Melanogenesis of avian neural crest cells in vitro is influenced by external cues in the periorbital mesenchyme

STEVEN CAMPBELL*

MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, UK

*Current address: Department of Medical Microbiology, Stopford Building, Manchester University, Oxford Road, Manchester, M13 9PL, UK

Summary

Avian melanoblast differentiation was studied by explantation of the neural tube and periorbital mesenchyme. Outgrowths from the mesenchymal explants consisted of a mixed population of melanocytes, melanoblasts and fibroblasts, whilst typical neural crest populations migrated from the neural tube explants. Cells that differentiated within explants of mesenchyme, produced elongate black eumelanosomes of normal ultrastructure which were identical to those found in the ocular connective tissues. However, melanoblasts that differentiated within outgrowths of mesenchyme or neural tube produced round brown melanosomes of highly abnormal ultrastructure. Some of these melanosomes contained a few disorganised melanosomal filaments whilst others had granular melanin with complete absence of filaments. This abnormality of phenotype was invariant over a range of culture conditions that modified cell behaviour, the timing of differentiation and the abundance of the pigmented cells. These experiments suggest that local factors in the mesenchyme are essential for the induction of melanogenesis in the presumptive connective tissue melanocyte.

Key words: periorbital mesenchyme, avian neural crest, defective melanogenesis in vitro.

Introduction

Neural-crest-derived melanocytes are found within the superficial epithelia and internal connective tissues and mesenteries of the pigmented vertebrate embryo (Horsfield, 1950; Noden, 1978). Within the avian embryo, melanocyte differentiation takes place during the course of organogenesis. The process of differentiation is therefore intimately associated with the development of the 'host tissue' at the terminal site of crest cell migration (Weston, 1970). Organ culture experiments have demonstrated that interaction of the host tissue with invading neural crest cells determines the nature of the derivative formed; as well as particular characteristics of its phenotype. In the case of the neurone, for example, coculture experiments have demonstrated that interaction of the dorsally migrating crest cells with the neural tube, somites and notochord (Cohen, 1972; Norr, 1973) are important in the induction of neuroblasts from their pluripotent precursors (Sieber-Blum & Cohen, 1980). Subsequent expression of particular phenotypic characteristics, such as neurotransmitter type (Patterson, 1978), is in turn under the extrinsic control of the tissue environment in which differentiation occurs (Le Douarin, 1980). During the course of melanocyte differentiation of the crest cell it seems likely that similar extrinsic controls may determine the fate of the pluripotent melanoblast precursor cell (Loring et al. 1982) and, subsequently, modulate melanosome production during the later stages of differentiation. The influence of the epidermis upon melanosome production has been clearly established by recombinatorial experiments with mouse skin and neural crest cells (Lamoreux & Mayer, 1975; Stephen-son & Hornby, 1985), and dermis and epidermis from animals of different genotype (Poole, 1975). Although the nature of this control remains obscure (Kinsley et al. 1975; Galbraith & Patriginani, 1976), a complex interaction between dermis and epidermis appears to take place (Poole, 1975), allowing certain pigment patterns to be expressed. Yet even when the integumental melanocyte produces a yellow pheomelanin by such interactions (Lamoreux & Mayer, 1975), the extrafollicular and extraepithelial melanocyte has been found to produce black eumelanin. Melanogenesis may therefore be controlled differently within the epithelial and extraepithelial environment. In vitro studies suggest that short range interactions between isolated neural crest cells and the extracellular matrix may cause the induction of melanocyte differentiation in the developing mesenteries and connective tissues (Derby, 1982; Perris & Lofberg, 1986; Perris et al. 1988). In order to
Melanoblast differentiation within the ocular connective tissues differs significantly from that occurring in the integument, for in this location the neural crest cells do not become intermingled with the keratinocytes of the ‘host’ environment, as they do during invasion of the developing epidermis (Teillet, 1971). The uveal melanoblasts differentiate from the migratory neural crest cells which colonize the internal spaces of the cephalic region. These internalized crest cells form a transitional ectomesenchyme (Horstadius, 1950), from which the melanocytes and fibroblasts of the cephalic connective tissues differentiate (Le Lièvre, 1978; Noden, 1978).

This study considers whether local factors within the mesenchymal environment influence the course of melanogenesis in the neural-crest-derived periorbital mesenchyme (POM). The experiments reported here were designed to compare the differentiation of premigratory crest cells in neural tube cultures (Cohen & Konigsberg, 1975; Loring et al. 1988) with their premigratory derivatives obtained by POM explantation (Campbell & Bard, 1985). Segments of the anterior mesenchyme were removed prior to the development of the scleral papillae and ossicles (Coulombre et al. 1962), and before the onset of melanogenesis (Campbell & Bard, 1985). Melanocytic and fibroblastic differentiation was then followed in cells that had migrated from the explants onto the substratum, and compared to those that differentiated in situ. Cell colour and melanosome ultrastructure were used as markers of melanocyte differentiation, and cell coat production (McBride & Bard, 1979) was used to identify differentiated fibroblasts.

Materials and methods

Gallus gallus embryos

Fertile eggs were supplied from a closed flock of randomly mated, J-line Brown Leghorn fowl kept at the Poultry Research Centre, Roslyn, Scotland. The integumental pigmentation of the adult and newly hatched birds corresponded to the ‘wild type’ Brown Leghorn/Red Jungle Fowl from which domestic fowl have probably been bred (Hutt, 1949). The adult male birds had areas of red-brown and black plumage and thus expressed pheomelanin and eumelanin respectively. Preliminary histological observations of the feather-germ melanocytes in the later half of embryogenesis demonstrated complex temporospatial variation of melanosome colour. These ‘wild type’ birds were therefore not considered useful for the examination of integumental melanogenesis.

Isolation of neural crest cells, periorbital mesenchyme, corneal fibroblasts, dorsal root ganglia

Premigratory trunk neural crest cells were isolated using the neural tube explantation technique (Cohen & Konigsberg, 1975). Isolation of the neural tube was performed according to the dissecting methods of Le Lièvre (personal communication), using a trypsin–pancreatin dissociation mixture (Bee & Thorogood, 1980). In some cases, whole trunk segments of five to six somites in length were used in order to maintain the crest cells within the embryonic environment. These segments consisted of ectoderm, neural tube, notochord and endoderm. Periorbital mesenchyme was isolated free of superficial ectoderm and pigmented retinal epithelium using the same dissociation mixture as described previously (Campbell, 1984; Campbell & Bard, 1985). Anterior eye segments were isolated and stripped of vitreous, lens and retina (care being taken to remove the entire pigmented retinal epithelium). The remaining mesenchyme and cornea were placed in a 0.2ml drop of trypsin–pancreatin (Difco 1:250 and Sigma) in Tyrode's solution without magnesium or calcium, prepared after the method of Bee and Thorogood (1980), for 6–8 min at room temperature, after which the dish was flooded with 2ml of fetal calf serum. The latter treatment was followed by blistering of the superficial epithelium, which facilitated its detachment with a bent microscalpel. Squares of mesenchyme (0.3–0.5 mm in length) close to, but not including, the corneal limbus, and similar pieces of cornea itself were isolated with small razor blade knives. Subsequent examination of all explants with an inverted microscope revealed no contamination of the mesenchyme by pigmented retinal epithelium. Older pieces of cornea were obtained in a similar fashion except that the endothelium was removed and the isolated stroma was reincubated in trypsin–pancreatin to facilitate cell outgrowth. Dorsal root ganglia were excised without enzyme treatment.

Explanation and culture

Explants were placed in thin films of growth medium in 30 mm nonvented Petri dishes (Nunclon) and sealed with silicone grease to allow humidification of the culture vessel and placed in an incubator at 37°C. After a period of 1–4 h a further 1:5 ml of medium was added. On some occasions, whole trunk segments were placed onto rat tail collagen gels (kindly provided by Dr Duncan Davidson) with or without trypsin–pancreatin treatment.

Explants were cultured in Ham’s F10 (Gibco) growth medium supplemented with 1 mM-glutamine, 10 mM-sodium bicarbonate, 20 mM-Hepes buffer, 100 units ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, fetal calf serum (FCS) or horse serum (HS) to a final concentration of 5, 10 or 15 %, and chick embryo extract (CEE) to a final concentration of 2 % or 5 %, as specified. A 50 % whole chick embryo extract was prepared after the method of Sieber-Blum & Cohen (1980), as described elsewhere (Campbell, 1984). The biological activity of the extract was tested by culturing 7- and 10-day dorsal root ganglion on rat-tail collagen-coated glass coverslips. Addition of extract to a final concentration of 2 % produced a marked increase in neuronal outgrowth like that observed by Cohen & Konigsberg (1975). The growth medium was changed after a period of 5 days and subsequently every 3 to 4 days. The presence of one neural tube per dish did not acidify the culture medium, indeed more frequent changes of medium caused reduced outgrowth from neural tube explants.

Microscopy and histochemistry

 Cultures were photographed using oblique or phase illumination on a Wild m40 inverted-phase microscope. Glycosaminoglycan-containing cell coats (McBride & Bard, 1979) were visualized as pericellular zones from which marker particles (Red Blood Cells) are excluded. The hyaluronidase sensitivity of the coats was examined by incubating the cultures in 50 l.u. ml⁻¹ bovine testicular hyaluronidase (Sigma) in saline at 37°C for 15 min prior to the addition of the marker particles. Tyrosinase (Dopa-oxidase) activity was
Melanocyte differentiation

detected by incubating glutaraldehyde-fixed cultures with L-Dopa (Sigma), or D-Dopa in the control, for a period of 4 h at 37°C, as described previously (Campbell & Bard, 1985). Melanin was distinguished from lipofuscin by bleaching with hydrogen peroxide or potassium permanganate, and staining in Nile Blue Sulphate, differentiated in ethanol. Specimens were observed with a Leitz Ortholux II microscope using excitation of less than 500 nm and long pass filtration transmitting over 515 nm. Melanin does not autofluoresce although lipofuscin does (Pearse, 1960).

Tissue melanocytes were examined by routine histological sectioning, followed by haematoxylin and eosin staining, or whole mounting after dehydration and clearing in xylene. Whole-mounted tissue was examined using Nomarski optics on a Zeiss Universal microscope.

Transmission electron microscopy

Cultured specimens and tissue pieces were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 4 and 20 h, respectively, postfixed in 1% osmium tetroxide, and routinely dehydrated and embedded in Araldite. Ultrathin 'silver' sections were cut and stained with uranyl acetate and lead citrate, and viewed on a Philips 300 microscope.

Results

Melanocyte differentiation within the Brown Leghorn embryo

The carcasses of 14- and 18-day embryos were examined directly at high power on the dissecting microscope (×120), with the inverted-phase microscope, or after whole mounting by Nomarski optics, in order to record the colour and tissue distribution of melanocytes in this breed of fowl. Within the ocular epithelia and all mesenchymal locations, the melanocytes were black in colour (Fig. 1). The internal melanocytes were distributed throughout the dermis, the connective tissues of the uvea and those surrounding the skeleton and skeletal muscles, and throughout the adipose tissue. Within the eye the onset of pigmentation varied according to location. Pigmented cells were first apparent within the stroma or epithelium of the corneal limbus at day 13 (stage 39, Hamburger & Hamilton, 1957) whereas those in the posterior uvea were already present on day 8 (stage 33).

Fig. 1. Dendritic black melanocytes characteristic of the cephalic connective tissues from whole-mounted pericranial connective tissues of the 18-day embryo. Nomarski optics, ×485, scale bar = 20 μm.

Fig. 2. A trunk neural tube cultured in medium supplemented with 10% F.C.S. and 5% C.E.E. after 36 h culture showing the explant (left) and the outgrowth of neural crest cells which have begun to aggregate. Phase optics, ×181, scale bar = 50 μm.

Fig. 3. An isolated population of overlapping crest cells after 13 days in culture in medium supplemented with 5% F.C.S. and 2% C.E.E., just after the onset of melanogenesis. Phase optics, ×189, scale bar = 50 μm.

Fig. 4. After 6 days of culture in medium supplemented with 10% F.C.S. the crest cells remained largely unpigmented. Many melanoblasts were present when visualized by L-Dopa staining. Oblique bright-field illumination, ×138, scale bar = 50 μm.

Fig. 5. After 5-5 days in 10% F.C.S. and 5% C.E.E. the crest cells have aggregated (left) and begun to pigment. Phase optics, ×170, scale bar = 50 μm.

Fig. 6. A mixed outgrowth obtained by maintaining the neural tube in situ, contains a small pigmented aggregate (arrow), refractile neuronal cell bodies with branched dendrites, some of which are adherent to a vesiculated cell of fibroblastic morphology. Phase optics, ×187, scale bar = 50 μm.
Neural tube cultures

Trunk neural tubes, or their dorsal segments in the cephalic region (Noden, 1975), were excised from all axial levels of the embryo and explanted onto tissue culture plastic. Crest cells began to migrate from the explant onto the substratum within the first day of culture (Fig. 2). In the majority of cases, the explants were removed mechanically after the method of Cohen & Konigsberg (1975) leaving behind a population of proliferative stellate cells. Differentiation was examined in a variety of culture media (Table 1), to determine pigment colour in conditions that affected differentiation and cell behaviour. The melanocytes that differentiated in vitro were stellate in shape (Fig. 3) and contained a pigment that was always light brown in colour, and thus very different from that found in vivo. Several histochemical tests were therefore performed in order to establish that the pigmented cells were melanocytes rather than aberrant crest derivatives which contained a neuronal lipofuscin (Nandy et al. 1978). The cultures stained positively with L-Dopa (Fig. 4) indicating the presence of tyrosinase activity. The pigment could be bleached with hydrogen peroxide or potassium permanganate even after Nile Blue Sulphate staining, and did not autofluoresce as lipofuscin is reported to do (Pearse, 1960).

In spite of the uniformity of pigment colour, the timing of differentiation, the abundance of the melanocytes and the social behaviour of the pigmented cells were all influenced by the culture conditions (Table 1). With higher concentrations of serum, differentiation was precocious with respect to that occurring in the embryo, and within the normal range at the lower supplement levels used. In the absence of embryo extract, the abundance of melanocytes was severely reduced as previously reported (Maxwell, 1976), although many tyrosinase-positive melanoblasts were present (Fig. 4). Melanocyte social behaviour appeared to be modulated independently of the timing of differentiation. At high supplementation concentrations, the unpigmented stellate cells began to aggregate within the first 48 h of culture on the surface of the neural tube and over the culture substratum. No pigmentation was observed at this stage. After a further 24 h, cells within the explant began to form pigment (Fig. 9). In all culture conditions examined (Table 2), the pigment formed within the explant was black in colour and distinct from the brown pigment found in neural tube cultures. The abundance of melanocytes appeared similar to that observed in the ciliary body of the late embryo and distributed throughout the explant when viewed in vertical section (not shown). A minority of those cells that differentiated within the explant started to migrate into the surrounding outgrowth 1 day after the onset of pigmentation. When many melanocytes migrated from the explants, and were thus in close contact, they began to aggregate rapidly (within 10 h).

Two other cell types were found within the outgrowth, as well as the migrating black melanocytes. Toward the periphery of the outgrowth, brown pigmented cells appeared during the third day of culture and then rapidly aggregated (Fig. 10). In order to confirm that the brown cells differentiated after migration onto the substratum, 6 explants were removed

<table>
<thead>
<tr>
<th>Table 1. Neural tube cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cultures</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>3</td>
</tr>
</tbody>
</table>

et al. 1980), were observed at low frequency around the edge of the outgrowth. If the explants were left in place for more than 48 h in the presence of embryo extract, neuronal processes emerged from the tube and isolated neurones were also found in the outgrowth (Fig. 6). Three morphological cell types (melanocyte, fibroblast and neurone) differentiated from the neural crest in the culture conditions used.

Trunk segment culture

Whole trunk segments (Fig. 7) were explanted onto collagen gels without enzymic digestion, or after incubation in trypsin–pancreatin for several minutes. Brown pigment, indistinguishable in colour from that found in neural tube cultures, was observed in cells within the explant (Fig. 8), and in isolated or aggregated cells in the outgrowth. Enzymic treatment did not alter the pigment colour or distribution.

Periorbital mesenchyme cultures

Segments of anterior mesenchyme were excised from embryos of 5–8 days (stage 27–32) and explanted onto tissue culture plastic (Campbell & Bard, 1985). Within the first 24 h, cells began to migrate from the explant onto the culture substratum. No pigmentation was observed at this stage. After a further 24 h, cells within the explant began to form pigment (Fig. 9). In all culture conditions examined (Table 2), the pigment formed within the explant was black in colour and distinct from the brown pigment found in neural tube cultures. The abundance of melanocytes appeared similar to that observed in the ciliary body of the late embryo and distributed throughout the explant when viewed in vertical section (not shown). A minority of those cells that differentiated within the explant started to migrate into the surrounding outgrowth 1 day after the onset of pigmentation. When many melanocytes migrated from the explants, and were thus in close contact, they began to aggregate rapidly (within 10 h).

Two other cell types were found within the outgrowth, as well as the migrating black melanocytes. Toward the periphery of the outgrowth, brown pigmented cells appeared during the third day of culture and then rapidly aggregated (Fig. 10). In order to confirm that the brown cells differentiated after migration onto the substratum, 6 explants were removed

<table>
<thead>
<tr>
<th>Table 2. Periorbital mesenchyme cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cultures</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>19</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>6</td>
</tr>
</tbody>
</table>
after 24 h of culture, and before the onset of pigmentation in the explant. Differentiation of the brown cells proceeded in an identical fashion in the absence of the explant. Variations in the culture medium (Table 2) had no obvious effect on the colour of the pigment formed, upon melanocyte frequency, or upon the timing of differentiation.

The most abundant cell type within the outgrowth was fibroblastic in shape, accumulated osmophilic lipid vesicles, and continued to proliferate and outgrow for at least 4 weeks. In order to substantiate that these unpigmented cells had acquired a mesenchymal character their ability to synthesize cell coats was examined. The fibroblastic cells from mesenchymal explants possessed well-formed cell coats (Fig. 11) which were sensitive to hyaluronidase activity. Glycosaminoglycan coats were also present on fibroblasts (Table 3) from the cornea and dorsal root ganglia. This supports previous observations that some fibroblasts possess cell coats (McBride & Bard, 1979). Coats were absent from the stellate neural crest cells, neurones, and the melanocytes (Fig. 12) which were found in mesenchymal or neural tube cultures.

**Table 3. Presence of cell surface coats**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Source</th>
<th>Donor age</th>
<th>Time in culture</th>
<th>Presence of the coat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts</td>
<td>P.O.M.</td>
<td>6-8 days</td>
<td>7 days</td>
<td>Present</td>
</tr>
<tr>
<td>Melanocytes</td>
<td>Neural tube</td>
<td>36 hours</td>
<td>7 days</td>
<td>Absent</td>
</tr>
<tr>
<td>Neurones</td>
<td>P.O.M.</td>
<td>5-8 days</td>
<td>7 days</td>
<td>Absent</td>
</tr>
<tr>
<td>Neural crest cells*</td>
<td>Neural tube</td>
<td>36 hours</td>
<td>7 days</td>
<td>Absent</td>
</tr>
<tr>
<td>Neurones</td>
<td>Ganglia</td>
<td>36 hours</td>
<td>7 days</td>
<td>Absent</td>
</tr>
<tr>
<td>Neurones</td>
<td>Neural tube</td>
<td>36 hours</td>
<td>7 days</td>
<td>Absent</td>
</tr>
<tr>
<td>Neurones</td>
<td>Ganglia</td>
<td>7 days</td>
<td>14 days</td>
<td>Absent</td>
</tr>
</tbody>
</table>

*In the absence of embryo extract where little pigment formation occurred.*

In order to provide an explanation for the observed colour variation between the black cells within the mesenchyme in vivo and in vitro, and the brown cells that differentiated in isolation.

**Melanogenesis in vivo**

The highly pigmented uveal tissues, which arise from the periorbital mesenchyme, were examined at 14 and 18 days of embryogenesis. The melanocytes of choroid,
sclera, ciliary body, iris and corneal limbus contained elongate melanosomes in various stages of formation (Figs 13 and 14), which corresponded to the normal eumelanogenic sequence (Jimbow et al. 1979) within the embryonic fowl.

The limbal epithelium of the cornea was a particularly useful location to observe the process of melanogenesis because it was a small well-defined area in which pigment could be observed by light microscopy. Round featureless premelanosomes, elongate intermediate stages (Fig. 14) containing arrays of melanosomal filaments and long fully melanized electron-dense organelles were all found in close proximity to each other. No evidence could be obtained for the existence of brown pheomelanosomes (Jimbow et al. 1979) in ocular connective tissue, ocular epithelia or the pigmented retinal epithelium, at either age examined.

One day after scant traces of pigment were first observed in the limbal epithelium of the cornea by light microscopy (see above), a mixture of organelles ranging from premelanosome to highly melanized structures were found (day 14), which indicated that the melanization process could be completed in one day.

Melanogenesis in neural tube cultures

The melanosomes present within the premigratory brown melanocytes were examined in the different growth media used after various times of culture. No differences were detected between the culture regimes although significant changes occurred over long time
periods of incubation. The first stages of melanosomal formation appeared normal (Jimbow et al. 1979). Vacuolar premelanosomes (Fig. 15) containing smaller vesicular inclusions were found in close proximity to coated vesicles and Golgi cisternae from which vesicle release was observed. Many cisternal stacks and associated premelanosomes were associated with the centrioles in a similar fashion to that described in vivo (Hogan et al. 1971). The later stages of melanogenesis were, however, different from that observed in vivo. Melanin deposition proceeded so slowly that the melanosomes were only partially melanized even after 2 weeks in culture and their substructure was also highly abnormal (Fig. 16). Many melanosomes contained a reduced number of melanosomal filaments, which intertwined around each other in an abnormal fashion (Fig. 17), instead of forming a dense linearly aligned array like that normally found in elongating organelles in vivo. Other cultured cells contained organelles with a partially melanized granular matrix from which the melanosomal filaments appeared to be almost entirely absent (Fig. 18). Although these melanosomes were brown in colour, their ultrastructure suggested that the extramesenchymal melanocytes contained organelles with a degenerate form of normal black eumelanosomes. Occasionally the melanosomal membrane was continuous between organelles, and giant melanosomes (Fig. 19) or large accumulations of melanosomes were present within the one inclusion, although no myelin bodies, characteristic of autophagocytosis (Jimbow et al. 1974), were found.

After an extended period in culture (3–5 weeks), the melanosomes became more distinct in the light microscope. This was accompanied by an increase in the melanization of individual organelles (Fig. 20), although no elongation was observed to occur even over such long time intervals.

**Melanogenesis in periocular mesenchyme cultures**
Mesenchymal explants were first examined ultrastructurally 1 day after the onset of pigment formation. At this time, cells within the explants contained numerous organelles in the various stages of melanogenesis (Fig. 21). These melanosomes appeared indistinguishable from those observed in the uveal tissues. The melanocytes that started to migrate after the onset of melanogenesis were those positioned above the fibroblasts at the edge of the explant. These cells, which appeared black by light microscopy, contained a mixture of organelles at various stages of formation. The pattern of melanogenesis within them seemed intermediate between the black melanosomes observed in connective tissue and the brown melanosomes found in premigratory crest cells. Elongate well-melanized organelles, and others with a less-melanized granular substructure, were present within the same cell (Fig. 22). Brown melanocytes found at the periphery of the explant were ultrastructurally indistinguishable from those present in the neural-tube-derived cells. They contained granular melanosomes (Fig. 23) and other organelles that contained a reduced amount of disorganized melanosomal filaments.

**Discussion**

The results presented here demonstrate that the mesenchymal environment has a profound influence on melanoblast differentiation and suggest that local factors within the mesenchyme are essential for the complete induction of black eumelanosomes. It seems likely that these factors were absent from the somitic and lateral mesoderm of the early embryo because the trunk segment explants did not support the development of black pigment. Furthermore, it is evident that the pigment-producing cells need to be continuously exposed to such local influences, because those cells that migrated from the explant after differentiation contained a mixture of melanosome types including those characteristic of the isolated cell. Although melanocyte-
stimulating hormone produces more rapid expression of melanin and greater commitment to melanocyte differentiation in vitro (Satoh & Ide, 1987), it seems very unlikely that the explants possessed or retained significant concentrations of soluble hormones. Direct melanocyte-fibroblast contact is probably not of prime importance, as melanocytes were frequently seen in close proximity to the fibroblasts within mesenchymal outgrowths. It seems more likely that the extracellular matrix (ECM) plays a role, for substratum-attached dermal ECM obtained from fibroblast cultures will stimulate melanocyte differentiation in premigratory crest cells (Derby, 1982). Similarly ECM from the subepidermal space of the axolotl embryo will stimulate tyrosinase activity (Perris & Lofberg, 1986; Perris et al. 1988). Indeed it seems reasonable to speculate that the highly pigmented tissues of the uvea not only lack a melanization inhibitory factor (Fukuzawa & Ide, 1988) but could contain matrix components which actively promote the differentiation of eumelanocytes.

Abnormalities of melanogenesis appear to be divorced from other aspects of melanocyte phenotype, for the elaboration of brown pigment does not seem to be linked to distinct in vitro behaviour patterns. Both black and brown cells aggregated in a fashion similar to that recorded by other workers (Twitty, 1945; Glimelius & Weston, 1981). The modulation of melanocyte social behaviour would therefore seem distinct from the control of melanogenesis in this system.

The isolated melanoblast of the Brown Leghorn embryo produced brown melanosomes that were distinct from those observed within the mesenchyme in vitro or within the embryonic ocular connective tissues and the black or brown feathers of wild-type birds.
(Jimbow et al. 1979). Three obvious defects existed in the process of melanosome formation. First, after formation of the premelanosome, its subsequent maturation was extremely slow in comparison to that occurring in the mesenchyme, which suggested a reduction in the rate of melanin synthesis. Second, the premelanosomes did not develop densely packed arrays of filaments. This phenomenon was probably related to the third defect; the absence of melanosomal elongation (Jimbow et al. 1979). Whether these differences in ultrastructure were accompanied by changes in the composition of the proteinaceous filaments (Zimmerman, 1982), and melamin heteropolymers (Prota, 1980) remains to be determined. Finally melanosomes occasionally aggregated together, or formed giant melanosomes, perhaps as a result of the membrane continuity which was sometimes observed between organelles.

In contrast to the pattern of melanogenesis described here quail neural crest cells grown in a very similar medium, and subcultured onto amnion basement membranes possessed or formed well-melanized organelles after a short time in culture (Erickson, 1987). This difference suggests that the factors influencing melanogenesis in vitro may be species specific. Nevertheless, only one other group of workers appear to have recorded the pigment colour produced by isolated postmigratory, or premigratory, fowl melanoblasts, and in that case the melanocytes also contained brown pigment (Greenberg & Pratt, 1977). However, in the example cited, the White Leghorn embryos used were likely to have been hypomelanotic (Jimbow et al. 1974), and so could not be relied upon to form typical melanosomes. Further work is therefore required to know whether embryonic melanoblasts from other breeds of fowl fail to produce eumelanosomes when removed from the influence of the epithelial, or connective tissue, environment.

Although this form of melanogenesis has not previously been observed in wild-type fowl melanocytes, there are two lines of evidence that suggest that the pattern of melanogenesis observed here was the result of defective gene expression in the isolated melanocytes. Melanocyte-specific mutations expressed within the fowl integument or mouse skin create abnormal melanosomes, which resemble the organelles observed in culture. The recessive b allele at the B locus in the fowl integument or mouse skin create abnormal-melanosomes. The recessive b allele at the B locus in the fowl integument or mouse skin create abnormal melanosomes, which are round in shape and have an apparent reduction in melanosomal development of the premelanosome, its subsequent maturation was extremely slow in comparison to that occurring in the mesenchyme, which suggested a reduction in the rate of melanin synthesis. Blastic differentiation of isolated crest cells, establishing that local external cues are required for the production of eumelanosomes by the extraepithelial melanocyte. However, the nature of these cues and their effect on gene expression have still to be described.

A summary of this work was presented to the IV European Workshop on Melanin Pigmentation. The author would like to thank Jonathan Bard for invaluable help and encouragement, and his comments on the manuscript, Christianne Le Lievre for tuition in embryological procedures, Allyson Ross for assistance with histology and EM, and Linda Bromley for commenting on the manuscript.

References


(Received 18 May 1989)