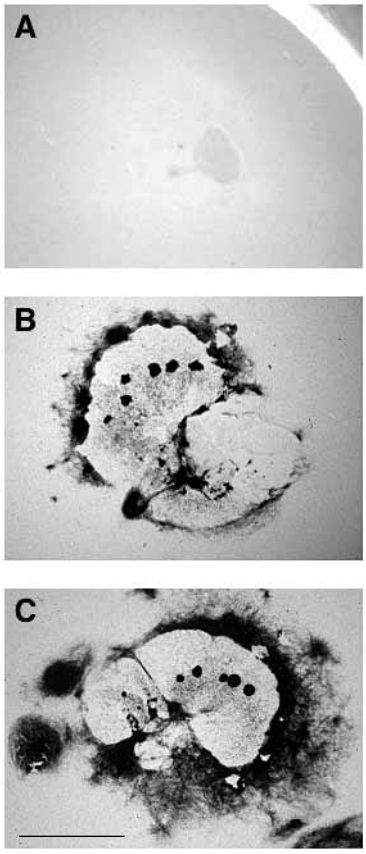

Fig. 9. Enhancement of pigment production by αMSH is dependent on ET. Low-power view of NC cultures incubated for 3 weeks with: (A) αMSH, which contained very few pigmented melanocytes (12±17 (*n*=4) melanocytes were present in these cultures); (B) ET1, which shows a large area of the culture contains pigmented cells; (C) ET1+αMSH, which shows an even larger area of the culture with pigmented cells. Bar, 2 mm.

the pigmentation pathway has been induced by ETs, resulting in more cells with visible levels of pigment. We examined this by treating cultures with dihydroxyphenylalanine (dopa, Ito and Takeuchi, 1984), which reacts with endogenous tyrosinase in mature melanocytes to form pigment. This enables the detection of mature melanocytes, which are capable of pigment production, including those that do not have visible pigment. By determining the percentage of pigmented cells in cultures before and after dopa treatment, the percent of total melanocytes (dopa$^+$) which were visibly pigmented could be determined. In ET-treated cultures, 25% of dopa$^+$ cells had visible pigment, compared to 68% in cultures treated with ET and αMSH (Table 1). This shows that αMSH acts to increase pigment levels in already mature melanocytes.

DISCUSSION

Co-regulation of melanocyte progenitor number by SLF and ET3

Studies of piebald-lethal and lethal spotting mutants (which carry mutations in the ETB receptor and ET3 genes, respectively) show that the critical time of action of ET signalling begins very early in melanocyte development, from as early as E10.5 and possibly before the onset of TRP-2 expression (Pavan and Tilghman, 1994; Yoshida et al., 1996). Likewise, c-kit$^+$ melanocyte progenitors were lost from Sl or W mutant embryos from as early as E11 (Steel et al., 1992; Wehrle-Haller and Weston, 1995). These studies thus infer that both SLF and ET3 are critical for progenitor development from the similar early times and thus that they act together to regulate progenitor number.

Table 1. Endothelin induces responsiveness to MSH which then regulates the degree of pigmentation in melanocytes

Treatment	% of total cells in culture		% of dopa$^+$ melanocytes which are pigmented
	pigmented	dopa$^+$ melanocytes	
Control	<0.1	<0.1	–
MSH	<0.1	<0.1	–
ET	12±4	43±10	25±8
ET+MSH	42±7	63±10	68±10

The results from our in vitro studies provide mechanisms of action for these two factors. We previously showed that SLF is a survival and proliferative factor for melanocyte progenitors (Reid et al., 1995). Now we show that both SLF and ETs regulate melanocyte progenitor number. Whereas either factor alone has activity, they show strong synergy in stimulating an increase in progenitor cell number. Part of this stimulation is due to proliferative effects of both factors on the progenitors; in the presence of both factors, the percent of cells incorporating [^{3}H]thymidine is 5-fold that of controls, to approximately 50% within a one hour period. This indicates that the combination of SLF and ET3 stimulates a high rate of proliferation of the progenitors. During revision of this manuscript, it was reported that ET3 is a potent mitogen for early avian NC precursors, which can give rise to melanocytes (Lahav et al., 1996), which is generally consistent with our findings.

Both factors also probably co-regulate the survival of the progenitors. There is almost no survival of progenitors in NC cultures with no added factors, and significant survival in the

presence of either ETs or SLF. In vivo, the loss of either signalling pathway (Steel et al., 1992; Wehrle-Haller and Weston, 1995; Pavan and Tilghman, 1994) results in almost complete loss of progenitors, which shows that there is no compensation of one factor for the other in melanocyte development. This indicates that there is tighter regulation of progenitor cell number within the developing embryo than within our NC cultures, albeit our observations reflect the in vivo findings. This additional regulation may be associated with very discrete spatial and temporal requirements for the two growth factors (see Yoshida et al., 1996, for discussion). Additional growth factors may also be present in our cultures that support the survival of the progenitors but which are not present in vivo.

ETs stimulate differentiation of progenitors into mature melanocytes

ETs not only act to regulate melanocyte progenitor cell number, but also induce pigment in these cells. These findings suggest that ETs stimulate differentiation of the melanocyte progenitors into fully mature melanocytes. In our culture system, we never see pigmented melanocytes in SLF alone and thus the addition of an extra stimulus is an absolute requirement for differentiation. We previously used phorbol esters to stimulate pigmentation in our cultures, which signal through activation of protein kinase C (Gschwendt et al., 1991). ETs activate the Ca^{2+}-messenger system, which involves both calmodulin and protein kinase C (Takuwa, 1993). In addition, the ET1 gene contains a phorbol ester responsive element (Tasaka and Kitazumi, 1994). Thus, the phorbol esters may stimulate melanocyte differentiation by mimicking ET signalling and/or by inducing endogenous ET production.

These observations and the findings that ET receptors are present on melanocyte progenitors strongly suggest that ETs act directly to induce differentiation of the melanocyte progenitors. In addition, the switching experiments show that it is possible to maintain the precursors in their undifferentiated state in the presence of SLF in vitro and, shortly after the addition of ETs, they begin to express visible pigment and thus differentiate.

Which ET receptor and ligand are involved in melanocyte development?

Studies on piebald lethal mice (Pavan and Tilghman, 1994), show that the ETB receptor is required for signalling in regulation of precursor cell number. ET3 and ET1 give the same dose response for production of pigmented melanocytes, suggesting that the differentiation signal is mediated through the ETB receptor, which binds both ET3 and ET1 with the same affinity (Sakurai et al., 1990). Our in situ hybridisation data show that, at the time that the NC cells are migrating out from the neural tube, only ETB receptor mRNA is expressed in trunk regions of the embryo. The majority of melanocytes are derived from this region of the neural tube (Le Douarin and Dupin, 1993) and our NC cells are derived from the trunk neural tube. Finally, the ETA receptor has recently been knocked out (Hosoda and Nakao, 1996) and results in a virtually identical phenotype to $ET1^{-/-}$ animals, which we find have no deficit in pigmentation at birth. Together, these findings strongly suggest that ETB receptor is the major receptor in melanocyte development in embryogenesis.

ET3 is also involved in the process of melanocyte development and recent studies of *ls/ls* mice indicate that ET3 is involved in regulation of progenitors in the dermis, but that it may not be involved at later stages (Yoshida, 1996). However, this is unclear since these studies only clearly define the first essential period when ET3 is required. Support for a role for ET3 at later stages comes from our finding of ET3 mRNA expression in skin at the initiation of pigmentation and at least through to postnatally, when there is a large increase in the amount of pigmentation in murine skin (Hirobe, 1984). In addition, tissue levels of ET1+ET2 are normal in ET3 knockout mice (Greenstein Baynash et al., 1994), indicating that these molecules do not entirely compensate for the loss of ET3. However, ET3 knockout mice have more pigment in the head and tail regions than ETB receptor knockout mice, and thus there may be compensation from the other ETs in melanocyte development, both during the regulation of melanocyte progenitors and in their differentiation.

ET1 does not appear to be essential for melanocyte development during embryogenesis since ET1 knockout mice show no pigmentation defects at birth. However, ET1 may play some role in melanocyte development, but the lack of this peptide may be fully compensated by ET3. The expression of ET1 in the skin during the initiation of pigmentation is consistent with this notion and it may be responsible for the pigment observed in the ET3 knockout mice. This expression in skin may also be related to the physiological role of ET1 in vascular homeostasis (Kurihara et al., 1994). We have no information on any role of ET2 and there are no *ET2* null mutants available. In addition, the other ETs may act postnatally to regulate the amount of pigment in melanocytes, analogous to αMSH (Yada et al., 1991; Imokawa et al., 1992).

Our results indicate that ET stimulates both progenitor proliferation and differentiation. How are these events separated? A number of observations may help to resolve this issue. In studies that used an anti-c-kit antibody to perturb melanocyte development in vivo, the critical period of SLF dependency was shown to end at E14.5 (Nishikawa et al., 1991). This was confirmed by in vitro studies (Morrison-Graham and Weston, 1993). In addition, the expression of SLF in the dermatome turns off by this time (Wehrle-Haller and Weston, 1995). Thus, at the time that differentiation of the progenitors begins, the melanocyte progenitors have matured to a stage of SLF independence and do not see SLF in vivo. ET is not strongly proliferative on its own and thus its main function at this stage (from E14 on) may be to stimulate the differentiation of these melanocyte progenitors. It is not known when expansion of the melanocyte progenitor pool ceases; however, the total number of melanocytes plus cells positive in the pre-melanin reaction does not increase after E17 (Hirobe, 1984). This indicates that progenitor proliferation has ceased at some time before E17 and is consistent with the idea that the main role of ET late in gestation is in differentiation of the melanocyte progenitors.

Regulation of pigmentation by αMSH

αMSH is involved in pigmentation by regulating levels of tyrosinase (Hirobe, 1992), the rate-limiting enzyme in the pigmentation pathway. Mice lacking functional receptors for αMSH have melanocytes that produce phaeomelanin (Robbins et al., 1993), the type of pigment produced in the presence of low levels of tyrosinase. This shows that αMSH is not required for mice to develop a fully functional, albeit low level, pig-

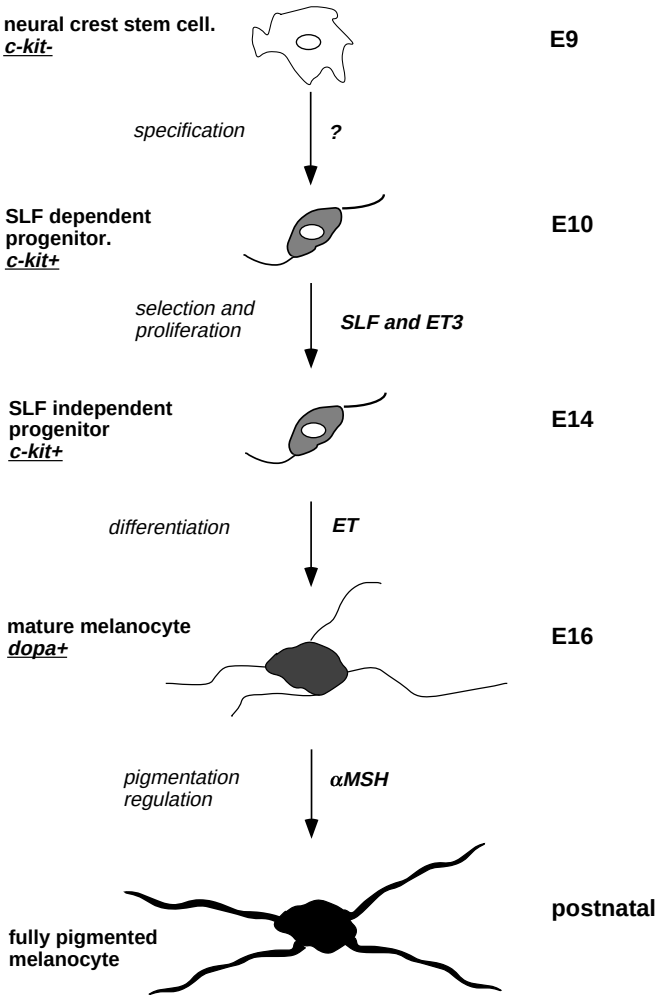

Fig. 10. Summary scheme of the roles of SLF, ET and αMSH in melanocyte development. Details are described in the discussion; numbers on the right indicate approximate times at which each step begins.

mentation pathway. This is entirely consistent with our conclusion that primary differentiation of melanocytes is regulated by ET.

We find that αMSH alone (either in the presence or absence of SLF) has no effect on pigmentation, but that it stimulates pigmentation in the presence of ETs. The most consistent interpretation for this finding is that the melanocyte progenitor cells in our cultures cannot respond to αMSH, but upon stimulation by ETs, these cells differentiate into mature melanocytes, acquire responsiveness to αMSH and increase their production of melanin. This conclusion is supported by the finding that a much greater proportion of melanocytes were visibly pigmented in the presence of ETs+αMSH compared to ETs. It would be interesting to determine whether ETs actually upregulate αMSH receptors on the melanocytes. Additionally, there may be interaction in the signalling pathways activated through ET and αMSH receptors, analogous to that described previously (Swope et al., 1995).

A pathway of melanocyte development

These findings can be incorporated into a pathway of melanocyte development whereby some of the steps are regulated by the factors that we have discussed here. There is considerable evidence that other factors are involved in melanocyte development (Yaar and Gilchrest, 1991; Halaban and Moellmann, 1993); however, the precise role of these factors has not been defined. Fig. 10 represents the respective roles of SLF, ET3 and αMSH in such a putative pathway. This pathway is entirely consistent with in vivo findings from the variety of mice with targeted and natural mutations in SLF, ET3 and αMSH signalling pathways. The regulation of the first step in the pathway, specification of melanocyte progenitors, is unknown, but the step is inferred from the findings that multipotential NC cells can give rise to melanocytes (Bronner-Fraser, 1995) and that c-kit$^+$ NC cells are melanocyte progenitors (Reid et al., 1995). Also shown are the approximate embryonic times at which each step begins, which are based on studies discussed herein. The time at which αMSH begins to act in melanocyte differentiation is inferred from observations that αMSH receptor signalling is required for eumelanin synthesis in vivo (see Robbins et al., 1993), and this pigment first appears at E16 (Hirobe and Takeuchi, 1977).

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