

**THE CONTROL OF MELANOGENESIS IN
EARLY AVIAN EMBRYOS**

by

Philippa Anne Hulley

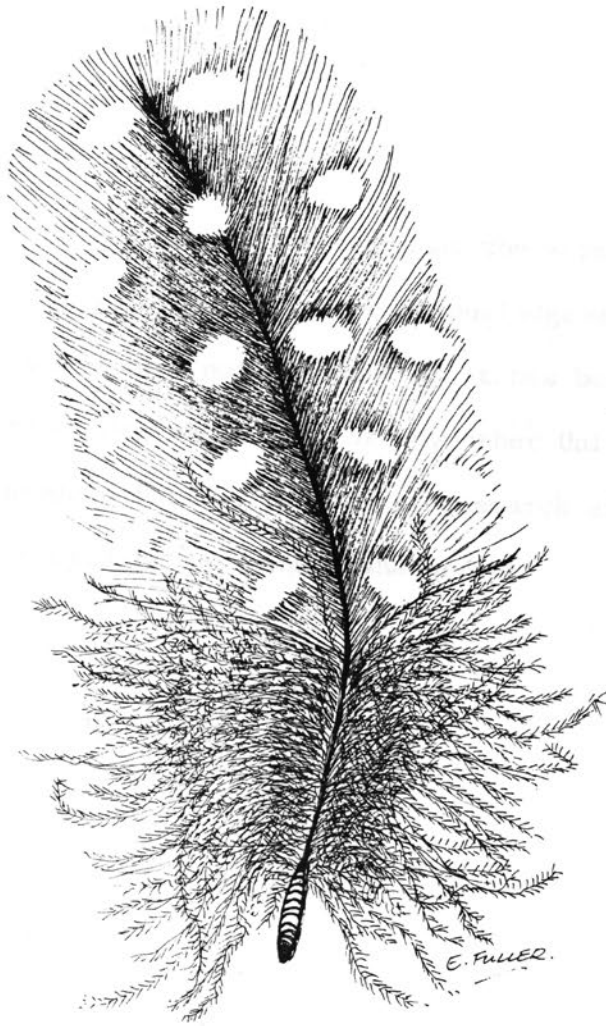
Submitted in fulfilment of the requirements for the degree
of DOCTOR OF PHILOSOPHY
in the Department of Anatomy and Cell Biology in the Faculty of Medicine at the
University of Cape Town.

July 1992

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ABSTRACT

The control of melanogenesis in early avian embryos

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The developing neural crest in avian embryos presents a unique system for the investigation of cell migration and differentiation. A variety of histological, cellular and molecular techniques have been used in this study to explore and clarify the exact sequence of events occurring during the determination and differentiation of neural crest cells into melanocytes, to discover where in the developing embryo these events take place and to investigate the mechanisms whereby these processes are controlled. Homo- and heterotypic combinations of white (White Plymouth Rock x Cornish Game) and black (Black Australorp x New Hampshire Red) chick epidermis and dermis have been used to demonstrate that premelanocytes migrate first through the sub-epidermal mesenchyme and then begin to cross into the epidermis from day 4. Results from these grafts and from tritium-labelling studies strongly suggest that there is little or no reverse migration from epidermis to dermis. This resolves the conflict of whether premelanocytes migrate immediately into the epidermis from the neural crest or spread out laterally around the body in the dermis before crossing into the epidermis.

Since the microenvironment is thought to play a key role in the control of neural crest cell diversification, the timing and route of migration may be critical factors in melanocyte differentiation. Pigmented melanocytes first become visible in the dermis at day 5 and tyrosinase activity (the key enzyme in melanin production) was detected in 5 day chick skin using a radiometric assay. *In situ*

hybridization of sections through 5 day embryos using Digoxigenin-labelled riboprobe revealed the presence of numerous tyrosinase mRNA-positive cells in the dermis and crossing into the epidermis. It is therefore likely that the dermis provides the first appropriate environment for melanocyte differentiation and not the epidermis as previously thought. However the role of the dermis may be permissive rather than active because when the dermis was replaced with gut mesenchyme, melanocytes still differentiated.

The presence of epidermis was shown to be critical for melanocyte differentiation. When 4 day dermis (prior to the appearance of melanin) was cultured in isolation from epidermis, melanocytes did not differentiate. Similarly, when epidermis was replaced with gut or chorionic epithelium, melanocytes largely failed to differentiate. Therefore the epidermis seems to be inducing terminal melanocyte differentiation, albeit at a distance from the migrating cells. Although the dermis does not seem to be essential for melanocyte differentiation, it appears to inhibit melanocyte differentiation in white pattern feathers. When the epidermis on the dorsal surface of quail wing buds was replaced with epidermis from a white chick, normally pigmented feathers formed but when the ventral wing epidermis was replaced, most of the resulting feathers were unpigmented. This is the normal colour pattern of quail wing feathers and the result therefore indicates that the ventral wing dermis is responsible for inhibiting melanocyte differentiation in the white pattern feathers of an otherwise pigmented bird.

The question of how melanocytes locate and physically migrate into the epidermis was addressed by developing a variety of novel organ culture procedures. When whole 4 day skin or heterotypic combinations of pigmented dermis with white epidermis were cultured on rafts floating in medium containing GRGDS, the cell-binding site peptide sequence of fibronectin, epidermal pigmentation was severely inhibited. When the laminin cell-binding site peptide, YIGSR, was similarly

used, normally pigmented epidermis and feathers developed. Therefore it is likely that melanocytes use fibronectin and not laminin when migrating through the dermis and into the epidermis. Melanocytes may also need to produce proteases in order to invade the epidermis since raft culture of skin in TLCK, a serine protease inhibitor, prevented epidermal melanisation. Finally, the ability of the epidermis to chemotactically attract melanocytes was investigated by assessing the change in distribution of melanocytes in dermis cultured on a filter above a well containing pieces of epidermis. The epidermis appears to be producing a diffusible factor which attracts melanocytes and is not mimicked by NGF, EGF, bFGF or retinoic acid. This is the first evidence that the epidermis may play a role in the homing of melanocytes.

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CHAPTER 1

The timing and route of migration of melanocytes in chick skin

1.1 Introduction

Melanocytes originate in the neural crest, a transient embryonic structure which forms from the dorsal lips of the neural tube during neurulation (Dorris, 1939). In the chick, this crest of cells stages a mass exodus from the neural tube between 50 and 55 hours of *in ovo* development (Weston, 1963). Neural crest cells stream out into different body regions, giving rise to derivatives as varied as melanocytes, cardiac septum, craniofacial cartilage, calcitonin cells of the thyroid, chromaffin cells of the adrenal medulla, sensory and autonomic neurons and Schwann cells (Le Douarin, 1982). This exceptional pluripotency and migrational ability singles out the neural crest as a unique system for the investigation of cell migration and differentiation.

Somewhere along the dispersal routes of the migrating neural crest, the cells become restricted to individual mature phenotypes through a process of differentiation. This maturation process could range from the programmed migration of a predetermined cell to the stochastic wanderings of a pluripotent progenitor cell which only undergoes developmental restriction once it reaches a recognised target organ. When blocks of neural crest are transplanted heterotopically between embryos of the same stage, the neural crest cells follow the migration routes and take on the phenotypic characteristics of the host site (Le Douarin, 1980). This suggests that the cells are multipotent and that the local tissue environment encountered by the migrating cells is undoubtedly playing a role in directing their movements and their differentiation. However, the extent of this influence can only be established once it is known precisely where and when neural crest cells (NCCs) migrate, and following that, where they undergo determination and differentiation.

1.1.1 Approaches to analysis of neural crest cell migratory pathways

The major problem experienced when trying to trace NCC migration pathways is that once the cells leave the neural tube, they resemble the mesenchymal cells amongst which they migrate. One approach to this problem has been to label neural tubes with a non-toxic marker such as tritiated thymidine (Weston, 1963), transplant the tubes into host embryos and observe the resulting dispersal of labelled cells. The major disadvantage of this technique is that the label rapidly dilutes with cell division, thus limiting the time over which dispersal can be accurately observed. Furthermore, only cells which are actively dividing during the labelling period will incorporate the radiolabel. Le Douarin and Teillet (1970) overcame this problem by transplanting quail neural tubes into chick embryos. Quail cells have distinctive nuclear heterochromatin which can be enhanced using the Feulgen-Rossenbeck staining technique, and this permits differentiation between chick and quail cells in embryonic chimeras. However, both of these techniques involve transplantation of tissue which perturbs the embryonic environment, probably affects the timing and possibly the routes of NCC migration and could also cause cellular rearrangements which might block melanocyte migration (Serbedzija et al., 1991). A further problem with embryonic transplants is that the grafts were sometimes left to heal for around 20 hours so that early NCC migration could not be examined (Erickson, 1988).

Detailed descriptions of NCC migration have come from the use of HNK-1 and NC-1, two equivalent monoclonal antibodies which recognise a carbohydrate surface epitope on most migrating avian NCCs (Tucker et al., 1984; Vincent and Thiery, 1984; Bronner-Fraser, 1986a; Loring and Erickson, 1987; Lallier and Bronner-Fraser, 1988). HNK-1 is an IgM monoclonal antibody raised against human leucocytes (Abo and Balch, 1981) and NC-1 is an IgM monoclonal raised against quail ciliary ganglion cells (Vincent et al., 1983). While fairly specific for NCCs, neither antibody labels all NCC derivatives and in particular, premelanocytes lose HNK-1 immunoreactivity almost as soon as they leave the neural tube (Vincent and Thiery, 1984; Loring and Erickson, 1987).

More recently, groups of NCCs have been labelled by microinjection of markers such as DiI (a fluorescent carbocyanine vital dye) into the lumen of the neural tube. The marker is picked up through the NCC end-feet, becomes intercalated with the cell membrane and labelled cells are thereafter readily traceable (Serbedzija et al., 1989, 1991). Similarly, when recombinant retroviruses containing the E.coli β -galactosidase (lac Z) gene are microinjected into the neural tube lumen, NCCs become infected and can be detected by a histochemical stain for lac Z (Frank and Sanes, 1991; Pomeranz et al., 1991). With both labelling methods, there is minor damage at the injection site and dye or virions can spread to adjacent tissue such as somites. A second problem is that not all cells pick up label, perhaps because they do not all extend end-feet into the lumen of the tube (Serbedzija et al., 1989). While the retrovirus is a heritable marker, DiI becomes diluted with cell divisions and is therefore only useful while studying the early stages of migration (Frank and Sanes, 1991).

1.1.2 Neural crest cell pathways

A summary of the results achieved using the above methods is given in Fig 1.1. The neural crest forms in a cranio-caudal wave along the embryonic axis (Hopkins-Fox, 1949) and migration follows the same pattern, cells emerging from the crest 3-4 somites back from the level of the most recently formed somite (Bronner-Fraser and Fraser, 1988). In avians, there are three pathways followed by truncal NCCs:

1. a dorsolateral pathway between the dermomyotome and the ectoderm (followed by presumptive melanocytes).
2. a ventral pathway through the rostral half of each sclerotome. Some cells remain in the somite and give rise to the dorsal root (sensory) ganglia and associated supporting cells. Other cells pass through and continue ventrally to form the adrenal medulla and the sympathetic ganglia.

3. Some ventrally migrating cells also pass between the somites to reach the level of the dorsal aorta and form sympathetic ganglia. These are only visible when whole mount embryos are viewed (Loring and Erickson, 1987).

Cephalic crest cell migration has not yet been studied in detail (For the rat see Tan and Moriss-Kay, 1986). In the chick, cells appear to migrate primarily beneath the ectoderm and not ventrally (Duband and Thiery, 1982). Some NCCs from the sacral and vagal levels give rise to parasympathetic ganglia of the gut (Teillet and Le Douarin, 1970; Serbedzija et al., 1991; Pomeranz et al., 1991).

1.1.3 Characterisation of the dorsolateral pathway

While the available NCC marking techniques have provided invaluable information on the precise pattern of ventral migration and the spatiotemporal population of ventral neural crest derivatives, the dorsolateral pathway has remained largely uninvestigated. This is primarily due to a lack of specific markers for premelanocytes. HNK-1 and NC-1 fail to recognise dorsolaterally migrating neural crest cells (Vincent and Thiery, 1984; Bronner-Fraser, 1986a; Loring and Erickson, 1987). Watterson (1942) observed possible premelanocytes in 3 to 4 day Barred Plymouth Rock embryos lying "partly in the epidermis and partly in the dermis" but could not provide direct proof of their phenotype. Bronner-Fraser and Fraser (1988) labelled NCCs by injecting them with lysinated rhodamine dextran and reported that a sub-population comes to lie beneath the epidermis in 4 to 4½ day chick embryos, but they make no mention of a dermal to epidermal crossing-over taking place at this stage presumably because dilution of label affects results after 2 days (Serbedzija et al., 1991). Weston (1963) radiolabelled cultured neural tubes with tritium and reimplanted them into early embryos. This method enabled him to trace the migration of presumptive pigment cells from the neural tube. He found that they entered the dorsal mid-line epidermis during stages 16-17 (51-64 hr) and by stages 21-23 (3½ -4 days) had spread within it as far as the dorsal margin of the wing bud. On the other hand, Teillet and Le Douarin (1970) grafted quail neural tubes into white chick embryos and observed a dorsolateral stream of premelanocytes moving out within the dermal mesenchyme and appearing in

melanocytes have been seen at 9 days in Black Australorps (Dorris, 1939), and at 10½ days in Barred Plymouth Rocks (Watterson, 1942), while red breeds are reported as never exhibiting pigment outside the epidermis (Dorris, 1939; Le Douarin, 1982; Sears and Ciment, 1988).

However, embryos from Black Australorp x New Hampshire Red chickens exhibit dermal melanisation from day 5, while epidermal melanisation begins to predominate from Day 6 (Hulley et al., 1991). In the light of the difficulties encountered when investigating early melanocyte migration and differentiation, it is particularly valuable to have discovered a breed of fowls in which visibly differentiated melanocytes are present during the time when the terminal migrational events are taking place. Stander made use of this discovery to map carefully the temporal and spatial distribution of pigmented melanocytes in 5 to 10 day embryonic chick skin (Standar, 1991; Hulley et al., 1991). She observed that at 5 and 5½ days, 70-80% of visibly pigmented melanocytes lie in the dermis. Thereafter there is an exponential increase in epidermal melanocyte number while dermal melanocyte numbers drop rapidly until at 10 days, only a scattered few remain. This new evidence strongly indicates that melanocytes spread out around the body in the dermal mesenchyme and cross over into the epidermis at around 5 days. However, it is unlikely that all melanocytes present in the skin at this stage are pigmented. Therefore a method had to be devised to confirm this result which would take unpigmented melanocytes into account. The results presented in this chapter represent an attempt to establish by experimental means the route taken by migrating melanocytes in the skin and the time of arrival of the first premelanocytes in dorsal epidermis.

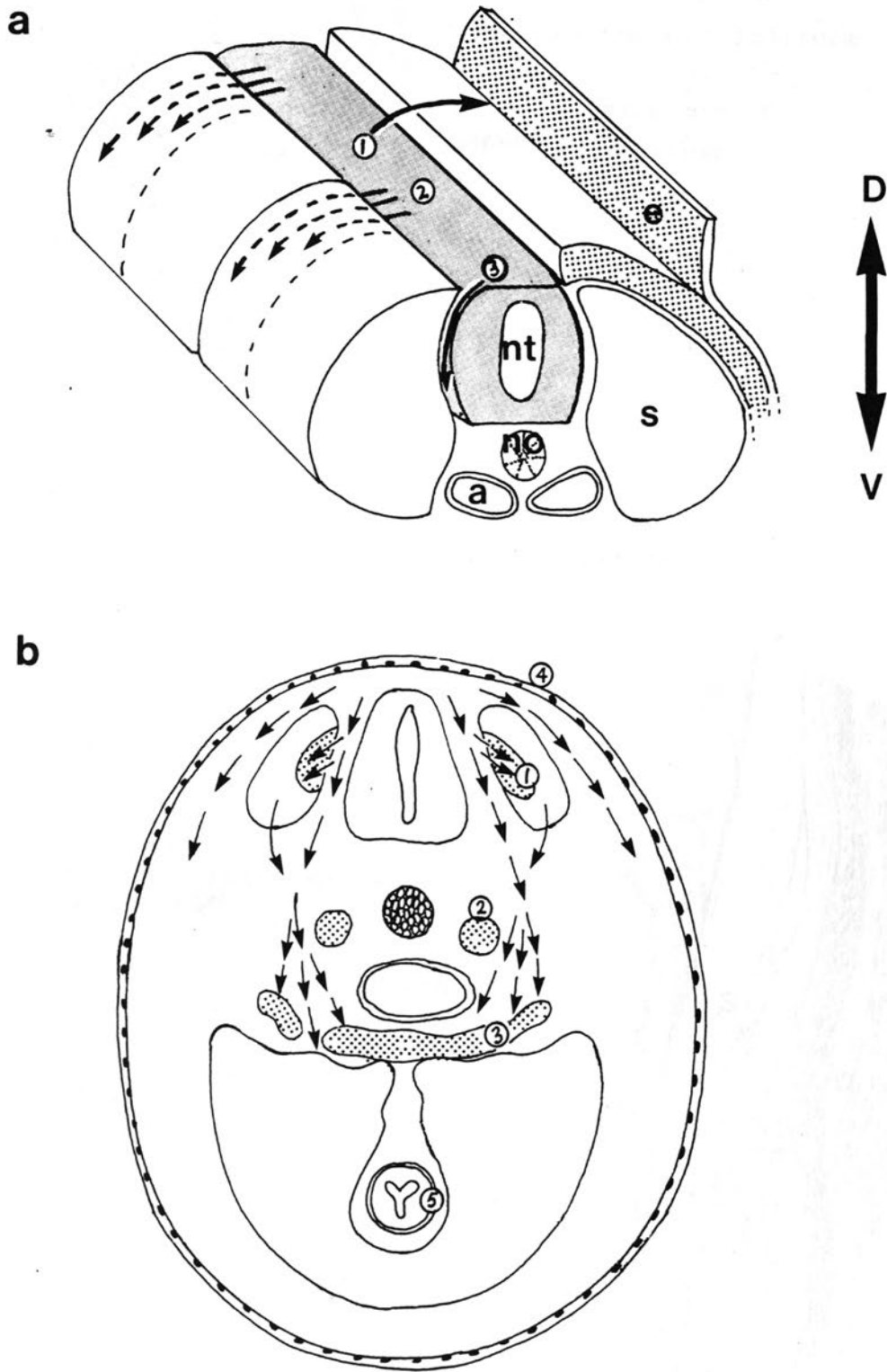


Fig 1.1

(a) Diagram summarising the pathways of migration of NCCs in the avian embryo. (1) a dorsolateral pathway between the ectoderm and somite; (2) a ventral pathway through the rostral half of each somite; (3) a ventral pathway between the neural tube and somite. e, epidermis; nt, neural tube; no, notochord; s, somite; a, dorsal aorta. (After Bronner-Fraser, 1986a).

(b) Diagram illustrating the major derivatives of trunk neural crest cells. (1) sensory ganglion (DRG); (2) sympathetic ganglion; (3) adrenomedullary cells; (4) melanocytes; (5) enteric ganglia. (After Bronner-Fraser and Fraser, 1988).

1.2 Methods

1.2.1 Materials

All embryos were staged according to Hamburger and Hamilton (1951) and the ages given in days refer to the Hamburger and Hamilton stage of development and not necessarily the period of incubation.

Black Australorp x New Hampshire Red chicks are pure black when hatched, except for a small white throat patch. According to Crawford (1990), this means the extended black locus, E, is dominant in the cross. Black Australorps are E/E and New Hampshire Reds e^{Wh}/e^{Wh} (dominant wheaten). White Plymouth Rock x Cornish Game chicks hatch with pale yellow down and develop into pure white feathered adults. Both white breeds are reported to carry the I allele, the so-called dominant "inhibitor of pigment" gene characteristic of White Leghorns (Crawford, 1990). In addition, earlier sources claim that the White Plymouth Rock is a recessive white (c/c) (Dorris, 1939; Hutt, 1949).

1.2.2 Grafting combinations of dermis and epidermis

White Plymouth Rock x Cornish Game (unpigmented feathers) and Black Australorp x New Hampshire Red (pigmented feathers) eggs were incubated for 3-10 days at 38°C in a humidified incubator. Dorsal skin was removed using iridectomy knives and treated with 1% trypsin (Difco 1:250) for 1 hr at 4°C to separate dermis from epidermis (Rawles, 1963). Enzyme activity was stopped by placing separated pieces in a 1 to 1 mixture of albumin and Ringers or foetal calf serum and Ringers (0.12 M NaCl, 5 mM KCl, 1 mM CaCl₂). Dermal and epidermal pieces were recombined (Fig 1.3a) on chick agar (Wolff, 1961) containing 10% horse serum, 10% foetal calf serum or chick embryo extract, penicillin (200 u/ml) and streptomycin (1 µg/ml), and were cultured for 24 hrs to allow reunion. The combinations of dermis and epidermis were then grafted onto the chorioallantoic membrane (CAM) of a white host embryo and allowed to develop for 7-10 days. They were subsequently removed, fixed in Bouin's fluid and processed for standard histological examination. Serial wax sections (5 µm) were lightly stained with hematoxylin

and eosin and examined for the presence and position of melanin.

1.2.3 Triple layer combinations

Pieces of 6-10 day white whole skin were cultured on top of pieces of 6 day red/black dermis, obtained as above. Combinations were cultured either on soft agar for 24 hours followed by CAM grafting and incubation for 7-10 days, or on a 0.45 μm pore size Millipore filter floating on Dulbecco's modified Eagles medium with 10% foetal calf serum, penicillin (200 u/ml) and streptomycin (1 $\mu\text{g}/\text{ml}$). Equivalent feather development was achieved using either method and therefore results have been pooled.

1.2.4 Tritium labelling

Pieces of skin from both red/black and white 6-day embryos were obtained and treated as above except that black skin was radiolabelled prior to separation of epidermis from dermis. Labelling was carried out in culture medium (Dulbecco's modified Eagle's medium) containing 10 $\mu\text{Ci}/\text{ml}$ [^3H]thymidine (Amersham International sp act 25 Ci/mmol) for 2 hr at 37°C. Labelled black epidermis was recombined with unlabelled white dermis and vice versa, and the combinations were grown on agar for 5 days. A second series of skin components were labelled overnight, recombined on soft agar and cultured for 3 days. Serial sections (5 μm) of all grafts were mounted on clean glass slides, coated with LM-1 emulsion for light microscopy (according to instructions, Amersham International), and exposed at 4°C for 10 days. Slides were developed using Ilford Phenisol developer for 5 min at room temperature, counterstained with haematoxylin, and examined for the position of labelled cells. Control sections of unlabelled tissue were treated in the same way to establish the level of chemography. Labelled cells were easily distinguished from randomly scattered background because silver oxide granules formed distinctive round clusters above the nuclei. The percentage of labelling was determined by counting the number of labelled cells in a group of 50 cells ($n=25$ sections selected randomly from all grafts).

1.2.5 Grafting of lateral skin

Lateral skin was removed from regions 1, 2 and 3 (Fig 1.3b) of 3 to 5½ day BAXNHR embryos with sharpened tungsten needles and grafted directly onto CAMs. Culture was terminated after 7-10 days and the grafts were fixed and processed as above and examined histologically for the presence of melanin.

1.2.6 Grafting of limb buds

BAXNHR eggs were incubated as above for 52 hr to 5 days. Forelimb and hindlimb buds were dissected off and grafted directly onto CAMs. Culture was terminated after 7-10 days and the grafts were fixed and processed as above and examined histologically for the presence and position of melanin.

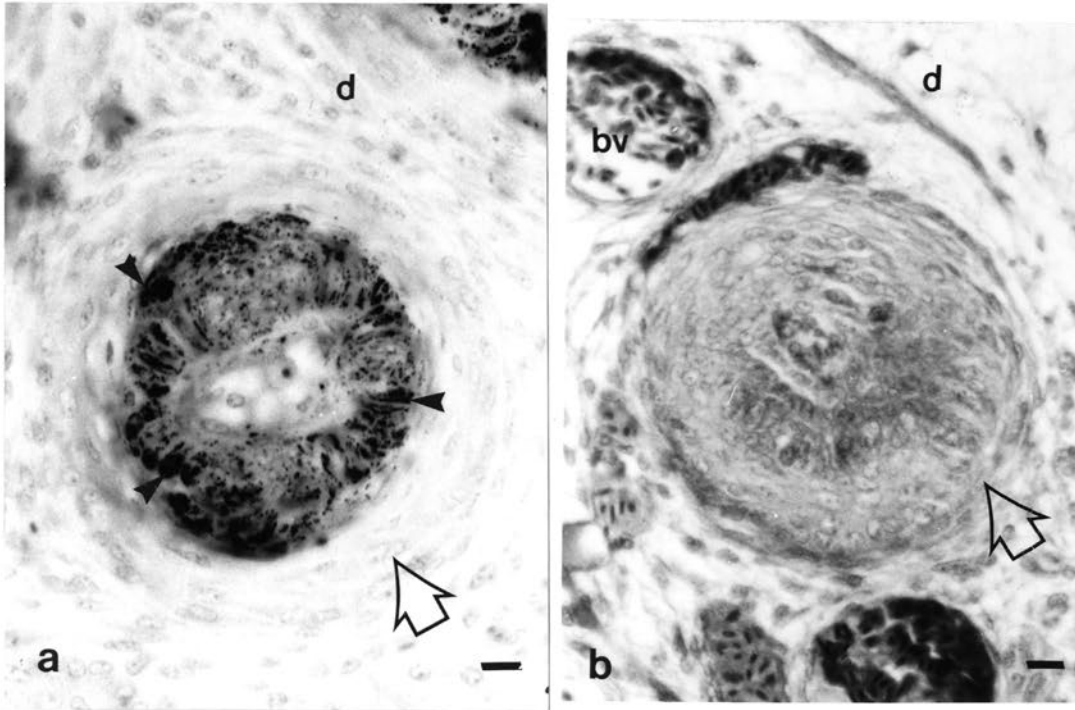


Fig 1.4

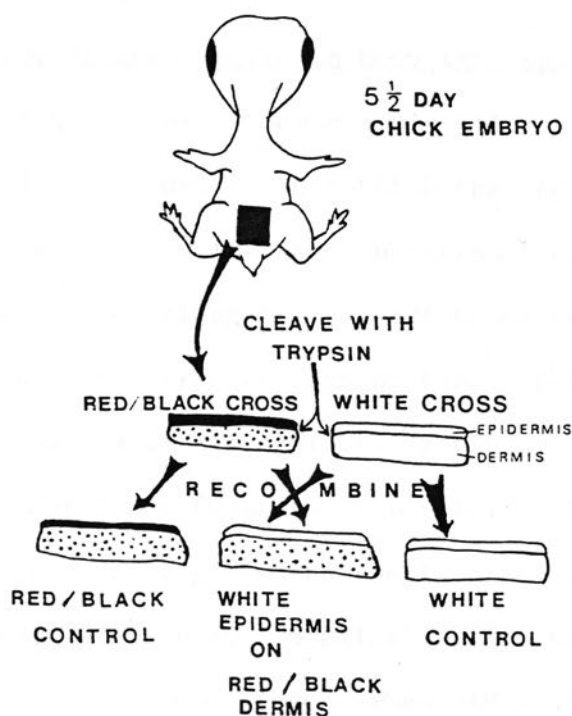
Feather rudiments produced from dermal-epidermal recombination grafts.

a. White epidermis combined with red/black dermis.

b. White epidermis combined with white dermis.

Arrowheads, melanin; open arrows, presumptive sheath cells; d, dermal connective tissue; bv, blood vessel. Bars=10 μ m.

a



b

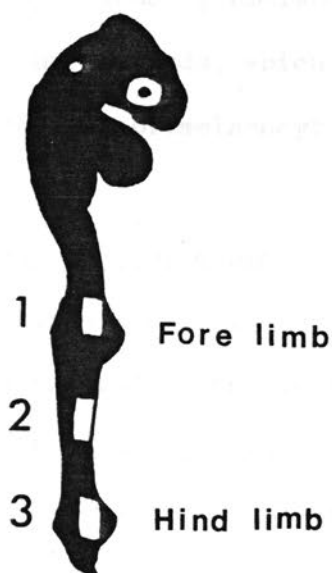


Fig 1.3

a. Scheme representing the different combinations of pigmented and unpigmented dermis and epidermis used to examine the dermal to epidermal cross-over of melanocytes.

b. Positions of lateral body regions in stage 20-28 embryos from which skin explants were removed prior to culturing on CAMs to establish the presence or absence of premelanocytes.

1.3 Results

1.3.1 Arrival of neural crest cells in body skin and limb buds

In the chick, neural crest cells commence migration between 50 and 55 hr in the trunk (Weston, 1963). In order to establish the approximate time of arrival and distribution of these cells in the skin in various body regions, skin was isolated from three regions along the lateral trunk (Fig 1.3b) and these and whole limb buds were grown for 7-10 days on CAMs. Lateral skin from embryos, Hamburger and Hamilton (1951) stages 20 to 28 (70 hr to 5½ days), consistently formed pigmented epidermis or feathers (Table 1.1). These results indicate that premelanocytes have already migrated to the lateral trunk skin, even in the most caudal region tested, by stage 20. Similarly, limb buds removed from stages 20 to 27 (70 hr to 5 days) produced pigment when CAM grafted, while those removed at stage 19 (68-72 hr) resulted in a mixture of pigmented and unpigmented grafts (Table 1.1). All limb bud grafts from stages 17 (52-64 hr) and 18 (65-69 hr) produced unpigmented grafts only. Thus, premelanocytes appear to arrive in the limb buds from stage 19 (68-72 hr) onwards, which suggests that lateral trunk skin is also likely to have received premelanocytes before day 3.

1.3.2 Melanocyte migration into the epidermis

Having established that melanocytes arrive in the skin by day 3, the next question was whether they were located in the dermis or epidermis at this stage. To test this, dorsal epidermis from 3, 4, 5 and 6 day red/black skin was combined with 6 day dermis from white skin and CAM-cultured for 7-10 days. The results, presented in Table 1.2, indicate that the feathers from 3 day-old epidermis were all unpigmented, whereas the majority of grafts with 4, 5 or 6 day epidermis formed pigmented feathers. These results indicate that the premelanocytes which have arrived in the skin by day 3 must be located in the dermis.

To test whether dermal melanocytes migrate into the epidermis, the following series of experiments was carried out. First, the dermis from 6 day red/black skin was recombined with epidermis from 6 day white skin. All 16 of the grafts thus obtained had pigmented feathers (Fig 1.4), indicating that there must have been a migration to the epidermis (Table 1.3). Similarly, when whole white skin (dermis and epidermis) was recombined with red/black dermis, all resulting grafts produced black feathers. The feathers formed in these grafts were not all as intensely black as in the previous combination, some being mixed black and white and a few pure white. Nevertheless, the result indicates that premelanocytes are able to traverse an extra thickness of dermis and a non-trypsinised basement membrane in order to enter the epidermis.

As an important control, 6 day red/black epidermis was combined with white dermis. All combinations of this type produced pigmented feathers (Table 1.3). The purpose of this combination was twofold. It was important to see whether the dermally melanised cells shown in the whole embryo counts of Stander (1991) could simply have been the result of melanocyte migration from the epidermis rather than of differentiation in the dermis (see also Hulley et al., 1991). In a few of these grafts there was a small number of pigmented dermal cells, all of which were clumped and pyknotic in appearance (similar to the melanophages illustrated in Hulley et al., 1991). The number of melanin containing dermal cells appeared to be far less than was commonly observed in the reverse combination of white epidermis with red/black dermis (Fig 1.5). The second aim of this control was to check that the white dermis did not have an inhibitory effect on the terminal differentiation of melanocytes already present in the 6 day epidermis. Feathers showing normal pigmentation formed, indicating no inhibition of developmental expression. In the above experiments, homotypic recombinations of red/black and white skin components were used as standard experimental controls (Fig 1.3). White/white combinations yielded unpigmented grafts only (Fig 1.4b and Table 1.3), indicating that under these experimental conditions, white skin is suitable for use as a pigment-free tissue source. Red/black epidermis and dermis were recombined to provide a comparison for the normal degree of feather pigmentation.

for use as a pigment-free tissue source. Red/black epidermis and dermis were recombined to provide a comparison for the normal degree of feather pigmentation.

To investigate further the possibility that melanocytes might migrate from the epidermis to the dermis, 6 day red/black skin components (dermis and epidermis) were tritium-labeled and recombined with unlabeled 6-day white skin components (Fig 1.6). Examination of autoradiographed serial sections revealed that labeled cells moved from the dermis into the epidermis in 74.7% of sections when dermis was labeled for 2 hr prior to recombination, and in 84.0% of sections when dermis was labeled overnight. In the reverse combination, however, labeled cells moved from epidermis to dermis in less than 0.98% of sections when epidermis was labelled for 2 hr and 3.9% of sections when epidermis was labeled overnight (Table 1.4 and Fig 1.6). Therefore although some melanocytes may move from epidermis to dermis, this appears to be exceptional and the numbers are not sufficient to account for the dermal melanocyte counts in whole embryos at the various ages counted in Hulley et al., (1991). The marginal increase in percentage of labelled cells crossing in both cases after overnight labelling indicates that a slightly higher degree of labelling can be achieved with a longer labelling period, but this does not alter the trend of the results and 3 out of 5 epidermal tissue pieces did not survive the extended culture apart from dermis. A shorter labelling period is therefore preferable.

Migration of melanocytes from the dermis to the epidermis appears to take place within a fairly narrow window of time. My results have shown that it commences at 4 days and it has been observed that few visibly pigmented melanocytes remain in the dermis after 8 days (Hulley et al., 1991). Is this because an age-related barrier develops in the epidermal BM which inhibits further melanocytes from entering or is the dermal complement of melanocytes totally depleted? To address this question, combinations of 6 day red/black dermis with 8,9 or 10 day white epidermis, and 8, 9 or 10 day red/black dermis with 6 day white epidermis were

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cultured on CAMs to allow feather development (Table 1.5). Somewhat surprisingly, all grafts developed heavily pigmented feathers, even those with an apparently unpigmented 10 day red/black dermis. This means that the dermis remains capable of seeding the epidermis with melanocytes even after epidermal cross-over is normally complete.

The epidermis seems to remain receptive to melanocytes beyond the apparent 4 to 8 day window during which crossing-over takes place (Hulley et al., 1991). However, when dermis and epidermis are split using EDTA, cleavage occurs along the epidermal basement membrane and most of the BM remains attached to the dermis (Peterson and Grainger, 1985). Although the red/black donor dermis has undergone a like separation, it is possible to graft the dermis upside down so there is no guarantee that an intact BM will be recreated. The period during which the BM is disrupted and BM repair is taking place may well be long enough to permit melanocyte invasion of the epidermis, thus artificially re-opening the window. To establish whether this is so, triple-layer grafts were constructed in which 6, 7, 8, 9, and 10 day whole white skin pieces (comprising epidermis, dermis and an intact BM) were recombined with 6 day red/black dermis (Table 1.5). Upon visual examination, the grafts containing 6 and 7 day white whole skin appeared to be obviously pigmented, while those containing 8, 9 and 10 day white whole skin appeared white. However when the grafts were subsequently serial sectioned and examined by light microscopy, grafts from all ages contained some degree of pigment. This was substantially less in those with 9 and 10 day white whole skin.

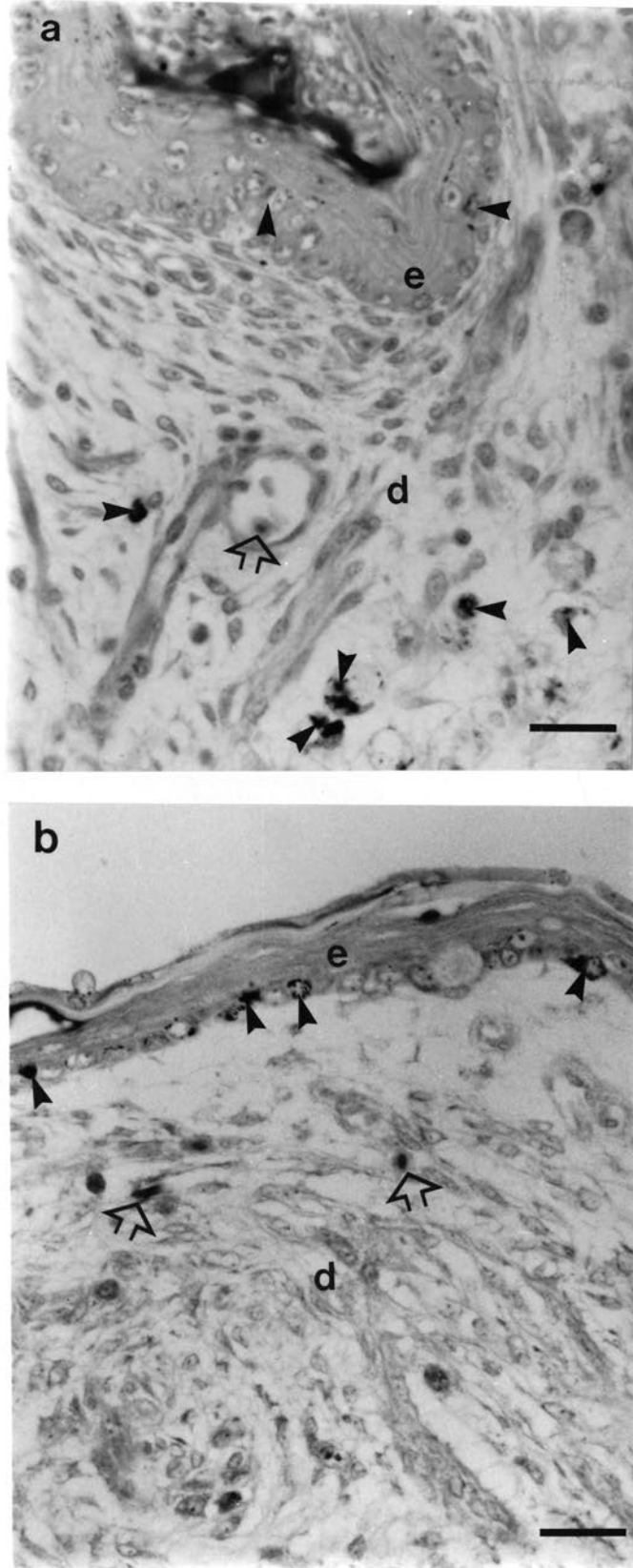


Fig 1.5

Dermal-epidermal recombination grafts showing melanin distribution.

(a) White epidermis combined with red/black dermis.

(b) Red/black epidermis combined with white dermis.

Arrowheads, melanin; e, epidermis; d, dermal connective tissue; open arrows, blood cells. Bars=10 μ m.

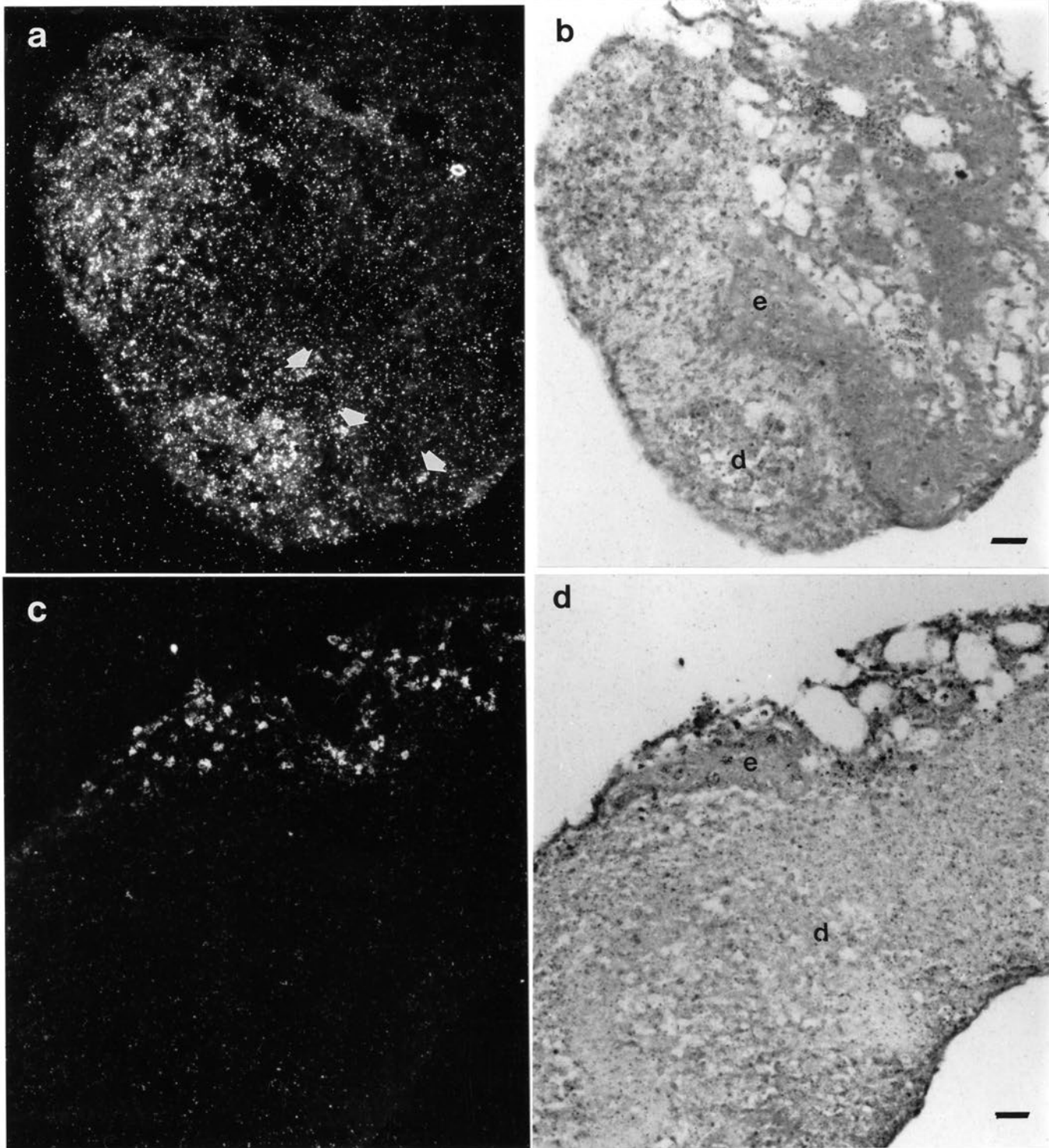


Fig 1.6

Dermal-epidermal recombination explants showing labelled cell distribution.. Bright clusters of granules represent single labelled nuclei (demonstrated best in c). (a). White epidermis combined with tritium-labelled red/black dermis (dark field). (c). Tritium-labelled red/black epidermis combined with white dermis (dark field). (b) and (d) bright field of (a) and (c) respectively. Arrowheads, labelled cells in epidermis; e, epidermis; d, dermal connective tissue. Bars=10 μ m.

TABLE 1.1
ARRIVAL OF PREMELANOCYTES IN LATERAL BODY SKIN AND LIMB BUDS

Stage *	17	18	19	20	22	24	25	27	28
Age	52-64h	65-69h	68-72h	70-72h	3½d	4d	4½d	5d	5½d
Region 1#	ND	ND	ND	X XX	ND -	X	XXX		
Region 2#	ND	ND	ND	X -	ND X	X	XXX		
Region 3#	ND	ND	ND	XXX	XX	ND X	X		
Fore limb#	00	0000	00	XXX	XX	XX ND	XX		
Hind limb#	0	0000	0X	X XX	XX ND	XX	ND		

X Individual pigmented grafts

0 Individual unpigmented grafts

- No surviving grafts

ND Grafts not done

* Stage of removal of limbs or lateral body skin (Fig 1.3b) from donor (Hamburger and Hamilton, 1951). Equivalent age given: h=hr, d=day.

Grafts were removed as shown in Fig 1.3b, CAM cultured, and examined histologically after 7-10 days for the presence or absence of pigmented epidermis/feathers.

Graft survival: Regions 1,2 and 3 = 96%, Limbs = 78%.

TABLE 1.2
ARRIVAL OF PREMELANOCYTES IN DORSAL BODY EPIDERMIS

Age of red/black epidermis (days) *	Age of white dermis (days) *	Total pigmented feathers#	Grafts with unpigmented feathers#	Grafts with feathers#
3	6	5	0	5
4	6	13	8	5
5	6	5	4	1
6	6	7	5	2

Pieces of epidermis and dermis were obtained and recombined as described in materials and methods

* Age of tissues at time of combination

After 7-10 days of CAM culture, grafts were examined histologically for the presence and position of melanin

Graft survival 87,5%.

TABLE 1.3
MIGRATION OF DERMAL MELANOCYTES TO THE EPIDERMIS.

Origin of tissue *	Age of tissue (days) #	Total grafts	Grafts with pigmented feathers@	Grafts with unpigmented feathers@
white epidermis combined with red/black dermis	6	16	16	0
white whole skin combined with red/black dermis	6	6	6	0
red/black epidermis combined with white dermis	6	7	5	2
white epidermis combined with white dermis	6	7	0	7
red/black epidermis combined with red/black epidermis	6	11	11	0

* Pieces of epidermis and dermis were obtained and recombined as described in materials and methods.

Age of epidermal and dermal pieces at the time of recombination.

@ After 7-10 days, grafts were examined for the presence of pigment in the epidermis and feathers by light microscopy.

Graft survival 84%.

TABLE 1.4
DIRECTIONAL MIGRATION OF MELANOCYTES IN CHICK SKIN

Tissue combination	Total no. of sections	No of sections exhibiting cross-over (>2 cells)	% Cross-over of labelled*
Labelled* red/black epidermis on white dermis#	715	7	0,98
White epidermis on labelled* red/black dermis#	241	180	74,70
Labelled* red/black epidermis on white dermis@	52	2	3,85
White epidermis on labelled* red/black dermis@	106	89	83,96

* Red/black skin components were labelled with tritiated thymidine prior to combination with white skin components. The percentages of labelling# remaining after 5 days in culture are epidermis 32,3 $\pm$ 12,5%; dermis 30,3 $\pm$ 16%.

Labelled for 2 hrs. Cultured for 5 days.

@ Labelled overnight. Cultured for 3 days.

Explant survival 78%.

TABLE 1.5

EFFECT OF DERMAL AND EPIDERMAL AGE ON TERMINAL MELANOCYTE MIGRATION

Age of with red/black dermis (days)*	Age of white epidermis (days)*	Age of pigmented whole skin (days)	T	Grafts with unpigmented feathers#	Grafts feathers#
6	8	ND	0	-	-
6	9	ND	2	2	0
6	10	ND	3	3	0
8	6	ND	2	2	0
9	6	ND	3	3a	0
10	6	ND	3	3	0
6	ND	6	6	4	2
6	ND	7	7	6	1
6	ND	8	5	2	3
6	ND	9	4	4	0
6	ND	10	4	3	1

Pieces of epidermis and dermis were obtained and recombined as described in materials and methods

* Age of tissues at time of combination

After 7-10 days of CAM culture, grafts were examined histologically for the presence and position of melanin.

a Two of these three grafts contained red feather melanin, as opposed to the normal dark brown melanin.

- Represents grafts which did not survive.

ND Not done.

T Total number of surviving grafts

Graft survival: epidermis/dermis combinations=73%, whole skin/dermis=100%.

1.4 Discussion

In an effort to resolve the conflict regarding the final migration pathway of melanocytes, I used combination grafts of white epidermis upon red/black dermis and demonstrated that in the chick, the dermis is capable of seeding the epidermis with viable melanocytes at 6 days of incubation, and will even accomplish this through a double thickness of dermis. Further, I have found that while whole skin and limb-buds form pigmented grafts when isolated at 68-72 hr, 3 day epidermis fails to produce pigmented feathers when cultured with white dermis as CAM grafts. This indicates that premelanocytes must be present in the dermal but not the epidermal components of the skin prior to 4 days. From 4 days onwards, red/black epidermis grown with white dermis produces pigmented feathers, indicating the presence of melanocytes in the epidermis at those stages. This provides the strongest evidence that premelanocytes are located in the dermis prior to the epidermis and that they begin to cross over into the dorsal epidermis between 3 and 4 days. My results are in agreement with Mayer (1973a), who reports that in the mouse, premelanocytes cross from dermis to epidermis between 11 and 14 days of gestation. He demonstrated this by combining epidermal and dermal components from black (C/C) and albino (c/c) mouse skins. Grafts of 11 day components only pigmented when the mesodermal tissue came from a black mouse and not with the reverse combination (that is, at this stage melanocytes are still located in the dermis). When 12 day black epidermis or dermis was used, pigmented grafts always resulted, showing that melanocytes were present in both the dermal mesenchyme and the epidermis.

The next question addressed here was whether melanocytes are capable of migrating from the epidermis into the dermis. This is a crucial question because it has bearing on the issue of whether the epidermis or dermis is the appropriate environment for melanocyte differentiation. Combinations of pigmented epidermis with white dermis or tritium-labelled, pigmented epidermis with unlabelled,

unpigmented dermis provide little support for this direction of migration. I therefore conclude that premelanocytes must pass through the dermis before entering the epidermis.

In these experiments, white skin has been regarded as a stable source of pigment-free tissue. It may be argued that pigmented feathers arising from combinations of white and red/black tissue could result from the activation (by contact with "normal" red/black tissue) of melanocytes already present in the white tissue and not from migration of melanocytes from the red/black tissue into the white tissue. The best evidence against this interpretation is the production of white feathers when a 3 day red/black epidermis was combined with a 6 day white dermis, while a 4 day red/black epidermis formed pigmented feathers under the same conditions. If co-culturing white dermis with epidermis from a pigmented breed was sufficient to override the hypothetical inhibition in the white dermis, the 3 day combination should have had pigmented melanocytes moving in from the 6 day white dermis.

To find out whether the dermal pool of melanocytes becomes exhausted during the seeding of the epidermis, 6 day white epidermis was combined with 8-10 day red/black dermis and cultured till feathers formed. All combinations gave rise to heavily pigmented feathers, indicating that as late as 10 days, the dermis contains a population of melanocytes which are capable of migrating into the epidermis. It seems likely that unpigmented melanocytes are forming a dermal reserve, perhaps for use when feathers are lost during moulting later on (Foulks, 1943).

It was established that the skin from our white cross-breed does become less receptive to donor melanocyte invasion with increasing age. While pigmented feathers formed from 6 day red/black dermis combined with both 8-10 day white epidermis and 6-10 day whole white skin, those grafts containing 9 and 10 day white whole skin were markedly less pigmented than the rest. There are three

possible reasons for this. The epidermis may be becoming less receptive to melanocytes with age, possibly caused by a thickening of the BM, or the underlying white dermis may be thickening and laying down ECM substances which inhibit melanocyte migration (see Chapter 3). It is impossible to distinguish between these two explanations because all the methods for separating epidermis and dermis seem to rely on cleavage along the BM (Woodley et al., 1983; Peterson and Grainger, 1985). Thirdly, if there is no age-related appearance of a physical barrier to melanocyte invasion of the epidermis, there are possibly a number of endogenous melanocytes (unpigmented and therefore invisible) in the white skin which may proliferate between days 8 and 10 and by some control mechanism, block further melanocyte entry. Evidence for this theory comes from studies which note that apparently unpigmented skin from White Leghorns and some red chick breeds is not receptive to donor melanocytes and an existing epidermal population of melanocytes is the reason most commonly put forward to explain this (Dorris, 1939; Willier and Rawles, 1940; Rawles, 1963). Red breeds in general initiate melanogenesis at a later stage than darker breeds (Dorris, 1939; Le Douarin, 1982). The pigmentary characteristics of our white cross-breed have not yet been established, but it seems unlikely that melanocytes present in 10 day whole epidermis will block further melanocyte entry while melanocytes in 10 day epidermis which has been split from the dermis fail to inhibit migration at all (see Table 1.5). It therefore seems likely that epidermis without an endogenous population of melanocytes remains receptive to donor melanocytes at all stages of development but that the dermis and/or epidermal basement membrane become increasingly difficult to penetrate after 8 days. By 11 days, the feather buds are so long that splitting techniques become unreliable and it is therefore difficult to test this theory further.

CHAPTER 2

The spatiotemporal pattern and control of melanocyte differentiation

2.1 Introduction

At the start of migration, the neural crest appears to be composed of a homogeneous population of cells. There is no way of knowing whether cells have already begun to diversify in response to internal genetic programs or whether they are developmentally unbiased and labile. However, when premigratory neural crest cells (NCCs) are labelled with β -galactosidase-expressing retroviruses by microinjection into the lumen of the neural tube, individual cells are seen to give rise *in vivo* to both homo- and heterogeneous clones. In the dorsal root ganglion, some clones contain only neurons or glia, others contain mixtures of neuron types, both neurons and glia or Schwann and satellite cells (Frank and Sanes, 1991). Using a complementary method, the vital dye, lysinated rhodamine dextran, was injected into individual premigratory NCCs. In some cases, an individual clonal progenitor cell gave rise to sympathetic neurons, premelanocytes, ganglionic satellite cells, adrenomedullary cells and neural tube cells (Bronner-Fraser and Fraser, 1988). These two studies clearly indicate that at least some NCCs are multipotent before their departure from the neural tube.

Further evidence of neural crest pluripotency comes from experiments involving *in vivo* manipulation of embryos. If premigratory neural tubes or neural tubes from which NCCs have already begun to migrate are transplanted into premigratory (young) host embryos, the same range of derivatives develop. However, if early or late migrating neural crest is grafted into a postmigratory host (old), the extent of the developmental potential is limited (Weston and Butler, 1966). This indicates that it is the embryonic environment which limits both the extent of migration and the range of developmental potential of NCCs and not the order in which cells leave the neural tube. When pieces of quail neural tube are transplanted heterotopically into chick embryos of equivalent age, cells appropriate to the new region develop, even though donor cells would not normally

give rise to those derivatives. For example, cranial NCCs were able to generate normal adrenal medulla when grafted to the mid-trunk and truncal NCCs from the adrenal medullary region were able to form enteric ganglia when grafted to the vagal level (Le Douarin, 1980). When cultured quail NCCs (both pigmented and unpigmented) are injected into the somite lumen of white chick embryos, they migrate along the dorso-ventral pathway and colonise the normal ventral derivatives such as dorsal root ganglia, sympathetic chain ganglia and adrenal medulla. Both pigmented and unpigmented quail cells which contribute to adrenergic derivatives begin to express catecholamines (Bronner and Cohen, 1979; Bronner-Fraser et al., 1980). It therefore seems that experimental rearrangements of the axial levels or migratory pathways of the neural crest have little effect on the formation of normal NC derivatives. This implies that at least initially, some NCCs are pluripotent and depend on cues from the local microenvironment for guidance of migration and differentiation and that there is a degree of lability even in cells which appear overtly differentiated (such as pigmented melanocytes).

2.1.1 Control of neural crest cell differentiation *in vitro*

Manipulation of *in vitro* culture conditions can give an indication of which environmental conditions are affecting the differentiation of various neural crest components. When 48-55 hr chick or quail neural tubes are explanted onto a variety of matrices including tissue culture plastic, poly-l-lysine, collagen and basement membrane components such as laminin, NCCs migrate out from the tube and form cell monolayers (Cohen and Konigsberg, 1975). Depending on the culture medium, melanocytes differentiate around day 4 in culture (Derby and Newgreen, 1982; Giss et al., 1982). Clonal cultures of individual cells show that single progenitors can produce colonies which are either totally pigmented or totally unpigmented and some colonies are mixed (Cohen and Konigsberg, 1975). The proportion of pigmented cells can be increased with the continued presence of neural tube (Loring et al., 1981; Derby and Newgreen, 1982), the addition to the

culture medium of at least 3% chick embryo extract (CEE, Glimelius and Weston, 1981), certain lots of foetal calf serum (FCS, Derby and Newgreen, 1982; Jordan et al, 1985), Buffalo rat liver conditioned medium (Giss et al., 1982), melanocyte stimulating hormone (α MSH, Satoh and Ide, 1987) and basic fibroblast growth factor (bFGF, Stocker et al., 1991). There is also a correlation between cell clustering and melanogenesis. When neural tubes are explanted onto agar, the emigrating NCCs do not spread onto the substrate but instead form clusters on the neural tube and become totally pigmented within 3-5 days (Glimelius and Weston, 1981). Substrates such as fibronectin which promote NCC dispersal delay melanocyte differentiation, while laminin promotes clustering and rapid onset of melanogenesis (Rogers et al., 1990). Mouse melanocytes differentiate in neural crest cultures in Ham's F10 culture medium plus TPA, dibutryl cyclic AMP, α MSH, calf serum and foetal calf serum (Tamura et al., 1987).

A proportion of the unpigmented cells arising from neural tube cultures become neurons- sympathetic (adrenergic), parasympathetic (cholinergic) and sensory. Chick embryo extract (at least 10-15%) and neural tube-conditioned medium both promote terminal adrenergic neuron differentiation (cells expressing intense catecholamine-specific histochemical fluorescence) after 5-6 days in culture (Cohen and Konigsberg, 1975; Howard and Bronner-Fraser, 1985; Ziller et al., 1987). If cultures are overlayed with a reconstituted basement membrane matrix, the proportion of catecholamine-positive adrenergic neurons increases 50-fold compared with untreated controls (Maxwell and Forbes, 1987). In contrast, cholinergic differentiation (cells expressing acetyl choline) is promoted by horse serum (Greenberg and Schrier, 1977; Ziller, 1986). A further difference between the two neuron types is that cholinergic neurons are competent to differentiate at the start of migration while adrenergic neurons need to proliferate before acquiring competence (Ziller, 1986). Quail NCCs in serum-free defined medium or medium with 10% CEE and 15% horse serum start expressing sensory neuron markers in 1-3 days (Ziller et al., 1987, Sieber-Blum, 1989).

2.1.2 Tissue factors affecting neural crest differentiation

While *in vitro* culture experiments have demonstrated that NCC phenotype can be dictated by certain factors, they give no indication of where these factors are encountered *in vivo*. If the migration pathway for parasympathetic neurons of the intestine is bypassed by co-culturing premigratory quail neural tubes with aneural chick gut on chorioallantoic membrane, cholinergic neurons develop in the enteric ganglia (Le Douarin, 1980). This supports *in vitro* culture results indicating that cholinergic neurons are differentiation competent at the start of migration (Ziller, 1986). In contrast, adrenergic neurons only develop in CAM-grafted gut when notochord is present on the co-cultured neural tube (Le Douarin, 1980). This requirement of notochord for adrenergic differentiation has also been demonstrated *in vivo*. When the neural tube is inverted so that the premigratory neural crest lies ventrally and the notochord dorsally, ventrally migrating NCCs do not encounter the notochord and no adrenergic neurons develop around the dorsal aorta. However, when the neural tube is inverted while leaving the notochord in its normal ventral position, adrenergic neurons develop. Furthermore, if the notochord is removed and the neural tube replaced with its normal dorsoventral orientation, adrenergic neurons still develop (Stern et al., 1991). This indicates that presumptive adrenergic neurons differentiate in response to either the notochord or the ventral neural tube region.

Sensory neurons *in vivo* appear to differentiate in response to a diffusible signal from the neural tube. When an impermeable silastin membrane is placed between the neural tube and ventrally migrating cells which have just emerged from the neural crest, the dorsal root ganglion (DRG) does not form. Sympathetic ganglia and adrenal medulla form as usual, and when the silastin is impregnated with neural tube extract, the DRG resumes development (Kalcheim and Le Douarin, 1986). The adrenal medulla also appears to receive developmental signals from neighbouring tissues.

Precursor cells of the adrenal medulla (pheochromocytes) appear in culture at the same time as sympathetic neurons but the proportion of pheochromocytes increases if cultures are treated with glucocorticoids (Vogel and Weston, 1990). Glucocorticoids are produced by the adrenal cortex which is of mesodermal origin and is immediately adjacent to the medulla.

The examples given so far indicate that some NCCs pick up their developmental cues from transient structures encountered along precise migration routes while others receive differentiation signals and survival factors from a permanent neighbouring tissue and yet others appear ready to differentiate right at the start of migration. A fourth developmental mechanism is that of target-mediated differentiation.

Cranial neural crest cells contribute to the cartilage and bone of the vertebrate face and will only give rise to bone if they encounter epithelium (mandibular or even limb bud). In the chick mandible, this epithelial-mesenchymal interaction takes place 2½-3 days before the first bone cells appear and if the epithelium of the target region is removed prior to the critical period, bone does not form (Hall, 1988). Tooth formation involves a more specific target-mediated neural crest cell differentiation. Cranial NCCs form the inner dentine producing layer of teeth, but only if they encounter mandibular arch epithelium (Lumsden, 1988).

2.1.3 Factors influencing melanocyte differentiation in vivo

Overt melanocyte differentiation is marked by the production of melanin and until recently, it has been thought that melanogenesis only occurs once NCCs have reached their target tissue, the epidermis (see Chapter 1). However, in a Black Australorp x New Hampshire Red breed, melanisation is first apparent in dermal melanocytes (Hulley et al., 1991). Therefore differentiation is unlikely to be of the target-mediated kind and more likely to occur in response to environmental factors encountered during migration. Since premelanocytes emerge from the neural crest and migrate along the dorsolateral pathway beneath the epidermis, they encounter tissues

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such as the dermal mesenchyme, the epidermal basement membrane and perhaps the dorsal aspect of the somites.

When extracellular matrix is extracted from tissues such as epidermis, dermis and gut mesenchyme and used as a substrate for avian NCC migration, only the matrix from dermis promotes melanocyte differentiation (Derby, 1982). In unpigmented quail feathers, however, it seems possible that the dermis is the tissue responsible for suppressing NCC differentiation into melanocytes. Unpigmented feather buds in organ culture fail to pigment as does isolated feather mesoderm, but when epidermis from these unpigmented feather buds is cultured, pigmented melanocytes migrate out from the explant (Richardson et al., 1990). The dermis also appears to control the region-specific development of pigment cells in amphibians. In newts, the dermis promotes melanophore differentiation except in the embryonic dorsal fin which remains unpigmented. In this region the dermis contains a high level of the glycosaminoglycan, hyaluronic acid, and this has been shown *in vitro* to inhibit melanophore differentiation (Tucker and Erickson, 1986).

The dermis also appears to play a major role in the control of coat colour in mice. The agouti pattern in mice is controlled by the genotype of the dermal component (Mayer and Fishbane, 1972). In another study, dermal-epidermal combinations between wild-type (C57BL/6J) mice and mice homozygous for a mutation at the *steel* locus (a mutation blocking the differentiation of neural crest-derived pigment cells) have shown that the lack of pigmentation is due to an incompetence of the dermal as well as the epidermal components of these mice to support melanocyte development (Mayer, 1973b). If the correct dermal conditions are not available, normal pigmentation does not occur. This implies that the epidermis alone cannot adequately support normal pigment cell development.

However in some cases the dermal role appears to be largely permissive. Neural crest cells from piebald mice form both pigmented and unpigmented hairs when combined with normal skin, but visibly pigmented melanocytes are never found in the dermis or inter-follicular epidermis. Normal controls have dermal and epidermal melanocytes, and this seems to suggest that the dermis is unable to support the more environmentally sensitive piebald melanocytes (Mayer, 1977). This may also be the case in red fowl breeds which never exhibit melanised pigment cells outside the feather epidermis (Dorris, 1939; Weiss and Andres, 1952), and in Japanese quails which are specifically reported as having melanocytes only in the feather primordia and not in the dermis or inter-feather bud epidermis (Sears and Ciment, 1988; Le Douarin, 1982).

2.1.4 Markers of melanocyte differentiation

To find out exactly which factor/s cause melanocyte differentiation, it is necessary to establish where and when NCCs first begin to express the melanocytic phenotype. Melanin is a clearly visible marker of melanocyte maturation but in most fowl breeds it is expressed only once premelanocytes reach the epidermis and even in early melanising breeds such as the BAXNHR, this is 3 days after neural crest emergence (Hulley et al., 1991). What is happening to the migrating dermal NCCs during these 3 days? Perhaps the first marker of melanocyte differentiation is the appearance of premelanosomes, membranous organelles derived from Golgi vesicles or rough endoplasmic reticulum (Jimbow et al., 1979; Hirobe, 1992). The second phase of melanocyte development is marked by the production of tyrosinase in the rough ER and Golgi apparatus. Tyrosinase is the enzyme which initiates melanin synthesis by catalysing the hydroxylation of tyrosine to 3,4-dihydroxy-phenylalanine (dopa) and the oxidation of dopa to dopaquinone. Dopaquinone is then converted to melanin by a series of autocatalytic reactions (Fig 2.1). Tyrosinase becomes active when it docks onto stage II melanosomes and the melanin produced is laid down on the melanosomal matrix proteins (Maul and Brumbaugh, 1971; Hirobe, 1992). Melanosomes

are transferred to feather barbule cells from day 11, thus giving rise to pigmented down feathers in chick embryos (Watterson, 1942).

Melanocyte differentiation prior to melanin formation has not yet been investigated. Differentiation can be traced using antibodies directed against melanosomal proteins or tyrosinase. There are no anti-chick melanosome or tyrosinase antibodies currently available, although there are several antibodies for mouse tyrosinase. These include the monoclonal TMH-1 (Tomita et al., 1985) and the polyclonal anti-mouse tyrosinase antibody 3819 (Dr V.J. Hearing). The gene for mouse tyrosinase has also been cloned (Ruppert et al., 1988) and this can be used to detect the expression of tyrosinase mRNA prior to enzyme formation. The experiments presented in this chapter were carried out in an attempt to clarify the timing and sequence of the events leading up to the differentiation of neural crest cells into melanocytes and to discover where in the developing embryo these events take place. Tyrosinase protein was used as a marker of melanocyte differentiation and the timing of its appearance in chick skin was established using a radiometric assay. Attempts were also made to detect tyrosinase in immunoblots of chick skin using polyclonal antibodies. In addition to this, an *in situ* hybridization protocol was established to examine the spatiotemporal pattern of expression of tyrosinase mRNA in chick skin melanocytes. Messenger RNA expression precedes expression of the coded protein and thus serves as an earlier marker of differentiation. *In situ* hybridization reveals the position of differentiating cells when gene transcription begins and may therefore indicate the position in the embryo of inducing tissue factors. The ultimate aim of the work presented here was to investigate the mechanisms controlling avian pigmentation. To this end, a series of tissue recombination experiments were carried out and the roles played by dermis and epidermis in melanocyte differentiation were explored.

Fig 2.1

Scheme of the melanin biosynthetic pathway. Note that tyrosinase can use both tyrosine and dopa as substrates. The relationship of the eumelanin (brown/black) and pheomelanin (red/yellow) synthetic pathways is also shown. After Hearing and Jimenez (1987).

2.2 Methods

2.2.1 Radiometric assay for tyrosinase activity

This assay was adapted from that published by Hearing and Ekel (1976) and gives a measure of tyrosinase activity by measuring the conversion of ^{14}C -tyrosine into acid insoluble melanin.

Pieces of dorsal skin were removed from 4 to 8 day Black Australorp x New Hampshire Red (BAXNHR) embryos using iridectomy knives. In the case of 4 and 5 day embryos this often included somites and neural tube since the skin adheres very tightly to the underlying tissue in these early stages. Organ tissue (including all abdominal organs and the heart) was used as a tyrosinase-negative control and was obtained from the same embryos. Approximately equal quantities of skin or organ tissue were manually homogenised in a 1:1 volume of 0.1M phosphate buffer with 1% Triton X-100 on ice. The homogenate was centrifuged at 10,000g for 5 mins and the supernatant used as a source of enzyme. Samples were adjusted to exactly equal amounts of protein using the Biuret dye-binding assay (Bradshaw, 1966).

A 10 μl aliquot of each supernatant was mixed with ^{14}C tyrosine (25 $\mu\text{Ci}/\text{ml}$, specific activity 513 mCi/mmol, Amersham), L-dopa (0.05 mM in 0.1M phosphate buffer) and an antibiotic mix in a 2:1:1:1 ratio. The antibiotic mix contained chloramphenicol (1 mg/ml), cycloheximide (1 mg/ml), bovine serum albumin (0.1 mg/ml) and penicillin (1000 iu/ml). This reaction mix was prepared in duplicate using supernatant from 4-8 day skin and organ extracts, together with blank samples which contained phosphate buffer instead of supernatant (to determine non-enzymatic incorporation levels), and then incubated for 3½ hrs at 37°C. Samples were individually spotted onto Whatman glass-fibre filter discs in 20 μl aliquots and washed in 10% trichloroacetic acid twice for 15 mins to remove unincorporated ^{14}C -tyrosine. The discs were dehydrated by washing for 5 mins each in 95% ethanol and acetone and then dried for 30 mins in scintillation vials at 50°C. 200 μl Soluene tissue solubiliser (Packard) was added

to lift the reaction products out of the filter and 5 ml of toluene scintillation fluid (Packard) was added prior to counting. Samples were counted using a Packard Tricarb liquid scintillation counter set for 10 min counts on the 14 carbon channel.

2.2.1a DEAE Sephacel purification of tyrosinase

A 10 ml syringe was blocked with glass wool, loaded with 8 ml of DEAE-Sephacel anion-exchange beads (Pharmacia) in 20 mM Tris buffer pH 8 and equilibrated with fresh buffer until the effluent OD₂₈₀ was zero. Skin and organ homogenates from 4-6 day BAXNHR embryos were prepared as above and 1 ml aliquots were mixed 1:1 with column buffer, loaded on the column and run under gravity. Unbound substances were washed through the column with Tris buffer pH 8 (first peak, Fig 2.2) until the OD of the effluent had dropped to <0.3. Bound substances were then eluted using a 0.3 M NaCl, 20 mM Tris buffer, pH 8, and emerged as a single peak (Fig 2.2). This second peak has been demonstrated to contain tyrosinase (Stander, 1991). The second peak fraction with highest OD from each sample was assayed as above.

2.2.2 Gel electrophoresis and Western blotting

Skin samples from 3-11 day BAXNHR embryos and skin and organs from 11 day white chick embryos (Cornish Game X White Plymouth Rock) were obtained, homogenised, centrifuged and corrected to equal protein as for the radiometric assay. Murine B16 melanoma cells were cultured in Dulbecco's modified Eagles medium with 10% foetal calf serum, penicillin and streptomycin. Cells were harvested using 0,02% trypsin, 0,05% EDTA, pelleted and homogenised in an equal volume of phosphate buffer with 1% Triton X-100. Protein concentration was determined by the Biuret assay as above.

Presence of tyrosinase in 11 day black skin was confirmed by subjecting supernatant from 11 day skin, organs and melanoma cells to non-SDS polyacrylamide gel electrophoresis (after Davis, 1964). Samples were loaded onto a 7,5% slab polyacrylamide gel in 0,025M Tris/glycine buffer and run at 75 V. The gel was washed for 30 mins in 0,1 M phosphate buffer and incubated in a 1 mg/ml solution of L-dopa in 0,1 M phosphate buffer at 37 °C until black bands appeared in the melanoma and black skin lanes.

Western blotting was carried out as described in the Appendix.

2.2.2a Detection of tyrosinase using polyclonal antibodies

See Appendix.

2.2.3 RNA extraction

All solutions were treated with 0,01% diethyl pyrocarbonate (DEPC) and autoclaved to destroy ribonucleases. Glassware was first siliconised by rinsing in 5% silicone in carbon tetrachloride, baked overnight at 80°C, soaked in 0,01% DEPC for 1 hr and then dry-sterilised at 120°C for 1 hr. Gloves were worn at all times.

Skins from 4-11 day BAXNHR embryos and 11 day skin and organs from white (CGxWPR) embryos were dissected off in chick saline and 1 ml quantities were homogenised in a Dounce glass homogeniser containing 5 ml NETS buffer (0,1M NaCl, 1mM EDTA, 10mM Tris, 0,5% SDS, pH 7,4). This homogenate was added to a further 5 ml NETS containing 50 µg/ml Proteinase K in a test tube and incubated in a waterbath at 37 °C for 30 min. The RNA was then purified from contaminating proteins by successive extractions, 2 with equal volumes of phenol, 1 with phenol:chloroform (1:1 ratio) and 2 with chloroform alone (chloroform contained 2% isoamyl alcohol). The supernatant was precipitated using one tenth volume of 3M sodium acetate and 2½ volumes of absolute ethanol (Analar grade, Merck) at -20°C overnight. Some DNA

precipitated instantly and was spooled out using a Pasteur pipette. RNA was pelleted by spinning at 12 000g for 20 mins at 4°C, the pellet was rinsed with cold 70% ethanol and vacuum-dried at room temperature. RNA thus purified is total RNA, containing messenger, ribosomal and transfer RNAs. RNA concentration was determined using a Shimadzu spectrophotometer at 260 nm ($OD_{260}=1$ corresponds to 40 μ g RNA) and purity was given by the OD_{260}/OD_{280} ratio.

RNA was also extracted using 4M guanidinium thiocyanate, 0,75M sodium citrate, 10% Sarcosyl, 0,1M β -mercaptoethanol (pH7) as the homogenisation buffer (Chomczynski and Sacchi, 1987), other steps the same as above.

2.2.3a Messenger RNA purification by Oligo-dT-cellulose column chromatography

Pelleted total RNA was dissolved in 1 ml column loading buffer (0,5M NaCl, 10 mM Tris-HCl, pH 7,5) and loaded onto 0,5g oligo-dT-cellulose beads in column loading buffer in a column (20 x 0,5 cm). Effluent was collected in 1 ml fractions, the first fraction being re-loaded to ensure optimal binding of mRNA, and followed with column loading buffer until the effluent OD_{260} approached zero. The buffer was then changed to a low-salt buffer (10mM Tris-HCl, pH 7,5). The first 2 effluent fractions generally contained all the released RNA and were precipitated with ethanol/sodium acetate as above. RNA was quantitated as above.

2.2.3b Northern blotting of RNA and mRNA

Denaturing slab mini-gels were made containing 1,1% agarose in MOPS buffer (0,2M 3-N-morpholine propane sulphonic acid, 0,05M sodium acetate, 0,01M EDTA) and 5% formaldehyde. RNA samples were mixed with loading buffer and run at 70 V for 1-2 hr. Gels were stained with $\pm 0,01\%$ ethidium bromide in MOPS buffer and photographed on a Spectroline transilluminator. The gel was rinsed in 10x SSC (0,15M NaCl, 0,015M trisodium citrate) and RNA was transferred overnight to Hybond-N+ nylon membrane (Amersham) by capillarity, using 10x SSC as blotting buffer. Transfer was

checked and ribosomal marker bands marked with a pin when viewing the membrane on the transilluminator. RNA was fixed into the membrane by baking at 80°C for 2 hr.

2.2.3c Hybridization of Northern blots

Northern blots were hybridized according to Davis et al. (1986). See Appendix for details.

2.2.4 Probe preparation

2.2.4a Preparation of nick-translated probe

See Appendix.

2.2.4b Preparation of multiprime probe

See Appendix.

2.2.4c Preparation of ^{35}S and digoxigenin-labelled riboprobe

See Appendix.

2.2.4d Quantitation of riboprobe

^{35}S probe was quantitated by counting 1 μl in 10 ml toluene as for the other probe types, and size was checked by running 1 μl on an RNA agarose gel, drying the gel and exposing to X-Ray film for 1-2 days. The probe band size was compared with ribosomal marker bands. Probe activity varied between 10^6 and 10^7 cpm/ μg template (note that since RNA is synthesised in this reaction, this is only a rough quantitation).

DIG probe was always resuspended in 50 μl of water, 0,5 μl placental ribonuclease inhibitor, and 3 μl was run on a 1,1% denaturing agarose gel and transblotted to Hybond N+ nylon membrane (Amersham). The membrane was baked for 2 hr at 80°C and

incubated for 30 min at room temperature (r.t.) in maleic acid buffer (0,1M maleic acid, 0,15M NaCl, pH 7,5) with Boehringer Mannheim blocking reagent and 2% normal sheep serum (NSS). The DIG kit alkaline phosphatase-conjugated polyclonal sheep anti-DIG antibody was applied in maleic acid, blocker, 2% NSS for 30 mins at r.t. The membrane was washed 2x15 min in maleic acid buffer, 1x5 min in kit buffer 3 (0,1M Tris, 0,1M NaCl, 0,05M $MgCl_2$, pH 9,5) and incubated in the dark with freshly prepared colour reagent until the probe band was clearly visible (usually less than 15 min). By comparison with ribosomal marker bands, the probe size could be established and the efficiency of the reaction compared with previous reactions to give a comparative quantitation.

2.2.5 In situ hybridization (ISH)

Slide preparation

Standard glass microscope slides were washed overnight in chromic acid, rinsed in tap water for 3 hr, rinsed overnight in distilled water and dried. A 2% solution of APTES was made in acetone and the slides soaked in it for 10-60 mins and then baked overnight at 80°C. Slides were rinsed for 10 min in 0,01% DEPC and then dry sterilised.

Tissue preparation

4 to 11 day old BAXNHR chick embryos were removed from the egg, rinsed in sterile chick saline and immediately fixed in one of the following fixatives: Bouin's solution (3 hr), 10% buffered formalin (overnight), 20% glutaraldehyde in phosphate buffered saline, pH 7,4 (1 hr) or 4% paraformaldehyde in 0,1 M sodium phosphate buffer, pH7,4 (20 min). Fixation was followed by standard paraffin wax (Paraplast) embedding, all steps carried out by hand and at r.t except for the final wax changes. Tissue was sectioned at 4, 5, 6 or 7 μm using a Leitz rotary microtome with a fresh disposable blade. Sections were transferred to APTES-coated slides with a dry paint brush, floated in DEPC-treated distilled water and dried overnight

at 40°C on a slide warming tray. Gloves and sterile glassware were used at all times.

Cryo-sections (10 μ m) of newborn (n=1) and 7 day (n=2) C57BL6 mouse skin were made using a SLEE microtome at -20°C. Tissue was prefixed in 4% paraformaldehyde for 2 hr, soaked in 25% sucrose solution in PBS for 2 hr and embedded in OCT compound. Sections were baked at 37°C overnight on APTES-coated slides.

B16 melanoma cells and 3T3 fibroblasts were seeded onto APTES-coated slides in culture dishes containing DMEM, 10% FCS, penicillin and streptomycin. When the cultures approached confluency they were fixed in 10% buffered formalin (overnight) or ethanol/acetic acid (3:1, 20 min), dehydrated through a graded ethanol series, air-dried and stored in sealed containers with silica gel until used.

Section pretreatment

NOTE: in every run with pmc0,5, duplicate slides were treated with :

1. antisense probe.
2. antisense probe with prior RNase treatment.
3. chemography control (hybridization mix with no probe added).

In every run with pmc0,1, the same treatments were duplicated with the addition of :

4. sense probe.

See Appendix

In situ hybridization using digoxigenin-labelled riboprobe

1. Fluorescein-conjugate

Attempts were made to obtain hybridization with this method using both wax and cryo-sections of chick and mouse tissue, fixed with Bouin's, paraformaldehyde,

glutaraldehyde, 10% buffered formalin and ethanol/acetic acid but none were successful, largely due to high levels of fixative-induced fluorescence and low signal intensity.

Melanoma and 3T3 cell monolayers (fixed in 10% formalin and dehydrated), were treated with 1mg/ml Proteinase K for 15 min and controls were digested with proteinase K and then with 40 µg/ml RNase A. All slides were acetylated as above, dehydrated and air-dried. DIG-labelled riboprobe (pmc0,5, from a standard 50 µl reaction yield) was mixed 1:3 with hybridization buffer (as for ³⁵S ISH) and cell monolayers were hybridized directly (20 µl/ slide under Parafilm) at 42°C overnight. Posthybridization washes were as follows: -2x30 min in 2xSSC at r.t.

-1x30 min in 0,1xSSC at 42°C

DIG detection:

- Block for 20 min in tris-buffered saline (TBS), 2% NSS at 37°C
- Incubate overnight in 1:2 fluorescein-conjugated polyclonal sheep anti-DIG antibody in TBS, 2% NSS at 4°C (20 µl/ slide)
- Wash for 2x10 min in TBS at r.t.

Slides were then mounted with buffered glycerine and viewed using a Leitz fluorescent microscope.

2. Alkaline phosphatase-conjugate

Paraformaldehyde- or Bouin's-fixed whole 4 to 8 day chick embryos (n=3 for each age group, n=10 for 8 day) and 7 day mouse skin (n=5) were wax embedded and sectioned at 6 µm. Dewaxed and dehydrated sections were directly and simultaneously treated with proteinase K and RNase A for 25 mins, followed by acetylation. As above, prehybridization (1 hr) and hybridization (overnight, 1:4-1:8 probe to hybridization

buffer ratio) were carried out at 42°C but with a new hybridization buffer (Boehringer Mannheim recommendation).

- 50% deionised formamide
- 25% 20xSSC
- 5% 100x Denhardt's solution
- 0,25 mg/ml tRNA

Posthybridization washes- 2x15 min in 2xSSC at 42-50°C

- 2x30 min in 0,1xSSC at 42-50°C

Blocking - 30 min in TBS, 2% NSS at r.t.

Alkaline phosphatase-conjugated sheep anti-DIG polyclonal

antibody was diluted 1:100 in TBS, 2% NSS and sections were incubated for 30 min at r.t.

- Washes
- 2x15 min in TBS at r.t.
 - 1x5 min in buffer 3

Colour reaction overnight in a sealed, moist Tupperware container in the dark.

TBS was later replaced with maleic acid buffer (maleic acid 0,1M, NaCl 0,15M, pH 7,5) and Boehringer Mannheim blocking reagent was added to both blocking and antibody steps (10% w/v in maleic acid buffer).

2.2.6 Dopa-enhancement of tyrosinase-positive melanocytes

Skin attached to somites and neural tube was dissected from 4 and 5 day BAXNHR embryos (n=2), fixed in 4% paraformaldehyde (in 0,2M phosphate buffer, pH 7,2) for 2 hr and rinsed in 0,1M phosphate buffered saline (PBS), pH 6,9 for 5 min. Tissue pieces were then incubated for 3 hr at 37,5°C in 0,1M PBS containing 5mM L-dopa (Sigma) and 0,12% glucose and processed for standard wax histology. (After Brumbaugh, 1971).

2.2.7 Grafting experiments

2.2.7a Replacement of dermis with gut mesenchyme

Neural tubes were dissected from 48 hr BAXNHR embryos using 0,01% collagenase in PBS, pH 7,4. White skin from 6 day white embryos was split using EDTA to give epidermis (n=4), and pieces of gizzard and stomach from the same embryos were similarly separated to give mesenchyme (n=14). Neural tube was placed upon mesenchyme and the combination was parcelled up in epidermis, incubated in chick saline for 1 hr to initiate recombination and the whole parcel was CAM grafted. As a positive control, neural tube was combined with white dermis and epidermis and as a negative control, epithelium was combined with gizzard mesenchyme (with no neural tube). After 10 days CAM culture, grafts were removed and examined for the presence and position of melanin.

2.2.7b CAM culture of potentially pigmented dermis

Pieces of skin from the dorsal trunk of 4½ day BAXNHR embryos were microdissected and the dermal and epidermal components isolated using a 15 min incubation in 0,2% EDTA in chick saline, followed by manual separation. Dermal pieces (approximately 2 mm²) were rinsed in chick saline and CAM grafted into 8 day white host embryos. As controls, pieces of whole skin (dermis and epidermis) were CAM grafted directly. After 7 days in culture, grafts were removed from host eggs, fixed, serially sectioned, stained with haematoxylin and eosin and examined for the presence of melanin.

2.2.7c Replacement of epidermis with gut or amniotic epithelium

Pieces of dorsal trunk skin from 6 day BAXNHR embryos and stomach and oesophagus from 6-10 day white embryos were removed. In all tissues, epithelium was separated from underlying mesenchyme using 1% trypsin (DIFCO 1:250) in chick saline at 4°C for 1 hr, followed by manual separation. Trypsin activity was inhibited by incubating tissue pieces in a 1:1 solution of FCS:chick saline for a few minutes. Epithelium

from oesophagus or stomach was combined with BAXNHR dermis on agar for 24 hr, followed by CAM grafting and culture for a further 7-10 days. In addition, amniotic membrane and dermis were collected from 6 day white embryos. The white dermis was covered with amniotic membrane and in turn placed on top of 6 day BAXNHR dermis. As a positive control, white epidermis was combined with white dermis on top of BAXNHR dermis. In all cases, dermis was orientated "epidermal-side" up ie. with normal orientation. These triple-layer combinations were cultured on agar for 24 hr and then CAM grafted and cultured for 7 days. All combinations were removed from host eggs, processed for wax histology, serial sectioned and examined for the presence and position of melanin.

2.2.7d Replacement of quail wing epidermis with white chick epidermis

Experiment 1

Quail (*Coturnix coturnix japonica*) embryos (3½-4 day) were removed from the egg and lightly stained with phenol red vital dye (diluted in chick saline). The dorsal or ventral surface of the wing bud was scratched with a tungsten needle to permit orientation and the bud was removed. The scratched epithelium was removed following a 15 min incubation in EDTA but the apical ectodermal ridge was retained to facilitate outgrowth. Dorsal trunk epidermis was removed from 6 day white chick embryos using EDTA and then draped over the denuded wing surface. The wing was carefully CAM grafted and cultured for 10 days. The grafts were photographed on removal from host embryos.

Experiment 2

As above, 4 day quail wings were marked and removed. Using EDTA to strip off ectoderm, small squares of dorsal or ventral mesenchyme were excised. These were placed on a Millipore filter, covered with 6 day white chick epidermis (dorsal trunk) and cultured for 2 weeks in DMEM with 10% FCS, penicillin and streptomycin.

Explants were fixed, wax embedded, serial sectioned and examined for the presence and position of melanin.

Experiment 3

Wing buds from 3½ day phenol red-stained quail embryos were removed and the whole epidermis peeled off after EDTA treatment. Taking care to maintain orientation, wing bud mesenchyme was transferred to agar dishes and positioned either ventral side up or dorsal side up. The uppermost surface of the explants was draped with 7 day white chick epidermis and after 24 hours, the recombined wing was CAM grafted. After 7 days in culture, the grafts were fixed in Bouin's solution, wax embedded, serially sectioned and examined for the presence and position of melanin.

Experiment 4

The dorsal or ventral surface of 4 day quail wing buds was marked with a spot of carbon powder and the wing removed from the embryo. Using EDTA, the whole epidermis was removed from the wing and the mesenchymal core was carefully transferred to a Millipore filter, either dorsal or ventral side up. The uppermost surface was covered with 8 day white chick epidermis and the explant cultured for 24 hours in DMEM, 10% FCS, penicillin and streptomycin. Explants were then CAM grafted for 7 days, fixed in Bouin's solution, wax embedded, serial sectioned and examined for the presence and position of melanin.

2.3 RESULTS

2.3.1 First appearance of active tyrosinase

The appearance of active tyrosinase in chick skin indicates a step in melanocyte differentiation prior to melanin formation. A measure of tyrosinase activity in tissue homogenate from 4-8 day BAXNHR chicks was obtained using the assay devised by Hearing and Ekel (1976). Tissue homogenate samples of equal protein content were prepared from chick skin and organs. Organ extract included gut, heart, liver and other internal organs and served as a melanocyte-free control. This assay uses the conversion of ^{14}C -labelled tyrosine to melanin as an indicator of tyrosinase activity and was repeated 4 times using crude homogenate. The cpm/ μg homogenate were converted to a percentage of maximum (8 day cpm/ μg) to facilitate comparison between assays. From the graph in Fig 2.3, it is apparent that tyrosinase activity increased dramatically between 6 and 8 days but was undetectable above organ control levels between 4 and 6 days (Table 2.1; Fig 2.3). At 7 days, skin tyrosinase activity averaged 68% of maximum, compared with organ activity at 39% of maximum. In order to partially purify tyrosinase and therefore to increase the sensitivity of the assay, tissue extracts from 4-6 day embryonic skin were subjected to DEAE sephacel column chromatography and then assayed as above. Tyrosinase activity increased approximately 3-fold at all ages when this method was used and was clearly detectable from 5 days (Table 2.2; Fig 2.3). This assay was repeated 3 times but tyrosinase activity was not consistently detected at 4 days. It therefore appears that tyrosinase activity first becomes detectable at 5 days, co-incident with the first expression of melanin in chick skin.

2.3.2 First appearance of immunodetectable tyrosinase

Tyrosinase enzyme undergoes post-translational modification and may be present in an inactive form before active tyrosine conversion commences. These inactive tyrosinase molecules should be immunodetectable on Western blots. Because there is no anti-chick tyrosinase antibody available, a polyclonal anti-mouse

tyrosinase antibody (antibody 3819, Dr V.J. Hearing) was tested on Western blots containing both chick skin and mouse melanoma tyrosinase to establish cross reactivity. Melanoma protein extract reacted strongly with the antibody, indicating that the conditions employed were adequate for immunodetection. However, chick skin extract reacted very weakly (Fig 2.4a) even when 11 day chick skin (which is heavily pigmented) was used and therefore earlier stages were not examined. A second polyclonal antibody raised by Stander (see Stander, 1991) against chick tyrosinase was tested on Western blots of 3-8 day BAXNHR skin and 11 day white skin and organs. This antibody reacted specifically to two proteins, one 67 kD and the other 43 kD in size (Fig 2.4b). These accord well with the expected sizes of the T₁ and T₃ isozymes of tyrosinase (see Fig 2.4c). There was also a graded increase in response between 3 and 8 days which could be attributed to a developmental increase in both the number and activity of melanocytes. However, although the antibody failed to react to organ tissue in initial runs, it was later shown to be non-specific, reacting to both skin and organs (Stander, 1991). Therefore no conclusions could be drawn about the timing of the appearance of immunodetectable tyrosinase in chick skin.

2.3.3 First appearance of tyrosinase mRNA

In order to determine whether the expression of tyrosinase could be detected in mRNA obtained from chick skin, two cDNAs thought to be specific for mouse tyrosinase (MTY811 and pMT4; Fig 2.14) were labelled with ³²P by random priming or nick translation and hybridized to Northern blots of chick skin RNA (total and mRNA). Despite many attempts and variations, listed below, this proved unsuccessful.

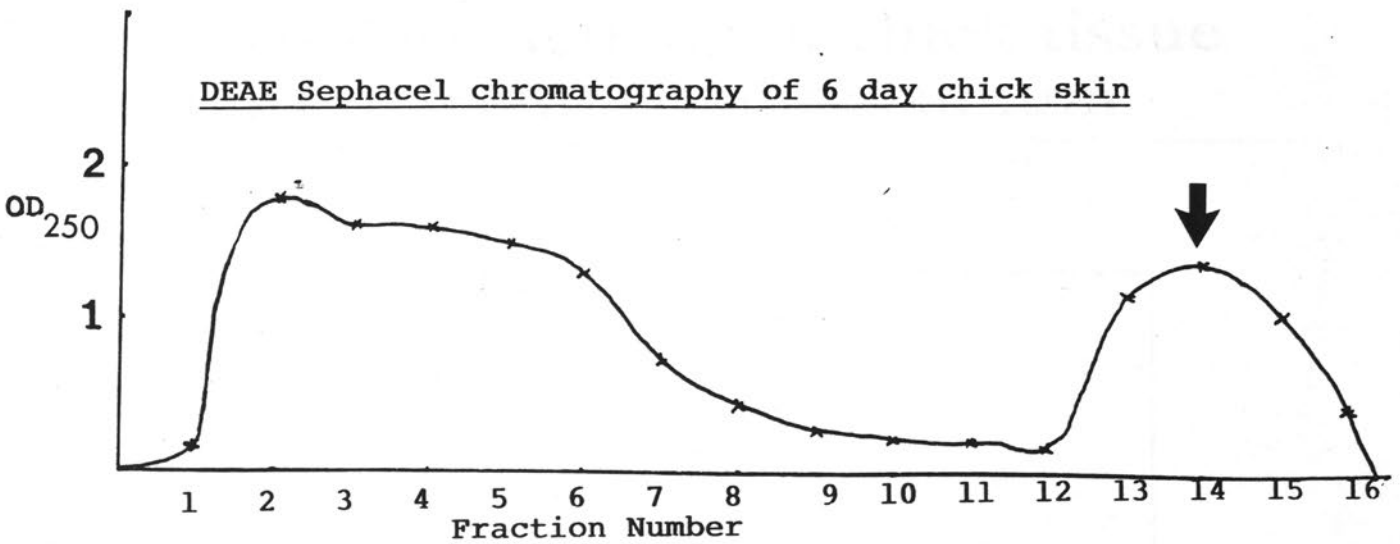


Fig 2.2

Graph showing protein content (OD₂₅₀) in 1 ml fractions eluted from a DEAE Sephacel column. Arrow indicates the tyrosinase-rich peak eluted when the buffer was changed to high salt (0,3M NaCl) buffer.

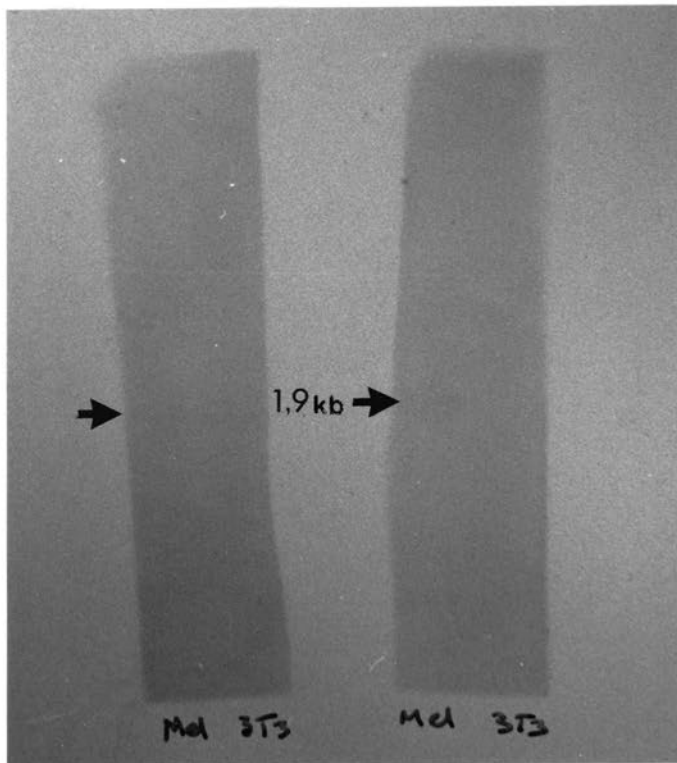


Fig 2.5

Detection of tyrosinase mRNA by hybridisation of a Northern blot with pmc0,5 riboprobe. Probe was labelled with digoxigenin and detected using chemiluminescence (alkaline phosphatase-conjugated anti-DIG antibody, AMPPD substrate, 25 min exposure). Blot A contains 1 μ g/lane total RNA; Blot B 10 μ g/lane total RNA; Mel, melanoma RNA; 3T3, fibroblast RNA.

Tyrosinase activity in chick tissue as percentage of maximum

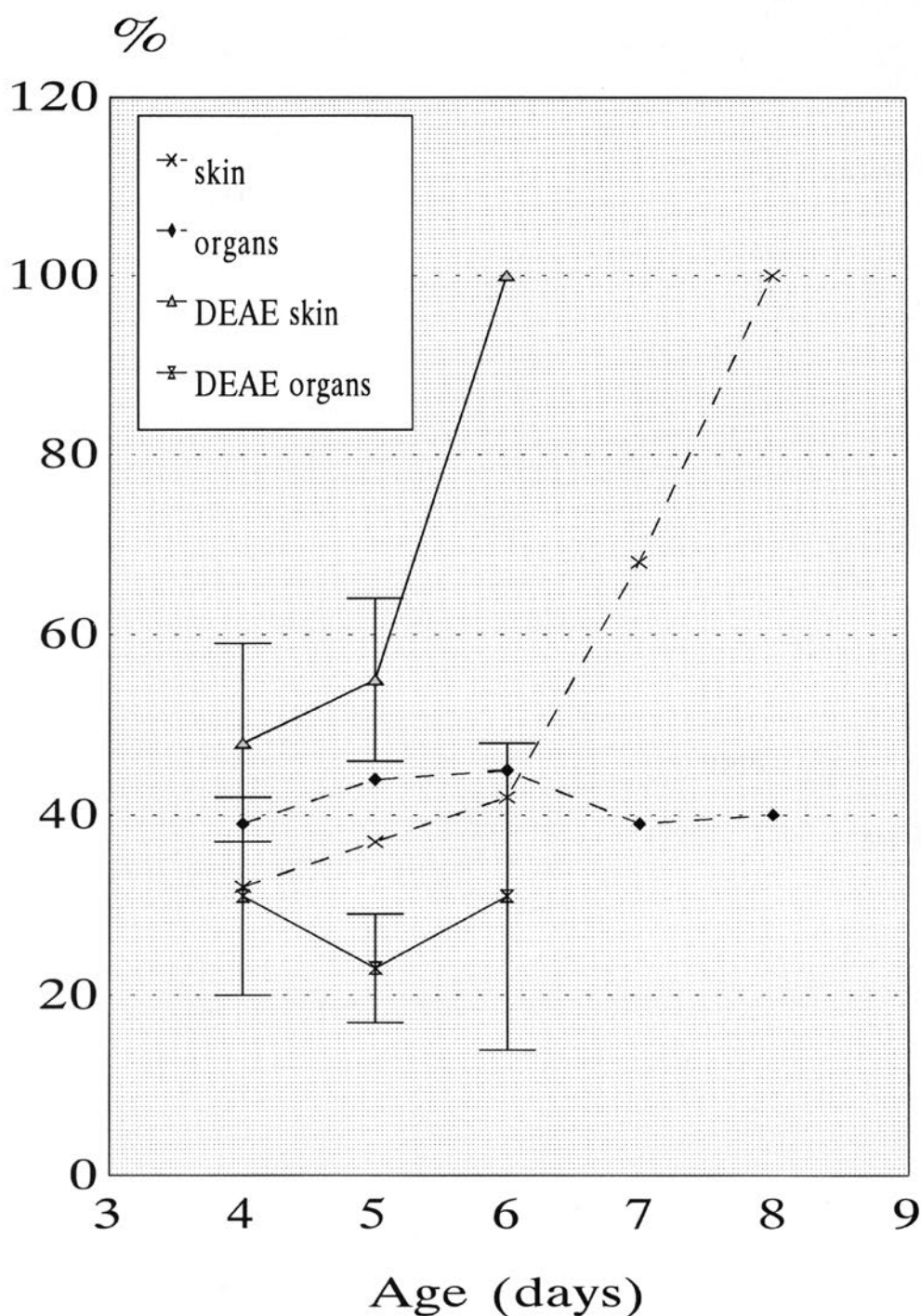


Fig 2.3

Radiometric assay of tyrosinase activity in 4-8 day chick skin and organ homogenates. DEAE skin and organs = tyrosinase was partially purified from crude 3-6 day homogenates by DEAE Sephacel column chromatography. Counts (cpm/ μ g) have been converted to percentage of maximum (counts for 8 day skin, crude homogenate; 6 day skin, DEAE homogenate). Means are given $\pm$ SEM, crude homogenate $n=4$, DEAE homogenate $n=3$. See appendix Table 2.2 for original counts.

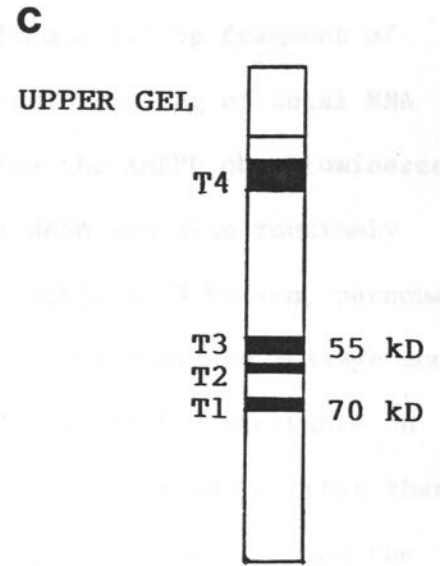
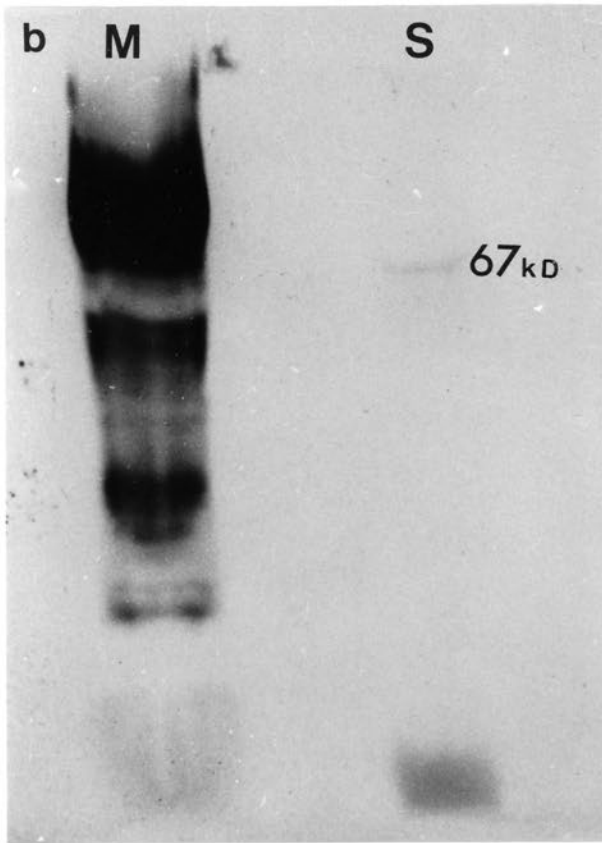
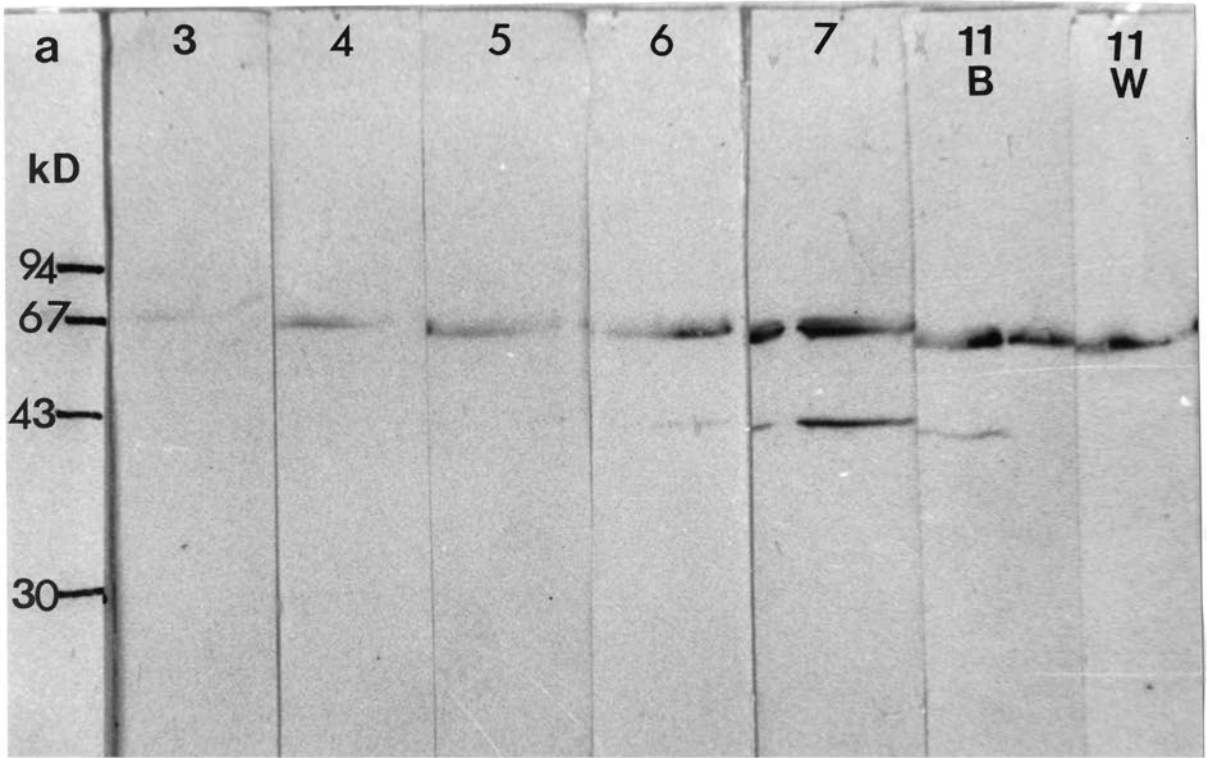


Fig 2.4

(a) Polyclonal anti-chick tyrosinase antibody reacted against a Western blot of 3-11 day black and 11 day white chick skin homogenate. (b) Polyclonal anti-mouse tyrosinase antibody reacted against a Western blot of murine melanoma (M) and 11 day black chick skin (S) homogenate. (c) Pattern of tyrosinase isozymes after electrophoresis of a crude melanocyte extract in the presence of non-ionic detergents and visualisation by histochemical dopa staining. Adapted from Hearing and Jimenez (1987).

2.3.3a Northern blots

The following variations in the stated protocol were carried out:

- up to 100 μ g of total RNA and 30 μ g of purified mRNA were loaded per lane
- prehybridization period was varied from zero to overnight and hybridization period ranged from 1 to 36 hr.
- hybridization temperature was varied from 37 to 50 °C and post-hybridization washes from 37 to 60 °C.
- posthybridization washes were extended from 10 to 45 min each.
- percentage of formamide in both prehybridization and hybridization mixes was dropped from 40% to 25% and 10%.
- exposure to X-ray was varied from 1 to 14 days.

Melanoma RNA was used as a positive control to establish Northern blot and hybridization conditions for all probes used, since melanomas contain abundant tyrosinase mRNA. DIG-labelled riboprobe generated from a 580 bp fragment of pmcTYR (pmc0,5) was hybridized successfully to as little as 1 μ g of total RNA extracted from mouse melanoma cells and detected using the AMPPD chemiluminescent amplification method (Fig 2.5). Melanoma tyrosinase mRNA was also routinely detected using nick-translated, ³²P-labelled pMT4 (C Viljoen, S Kidson, personal communication). It was eventually concluded that either tyrosinase message could not be extracted from chick skin in sufficient quantities to be detectable on Northern blots (due to the paucity of melanocytes/tyrosinase mRNA) or that there was a very low level of cross-reactivity between chick tyrosinase RNA and the mouse cDNA.

2.3.3b In situ hybridization

In situ hybridization provides perhaps the best method for detecting rare messages in a heterogeneous cell population since there is no dilution of mRNA in the melanocyte *in situ*. Riboprobe is the most sensitive type of probe for *in situ* hybridization because it is single stranded and there is therefore no

competition for the antisense probe strand between mRNA and the sense strand (as in double stranded DNA probes). The 580 bp truncated 5' end of pmcTYR was chosen as the region most likely to cross hybridize with chick tyrosinase mRNA because it is the most highly conserved region of mouse, human, *Neurospora* and *Streptomyces* tyrosinase genes (Muller et al., 1988). Radio-isotope-labelled probes are reported to be the most sensitive detectors of RNA in tissue sections (Angerer et al., 1990) and therefore numerous attempts were made to label melanocytes in tissue sections of chick skin using ³⁵S-labelled pmc0,5 riboprobe (Fig 2.14). The most successful attempt is shown in Fig 2.6 and it is immediately apparent that the background noise in the dermis and the "positive" signals in the epidermis are of similar intensity. It does seem that the epidermal signal correlates in many cases with melanocytes but it proved impossible to increase the signal intensity. All the method modifications suggested by reviewers such as Angerer et al., (1990) were tried. These included :

- proteinase treatment for 0 to 45 mins
- acetylation
- post-hybridization RNase treatment
- wash temperature was ranged from room temperature to 65°C
- wash times were varied between 10 and 120 min
- SSC concentration ranged from 4x to 0,1xSSC
- washes were also carried out with 50% formamide in 2xSSC
- dithiothreitol was added to all post-hybridization washes
- emulsion exposure was varied between 1 and 4 weeks

A second problem was that melanin granules were very difficult to distinguish from autoradiographic granules. Chick melanosomes are dark brown and rod-shaped rather than black and round and also lie beneath the plane of developed emulsion but signal detection was never very satisfactory.

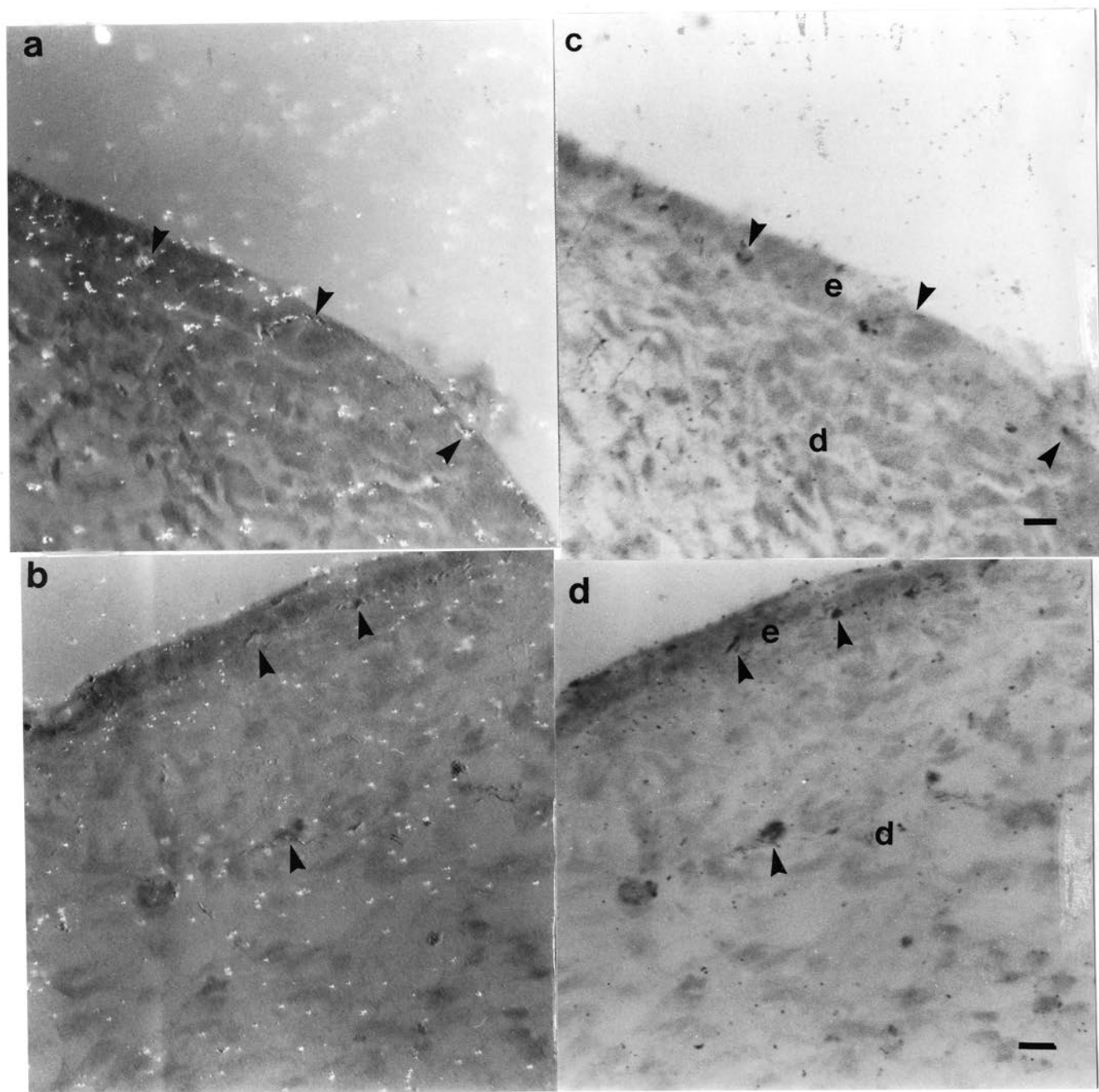


Fig 2.6

In situ detection of tyrosinase mRNA in 8 day chick skin melanocytes using ^{35}S -labelled pmc0,5 riboprobe. (a) Antisense probe (DIC microscopy). (b) RNase control (DIC microscopy). (c) and (d) Bright field of (a) and (b) respectively. e, epidermis; d, dermis; arrows, melanocytes. Bar=20 μm .

Since ^{35}S -labelled riboprobe had proved to be unsuitable for detection of tyrosinase mRNA in chick embryos, pmc0,5 riboprobe was labelled instead with digoxigenin (DIG). *In situ* hybridization conditions for fluorescein detection of DIG-labelled riboprobe were established using hybridization to mouse melanoma cells grown on slides. As shown in Fig 2.7, melanoma cells hybridized with antisense DIG-labelled probe were shown to be tyrosinase mRNA positive using a fluorescein-conjugated anti-DIG antibody. Fibroblasts were negative as were RNase treated controls. However, when this protocol was applied to both wax and cryo-sections of mouse skin and whole chick embryos, no signal was detected above background fluorescence. In an attempt to reduce background fluorescence, a number of fixatives were investigated. Bouin's fluid, ethanol/acetone, 1% glutaraldehyde and 4% paraformaldehyde all caused severe background fluorescence in tissue sections and cell cultures. Buffered formalin (10%) proved to be the best fixative in terms of reduced background fluorescence but this was still high enough to mask any faint hybridization which might have occurred in tissue sections.

Because of this failure to detect positive hybridization of melanocyte mRNA in tissue sections using the fluorescein-DIG method, the alkaline phosphatase method of detection was then tested. Fluorescence would have had advantages over both ^{35}S and alkaline phosphatase-DIG methods because both these methods give end-products which can be confused with melanin in heavily pigmented cells. Initially, DIG-labelled pmc0,5 riboprobe was applied to sections of mouse skin, because pmc0,5 was generated from the mouse tyrosinase gene and therefore served as a positive control to check hybridization conditions. Using the alkaline phosphatase method of DIG detection, melanocyte-specific hybridization of tyrosinase mRNA was demonstrated in hair bulbs of 7 day (post-natal) mouse skin fixed with 4% paraformaldehyde (Fig 2.8a). There were melanin containing cells in the hair bulbs and a few outside in the dermis. Using the conditions established above,

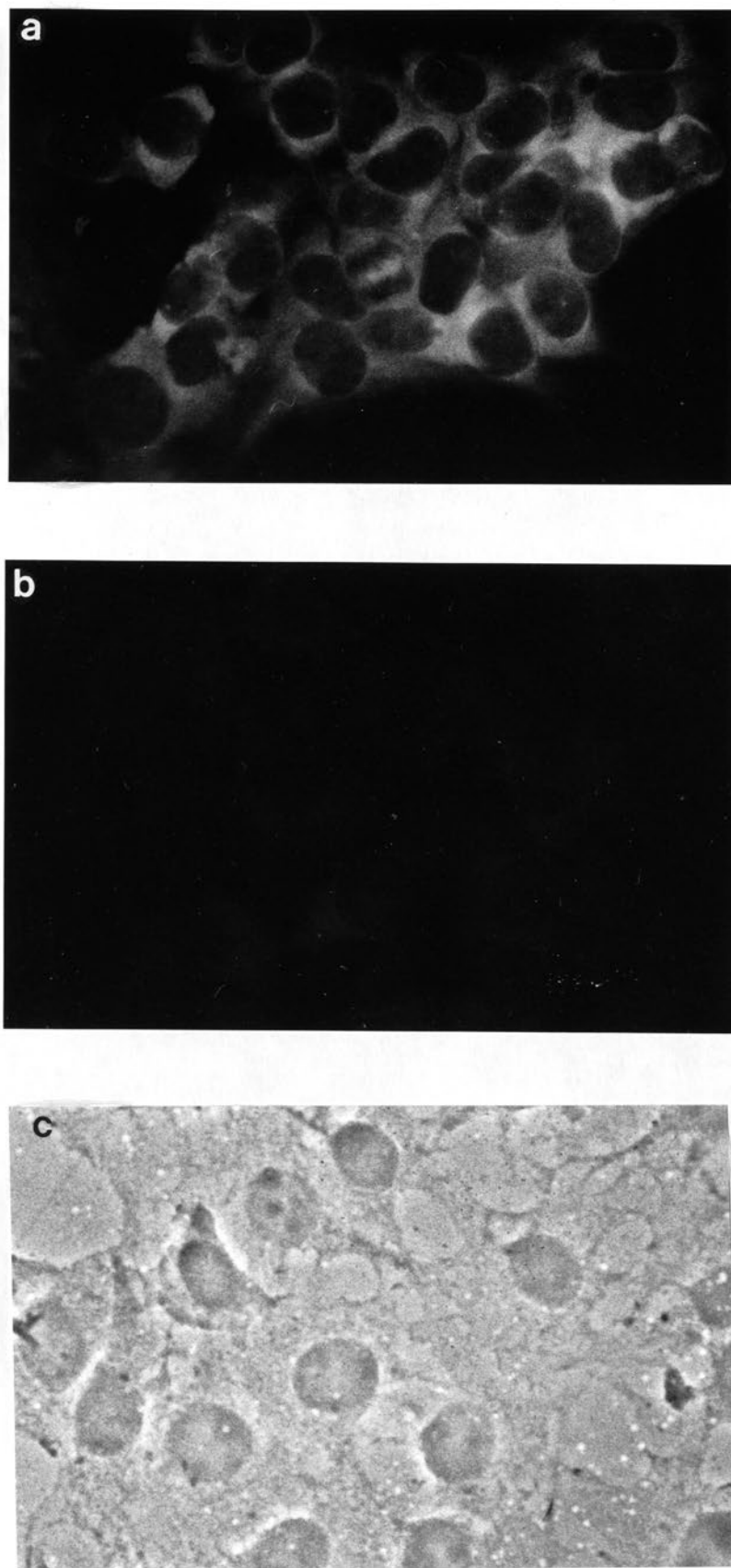


Fig 2.7

In situ detection of tyrosinase mRNA in cell monolayer cultures using DIG-labelled pmc0,5 riboprobe and fluorescein-conjugated anti-DIG antibody. (a) Murine melanoma cells (fluorescence). (b) 3T3 fibroblasts (fluorescence). (c) 3T3 fibroblasts (phase contrast).

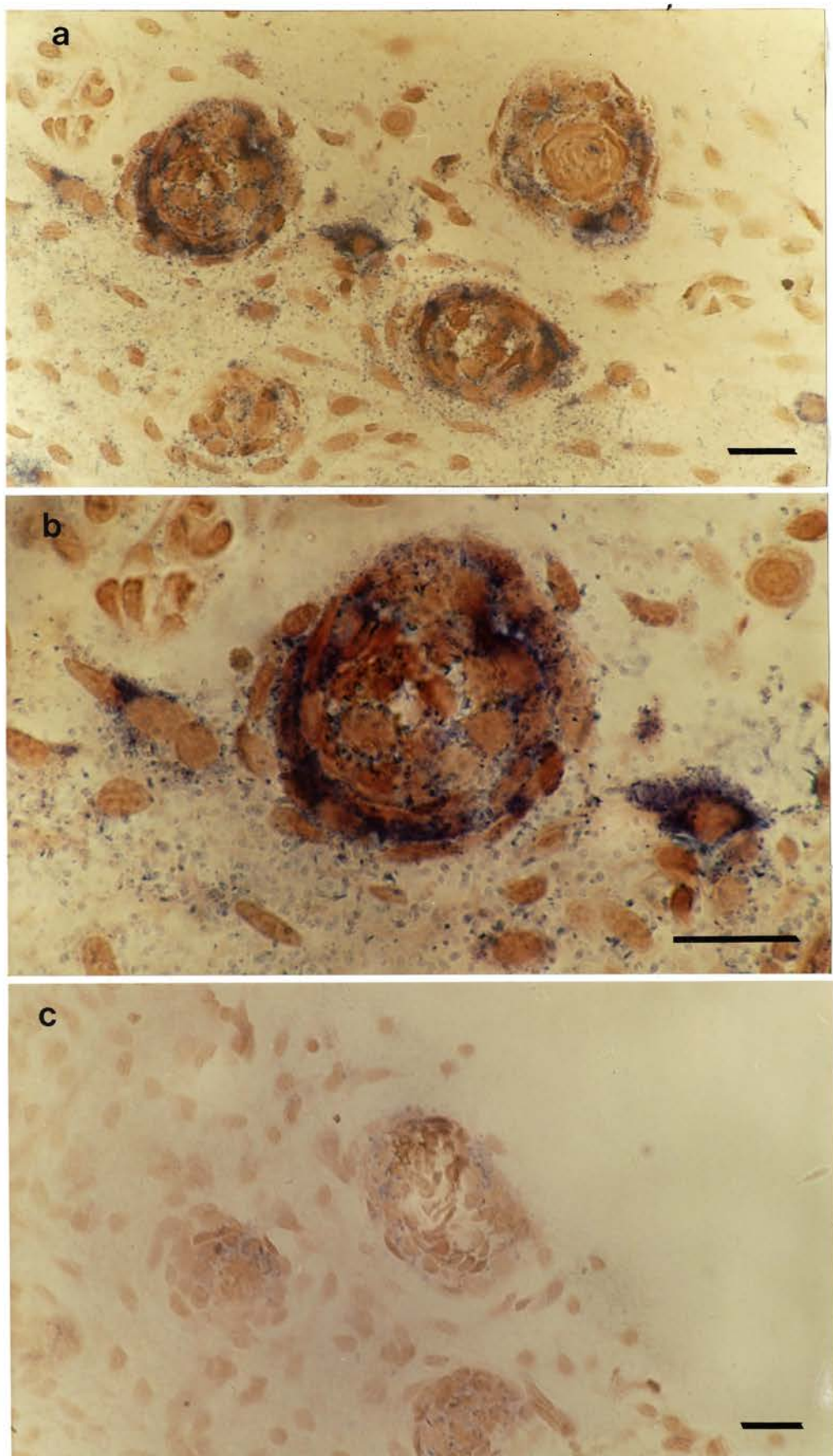


Fig 2.8

In situ detection of tyrosinase mRNA in 7 day (post-natal) mouse hair follicles using DIG-labelled pmc0,5 riboprobe and alkaline phosphatase-conjugated anti-DIG antibody. (a) Antisense probe.(b) High power of (a).(c) RNase control. Bar=20 μ m

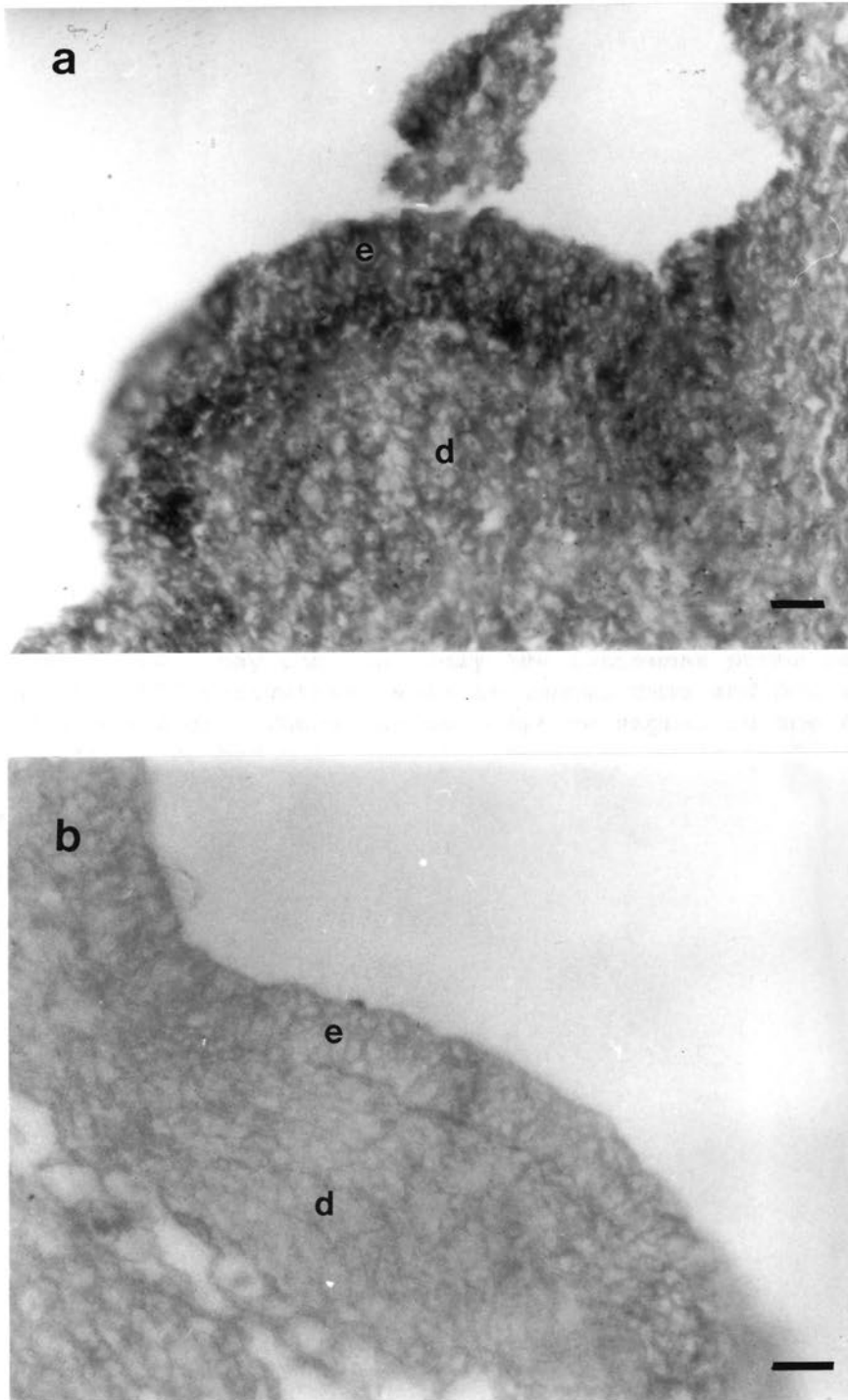


Fig 2.9

In situ detection of tyrosinase mRNA in 8 day embryonic chick skin using DIG-labelled pmc0,5 riboprobe and alkaline phosphatase-conjugated anti-DIG antibody. (a) Antisense probe. (b) RNase control. e, epidermis; d, dermis. Bar=20 μm.

sections from a Bouin's-fixed, 8-day chick embryo were hybridized with pmc0,5. Melanocytes in the epidermis were labelled, there was no dermal label (Fig 2.9a) and RNase-treated sections were negative (Fig 2.9b). This is the first indication that the mouse tyrosinase gene is sufficiently homologous to chick mRNA to cross-react.

At this stage, a sequence of chick DNA thought to be part of the chick tyrosinase gene was cloned using pmcTYR. Upon sequencing, however, it turned out to be a 100 bp piece from exon 3 of the mouse tyrosinase gene (C Viljoen, personal communication). It is referred to here as pmc0,1 and was used to make both sense and antisense DIG-labelled probes. These were hybridized to sections of 4-6 day chick embryos fixed with 4% paraformaldehyde. As shown in Fig 2.10a and b, scattered dermal cells in 5 day embryos and both dermal and epidermal cells in 6 day embryos (Fig 2.11a and b) were positive for tyrosinase mRNA. Although there was very little visible melanin at these stages, the location was typical of migratory premelanocytes and one cell is shown crossing from dermis to epidermis in the 5 day embryo (Fig 2.10b). Sections of 4 and 5 day embryos were also incubated with dopa, a method which allows detection of tyrosinase-positive melanocytes prior to the formation of visible melanin (Brumbaugh, 1971). Dopa-positive cells were apparent in the dermis and epidermis in 5 day embryos (Fig 2.10c) and compare well in size and position with the cells shown to be positive by *in situ* hybridization (Fig 2.10a). There were also occasional, faintly dopa-positive cells in the periderm of 4 day embryos (Fig 2.10d). Unfortunately this layer was invariably lost during *in situ* hybridization procedures, so co-localisation of tyrosinase mRNA could not be checked. In both 5 and 6 day embryos, only the antisense probe bound to cells in the dermis and other NCC derivatives such as neural tube and DRG were completely negative (Fig 2.11c and d). There was no positive signal in the 4 day embryo or RNase-treated controls probed with pmc0,1. Positive cells in 5 and 6 day controls hybridized with sense probe were limited to abdominal organs such as liver, and cartilage matrix

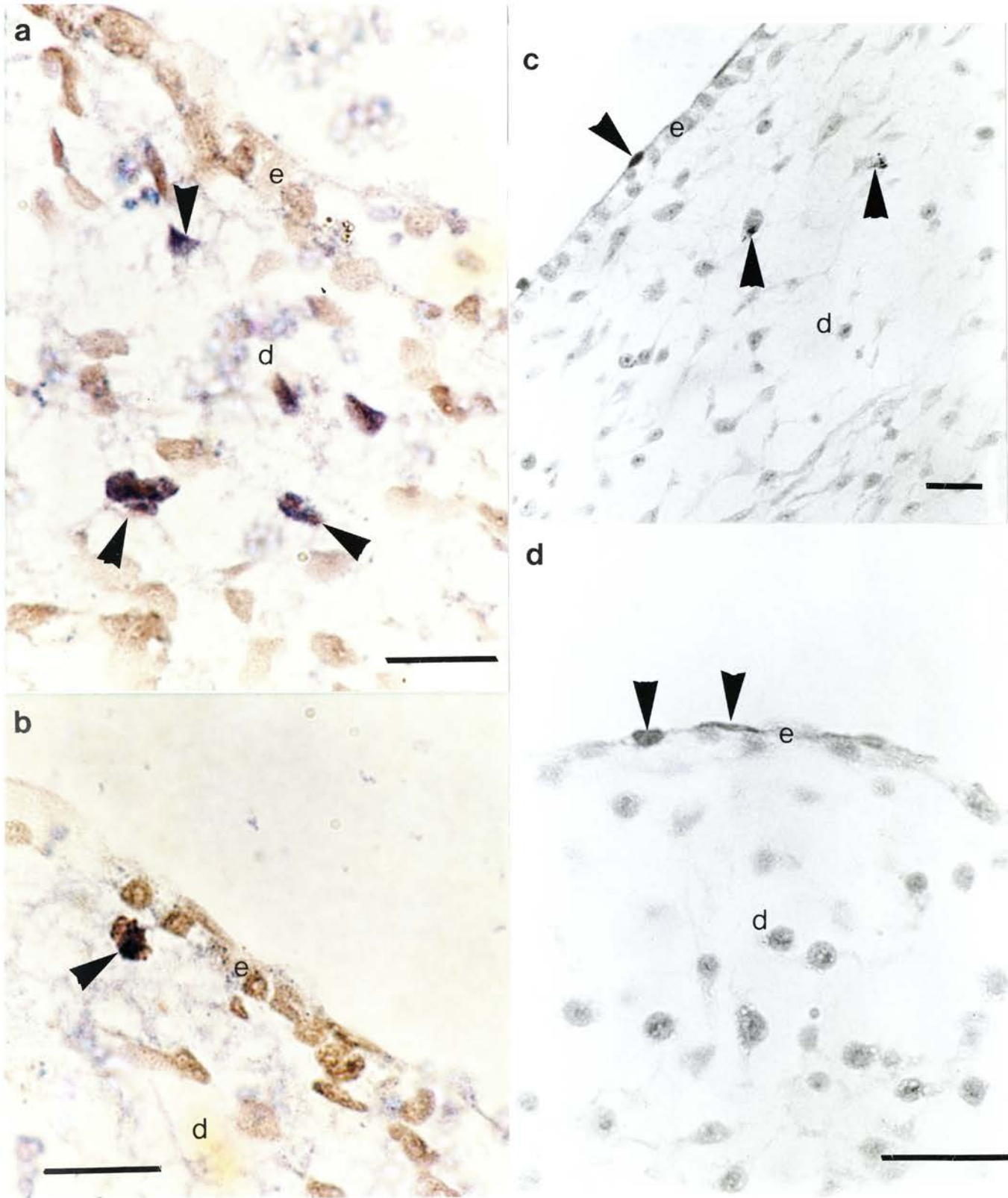


Fig 2.10

(a) and (b) *In situ* detection of tyrosinase mRNA in 5 day embryonic chick skin using DIG-labelled pmc0,1 riboprobe (antisense probe) and alkaline phosphatase-conjugated anti-DIG antibody. (c) and (d) Histochemical detection of tyrosinase by dopa staining. (c) 5 day, (d) 4 day. e, epidermis; d, dermis; arrows, positive cells. Bar=20 μ m.

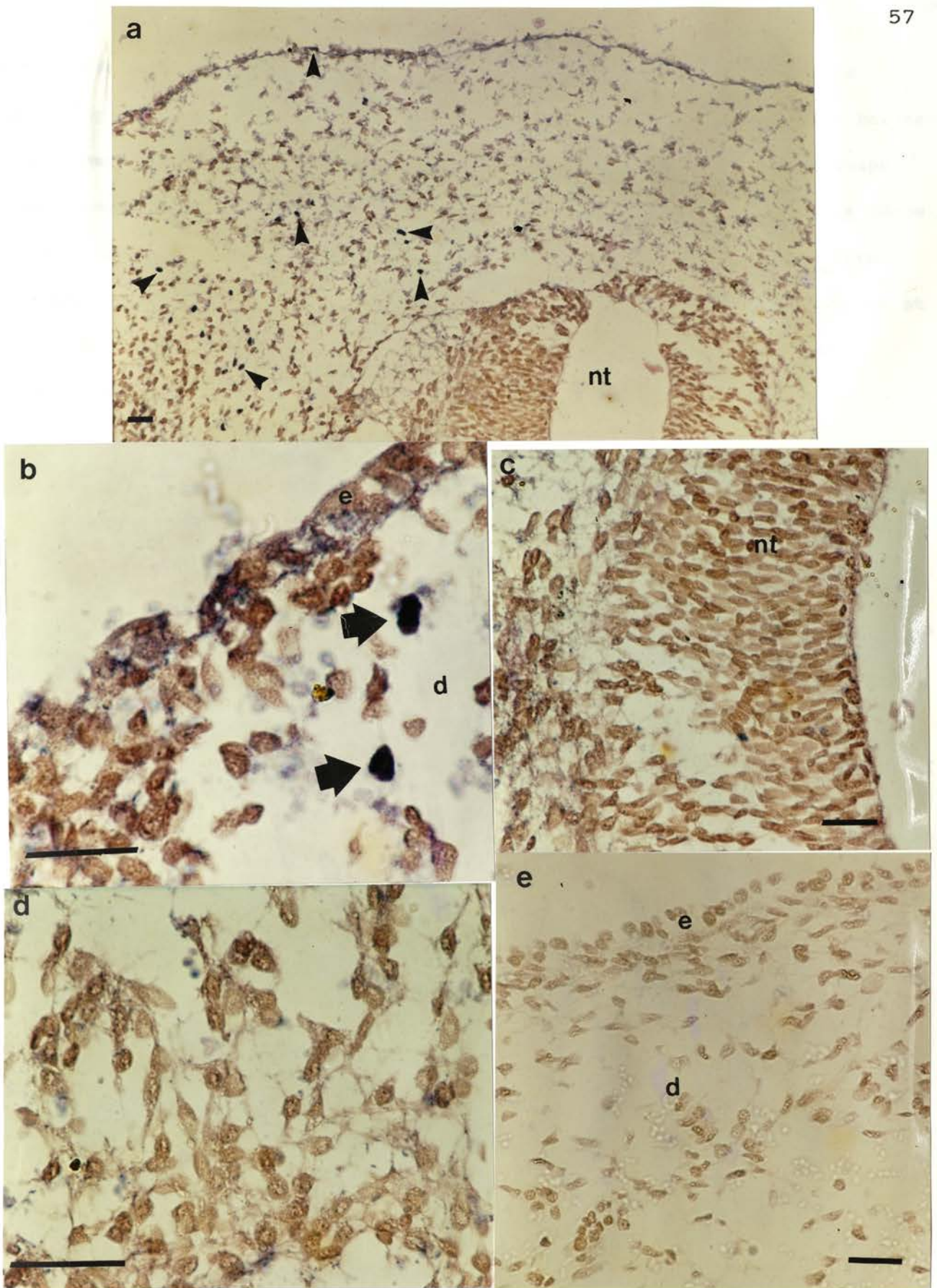


Fig 2.11

In situ detection of tyrosinase mRNA in a 6 day chick embryo using DIG-labelled pmc0,1 riboprobe and alkaline phosphatase-conjugated anti-DIG antibody. (a-d) antisense probe. (a) dorsal region. (b) skin. (c) neural tube. (d) dorsal root ganglion. (e) skin, sense control. e, epidermis; d, dermis; nt, neural tube, arrows, positive cells. Bar=20 μ m.

(also positive in sections probed with antisense riboprobe). This is perhaps because the glandular tissue is very rich in endogenous alkaline phosphatase enzymes (Ross and Romrell, 1989). The cartilage effect was not cell-related and perhaps reflects endogenous alkaline phosphatase activity in the calcifying cartilage (Ross and Romrell, 1989). These results signify that tyrosinase mRNA appears in dermal premelanocytes at 5 days and is present in both dermal and epidermal melanocytes at day 6. With improvements in the technique, it may become possible to detect tyrosinase mRNA in 4 day chick embryos and in greater abundance at 5 days.

2.3.4 Replacement of epidermis with gut or amniotic epithelium- effect on melanocyte differentiation

Having established that many melanocytes begin to differentiate while still in the dermis, the next question asked was whether the dermis or the epidermis provides the critical factors for melanocyte differentiation. To test the role played by the epidermis in melanocyte differentiation, 6 day BAXNHR dermis (as a source of melanocytes) was co-cultured with epithelium from oesophagus and stomach, instead of epidermis. Scattered dermal melanocytes were visible in 3 grafts while 5 were completely unpigmented (Fig 4.9a, chapter 4). None of the 8 grafts contained any epithelial melanin, even those containing stratified oesophageal epithelium; for this reason in Table 2.2 grafts are combined in the row for stomach epithelium. When amniotic membrane was co-cultured with white dermis on top of BAXNHR dermis, 7 out of 8 grafts were completely unmelanised but one developed both epithelial and dermal melanocytes. In the positive controls, white dermis and epidermis were cultured on top of BAXNHR dermis. All 6 grafts developed heavily pigmented feathers. This experiment was in fact designed to investigate melanocyte migration as detailed in Chapter 4, but it was included here since the results also have relevance in the context of melanocyte differentiation. There were two problems with the design of this experiment, the first being that 6 day dermis already contains some differentiated melanocytes and these may have survived to give rise to

the 4 pigmented grafts (3 with gut epithelium, 1 with amnion). In addition, tissue pieces in this experiment were recombined on agar and cultured in vitro for 24 hr prior to CAM grafting and the serum present in the agar may have influenced the results. It does seem, however, that the presence of epidermis is important for the survival and proliferation of melanocytes and that other epithelia provide poor substitutes.

2.3.5 Replacement of dermis with gut mesenchyme- effect on melanocyte differentiation

If the epidermis is critical for melanocyte differentiation, what role does the dermis play in the development of pigmentation? The first approach taken to address this question was to discover whether dermis alone could support melanocyte differentiation. Dermis from 4 day chick embryos (containing no pigmented melanocytes) was separated from whole skin using EDTA, rinsed in chick saline and CAM grafted without further culture to avoid possible induction by serum components. No melanocytes differentiated in the 7 grafts (Table 2.2b) but it was difficult to assess whether a specialised dermis or merely mesenchyme survived (Fig 2.12d). Grafts were identified by a thickening in the CAM mesenchyme and this frequently contained cartilage. The 2 pieces of whole 4 day skin cultured both produced well melanised feathers. This result suggests that dermis in isolation from epidermis is not able to support melanocyte differentiation.

Secondly, a method was devised in which the dermis was replaced with gut mesenchyme so that neural crest cells would emerge from the neural tube into a non-dermal mesenchyme adjacent to normal epidermis. Pieces of neural tube (BAXNHR, as a source of premelanocytes), gut mesenchyme (white) and epidermis (white) were parcelled together and allowed to begin recombination in chick saline but after 1 hr they were CAM grafted for the remainder of the culture period. This method was designed to avoid use of sera and artificial culture media. As shown in Table 2.2c, 6 of 8

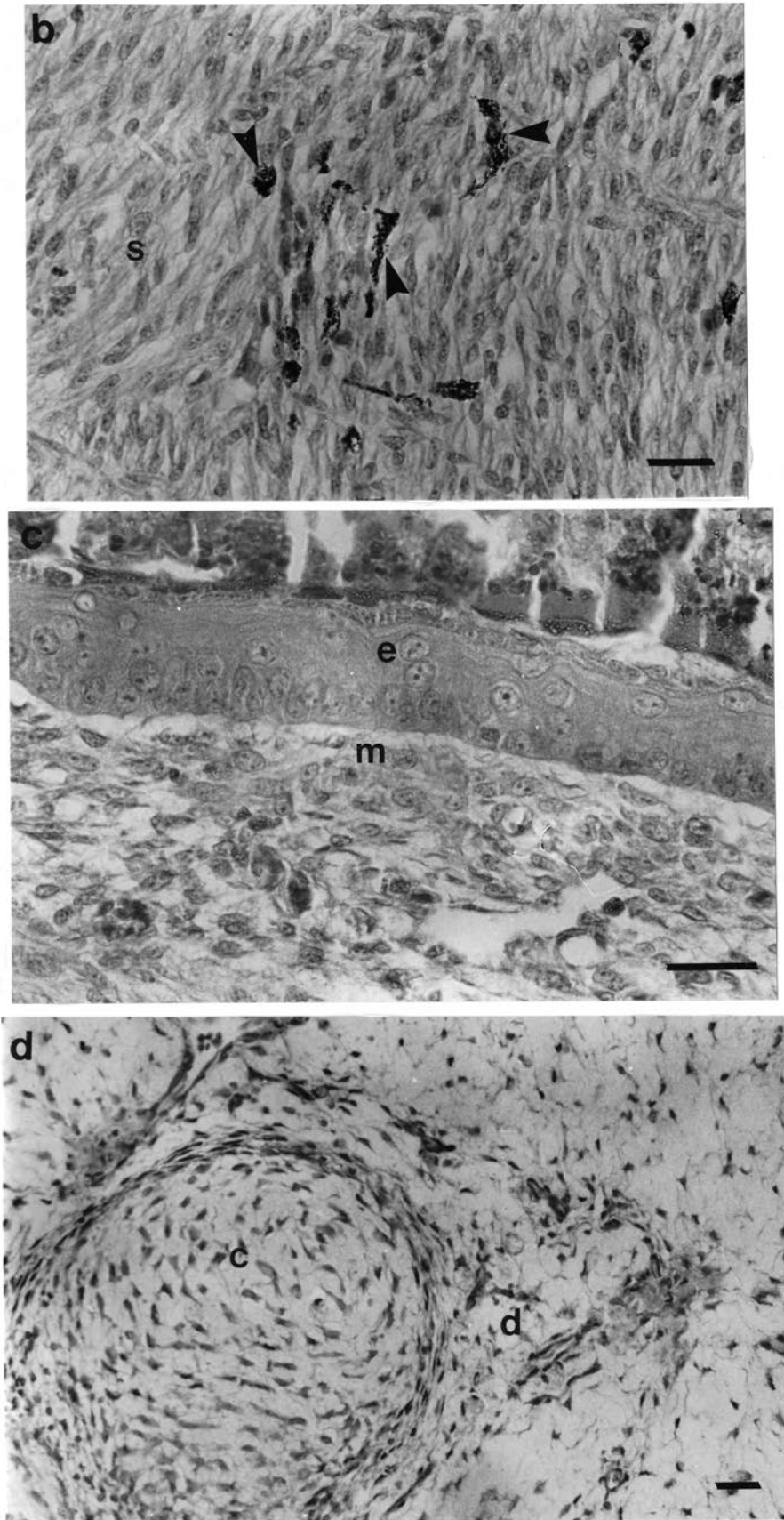


Fig 2.12

CAM cultures of tissue combinations. (a) Stomach epithelium and BAxNHR dermis (see Fig 4.9a). (b and c) BAxNHR neural tube, white gut mesenchyme and white epidermis. (d) dermis alone. arrows, melanin; s, smooth muscle, e, epidermis; m, mesenchyme, d, dermis; c, cartilage. Bars=20 μm.

surviving grafts contained numerous pigmented melanocytes in amongst the muscle, fat and connective tissue of the gut (Fig 2.12b). Epidermis was found in all grafts but was thin, flat and unmelanised (Fig 2.12c). The 2 unpigmented grafts both contained gizzard mesenchyme although this is probably not significant because melanocytes differentiated in the only other graft with gizzard mesenchyme. Controls without neural tubes contained no melanocytes, indicating that the white chick gut at the stages used does not contain cells with melanogenic potential. This is an important control because vagal NCCs which give rise to the enteric ganglia have migrated as far as the umbilicus by day 5 (Le Douarin and Teillet, 1973) and although we have never observed melanocytes in our white breed, these neuroblasts could potentially give rise to melanocytes. Embryos from both white and BAXNHR 9 and 19 day embryos were examined for the presence of gut melanocytes. Only 9 day BAXNHR had melanocytes present in gut connective tissue and mesenteries, but these were not present in white chick gut at 9 days or in either breed at 19 days. This disappearance of extra-epithelial melanocytes just prior to hatching has been reported in the Barred Plymouth Rock (Hopkins-Fox, 1949). Controls which contained white dermis instead of gut mesenchyme all formed pigmented feathers (4 out of 4). It was therefore concluded that under the culture conditions used, NCCs were able to migrate into the epidermis and differentiate into melanocytes when dermis was present but although they differentiated when dermis was replaced with gut mesenchyme, they were unable to migrate into the epidermis.

2.3.6 Melanocyte differentiation in dorsal vs ventral quail wing mesenchyme

The experiments detailed above indicate that the control of melanocyte differentiation in a fully pigmented bird is possibly due to an epidermal signal which is received by the migrating premelanocytes while they are still in the dermis. However, birds such as the Japanese quail exhibit regular plumage patterns composed of both dark and white feathers. White feathers have been shown to contain undifferentiated neural crest cells (Richardson et al., 1989).

Which component of the skin is responsible for this suppression of melanocyte differentiation? In an effort to resolve this question, the mechanism regulating quail wing pigment patterning was investigated. Quail wings early in development exhibit predominately melanised feathers on the dorsal surface and white feathers on the ventral surface (Richardson et al., 1989). In Expt 1, epidermis was removed from either the dorsal or ventral surface of 3½ day quail wing buds and the epithelium replaced with white chick epidermis from the dorsal trunk region. All steps were carried out in chick saline to avoid contact with culture medium and sera and the recombinants CAM grafted. If the wing bud dermis is controlling melanocyte differentiation, either by promoting melanisation in dorsal feathers or by inhibiting melanisation in ventral feathers, replacement of epidermis should have no effect on the normal colour of feathers. Ventral feathers should be largely white and dorsal largely black. The results in Table 2.4 indicate that all 3 surviving wings with replaced ventral epidermis developed mostly white ventral feathers, while the single survivor with replaced dorsal epidermis developed a mixture of black and white feathers (Fig 2.13a and b). This suggests that the ventral mesenchyme certainly could be causing inhibition of melanocyte differentiation. However, if this is the case, the dorsal replacement should have had predominantly pigmented feathers. Unfortunately, only 1 graft survived and it was ambiguously pigmented (Fig 2.13a).

In vitro culture techniques were used in Expt 2 in an attempt to improve survival. However, feather development did not occur and the surviving 3 dorsal and 7 ventral mesenchyme/epidermis co-cultures were totally unpigmented. Previous experiments involving older tissue had exhibited good feather development and growth *in vitro*, so the poor growth apparent in the present experiment can perhaps be attributed to tissue age at the start of the culture period.

In the third run, whole wing buds were denuded of epidermis and placed dorsal or ventral side down on agar. The upper surface was covered with white chick epidermis and after 24 hr to allow recombination, the wings were CAM grafted. All 3 of the surviving grafts in which the dorsal surface had been covered with chick epidermis developed pigmented feathers. Three grafts with replaced ventral epidermis developed white feathers only and one developed black feathers. With this technique, it is relatively easy to lose the dorso-ventral orientation of the wing once it is placed on agar and the one darkly pigmented wing with ventrally replaced epidermis might therefore be a dorsal replacement. This method was re-used in Expt 4 except that dorsal and ventral epidermal surfaces were marked with carbon before removal from the embryo to reduce the chance of losing dorso-ventral orientation. Of the 8 surviving dorsal mesenchyme/epidermis combinations, 7 produced black feathers and 1 white (Table 2.4). All 7 grafts with pigmented feathers contained dermal melanocytes. Ventral mesenchyme/epidermis combinations gave rise to 7 grafts with white feathers only and 4 with mixed black and white feathers. Dermal melanocytes were only present in the 4 grafts with black feathers. Quail melanocytes were readily identifiable amongst the chick epithelial cells because of their nucleolar heterochromatin (Fig 2.13c and d). Overall, the 4 experiments indicate a consistent tendency for dorsal mesenchyme to promote/permit feather pigmentation and of ventral mesenchyme to inhibit melanocyte differentiation.

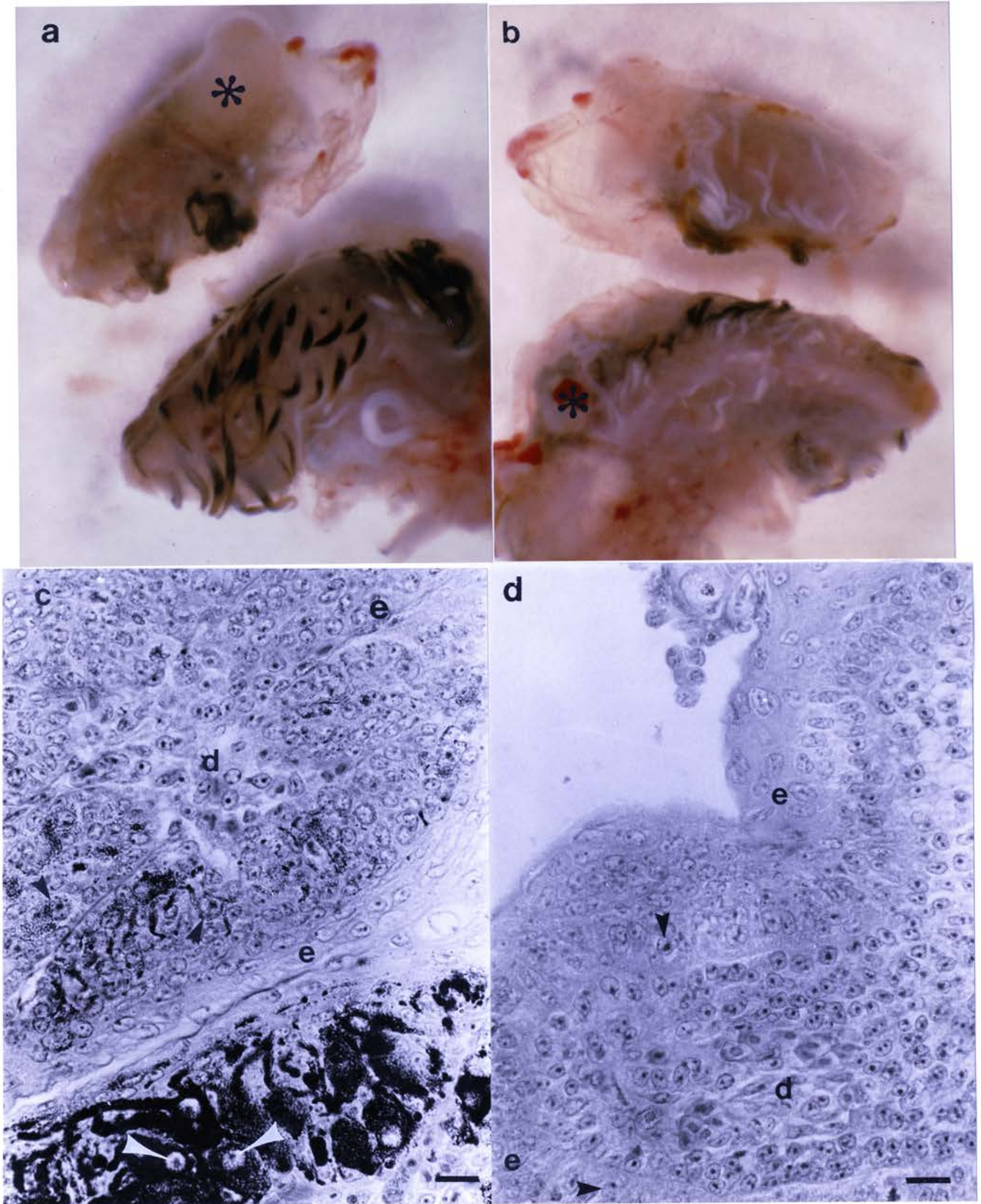


Fig 2.13

Replacement of quail wing epidermis with white chick epidermis. (a) dorsal wing surface, * = replaced epid. (b) ventral wing surface, * = replaced epid. (c) Feather buds from dorsal-replacement TS. (d) Feather bud from ventral replacement. Arrows, quail nucleoli; e, chick epidermis; d, quail dermis. Bars=20 μ m

Table 2.1

DETECTION OF TYROSINASE ACTIVITY IN EMBRYONIC CHICK SKIN

		Tyrosinase activity given as % of maximum				
		4 day	5 day	6 day	7 day	8 day
Skin	expt 1	16	15	36	70	100
	expt 2	62	51	63	94	100
	expt 3	38	44	49	82	100
	expt 4	12	-	18	25	100
Average		32±12	37±9	42±24	68±15	100
Organs	expt 1	29	10	46	19	41
	expt 2	50	77	69	60	64
	expt 3	43	44	41	45	46
	expt 4	33	-	22	32	9
Average		39±5	44±16	45±10	39±9	40±12
Skin	expt 5	63	67	100		
	expt 6	53	61	100		
	expt 7	27	37	100		
Average		48±11	55±9	100		
Organs	expt 5	21	22	22		
	expt 6	53	34	63		
	expt 7	18	13	7		
Average		31±11	23±6	31±17		

Skin and organ homogenate was prepared from 4-8 day BAXNHR chick embryos, adjusted to equal protein quantities and the tyrosinase activity assayed by measuring the incorporation of ^{14}C tyrosine into insoluble melanin. In experiments 5-7, tissue homogenate was first purified by DEAE Sephacel column chromatography to concentrate tyrosinase. The counts for each sample within an experiment have been calculated as a percentage of the counts for 8 or 6 day skin (maximum count) to facilitate comparison between separate assays run at different times and adjusted to slightly different protein concentrations. Values expressed $\pm$ SEM.

Table 2.2

THE ROLE OF DERMIS AND EPIDERMIS IN MELANOCYTE DIFFERENTIATION

	Mesenchyme source*	Epithelium source*	Neural tube source	Epithelial melanin	Mesenchymal melanin	No melanin
a	6 day black and 6 day white skin	6 day white skin	not present	6 (6)	6	0
	6 day black skin	6 day white stomach	not present	0	3	5
	6 day black and 6 day white skin	6 day white amnion	not present	1	1	7
b	4 day black skin	4 day black skin	not present	2 (2)	0	0
	4 day black skin	not present	not present	0	0	7
c	6 day white skin	6 day white skin	55 hr black skin	4 (4)	4	0
	6 day white gizzard	6 day white skin	55 hr black skin	0	1	2
	6 day white gizzard	6 day white skin	not present	0	0	2
	6 day white stomach	6 day white skin	55 hr black skin	0	6	0

() Figures in brackets indicate number of grafts with melanised feather epidermis.

* Tissue components were separated using EDTA or trypsin and combinations either CAM grafted directly or grafted after a 24 hr period on agar. After 7-10 days incubation, grafts were fixed, wax embedded and serial sectioned. Sections were scored for the presence and position of melanin.

Graft survival: With neural tube = 81%, other = 100%.

Table 2.3

THE ROLE OF THE DERMIS IN THE PIGMENT PATTERNING OF QUAIL WINGS

Experiment number	Epidermis source*	Dermis source#	Melanised ^e feathers	White ^e feathers	White ^e epidermis
1	white chick	dorsal quail wing	0	1	0
	white chick	ventral quail wing	0	3	0
2	white chick	dorsal quail wing	0	0	3
	white chick	ventral quail wing	0	0	7
3	white chick	dorsal quail wing	3	0	0
	white chick	ventral quail wing	1	3	0
4	white chick	dorsal quail wing	7	1 (1)	0
	white chick	ventral quail wing	4	7 (3)	0

* Epidermis was separated from dorsal trunk skin of 6-8 day white chick embryos using EDTA.

Dermis was provided by removal of epidermis from 3½-4 day quail wing buds using EDTA. In Expt 2, pieces of dermis were excised and cultured on filters floating in culture medium for 2 wks. In the other Expts, epidermis was draped over either the ventral or dorsal wing surface and the whole bud CAM grafted and cultured for 7-10 days. In all cases, cultured tissue was fixed, wax-embedded, serial sectioned and examined for the presence of pigmented or unpigmented feathers.

@ Grafts were scored as having melanised feathers when the majority of feathers on the graft were melanised and as having white feathers when the majority of feathers on the graft were white. In Expt 1, only the surface with replaced epidermis was scored.

() Brackets indicate slightly degenerate feathers which were nevertheless obviously pigment-free.

- Indicates no survival.

Graft survival: Expt 1= 50%, Expt 2= 12%, Expt 3= 78%, Expt 4= 79%.

2.4 DISCUSSION

2.4.1 Melanocytes exhibit tyrosinase activity from day 5

The timing of the various steps in melanocyte differentiation prior to the first appearance of visibly melanised cells from 5 days is unknown. Perhaps the first marker of melanocyte differentiation would be the synthesis of melanosomes, membranous organelles on which melanin is deposited. However, fully melanised melanosomes are transferred to keratinocytes in the epidermis and are thereafter not exclusive to melanocytes. Tyrosinase is the enzyme credited with catalysis of tyrosine to melanin although there are several autocatalytic steps and the role of dopachrome tautomerase is unknown (Hearing and Jimenez, 1989; Hearing and Tsukamoto, 1991). Tyrosinase is therefore an unambiguous marker for differentiated melanocytes. The appearance of active tyrosinase is likely to be preceded by the appearance of immature and perhaps inactive forms of the enzyme and earlier yet, the mRNA for tyrosinase would be transcribed from the melanocyte DNA. I have attempted to trace the timing of these steps in melanocyte differentiation using a radiometric assay for active tyrosinase, immunoblotting with polyclonal anti-chick and anti-mouse tyrosinase antibodies for inactive tyrosinase and Northern blots and *in situ* hybridization to detect the first appearance of tyrosinase mRNA in tissue sections of chick embryos. *In situ* hybridization also yielded information on the position of melanocytes as they differentiated.

Tyrosinase activity was detected in 5-8 day embryonic chick skin using a sensitive, specific radiometric assay (Fig 2.3). However it was not detectable at 4 days using this assay and it was therefore concluded that the appearance of tyrosinase activity coincides with the appearance of visible melanin at 5 days. If tyrosinase is present in melanocytes prior to 5 days, it might be present in an inactive but immunodetectable form. To test this idea, it was first necessary to obtain a suitable antibody. A polyclonal antibody directed against mouse tyrosinase barely detected chick tyrosinase in Western blots of 11 day chick skin

(Fig 2.4a). There are two possible reasons for this result, the first being that tyrosinase is not an abundant protein, contributing less than 0.1% of total melanocyte protein, and chick skin contains relatively few melanocytes (Hearing and Jimenez, 1989). Secondly, chick tyrosinase may only be partially homologous to mouse tyrosinase and this may affect antibody recognition.

Since the mouse tyrosinase-specific antibody had proved to be unsuitable for the detection of tyrosinase in early chick embryos, a polyclonal antibody raised in our laboratory and thought to be specific for chick tyrosinase (Stander, 1991) was tested on Western blots of 4-8 day skin and 8 day organs. As shown in Fig 2.4b, there were several indications that it was tyrosinase-specific. Firstly it reacted against skin homogenate and not organs, and it reacted against protein bands at 67 kDa and 43 kDa which correlate well with the sizes of T4 and T3 isoforms of chick (Yamamoto and Brumbaugh, 1984) and mouse tyrosinase (Fig 2.4c, Hearing and Jimenez, 1987). It showed a graded increase in response to samples of equal protein content from 3 to 8 day chick skin, and interestingly, the lower 43 kDa band was absent in white chick skin. The initial conclusion was that tyrosinase was present in 3 day chick skin. However, subsequent experiments revealed that the antibody was non-specific (Stander, 1991), and although there is probably some anti-tyrosinase immunoreactivity present in this serum, the detection of tyrosinase in 3 and 4 day skin was probably false.

Since the antibodies available could not effectively be used to trace the time of the first appearance of tyrosinase in chick melanocytes, a cDNA sequence thought to code for mouse tyrosinase (termed pMT4) was used as a probe and many fruitless attempts were made to hybridize this to both total and mRNA on Northern blots. During the course of these experiments, a paper was published which showed that pMT4 codes for the brown (b) locus protein (Jackson, 1988) and this is only expressed in abundance in transformed melanocytes (Hearing and Jimenez, 1989). We therefore obtained the c-lens gene sequences MTY811 (Kwon et al., 1988) and

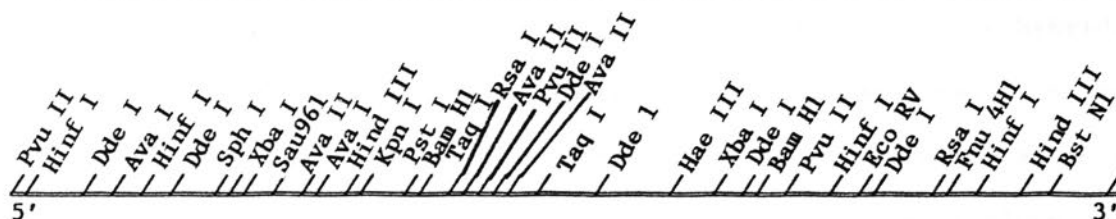
pmcTYR-1 (Ruppert et al., 1988)(Fig 2.14). MTY811 was used to generate ^{32}P -multiprime probe and hybridized to Northern blots of total and purified mRNA from chick skin, without success. This lack of success was attributed to the very low levels of tyrosinase mRNA transcripts which are present in tissue extracts due to the rarity of the message and the relative paucity of melanocytes as compared to other skin cells. Single stranded probes such as riboprobe are far more sensitive than double stranded probes because the latter have a tendency to self-reassociate during hybridization whereas antisense riboprobe can only hybridize with the complementary mRNA in tissue. Therefore a 580 bp fragment from the 5' end of pmcTYR1 was cloned into pGEM and used to generate both ^{32}P -labelled and DIG-labelled antisense riboprobe. Although both types of riboprobe successfully detected melanoma RNA in Northern blots (Fig 2.5), chick tyrosinase mRNA remained undetected. It is unlikely that this failure to detect chick tyrosinase mRNA was due to a lack of homology between mouse and chick tyrosinase gene sequences because the mouse sequence has been shown to cross-hybridize to chick genomic sequences on Southern blots (Kidson and Viljoen, personal communications). It therefore seems that tyrosinase mRNA is expressed in quantities too low to be detected using Northern blots. Two attempts were made to detect tyrosinase mRNA in RNA extracts from 11 day chick skin using RNase protection assays with ^{32}P -labelled pmc0,5 riboprobe. Although tyrosinase mRNA was detected in $10\mu\text{g}$ samples of total melanoma RNA, nothing was seen when up to $150\mu\text{g}$ of chick skin RNA was used. However there was no time to optimise the method and the results are not presented here.

Perhaps the most sensitive method for detection of a rare mRNA species in a heterogeneous cell population is *in situ* hybridization. Since the cells of interest are hybridized *in situ*, there is no dilution of mRNA due to extraction procedures. This method was therefore applied to sections through chick embryos, using pmc0,5. Initially, ^{35}S pmc0,5 riboprobe was hybridized to melanised chick skin. Attempts to raise the level of signal above

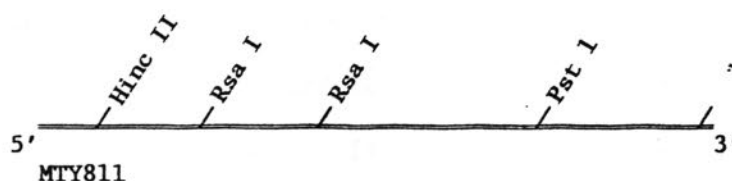
Restriction maps of putative tyrosinase cDNAs

bp 0 200 400 600 800 1000 1200 1400 1600 1800 2000 2200 2400

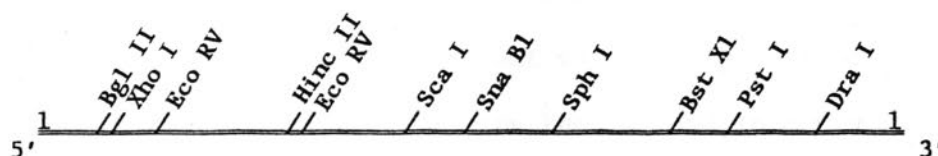
1 1 1 1 1 1 1 1 1 1 1 1 1



A. pMT4



B. MTY811



C. pmcTyr1

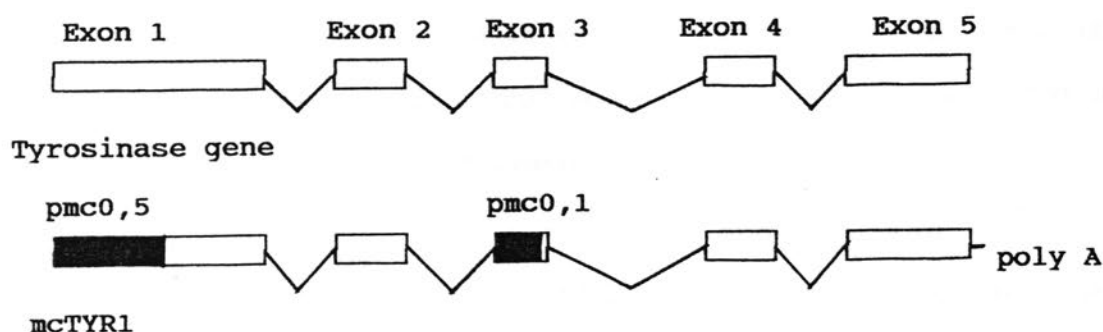


Fig 2.14

A. Restriction map of pMT4 murine tyrosinase cDNA (Shibahara et al., 1986). Sau96 is Sau 961, Fnu4H is Fnu 4H1, restriction enzyme numerals greater than 1 have been converted to arabic eg. Hind III is Hind3. B. Partial restriction map of MTY811 murine tyrosinase cDNA (Kwon et al., 1988). C. Partial restriction map of pmcTyr1 murine tyrosinase cDNA (Ruppert et al., 1988). D. Organisation of the mouse tyrosinase gene showing the composition of the major transcript, mCTYR1 (Ruppert et al., 1988). Exons are shown as open boxes, introns as disconnected lines. The shaded regions represent the positions of the 580 bp fragment called pmc0,5 and the shorter 100 bp pmc0,1, both used as riboprobe in the present study.

background proved to be unsuccessful. It also proved to be very difficult to distinguish between black autoradiographic granules and dark brown melanin granules. The newly available digoxigenin-fluorescein labelling technique was tried next since the yellow-green fluorescence would not be masked by melanin granules. Cultured melanoma cells exhibited strongly positive hybridization signals when hybridized with DIG-labelled riboprobe generated from pmc0,5 (Fig. 2.7) but even faint fixative-induced fluorescence in chick and mouse tissue sections masked any positive hybridization. Finally, the alkaline phosphatase DIG detection system was tested since this involves the cumulative production of a purple-blue product by bound enzyme and might therefore be more sensitive. In addition, the reaction product would be distinguishable from melanin on lightly melanised sections. This method was first tested on 7 day post-natal mouse skin because the probe was known to be specific for mouse tyrosinase mRNA and at 7 days, the newborn mouse hair follicles are growing and melanising rapidly. Alkaline phosphatase reaction product was clearly apparent in the base of the hair follicles where melanocytes are located and in a few scattered dermal cells, some of which contained melanin. Using the conditions established for mouse skin, tyrosinase mRNA-positive cells were then detected in the epidermis of 8 day embryonic chick skin, indicating that mouse and chick tyrosinase mRNA were sufficiently homologous to cross-hybridize.

The method thus established was applied to sections of younger chick embryos in an attempt to establish the earliest time of expression of the melanocytic phenotype in chick skin. Sense and antisense DIG-labelled riboprobe (termed pmc0,1) were generated from a 100 bp sequence cloned from chick DNA and thought to be part of the chick tyrosinase gene. It was hoped that this would be possibly more specific than the mouse sequence and that the smaller probe size would improve probe penetration into the tissue. However, after the hybridizations had been carried out, this sequence was found to be a smaller piece of pmcTYR from exon 3. Like exon 1, exon 3 is highly conserved in the

mouse, human, *Neurospora* and *Streptomyces* tyrosinase gene (Muller et al., 1988). The antisense probe hybridized to dermal and epidermal cells in sections of 5 and 6 day chick embryos (Fig 2.10 and 2.11), but there was no detectable hybridization in 4 day embryos. Positive cells were mostly located in the dermis at 5 days and in both dermis and epidermis at 6 days, in agreement with the counts of pigmented melanocytes at these ages (Stander, 1991; Hulley et al., 1991). Dopa enhancement revealed tyrosinase positive cells in the dermis of 5 day embryos which appear to be similar in position and size to those positive for tyrosinase mRNA. Endogenous alkaline phosphatase is present in cartilage, neutrophils and some macrophages (Ross and Romrell, 1989) but only the cartilage stained positive in RNase-treated sections and sections with antibody but no probe. It therefore seemed unlikely that the positive dermal cells in Fig 2.10 and 2.11 were white blood cells, and furthermore, white blood cells are seldom found in the epidermis or crossing into it (Fig 2.10b). The number of cells positive for tyrosinase mRNA also correlated well with counts of pigmented melanocytes at the same ages. Less than 1 melanocyte per 7 μm -thick section was counted in the mid-trunk region of 5 day chick embryos (Stander, 1991; Hulley et al., 1991), whereas tyrosinase mRNA was detected by *in situ* hybridization in approximately 10 cells per equivalent 5 μm thick-section at 5 days. It seems likely that cells would begin to express tyrosinase mRNA some time before the enzyme itself was produced and even longer before the final end-product of the enzyme reaction, melanin, was formed.

From these results it was apparent that the tyrosinase enzyme was active in the skin of BAXNHR chick embryos from 5 days and that melanocytes expressing tyrosinase mRNA were predominately dermal at this stage. Transcription of the tyrosinase gene may begin earlier than 5 days but this appears to be beyond the limits of sensitivity of the available detection systems. This result signifies that it is the dermis which provides the appropriate environment for the initiation of melanocyte differentiation, and not the epidermis as previously

suggested (Watterson, 1942; Hopkins-Fox, 1949; Le Douarin, 1982; Sears and Ciment, 1988). However, although the dermis may permit or instruct melanocyte differentiation, it seems unable to support melanocyte survival after 8 days. Pyknotic dermal melanocytes were seen in 8 day skin and these cells became more frequent in 9 and 10 day skin (Stander, 1991; Hulley et al., 1991) and it is thought that melanocytes remaining in the dermis at this time may become trapped in an environment which is no longer favourable, die, and be ingested by macrophages. It is possible that melanocytes become dependent with age on an epidermally-produced growth factor such as basic fibroblast growth factor (bFGF). bFGF is a natural growth factor and mitogen for melanocytes, is produced by the basal keratinocytes of human skin, and close contact between keratinocytes and melanocytes is essential for melanocyte survival in culture (Halaban et al., 1988). A similar situation exists in the developing dorsal root ganglion where sensory neurons begin to differentiate and send out neurites at 6 days in culture but start to die at 9 days unless given nerve growth factor (Ernsberger and Rohrer, 1988).

2.4.2 Role of the dermis and epidermis in melanocyte differentiation

The results presented above indicate that many melanocytes begin to express markers of differentiation such as the messenger RNA coding for tyrosinase while they are still in the dermis. This could mean that the dermis is playing an inductive role in melanocyte development. Alternatively, the epidermis could be releasing diffusible factors into the dermal matrix which then act on melanocytes to trigger the first steps in differentiation. In order to investigate the question of which tissue is predominately influencing melanocyte differentiation, a series of tissue co-culture experiments were carried out. The effect of dermis and epidermis on melanocyte differentiation was first tested by replacing them with mesenchyme or epithelia from other body regions. When the dermis was replaced with mesenchyme from gut sources so that neural crest cells emerged from the neural tube directly into a non-skin mesenchyme which was associated with epidermis, melanocytes

developed in 7 out of 9 cases but failed to populate the epidermis. This result indicates that the dermis *per se* is not necessary for melanocyte differentiation and that the gut mesenchyme provides the same permissive environment as the dermis. In support of this conclusion, I have observed that in BAXNHR embryos, melanocytes normally develop in the mesenteries and gut connective tissue by 9 days although they disappear by day 19. The fact that melanocytes in gut mesenchyme fail to reach the epidermis may indicate that the dermis plays a critical role in the terminal migration of melanocytes or that gut mesenchyme is unable to maintain epidermal development and so the normal homing signals for melanocytes are not transmitted.

It should be noted that the neural crest cells which have entered gut mesenchyme and differentiated into melanocytes in the present experiments were from the mid-trunk level of the neural crest. Recent lineage studies using quail-chick chimeras, retroviral-labelling and microinjection of fluorescent dye have shown that only NCC's from the vagal and sacral levels contribute to the enteric neural crest derivatives such as the parasympathetic ganglia of the gut (Le Douarin and Teillet, 1973; Pomeranz et al., 1991; Serbedzija et al., 1991) and that trunk NCCs do not reach the gut for some reason. However, if truncal neural crest cells are grafted to vagal levels, they do colonise the gut (Le Douarin, 1980), indicating either that the pathway to the gut is only accessible at that level (see Tucker et al., 1986) or that they acquire a more invasive phenotype at their transplantaion site. The fact that melanocytes injected onto the dorso-ventral pathway anywhere along the trunk are able to migrate to the parasympathetic enteric ganglia (Bronner and Cohen, 1979; Bronner-Fraser et al., 1980) and that melanocytes in my co-culture experiments are also able to penetrate the gut mesenchyme indicates that some neural crest derivatives are more invasive than others and that this characteristic may be acquired in the vagal region or as cells differentiate into melanocytes under the influence of the epidermis.

To establish whether, in the absence of epidermis, melanocytes would still differentiate, epidermis was replaced with stomach epithelium or with amniotic epithelium. Out of 17 grafts, 12 showed no melanisation after 7-10 days in culture and only 1 of the amniotic epithelium grafts exhibited epidermal melanisation, whereas all controls developed intensely black feathers. It is important to note that in this series of experiments, the melanocyte source was not neural tube but 6 day dermis which already contains some differentiated melanocytes, and therefore these must have degenerated or dedifferentiated in the totally unpigmented grafts. The melanocytes present in the 4 dermally pigmented grafts may have differentiated before the graft was constructed, and would therefore not be the result of *de novo* dermal or epithelial induction. Alternatively, it should be remembered that until 6 days, those melanocytes were in dermis underlying epidermis and it may be that the epidermis was at that time providing a diffusible signal which resulted in continued development of some melanocytes even after the epidermis had been replaced. This experiment indicates that epidermis is necessary for normal levels of melanocyte differentiation. It should, however, be repeated using either 4 day dermis or neural tube as the source of melanocytes, since both tissues contain premelanocytes only and would give a less ambiguous result.

A second approach to examining the role of the dermis in melanocyte differentiation would be to culture it in isolation from a stage when only premelanocytes were present. Intact skin from 4 day embryos contained no visibly differentiated melanocytes but when CAM grafted, normally pigmented feathers developed. However when 4 day dermis was cultured in isolation for 7 days, there was no evidence of melanocyte differentiation. From this it appears that melanocytes are dependent on an epidermal signal for differentiation, unless the melanocytes are differentiating and then degenerating in the absence of necessary maintenance growth factors from the epidermis. This would correlate with the gradual disappearance of dermal melanocytes from 8 days in whole embryos (Hulley et al., 1991). Alternatively,

premelanocytes may need a continuous epidermal signal until 6 days, after which time they can maintain a differentiated state in the absence of epidermis. If this is the case, melanocytes isolated from epidermis at 4 days would not have had sufficient time to become stably determined. One problem with this method is that the dermal mesenchyme tended to merge with the CAM mesenchyme except where cartilage developed due to the inclusion of some sub-dermal mesenchyme. It is possible that no true dermis survives in these grafts. Unfortunately, this method could not be used to test the role of epidermis alone because epidermis does not survive in the absence of a supporting mesenchyme (my observations; Dodson, 1967).

In a final attempt to elucidate the roles played by dermis and epidermis in melanocyte differentiation, I investigated the mechanisms underlying production of unpigmented pattern feathers in the quail, an otherwise pigmented bird. The cause of inhibition of melanocyte differentiation in white pattern feathers of the quail is unknown. Normally, quail wings are heavily pigmented dorsally but are largely unpigmented ventrally, though a few black feathers are seen (Richardson et al., 1989). Richardson's group found that when a quail wing was grafted onto a guinea fowl embryo (uniformly pigmented bird) prior to NCC migration, a normal quail pattern developed in the grafted wing (Richardson et al., 1991). This means that the guinea fowl NCCs were influenced by a positional prepatter in the quail wing and responded by producing a quail and not a guinea fowl melanocyte differentiation pattern. In an effort to establish whether the dermis or the epidermis of the wing was responsible for this pigment pattern, I carried out a series of grafting experiments in which dorsal or ventral quail wing epidermis was replaced with white chick epidermis from a different body region. The results are given in Table 2.3 and they strongly suggest that it is the dermis which is responsible for suppression of melanocyte differentiation in the unpigmented regions of quail embryos. When the dorsal epidermis from quail wing buds was removed and replaced with white chick epidermis, most grafts developed heavily pigmented feathers- the normal pattern for

quail wings. Likewise, when the ventral wing epidermis was replaced, the majority of grafts developed the normal pattern of white feathers. This means that removal of the epidermis had no effect at all on the development of normal quail patterning and that the dermis played the critical role.

Melanocytes have been shown to respond to positional signals (Richardson et al. 1989), and have demonstrated that it is the dermal and not the epidermal component of the skin which provides this positional information. The dermis may be receiving this information from underlying skeletal elements and passing it on to the epidermis which brings it into effect. However, the dermis appears to be the crucial mediator. This finding may relate to the discovery that the unpigmented ventral skin of *Xenopus* and *Rana* tadpoles produces a strong inhibitor of melanisation, capable of suppressing melanisation induced by α -MSH and serum melanisation factors. Dorsal skin does not produce this factor and is heavily pigmented (Fukuzawa and Bagnara, 1989).

The control of avian melanogenesis is likely to be highly complex, and there are many more questions which need to be investigated before a clear picture can emerge. If the epidermis is the major promoter of melanocyte differentiation, it would be very interesting to know why in red breeds and quails, pigmented melanocytes are never seen outside the epidermis and do not appear till at least 9 days (Dorris, 1939; Weiss and Andres, 1952; Le Douarin, 1982; Sears and Ciment, 1988), whereas in our BAXNHR breed, melanogenesis begins from 5 days and is predominantly dermal until after 6 days (Hulley et al., 1991). Dorris (1939) suggested that black chick breeds have independently differentiating melanocytes, while red breed melanocytes are dependent on the epidermis for differentiation. A recently reported antibody, MEBL-1, appears to bind specifically to all cell membranes within chick premelanocytes, and it begins to pick up premelanocytes as they lose reactivity to HNK-1 (Kitamura et al., 1992). If this is an indication of melanocyte differentiation, it

correlates well with my in situ hybridization studies which show that tyrosinase mRNA expression begins in dermal melanocytes from day 5, and observations of isolated, pigmented dermal melanocytes in 5 day chick embryos (Stander, 1991; Hulley et al., 1991). Both MEBL-1 and riboprobe for tyrosinase should be used on quails and red chick breeds to see if the early differentiation pattern differs between breeds.

If the epidermis is producing a diffusible differentiation signal for melanocytes, transfilter co-culture of epidermis with 4 day BAXNHR dermis should result in dermal melanocyte differentiation. However this would have to be done in culture and the medium and serum could well affect the result. Melanocyte differentiation may turn out to be a multi-step process, starting in the dermis with signals necessary for initiation of melanogenesis. Melanocytes may become growth factor dependent (either for survival or for stabilisation of phenotype) after 8 days and begin to degenerate or dedifferentiate unless they are located in an epidermal environment.

Melanogenesis in some breeds, particularly red melanin production, may require this second phase of differentiation signals before starting. Production of white feathers in plumage patterns may be caused by the absence of the first phase dermal signal for melanocyte differentiation.

CHAPTER 3

The role of fibronectin, laminin and serine proteases in the terminal migration of melanocytes

3.1 Introduction

The sub-group of neural crest cells (NCCs) which migrate away from the neural tube along the dorsolateral pathway give rise to melanocytes of the skin. In order to reach their epidermal target, they have to spread laterally around the body, migrate towards the epidermis and cross the epidermal basement membrane. At present it is not known how melanocytes physically negotiate their way towards and into the epidermis. It is perhaps relevant to note at this stage that any investigation of the normal processes involved in cell migration through extracellular matrix (ECM) and basement membranes might add to our understanding of the mechanism of metastasis. Many studies, some of which will be discussed below, have implicated the ECM and matrix-degrading proteases in the migration of NCCs. Few of these, however, have directly considered the role of either ECM or proteases in the terminal migration of melanocytes through the dermis and into the epidermis, and it is this topic that forms the focus of this chapter.

3.1.1 Role of the ECM in pigment patterning

The critical role played by ECM components of the skin in normal pigment cell migration and subsequent pigment patterning is clearly illustrated by vertebrate colour mutants. For example, in white larval axolotls pigment cells move slowly or not at all (Keller and Spieth, 1984), and yet, when sub-epidermal matrix from a dark embryo is placed next to the presumptive neural crest of a white embryo, it triggers NCC migration into the subepidermal space (Lofberg et al., 1989). These mutant white axolotls have since been shown to possess a sub-epidermal ECM which is transiently defective as a substrate for NCC migration (Lofberg et al., 1989; Perris et al., 1990). The fact that this pigment defect is reversible by a simple matrix transplant means that a faulty ECM composition alone is the cause of a total failure of pigment development.

In the same way, mouse mutants with coat colour irregularities offer an invaluable source of information on what directs normal pigment cell migration. Studies suggest that ECM can influence developmental events in two ways, firstly by supporting cell differentiation and secondly, by providing cells with a migration substrate. *Steel* mice are white because of an altered ECM in the skin environment, possibly related to the absence of the *c-kit* receptor ligand, *dominant spotting* mutants are white, express a defect in the *c-kit* oncogene and exhibit abnormal migration with uncertain cause, *piebald* mice have white spots due to regional defects in NCC development and/or migration and have an altered skin environment, *lethal spotting* causes regional defects in NCC development and gut NCCs exhibit faulty migration due to an altered ECM, and *patch* results in an altered composition of the cranial interstitial matrix which causes abnormal migration and/or differentiation of melanocytes (Reviewed by Morrison-Graham and Weston, 1989). Very little work has been published on colour mutants in avians, although the dark and light feathers of quail embryos have both been shown to contain NCCs, thereby excluding differential migration as a possible cause of these pigment differences (Richardson et al., 1989).

3.1.2 Possible NCC migration substrates

In order for a matrix component to be a neural crest cell migration substrate, it must be present along NCC migration pathways at the time of migration and must be shown to be involved with the *in vivo* movement of the cells. Components which fulfil these criteria are the glycoproteins- fibronectin (FN), laminin (LN), tenascin (TN), many of the collagens and the proteoglycans, including chondroitin and chondroitin 6 sulphate (ChSO_4), hyaluronic acid (HA) and heparin sulphate. Demonstration of active involvement in NCC migration is easily performed *in vitro* using cultured neural crest cells, but *in vivo* involvement is more difficult to demonstrate and is most commonly attempted using antibodies either to the

substrate or to specific cell surface receptors. Enzymatic perturbation of substrates and treatment with competitive peptide fragments are also frequently used methods. 3.1.3 Fibronectin

FN is the most effective migration substrate for avian (Newgreen et al., 1982; Rovasio et al., 1983; Erickson and Turley, 1983; Erickson and Tucker, 1986), mammalian (Mackie et al., 1988) and amphibian (Epperlein et al., 1988; Perris and Johansson, 1990) NCCs in vitro. In avians, FN is spatiotemporally distributed along the major migration pathways followed by NCCs (Newgreen and Thiery, 1980; Mayer et al., 1981; Duband and Thiery, 1982; Brauer and Markwald, 1988; Erickson, 1988), and likewise in amphibians (Epperlein et al., 1988), and mice and rats (Mackie et al., 1988). In addition the cell-binding fragment pentapeptide sequence of FN (Fig 3.1a), GRGDS (FNpep), blocks migration of NCCs in vitro on FN substrates (Boucat et al., 1984; Perris et al., 1989). This peptide sequence is the functional region of the major cell-binding sequence of FN and can be used as a competitive inhibitor, binding to cell surface receptors and thus preventing the cell from adhering to FN substrates (Dufour et al., 1988). When peptides containing the GRGDS (FNpep) sequence are microinjected into avian embryos in the region of the premigratory cranial neural crest, NCC migration is partially inhibited (Boucat et al., 1984). Overall, these results strongly support FN as the most probable migration substrate for avian NCCs.

There are, however, a few difficulties with this theory. One is that cranial NCCs enter a FN-free space at the mesencephalic-rhombencephalic level and migrate readily through it (Duband and Thiery, 1982). Furthermore, Brauer and Markwald (1988) report the absence of FN in the sub-ectodermal pathway taken by premelanocytes. However Brauer and Markwald (1988) were using White Leghorn chicks which have abnormal melanocytes and FN is reported in the sub-ectodermal pathway of the chick by Erickson (1988). A second problem is that FN is also found in many embryonic regions which are never populated by NCCs (Krotoski et al., 1986; Tucker and Erickson, 1986; Dufour et al., 1988) such as the perinotochordal space

(Brauer and Markwald, 1988; Stern et al., 1991), the entire somite, caudal as well as rostral halves and the extraembryonic membranes (Mackie et al., 1988). If FN is the only ECM matrix molecule used by the migrating neural crest *in vivo*, why are there no NCCs in certain FN-rich regions and how can NCCs migrate in the absence of FN? The most likely answer is that NCCs are capable of migrating on multiple substrates. In fact, different combinations of ECM molecules may play crucial roles in directing different NCC sub-populations to different body regions and even inducing a variety of phenotypes.

There are several ways by which FN can be rendered unsuitable as a migration substrate. Firstly FN has at least 4 cell binding sites (Fig 3.1a), not all of which are present on all FN molecules due to alternative splicing of FN mRNAs (at least 8 distinct FN polypeptides in chicks, 20 in humans) (Dufour et al., 1988). This means that NCCs, which prefer migrating when both the RGDS and high affinity binding sites are present, may be excluded from certain FN-rich regions because there are regional differences in FN conformation. As an example of this, Brauer and Markwald (1988) noticed that although the glycoprotein particles along trunk NCC pathways all contained FN, all were not able to bind an anti cell-binding site antibody. This is probably caused by alternative splicing of FN molecules. In fact many cell lines exhibit FN-dependent cell adhesion but only some FN-dependent cell migration. Both appear to be mediated by the same cell surface receptor and involve the RGDS sequence on the FN molecule but additional factors seem necessary to make a cell susceptible to the dynamic function of the FN receptor (Straus et al., 1989).

The ability of FN to support cell migration can also be modulated by other ECM molecules. Cartilage type chondroitin sulphate proteoglycans impair NCC migration on FN, LN, vitronectin and collagens I, III, IV and VI by interfering with a cell surface receptor (Perris and Johansson, 1990). This is the suggested reason for NCC avoidance of the perinotochordal ECM, as it is rich in ChS04

proteoglycans (Stern et al., 1991). Migration on FN can also be enhanced by addition of a slightly less adhesive molecule to a dominantly FN substrate. For example, heparin sulphate proteoglycan stabilises neurite outgrowth and extension on FN and LN (Chernoff, 1988) and tenascin appears to improve NCC migration on FN (Mackie et al., 1988). This phenomenon is not exclusive to FN; for example, addition of ChSO₄ proteoglycans to NCCs migrating in collagen gels causes an increase in speed of migration (Tucker and Erickson, 1984).

3.1.4 Laminin

Since FN is not entirely satisfactory as the key substrate for NCC migration, other substrates should also be considered. Laminin is primarily distributed in the basal laminae bordering NCC migration pathways (Rogers et al., 1986). Avian NCCs migrate readily on low concentrations of LN *in vitro* (Newgreen, 1984), particularly when it is complexed with nidogen, collagen IV and heparin sulphate proteoglycan (all associated with basement membranes) (Perris et al., 1989). The laminin B1 chain (Fig 3.1b) contains the pentapeptide YIGSR (Tyr-Ile-Gly-Ser-Arg), which is the principle site of LN-mediated cell attachment, migration and receptor binding (Graf et al., 1987). When NC cells cultured on LN substrates are treated with the YIGSR pentapeptide (LNpep), there is no inhibitory effect (Perris et al., 1989). However Bilozur and Hay (1988) report that trunk neural crest cells migrate extensively and optimally in 3-dimensional BM matrix gels, and this migration could be totally blocked with 400 µg/ml YIGSR (LNpep), strongly suggesting LN as the active molecule in BM matrix. It is important to note that NCCs can react differently to the same substrate depending on the planar or 3-D nature of the culture system. Perhaps the conformation of the LN molecule is changed when it is used to coat culture dish surfaces (planar substrates), thus rendering the cell-binding site unavailable/ unuseable.

The strongest evidence implicating LN as a NCC migration substrate *in vivo* comes from studies using cranial crest cells. YIGSR (LNpep) has not been used to block

in vivo NCC migration but two other methods have successfully demonstrated an *in vivo* role for LN in cranial NCC migration. The inhibitor of neurite outgrowth (INO) antibody causes a functional blockage of the LN-heparin SO₄ complex and it interferes *in vivo* with cranial but not trunk NCC migration. Further, it selectively affects only a sub-group of cranial NCCs (Bronner-Fraser and Lallier, 1988). Perhaps this is because cranial migration is more dependent on basal laminae which are rich in LN. Migrating NCCs have high levels of cell surface galactosyltransferase which they use to migrate on LN. Inhibition of this enzyme has little effect on trunk NCC migration but cranial NCCs are impeded, those that normally would migrate furthest being most inhibited (Runyan et al., 1986). It is possible that LN does not promote trunk NCC migration. It disappears from the basement membrane of the neural tube at the time of trunk crest cell emergence and reappears in the developing ganglia, perhaps signifying a role in halting migration rather than promoting it (Duband and Thiery, 1987).

This discrepancy in preference for substrates between cranial and trunk NCCs is a recurring theme in the literature. In fact, if the sub-ectodermal ECM is extracted and used as a culture substrate, both cranial and trunk crest cells preferentially migrate on the cranial substrate (Brauer and Markwald, 1987). Essentially this suggests that NCCs probably encounter different ECM components in different body regions and use different receptors on each. Therefore cells may use a repertoire of receptors in order to migrate to any target region, or different sub-populations of NCCs may select certain pathways depending on which receptors they have. Movement on LN is also likely to be modulated by other matrix molecules. LN and nidogen show greater motility promoting activity than LN alone and addition of collagen IV further increases motility (Perris et al, 1989). Association with other BM components may result in organisation of the LN molecule into its optimal attachment or motility promoting configuration.

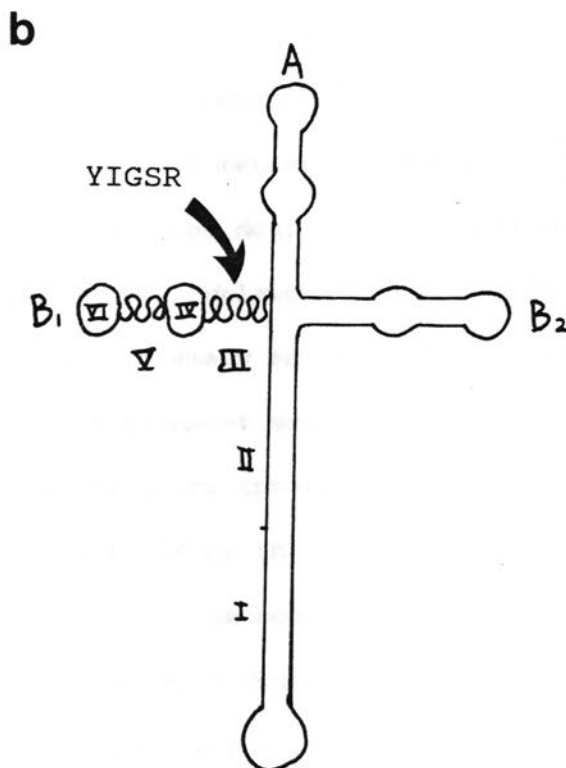
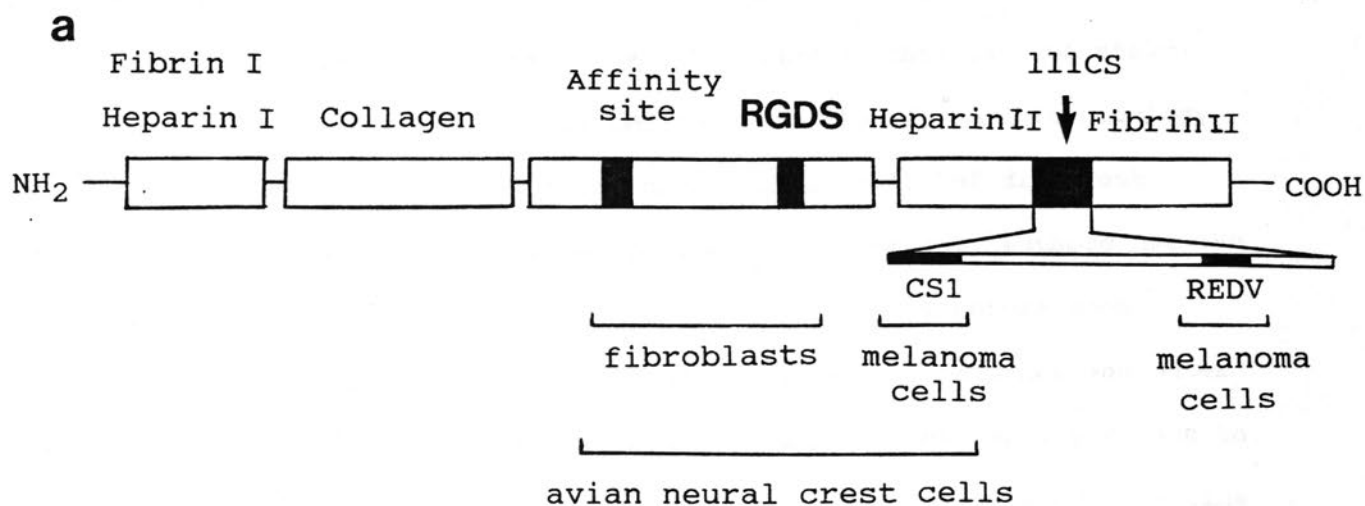


Fig 3.1

(a) Fibronectin molecule showing different cell-binding sites. Avian neural crest cells use the high affinity and RGDS sites for attachment, spreading and motility and may use the CS1 sequence in domain IIICS for attachment and motility. From Dufour et al. (1988).

(b) Laminin molecule showing cruciform shape. The YIGSR cell-binding sequence is located in domain III of the B chain. A, B₁, B₂, chains; i-vi, domains. After Panayotou et al. (1989).

3.1.5 Involvement of proteases in terminal melanocyte migration

As discussed in Chapter 1, melanocytes do seem to be crossing an intact basement membrane in order to lodge in the epidermis. How they do this is completely unknown. Before considering this, it is necessary to clarify some terminology. Basement membranes normally form barriers which prevent cells of different tissues from mixing (Schittny and Yurchenco, 1989). In general, authors use the term basal lamina (BL) when referring to the 3-layered ECM structure seen underlying epithelia at the TEM level (central lamina densa and inner and outer lamina rara). The more commonly used 'basement membrane' (BM) usually refers to the basal lamina plus adjacent ground substance and fibres, visualised as a line underlying epithelia with the light microscope.

Basement membranes are said to form an impenetrable barrier to all cells except leucocytes, with the notable exception of metastatic cancer cells. In fact, most of the published studies on cell migration deal with the extraordinary migratory capabilities of transformed cell lines. Melanoma cells are the malignant counterpart of melanocytes and are extremely metastatic, capable of rapid migration and efficient invasion of basement membranes. It is attractive to hypothesize that these characteristics are inherent properties of the developing melanocytes, which have been exaggerated by the malignant state.

One problem with this theory is that it has been frequently observed that during the course of migration neural crest cells migrate along BMs but do not pass through them. These observations come from experiments in which cells have been (1) labelled with tritiated thymidine, re-injected into embryos and their subsequent movements traced (Weston, 1963), (2) cultured till melanisation has occurred and the melanin used as a specific cell marker to demonstrate cell locations at different stages (Erickson, 1987) or (3) simply labelled *in situ* with NCC-specific antibodies such as HNK-1 and their general positions observed

(Bronner-Fraser, 1986a). In each of the above methods, sequential embryonic stages have been examined in order to trace the routes taken by migrating cells, particularly with reference to which tissue regions they enter and which they appear to avoid. BMs consistently appeared to pose barriers to NCC migration, but it should be noted that this type of study does not test active involvement of BMs in NCC migration.

Active invasion of BMs by NCCs has not yet been demonstrated, but it has been directly tested by injecting cultured quail neural crest cells, both early and pigmented, into the neural tube lumen of chick embryos *in ovo*. In this position they are completely surrounded by embryonic basal laminae and have never been seen to escape (Erickson, 1987). Likewise, when placed on human placental amnion, cultured quail neural crest cells failed to penetrate the BM. Possibly these NCCs failed to develop the invasive phenotype because culture conditions did not provide the appropriate developmental cues. Melanocytes that have begun to differentiate *in vivo* may well be capable of digesting basement membranes, unlike earlier neural crest cells or cultured melanocytes.

The barrier-like behaviour of BMs can be explained in terms of BM composition. They are composed primarily of FN, LN, collagen IV, nidogen and heparin sulphate proteoglycan (Kefalides et al., 1979; Timpl et al., 1979; Martins-Green and Erickson, 1987; Payette et al., 1988; Perris et al., 1989; Schittny and Yurchenco, 1989). FN is regarded by some authors as being part of the BM (Martins-Green and Erickson, 1987, O'Shea, 1987) while others prefer to consider it an associated molecule (Bilozur and Hay, 1988; Payette et al., 1988). LN, FN and collagen IV are all highly adhesive matrices for NCCs while nidogen promotes migration on LN and as reviewed above, all of these molecules are adhesive substrates for NCCs. While at low concentrations, these molecules support cell migration *in vitro*, higher concentrations can halt migration (Newgreen, 1984). *In vivo* in the *ls/ls* mutant mouse, NCCs fail to invade the gut wall because of an

excessive accumulation of LN and collagen IV in the BMs of gut muscle fibres (Payette et al., 1988).

It is known that highly invasive melanoma cells use a plasminogen activator-dependent cascade to penetrate BMs, with type IV collagenase as the final lytic enzyme (Mignatti et al., 1986; Reich et al., 1988; Paneyotou et al., 1989). It is also known that neural crest cells begin to produce increasing quantities of the serine protease, urokinase type plasminogen activator (uPA), as they differentiate into melanocytes *in vitro* (Erickson, 1988), and they are thought to produce a metalloproteinase specific for collagen type IV (Erickson and Isseroff, 1989). Lending support to this theory is the observation that premelanocytes in the chick lie next to the basement membrane for a while before crossing into the epidermis and it is conceivable that at this time they are secreting proteases (Erickson, 1987). Migration of NCCs on tissue culture plastic and in 3-dimensional collagen gels can be reduced by addition of PA/plasmin inhibitors (Erickson and Isseroff, 1989). These *in vitro* studies strongly indicate a role for serine and metalloproteinase enzymes in NCC migration, both through the dermal ECM and the epidermal BM, but this role has not yet been investigated *in vivo*.

3.1.6 Aim and approach

An examination of the studies discussed above provides several points which need to be studied further. Both FN and LN seem to be involved with cranial NCC migration *in vivo*. However trunk NCCs do not appear to migrate on LN *in vivo*, and the *in vivo* role of FN in both trunk NCC and terminal melanocyte migration has not yet been examined. Furthermore, there is as yet no direct evidence of protease action during the terminal migration of melanocytes. It seems that NCC migration is affected by the type of culture system as well as the substrate used. NCC migration on planar FN substrates was inhibited by treatment with GRGDS (FNpep) but the peptide had no effect on NCC migration in 3-dimensional

gels containing FN. In contrast, YIGSR (LNpep) failed to perturb NCC migration on planar LN substrates but totally inhibited migration in 3D basement membrane matrix gels containing LN. Therefore care must be taken when designing *in vitro* experiments to ensure that physical conditions are as close as possible to the *in vivo* situation. Likewise, it is dangerous to directly equate the behaviour of cultured NCCs with *in vivo* developmental counterparts.

There are several advantages to using the pentapeptide cell binding fragments GRGDS (FNpep) and YIGSR (LNpep) to perturb cell migration. They are specific to FN and LN respectively (note, RGDS is found in over 100 other ECM molecules including vitronectin, fibrinogen and collagen I, but it does not seem to function in a cell binding capacity in any of these (Dufour et al., 1988; Gehlsen et al., 1988; Donaldson et al., 1988)). Because the peptides are over 100-fold smaller than an antibody molecule, they diffuse readily through embryonic tissue (Boucat et al., 1984). In addition, both YIGSR (Iwamoto et al. 1987) and GRGDS (Humphries et al., 1988) have been effectively used to block melanoma metastasis into the lungs in mice. This indicates that cells must bind to FN and/or LN before they can degrade basement membranes. It therefore seemed that these peptides could be usefully applied to examine the terminal migration of melanocytes into the epidermis. The same system could also be used to apply protease inhibitors to the migrating melanocytes *in situ*, thus investigating the role of protease enzymes in terminal melanocyte migration.

Therefore in this study, 4-6 day chick skin explants have been treated with GRGDS (FNpep) and YIGSR (LNpep) in an attempt to determine whether they block terminal melanocyte migration. In addition, two serine protease inhibitors have been added to cultures of whole skin in an attempt to establish whether proteases are involved in melanocyte translocation into the epidermis. If migrating melanocytes have to cross an intact BM before reaching the epidermis, they must degrade some of the more adhesive components in order to pass through.

Additionally, even migration up through the dermis may require degradation of ECM. Serine proteases are widely used by migrating cells for this purpose, and hence blockers of serine protease activity should interfere with migration. TLCK (tosyl lysine chloromethyl ketone) is a general serine protease inhibitor (Barrett and Salveson, 1986), a vertebrate trypsin inhibitor (Ficq, 1975) and is known to inhibit plasmin (the enzyme activated by plasminogen activator) (Barrett and Salveson, 1986). TAME (tosyl arginine methyl ester) is a competitive substrate for plasmin (Barrett and Salveson, 1986) and for trypsin-like enzymes (Ficq, 1975).

3.2 Methods

3.2.1 Expt 1. To determine whether melanocytes migrate into the epidermis using fibronectin and laminin as substrates.

In all the following experiments, pieces of chick skin were cultured under the conditions indicated below. Variations are described and explained in the results section.

Strips of skin were obtained from the dorsal surface of the trunks of chick embryos by microdissection. Six day BAXNHR chicks served as donors of pigmented skin tissue (tissue containing normal premelanocytes or melanocytes) unless otherwise specified, since dermis at this stage is known to contain many melanocytes and skin is very easy to microdissect. White/unpigmented skin tissue (tissue free of normally pigmenting melanocytes) was obtained from 6 day White Plymouth x Cornish Game chicks (referred to as white). Embryos were staged according to Hamburger and Hamilton (1951) and ages are given in days of in ovo development.

Dermis and epidermis were separated using either 1% trypsin (DIFCO 1:250) in chick saline for 1 hour at 4°C followed by 5 min incubation in 50% FCS in chick saline to inactivate trypsin, or 0,25% EDTA in chick saline or PBS, pH 7.5, for 20 mins at 37°C. Both methods cleanly separate dermis from epidermis, but the EDTA method has a shorter incubation time and was therefore preferred. In all experiments, the culture medium used was Dulbecco's modified Eagle's medium (DMEM) containing 100 units/ml penicillin and 100 µg/ml streptomycin (Pen-Strep) and foetal calf serum (State Vaccine Institute, Pretoria) at the concentrations indicated for each experiment. Medium was changed every 3 days. Skin explants were most commonly cultured on nitrocellulose filters from Millipore (0,22 µm pore size). Cultures were incubated at 37°C, 5% CO₂ and 50-80% humidity. The pentapeptides GRGDS (FNpep) and YIGSR (LNpep) were obtained from Sigma and dissolved in DMEM at final concentrations between 200-1000 µg/ml.

3.2.2 Expt 2. To investigate whether melanocytes use proteases for translocation into the epidermis.

Pigmented and unpigmented skin was obtained from 6 day embryos of the pigmented BAXNHR and white WPRxCG breeds respectively. Dermal and epidermal components were obtained using 1% cold trypsin. The serine protease inhibitors TAME (tosyl arginine methyl ester) and TLCK (tosyl lysine chloromethyl ketone) were obtained from Sigma and diluted in culture medium from a stock (in 0,1% DMSO) for use at a final concentration of 100 $\mu\text{g/ml}$. Explants were cultured in DMEM containing 10% foetal calf serum, penicillin and streptomycin (as in Expt 1). Controls were treated likewise but without protease inhibitors.

3.2.3 Analysis

After culture, all explants were fixed in Bouin's fixative for 3 hours, dehydrated and wax embedded. Those on filters were left *in situ* as the filters proved quite easy to section and provided orientation. Serial sections (5 μm) were cut at right angles to the filter using a Leitz rotary microtome, sections were lightly stained with Mayer's haematoxylin and eosin and examined for the presence of epidermal melanin. Every alternate section was examined and scored for the presence or absence of feather or inter-plumar epidermal melanin. Presence of melanin was graded as "heavy" if tissue was extensively melanised and "slight" if melanin was sparse. In these experiments, dead or degenerating grafts fell off their filters during processing and are therefore not represented.

3.3 Results

3.3.1 Do melanocytes migrate into the epidermis using fibronectin and laminin as substrates?

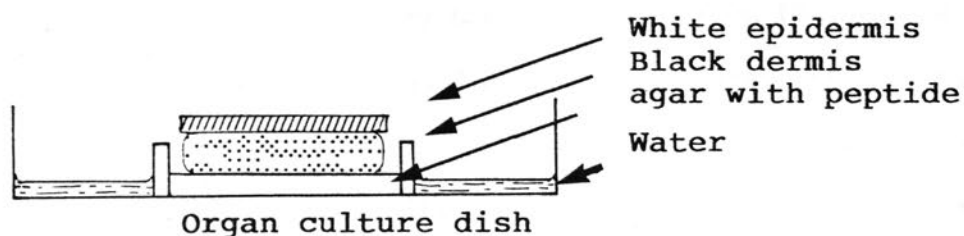
The first objective of these experiments was to devise culture conditions that would allow premelanocytes and melanocytes to continue to migrate along their normal routes into the epidermis and in this way to provide the migrating cells with the exact ECM and cellular components that they would normally encounter. Having achieved this, the second objective was to introduce into this culture system potential migration inhibitors and to analyze their effects on terminal melanocyte migration. Such an *in vitro* system would have the advantage of permitting precise modification of the culture environment and in particular, would make it possible to locally apply substances which might be extremely damaging to other developmental systems if the whole embryo were treated. In order to achieve these objectives, the following basic criteria had to be met: tissue pieces had to grow and develop normally, melanocytes had to survive and be able to move along their normal migratory routes, and culture volumes had to be minimised because the peptide fragments were prohibitively expensive. In addition, the epidermis had to be melanocyte-free at the start of the culture period so that migration or failure to migrate could be unambiguously demonstrated. In each of the following experiments, the attempts to fulfil these criteria will be discussed and the results presented.

Expt 1.1

Dorsal skin from 6 day BAXNHR and white chicks was split into dermis and epidermis using trypsin and the separated tissue pieces were incubated overnight at 37°C in 1 mg/ml GRGDS or YIGSR in chick saline (most studies use peptides in the range 0.1-1 mg/ml). White epidermis was then recombined with BAXNHR dermis and cultured for 72 hours on chick agar (Chapter 1) containing 200 µg/ml GRGDS (FNpép) or YIGSR (LNpép) as indicated below. White epidermis was combined with dermis from a pigmented source in order to ensure that no melanocytes were

present in the epidermis at the start of the culture period. As a control, tissue recombinants were cultured without peptides.

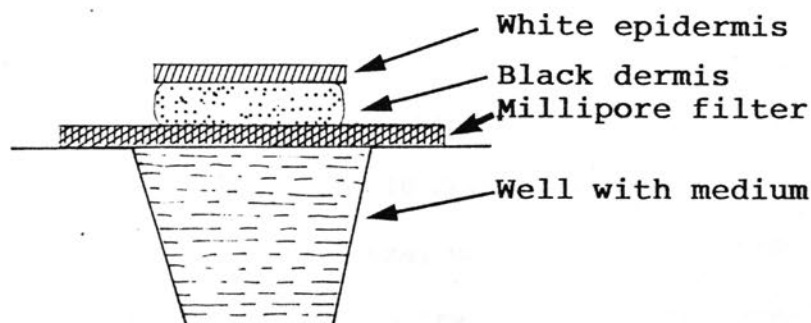
The above culture system did not prove to be satisfactory as 7 out of 18 explants were lost due to failure of the tissue components to reunite. The results from the surviving 11 explants are indicated in Expt 1.1 (Table 3.1). Furthermore the short culture period used (72 hrs) did not allow time for feather formation (only 2 out of 11 explants), with the resulting melanocyte proliferation and melanin deposition, to occur. Despite these problems the following results were obtained. GRGDS (FNpep) treatment resulted in 4 out of 5 explants with unmelanised or lightly melanised epidermis, while YIGSR (LNpep) treatment produced 4 out of 5 heavily melanised explants. The only surviving control explant was unmelanised, which makes any interpretation of results unreliable. However, YIGSR (LNpep) does not seem to have any effect on melanocyte migration at the concentration used (200 $\mu\text{g}/\text{ml}$), while GRGDS (FNpep) appears to be partially inhibiting migration. It would appear that the major drawback of the agar culture technique was that the tissue-medium interface is restricted to the basal surface of the explant, thus reducing access of the surface layers to nutrients and additives such as peptides. Renewal of the culture medium is impossible, short of removing the entire explant to a new agar plate.



Expt 1.2

In view of the above problems, it was felt that a liquid culture medium would allow a greater medium-tissue interface, facilitate diffusion of peptides and would also be easier to replenish. Tissue pieces were recombined as before and

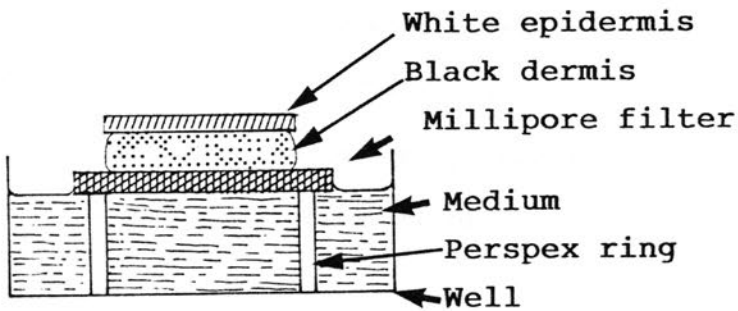
cultured directly in DMEM with 10% FCS, and with GRGDS included at an increased concentration of 400 $\mu\text{g}/\text{ml}$ for an extended culture period of 7 days. Medium was changed daily. Cultures rested on 13 mm diameter filters supported above 25 μl of medium in a 5 mm diameter micro-well (60-well culture plate, Falcon) as indicated below. These miniwells take only 20 μl of medium at a time, which conserves peptide but evaporates very rapidly. There were only two surviving explants (Table 3.1) from this experiment due to failure of recombination and dehydration. These were a control explant with heavily pigmented feathers and a GRGDS (FNpep) treated explant with unpigmented epidermis (Table 3.1). Obviously the sample number is too small but GRGDS again appears to be inhibiting melanocyte migration.



Expt 1.3

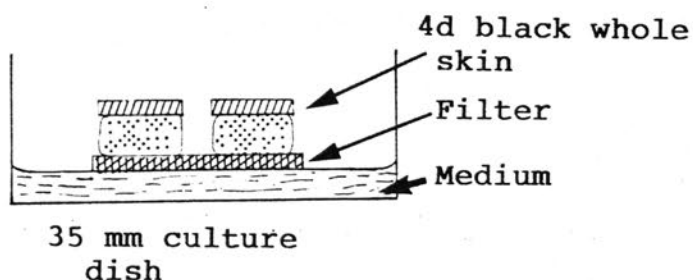
In the third experiment the culture technique was modified in the following way. Tissue recombinants were cultured directly in DMEM with 10% FCS, including either GRGDS at 500 $\mu\text{g}/\text{ml}$ or YIGSR at 300 $\mu\text{g}/\text{ml}$ (because end of stock) for 7 days. Pigmented dermis was taken from 5-day Loman Brown embryos (BAXNHR temporarily unavailable) and white epidermis from 7 day white embryos. Filters were supported by a perspex ring cut from a 5 mm syringe in the bottom of a 15 mm diameter well of a Falcon 24-well culture plate. Evaporation was slower in this larger well with better explant growth (2 explants developed feathers) but ideally the filter with the explants needs to be able to drop as the level of the medium falls. In this experiment, controls were more pigmented (2 out of 2 heavily pigmented), indicating that normal migration of melanocytes into the epidermis could take place. GRGDS treatment resulted in 1 unpigmented explant

epidermis and 1 heavily pigmented explant epidermis. The only 2 surviving YIGSR (LNpep) explants had pigmented epidermis, indicating either that the peptide concentration was still too low to inhibit migration effectively or that perhaps LN does not play a role in premelanocyte migration. However, 7 out of 15 explants were lost due to failure of recombination, which means that the recombination technique is not satisfactory.



Expt 1.4

In this experiment, whole (unsplit) skin pieces were cultured in DMEM containing 30% FCS and GRGDS at 500 $\mu\text{g}/\text{ml}$ for 10 days. Because of the repeated loss of explants due to recombination failure, unsplit 4½ day BAXNHR (red/black) skin was used on the assumption that the very few epidermal premelanocytes present at this stage (Hulley et al., 1991) would be unable to significantly pigment the epidermis and that the explant survival rate would increase. Not having to perform the time consuming splitting of skin allowed larger sample numbers to be readily obtained. The whole skin pieces from 4½ day BAXNHR embryos were cultured on 13 mm diameter filters floating in 1 ml medium in a 35 mm petri dish (Costar). Provided not more than 1ml of medium was used, the filters did not sink. They remained at the liquid-air interface- apparently optimal for skin development (Peterson and Grainger, 1985). This technique was subsequently used for all filter culture experiments.



With these improvements, GRGDS caused a significant reduction in pigmentation while controls were nearly 100% pigmented. Table 3.1 shows that 10 of 11 surviving skin explants treated with GRGDS had unpigmented epidermis. Of the control explants treated with medium alone, 23 out of 26 were heavily pigmented and the remaining 3 were slightly pigmented. This shows a substantial inhibition of normal epidermal melanisation caused by GRGDS-mediated interference with either premelanocyte migration or differentiation. Most explants contain dermal melanocytes (Fig 3.2c) even though the epidermis remained unpigmented, which argues against a GRGDS-based inhibition of differentiation.

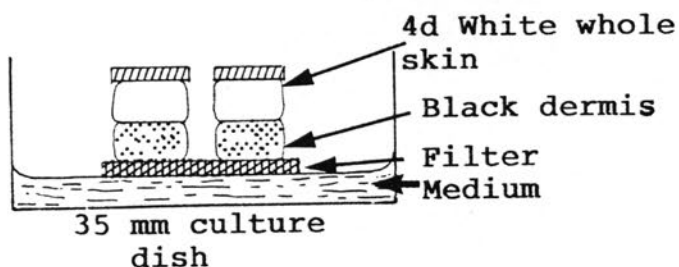
It should be noted that while 22 out of 26 control explants in this experiment formed feathers with normal morphology, none of the GRGDS treated explants did (Table 3.1). It therefore seems that GRGDS may be interfering with normal feather development. This disruption of normal developmental processes in the epidermis may affect melanocyte proliferation, and should be kept in mind when assessing the effect of GRGDS on melanocyte migration. Fig 3.2a and 3.2b show examples of feathers which have developed in control cultures. These compare favourably with similar feathers in CAM-grafted skin explants. Fig 3.2b is a longitudinally sectioned feather from a lightly melanised explant showing extensive keratinisation of the feather filament. Fig 3.2c-e are examples of GRGDS (FNpep) treated tissue, showing an example of a lightly melanised epidermis (3.2c), and 2 distorted "feathers" (3.2d and e). Note the accumulation of ECM in 3.2c which is typical of many of the GRGDS treated grafts. There is a malformed feather under the epithelium and at least two melanocytes are visible in the epidermis (having crossed over before or during culture) while several melanocytes remain in the dermis. The whorl-like epithelial structures in 3.2d represents a malformed feather with central cavities for the dermal pulp core and invading blood vessels. The epithelial cells in 3.2e are totally disorganised but appear to be producing keratin, and as in 3.2d, they are typically

unpigmented.

Treatment of 4 day whole skin with 500 $\mu\text{g/ml}$ YIGSR (LNpep) appeared to have no effect on either epidermal pigmentation or feather formation. Heavily pigmented feathers formed in 13 out of 15 explants and were morphologically identical to control feathers (result not shown).

Expt 1.5

In this experiment, an unsplit 6 day white skin was placed on top of a 6 day pigmented dermis, making a triple-layered explant. This was intended as the ultimate test to determine whether premelanocyte migration can be blocked by GRGDS (FNpep). Melanocytes from the red/black dermis would have to migrate through the additional white dermis to reach the epidermis, in the presence of GRGDS (FNpep). Previous CAM-grafted triple layer cultures (Chpt.2) had exhibited 100% pigmented feather formation, indicating that a double dermis does not block feather melanogenesis. Tissue recombinants were cultured in DMEM with 15% FCS and 500 $\mu\text{g/ml}$ GRGDS for 14 days. As in Expt 1.1, explant survival and pigmentation was good, 5 out of 7 controls producing heavily pigmented feathers. There were no heavily melanised GRGDS treated explants and 3 out of 7 produced unmelanised feathers. However, 4 of the 7 explants were lightly pigmented, indicating that GRGDS did not totally block melanocyte migration in the three-layered explants.



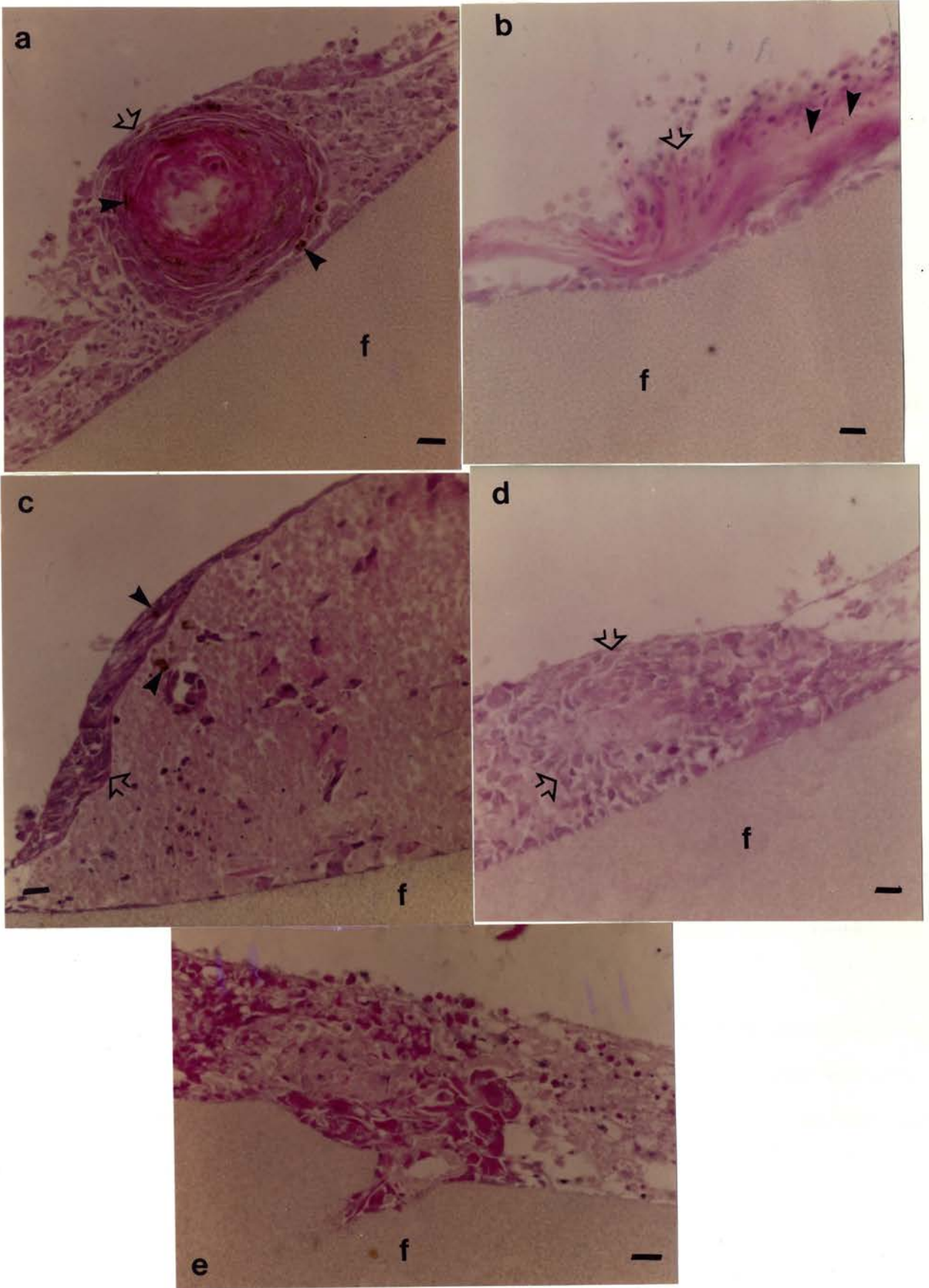


Fig 3.2

(a) Heavily melanised feather (control) T.S. (b) Lightly melanised feather (control) L.S. (c) Lightly melanised epithelium (500 µg/ml GRGDS). (d) Unmelanised distorted "feather" (500 µg/ml GRGDS). (e) As above (500 µg/ml GRGDS). Arrows, melanin; open arrows, epidermis; f, filter. Bar=20µm.

Summary of Expt 1

Although experimental conditions and explant survival were not optimal in all runs of Expt 1, the results show a consistent trend. Of a total of 37 control explants, 31 were heavily melanised. This indicates that the experimental conditions permit normal skin survival and pigmentation. Feathers formed in 28 of the 31 pigmented control explants and also in 2 of the unpigmented controls. Therefore experimental conditions also permit normal development to continue after the skin is removed from the embryo. A highly significant trend is also apparent in explants cultured in the presence of GRGDS (FNpep). Of 26 surviving explants, 24 contained little or no epidermal melanin and 8 of these explants formed feathers. Therefore it seems that terminal melanocyte migration can be inhibited when a synthetic peptide competes for cell surface receptors with the cell-binding sites on endogenous FN molecules present in the dermis.

In contrast, the YIGSR cell binding site sequence of LN has no effect on melanocyte migration at the concentrations used. Of 22 surviving explants, 19 were heavily pigmented and 2 lightly pigmented. Even at the 500 $\mu\text{g/ml}$ concentration found to most effective for GRGDS (FNpep), YIGSR (LNpep) did not block melanocyte migration into the epidermis. Feathers formed in 15 out of 22 explants, indicating that YIGSR did not interfere with feather development.

3.3.2 Expt 2 -Do melanocytes use proteases while migrating into the epidermis ?

The organ culture techniques devised in Expt 1 were also used to test whether serine proteases are involved in the terminal migration of melanocytes through the dermis and into the epidermis.

Expt 2.1

This first experiment was carried out at the same time as experiment 1.1, and proved to have the same inadequacies. Pigmented and unpigmented skin was obtained from 6 day embryos of the pigmented BAXNHR and white breeds

respectively. Dermal and epidermal components were obtained using 1% cold trypsin. Pigmented dermis and unpigmented epidermis were cultured for 2 hrs in DMEM containing 100 $\mu\text{g/ml}$ TAME or TLCK and then combined on 1 ml Falcon organ culture dishes (containing agar and protease inhibitors- 100 $\mu\text{g/ml}$) as shown for Expt 1.1 and cultured for 24, 48 or 72 hours. Controls were treated likewise but without protease inhibitors. Many grafts were lost due to tissue pieces failing to recombine and controls were poorly pigmented (1 out of 3 controls lightly pigmented) owing to the brief culture period (24-72 hrs) and restricted medium-tissue interface. However, 6 out of 6 TLCK-treated explants were unpigmented, compared with 3 out of 6 TAME treated explants (Table 3.2, Expt 2.1), which perhaps indicates that TLCK is a more effective blocker of melanocyte migration than TAME.

Expt 2.2

Whole 4½ day skin from BAXNHR embryos was explanted onto a 23 mm, 0.45 μm pore size Millipore filter, floating in 1 ml of DMEM with 10% FCS and antibiotic in a 25 mm tissue culture dish (Costar). Controls contained no protease inhibitor. TAME and TLCK were used at 200 $\mu\text{g/ml}$. Medium was changed every two days and culture was terminated after 7 days. All surviving controls produced well pigmented feathers (3 out of 3), as did cultures treated with TAME (6 out of 6). This indicates that conditions for tissue survival and feather morphogenesis were favourable. At 200 $\mu\text{g/ml}$, TLCK again produced largely unpigmented explants (5 out of 6. See Table 3.2), this time with evidence of tissue degeneration in some cultures. However one heavily pigmented feather formed, indicating that conditions were not totally effective in blocking melanocyte migration. It should be remembered that 4½ day whole skin, which does contain a few epidermal premelanocytes, was used in this experiment.

Expt 2.3

This experiment was performed under exactly the same conditions as 2.2 except that TLCK and TAME were used at 300 µg/ml to determine whether melanocyte migration could be more effectively inhibited at higher concentrations. All control explants produced pigmented feathers as before (6 out of 6). At this concentration, TLCK was clearly cytotoxic with visibly necrotic explants falling off their filters during processing. TLCK can cause blockage of protein synthesis and this is probably the cause of tissue death (Ficq, 1975). TAME still failed to inhibit migration, as 7 out of 7 explants developed heavily pigmented feathers. Since TAME functions as a competitive substrate for trypsin-like enzymes (Ficq, 1975), it is possibly still being used at a less than effective concentration.

Summary of Expt 2

As in Expt 1, the filter culture technique provided better conditions for explant survival and growth than agar cultures. When results were totalled, 10 out of 12 control explants were pigmented. In contrast, only 1 out of 12 explants treated with TLCK was pigmented. These results indicate that TLCK may well be interfering with serine protease action and the subsequent migration of melanocytes into the epidermis (Fig 3.3). Normal epidermal development into feathers is not inhibited at the lower concentrations of TLCK used while melanisation is far less extensive than in equivalent control and TAME-treated explants. TAME does not seem to be inhibiting the enzyme/s involved in terminal melanocyte migration. Control and TAME-treated explants produced epidermis and feathers with similar morphology to those in Expt 1 (Fig 3.2a). However the GRGDS-induced accumulation of ECM (Fig 3.2c) was not seen with either TLCK or TAME.

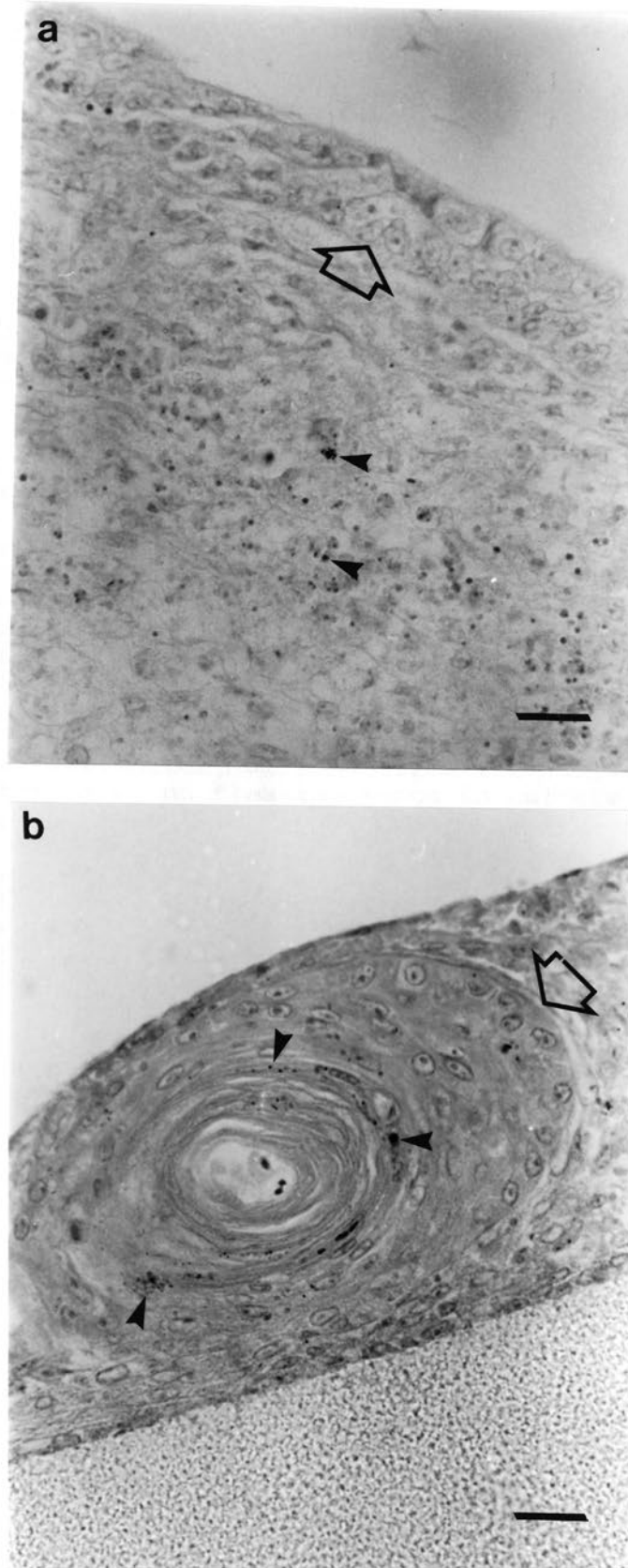


Fig 3.3
Skin explants cultured in (a) 200 $\mu\text{g/ml}$ TLCK, (b) 200 $\mu\text{g/ml}$ TAME. Arrows, melanin; open arrows, epidermis. Note the absence of epidermal melanin in (a). Bar=20 μm .

Table 3.1

EFFECT OF GRGDS AND YIGSR ON MELANOCYTE MIGRATION IN CHICK SKIN

Expt no.	Type of explant*	Treatment	Conc. $\mu\text{g/ml}$	Numbers of explants			
				Total	No melanin	Slight melanin	Heavy melanin
1	split	Control		1	1	0	0
		GRGDS	200	5	1	3	1
		YIGSR	200	5	0	1	4 (2)
2	split	Control		1	0	0	1 (1)
		GRGDS	400	1	1	0	0
3	split	Control		2	0	0	2
		GRGDS	500	2	1 (1)	0	1 (1)
		YIGSR	300	2	0	0	2
4	whole	Control		26	0	3	23(22)
		GRGDS	500	11	10	1	0
		YIGSR	500	15	1	1	13(13)
5	triple	Control		7	2 (2)	0	5 (5)
		GRGDS	500	7	3 (3)	4 (4)	0
Total		Control		37	3 (2)	3	31(28)
		GRGDS		26	16(4)	8 (4)	2 (1)
		YIGSR		22	1	2	19(15)

Pieces of skin (whole or recombined) were cultured for varying periods (indicated in the text) on floating rafts in culture media containing the pentapeptide sequences GRGDS (FNpep) or YIGSR (LNpep) or no peptide. Explants were examined histologically for the presence of epidermal melanin and feathers. Numbers represent total explants scored for the presence or absence of epidermal melanin. Figures in brackets represent those with feathers.

* variations are detailed in Results.

Explant survival : 1=61%, 2=33%, 3=67%, 4=100%, 5=88%.

Table 3.2

EFFECT OF SERINE PROTEASE INHIBITORS ON MELANOCYTE MIGRATION IN CHICK SKIN

Expt no.	Type of explant*	Treatment	Conc $\mu\text{g/ml}$	Number of explants			
				Total	No melanin	Slight melanin	Heavy melanin
1	split	Control		3	2	1	0
		TAME	100	6	3	3	0
		TLCK	100	6	6	0	0
2	whole	Control		3	0	0	3 (3)
		TAME	200	6	0	0	6 (6)
		TLCK	200	6	3	2	1 (1)
3	whole	Control		6	0	2 (2)	4 (4)
		TAME	300	7	0	0	7 (7)
		TLCK	300	0	0	0	0
Total		Control		12	2	3 (2)	7 (7)
		TAME		19	3	3	13 (13)
		TLCK		12	9	2	1 (1)

Skin pieces (whole or recombined) were cultured for varying periods (indicated in the text) on agar or on floating rafts in culture media containing the serine protease inhibitors, TAME and TLCK or no inhibitors. Explants were examined histologically for the presence of epidermal melanin and feathers. Numbers represent total explants scored for the presence or absence of epidermal melanin. Figures in brackets represents those with feathers. Zero (0) indicates no surviving explants.

*variations are detailed in Results.

Explant survival: 1=83%, 2=76%, 3=62% (TLCK =100% mortality).

3.4 DISCUSSION

3.4.1 Use of FN and LN as migration substrates by melanocytes migrating into the epidermis

After leaving the neural crest, the melanocytic component of the neural crest migrates dorso-laterally beneath the epidermis and above the somites and so spreads out around the body. It is not known how far laterally this movement reaches before the secondary migration of melanocytes towards and into the epidermis begins (Weston, 1963; Erickson, 1988). It has been shown that premelanocytes first start crossing from dermis to epidermis at 4 days and that this movement is largely completed by day 8 (Hulley et al., 1991). This chapter examines the question of how these cells physically negotiate their way through the dermal mesenchyme and into the epidermis. Do they follow pathways composed of specific ECM components, and do they use protease enzymes to move through the ECM and particularly to penetrate the basement membrane of the epidermis? These questions surrounding the terminal migration of melanocytes have not yet been addressed and there are several problems to be overcome when attempting to answer them as outlined below.

In vivo, migrating cells move through a three dimensional latticework of ECM fibrils and a complex ground substance composed of proteoglycans with differing densities and hydrophilic properties. However, many *in vitro* migration studies have simply involved the placing of cells on a planar (2-D) substrate in a culture dish and extrapolation of the results to the *in vivo* situation. A second and more satisfactory approach has involved the use of 3-D gels composed of collagen or BM-matrix. This at least permits cells to assume a more natural migratory morphology, that is, more rounded than flat, but the choice of migratory substrates is still limited to the primary gel components. The cells usually continue migrating (they will even migrate on tissue culture plastic and even plain glass), but they are undoubtedly lacking many normal cues and stimuli. For example, NCCs will migrate on LN substrates but will migrate further and

faster if nidogen and collagen IV are added to the substrate (Perris et al., 1989). Presumably this is because optimal migration conditions are provided by complex substrates.

Thus when interpreting results of migration studies it is important to note the different effects that planar and 3-D culture conditions have on NCC migration. NCC migration on planar FN substrates was inhibited by treatment with GRGDS (FNpep) (Boucat et al, 1984; Perris et al., 1989) but the peptide had no effect on NCC migration in 3-dimensional gels containing FN (Bilozur and Hay, 1988). In contrast, YIGSR (LNpep) failed to perturb NCC migration on planar LN substrates (Perris et al., 1989) but totally inhibited migration in 3D basement membrane matrix gels containing LN (Bilozur and Hay, 1988). Furthermore, when serine protease inhibitors were incorporated into collagen gels, they reduced NCC migration, but the same inhibitors had no effect on migration on tissue culture plastic (Erickson and Isseroff, 1989).

A possible explanation for all of these discrepancies is that NCCs are able to migrate, although perhaps not optimally, on most substrates without necessarily using specific cell-binding sites (hence migration on plastic substrates and on LN substrates when the YIGSR site is clearly not involved). LN normally has a cruciform structure (Fig 3.1a) with the YIGSR cell-binding site located on a short arm and close to the centre of the molecule. If the molecule was folded up during substrate preparation, the cell-binding site could become physically unavailable to the NCCs. Future experiments may show that this is less likely to happen in a 3-D gel. FN is a linear molecule, subject to polymerisation, and this is perhaps why its cell-binding sites remain available when it is used as a planar substrate. It has also been shown that FN bound to collagens I,II or IV is a less effective NCC migration substrate than FN alone (Perris et al., 1991), that is, association of FN with collagen may cause a conformational change in the FN molecule which reduces its ability to support cell migration (as in Bilozur

and Hay, 1988). Furthermore, both FN and LN enhance NCC migration in collagen gels but only if they are added to the collagen before it polymerises (Perris et al., 1991). This implies that a specific supramolecular organisation of these molecules determines their ability to support NCC migration.

Probably the ideal approach to studies of cell migration is to attempt to disrupt *in vivo* migration by injecting developing embryos with antibodies or peptides capable of blocking the cell binding regions of substrate molecules. However, these molecules do not spread far from the injection site, are fairly rapidly cleared from the system by normal metabolic processes and cannot be exclusively targeted to one group of cells migrating at a specific stage in a particular region (Boucat et al., 1984; Bronner-Fraser, 1986b). Therefore other developing systems are liable to be disrupted with unpredictable consequences. Protease enzymes, for example, are used throughout the developing embryo for a multitude of differing purposes, so injection of protease inhibitors could have lethal consequences. For instance, even *in vitro*, phenanthroline (a potent inhibitor of collagenase IV) caused substantial cell death as well as inhibition of migration (Erickson and Isseroff, 1989). This is probably why no *in vivo* microinjection studies using protease inhibitors have been published.

The experimental design presented in this chapter is an attempt to overcome the above problems. It closely approximates the *in vivo* situation of the migrating melanocyte population and also serves to isolate them, complete with their immediate environment, from the rest of the developing embryo. By placing pieces of whole skin (dermis and epidermis) on filter rafts in culture medium at the start of the culture period, the melanocytes are surrounded by all the dermal components that they would normally encounter while migrating towards and into the epidermis. They can also be immersed in medium containing cell-binding site peptides or protease inhibitors, and because the skin is isolated, only the developmental processes which normally occur in skin are subject to possible

disruption. Obviously the culture medium only approximates the extracellular fluids normally found in the skin, but the normal dermal and epidermal cells are still present and are therefore able to modify this medium by secreting growth factors or other substances into the migrating melanocyte pathways. A measure of the success of this culture system is the formation of feathers with normal morphology and pigmentation (Fig 3.2a).

If FN is the key substrate used by melanocytes en route to the epidermis, treatment with a competitive dose of GRGDS (FN_{pep}) should substantially interfere with normal epidermal pigmentation. As shown in Table 3.1, 500 $\mu\text{g/ml}$ GRGDS causes a complete block of epidermal pigmentation in 10 of 11 explants, compared with 8 out of 8 heavily pigmented controls. There are three possible explanations for this result. Firstly, the GRGDS peptide may be interfering with melanocyte differentiation. This is unlikely because these explants contain many dermal melanocytes (Fig 3.2c), indicating that melanocyte differentiation has occurred. Secondly, since GRGDS at 500 $\mu\text{g/ml}$ prevents normal feather formation (Fig 3.2c, d and e), it could be interfering indirectly with associated melanocyte proliferation. However, epidermal melanocytes in the BAXNHR breed become visibly pigmented from day 5, which is 3 days earlier than the start of feather elevation at 8 days (Hulley et al., 1991), and therefore pigmentation is not entirely dependent on feather formation. Pigmented epidermis is thus the first indicator of successful melanocyte migration from the dermis into the epidermis and this is blocked in the majority of GRGDS-treated explants (Table 3.1, Expt 1.4). A third explanation (which seems the most likely at this stage), is that the pentapeptide successfully competed with endogenous FN for the cell-binding sites on the migrating premelanocytes, thus preventing them from completing their terminal migration. This suggests that FN is indeed a major substrate involved in melanocyte migration through the dermis and into the epidermis and adds to the growing body of evidence supporting FN as a primary NCC migration substrate in avian embryos.

Rather surprisingly, 4 of the 7 surviving GRGDS treated 3-layered cultures (Expt 1.5), in which whole white skin was cultured on top of BAXNHR dermis, were slightly melanised. This means that melanocytes from the BAXNHR dermis were able to migrate through a second thickness of dermis in the presence of 500 $\mu\text{g/ml}$ GRGDS to reach the epidermis. Previously this concentration of peptide was shown to be sufficient to block migration in whole BAXNHR skin (Expt 1.4). The triple-layer combination contains nearly twice as much tissue (and hence FN) as the double-layered whole skin, so perhaps the GRGDS concentration was effectively less due to the extra tissue involved, that is, the ratio of peptide to endogenous FN was too low to effectively compete for the melanocyte receptors. A few melanocytes are also visible in the epidermis of 1 of the 11 surviving explants in Expt 1.4 (Fig 3.2c). This could mean that GRGDS was not totally effective in inhibiting melanocyte migration into the epidermis or simply that at the start of culture, there were already a few epidermal melanocytes. It should be remembered that although no melanocytes are visible at 4 days in chick skin, a few premelanocytes have crossed into the epidermis (Hulley et al, 1991), and these would therefore not be affected by GRGDS. For practical reasons older embryos are far easier to skin than 3 day chicks which do not yet have epidermal premelanocytes.

The next question addressed was whether LN was used as a substrate by melanocytes migrating into the epidermis. All 7 of the surviving skin explants treated with YIGSR (LNpep) at 200 or 300 $\mu\text{g/ml}$ produced pigmented feathers or epidermis. When the peptide was used at 500 $\mu\text{g/ml}$, 13 out of 15 explants produced normally pigmented feathers. Therefore migrating melanocytes do not seem to need to bind to LN in order to enter the epidermis. In a recent paper, Loeber and Runyan (1990) successfully blocked mesenchymal cell migration in 3-D collagen gels containing FN or LN using 300-1000 $\mu\text{g/ml}$ GRGDS or YIGSR respectively. It may be argued that YIGSR was not used at a sufficiently high concentration to

successfully compete against endogenous YIGSR sequences in this study. However, even at the lowest concentrations used, GRGDS consistently produced explants with less epidermal melanin than controls or YIGSR treated explants. This may indicate that LN is less involved in trunk NCC migration than FN and, in fact, it is well documented that cranial NCCs use LN in preference to FN as a migration substrate *in vivo* (Runyan et al., 1986; Bronner-Fraser and Lallier, 1988).

3.4.2 How do NCCs migrate on FN and/or LN?

Avian NCC migration on FN and LN seems to occur through complementary mechanisms involving recognition of multiple motility promoting sites (Perris et al., 1989). The HNK-1 antibody recognises a carbohydrate moiety found in cell adhesion molecules (CAMs) and on the surface of early migrating crest cells. *In vitro*, HNK-1 interferes with NCC binding to LN but not FN, and it interferes with cranial NCC migration *in vivo* (Bronner-Fraser, 1987). Again this implies a greater use of BM components (possibly LN) in cranial NCC migration than trunk. The CSAT antibody raised against the integrin receptor for both FN and LN interferes partially with trunk NCC emigration (Bronner-Fraser, 1986b). Interestingly, when both CSAT and HNK-1 are microinjected into the neural tube, a far greater degree of disrupted migration is seen than with either molecule alone. This additive effect implies that they act on different sites (Bronner-Fraser, 1987). Chick NCC-derived neurite outgrowth and adhesiveness are also affected by HNK-1 treatment. On LN both outgrowth and spreading are discouraged while on FN only outgrowth is affected (Sanders and Cheung, 1988). Available evidence suggests that there are age or differentiation related changes in NCC surface receptors that go hand in hand with the orchestrated matrix modulation proceeding around the migrating sub-populations. When cultured chick dorsal root ganglion cells are reinjected into host embryos following treatment with the phorbol ester, TPA, there is a marked increase in cell invasiveness compared with untreated cultured cells (Sears and Ciment, 1988). TPA seems to be

reversing a developmental restriction in responsiveness to ECM cues in the environment. Prior to leaving the neural tube, NCCs express NCAM and N-cadherin, both of which are rapidly lost once migration commences. The migrating cells express high levels of substrate adhesion molecules (SAMs) such as FN receptors (Tucker et al., 1988) but when migration stops and reaggregation such as ganglion formation occurs, CAMs are re-expressed. TPA may be inducing a re-expression of SAMs in the immobile dorsal root ganglion cells or even inducing *de novo* expression of a matrix receptor normally expressed in other sub-populations of crest cells. However, the epidermal melanocytes are one NCC sub-population which do not reaggregate upon terminating migration but lie in a dispersed fashion amongst the basal epidermal cells. Obviously they cannot be re-expressing NCAM in the same way as the neural derivatives. Therefore before the mechanisms controlling NCC migration can be fully elucidated we need (1) a more detailed understanding of the receptor expression pattern on developing NCCs and (2) information on the molecular biology of the various ECM components encountered.

3.4.3 Other migration substrates

Although most of the NCC migration studies carried out to date centre around the roles of FN and LN, it is becoming increasingly clear that other matrix molecules are also involved. Ignoring these will produce an overly simplistic picture of NCC migration. Therefore the potential roles of the proteoglycans, chondroitin sulphate and hyaluronic acid, the glycoprotein, tenascin and the fibrillar protein, collagen are briefly considered.

Chondroitin sulphate-Chondroitin sulphate (ChSO_4) is one proteoglycan which appears to play a negative role in NCC migration. In culture, addition of ChSO_4 to any substrate causes inhibition of NCC migration (Newgreen et al., 1982; Erickson and Turley, 1983; Tucker and Erickson, 1984). Mutant white axolotls have significantly more ChSO_4 in the sub-epidermal region than black embryos and their premelanocytes fail to migrate away from the mid-line (Lofberg, 1989;

Perris and Johansson, 1990). In the chick, NCCs are never found in the caudal halves of the somites or around the notochord and both of these regions are rich in ChS04 (Oakley and Tosney, 1991; Perris et al., 1991). However in the mouse, the ECM surrounding migrating melanoblasts and sympathoblasts is rich in ChS04 produced by the surrounding cells (Derby, 1978). This seems to contradict the role of ChS04 as a migration blocker in other vertebrates. A recent paper indicates that ChS04 is the molecule responsible for preventing sensory neurites from the dorsal root ganglion from approaching the epidermis (Fichard et al., 1991). It clearly does not block melanocyte invasion, so perhaps different sub-populations of NCCs respond differently to the same ECM molecule as they differentiate.

Hyaluronic acid-Hyaluronic acid (HA) is found in most of the spaces into which NCCs migrate (Derby, 1978). It is an enormous, hydrophilic glycoprotein which remains linked to the surface of the cell throughout secretion, acting as a precisely local space filler (Erickson, 1988). When added to collagen gels *in vitro*, HA enhances the speed of NCC migration and increases the spaces between collagen fibres (Tucker and Erickson, 1984). Its involvement has been demonstrated in two key phases of neural crest migration, the first being an accumulation between the neural tube and the overlying ectoderm in mouse embryos (Derby, 1978), which appears to create space for the emerging NCCs to migrate through. Secondly, in the developing chick cornea, hyaluronan is secreted between the external epithelium and the endothelium, creating a space which is then invaded by NC-derived mesenchyme (Toole and Trelstad, 1971). Therefore the role of hyaluronic acid in NCC migration increasingly appears to be a passive one.

Tenascin-It is currently not clear whether tenascin is involved with NCC migration at all. It has been shown to serve as an *in vitro* substrate for NCC migration in amphibians (Epperlein et al., 1988) and avians (Halfter et al.,

1989), and its distribution correlates temporally and spatially with NCC migration in amphibians (Epperlein et al., 1988, Mackie et al., 1988). Thus far in the chick, TN has been correlated with NCC migration into the cornea (Kaplony et al., 1991) and the rostral half of the somites (Stern et al., 1986; Tan et al., 1987; Halfter et al., 1989; Mackie et al., 1988). In the cornea, it appears in the primary stroma immediately prior to invasion by a wave of NCCs destined to form the corneal endothelium, and remains at high levels until completion of the subsequent invasion of NCC derived fibroblasts which secrete collagen and form the secondary stroma (Kaplony et al., 1991). However Norris et al., (1989) have shown that tenascin only localises in the rostral somite halves after migration has commenced so it is unlikely to be causing invasion.

Collagen-No clear role for the collagens in NCC migration has yet emerged. In *vitro*, avian NCCs migrate on both planar collagen substrates (Erickson and Tucker, 1986; Sanders et al., 1988) and in 3-dimensional collagen gels (Tucker and Erickson, 1984; Bilozur and Hay, 1988). Types I and IV are the most effective *in vitro* substrates while types II and V are unable to support motility at all (Perris et al., 1991). In the developing chick, collagens II, V and IX are preferentially localised in regions where NCCs are absent such as the perinotochordal space (Perris et al., 1991) which, with the *in vitro* data, suggests a negative role in NCC migration. An apparently continuous sub-epithelial layer of collagen types I, II and IV is present throughout the trunk from stage 16 (Fitch et al., 1989). This places it firmly in the way of the migrating pre-melanocyte population of NCCs. In fact, type IV is lost from the neural tube BM at the time of neural crest emergence and although it is seen in all BMs lining NCC pathways, it is not found in large amounts amongst the migrating cells, whereas collagen I is abundant along migratory pathways. When sensory and sympathetic neural crest cells aggregate to form ganglia, collagen I disappears from amongst the NCCs and collagen IV reappears (Duband and Thiery, 1987). This could mean that collagen I supports migration while collagen IV

inhibits it, but the correlation is probably co-incidental, particularly as collagen IV is a good *in vitro* migration substrate. An *in vivo* role for collagen has yet to be directly tested.

3.4.4 How do melanocytes cross the epidermal basement membrane?

The first BM encountered by migrating NCCs is that of the neural tube and they must emerge through this in order to commence migration. It was initially suggested that gaps appear in the BM prior to emigration (Tosney, 1982) but later shown that in fact the BM does not become intact until after migration (Martins-Green and Erickson, 1987), and that type IV collagen and laminin disappear from the forming BM just before migration starts (Duband and Thiery, 1987). An overabundance of these molecules seems to arrest migration (Payette et al., 1988), so their removal would facilitate passage through the BM. It has been suggested that the reason laminin and collagen IV disappear is that they are degraded by proteases secreted by the emerging crest cells (Martins-Green and Erickson, 1987). NCCs may therefore be playing an active role in disrupting the BM, but whatever the case, the first BM encountered by NCCs is not yet intact and is easily crossed.

The second BM encountered by migrating melanocytes is the epidermal BM. The integrity of this BM has not been studied at TEM level using immunocytochemical techniques to demonstrate the arrangement of different BM molecules, but numerous ICC studies at the level of light microscopy (Sanders, 1979; Mauger et al., 1982; Duband and Thiery, 1987; O'Shea, 1987; Payette et al., 1988; Fitch et al., 1989; Bortier et al., 1989) and preliminary, unpublished TEM investigations of BM morphology (this laboratory) give no indication of breaks in the epidermal BM over the period of melanocyte invasion. How are the melanocytes crossing? In general, invasive cells use two classes of endoproteinases to breach BMs- the serine and metalloproteinases (Reviewed by Pittman, 1990). Of these enzymes, the plasminogen activator/plasmin family and collagenase IV (serine and metallo

proteinase respectively) together are capable of degrading FN, LN, the collagens and the proteoglycans (Alexander and Werb, 1989), many of which are BM components.

NCCs are known to produce the serine protease, plasminogen activator during migration *in vitro* (cranial- Valinsky and Le Douarin, 1985; trunk- Erickson and Isserhof, 1986). Plasminogen activator in turn produces plasmin, a broad spectrum protease capable of cleaving most ECM components except collagen IV. The metalloproteinase collagenase IV is necessary for this but collagenase has to be activated by the plasminogen activator/plasmin system before it can act on BMs (Mignatti et al., 1986; Reich et al., 1988; Panayotou et al., 1989). This suggests that the enzymatic cascade essential for cell passage through a BM can be halted by blocking PA or plasmin alone (Mignatti et al., 1986; Alexander and Werb, 1989). However, as far as is known, no attempts to block NCC migration *in vivo* using protease antagonists have been made, undoubtedly due to the lethal consequences such treatment would have on other developing systems. The tissue culture system devised for Expt 1 is ideal for testing the effects of protease inhibitors on terminal migration of melanocytes because it allows continued development of the skin in isolation from vulnerable systems in the rest of the body.

Of the two serine protease inhibitors tested in this culture system, only TLCK seemed to significantly reduce melanocyte migration into the epidermis. TLCK is a known inhibitor of plasmin (Barrett and Salveson, 1986), but it is possible that other enzymes such as trypsin and thrombin are also involved in BM breakdown. This could explain why TLCK is not totally effective as a migration blocker. Furthermore, blocking the action of plasmin may not entirely block the action of collagenase IV. It is known that laminin domain I stimulates synthesis of metalloproteinases by melanoma cells, which in turn are capable of cleaving collagen IV in the BM (Turpeeniemi-Hujanen et al., 1986; Panayotou et al., 1989).

At the time when premelanocytes are lying directly beneath the epidermis prior to crossing over, they may be in contact with the exposed laminin core of the developing BM. It would therefore be of great interest to know whether laminin is also capable of stimulating collagenase activity in melanocytes. This, working in conjunction with the known secretion of plasminogen activator, would provide a very neat way of temporarily arming melanocytes with the necessary mechanism for crossing an intact BM. If the acquisition of this mechanism is strictly dependent on age and position in the embryo of the melanocytes, it would not be surprising if under different conditions (Erickson, 1987; Gehlsen and Hendrix, 1987), BMs pose an impenetrable barrier to cultured neural crest cell migration.

TAME, a competitive inhibitor of trypsin-like enzymes, had no effect on epidermal pigmentation. Either it was not used at a concentration high enough to elicit a response or it blocked enzymes not involved in terminal melanocyte migration. It would be of great interest to test the effects of other specific plasminogen activator inhibitors and blockers of the metalloproteinase, collagenase IV, using this whole skin culture technique. However, many of these inhibitors are cytotoxic except at very low levels (Erickson and Isseroff, 1989; personal communication, Boehringer Mannheim) and therefore antibodies directed against the active sites of specific enzymes might be more effective. The major drawback of this method is that antibodies are very large molecules and might not penetrate the tissue sufficiently.

In conclusion, this study indicates that both fibronectin and serine protease enzyme/s may be necessary for the terminal migration of trunk NCCs as melanocytes in chick skin and that laminin is not used as a substrate. The experiments have been carried out under conditions which closely approximate the *in vivo* situation, insofar as normal feathers develop during the period of culture. The

system developed here lends itself to further examination of the roles played by both substrate molecules and proteinase enzymes in the terminal migration of melanocytes, but further experiments are necessary before any definite conclusions can be drawn. These could include :

Controls using a peptide with point mutation or a reverse peptide eg. SDGRG

In the case of the peptide inhibitors, a control with DMSO alone.

A statistical assessment (based on a larger sample number than in the present pilot study) should be devised to verify the significance of differences.

YIGSR is a larger peptide than GRGDS, since tyrosine has a ring structure, and also carries a different charge and hydration shell. These factors could limit its penetration into the tissue.

The effects of the protease inhibitors are inconclusive. In theory, both should inhibit melanocyte migration, and even if they did, this could be due to indirect causes eg. a failure of leukocytes to clear up accumulating debris resulting from toxicity. As suggested in the discussion, a wider range of inhibitors should be tested, possibly including antisense strategy.

CHAPTER 4

The role of the epidermis in the homing of melanocytes

4.1 Introduction

4.1.1 Why do melanocytes migrate towards and into the epidermis?

The presumptive melanocyte population is amongst the last group of cells to leave the neural tube in the chick (Loring and Erickson, 1987; Serbedzija et al., 1989). As detailed in Chapter 1, the early migration of premelanocytes has been traced using grafts of tritium-labelled neural crest (Weston, 1963), grafts of quail neural crest in chick hosts (Teillet and Le Douarin, 1970), labelling of individual premigratory NCCs by microinjection with fluorescent DiI (Serbedzija et al., 1989) and more recently the position of premelanocytes prior to 4 days has been examined using a premelanocyte-specific antibody, MEBL-1 (Kitamura et al., 1992). These studies indicate that upon leaving the neural crest, dorsally migrating NCCs spread out laterally around the body in the sub-epidermal mesenchyme and some of these cells begin to appear in the epidermis from day 5. In chapter 2, it was demonstrated by means of heterochronic dermo-epidermal recombination grafts that unpigmented melanocytes start crossing into the epidermis as early as day 4 in mid-dorsal trunk skin. Therefore it is apparent that at some stage prior to day 4, premelanocytes begin to migrate towards and into the epidermis. It is not known how extensive the lateral spread around the body is before secondary migration towards the epidermis begins (Erickson, 1988). This chapter addresses the question of what could be causing this secondary migration towards the epidermis.

4.1.2 What are the forces governing NCC migration?

NCCs are inherently motile cells, migrating even when they are microinjected into ectopic sites in the embryo (Erickson, 1988) and it is likely that environmental factors play a major role in directing this motility. NCCs will only migrate on certain adhesive ECM components as reviewed in chapter 3. This preference could define specific pathways, cells staying preferentially on the more adhesive

matrix components and avoiding areas containing less adhesive ECM (as demonstrated by the *in vitro* experiments of Hammarbach et al., 1988). Erickson and Tucker (1986) state that there is no indication of positive or negative haptotactic (adhesive) gradients affecting the early migration of crest cells away from the neural tube. However, studies carried out by Duband and Thiery (1987) suggest that negative adhesion gradients may act to halt the migration of NCCs, so permitting ganglion formation. A local accumulation of very adhesive ECM components such as type IV collagen and LN, accompanied by a loss of motility promoting collagen I and FN leads to an increasing environmental resistance to migration, with subsequent formation of the dorsal root ganglia. Further examples of negative haptotaxis are the avoidance by NCCs of the notochord (Oakley and Tosney, 1991) and the caudal halves of the somites (Stern et al., 1986). In both cases, NCCs are prevented from approaching the tissue regions by an accumulation of non-adhesive ECM components such as chondroitin sulphate proteoglycans (Tan et al., 1987; Perris et al., 1991).

However, there are indications that different sub-groups of NCCs interpret matrix signals in different ways. Very recently, chondroitin sulphate has been implicated in preventing NCC-derived dorsal root ganglion (DRG) nerve fibres from approaching the epidermis too closely (Fichard et al., 1991). This is interesting because it means that one NC cell type, the melanocyte, can ignore a matrix signal which halts migration in another type, the DRG fibre. A second example is the role played by collagen IV and LN. These seem to act as a stop signal for migrating DRG cells (Duband and Thiery, 1987). Basement membranes (containing both collagen IV and LN) seem to deflect some migrating NCCs. Cranial NCC migration is arrested for 6-8 hours at the apposed BMs of the surface ectoderm and neural tube at the prosencephalic and median rhombencephalic levels, but resumes as soon as the gap between them widens with age (Duband and Thiery, 1982). However, when melanocytes encounter the epidermal BM, they are neither halted nor deflected but instead penetrate the BM and cease migration only once

they are located amongst the epidermal keratinocytes. The BM of the epidermis appears to be intact during the 48 hr to 8 day period during which premelanocyte migration into the epidermis is taking place (Duband and Thiery, 1982; our unpublished observations). This raises the question of how neural crest cells maintain their directionality of movement in the face of BM components which are interpreted elsewhere as a stop signal, namely collagen IV and LN.

The most accepted mechanism governing the directional migration of NCC away from the neural tube is contact guidance (Tucker et al., 1988; Halfter et al., 1989). Essentially, cells stop advancing when they contact one another and send out processes in other directions in search of cell free space. In a closely packed group of cells such as the early migrating crest, this should produce radial outward migration away from the area of highest cell density. This is apparent in the migrating neural crest near the neural tube *in vivo* and contact inhibition of migration has also been seen in NCC cultures (Erickson, 1985). In the chick, NCCs disperse as compact masses of cells down through the somites and between somites and neural tube, unlike the rat where NCCs move in diffuse streams through the mesenchyme (Tan and Morriss-Kay, 1986). However by the time melanocytes are seen in the early dermis, they are widely dispersed (Hulley et al., 1991). This situation seems similar to that seen in *Xenopus* (Krotoski et al., 1988) and in axolotls where pigment cells migrate from dorsal to ventral in a highly directional manner in the absence of cell contact and of directional alignment of ECM fibres (Keller and Spieth, 1984). Obviously under these conditions, contact guidance is not playing a role in maintaining directionality of migration. Therefore while contact guidance may influence early neural crest migration, it is unlikely to have any effect on the terminal migration of melanocytes.

Evidence suggests that cells will move along NCC pathways as long as they fulfill certain basic surface criteria. In fact, when latex beads of NCC size are injected onto the ventral pathway by way of the somite, they translocate either with or without NCCs and end up in much the same places (Bronner-Fraser, 1986c). This indicates that the guidance system behind ventral NCC migration is partially passive, cells being carried along by growth of the surrounding tissues or by changes in tissue consistency. Interestingly, FN or LN coated beads, fibroblasts and somite cells will not move when similarly injected, indicating some role for surface receptors/SAMs (Bronner-Fraser, 1986c). However cells are never seen to move along the dorsolateral pathway into the skin, perhaps because they are injected too low down. Erickson (1985) injected cultured pigmented NCCs onto the dorsolateral pathway and into the sclerotome and found that they migrated normally in each context (that is, into the epidermis or ventrally to form DRG, sympathetic ganglia etc.). However when cells were fixed prior to injection, they failed to migrate dorsolaterally but transferred normally down the ventral pathway. This strongly indicates that cells can be carried along passively on the ventral pathway but that this is not the case sub-epidermally.

NCCs appear to be susceptible to negative guidance as well as positive. NCCs appear to populate their derivatives in a ventral to dorsal order with premelanocytes migrating last (Serbedzija et al., 1989). They suggest that this may be caused by NCCs filling up available ventral spaces, so forcing cells to occupy more dorsal positions or alternatively that the environment becomes non-permissive for migration in a ventral to dorsal order. Evidence supporting the former comes from Mintz (1967) and Huszar et al. (1991) who suggest that mouse skin has a limited capacity for melanocytes. Once places are filled, no further cells can enter the epidermis. Evidence for a ventral to dorsal change in migratory substrates is that cells trying to gain entry to the somites 9-12 hours after the onset of migration are severely inhibited (Weston and Butler, 1966). This indicates that late entry into the somites is actively blocked by endogenous

changes in the somite ECM (Newgreen et al., 1990). An accumulation of something like cartilage-specific chondroitin sulphate proteoglycan under the skin may likewise encourage premelanocytes to move towards the epidermis.

4.1.3 Role of chemoattractants

One mechanism which might provide the directional cues for the epidermal homing of melanocytes is chemotaxis. The epidermis certainly seems to play a role in peripheral nerve guidance. When embryonic chick limb ectoderm is laser ablated immediately prior to arrival of the peripheral nerves, cutaneous nerve plexi and the branches that supply them do not develop (Martin et al., 1989). Therefore in the absence of a target skin patch, the nerve branch does not even begin to form, indicating that a long range interaction between target and fibre is not taking place. Chemotaxis is a far more satisfactory explanation for this than contact guidance because the latter requires direct physical contact between target and fibre while in this case, the epidermis is affecting nerve process growth from a distance. Melanocytes find their way into the epidermis even when they are injected into the vascular system of white host chicks (Weiss and Andres, 1952). Presumably they arrive in the dermis through ruptured capillaries and they may then be attracted chemotactically by the epidermis. Mouse whisker skin emits a chemoattractant which guides trigeminal neurites in to their target (Lumsden and Davies, 1983). NGF is produced by chick wing skin at the age of nerve invasion and may be the chemoattractant (Rohrer et al., 1988). Nerve fibres could also be guided in by Schwann cells which move ahead of developing axons and express NGF receptors (Scarpini et al., 1988). Since Schwann cells and most peripheral nerves are of neural crest origin (Le Douarin, 1982) and since they may be chemotactically attracted towards the epidermis by growth factors, it is possible that melanocytes may be guided likewise.

Growth factors act as chemoattractants for a variety of cell types. Basic FGF acts as a chemoattractant for astroglial cells (Westermann et al., 1990). EGF and also the CDPGYIGSR peptide from laminin which has EGF-homology and partial TGF β -homology are chemotactic for melanoma cells (Graf et al., 1987). EGF is a chemoattractant for normal dermal fibroblasts (Adelmann-Grill et al., 1990) as is PDGF (Adelmann-Grill et al., 1989). TGF β is a potent chemoattractant for fibroblasts and epithelial cells (Moses et al., 1990). On the other hand, retinoic acid serves to block melanoma migration *in vitro* and might exert the same effect on migrating melanocytes (McGarvey and Persky, 1989). These are all small molecules capable of diffusing freely through tissue spaces- an important criterion for a chemotactic agent.

If a growth factor is responsible for melanocyte homing, its receptors should be present on at least some NCCs. There is much evidence of growth factor involvement in NCC survival and differentiation. Placing a silastin membrane between the neural tube and the developing DRG results in selective death of NCCs, while impregnating the silastin membrane with bFGF results in rescue (Kalcheim, 1989). Proliferating chick melanocytes have NGF receptors (Rakowicz-Szukczynska et al., 1988) as do newborn human foreskin melanocytes (Thomson et al., 1988), while the survival of both sensory and sympathetic neurons in culture is promoted by NGF (Raynaud et al., 1988). bFGF is a natural mitogen for melanocytes (Halaban et al., 1988; Gordon et al., 1989) and can induce melanogenesis in cultured early DRG and peripheral neurons (Stocker et al., 1991). Furthermore the pigment defect in *steel* mouse skin appears to be due to a defective gene for MGF, (mast cell growth factor, Halaban, 1991), and the *Patch* mutant mouse has a deleted PDGF gene (Stephenson et al., 1991). Although bFGF may be the growth factor responsible for maintaining epidermal melanocytes, it is not released by basal keratinocytes but only acts on melanocytes when they are in direct contact with keratinocytes (Halaban et al., 1988; Herlyn et al., 1988). Interestingly, keratinocyte-conditioned medium causes a 4-fold increase in

melanocyte yield within 24 hours in culture and this activity is caused by a low molecular weight fraction which cannot be substituted for by bFGF (Gordon et al., 1989). Quail NCCs express receptors for EGF and proliferate when treated with EGF in culture (Erickson and Turley, 1987), and TGF α is known to be produced by keratinocytes and also acts as a melanocyte mitogen (Ellem et al., 1988). Therefore keratinocytes do produce more than one growth factor for melanocytes and at least one of these is released into the ECM and would therefore become available to migrating melanocytes in the dermis.

Most authors do not favour chemotaxis as an explanation for guided migration of neural crest cells but only a few studies have directly addressed this question. Willier et al. (1937) noticed that when neural tube from a black chick breed was grafted into the base of a white chick wing, the resulting black patch extended towards the tip of the wing. Upon further investigation of this phenomenon, Wachtler (1984) found that when a neural crest cell source was grafted into the base of the developing chick wing bud, epidermal melanocytes were found exclusively distal to the graft. Furthermore, if an impermeable barrier was interposed between the apical ectodermal ridge (AER) and the graft, melanocytes migrated both distally and proximally from the graft, but if a permeable filter was similarly placed, melanocytes once more were only found distal to the graft. Under all three experimental conditions, other NCC-derivatives such as Schwann cells migrated proximally and distally from the graft. This seems to indicate that melanocytes in the limb bud are responding to a diffusible chemotaxic agent emitted by the AER by migrating in a specific and directional manner. Very recently, it has been found that the AER and the zone of polarising activity both produce a higher level of growth factor activity than other regions of the limb bud (Munaim et al., 1991); it therefore seems likely that a growth factor is the chemotaxic agent responsible for directed melanocyte migration.

In summary, while many studies have been directed towards understanding the factors controlling the initial migration of NCCs, relatively few have addressed the mechanism of terminal migration. It seems probable that these inherently motile cells will continue to move along a supportive substrate pathway once directionality has been established by contact inhibition of movement at the start of migration. Stop signals in the form of temporal matrix changes could orchestrate a mass cessation of movement, resulting in the formation of sensory and sympathetic ganglia. However, in the case of terminal melanocyte migration, the cells are scattered amongst mesenchymal cells and must individually migrate towards and into the epidermis. Emission of a chemotaxic agent by the target epidermis would provide an attractive and feasible alternative mechanism. It might also provide an explanation for the formation of exotic pigment patterns. Therefore this present study was carried out, firstly to test the epidermis for chemotaxic properties and secondly, to attempt to match this activity with growth factors known to act on melanocytes.

4.2 Methods

4.2.1 Chorioallantoic membrane culture

Embryonic ages are given as days *in ovo*, according to Hamburger and Hamilton (1951).

Tissue pieces were obtained from 6 day BAXNHR (pigmented) or WPRxCG (white) embryos. Separation of epithelium from underlying mesenchyme was achieved by incubating whole tissue pieces in 1% trypsin (1:250 DIFCO) at 4°C for 1 hr, followed by incubation in 50% horse serum or 50% fresh albumin in chick saline to inactivate the trypsin. Epithelium was then manually peeled off the mesenchyme. The following tissue recombinants were made. (i) Oesophageal or gizzard endoderm was combined with BAXNHR dermis (Fig 4.1a). (ii) Amniotic membrane epithelium was combined with white dermis on top of BAXNHR dermis (Fig 4.1b). Whole white skin was also recombined with BAXNHR dermis (Fig 4.1c). All recombinations were performed on chick agar and the resulting explants cultured on agar at 37,5 °C for 24 hours to initiate reassociation. After 24 hrs, explants were grafted onto 8 day chorioallantoic membranes in white host eggs and cultured at 37,5 °C for a further 7-10 days. Grafts were then removed and fixed in Bouin's fluid for routine wax embedding. Grafts were serially sectioned using a Leitz rotary microtome at 5 µm and stained with Mayer's haematoxylin and eosin and examined for the presence and position of melanin.

4.2.2 Transfilter culture

Experiment 1

Dermis from 6 day BAXNHR embryos was isolated from whole skin pieces by trypsin digestion as above. Dermal pieces of uniform thickness and approximately 2 mm² were placed, "epidermal" side up, on 0,45µm pore size Millipore filters. Filters were rested above miniwells containing Dulbecco's modified Eagles medium (DMEM) with 10% horse serum, penicillin (µg/ml) and streptomycin (u/ml). The following

variations in culture medium were used:

1. DMEM + horse serum alone.
2. DMEM without serum.
3. DMEM + serum containing pieces of epithelium.
4. DMEM containing epithelium as above, without serum.
5. DMEM + serum containing pieces of CAM.
6. DMEM containing CAM, without serum.

Explants were cultured at 37,5 °C, with air containing 5% CO₂ and 50-90% humidity for periods of 24, 48 and 72 hrs. Duplicate explants were cultured for each experimental variation. Filters with attached explants were fixed in Bouin's fluid and processed for routine wax histology. Serial 5 µm sections were made through the explants at right-angles to the filters and stained lightly with Mayer's haematoxylin and eosin.

Analysis

The central region of each explant was chosen for analysis to avoid the flattened edges. This region spanned 30 consecutive sections where the explant was thickest and every third section was counted. Each section was divided into 3 equal regions (Fig 4.2a), nearest the filter (Region 1), the central region (Region 2) and near the original epidermal border (Region 3), and the number of melanocytes in each of these regions was counted. Therefore in each explant, the 3 regions were counted in 10 sections at 15 µm intervals (to avoid counting the same cell twice). Data is presented as number of melanocytes for each region (Table 4.2).

Experiment 2

Skin pieces were obtained from 6 day white and BAXNHR embryos and split as before. Uniformly sized (2 mm²) pieces of BAXNHR dermis were placed on top of slightly larger pieces of white dermis on Millipore filters above miniwells and cultured for

5 days as above (Fig 4.2b). Culture medium was DMEM with 10% horse serum, antibiotics as above and the following variations:

1. DMEM + horse serum alone.
2. DMEM + horse serum containing pieces of epithelium.
3. DMEM + horse serum with 10 ng/ml EGF.
4. DMEM + horse serum with 10 ng/ml NGF.
5. DMEM + horse serum with 10 ng/ml bFGF.
6. DMEM + horse serum with 10^{-8} M retinoic acid.

Each experimental variation was carried out with 5 explants. Culture medium was replaced daily until culture was terminated. Explants were fixed and processed as above.

Analysis

The central region of each explant was chosen for analysis (to avoid the flattened edges). This region spanned 150 consecutive sections where the explant was thickest and every third section was counted. Each section examined was divided into 2 equal regions (Fig 4.2b), nearest the filter (Region 1) and near the original epidermal border of the BAXNHR dermis (Region 2), and the number of melanocytes in each of these regions was counted. Therefore in any explant, the 2 regions were counted in 50 sections at 15 μ m intervals from each other. Data is presented as number of melanocytes for each region (Table 4.3).

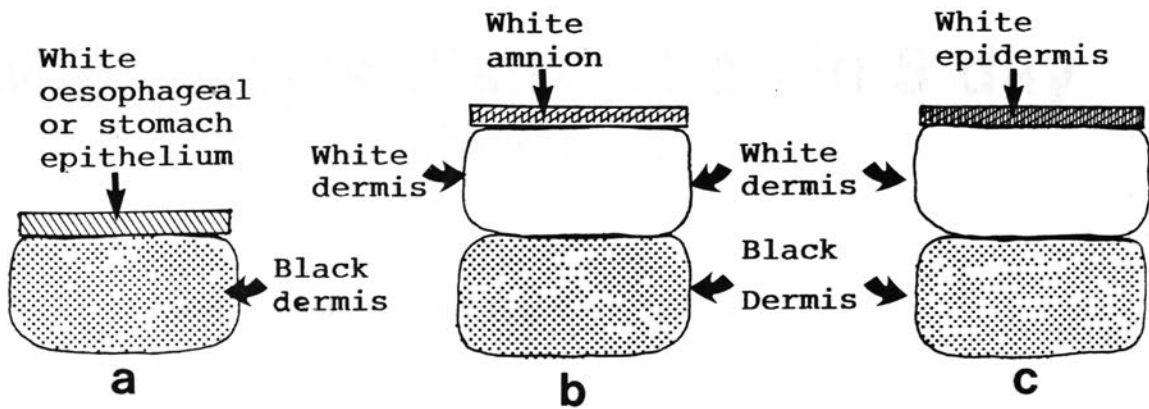


Fig 4.1

Scheme representing the different combinations of epithelium and dermis used to examine the terminal migration of melanocytes.

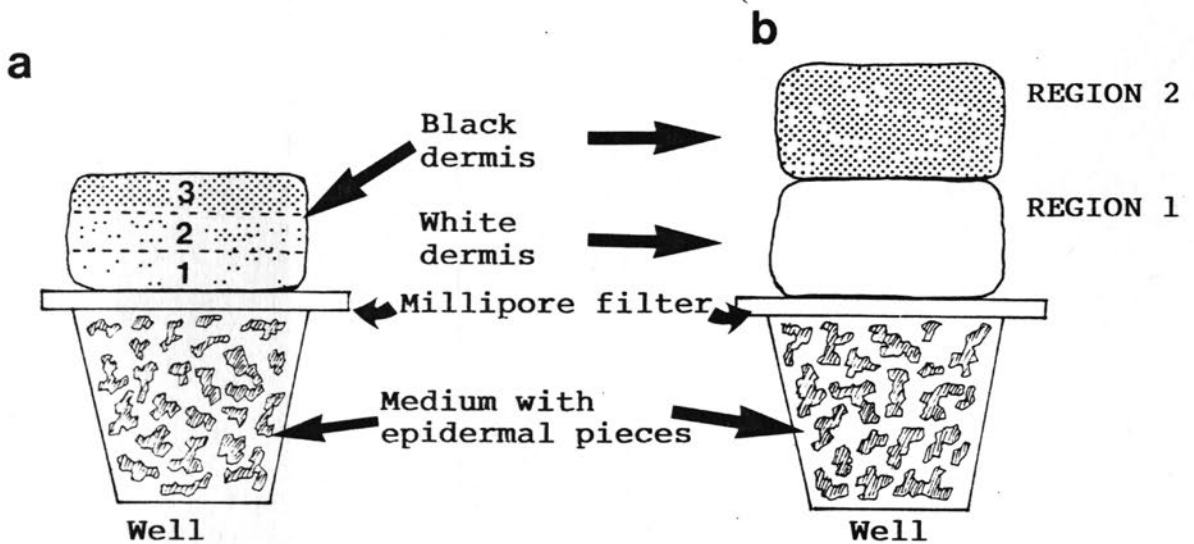


Fig 4.2

(a) Transfilter culture system used in Expt 1. Regions 1, 2, 3 indicate the sub-divisions of the dermis in which melanocytes were counted. (b) Transfilter culture system used in Expt 2. Regions 1 and 2 indicate the regions of the explant in which melanocytes were counted. Note that the regions are of equal size and do not necessarily correspond to the original white and black dermal components.

Culture medium components were varied as detailed in Methods.

Melanocyte distribution in 5 day chick skin

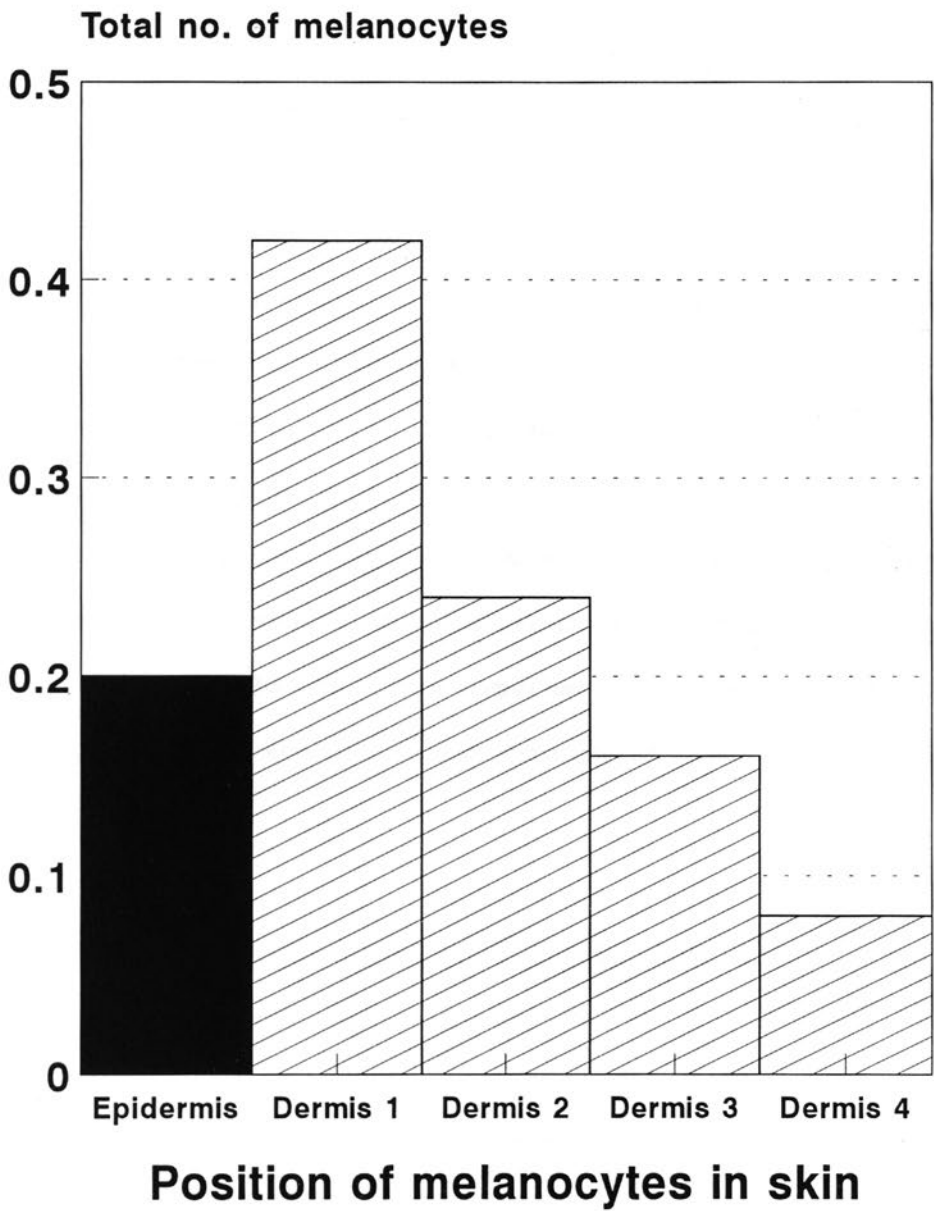


Fig 4.3
 Melanocyte counts in the dorsal half of cross-sections through the body region of 5 day chick embryos; n=4 embryos (32 sections in total). Dermal melanocyte counts were subdivided into groups according to the visually estimated perpendicular distance of dermal melanocytes from the base of the epidermis. Dermis 1 = 0-24 μm ; dermis 2 = 36-60 μm ; dermis 3 = 72-108 μm ; dermis 4 = 108+ μm . From Stander (1991).

4.3 RESULTS

4.3.1 Melanocyte distribution in whole embryos

An examination of cross sections through the trunk of 5½ day BAXNHR chick embryos revealed the pattern of melanocyte distribution illustrated in Fig 4.3 (Stander, 1991; see also Hulley et al., 1991). The majority of melanocytes are located in the dermis, distributed along a gradient, with more melanocytes close to the epidermis than further away. This distribution could come about in two ways- in response to a differentiation inducer emitted from the epidermis (those cells closest to the source responding in larger numbers), or as a result of migration with time towards the epidermis (with the older and therefore more pigmented cells lying nearest to the epidermis). In chapter 1, it was shown that melanocytes do indeed first move through the dermis before crossing into the epidermis. However, as progressively older embryos were examined it became apparent that there were less and less dermal melanocytes and these disappeared altogether after 10 days (Hulley et al., 1991). It therefore seems probable that the pattern of distribution of melanocytes in 5½ day embryos comes about as a result of directional migration towards the epidermis (homing) and not as the result of a differentiation gradient acting on randomly dispersed melanocytes. To test this hypothesis, the ability of melanocytes to migrate towards non-skin epithelia and through additional dermal tissue was examined.

4.3.2 Do melanocytes migrate into inappropriate epithelia?

If it is true that the epidermis is producing some kind of diffusible homing signal (chemoattractant) for melanocytes, they should be able to find their way through an extra layer of dermis and still reach the epidermis. When whole white skin was cultured on top of BAXNHR dermis (as a source of melanocytes), pigmented feathers resulted in 6 out of 6 cases (Table 4.1). As a control, amniotic epithelium was placed on white dermis and cultured on top of BAXNHR dermis. Only 1 out of 8 grafts exhibited epidermal pigment. As a further control, epithelium from the gut (oesophagus and gizzard) was cultured on top of BAXNHR dermis. No extra white

dermis was used in this experiment in case the dermis had been the inhibiting factor in the amniotic epithelium triple-layer combinations. All 8 surviving grafts had unpigmented epithelium (Fig 4.9a). These results indicate that epidermis has by far the strongest ability to promote directional migration of melanocytes. The epithelium of amniotic membrane seems to have a limited ability to promote melanocyte migration and differentiation, and stomach and oesophageal epithelia seem completely unable to support melanogenesis.

4.3.3 Does epidermis produce a diffusible chemoattractant for dermal melanocytes?

The above method does not clearly discriminate between melanocyte migration into an epithelium and melanocyte survival in that epithelium, that is, if melanocytes were unable to survive in a gut epithelium environment, they would also score negative on the count of directional migration. To eliminate this ambivalence, the following experiments were carried out. Pieces of dermis were isolated and cultured on filters above wells containing pieces of epithelium or CAM. In this way, there was no direct contact between tissues but diffusible factors were free to move through the 0.45 μm pores of the filters and diffuse into the dermis above. A gradient of the putative signal was maintained because the source of factors was separated from the migrating melanocytes and was continuously secreting factors throughout the culture period. In order to establish whether any change in melanocyte distribution occurred, the dermis was carefully placed epidermis-side up on the filter, so that the original epidermal-dermal border region containing most melanocytes was positioned furthest away from the epidermis under the filter. Therefore melanocytes had to completely reverse their direction of migration in order to move closer to the filter and the source of chemoattractant. This inversion of dermis would also have negated the effects of any adhesive matrix gradients underlying the epidermis.

Effect of epidermis vs CAM

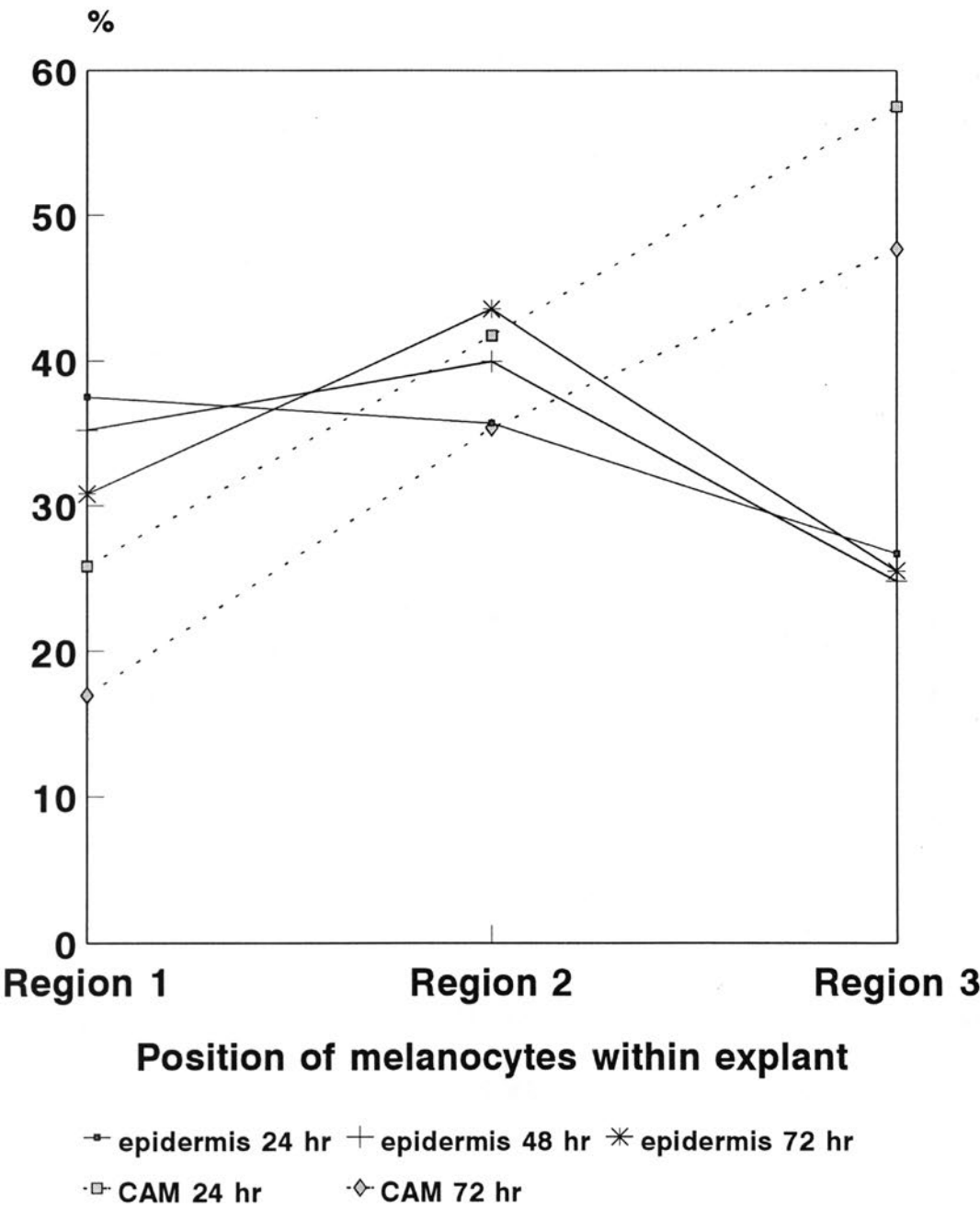
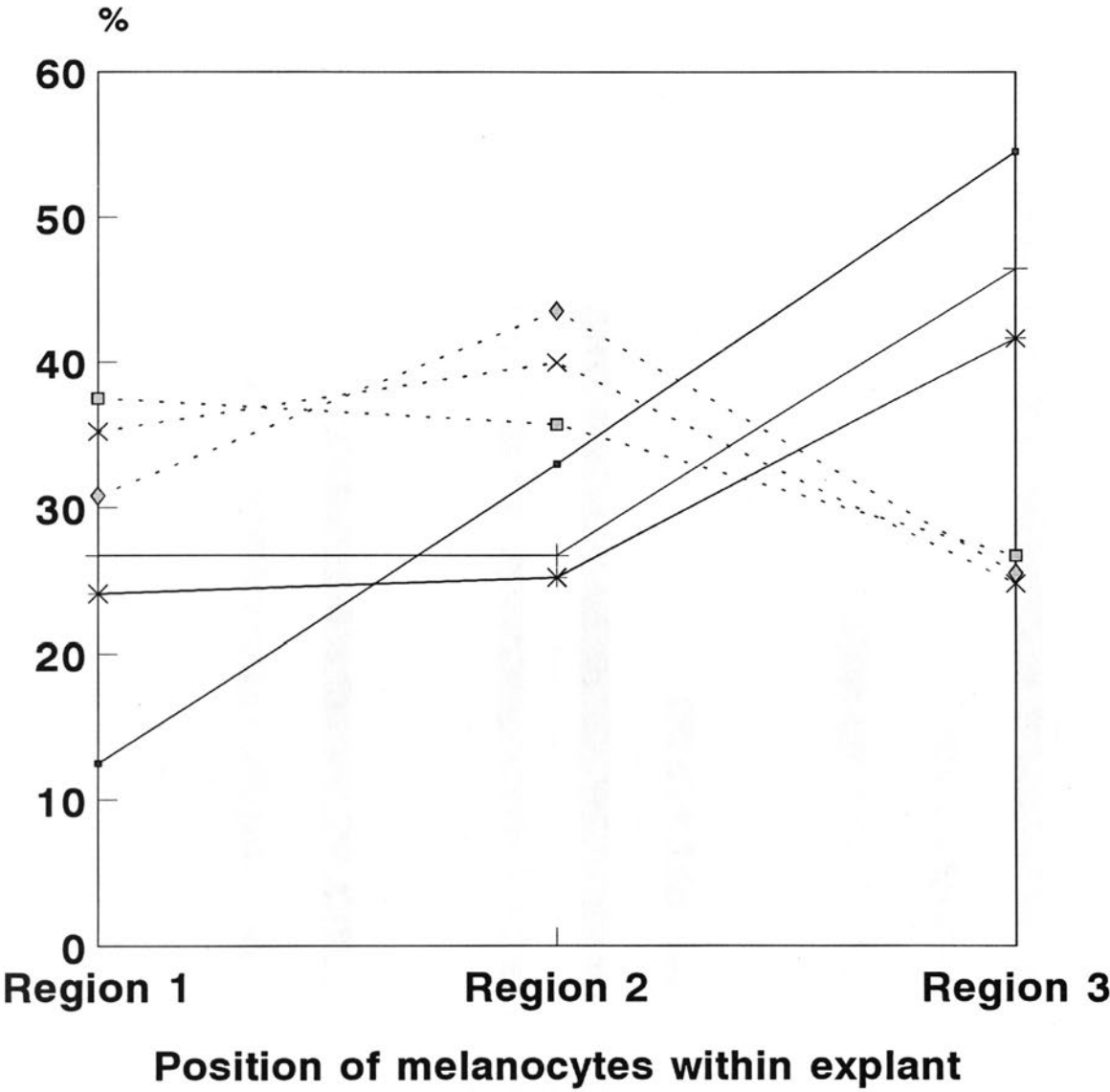


Fig 4.4
Melanocyte counts in pieces of BAxNHR dermis cultured on filters above a well containing medium with either pieces of epidermis or pieces of chorioallantoic membrane (CAM). Dermal melanocyte counts were sub-divided into three equal regions according to the visually estimated perpendicular distance from the filter to the top of the dermis. Region 1 = nearest to the filter, region 2 = centre of the explant, region 3 = the original epidermal border. The means of the two samples in region 3 are significantly different, $p = 0,005$.

Effect of epidermis on melanocyte migration



—■— epidermis 24 hr + epidermis 48 hr * epidermis 72 hr
- - - □ - - - ep + serum 24 hr - - - X - - - ep + serum 48 hr - - - ◇ - - - ep + serum 72 hr

Fig 4.5
Melanocyte counts in pieces of BAxNHR dermis cultured on filters above a well containing medium with either pieces of epidermis and 10% horse serum or epidermis alone. Dermal melanocyte counts were sub-divided into three equal regions according to the visually estimated perpendicular distance from the filter to the top of the dermis. Region 1 = nearest to the filter, region 2 = centre of the explant, region 3 = the original epidermal border. The means of the two samples are significantly different in all three regions, $p < 0.05$. (see appendix for data).

Melanocyte survival

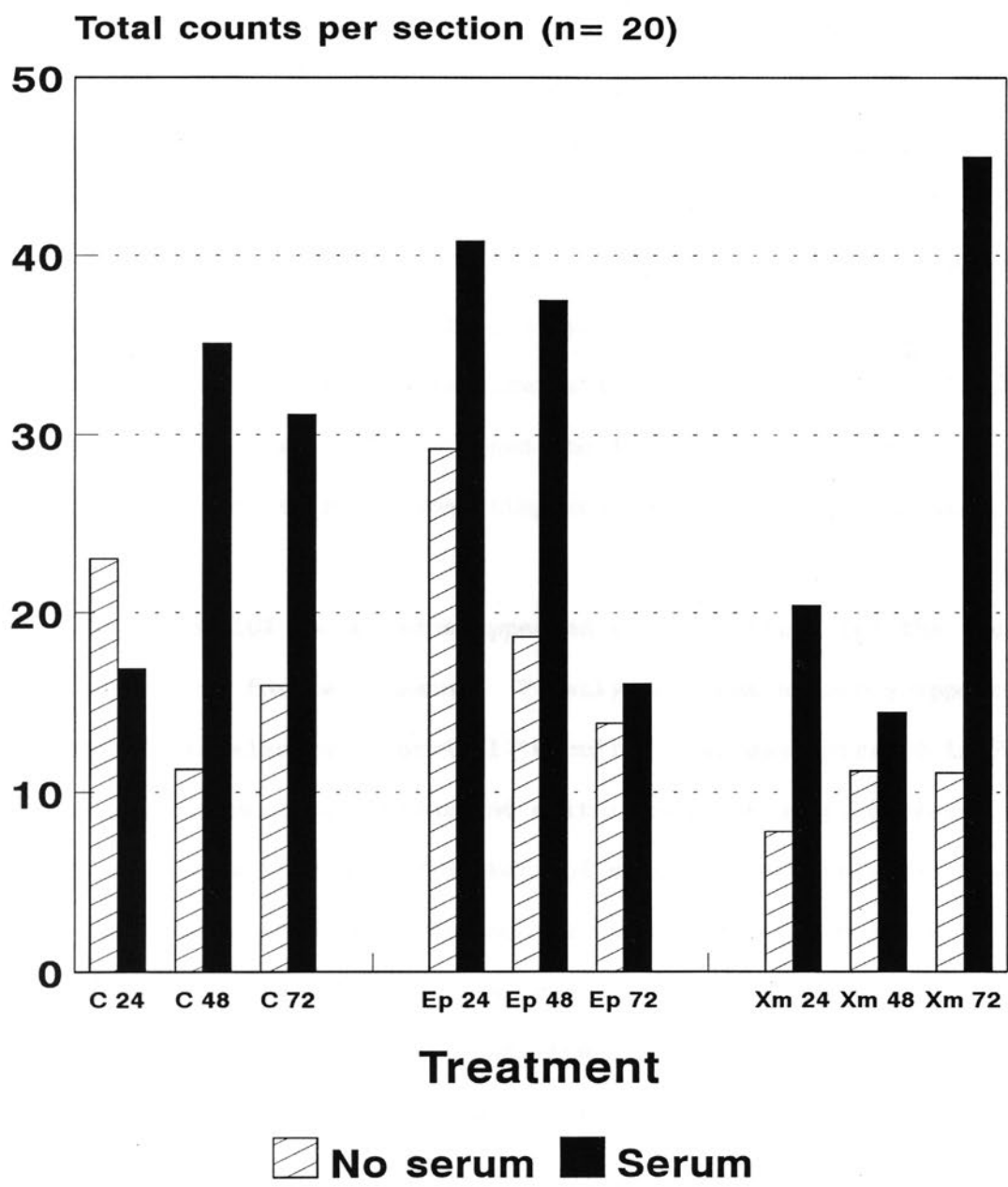


Fig 4.6
Melanocyte survival in trans-filter cultures of BAxNHR dermis. C = medium alone; Ep = treatment with pieces of epidermis; Xm = treatment with pieces of chorioallantoic membrane. Culture period indicated as 24=24 hr, 48=48 hr, 72=72 hr.

Fig 4.4 shows the distribution of melanocytes in dermis exposed to pieces of epidermis, compared with exposure to pieces of CAM (after culture for 1-3 days in medium containing horse serum). In the region closest to the filter (region 1), there were 13% more melanocytes in the explant treated with epidermis than that treated with CAM. In region 3, the old epidermal border, there are 27% less melanocytes in the epidermis-treated explant than in the CAM-treated explant (average of the three culture periods). In the central region, the 72 hr epidermis-treated explant contained most melanocytes, followed by the 48 hr culture and then the 24 hr culture. This graph seems to indicate a trend of melanocyte migration away from region 3 and towards region 1 (away from the old epidermis region and towards the new epidermis underneath the filter) was underway at the time of culture termination. Only one 48 hr culture treated with CAM membrane survived. It contained the least melanocytes of all explants (see Fig 4.6), appeared to be degenerating and was therefore discounted.

The addition of 10% horse serum appeared to be critical for the success of the above experiment for two reasons. Firstly, absence of serum appeared to be detrimental to melanocyte survival in culture, as demonstrated in Fig 4.6. Under all the experimental conditions and culture periods used, explants with serum exhibited consistently higher numbers of melanocytes than non-serum counterparts. Horse serum was chosen in preference to foetal calf serum as it is from an adult source and might therefore contain lower levels of embryonic chemoattractants such as growth factors. The second effect of serum in this experiment was to permit epidermis to exert its homing effect. Fig 4.5 shows the distribution of melanocytes in dermis treated with culture medium containing epidermis in the presence and absence of serum. Without serum, the pattern of distribution is similar to that obtained with CAM treatment in the presence of serum. In region 3, there were 22% more melanocytes in the explant treated with epidermis alone than that with epidermis and serum, while in region 1 there were 13% less melanocytes in the explant treated with epithelium alone, compared with

epithelium and serum. Therefore the distribution of melanocytes has remained unchanged, with the bulk of melanocytes lying adjacent to the old epithelial border.

4.3.4 What is the directional signal?

Growth factors have been shown to be chemotaxic agents for a variety of cell types (see introduction). To test the idea that a growth factor might be the homing factor released by the epidermis which attracts migrating melanocytes, pieces of dermis were cultured above wells containing NGF, EGF, bFGF or RA and examined for any subsequent alteration in melanocyte distribution (Expt 4.2.2). In order to make melanocyte movements clearly apparent, BAXNHR dermis was cultured on top of white dermis (Fig 4.9b). As explained previously, melanocytes are mostly located along the top of the dermis (adjacent to the old epidermal border). The explants were analysed in 2 halves, the top region corresponding approximately to the BAXNHR dermis and the half next to the filter corresponding to the white dermis. Melanocytes found near the filter will have reversed their direction of migration through the BAXNHR dermis and in addition, will have moved some distance through the white dermis. Signal polarity was maintained by daily culture medium changes. Explant numbers and the overall region analysed were greatly increased compared with Expt 4.2.1.

As shown in Fig 4.7, only the positive control (treatment with pieces of epidermis) showed more melanocytes in region 1 than 2, providing evidence of extensive melanocyte movement into the white dermis, that is, towards the filter and source of chemoattractant (see also Fig 4.9c). Although some melanocytes moved through into the white dermis under all other treatment conditions, this was not greater than movement towards medium alone. This result is also illustrated in Fig 4.8, which shows the results for individual explants.

Melanocyte homing response

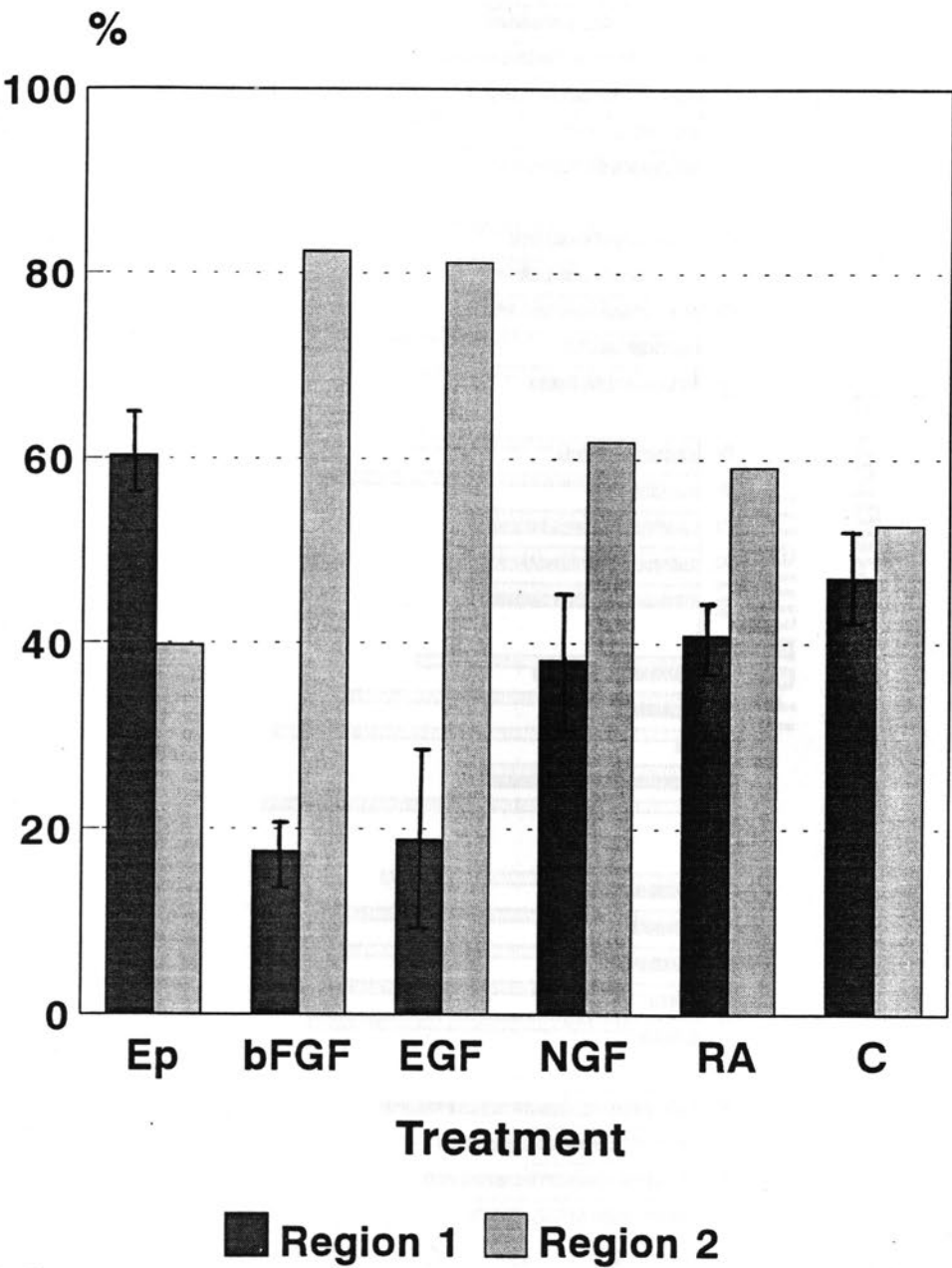


Fig 4.7
Melanocyte counts in pieces of BAxNHR dermis combined with white dermis and cultured above a well containing medium with additives as indicated. Ep=epidermis; bFGF=basic fibroblast growth factor; EGF=epidermal growth factor NGF=nerve growth factor; RA=retinoic acid; C=medium only. Explant melanocyte counts were sub-divided into two equal regions according to the visually estimated perpendicular distance from the filter to the top of the explant. Region 1 = nearest the filter (largely white dermis); Region 2 = upper half (largely BAxNHR dermis). Melanocyte counts are given as means $\pm$ SEM, n=5 (epidermis n=4). See appendix for data.

Homing response in melanocytes

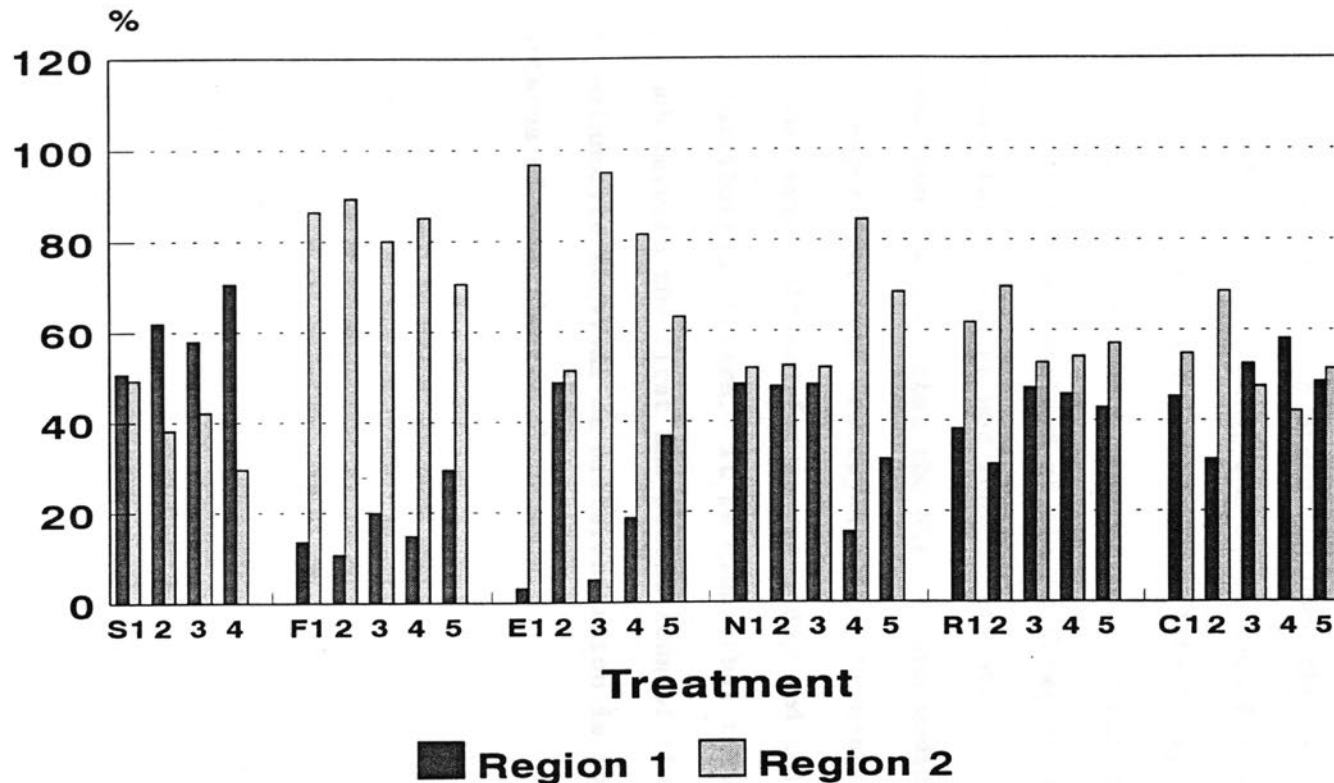


Fig 4.8
Melanocyte counts in pieces of BAXNHR dermis combined with white dermis and cultured above a well containing medium with additives as indicated. S=epidermis; F=basic fibroblast growth factor; E=epidermal growth factor N=nerve growth factor; R=retinoic acid; C=medium only. Explant melanocyte counts were sub-divided into two equal regions according to the visually estimated perpendicular distance from the filter to the top of the explant. Region 1 = nearest the filter (largely white dermis); Region 2 = upper half (largely BAXNHR dermis). Each region was counted in 50 sections.

It appears in Fig 4.7 that NGF produces a melanocyte distribution similar to medium-only control and retinoic acid-treated explants, while bFGF and EGF showed a larger difference in melanocyte number between regions 1 and 2. There are two possible explanations for this difference in effect between the 3 growth factors. Firstly, melanocytes normally stop migrating once they are located amongst the basal keratinocytes of the epidermis, and since both EGF and bFGF are produced by the basal keratinocytes, they may constitute a "stop" signal for migrating melanocytes. This would limit the number of melanocytes migrating from region 2 to region 1. NGF may not be involved at all in terminal melanocyte migration and would therefore neither stimulate nor inhibit migration. An alternative explanation stems from the fact that the NGF, RA and medium-only control explants from the initial experiment were destroyed during processing and were repeated at a later date. They were cultured for an extra day and this might explain the difference in distribution pattern. It is clear that total melanocyte numbers did not vary much between the first and second runs of the experiment (Table 4.3), so poor melanocyte survival or differentiation is not the cause of the different patterns of distribution observed.

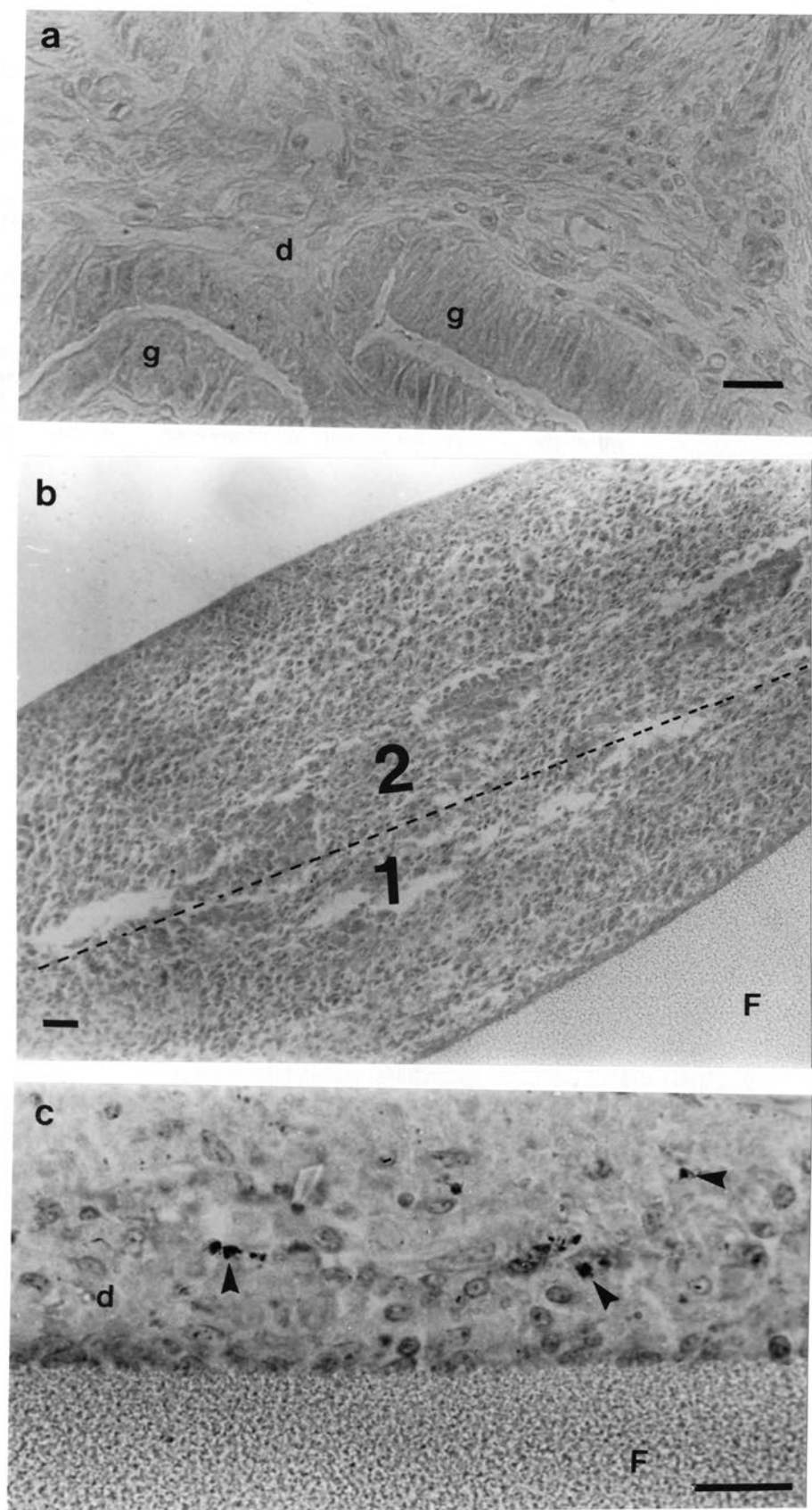


Fig 4.9

(a) CAM graft of stomach epithelium combined with BAXNHR dermis.

(b) Filter culture of BAXNHR dermis on top of white dermis, treated with 10 ng/ml bFGF. (c) As above, treated with pieces of epidermis. g, epithelial

glands of stomach; d, dermis; 1 and 2, regions counted; F, filter; arrows, melanin. Bar = 20 μ m.

Table 4.1

EFFECTS OF INAPPROPRIATE EPITHELIA AND EXTRA DERMIS ON MELANOCYTE MIGRATION

Epithelium	Mesenchyme 1	Mesenchyme 2	Graft epithelium	
			Pigmented	Unpigmented
Gut endoderm	BAXNHR dermis	-	0	8
Amnion	white dermis	BAXNHR dermis	1	7
White epidermis	white dermis	BAXNHR dermis	6	0

Tissue components were separated using trypsin and then recombined on agar for 24 hrs. Recombinants were grafted onto CAMs and grown for 7-10 days. Grafts were fixed and examined for the presence of epidermal melanin. BAXNHR= pigmented chick breed. Graft survival = 100%.

Table 4.2

DISTRIBUTION OF MELANOCYTES IN DERMIS FOLLOWING EXPOSURE TO POSSIBLE
CHEMOATTRACTIVE TISSUE SOURCES

Treatment	Culture period (hrs)	Total melanocyte counts			% melanocyte counts		
		Region 1	Region 2	Region 3	Region 1	Region 2	Region 3
Epidermis	24	7.3	19.2	31.7	12.5	33.0	54.5
	48	5.0	5.0	8.7	26.7	26.7	46.5
	72	9.2	7.0	11.6	24.1	25.2	41.7
Ep + serum	24	30.6	29.1	21.8	37.5	35.7	26.7
	48	26.3	29.9	18.5	35.2	40.0	24.8
	72	9.9	14.0	8.2	30.8	43.6	25.5
CAM	24	3.6	2.8	1.4	46.2	35.9	17.9
	48	3.0	4.9	3.3	26.8	43.8	29.4
	72	4.9	3.3	2.9	44.1	29.7	26.1
CAM + serum	24	10.5	17.0	13.2	25.8	41.8	57.5
	48	8.8	11.5	8.6	30.4	39.8	29.8
	72	15.4	32.1	43.3	17.0	35.4	47.7
Medium	24	18.7	9.7	17.3	40.9	21.2	37.9
	48	7.1	6.2	9.2	31.6	27.6	40.8
	72	10.7	8.7	11.4	34.7	28.2	37.0
Med + serum	24	9.1	12.9	11.5	27.2	38.5	34.3
	48	25.4	26.7	18.0	36.2	38.1	25.7
	72	19.3	18.1	24.5	31.2	29.2	39.6

Pieces of BAXNHR dermis were cultured on 0,45 μ m pore size Millipore filters above wells containing medium alone or medium with pieces of epidermis or CAM. Melanocytes were counted in three regions: (1) near the filter, (2) centrally, (3) near the original epidermal border. Culture variations were prepared in duplicate and counts represent total melanocytes from both samples. CAM= chorioallantoic membrane, Ep= epidermis, Med= medium, BAXNHR= pigmented chick breed.
 Explant survival = 94%.

Table 4.3

DISTRIBUTION OF DERMAL MELANOCYTES FOLLOWING EXPOSURE TO POTENTIAL CHEMOATTRACTANTS

Treatment	Explant number	Total melanocyte counts*		% melanocyte counts	
		Region 1	Region 2	Region 1	Region 2
Epidermis	1	662	645	50.7	49.3
	2	575	354	61.9	38.1
	3	212	154	57.9	42.1
	4	201	84	70.5	29.7
	5	0	0	0	0
bFGF	1	58	373	13.5	86.5
	2	44	372	10.6	89.4
	3	122	491	19.9	80.1
	4	76	436	14.8	85.2
	5	30	72	29.4	70.6
EGF	1	14	443	3.1	96.9
	2	689	725	48.7	51.3
	3	9	170	5.0	95.0
	4	137	598	18.6	81.4
	5	172	295	36.8	63.2
NGF	1	391	420	48.2	51.8
	2	214	235	47.7	52.3
	3	487	526	48.1	51.9
	4	59	324	15.4	84.6
	5	145	315	31.5	68.5
RA	1	326	528	38.2	61.8
	2	220	505	30.3	69.7
	3	191	214	47.2	52.8
	4	247	292	45.8	54.2
	5	728	967	42.9	57.1
Medium	1	708	859	45.2	54.8
	2	190	415	31.4	68.6
	3	986	892	52.5	47.5
	4	377	273	58.0	42.0
	5	315	333	48.6	51.4

Pieces of BAXNHR dermis were cultured on top of pieces of white dermis on 0,45 μ m pore size Millipore filters. These lay above wells containing medium alone or medium with epidermal pieces, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), nerve growth factor (NGF) or retinoic acid (RA). Concentration of growth factors was 10 ng/ml in all cases and RA was used as at 1×10^{-8} M. After 5 days in culture with daily medium renewal, explants were fixed and sectioned at right angles to the filters. Melanocytes were counted in 2 regions of equal size, (1) close to the filter and (2) the top half of the explant. These regions correspond approximately to the white dermis and the BAXNHR dermis respectively.

* Total number of melanocytes in 50 consecutive sections through explant.

Explant survival = 97%.

4.4 DISCUSSION

4.4.1 The role of the epidermis in the homing of melanocytes

The forces governing the directional migration of NCCs are not well understood. In the chick, the ventrally moving cells migrate as sheets of cells rather than as individual cells (Perris et al., 1991), and therefore their directionality of movement may be governed by factors such as contact guidance, passive physical forces due to surrounding tissue growth and density, and orchestrated matrix changes as discussed in the introduction. On the other hand, migrating sensory neurites from the neural crest-derived DRG will not approach their target skin patch if the overlying epidermis is absent (Martin et al., 1989). It therefore seems that the epidermis may be chemotactically attracting neurites. In the light of the results presented here, it seems likely that the directional migration of dermal premelanocytes is also in response to a diffusible homing factor/chemoattractant released by the epidermis. This is the first direct evidence of epidermal homing of melanocytes and evidence in support of this conclusion comes from two different sets of experiments. In the first set, directional migration towards epidermis was tested in transfilter cultures by exposing pieces of dermis to diffusible factors emanating from pieces of epidermis. By analysing the distribution of dermal melanocytes after this treatment, it was possible to show that there was a significant movement of melanocytes towards the epidermis (as compared to controls where the epidermis was replaced with CAM).

In the second set of experiments, the chemotaxic role of epidermis was again demonstrated under more rigorous conditions. Dermis containing melanocytes was inverted, placed on top of white dermis, and the combination was cultured on a filter above a well containing medium plus epidermis or one of a variety of growth factors. Melanocytes would have had to reverse their original migratory direction, migrate back through the BAXNHR dermis and then into the underlying white dermis before reaching the filter. The results demonstrated that only epidermis-treated explants were able to cause a significant reversal, while in explants treated with

bFGF, EGF, NGF, RA or plain medium, this reversal was not observed. These results suggest that although all 3 growth factors are mitotic for melanocytes or produced in the skin, they do not appear to be chemotaxic for melanocytes. Other growth factors such as MGF (mast cell growth factor), PDGF (platelet derived growth factor) and TGF β (transforming growth factor β) were not tested and might be found to be homing factors. MGF is of particular interest since it is a mitogen for melanocytes and mast cells and is coded for by the *Steel* locus in mice. A defective gene at this locus produces an animal deficient in melanocytes and mast cells and it appears that the defect affects both melanocyte migration and differentiation (Nakayama et al., 1988; Halaban, 1991). It has been recently suggested that because the PDGF receptor can be detected earlier in development than those of MGF or bFGF, PDGF may be involved with melanocyte migration and the others with differentiation (Halaban, 1991). This idea is supported by the finding that PDGF restores normal pigmentation capabilities to *Steel* mouse melanocytes (Morrison-Graham et al., 1990) and that another murine piebald gene, *Patch*, is the gene for the PDGF β receptor subunit (Stephenson et al., 1991). TGF β is another possible homing factor because it is produced by keratinocytes and is also a melanocyte mitogen (Ellem et al., 1988). Very recently, TGF β has been shown to stimulate human melanocyte migration *in vitro* (Morelli et al., 1992).

A second possible reason that no homing response was obtained with the three growth factors could be because growth factor concentrations on both sides of the filter rapidly equilibrated and the daily changes of medium were insufficient to maintain the concentration gradient. To determine if this is the case, some type of ion-release bead soaked in growth factor, or a cell line producing high quantities of each growth factor could be used as a gradient source. It is notable that melanocyte numbers do not appreciably vary with or without growth factors in Expt 4.3. This could reflect an adequate growth factor supply in the horse serum or produced by the dermal cells. Alternatively, melanocytes at this stage may not be

growth factor-dependent and may acquire dependency as they differentiate in the epidermis. This happens in certain neuronal populations- basal forebrain cholinergic neurons in the rat become dependent on target-derived NGF for survival during the early post-natal period (Hatanaka et al., 1988). NGF is similarly involved in the target-controlled survival of the sensory neurons which innervate embryonic mouse skin (Davies et al., 1987).

Finally, growth factor concentrations may have been too low to attract melanocytes. The concentration of 10 ng/ml was chosen because:

- 10 ng/ml of EGF causes optimal chemotactic migration of fibroblasts (Adelmann-Grill et al., 1990)
- 1 ng/ml of EGF provides the saturating dose for keratinocyte mitosis (Gospodarowicz et al., 1990)
- bFGF optimally stimulates mitosis and production of plasminogen activator by endothelial cells at 10 ng/ml (Flaumenhaft et al., 1989)
- bFGF stimulates keratinocyte mitosis optimally at 1 ng/ml (Gospodarowicz et al., 1990)
- bFGF has been assayed at a maximum of 18 ng/mg protein in the embryonic chick nervous system (Kalcheim and Neufeld, 1990).

Retinoic acid inhibits melanoma migration at $1 \times 10^{-6} \text{M}$ (McGarvey and Persky, 1989) and promotes melanoma melanogenesis at 10^{-7}M (Dr S Kidson, personal communication). Further experiments using higher concentrations of factors might elicit a homing response.

The culture system devised here has proved adequate for demonstrating chemotaxis of one group of cells towards the tissue source of a diffusible homing signal. Unlike more classical migration assay systems such as the Boyden chamber, the use of whole dermis provides the premelanocytes with the normal spectrum of ECM, fibres and cells normally encountered during terminal migration, except that the ~~epidermis is~~

epidermis is separated from the dermis by a Millipore filter. The method also ensures that melanocytes have passed through all prior steps of development without manipulation or interruption and are therefore identical to *in vivo* counterparts, at least at the start of the culture period. This means that the melanocyte migration assayed here more closely approximates the *in vivo* situation than would cultured crest cells in a Boyden chamber. The question of which (if any) growth factor is responsible for melanocyte chemotaxis would be answered if another growth factor such as PDGF induced the same response as epidermis under the established conditions described here, or if bFGF, EGF or NGF evoked a similar response when tested under the slow-release conditions suggested above. The major drawback of this migration assay system is the time involved in making and counting serial sections. This limits the sample number of each experiment.

4.4.2 Implications for pattern formation

It has become generally accepted that neural crest cells follow specific pathways determined by their migratory environment, rather than by intrinsic properties of the cells themselves (Le Douarin, 1980; Erickson, 1988). However, recent findings indicate that while this may be the case in early migrating crest cells, it is not necessarily true for NCCs undergoing terminal migration. When early chick dorsal root ganglion and peripheral Schwann cells are treated with TPA (tetradecanoyl phorbol acetate) and bFGF, they become melanised. When reintroduced into embryos, they migrate into the overlying epidermis (Stocker et al., 1991). This implies that developmental restriction to the melanocyte lineage is accompanied by a change in responsiveness to local guidance cues so that the injected cells localise in the epidermis rather than re-forming a DRG. It was further found that TGF β prevents the melanogenic effects of TPA and bFGF. Black and white pigment patterns in the skin may therefore be caused when migrating premelanocytes encounter different growth factors along their individual migration routes. In this way, bFGF encountered in the sub-epidermal mesenchyme or BM might create black epidermis, while TGF β encountered in adjacent

mesenchyme or BM might inhibit melanocyte differentiation and so cause white epidermis. Further evidence for this theory comes from studies carried out using chimeras of *Steel* and wild-type mouse embryos. While melanocyte precursors are found homogeneously throughout the dermis of chimeric mice, the *Steel* epidermal patches prevent invasion by migrating melanocytes and remain unpigmented (Nakayama et al., 1988). This agrees with the finding presented in this chapter that the epidermis plays a role in directing melanocyte invasion. As already mentioned, the *steel* locus codes for MGF which is a melanocyte and mast cell mitogen (Halaban, 1991) and this lends credibility to the idea that growth factors are the melanocyte homing factors emitted by the epidermis.

By the time different NCC derivatives have reached their terminal migratory stages, they show a varied response to the same ECM product. As detailed in Chapter 3, NC-derived sensory neurites are prevented from closely approaching the epidermis by the presence of chondroitin sulphate in the mesenchyme immediately adjacent to the epidermal BM (Fichard et al., 1991). Melanocytes, however, pass straight through this region. Differentiation is therefore being accompanied by a change in receptor conformation so that cells of the same origin are directed to different targets. If NCCs do exhibit a change of receptor type and density as they differentiate, then melanocytes at the stage of epidermal invasion may develop receptors for specific growth factors provided exclusively by epidermal keratinocytes or lose receptors which permit migration on specific substrates. This would neatly explain why after 8 days some melanocytes fail to migrate into the epidermis and degenerate in the dermis. There seems to be a mechanism by which the epidermis limits invasion to a discrete number of melanocytes only. When there are host melanocytes, whether pigmented or not, in the epidermis, only a breed specific number of donor melanocytes successfully enter the epidermis (Mayer, 1973a). For example, when cultured C57Bl/6 melanocytes are injected into melanocyte-deficient W mutant mouse embryos, they make an extensive contribution to coat colour, but when injected into Balb/c mice they only contribute to

limited regions (Huszar et al., 1991). This could be explained in terms of saturation of available secreted growth factor, that is, the keratinocytes are only able to produce a limited amount of growth factor for melanocytes and once the melanocytes have matured to the stage of dependency on this growth factor, they will only be able to survive in close proximity to the keratinocytes. The number of surviving melanocytes would be limited by the amount of growth factor secreted by the epidermis. Alternatively, melanocytes might not develop growth factor dependence but may receive growth factor from an extracellular fluid source while they are migrating and when this source is terminated with age, only those which are successfully located in the epidermis will remain fully differentiated.

Recent clonal analysis of NCCs indicates that they do not reach their differentiation sites in an identical state of commitment (Baroffio et al., 1991). The authors suggest that the role of the microenvironment encountered at the sites of differentiation may be in directing multipotent progenitors towards a specific differentiation pathway as well as promoting the survival of subsets of fully determined NCCs. I further suggest that the microenvironment encountered during terminal migration of melanocytes provides guidance cues which actively encourage epidermal targeting.

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APPENDIX

1. Western blotting

For Western blotting, 7,5% polyacrylamide gels containing 0,4% SDS were prepared and run in Tris/glycine buffer at 120 V (after Laemmli, 1970). Molecular weight markers were run in the outer lanes of all gels, cut off and stained with Coomassie brilliant blue (Sigma). The separated proteins were transferred (after Towbin et al., 1979) from gel to nitrocellulose paper (Hybond-C, Amersham) at 100-300 mA, approx. 40 V and 4 °C overnight in a Consort blotting chamber filled with blotting buffer (20 mM Tris, 150 mM glycine, 20% methanol, pH 8,3). Transblotted molecular weight markers were cut off, washed in 0,1M phosphate buffer for 10 mins, stained with 0,1% Indian Ink and 0,1% Tween in phosphate buffer for 1½ hr and rinsed in distilled water.

2. Detection of tyrosinase using polyclonal antibodies

All steps were carried out on a rotary shaker at room temperature. Filter was :

1. Blocked for 3 hr in Tris buffered saline (TBS), 3% fat-free milk, 3% Tween, 1:100 goat serum.
2. Washed twice for 15 mins in TBS, 3% milk.
3. Incubated overnight in rabbit anti-mouse tyrosinase primary antibody, diluted to 1:500 in TBS, 3% milk (polyclonal antibody 3819, Dr V.J. Hearing).
4. Washed twice in TBS, 0,05% Tween for 15 mins.
5. Incubated for 30 mins in biotinylated goat anti-rabbit IgG secondary antibody (Vectastatin ABC kit). Diluted 1 drop in 20 mls TBS, 0,05% Tween.
6. Washed twice for 15 mins in TBS, 0,05% Tween.
7. Incubated for 30 mins in 20 ml TBS with 4 drops each of solutions A (avidin DH) and B (biotinylated horse radish peroxidase H). A and B were pre-mixed 30 mins prior to use.
8. Washed twice for 15 mins in TBS.

9. Incubated in peroxidase substrate, 2 ml of 3 mg/ml chloronaphthol in methanol, 10 ml TBS, 100 μ l 1M imidazole, 6 μ l 30% hydrogen peroxide until bands became clearly visible.

A polyclonal guinea pig anti-chick tyrosinase antibody (Cornelia Stander) was also used in step 3, diluted 1:100 without goat serum. In this case the secondary antibody in step 5 was a peroxidase-conjugated rabbit anti-guinea pig IgG (Zymed), diluted 1:300. Steps 6 and 7 were omitted when using this secondary antibody.

3. Preparation of nick-translated probe

PMT4 template DNA (1 μ g, in pBR322, Shibahara et al., 1986) was linearised with Hind III and then mixed with 2,5 μ l nick-translation buffer, 0,2M dATP, dGTP and dTTP, 5 μ l 32 P-dCTP (specific activity **, Amersham) in 25 μ l. This mixture was partially digested with a 1/10 000 dilution of DNase I (1 mg/ml) for 10 mins and then the nicks repaired with 1,5 u/ μ l DNA polymerase I for 1 hr at 15°C (after Rigby et al., 1977). Unincorporated 32 P-UTP was removed by passing the reaction mix over a 10ml bed volume sephadex G50 column, buffer Tris/EDTA (10mM/0,1mM, pH 7,5). Radioactivity was monitored with a Geiger counter and the first peak collected. The second peak contains unincorporated nucleotides and was discarded. Collected probe was then precipitated with ethanol, sodium acetate and 1 μ l tRNA. Once probe DNA had been precipitated and resuspended, 1 μ l was counted in 10 ml toluene scintillant with a scintillation counter programmed for 1 min counts on the 32 P channel. Probes routinely gave $>1 \times 10^7$ cpm/ μ g DNA.

4. Preparation of multiprime probe

MTY811 murine tyrosinase (Kwon et al., 1988) template DNA (25 ng) was denatured by boiling for 2 mins and then was added to 10 μ l multiprime buffer (containing other deoxynucleotides), 5 μ l primer (containing random hexanucleotides and bovine serum albumen), 5 μ l 32 P-dCTP and 2 μ l Klenow fragment of DNA polymerase I in 50 μ l

on ice. This reaction mixture was incubated for 3-24 hr at 37°C in a water-bath and then unincorporated nucleotides were removed using a sephadex column as above. PMT4 insert was also cut out, electrophoresed and electroeluted from the gel fragment prior to use as multiprime template.

5. Preparation of ^{35}S and digoxigenin labelled riboprobe

pGEM containing 580 bp (referred to as pmc0,5) of the 5' end of exon 1, pmcTYR-10217 (full length mouse tyrosinase cDNA, 1,9 kb, Ruppert et al., 1988) or pGEM containing a smaller 100bp fragment (from exon 3, referred to as pmc0,1) were linearised with EcoRI. Linearised template (1 μg of pmc0,5, 4 μg of pmc0,1) was added to 4 μl 5x transcription buffer, 2 μl 100mM DTT, 0,5 μl placental ribonuclease inhibitor, 10mM CTP, ATP and GTP, 0,36 mM UTP, 5 μl ^{35}S -UTP (10 $\mu\text{Ci}/\text{ml}$, specific activity 250 mCi/mmol, Amersham) and 1 μl SP6 polymerase (after Melton et al., 1984). In the case of DIG-labelled probes, the UTP was added as a 3,5 mM DIG-UTP:6,5 mM UTP mix. The reaction mixture was incubated at 37°C for 1½ hr in a water-bath. Thereafter, template DNA was removed by digesting the mixture with 1 μl DNase (RNase-free) for 15 min at 37°C and the enzymes removed by phenol/chloroform extraction. ^{35}S probe was precipitated with 6M ammonium acetate and ethanol. DIG probe cannot be phenol extracted and template DNA was therefore not removed. Instead, probe was precipitated with 4M LiCl which specifically precipitates RNA.

6. Hybridization of Northern blots

a. With ^{32}P nick-translated PMT4

All steps were carried out in a shaking waterbath.

Prehybridization - 2 hr at 42°C.

- 40% deionised formamide
- 25% 20x SSC
- 5% 100x Denhardt's solution (2% each
polyvinylpyrrolidone, Ficoll and BSA.
- 2% 1M Tris, pH 6,8
- 0,1% SDS
- 1% 10 mg/ml salmon sperm DNA

Hybridization

- overnight at 42°C.
- probe was denatured in boiling water for 90
sec and held on ice.
- 1×10^6 cpm/ $\mu\text{g}/\text{ml}$ hybridization mix probe was
added to a packet containing the Northern
blot
filter.
- sealed packet was weighted down and
incubated.

Posthybridization washes -2x 20 min in 2x SSC, 0,1% SDS at r.t.
 -2x 20 min in 0,2x SSC, 0,1% SDS at
 42°C/60°C

Exposure -air dry filter and mount on filter paper.
 -expose to a preflashed X-ray film at -80°C for ± 7
 days

b. With ^{32}P multiprime MTY811

Prehybridization and hybridization as above.

c. With DIG-pmc0,5 riboprobe

All steps were carried out in a shaking waterbath.

Prehybridization - 2 hr at 65°C

- 50% deionised formamide
- 5xSSC
- 2% blocker
- 0,1% sarcosyl
- 0,025 SDS

Hybridization

- overnight at 65°C
- 10 µl DIG-pmc0,5 in 4 ml prehyb. buffer

Washes

- 2x10 min in 2xSSC, 0,1% SDS at r.t.
- 1x20 min in 0,1xSSC, 0,1% SDS at 65°C

Blocking

- 30 min in maleic acid, blocker, 2% NSS, r.t.
- 30 min in 1:10 000 alkaline phosphatase-conjugated polyclonal sheep anti-DIG antibody in blocking solution at r.t.

Washes

- 1x5 min in blocking solution
- 4x5 min in maleic buffer, Tween.
- 1x5 min in buffer 3

AMPPD

- 1x5 min at 10 µl/ml AMPPD in buffer 3

Filter was lightly blotted, sealed in a plastic packet, incubated overnight in the dark at 4°C and then exposed to a pre-flashed X-ray film for 30 min at r.t.

7. In situ hybridization

Section pretreatment

1. Rehydration

Sections were dewaxed in 2x10 min Xylol, 1x1 min each in 100% ethanol, 90% ethanol and 70% ethanol, and airdried. At all steps, 70% ethanol was buffered with ammonium acetate (0,3M, pH 8).

2. Proteinase K digestion

Sections were digested with 100 μ l/section 1 mg/ml proteinase K in buffer (50mM Tris-HCl, 10mM EDTA, 10 mM NaCl, pH 7,4) for 30 min at 37°C. Cell monolayers were digested for 15 min only.

RNase treatment

Control slides were incubated with 100 μ l/section 40 μ g/ml RNase A at 37°C for 30 min.

3. Acetylation

Proteinase K and RNase A were rinsed off with DEPC-treated water and the slides buffered in 0,1M triethanolamine in water. Acetic anhydride was added to give a final concentration of 0,25%, mixed gently and the slides were acetylated for 10 mins.

NOTE: Steps 1-3 were used for all tissue and probe types. The following steps apply to ³⁵S labelled probes and wax-sections only.

Prehybridization

Slides were dehydrated through 70%, 90% and 100% ethanol (1 min each) and air dried. Slides were incubated in 100 μ l/section prehybridization mix for 1 hr at 42°C.

Prehyb mix: 50% deionised formamide

0,5g dextran sulphate

50 μ l Tris (1M, pH 8)

10 μ l EDTA (0,5M)

50 μ l Denhardt's solution

50 μ l dithiothreitol (1M)

50 μ l salmon sperm DNA (10 mg/ml)

25 μ l tRNA (10 μ g/ml)

565 μ l water

Hybridization

Prehybridization mix was tipped off, the sections were covered with 20 μ l hybridization mix under parafilm and incubated overnight at 42 °C. Sulphur probe was used at 1×10^5 cpm/20 μ l prehyb mix. The slides were incubated flat on 4xSSC-soaked paper towel in a sealed Tupperware container with a humidifying petri dish containing formamide/4xSSC (1:1).

Posthybridization washes

- 2x30 min washes with 4xSSC at r.t.
- 30 min digestion with 40 μ g/ml RNase at 37°C
- 1x30 min wash in 2xSSC at 37°C
- 1x30 min wash in 0,1xSSC at 37 °C

Emulsion coating

Slides were dehydrated through 70%, 90% and 100% ethanol and air-dried.

All steps were carried out in a clean dark-room, light filtered with a Kodak dark brown filter. LM-1 emulsion (Amersham) was melted in a 43°C waterbath and poured into an acid-washed, glass slide-dipping chamber (made from a flattened 5 ml measuring cylinder). Slides were dipped vertically, drained onto tissue paper and laid flat on a 4°C cooling slab for 10 mins. Thereafter slides were transferred to a wooden slide rack and air-dried for 2 hr. Exposure took place in light-tight boxes containing silica gel at -80°C.

Processing conditions

After 2-3 weeks, slides were warmed to room temperature and developed in Ilford Phenisol developer for 5 min. Slides were passed through a stop solution of 0,5% acetic acid and then fixed for 8 min in fresh 30% sodium thiosulphate. After washing for 20 min in deionised water, slides were lightly counterstained with Mayer's haematoxylin or 1% methylene blue, dehydrated and mounted with DPX.

Table 2.2

		Tyrosinase activity in cpm/ μ g				
		4 day	5 day	6 day	7 day	8 day
Skin	expt 1	905	810	1955	3815	5465
	expt 2	2648	2200	2716	4032	4296
	expt 3	1385	1585	1785	2947	3563
	expt 4	653	-	952	1222	5338
Organs	expt 1	1615	550	2530	1065	2220
	expt 2	2144	3316	2968	2600	2756
	expt 3	1572	1578	1475	1625	1678
	expt 4	1752	-	1149	669	473
Skin	expt 5	7392	7852	11760		
	expt 6	984	1132	1858		
	expt 7	952	1320	3586		
Organs	expt 5	2437	2603	2619		
	expt 6	984	624	1164		
	expt 7	628	470	244		

Skin and organ homogenate was prepared from 4-8 day BAXNHR chick embryos, adjusted to equal protein amounts and the tyrosinase activity assayed by measuring the incorporation of ^{14}C tyrosine into insoluble melanin. In experiments 5-7, tissue homogenate was first purified by DEAE Sephacel column chromatography to concentrate tyrosinase.

Data for Fig 4.4 and Fig 4.5

Region		Epi	Episer	Xm	Xmser	med	medser
1	mean	21.1	34.5	39.0	21.4	35.7	31.5
	SEM	5.35	2.40	7.52	4.4	3.35	3.19
2	mean	28.3	39.7	36.5	38.6	25.7	35.3
	SEM	2.92	2.80	5.00	3.2	2.69	3.71
3	mean	47.6	25.7	24.5	52.6	38.6	33.2
	SEM	4.57	0.68	4.19	6.90	1.40	4.95

Mean melanocyte counts are given for each region, n=3. See chapter 2 for experimental details. p values are as follows:

	Region 1	Region 2	Region3
epi vs episer	p=0,048	p=0,025	p=0,004
epi vs Xm	ND	ND	p=0,005

Data for Fig 4.7

Region 1

	Epid	bFGF	EGF	NGF	RA	Med
Mean	60.3	17.6	22.4	38.2	40.9	47.1
SEM	4.77	3.69	9.96	7.30	3.42	5.00

Mean melanocyte counts are given for each region, n=5 (epidermis n=4). See chapter 2 for experimental details. p values for points of interest are as follows:

Region 1

Epidermis vs

	bFGF	EGF	NGF	RA	Med
p	<0.001	0.01	0,03	0.006	0.07

All p values obtained using unpaired Student T test
EPISTAT Statistical program, version 3.0, 1984.