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MGF (KIT Ligand) Is a Chemokinetic Factor for Melanoblast Migration into Hair Follicles

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Melanoblasts, the precursors of the pigment-producing cells of the skin and hair, are derived from the neural crest and migrate to the skin around 12 days of gestation in the mouse. In adult mice almost all the melanoblasts are confined to the hair follicles except for the epidermal layers of nonhairy skin. The receptor tyrosine kinase, KIT, is necessary for the survival, proliferation, and migration of melanoblasts. We have utilised an organ culture for embryonic skin taken from Dct-lacZ transgenic mice. The early patterning of the follicles and developing skin layers is retained within the cultures and the lacZ reporter allows visualisation of the melanoblasts within their native tissue environment. Soon after initiation of hair follicle development, melanoblasts localise in the follicles. Inhibition of follicle formation demonstrates that this localisation is an active process; in the absence of follicles, the melanoblasts proliferate but remain associated with the basement membrane. Implantation of beads releasing MGF, the ligand of KIT, does not result in melanoblast migration towards the bead, rather their localisation to the follicles is accelerated. Addition of soluble MGF induces the same effect; KIT therefore promotes melanocyte movement and acts as a chemokinetic, or motogenic, receptor. The melanoblasts must be guided to their correct location by other chemotactic signals or move at random and locate by ceasing movement when the follicle is engaged.

Key Words: melanoblasts; melanocytes; skin; Steel factor; hair follicles; Dct-LacZ.

INTRODUCTION

Melanoblasts provide an excellent paradigm of developmental processes. These cells, the precursors of vertebrate melanocytes, differentiate from the neural crest and migrate dorsolaterally through the developing dermis to all regions of the body. They move from the dermis to the epidermis in the mouse embryo between E11 and E13 and subsequently become specifically localised to the hair follicle (Mayer, 1973). Melanocytes in the follicle produce melanin granules, which are transported to surrounding keratinocytes, giving rise to pigmented hairs (Silvers, 1979). In regions of the skin that are covered with hair almost all the melanocytes are localised in the hair follicles and as a consequence the skin is unpigmented. In the few regions of glabrous (nonhairy) skin, melanocytes remain in the epidermis and these locations (such as the tail, pinna, and snout) are pigmented. It appears that the presence of hair follicles may trigger specific events that lead to melanocyte localisation within the follicles.

The development of hair follicles from the skin is well documented (Hardy, 1992; Panaretto, 1993; Oro and Scott, 1998). An aggregation of dermal mesenchymal cells known as a placode (stage 1) initiates the downgrowth of a small group of adjacent basal epithelial cells into the mesenchyme (stage 2). As the epithelial follicle bud elongates, its base surrounds the mesenchymal cluster to form the dermal papilla. A primitive hair known as a hair matrix (stage 4) then begins to grow from the epithelial cells next to the dermal papilla and by rapid cell division, elongation, and intracellular keratinisation, a keratinised hair eventually emerges (stage 8). In the hair follicle, melanocytes are located in the inner and uppermost layer of the hair matrix, in contact with the dermal papilla (Billingham and Silvers, 1960; Hirobe, 1995). It is only during the hair growth phase (anagen) that melanocytes transfer melanosomes containing melanin granules to the dividing keratinocytes of the growing hair.

Identification of the genes affected in mouse coat colour mutations has shown a role for several transcription factors, receptors, and ligands for melanoblast development. These include the SRY-box factor SOX10, the paired box transcription factor PAX3, the basic helix-loop-helix leucine zipper transcription factor MITF, the endothelin receptor B, its
ligand endothelin 3, and the receptor tyrosine kinase KIT and its ligand mast cell growth factor (MGF) (reviewed in Jackson, 1997). Endothelin 3 appears to be necessary very early in melanoblast differentiation, possibly before the melanoblast lineage has separated from other neural crest lineages (Stone et al., 1997; Shin et al., 1999). KIT stimulation by MGF, however, is important only for melanoblasts and not for other neural crest cells. Analysis of mice mutant in Kit or Mgf has indicated that the products of these genes are not needed for differentiation of melanoblasts, but are necessary for their survival, proliferation, and initial migration from the crest (Steel et al., 1992; Murphy et al., 1992; Cable et al., 1995; Wehler-Haller and Weston, 1995; MacKenzie et al., 1997). Further investigations by injecting blocking antibodies against KIT into pregnant mothers have shown that KIT is necessary later in melanoblast development during their movement from the dermis to the epidermis and subsequent localisation to the follicle (Nishikawa et al., 1991).

DCT (DOPAchrome tautomerase or tyrosinase-related protein-2) is a marker for cells of the melanoblast lineage. Presumptive neural-crest-derived melanoblasts can be identified by XGal staining in Dct-lacZ transgenic mice from E10.5 (MacKenzie et al., 1997). Their subsequent migration on a dorsolateral pathway from the neural crest to the epidermis via the dermis can be followed up to E16.5. The XGal-stained melanoblasts have begun to populate all regions of the mouse embryo at E13.5 and by E16.5 have entered the hair follicles. The expression of the transgene is still detectable in adult skin and hair follicles.

To investigate factors regulating migration of melanoblasts from the epidermis to the hair follicles and their subsequent differentiation to become active melanocytes in the hair matrix, we have cultured embryonic skin tissues from Dct-lacZ transgenic mice. The cultured explants mirror the development of normal embryonic skin from E13.5 to postnatal day 4, including the migratory behaviour of the melanoblasts. Furthermore, the explant system is amenable to manipulation, allowing us to demonstrate that melanocyte movement into follicles is an active process and that although MGF stimulates movement of melanoblasts into the follicles, it does not act as a chemotactic factor. Rather MGF appears to stimulate melanoblast movement overall, and another mechanism is required to promote localisation to follicles.

MATERIALS AND METHODS

Mice

The Dct-lacZ mice have been previously described (MacKenzie et al., 1997) and were maintained under SPF conditions. The time of gestation of embryos was calculated by taking noon of the day of detection of a vaginal plug (E0.5) and confirmed by noting the external appearance of the embryo (according to Kaufman, 1992).

Skin Organ Culture

The organ culture system was adapted from Kashiwagi et al. (1997). In brief, the E13.5 Dct-lacZ skin pieces were spread epidermal side up onto a Nucleospore filter (Corning-Costar) coated with reduced growth factor Matri-Gel (Becton-Dickinson) or collagen type 1 from calf skin, pH 3.7 (Elastin Products Co. Inc., USA). The same site was used consistently as the source of cultured tissue from all embryos. After a defined culture period of 4 h to 12 days, the lacZ-positive cells were detected using XGal as described previously (MacKenzie et al., 1997).

Growth Factors and Preparation of Beads

Epidermal growth factor (Sigma) and cyclopamine (kind gift from Dr. William Gaffield) were added directly to the culture media at different concentrations ranging from 15 to 30 ng/ml and 1 to 2 μM, respectively. Ten millimolar stock solutions of cyclopamine in 95% ethanol were stored at −20°C. For slow release bead preparation, MGF (Steel factor; First Link, UK Ltd.) was used to coat Affi-Gel blue beads (Bio-Rad; 100–200 μm in diameter). After overnight washing in PBS, the beads were added to a 10-μl aliquot of MGF (final concentration of 2–10 ng/μl) and incubated for 1–3 h at room temperature or for 1 h at 37°C. Beads were placed in the top layer of the skin explant for 4–96 h and incubated at 37°C. PBS-soaked control beads were implanted in a similar fashion.

Histology

The explants or E16 Dct-lacZ embryos were fixed in 4% paraformaldehyde after XGal staining and paraffin embedded. Five-micrometer sections were counterstained with eosin for verification of the skin layers. Four sections were counted from each explant and double counting of individual melanoblasts was avoided by counting only alternate sections. Each experiment includes data from at least five separate explants for each condition analysed. To determine the melanoblast cell number for each treatment, the total number of cells present in four microscopic fields of 0.142 mm² per explant was averaged. To ensure the study was unbiased all histological analyses were performed blind to treatment.

RESULTS

Embryonic Skin Organ Culture System

In order to examine melanoblast localisation during their colonisation of the hair matrix, we utilised an organ culture for skin tissues (Kashiwagi et al., 1997) taken from the dorsum of Dct-lacZ embryos. Induction of the first hair follicles in embryonic mouse back skin occurs at E14.5 when the tylotrich pelage hair follicles form (Hardy, 1992; Hardy and Vriend, 1996). We therefore cultured skin from E13.5 transgenic embryos, which contains no hair follicles, for a period of up to 12 days during which time follicles form and begin synthesising hairs. Staining of the tissue pieces with XGal after defined culture periods reveals the lacZ-positive melanoblasts (Fig. 1).

In the initial skin samples and during the first 24 h of culture, the melanoblasts appear randomly distributed (Fig.
Small clusters of stained cells localised to developing hair follicles are first evident at 48 h. After 72 h in culture, a regular spatial pattern of hair follicles which are populated by melanoblasts is established. After 96 h, melanoblasts are still found both in the hair follicles and in interfollicular regions. Melanocytes undergo characteristic changes in shape during the 96-h growth period. At the onset of culturing, the melanoblasts appear round and discrete; however, after 4–24 h in culture, they become more dendritic and spindle-like (compare Figs. 1A and 1C). This characteristic spindle shape persists up to 12 days in culture, consistent with the observation that active melanocytes in the hair bulb, which secrete pigment granules, are highly dendritic (Hirobe, 1995).

The stained explants were embedded in paraffin and sectioned to examine the morphology of the developing skin and the location of melanoblasts within the organ culture. Comparison of sections from 72-h cultures and an
age-matched E16 Dct-lacZ embryo (Figs. 2B and 2E) shows that the development of the epidermal layers and the density and distribution pattern of the XGal-stained melanoblasts are very similar. A few very early stage follicles are detected in sections from 48-h cultures (Fig. 2A). The downgrowth of the developing hair follicles into the dermal layers can be clearly observed from 72 h in culture (Fig. 2B). After 96 h the primary follicles that develop in the explant have invaginated farther into the dermal layers, but more recently formed shorter, secondary follicles still contain melanoblasts. At this time, approximately 20% of the hair follicles observed in the cultures had reached stage 4 of morphogenesis. Thus the development of the explant layers is essentially identical both histologically and temporally to skin isolated from an age-matched mouse embryo.

Spatial Localisation of Melanoblasts in the Developing Skin

Having established that the Dct-lacZ skin tissue explants provide a good model of in vivo development, we next examined the number and location of the melanoblasts in the skin tissue. An estimate of melanoblast number in the explants during the 96-h culture period was established by counting stained cells from paraffin sections in defined microscope fields. The population density of melanocytes increased two- to threefold from the time of dissection to 48 h in culture. After this point the density remained constant to 96 h although the skin explant itself became thicker and more complex. Following long-term culture of the explants the overall melanocyte density falls approximately twofold (the 4- and 12-day cultures contain on average 27 and 14 melanocytes, respectively, within defined microscopic fields).

The proportion of stained cells in the four distinct skin layers, epidermis, epidermal/dermal junction (basement membrane), dermis, and hair follicles, of the explants was determined, in order to characterise the migration of melanocytes from the epidermal layer into the hair follicles (Fig.

**FIG. 2.** Morphogenesis of embryonic Dct-lacZ transgenic mouse skin compared with that in vivo. Histology of organ-cultured skin after (A) 48 h, (B) 72 h, (C) 96 h, and (D) 12 days in culture compared to (E) developing transgenic Dct-lacZ mouse skin at E16. The tissue sections were counterstained with eosin. Development of the embryonic skin in culture and the distribution of the melanoblasts are morphologically and temporally similar to those in vivo. The majority of blue-stained melanoblasts are found associated with the apical side of the basement membrane after 48 h in culture. Melanoblasts localise to the invaginating follicles at 72 and 96 h. After 12 days in culture the melanocytes are almost exclusively localised to the region above the dermal papilla. The melanin produced by the active melanocytes is also evident. Similar staining was observed in at least five different independent cultures for each time point. Scale bar, 100 μm (A–C, E) and 30 μm (D).
3). To distinguish between melanoblasts in the epidermis or at the epidermal/dermal junction at early stages (up to 48 h) when the developing keratinocyte layers are thin, stained cells were considered to be in the epidermal layer if they did not touch the basement membrane (see Fig. 2A). At the time of dissection (E13.5), the melanoblasts have just initiated their penetration of the epidermis from the dermal mesenchyme. Over the next 48 h the fraction of melanoblasts in the epidermis and dermis decreases as they become localised to the apical side of the basement membrane (Figs. 2 and 3). Localisation of the melanoblasts to rudimentary hair follicles begins after 48 h in culture and by 96 h approximately 50% of the melanoblasts are present in the follicles. At this time total melanocyte numbers in the upper epidermal layers and in the dermis are substantially reduced. Nevertheless, almost 40% of the stained cells remain associated with the basement membrane, reflecting the interfollicular melanoblasts seen in the intact cultures after 96 h.

With careful maintenance of the air–liquid interface, the explants can be kept in culture for up to 12 days (Fig. 2D). The morphogenesis of the skin explants during days 4–12 is again histologically and temporally identical to the process in vivo although some increased proliferation of the interfollicular epidermis is observed as reported previously (Kashiwagi et al., 1997). Melanin granules are observed in the hair follicles after 6–8 days in culture and in the majority of explants, pigmented hair shafts are formed. In the mature follicles, the melanocytes appear localised to the basal layer of the hair matrix in close proximity to the dermal papilla (Fig. 2D), consistent with what has been observed previously (Hirobe, 1995). Therefore, the tissue excised from E13.5 embryos contains all the developmental cues necessary for the differentiation of primitive melanoblasts into active melanocytes capable of producing pigment.

After 12 days in culture ~90% of all stained cells are found in the follicles (Fig. 3) and the interfollicular melanocytes associated with the basement membrane, in the upper epidermal layers, and in the dermis are reduced compared to 4 days in culture. This may be due to the continual migration of melanocytes from the basement membrane to the follicle or a selective reduction of those present in the dermis and basement membrane due to the down-regulation of growth factors necessary for their survival. Interestingly, by 12 days only 4–10 melanocytes are found per follicle, a significant reduction of the 14–20 stained cells observed localising to each follicle after 4 days in culture (compare Figs. 2C and 2D).

**Localisation of Melanoblasts to Hair Follicles Is an Active Process**

Is the observed localisation of melanoblasts to hair follicles due to active migration of the cells to the follicles, or is there a stimulus that causes melanoblasts within follicles to proliferate more than those outside, thereby resulting in a preferential accumulation? The development of hair follicles can be almost completely inhibited by treating the explant with epidermal growth factor (EGF) without affecting the stratification and keratinisation of the epidermis (Kashiwagi et al., 1997). EGF specifically inhibits the appearance of the epidermal thickening known as the hair placode at the first stage of follicle formation. Addition of EGF beyond E14.5 when cells are committed to forming follicles does not inhibit their formation.

We treated explants with 30 ng/ml EGF throughout the culture period, stained with XGal after 96 h, and examined serial paraffin sections of the samples. The EGF-treated explants showed a significant reduction in the number of follicles formed compared to untreated controls. On average, the numbers of follicles observed were reduced from four or five per microscope field in untreated controls to fewer than one per field in the treated cultures. Following inhibition of hair follicle formation (Figs. 4A, 4B, and 4D) the proportion of melanoblasts located at the epidermal/dermal junction is significantly increased compared to untreated controls (P < 0.00005 by t test assuming unequal group variances). Interestingly, we also see a significant reduction in the number of melanocytes found in the epidermis of the treated cultures (Fig. 4D). We do not know the reason for this reduction; it may be a secondary effect of EGF action on the structure of the skin explants. Table 1 demonstrates that the overall number of melanoblasts in the explants remains unchanged by treatment with various different concentrations of EGF. The number of melanocytes is therefore unaffected by the presence or absence of follicles and we can thus exclude the possibility that melanocyte concentration is due to enhanced proliferation of these cells at this location. Melanoblasts, in the absence of hair follicles, move onto the epidermal/dermal junction, but without a migratory target remain there.

To observe whether melanoblasts can enter rudimentary hair germs, at the second stage of follicle formation, E13.5, skin explants were treated with the alkaloid cyclopamine (Gaffield and Keeler, 1996), which has been shown to block Sonic hedgehog (SHH) signal transduction in neural plate explants and vibrissa pads (Cooper et al., 1998; Incardona et al., 1998; Chiang et al., 1999). SHH signalling is essential for controlling follicle ingrowth and morphogenesis. Lack of SHH blocks hair follicle development at the bud stage after initial epidermal–dermal interactions. SHH is expressed by the epidermal cells as they invaginate into the dermis with expression of Patched, a putative SHH receptor detected in the adjacent mesenchymal tissue (Bitgood and McMahon, 1995; Iseki et al., 1996; Oro et al., 1997; Motoyama et al., 1998). Rudimentary hair germs composed of epidermal placodes and associated dermal condensates are detected in both control and SHH-deficient embryos at E14.5, but subsequent stages of follicle development are blocked in the mutants (St-Jacques et al., 1998; Chiang et al., 1999).

Cyclopamine-treated explants were stained with XGal after 96 h in culture and sectioned to reveal follicle struc-
ture and melanoblast localisation. Follicle invagination was blocked in the cyclopamine-treated explants at stages 1 and 2 of morphogenesis and the hair germs penetrated less deeply into the dermis (Fig. 4C). However, the treatment did not disrupt normal skin development, noted by the appearance of granular and cornified cell layers. There was no difference in the density or spacing of rudimentary hair germs observed per micrometer of tissue in the treated explants compared to the developed follicles in the untreated controls. Melanoblast numbers were unaffected by the treatment (Table 1) and the rudimentary hair buds of the treated explants contained melanoblasts after 96 h, albeit in a reduced proportion compared to those in the untreated follicles (Fig. 4D). An increased proportion of melanoblasts is found at the epidermal/dermal junction and fewer localise in the rudimentary hair germs. Thus, melanoblasts remain associated with the basement membrane in the complete absence of hair follicle initiation, but are capable of localising to the early hair buds once invagination has commenced.

**MFG Enhances Migration of the Melanoblasts into Hair Follicles**

Do specific signals emanate from the hair follicle and promote melanoblast migration and localisation during the critical period of E13.5–E16.5? One candidate for inducing a motogenic response in melanocytes is MGF (also known as Steel factor), the ligand for the tyrosine kinase receptor, KIT. Active MGF signalling is required for migration of melanoblasts from the neural crest (Wehrle-Haller and Weston, 1995). In addition, KIT function appears essential for melanoblast survival and proliferation during embryogenesis (Steel et al., 1991; Murphy et al., 1992; Cable et al., 1995; Wehrle-Haller and Weston, 1995; MacKenzie et al., 1997) and for migration of the dermis to the epidermis (Mayer, 1973; Nishikawa et al., 1991).

To test for KIT expression by the melanoblasts in the organ cultures, which may thus have a role in their migration to the follicles, we assessed the effect of adding ACK2 KIT blocking antibody to the explants. No melanoblasts survive in the skin biopsies after 4 days in culture if ACK2 antibody is added at the initiation of growth or 24 h later and maintained throughout the culture period (data not shown). However, if the antibody is added after 48 or 72 h of growth, any melanoblasts that are already in the follicles at these time points can survive and appear to be KIT-independent as previously observed in vivo (Nishikawa et al., 1991; Yoshida et al., 1996a). Thus melanocytes localising to the follicles appear to express the KIT receptor and are thus responsive to its ligand MGF.

If MGF is a chemoattractant for melanoblasts, we reasoned that addition of a localised source of the factor to the skin culture should cause an accumulation of melanoblasts around the source. We implanted slow-release Affi-Gel beads coated with MGF into upper layers of skin biopsies and stained with XGal after 96 h in culture. The developing tissue grew around the beads, trapping them in the dermal layers. Melanoblasts were not attracted towards the high source of MGF in the implanted bead, but interestingly many more melanoblasts became specifically localised to the follicles compared to control cultures containing PBS-soaked beads (Figs. 5 and 6A). Indeed, addition of soluble MGF directly to the culture medium achieved the same effect (Figs. 5 and 6B). Approximately twofold more melanocytes are observed in the follicles in the presence of slow-release beads soaked in 10 ng/μl MGF or 0.2 ng/μl exogenous MGF added directly to the culture. The MGF-treated skin biopsies cultured for 4, 24, 48, 72, and 96 h show increased numbers of melanoblasts localising to the developing follicles from 48 h (Fig. 6B) but no differences in the number of melanoblasts observed per microscopic field or morphology of the melanocytes compared to untreated controls. Follicle morphogenesis is also unaffected by the MGF treatment with no observed differences in the follicle density or spacing during the 96 h of culture.

We considered the possibility that any potential MGF chemotraction for melanoblasts to the implanted bead is

**FIG. 3.** Localisation of melanoblasts in skin explant cultures from Dct-lacZ embryos after defined culture periods of 0, 4, 24, 48, 72, and 96 h and 12 days. The blue-stained cells are found in the epidermis, epidermal/dermal junction, dermis, or hair follicle during this time period. The proportion of cells present in each location was determined from the mean of the total number of stained cells in four defined microscopic fields counted from five independent cultures for each time point and expressed as a percentage of the total number of lacZ-positive cells. At the time of dissection (0 h) the melanoblasts are migrating from the dermis to the epidermis. Localisation to the follicles appears to express the KIT receptor and are thus responsive to its ligand MGF.

**FIG. 4.** Localisation of melanoblasts in skin explant cultures from Dct-lacZ embryos following 96 h in culture after treatment with 10 mM cyclopamine and 30 ng/ml EGF. Histology of organ-cultured skin upon addition of (A) no factor, (B) 30 ng/ml EGF, and (C) 10 mM cyclopamine. (D) The proportion of cells present in each location determined from the mean of the total number of stained cells in four defined microscopic fields counted from five independent cultures for each treatment and expressed as a percentage of the total number of lacZ-positive cells. The 5-μm paraffin tissue sections were counterstained with eosin. Development of the epidermal layers is unaffected by the addition of the factors. Melanoblasts can localise to the stage 2 hair germs formed after the inhibition of Shh signalling, whereas complete lack of follicle formation induced by EGF treatment forces the melanoblasts to remain at the basement membrane. Similar staining was observed in at least five different independent cultures for each time point. Scale bar, 100 μm (A–C). Error bars, SEM.
masked by stronger signals emanating from the developing hair follicles. We examined cultures containing embedded MGF-soaked beads after 4–72 h of growth. In these early stage cultures, few follicles are present but there was nevertheless no increase in melanoblast density around the bead compared to the rest of the culture (data not shown).

Furthermore, we examined cultures treated with 30 ng/ml EGF to inhibit follicle formation and which also contained embedded MGF-coated beads. These cultures are identical to those treated with EGF alone (Fig. 5E). The melanoblasts are evenly dispersed throughout the tissue biopsy with no locally dense areas around the bead. Moreover, the morphology of the melanoblasts in the dual-treated cultures is similar to untreated controls. Thus a localised source of MGF even in the absence of follicles does not appear to attract melanoblasts in developing skin.

It appears that melanocytes associated with the basement membrane show increased motility in the presence of high concentrations of MGF and become more rapidly localised in the follicles. However, as melanoblasts are not attracted...
to a source of MGF, and localise in response to the factor directly added to the culture medium, we rule out MGF as a chemotactic factor produced by the follicles. The data indicate that it is a chemokinetic factor, which appears to stimulate melanoblast migration, but there is another mechanism that ensures correct localisation. We do not know if localisation is in response to another factor emanating from that site (and to which melanoblasts are accelerated by MGF) or whether the melanoblasts move at random on the basement membrane and through the hair follicles and are signalled to stop migrating by follicular factors. In this case MGF would accelerate melanoblast localisation by accelerating their random movements.

**DISCUSSION**

**Organ Culture Accurately Reflects in Vivo Development**

The Dct-lacZ transgenic mice provide an assay for melanoblast distribution during embryonic development (MacKenzie et al., 1997). We have used an organ culture system in order to model and manipulate some of these events in vitro. The cultured skin biopsies from E13.5 transgenic mice provide an assay for melanoblast proliferation. Treatment with EGF, transgenic embryos develop in vitro histologically and temporally very similar to their development in vivo. The early patterning of the follicles is retained in the culture and melanoblasts can be easily identified in the skin layers. Moreover, the number and distribution of XGal-stained melanoblasts in cultured skin tissues and age-matched Dct-lacZ embryos are very similar. In contrast to many previous studies that have assessed melanoblast proliferation and differentiation using dissociated epidermal ectoderm cultures (Mayer, 1980, 1982) or disaggregated mouse epidermal cell suspensions (Hirobe, 1992, 1994), our culture of whole skin explants allows the manipulation and visualisation of melanoblasts within their native tissue environment.

During the initial culture period, the segregation of melanoblasts between the dermal and the epidermal layers is in agreement with that previously found by Mayer (1973) using skin recombination experiments. Over the next few days, the melanoblasts are redistributed from the epidermis to initially reside on the apical side of the basement membrane but thereafter in the follicles. Melanoblasts have the capacity to enter follicles at very early stages of follicle morphogenesis. Most notably they are found in the stage 2 and 3 follicles after 48 h in culture and continue to localise there during the downgrowth of follicles, such that after 96 h in culture approximately 50% of the stained cells are found in the follicles. After prolonged culture of up to 12 days a population of melanocytes, capable of producing melanin, is retained in the innermost layer of the hair matrix and the cells are no longer detected elsewhere.

The number of melanoblasts increases at two distinct time points during skin explant culture and is followed by a decrease. The number of stained cells per microscope field increases during the initial 24 h in culture and thereafter the density remains constant, in good agreement with the numbers of epidermal melanoblasts and melanocytes observed in pieces of skin excised from mouse embryos (Hirobe, 1984). A given area of skin can support only a limited density of melanoblasts (Rawles, 1947; Mayer, 1970) and colonisation of the epidermal layers in culture by E14.5 appears to prevent the entry of additional melanoblasts. Although the melanoblasts redistribute between the epidermis and the dermis during this period, their overall numbers remain constant. A second increase in melanocyte numbers occurs after 6 days in culture, consistent with the increase seen after birth in mouse skin (Hirobe and Takeuchi, 1977, 1978). By 12 days, when over 90% of the melanocytes are found in the follicles, the total number has significantly decreased (P < 0.011 by t test).

**Hair Follicles Provide a Localisation Signal**

Multiple signals and stimuli mediate the epithelial-mesenchymal interactions necessary for hair follicle morphogenesis during a series of stages previously defined (Hardy, 1992; Paus et al., 1999). We could specifically inhibit stages 1 and 2 of morphogenesis in the skin culture system and observe the effects on melanoblast numbers and distribution. Treatment of skin explants with EGF, transforming growth factor-α, or transforming growth factor-β1 inhibits the initiation of hair germ formation (Kashiwagi et al., 1997; Foitzik et al., 1999). Sonic hedgehog regulates the mesenchymal component of the hair follicle and is thus required for the progression of follicle morphogenesis past the hair germ stage of development. This process can be blocked using the SHH-inhibitor cyclopamine. Neither EGF nor cyclopamine treatment disrupts skin development apart from their effect on follicles.

Inhibition of follicle development had no effect on melanoblast proliferation but profoundly affected their localisation. We can thus exclude a mechanism of follicle localisation in which melanoblasts are stimulated to proliferate in the follicles and thereby accumulate by a passive mecha-

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**TABLE 1**

Inhibition of Hair Follicle Formation Has No Effect on Melanocyte Proliferation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean No. melanocytes (±SD)ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>17.1 (±5)</td>
</tr>
<tr>
<td>EGF (15 ng/ml)</td>
<td>18 (±2.4)</td>
</tr>
<tr>
<td>EGF (20 ng/ml)</td>
<td>16.8 (±1.3)</td>
</tr>
<tr>
<td>EGF (30 ng/ml)</td>
<td>17 (±4.7)</td>
</tr>
<tr>
<td>Untreated</td>
<td>20.9 (±0.7)</td>
</tr>
<tr>
<td>Cyclopamine</td>
<td>20.4 (±1.2)</td>
</tr>
</tbody>
</table>

a Mean of total cell number counted within four 0.142-mm² microscopic fields.

b Five independent cultures were analyzed for each treatment.
MGM and Melanoblast Migration

nism. The complete lack of follicles in the EGF-treated explants resulted in melanoblasts remaining associated with the basement membrane but their numbers were not affected. The follicles, then, must produce a signal that causes active movement of melanoblasts towards them. This signal is produced early in follicular development; melanoblasts can be seen in the developing hair buds after 48 h in untreated cultures. Furthermore, by using a specific inhibitor of SHH, we are able to block development at stage 2 and these hair buds also attract melanoblasts. Initiation of the invagination of the epithelium into the surrounding dermis appears to be sufficient to localise melanoblasts to the developing follicle. These melanoblasts are able to differentiate into pigmented melanocytes because skin from embryonic Shh mutant embryos, if grafted onto the back of nude mice, will generate hairless but pigmented skin (St-Jacques et al., 1996).

Mechanism of Melanoblast Localisation to Hair Follicles

What are the mechanisms regulating the specific migration and localisation of melanoblasts from the epidermis to the hair follicle? Analysis of melanoblasts in Kit mutant embryos reveals that KIT stimulation is necessary for melanoblast survival between E10.5 and E11.5, for proliferation of melanoblasts after E11.5, and also for the early migration of melanoblasts from the neural crest to a region above the somites. However, mutant analysis cannot inform us as to migratory events later, as melanoblasts in the severe Kit mutants that fail to migrate do not survive to later stages. Administration of Kit blocking antibodies by injection into adult mice or into embryos in utero by injection into pregnant females shows that KIT is needed as a survival signal for proliferating melanoblasts in the epidermis (Nishikawa et al., 1991; Yoshida et al., 1996a). During postnatal life, KIT is also required for melanocyte activation concomitant with the hair cycle. The melanoblasts that enter developing hair follicles around E14.5, however, appear to survive without a KIT signal until they are reactivated upon initiation of the first wave of the hair cycle after birth (Nishikawa et al., 1991; Yoshida et al., 1996a). Moreover, KIT blocking antibody does not affect the survival of follicular melanoblasts in our cultures, whereas interfollicular melanoblasts fail to survive.

As KIT signalling has been shown to be necessary for migration from the neural crest, it is a candidate for the follicle localisation signal. We asked whether the KIT ligand, MGF, provides a chemotactic signal for melanoblast localisation to the follicle in our organ cultures. High levels of Mgf expression are detected in the dorsal region of the somites, over which the presumptive melanoblasts migrate from the neural crest (Matsui et al., 1990; Manova and Bachvarova, 1991). Transcripts are also detected in the ectoderm of whisker follicles and the dermis at E12.5. Around E16.5, it can be detected in both the dermis and the epidermis of skin (Motro et al., 1991; S.A.J., unpublished observations). Using Mgf-lacZ transgenic mice expression is detected in the initial mesenchymal condensations, which form the dermal papillae of hair follicles (H. Yoshida, personal communication), consistent with a role for the morphogen in actively guiding the melanoblasts into the follicle. Moreover, lacZ expression is restricted to the dermal papilla region of the skin after birth (Yoshida et al., 1996b).

However, implantation of beads soaked in MGF into the epidermis of the skin biopsies did not direct melanoblasts towards this source of morphogen. Instead, more melanocytes became localised in the hair follicle after 96 h in culture, compared with untreated controls. Addition of soluble MGF to the medium has the same effect of accelerating follicular localisation from 48 h. Even when any potentially opposing follicle morphogenetic signals are masked by treatment with EGF or in biopsies cultured prior to the onset of morphogenesis, melanoblasts were still not attracted towards the MGF-coated beads. It appears that increased concentrations of MGF stimulate melanoblast motility on the basement membrane and promote active localisation. If there is a chemotactic factor that actively attracts melanoblasts to the follicles, then it is not MGF. Alternatively there may not be a chemotactic factor; localisation may come about by other guidance cues or even by random migration, but once in the follicle the melanoblasts stop migrating and remain there. Perhaps the soluble form of MGF induces increased acceleration of the melanoblasts while transmembrane MGF results in localisation. Adhesion molecules within the local microenvironment may also direct melanoblasts into the follicles. Coordinated expression of E- and P-cadherins may act to guide melanoblasts from the dermis to the follicle (Nishimura et al., 1999).

After 96 h in untreated cultures only about 50% of the melanoblasts are localised to follicles. Over the next several days this proportion increases, but it appears that this is due not to further migration to the follicle but to death of interfollicular melanoblasts as overall melanoblast numbers decreased. We suggest that MGF is acting as a survival factor up to 96 h after which its expression stops in the epidermis, but continues in the follicle, resulting in differential death of those melanoblasts outside the follicles. Support for this comes from transgenic mice, which have ectopic expression of Mgf in epidermal keratinocytes. These animals retain interfollicular melanocytes and have pigmented skin (Kunisada et al., 1998).

In summary, we have demonstrated a previously unknown function of MGF and the KIT receptor. It is known that MGF promotes migration of early melanoblasts from the neural crest. We now show that it also promotes migration later in development. It does not, however, act as a chemotactic factor, rather it is chemokinetic and accelerates movement of melanoblasts, which are guided or maintained in their correct location by other mechanisms.
FIG. 6. Localisation of melanoblasts in skin explant cultures from Dct-lacZ embryos following 96 h in culture after treatment with (A) slow-release Affi-Gel blue beads soaked in various concentrations (1–10 ng/µl) of MGF and (B) exogenous MGF (0.2 ng/µl) added directly to the cultures after defined culture periods of 24, 48, 72, and 96 h compared to untreated controls. The blue-stained cells are found in the epidermis, epidermal/dermal junction, dermis, or hair follicle during this time period. The proportion of cells present in each location was determined from the mean of the total number of stained cells in four defined microscopic fields counted from five independent cultures. The proportion of melanoblasts found in the follicle increases after treatment with MGF from 48 h compared to untreated controls. Error bars, SEM.
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