Birds Of A White Feather: A Congenital Hypopigmentary Disorder In The Chick

By Dominique Koubovec

Thesis presented for the Degree of Masters (Medicine) in

Cell Biology in the Department of Anatomy and Cell Biology in the

Faculty of Health Sciences

UNIVERSITY OF CAPE TOWN

March 1999

I, Dominique Johanna Barbara Maria Koubovec, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the work nor any part of it has been, is being, or is to be submitted for any other degree in this or any other university. I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate Signature Removed

March, 1999

Acknowledgements

Firstly, I would like to thank my supervisor, Professor Susan Kidson, for remarkable supervision, guidance and inspiration.

I would also like to extend a sincere thank you to Professor Rawdon, who supervised me temporarily, and who was always available with invaluable advice.

I owe a particular vote of thanks to my colleagues in the laboratory, who have in the past three years become my closest friends. A very special thank you to Toni Wiggins for her endless 'life-saving' support and boundless 'motherly' advice.

To my mother, a very special thank you for her patience and financial assistance throughout this degree.

To Geoffrey, many thanks for invaluable technical assistance.

Lastly, I am particularly grateful to Murray, who on so many occasions, bravely held the fort. Thank you for your technical advice and nine months of much endurance, support and most importantly, understanding.

I wish to dedicate this thesis to the memory of my dear younger brother, Rudi (17th June 1978 to 22nd September 1994), who died tragically. And to my loving father (12th December 1934 to 1st February 1997), who constantly encouraged me and who genuinely believed in my strengths.

Abstract

Dominant white, a mutation of the *I* gene, leads to amelanosis and is common to many commercial breeds of fowl, including Pile Games, White Plymouth Rocks and White Leghorns. Despite much investigation on the cellular mechanisms of *Dominant white*, its mode of action is still poorly understood. The aim of this study is to elucidate the molecular basis of amelanosis in WPR X PG chickens by addressing the following two questions: are melanocytes present in the skin regions and feather follicles in normal numbers of 8- to 13-day white chick embryos? If melanocytes are present in normal numbers, are they unable to synthesise pigment because of a defect in melanocyte differentiation?

Two approaches were used to answer the first question. MelEM, a monoclonal antibody, shown to react specifically to quail melanocytes, was found to be unsuitable for localisation of chicken melanocytes. Secondly, *in situ* hybridisation with *tyrosinase* and *tyrosinase related protein-2* (*TYRP2*) probes was carried out to quantitate the number of melanocytes at different stages of development. The results indicate that *tyrosinase* - and *TYRP2* -expressing melanocytes are present in 10-day white chick skin and feather buds in normal, if not greater numbers than in the control (Black Australorp) breed. This suggests that amelanosis is not due the failure of migratory melanoblasts to reach the developing feathers, nor is it due to the selective elimination of melanocytes during migration. The results further showed that with increasing developmental age (12- and 13-days), there is a decline in the number of *tyrosinase* - and *TYRP2* -expressing melanocytes in the white chick breed in comparison to the black breed. This suggests that white skin melanocytes either downregulate *tyrosinase* and *TYRP2* gene expression yet remain viable, or they undergo cell death. At 17-days, the results showed an absence of gene expression in both the black and white follicles due to the normal process of feather development.

Thus, although WPR x PG melanocytes are present in normal numbers in 10-day skin and feather follicles, they never melanise. To address this issue, black and white neural crest cells were cultured in conditions resembling their respective skin environments. Firstly, black neural crest cells grown in defined medium with either black or white skin extract were able to synthesise melanin. This suggests that white skin contains the appropriate signals necessary to induce melanogenesis of black melanocytes. This in turn suggests that the white melanocyte itself is intrinsically defective. To test this, white chick neural crest cells were grown in defined medium in the presence of black or white skin extract. The results showed that white cells were able to respond to signals in extracts of skin from both breeds and became melanised, suggesting that white melanocytes are not intrinsically defective. Due to the intricate nature of this study and

subsequent experimental limitations encountered, these contradictory results could not be completely resolved. However, a testable model in which the *I* gene is postulated to encode c-kit is presented.

Table of Contents

List of Figures

List of Tables

National Geographic (1998)

1. Introduction

"We thought it was a lump of snow," recalls Gerald Kooyman, a biologist at the Scripps Institution of Oceanography. He and his son, Carsten, were counting chicks, nearly 20 000 of them, in an emperor penguin colony near Cape Washington in December 1996. But the lump moved - it was a young amelanotic penguin, only its brown eyes were normally coloured... (National Geographic, 1998). White plumage accompanied by normally pigmented eyes is a prevalent characteristic amongst avians. "*Dominant white*", as the name implies, is a dominant mutation of the *I* gene, and is also common to many commercial breeds of fowl such as Pile Games (PG), White Plymouth Rocks (WPR) and White Leghorns (WL). Despite much investigation into the molecular mechanisms of the *Dominant white* mutation, the cause of this phenotype remains unresolved.

White Plymouth Rock x Pile Game chickens have normally pigmented eyes yet have no pigment in their feathers and skin, making them a useful experimental model for investigating the involvement of "*Dominant white*" in pigmentation. The purpose of this study, therefore, is to further our understanding of the cellular mechanisms leading to amelanosis in this white chicken, by building on previous studies and making use of recently cloned avian melanocyte-specific markers.

1.1 Introduction to pigment cell biology

The pattern of pigmentation in birds is remarkably varied and beautiful. Pigments of the chicken include two general types, the carotenoids and the melanins. The carotenoid, xanthophyll, gives the yellow colouration to the skin, fat and egg yolk, while the melanins are responsible for feather colouration and dark pigments of the eyes, skin and connective tissue (Smyth, 1990). Melanin is produced in pigment cells known as melanocytes, located mainly in the outer body covering and eyes. In chickens, melanocytes in the body skin are rare, but they are numerous within the epithelium of germinal tips of embryonic, neonatal or regenerating feathers (Bowers, 1988). An important function of melanin in birds is to provide a protective camouflage patterning against predators and to play a role in social communication.

1.1.1 Origin and migration of melanocytes

Melanocytes originate in the neural crest, a temporary embryonic structure formed from the dorsal folds of the of the developing neural tube. The neural crest is an irregular, flattened mass of

cells, which transiently resides between the neural tube and the overlying ectoderm (Balinsky, 1970). In the trunk of vertebrate embryos, this temporary location of the crest cells is known as the 'migrating staging area' (MSA) (Wehrle-Haller and Weston, 1995). Migration of the neural crest cells begins as soon as the neural tube closes and occurs along precise migratory pathways. The neural crest cells follow these pathways to reach various locations in the embryo where they differentiate into a variety of different cell types, including: melanocytes; neurons and supporting glial cells of the sensory, sympathetic and parasympathetic nervous systems; the epinephrine-producing (medullary) cells of the adrenal gland as well as the skeletal and connective tissue components of the head (Gilbert, 1991). In chick embryos, trunk neural crest cells follow three distinct pathways (Fig. 1.1). Presumptive melanocytes follow one of these pathways, the dorsolateral pathway, between the somites and the overlying ectoderm.

Of the total neural crest cell population, the cells destined to become melanocytes follow the dorsolateral pathway and are the last of the neural crest cells to leave the MSA (Wehrle-Haller and Weston, 1995). In the chick, these presumptive melanocytes leave the MSA at approximately 50-55 hours of *in ovo* development (Hulley et al, 1991). An important issue in development is how these pluripotent neural crest cells are instructed to remain in the MSA and then follow the dorsolateral pathway to become melanocytes. A favoured explanation is that different exogenous factors (or a combination of factors) produced by the local environment induce the expression of melanocyte-specific genes before migration begins. Consistent with this notion are observations that neural crest cells migrating along the dorso-lateral pathway seem already to be committed to a melanocyte fate (Erickson and Goins, 1995). In agreement with this study, Wakamatsu et al (1998) also found that melanocyte precursors are specified before they disperse on the dorsolateral pathway.

Environmental signals also guide and support the presumptive melanocytes as they spread around the body in the subepidermal mesenchyme before entering the epidermis. One of these signals is steel factor, a protein ligand which binds to a melanocyte-specific membrane tyrosine kinase receptor, c-kit (Nishikawa et al, 1991). Another signal required during stages within the dorsolateral migratory pathway is endothelin 3 (Yoshida et al, 1996). The effects of other signals such as neurotrophins, alpha melanocyte stimulating hormone (αMSH), basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGFβ) on neural crest cells have also been studied *in vitro* (Langtimm-Sedlak et al, 1996; Bowers et al, 1997; Stocker et al, 1991; Rogers et al, 1992). Although it is still not clear how these signals function *in vivo*, more is known about their affects on melanocytes *in vitro* (see section 1.3).

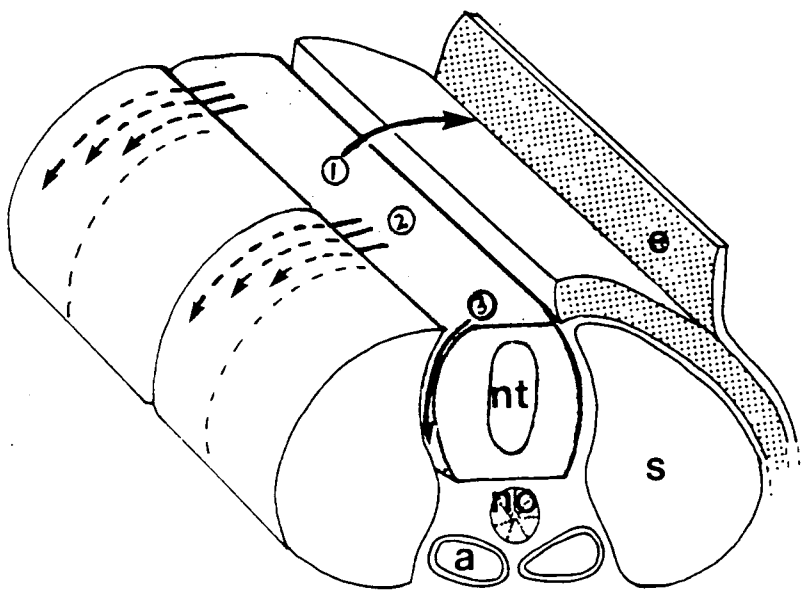


Figure 1.1: Diagram illustrating the pathways of neural crest cell migration in the trunk of the chick embryo (After Bronner-Fraser, 1986).

1. a dorsolateral pathway between the somites (s) and the ectoderm followed by presumptive melanocytes.
2. a dorsoventral pathway through the rostral half of the somite.
3. a ventral pathway between the neural tube (nt) and the somite.

After neural crest cells have migrated in the subepidermal mesenchyme, precursor melanocytes then pass from the dermis through the basement membrane and into the epidermis between days 4 and 10 in the chick. Many of these precursor melanocytes differentiate into mature melanocytes whilst still in the dermis, before entering the epidermis. In a pigmented breed of chickens, Black Australorp, visibly pigmented melanocytes first appear at day 5 of embryonic development. However, not all melanocytes initiate pigment production in the dermis. Some unpigmented premelanocytes have already entered the epidermis by day 4 where they also begin to differentiate around days 5 and 6. With further development, epidermal melanocyte numbers increase exponentially, while dermal melanocyte numbers drop rapidly, as melanocytes eventually become located only in the feather follicles (Hulley et al, 1991).

1.1.2 Feather morphogenesis

Down feathers start to develop from embryonic day 8. Feather buds are first visible as ectodermal placodes underlined by dermal condensations (Fig. 1.2). The feather bud increases in length and invaginates into the skin to form a follicle (Fig. 1.2). At the base of the follicle, a ring of epidermal germinative tissue, referred to as the collar, develops and produces the material that will become the new feather (Lucas and Stettenheim, 1972). In other words, subsequent feather growth is the result of cell proliferation at the collar region of the follicle base. Between days 8 and 18, as a tube of epidermis is pushed upwards by the cell proliferation in the collar, the feather follicle undergoes further morphogenesis (Fig. 1.2). At day 10, the intermediate cell layer of the epidermis (between the germinative and superficial layers) starts to become arranged into 10 to 11 groups, which form the barbs separated by marginal plates bilaterally (Lecoin et al, 1995). The barb plate epithelium later keratinises. The marginal plate epithelium undergoes programmed cell death to give rise to the spaces between the barbs (Ting-Berreth and Chuong, 1996).

The melanocytes previously distributed randomly in the epidermis, become aligned in longitudinal rows within each ridge. They are located with their cell bodies at the apex (nearest the pulp) and their processes extended along the ridge margin towards the periphery of the follicle (Fig. 1.2). At the periphery, melanocytes deliver their pigment granules to the outermost barbule cells first (Watterson, cited in Lecoin et al, 1995). The amount of transferred melanin probably determines the degree of pigmentation of the feathers, as suggested by Jimbow et al, (1976).

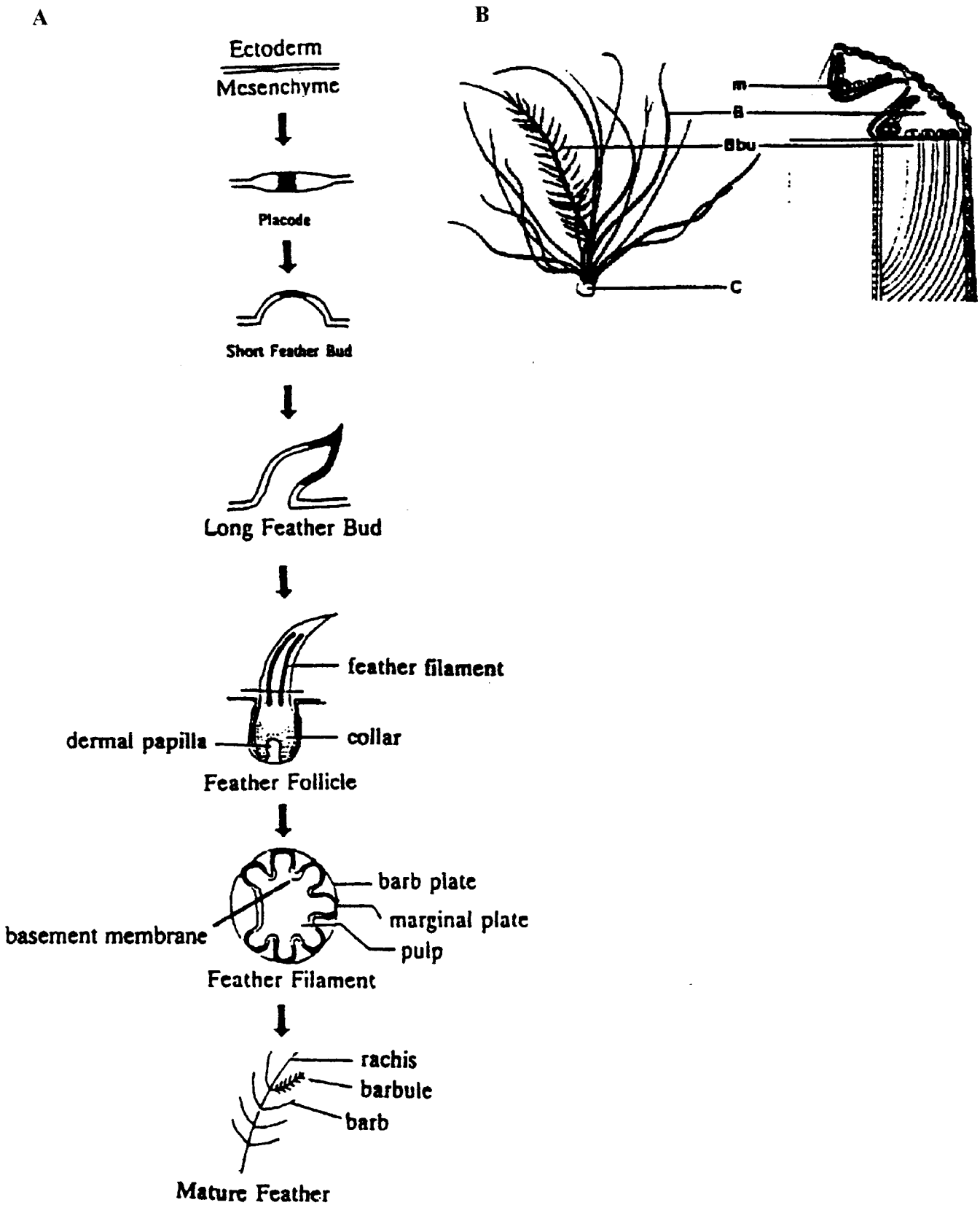


Figure1.2: A. Schematic diagram illustrating the different stages of feather development (After Ting-Berreth and Chuong, 1996). B. Schematic drawing of a down feather (modified from Lecoin et al, 1995).
The melanocytes (m) are located at the inner side of the barb ridge (B) and deliver their pigment granules to the outermost barbule cells (Bbu). C: calamus.

In contrast to epidermal or hair melanocytes, feather melanocytes become incorporated into the keratinising epithelium after their melanosomes have been transferred into keratinocytes. With barb keratinisation, they retract their processes, round up and eventually degenerate. These feather melanocytes are therefore only active during the initial phases of the feather cycle. As feathers regenerate, new melanocytes appear from a dermal pool of melanoblasts in the dermal papilla at the base of the feather and pigmentation of the new feathers begins again (Lucas and Stettenheim, 1972).

1.1.3 Melanin biosynthesis and its regulation

During melanocyte differentiation, tissue-specific genes encoding a variety of enzymes and structural proteins involved in the melanogenic process are expressed. Such genes include three members of the *tyrosinase related protein* (*TRP*) gene family; including *tyrosinase*, *tyrosinase related protein-1* (*TYRP1*) and *tyrosinase related protein-2* (*TYRP2*). Although the *TRP* gene family is well investigated in human, mouse, goldfish and axolotl, relatively little is known about these genes in avians.

Tyrosinase is the rate limiting enzyme in the melanogenic pathway. It initiates a cascade of reactions resulting in the conversion of the amino acid tyrosine to the final product, the melanin polymer (Fig. 1.3). The first reaction in the melanogenic pathway (catalysed by tyrosinase) is hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). Tyrosinase also catalyses oxidation of DOPA to DOPAquinone and in addition, is involved in the oxidation of DHI to indole-5,6-quinone further down the melanogenic pathway (del Marmol and Beerman, 1996). Chick *tyrosinase* cDNA was recently cloned and encodes a protein with a deduced molecular weight of 58kDa (April et al, 1996).

TYRP1 and *TYRP2* also play a role in the melanogenic pathway. Although the function of TYRP1 has been the subject of much controversy, recent experiments on mouse Tyrp1 provided evidence that the protein functions as DHICAoxidase, converting DHICA to indole-5,6-quinone. The chicken *TYRP2* gene encodes a deduced protein of 516 amino acids and shares 75.3% and 75.2% similarity with the mouse and human *TYRP2* proteins respectively (April et al, 1998). *TYRP2* has DOPAchrome tautomerase activity and is involved in the conversion of DOPAchrome to DHICA. This enzyme plays an important role in regulating the ratio of DHICA over DHI, which may contribute to the different properties, such as solubility, flocculence and colour of the melanin formed. Two types of melanins are synthesised: eumelanins are the black and brown pigments, while phaeomelanins constitute the yellow and reddish pigments (del Marmol and Beerman, 1996).

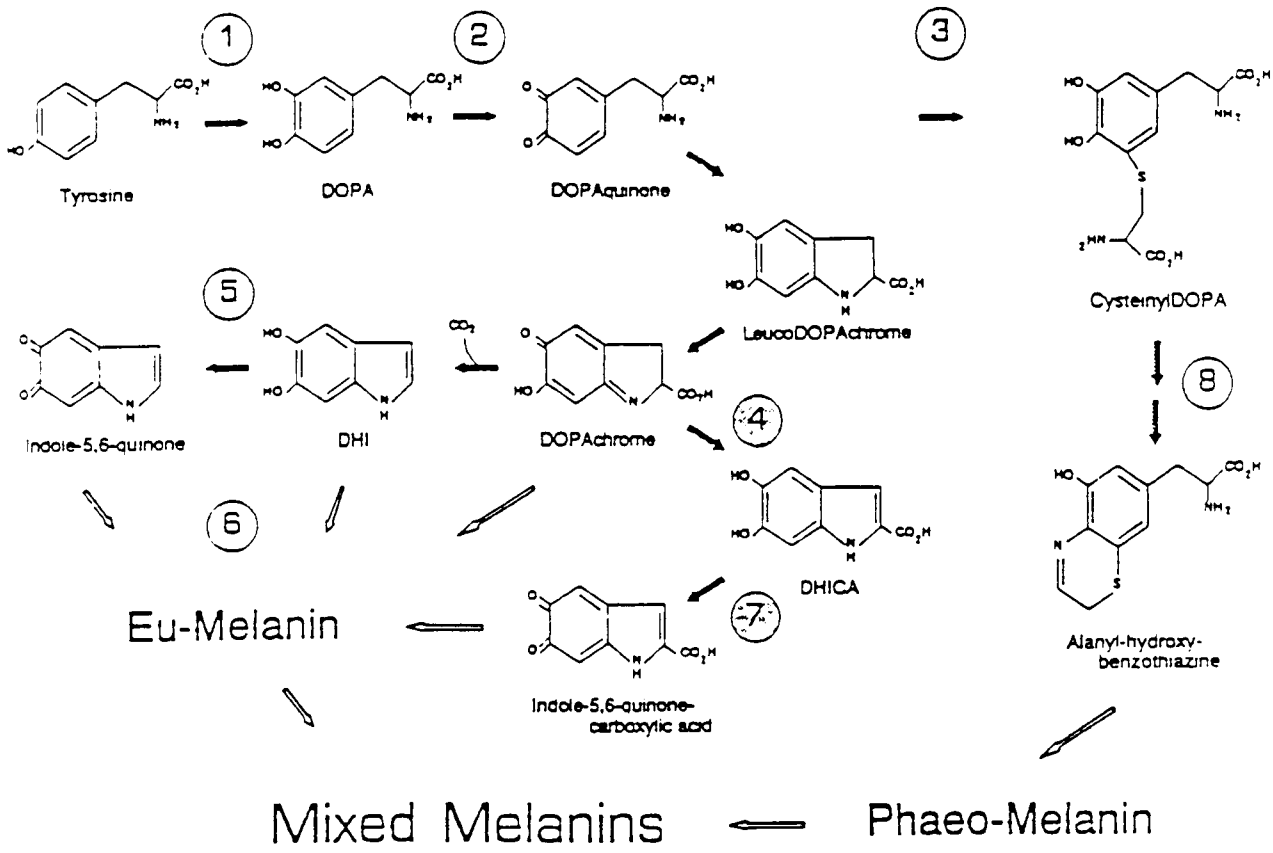


Figure 1.3: The melanin synthesis pathway (modified from Hearing, 1993).

1. Tyrosinase, the rate-limiting enzyme, catalyses the conversion of tyrosine to DOPA.
2. Tyrosinase can also catalyse the DOPA oxidation reaction.
3. In the presence of glutathione or cysteine, DOPAquinone is directed to the production of cysteinylDOPAs. In the absence of glutathione or cysteine, DOPAquinone is converted to leucoDOPA-chrome, which then rearranges to form DOPAchrome.
4. DOPAchrome is diverted to DHICA in the presence of DOPAchrome tautomerase (TYRP2) or divalent metal cations. In the absence of these factors, DOPAchrome forms DHI.
5. DHI is oxidised to indole-5,6-quinone, which is catalysed by tyrosinase or peroxidase.
6. The indoles contribute to eumelanin formation.
7. DHICA is oxidised to indole-quinone, thought to be catalysed by TYRP1.
8. Cysteinyl DOPA is incorporated into phaeomelanin.

1.1.4 Melanosome ontogeny and its ultrastructure

The above cascade of reactions in the melanogenic pathway takes place within precursor pigment granules referred to as premelanosomes. Each premelanosome undergoes a maturation process composed of four stages. During stage I, the premelanosome is spherical with absent or ill-defined matrix filaments. In stage II, the premelanosome is ellipsoid with a well-defined filamentous matrix. It is at this stage that the melanogenic pathway is initiated, resulting in melanin deposition onto the matrix within the premelanosome. This occurs as tyrosinase, and probably TYRP1 and TYRP2, synthesised in the rough endoplasmic reticulum and post-translationally modified in the Golgi, fuse with the stage II premelanosomes (Maul and Brumbaugh, 1971). By this stage, L-tyrosine, the melanin precursor, has also entered the melanosome. The transport of tyrosine was thought to be regulated by an integral melanosomal membrane transporter protein encoded by the P gene (pink-eyed dilution locus) (Rinchik et al, 1993). However, it is now thought to be a regulator of pH in the melanosome (Puri and Brilliant, 1998).

The pigment granule is now referred to as a melanosome and progresses through the last two stages in its maturation process. During stage III, melanosomes display electron-dense melanin on their matrix filaments, and at stage IV, the melanosomes are mature with their matrix fully concealed by melanin deposits (Fitzpatrick et al, 1969). Once the melanosomes are fully matured, they are transferred along the dendrites of the melanocyte into the neighbouring epithelial cells of the feather follicles (Nakagawa et al, 1984).

1.2 Amelanosis in the White Chicken

Many poultry fanciers, motivated by the aesthetic appeal of plumage colour, still breed for the beauty and technical excellence of colouration. However, pigmentation is also of economic importance to the commercial poultry industry. Genetically determined choices such as yellow versus white skin, and coloured versus white plumage are useful to satisfy the preferences of specific markets. For example, white plumage is economically desirable to the meat industry because it leaves no residual melanin in the skin after feather removal (Smyth, 1990). As a result of industry and nature, white plumage (amelanosis) is particularly widespread in avians. In chickens, white varieties include Dorkings, Orpingtons, Minorcas, Wyandotes, Plymouth Rocks, Leghorns, Pile Games, Cochin Bantams and Langshans (Hutt, 1949). Because of the complexity of melanogenic control, the aetiology of amelanosis in chickens is still not completely understood. This study focuses on the White Plymouth Rock x Pile Game white chicken breed which is

heterozygous for *Dominant I (I/i)* and for *tyrosinase (C/c)*. A summary of the key information on these loci is given below.

1.2.1 The *Dominant white (I)* locus

'Dominant white' (I), as the name implies, is a dominant mutation of the *I* gene, common to many commercial breeds of fowl such as Pile Games (PG), White Leghorns (WL) and White Plymouth Rocks (WPR). *Dominant white* affects melanin production in neural crest-derived melanocytes, resulting in completely white plumage (Bitgood and Smyth, 1991). It was therefore assigned the gene symbol *I* for inhibitor of black pigment by Hurst (1905). Iridial and retinal pigment epithelial cells however, remain unaffected and synthesise enough melanin to give the eye its black colour (Bitgood and Smyth, 1991). *Dominant white* is currently believed to be multi-allelic, with three loci described, $I > I^{d}$ *(dun)*$> i^{+}$ (wild-type) (Smyth, 1990).

The *I* gene suppresses eumelanin production, but does not affect phaeomelanin synthesis. Brumbaugh (1971) studied *Dominant white* melanocytes ultrastructurally and reported that eumelanocytes assembled a larger number of premelanosomes during the early stages of differentiation than the threshold amount produced by phaeomelanocytes (Brumbaugh, 1971). Brumbaugh (1971) further proposed that phaeomelanocytes remained unaffected by the *Dominant white* mutation because they were capable of increasing their premelanosome numbers later in development, unlike eumelanocytes.

Although the *I* gene has not been identified or cloned in mice, the mammalian equivalent is possibly represented by the *Dominant white* mutation in pigs. In addition, although early developmental events have not been examined, it has been shown that the white phenotype of these pigs corresponds to an absence of melanocytes in the hair follicles. In a recent study, Moller et al (1996) investigated *c-kit* as the candidate gene for the *Dominant white* mutation in pigs. *C-kit* encodes the steel/mast/stem cell growth factor receptor which plays an important role in melanogenesis, hematopoiesis and germ cell development in the mouse (Nocka et al, 1990). PCR amplification of a genomic fragment corresponding to *c-kit* was carried out and the resulting amplification products screened for single-strand conformation polymorphism (SSCP) by electrophoresis. One of the SSCP markers, denoted *KIT(SSCP1),* showed different patterns in white and coloured animals. The KIT(SSCP1) polymorphism was further analysed in a sample of more than 90 unrelated animals representing white (*Dominant white*) as well as coloured breeds. The analysis revealed a very strong association between the allele *KIT(SSCP1)* and the *Dominant white* mutation, since all white animals carried the allele, which was absent in all the coloured breeds. Further investigation of this polymorphism by cloning and sequencing revealed the presence of a duplication of the *c-kit* gene (Moller et al, 1996). Because of overt similarities

between white pigs and white chickens, the possibility exists that *Dominant I* is in fact the *c-kit* gene. Furthermore, Ruyter-Spira et al (1997) performed bulked segregant analysis using microsatellite markers for the preliminary mapping of the *Dominant white* locus in the chicken. The *I* locus was mapped on linkage group 22 of the East Lansing reference family at a distance of 2cM from MCW188.

1.2.2 What is the molecular basis of amelanosis?

There are a number of mechanisms which could account for the *Dominant white* phenotype, including: 1) a failure of melanoblasts to migrate and reach the skin regions; 2) a reduction in melanocyte survival during migration or in the feather follicle; 3) a block in melanocyte differentiation (melanocyte-specific gene expression); and 4) defects in enzymes that are critical for normal melanin synthesis. Evidence for each of these theories will be discussed below and is also summarised in Table 1.1 for ease of reference.

I (for inhibitor) was originally assigned as the gene symbol for *Dominant white,* as long ago as 1940, because it was thought that the characteristic white plumage of WLs was essentially the consequence of a physical **inhibitor** of pigment synthesis. To test this idea, Hamilton (1940) carried out an experiment in which skin melanocytes from 6- to 7-day wild-type embryos were cultured in embryo extract or plasma from WL embryos. The results showed abundant pigment production, indicating that a diffusible inhibitor was not present in either WL extract or plasma. To further investigate the lack of melanin synthesis in WLs, Hamilton (1940) then cultured melanocytes from 6- and 7-day embryonic skin from WL as well as from pigmented (control) embryos. The results clearly showed that WL melanocytes were capable of synthesising melanin *in vitro*. However, they were reported to have a "much lower viability and a higher sensitivity" to adverse environmental conditions compared with melanocytes obtained from pigmented breeds. Furthermore, split preparations of WL feathers examined with light microscopy showed a smaller number of differentiated melanocytes than in pigmented feathers. From these observations, Hamilton (1940) proposed that the apparent decrease in the number of differentiated WL melanocytes *in vivo* was due to the fact that many of them died in the melanoblast stage, before reaching the skin regions. In other words, a reduced survival of migratory melanoblasts to the skin regions was thought to account for the lack of pigmentation (see Table 1.1).

Willier and Rawles (1940) used a different approach to investigate the lack of pigment in WLs. Skin grafting experiments were used to assess whether normal melanocytes which invade white follicles are able to survive and make melanin. A small piece of skin ectoderm or mesoderm was grafted from the donor embryo to a host embryo of the same age (76-108 hours incubation) at the base of the right wing bud. Black melanocytes which migrated into white feather germs were able

to survive and differentiate, indicating that the white environment is functional and supportive of melanogenesis. This supports the conclusion drawn by Hamilton (1940) that a diffusible inhibitor was not characteristic of a *Dominant white* mutation. Reciprocal grafts showed that WL melanocytes were unable to melanise in wild-type host tissue. In addition, the white feathered areas caused by WLs were smaller than the white feathered areas caused by other white breeds of fowl (such as Sylkies) in hosts of the same breed. Because the white environment can support black neural crest cell differentiation, it was concluded that WL feathers are white because of an intrinsic defect in melanocyte precursors. This defect results in an inability of these precursors to migrate properly (Willier and Rawles, 1940; see Table 1.1).

Table 1.1: Summary of results and mechanisms that account for amelanosis.

Reference	**Technique**	**Migration**	**Survival**		**Melanocyte differentiation**	**Normal melanin synthesis machinery**
			mign	**skin**		
Hamilton (1940)	cell culture, light microscopy	×	×		✓	✓
Willier and Rawles (1940)	tissue transplants	×	×			
Brumbaugh (1971)	electron microscopy	✓			(✓)	×
Jimbow et al (1974)	electron microscopy, DOPA reaction	✓		×	(✓)	×
Bowers et al (1992)	electron microscopy, cell culture	✓		×	(✓)	
Marco (1994)	electron microscopy, DOPA reaction	✓		✓	(✓)	✓

× = failure or abnormal

✓ = normal , (✓) = process is initiated

mign = migration

In a more recent investigation of the effects of *Dominant white*, Brumbaugh (1971) carried out an ultrastructural examination of two week old regenerating feather material from the breast and hackle regions of WL males. *Dominant white* melanocytes contained irregularly shaped and poorly assembled, unmelanised melanosomes at significantly lower numbers than normal melanocytes. Very few melanised melanosomes were encountered, and those observed showed evidence of disorganisation. These results indicate that *Dominant white* melanocytes are capable of initiating melanogenesis. Furthermore, the presence of melanocytes in feathers indicates that at least some melanocytes arrive in the skin, but it does not indicate whether they all arrive (see Table 1.1). Although viability was not tested in this investigation, based on these ultrastructural observations, Brumbaugh (1971) suggested an alternative explanation to the "viability and early death" hypothesis proposed by Hamilton (1940). He stated: "*Dominant white* melanocytes **appear** to die sooner than the standard melanocytes because they have transferred and dispersed their few, poorly assembled granules into the epidermal cells, and are therefore no longer dark enough to be identified" (Brumbaugh, 1971). In other words, it is possible that *Dominant white* melanocytes remain viable in the skin but because of defective enzymes necessary for normal melanin synthesis, are unable to synthesise melanin (see Table 1.1). Since molecular techniques for tracking these cells were unavailable at the time, the possibility that living, undifferentiated melanocytes were present in the skin in normal numbers could not be investigated.

In support of Brumbaugh's (1971) studies, ultrastructural examination of 10-day WL skin by Jimbow et al (1974) showed melanocytes containing reduced numbers of immature, unmelanised melanosomes, with only a few that were completely melanised. In addition, many small membrane-delimited vacuoles were reported to contain cytoplasmic organelles such as mitochondria, rough endoplasmic reticulum, and ribosome like granules. Jimbow thought that these vacuoles were autophagosomes and concluded that this was indicative of melanocytic degradation (Jimbow et al, 1974). In the same study, Jimbow et al (1974) attempted to determine whether melanocytes arrive in the skin regions in normal numbers. For this, a tyrosinase-specific histochemical technique, the DOPA reaction, was used on 9-day WL skin. The results showed DOPA-positive melanocytes to be present in WLs in normal numbers compared with the control pigmented breed, but those in WLs had fewer and shorter dendrites. Jimbow et al (1974) did not state how this quantitation was carried out and it was assumed that these conclusions were based on visual assessment of different follicles. In addition, the intensity of the DOPA reaction was weaker in WLs than it was in the control breed. It was therefore concluded that melanocytes arrive in the skin regions in normal numbers, and only begin to die after day 9 (see Table 1.1).

Consistent with the melanocyte degeneration observed by Jimbow et al (1974), Bowers et al (1992) suggested that WL feather melanocytes undergo premature degeneration in the collar regions prior to their migration into and subsequent melanisation of the feather. This hypothesis was based on ultrastructural examination of the collar region of WL feathers which showed adendritic, degenerating melanocytes containing autophagosomes. In addition, few, if any melanosomes were transferred to the surrounding keratinocytes. Since cultured WL melanocytes have been shown to be more sensitive to WL conditioned medium than wild-type melanocytes, it was further hypothesised that the somewhat avascular feather capsule must allow the build up of a toxic substance which causes the premature death of the melanocytes. In addition, the degree of sensitivity of the melanocytes to this toxic substance depends on their genotype since grafted wild-type donor melanocytes survive in WL host feathers and are able to melanise (Bowers et al, 1992).

In a recent study on the fate of *Dominant white* melanocytes in WPR x PG chickens, Marco (1994) carried out an ultrastructural examination of 9 and 10-day chick skin. In support of the findings presented by Jimbow et al (1974), it was found that WPR x PG melanocytes contained morphologically normal premelanosomes but that these occurred in fewer numbers than in black skin melanocytes, and did not mature and become melanised. Consistent with this result, Brumbaugh and Lee (1975) also reported fewer melanogenic organelles in *I/I* melanocytes compared with *i/i* melanocytes. In contrast to the findings of Jimbow et al (1974) however, Marco (1994) found no evidence of melanosomal or melanocytic degradation in WPR x PG skin. This is consistent with a recent study which investigated the possibility that melanocytes might be dying by apoptosis in 10-day WPR x PG skin. The TUNEL reaction was utilised and the results consistently showed no traces of cells dying by apoptosis at this stage of development (Koubovec, 1996).

Marco (1994) also used the DOPA reaction, and found tyrosinase-containing melanocytes in 8- to 10-day WPR x PG skin. However, by 11- to 13-days, skins were DOPA-negative, indicating that tyrosinase activity was no longer detectable. These results were further confirmed using a sensitive radiochemical assay that measures melanogenic activity. Low levels of melanogenic activity were detected in 9-day skins but at 13-days, there was no increase as development proceeded. In contrast, black skin had 50% more activity at 9-days, which increased 8.5-fold by day 13 (Fig. 1.4). In contrast to these results, Brumbaugh and Lee (1975) measured dopa oxidase activity of skin melanocytes of single mutant (*I/I*$^{+}$) stocks and found no reduction in enzymatic activity.

In conclusion, the presence of immature melanocytes in the skin and feather follicles of WPR x PG embryos indicates that at least some melanocytes reach the skin regions, survive and initiate melanogenesis (see Table 1.1). However, it still remains unclear whether melanocytes reach the skin regions in normal numbers. Furthermore, to complicate matters, Marco (1994) found that in cell culture environments (in serum-supplemented medium), WPR x PG melanocytes not only survived as long as black melanocytes, but also produced pigment.

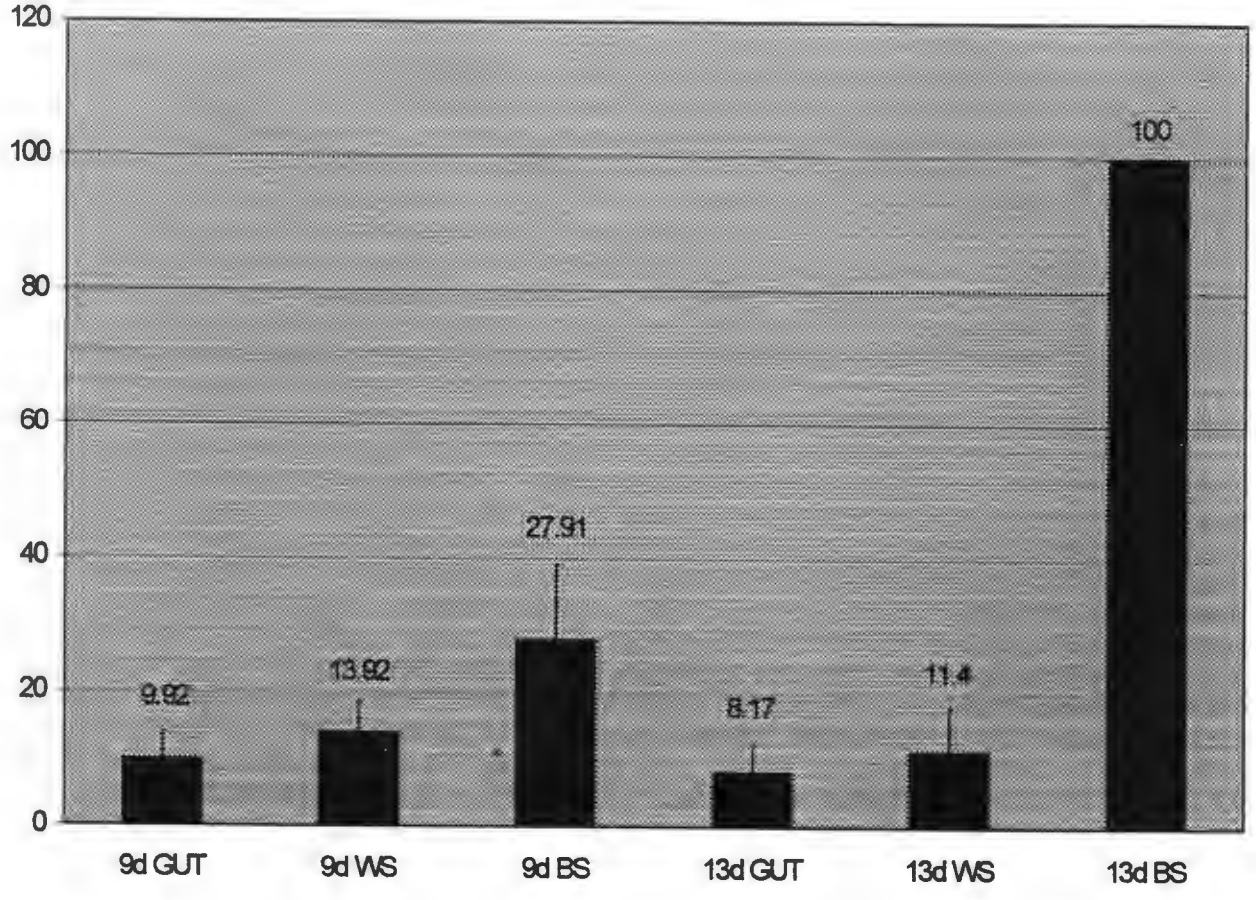


Figure 1.4: Radiometric assay of tyrosinase activity in 9- and 13-day Black Australorp x New Hampshire Red and White Plymouth Rock x Pile Game embryonic chick skin and organ homogenates. Counts (cpm/10μg homogenate) have been converted to percentage of maximum (counts for 13-day black skin). Means given ± SEM, n=3. BA = Black skin. WS = White skin. GUT = control to determine baseline levels of [^{14}C] tyrosine incorporation in tissues (gut) known to be tyrosinase-negative (Marco, 1994).

This is the same result as those of Hamilton (1940) who found that cultured WL melanocytes were able to survive and melanise. This indicates that white chick melanocytes have the potential to become fully melanised when in a supportive environment. This result in turn suggests that the underlying defect may not be intrinsic in the melanocyte itself but be a consequence of a defective tissue environment which does not allow for melanogenesis *in vivo*.

1.2.3 The *recessive white (c)* locus

In addition to *Dominant white,* White Plymouth Rocks carry an autosomal recessive mutation at the c locus known as "recessive white" (Smyth, 1990). *Recessive white* is the most common form of white plumage that is inherited as a recessive mutation. Like *Dominant white, recessive white* was another of the earliest animal traits to be investigated following the rediscovery of Mendel's classical work (Smyth, 1990). As in mammals, the c locus in chickens is multi-allelic, with 4 alleles described with an order of dominance: C^+ (wild-type) > *c* (recessive white) > c^{re} (red-eyed) > c^a (autosomal albino) (Brumbaugh et al, 1983; Smyth et al, 1986). Adult plumages of all three *c* mutants are typically white. Furthermore, c^a chickens have unpigmented eyes, whereas *c* and c^{re} mutants have pigmented and red eyes respectively. *c* alleles affect tyrosinase activity, but whether they represent structural or regulatory mutations of the *tyrosinase* gene remains to be established (Smyth, 1990). Immunoblotting assays with anti-chicken tyrosinase mouse serum by Oetting et al (1985a) showed that the *c* and c^a mutant alleles in albino fowls produce tyrosinase-like molecules which possess no enzyme activity. This suggests that the mutations in the *c* genes are in the coding region. Chickens heterozygous for *C/c* would therefore be expected to display half the enzyme activity of that of normal homozygotes *(C/C)*.

1.3 The role of growth factors in melanocyte development

In view of the evidence presented above, which suggests that the milieu in which the melanocyte finds itself may be central to its survival and differentiation, a full understanding of the role of growth factors implicated in melanocyte development is necessary.

1.3.1 Steel factor

Steel factor, also known as stem- or mast cell growth factor, and its receptor, c-kit, play an important role in melanocyte development. In the mouse, *steel* mRNA expression is transiently localised to the dorsal dermatome. This expression occurs approximately 6-12 hours before the onset of melanocyte precursor dispersal (E11) on the dorsolateral path towards the dermatome.

Steel seems to be required for the initial survival of melanocyte precursors in the MSA, since in *steel null* embryos, precursor melanocytes in the MSA do not migrate and eventually disappear. Once melanocyte precursors have dispersed in the dermal mesenchyme, steel factor is no longer produced by the dermatomal cells in the vicinity of the MSA (Wehrle-Haller and Weston, 1995). However, *steel* continues to be expressed by dermal cells. In the dermis, it is spliced differently to steel found in the vicinity of the MSA. In the dermis, the splice-variant is not cleaved off to become a soluble factor as in the mesenchymal cells in the MSA, but remains on the cell surface. This membrane-bound steel is thought to play a role in survival of proliferating precursor melanocytes, since *steel-dickie* homozygote mutants which lack the membrane-bound factor lack melanocytes and therefore have no coat colouration (Wehrle-Haller and Weston, 1995).

C-kit is first expressed by mouse precursor melanocytes whilst still in the MSA and continues to be expressed throughout the migration of the precursor melanocytes to the hair follicles. Interestingly, however, precursor melanocytes do not require c-kit throughout their entire journey, but alternate through stages of c-kit dependence and independence. As the cells migrate through the dermis, they require c-kit for their survival. Just before entering the epidermis, melanoblasts become independent of c-kit. Upon entering the epidermis, however, proliferating melanocytes again become c-kit dependent. Finally, once the melanocytes enter the hair follicles, they form part of a different microenvironment composed of follicular epithelium, and do not require c-kit for their survival (Yoshida et al, 1996).

In a recent study on the expression pattern of *steel* and *c-kit* in avian development, *steel* was first shown to be expressed at embryonic day 4 (E4), about two days after the neural crest cells leave the neural primordium. Interestingly, unlike the mouse, expression of the *steel* gene was restricted to the epidermis throughout development in all breeds studied (Lecoin et al, 1995).

At E4, rare and scattered *c-kit* -positive cells appeared in the dorso-lateral pathway of neural crest cell migration. By E5, the number of *c-kit* -positive cells had increased significantly in the subepidermal mesenchyme, and some *c-kit* -positive cells were present in the dorsal ectoderm. Within the next four days, proliferating *c-kit* -positive cells were observed throughout the epidermis, and were especially numerous at the base of the feather buds. At E9- E10 in avians, the *steel* gene was still expressed all over the epidermis although more intensely at the top of the feather buds. This coincided with the distribution of numerous *c-kit* -positive cells located near the feather buds, with ones at the top of the feather buds becoming pigmented. With elongation of the feather buds between E11 and E14, and subsequent migration of melanocytes into the developing barb ridges, *steel* expression was found only in the feathers and no longer in the rest of the skin (Lecoin et al, 1995).

The results show that while *c-kit* -positive melanocytes migrate through the subepidermal mesenchyme, there is no concomitant *steel* expression in the dermis (Lecoin et al, 1995). To examine this issue, Guo et al (1997) looked for *steel* expression and cultured quail neural crest cells and immunostained them with antibodies generated against steel. The results showed that neural crest cells themselves express *steel*. This suggests that steel could act in an autocrine or paracrine fashion to influence melanogenesis in nearby cells and that neural crest cell populations play a more active role in the determination of their own cell fate. This is consistent with the earlier observations by Weston and colleagues (1988), who showed that the ability of cultured avian neural crest cells to give rise to melanocytes depended on the period of time in which the cells were in close physical apposition to each other. Neural crest cells that formed tight clusters for periods of at least 36 hours gave rise to numerous pigmented cells. However, neural crest cells prevented from forming clusters or in which the clusters were dissociated within the 36-hour period, contained significantly fewer melanocytes. Although these studies did not identify the factor(s) involved, they are consistent with an autocrine mechanism of steel action (Vogel and Weston, 1988).

In contrast to Lahav et al (1994) who reported that steel factor was necessary for the survival of melanocytes, Guo et al (1997) found that steel factor was not necessary as an early survival signal for avian neural crest cells. Depriving neural crest cells of steel during the initial 5 days in culture did not result in an irreversible loss of most steel-dependent cells as would be the case if steel was a survival factor. Furthermore, lack of the growth factor did not prevent subsequent pigmentation of crest cells induced by late additions of steel. These results indicate that instead of affecting crest cell survival, steel acts to induce differentiation of cells into melanocytes. This however, does not exclude the possibility that committed melanoblasts become dependent on steel for their survival at later stages of development.

Since prior *in vitro* studies, like that of Lahav et al (1994), were performed in complex culture media, Langtimm-Sedlak et al (1996) re-evaluated the role of steel on quail neural crest cells in a defined (serum-free) environment. When present throughout the entire culture period, steel was found to cause an increase in the number of colonies containing sensory neuron precursors, indicating a trophic role in sensory neuron development. However, steel had no detectable effect on melanogenesis since the number of pigmented cells per pigmented colony remained the same. This indicates the requirement of another factor that acts in tandem with steel to affect melanogenesis.

Since Yaar et al (1991) and Di Marco et al (1991) showed that cultured human keratinocytes produce biologically active NGF (nerve growth factor), it is possible that in the chick presumptive melanocytes also encounter neurotrophins when they arrive in the developing skin. This is further

supported by the report that chick melanocytes have been found to express NGF receptors in the dermal mesenchyme and in the feather follicles (Heuer et al, 1990). To investigate this theory directly, Langtimm-Sedlak et al (1996) tested the combination of steel and a number of neurotrophins (NGF, BDNF, NT-3) on quail neural crest cells in culture. Steel factor with any neurotrophin tested was found to promote survival of committed melanogenic cells, since the number of pigmented cells per pigmented colony was significantly increased (Langtimm-Sedlak et al, 1996). It is possible therefore, that the effects on neural crest cells observed by Lahav et al (1994) were due to the combination of steel and a neurotrophin present in the serum.

1.3.2 Endothelin 3 (EDN3)

EDN3 is also thought to play a role in the development of melanocytes. It stimulates a signalling pathway by binding to its receptor, the endothelin-B receptor (EDNRB), on neural crest cells. In a recent study on avians, high levels of EDNRB gene expression was observed in neural crest cells at the onset of their dorsolateral migration at around days 2 and 3. This expression was reported to cease as these precursor melanocytes began expressing *c-kit* at around day 4. These results suggest that EDNRB/EDN3 interaction influences precursor melanocytes early in development, before the c-kit/steel signalling pathway begins to perform its role.

To establish its function in melanocyte development, EDN3 was added to cultured avian neural crest cells. It was shown to stimulate dramatically the proliferation of these cells (Nataf et al, 1996). This is consistent with the study by Lahav et al (1996) where EDN3, added to cultured avian neural crest cells in serum-supplemented medium, was also found to strongly stimulate proliferation and survival of the cells whilst inhibiting the onset of melanogenesis. Furthermore, after removal of EDN3 from the culture medium, cell proliferation and viability diminished drastically. Interestingly, this phase of expansion, which could be prolonged for a few weeks if the cells were replated regularly, was then followed by both a decrease in cell proliferation and the onset of melanocyte differentiation (Lahav et al, 1996). These results suggest that *in vivo*, EDN3 plays a crucial role in expanding the population of precursor melanocytes to a large number of cells at the onset of their migration. Since these precursors have to undergo an expanded migration to the entire skin area, this early growth of neural crest cells seems crucial to ensure the survival of a sufficient number of these cells (Nataf et al, 1996). Once a sufficient number of cells has been obtained, cells are then induced to begin their differentiation into melanocytes.

The effects of EDN3 were also examined in serum- and chick embryo extract-free cultures. During the first days in culture, EDN3 also increased neural crest cell proliferation, and inhibited melanocyte differentiation. However, the effect on cell survival and differentiation was severely

compromised, suggesting a requirement for additional factors present in the serum or embryo extract acting in combination with EDN3 to permit prolonged survival followed by enhanced differentiation of melanocytes. A possible candidate for the additional factor was steel, since it has previously shown to favour the survival of avian neural crest precursors (Lahav et al, 1994). Lahav et al (1996) therefore cultured cells in steel supplemented medium in the presence and absence of EDN3. Analysis of cultures, however, revealed no difference between those treated with EDN3 or steel plus EDN3. Therefore, steel was not the factor in the enriched medium that co-operated with EDN3 to enhance survival and melanocyte differentiation (Lahav et al, 1996).

In the mouse embryo, mutations in either EDN3 or EDNRB receptor genes show gross pigment defects. For example, *piebald-lethal* mice *(s^l)*, which lack functional EDNRB receptors, show a marked reduction in the number of melanocyte progenitors. Comparing s^l with wild-type, neural crest cells expressing *tyrosinase related protein-2 (TYRP2)*, a lineage-specific marker for melanoblasts, are almost entirely lacking in s^l while they are detectable as early as E10 in wild-type embryos (Nataf et al, 1996). Endothelin signalling thus seems to be involved in the regulation of melanocyte progenitor number early in development, which is similar to its activities in the chick. Reid et al (1996) investigated the effects of EDN3 *in vitro* by incubating neural crest cultures with EDN3 for a few days. Melanocyte progenitors, identified with an anti-c-kit antibody, were reported to increase significantly in number.

1.3.3 Alpha melanocyte stimulating hormone (αMSH)

Little is known about the effect of αMSH on avian melanocytes *in vivo*. Birds lack a pars intermedia in their pituitary gland and it was unknown for years whether αMSH was even produced in birds (Sherbrooke et al, cited in Bowers et al, 1997). However, recent cytochemical and bioassay evidence suggests that αMSH secreting cells are present in the pars distalis in the bird pituitary gland (Hadley, 1992). *In vitro* studies have shown that αMSH stimulates melanogenesis in chicken melanocytes (Bowers et al, 1992). Visible pigmentation in these cells was greatly increased in the presence of the hormone suggesting that mature melanocytes possess αMSH receptors. This is consistent with the work of Satoh and Ide (1987), where quail neural crest cells cultured in the presence of αMSH showed accelerated melanogenic differentiation and increased commitment of neural crest cells to the melanogenic lineage. Furthermore, recent investigations showed that adenosine-3′,5′-cyclic monophosphate (cAMP) is the second messenger for αMSH in these cells. A direct inhibitor (Rp-c-AMPS) of cAMP was reported to inhibit completely the stimulatory effect of αMSH on pigmentation in these avian *in vitro* melanocytes (Bowers et al, 1997).

The role of αMSH in the mouse is more clearly defined. αMSH regulates melanocyte differentiation in the mouse epidermis by inducing tyrosinase activity, melanosome formation, translocation of melanosomes, and increased dendritogenesis (Hirobe, 1992). Furthermore, cAMP similarly induced the differentiation of melanocytes both *in vivo* and *in vitro* suggesting that the action of αMSH (likewise in avians) may be mediated through cAMP (Hirobe and Takeuchi, cited in Hirobe, 1992).

In a more recent study, αMSH was found to stimulate a rapid increase in *microphthalmia (mi)* mRNA and protein levels in cultured human and mouse melanoma cells and primary human neonatal melanocytes shown by northern and western blot assays. Mi is a transcription factor involved in the regulation of tyrosinase and TYRP1 (Bentley et al, 1994), thus explaining the upregulatory effect on tyrosinase protein described above. The results further showed that cAMP, triggered by αMSH, leads to rapid induction of the *Mi* promoter. This induction is dependent on an intact CRE (cAMP-responsive element) in the promoter (Price et al, 1998). The role of αMSH and mi in avian melanocytes remains to be investigated.

1.3.4 Basic fibroblast growth factor (bFGF)

Little is known about the effect of bFGF on avian melanocytes. *In vitro* studies have shown that neural crest-derived cells from embryonic quail dorsal root ganglia (DRG) and peripheral nerve, which do not normally give rise to melanocytes, become committed to melanogenesis following treatment in culture with bFGF (Stocker et al, 1991). Similarly, treatment of these cells with the phorbol ester, 12-0-tetradecanoyl phorbol-13-acetate (TPA), also gave rise to melanocytes. In contrast, Oetting et al (1985b) used TPA as a reversible inhibitor of melanogenesis. Melanogenesis observed in cultures by Stocker et al (1991) supports the notion of a melanocyte/Schwann cell progenitor, first proposed by Nichols and Weston (1977), that melanocytes and Schwann cells (associated with peripheral nerves and ganglia) are derived from a common bipotent intermediate in the neural crest lineage.

To determine whether these effects of bFGF and TPA depended on other, unknown factors present in the medium, experiments were repeated using a serum-free and embryo extract-free defined medium (DM). Results showed that neither the DM alone, nor DM plus various concentrations of bFGF, were able to induce pigmentation. In contrast, DM supplemented with TPA alone induced pigmentation in 17% of the cultures. These results suggest that bFGF requires some unknown serum factor (which can be replaced by TPA) to induce pigmentation under these conditions. Furthermore, bFGF was found to influence the survival of non-neuronal cells in these cultures. Cultures in DM without bFGF often contained cells that were poorly attached to the substratum and contained vacuoles (Stocker et al, 1991).

1.3.5 Transforming growth factor beta (TGFβ)

TGFβ, another growth factor involved in the regulation of neural crest cell differentiation, is thought to inhibit ectopic melanogenesis along the ventral neural crest migratory pathway (Guo et al, 1997). *In vitro*, TGFβ was found to inhibit the TPA- or bFGF-induced pigmentation of DRG cultures described above (Stocker et al, 1991). Consistent with this result, Rogers et al (1992) cultured quail neural crest cells in the presence and absence of TGFβ and reported a dramatic decrease in the number of melanocytes in treated cultures. Furthermore, with continuous TGFβ treatment, many cells increased in area, showed fibronectin immunoreactivity, and resembled cells derived from the cephalic neural crest (Rogers et al, 1992).

1.4 Approach and Aim

In the present study, the following specific questions are addressed.

1. Are melanocytes present in normal numbers in the skin regions and feather follicles of 8- to 13-day WPR x PG embryos?

2. If present in normal numbers, are melanocytes unable to synthesise pigment because of a defect in melanocyte differentiation?

Two approaches were used to answer the first question. Firstly, an immunocytochemical procedure was carried out which uses antibodies as markers to detect proteins specific to melanocytes. Secondly, *in situ* hybridisation was performed with melanocyte-specific molecular markers including *tyrosinase* and *tyrosinase related protein-2* (*TYRP2*). The process of melanocyte differentiation and the role of growth factors was addressed in cell culture experiments using serum-free conditions and skin extract from black and white chick embryos.

2. Materials And Methods

2.1 Animals

Fertile fowl eggs were obtained from Elsenberg Poultry Farm, Poultry Science Department, University of Stellenbosch. Eggs were incubated at 37°C in a humidified chamber and staged according to Hamburger and Hamilton (1951). Quail embryos, White Plymouth Rock x Pile Game (WPR x PG) and Black Australorp (BA) chick embryos were used in the present study.

2.2 Histological procedures

Embryos were fixed in fresh 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) at 4°C overnight for routine histology, immunocytochemistry (ICC) and *in situ* hybridisation (ISH). Larger embryos were cut in order to make penetration of the fixative more effective. For ISH, embryos were either processed and paraffin wax embedded (Appendix III). For ICC, embryos were cryoprotected (15% sucrose in PBS at 4°C for 2 hours, 30% sucrose in PBS o/n at 4°C) and embedded in 7% gelatin in 20% sucrose in PBS for 2 hours at 37°C for ICC. After setting the gelatin at 4°C the tissue was frozen in liquid nitrogen.

Wax sections (7μm) were cut on a Reichert-Jung microtome, and collected on aminopropyltriethoxysilane (APTES) coated slides (Appendix III). Sections were incubated at 60°C for 30 minutes and stored at 4°C until required. Frozen sections (7μm) were cut with a cryostat (Anatomical Pathology Department, UCT) and collected on APTES coated slides (Appendix III). They were subsequently air dried and stored in a sealed container at -80°C until required.

2.3 Immunocytochemistry

2.3.1 Procedure for immunocytochemical staining

After cryostat sectioning, slides were washed in phosphate buffered saline (PBS, pH 6.9) at room temperature for 5 minutes. Primary antibody (10μl MelEM) was applied to frozen sections and incubated at room temperature overnight. The following day slides were washed in PBS three times for 5 minutes. A 1 in 10 dilution of the secondary antibody (rabbit anti-mouse-FITC),

selected from numerous trial runs, gave the strongest fluorescence with the lowest background. Secondary antibody (10μl) was applied onto each section and left for 1 hour in a humidified chamber in the dark. Slides were then washed in PBS four times for 5 minutes to remove unbound antibody, and then mounted in buffered glycerol. Coverslips were sealed with clear nail varnish to prevent sections from dehydrating.

Controls: Negative controls were included in every ICC reaction carried out, and were treated identically to the experimental samples except that the primary antibody was substituted with PBS.

2.4 Gene constructs and preparation of template DNA for *in situ* hybridisation

Three gene constructs were used for the synthesis of digoxigenin-labeled riboprobes: a chicken *tyrosinase* clone (B8.3, April et al, 1996) and two chicken *TYRP2* clones (clones #196/7) (April et al, 1998). Approximately 10-20μg of each clone was digested with the appropriate restriction enzyme (RE) for preparation of linear template DNA. Proteins were removed from the sample by standard phenol:chloroform isoamyl alcohol extraction and precipitated (Appendix II). Samples were then quantified by electrophoresis using known amounts of lambda DNA (Boehringer Mannheim) (Appendix II).

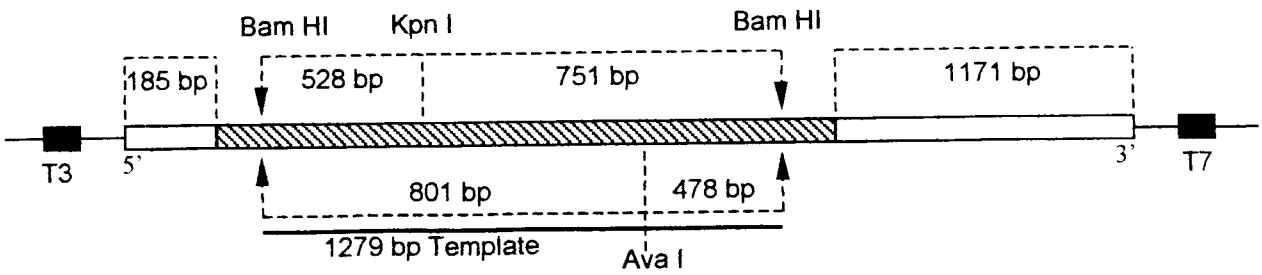
2.4.1 Chicken *tyrosinase* cDNA

The full length chicken *tyrosinase* cDNA consisting of 1909 bp was cloned into the EcoRI-XhoI site of pBluescript II SK (+/-) (2.96 kb) (Appendix I) (April et al, 1996) (Fig. 2.1). Hind II was used to linearise the plasmid, producing a template of 636 bp for antisense riboprobe synthesis using T7 RNA polymerase. For sense riboprobe synthesis using T3 RNA polymerase, digestion with Hind II produced a template of 1.287 kb.

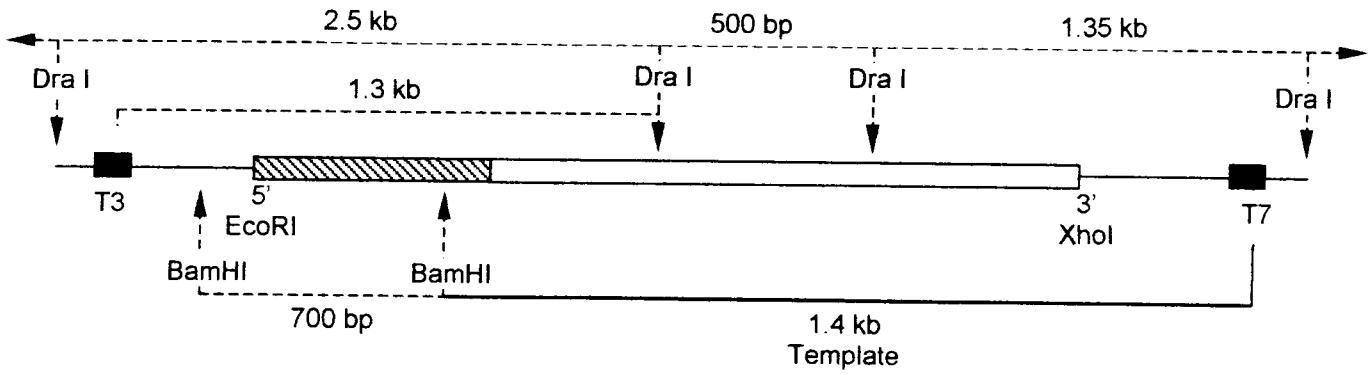
2.4.2 Chicken *TYRP2* cDNA

Two clones containing restriction fragments encoding chicken *TYRP2* were isolated by strong cross-hybridisation to mouse *TYRP2* cDNA on a Southern blot (April et al, 1998). The two fragments, one partial (clone 196) and one full length (clone 197), were cloned into the EcoRI-XhoI site of pBluecript II SK (+/-) (Appendix I) (Fig. 2.1).

A. Chicken *TYRP2* clone 197 (2.9 kb)



B. Chicken *TYRP2* clone 196 (2.1 kb)



C. Chicken *tyrosinase* (1.9 kb)

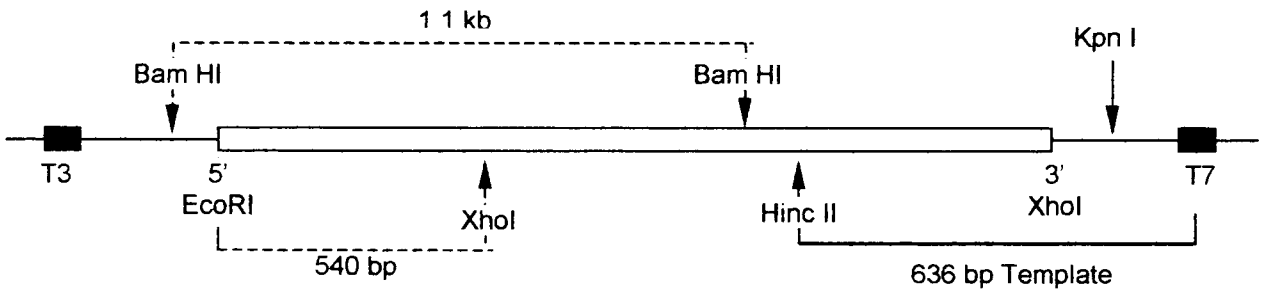


Figure 2.1: Restriction enzyme maps of the gene constructs used in this study. Templates used for antisense riboprobe are indicated. The hatched region represents the coding region of the cDNA.

Clone 196:

Clone 196 (created by C. April) contains a 2.1 kb fragment of chicken *TYRP2* cDNA consisting mainly of untranslated DNA. At the beginning of the present study, details regarding this untranslated portion were not known. This clone was chosen for riboprobe synthesis because the untranslated region was thought to be specific to *TYRP2* only. The coding region was avoided because of possibilities of cross-hybridisation to the other *TRP*'s (April, pers. comm.). Digestion with Bam HI yielded a template of 1.4 kb for the synthesis of antisense riboprobe using T7 RNA polymerase. Sense riboprobe was synthesised from a 1.3 kb Dra I digestion fragment using T3 RNA polymerase. However, subsequent analysis of the chick *TYRP2* sequence by April et al (1988) revealed a stretch of 177 nucleotides within the untranslated region sharing 87% sequence identity with a region downstream of the chicken *T-cell receptor alpha chain constant region* and upstream of the *defender against cell death 1* genes (Wang et al, 1997). Because of these possibilities of cross-hybridisation, this probe was not used in subsequent ISH reactions, and a second *TYRP2* antisense riboprobe was synthesised off a different clone.

Clone 197:

Clone 197 contains the full-length 2.938 kb chicken *TYRP2* cDNA cloned and sequenced by April et al (1998) and consists of 185 bp of 5′ untranslated DNA, 1563 bp of coding region and 1171 bp of 3′ untranslated DNA. A large portion of the coding region (1.279 kb Bam HI digestion fragment) of the insert of clone 197 was subcloned into pGem-3 (Appendix I) and used for the synthesis of another *TYRP2* antisense riboprobe. Digestion with Eco RI yielded a template of 1.279 kb for the synthesis of antisense riboprobe using SP6 RNA polymerase. Sense riboprobe was synthesised from a 1.279 kb Hind III digestion fragment using T7 RNA polymerase.

2.5 Subcloning of 1.279 kb *TYRP2* insert into pGem-3

The 1.279 kb Bam HI digestion fragment of *TYRP2* clone 197 (April et al, 1998) was subcloned into pGem-3 (Appendix I) according to methods by Sambrook et al (1989), and Davis et al (1986). Briefly, pGem-3 was linearised with Bam HI and dephosphorylated to prevent religation of compatible ends. Linear, dephosphorylated vector was electrophoresed and agarose removed from the DNA with Geneclean (GENECLEAN II Kit, BIO 101 Inc.) according to kit instructions. Similarly, clone 197 was digested with Bam HI to release the 1.279 kb insert, separated from its vector by gel electrophoresis, and cleaned with Geneclean. Both the 1.279 kb insert and the linear pGem-3 vector were quantified by electrophoresis using known amounts of lambda DNA

(Boehringer Mannheim) and ligated in a 3:1 insert to vector ratio (Davis et al, 1986). 200µl of competent bacterial cells (XI1-Blue) were transformed with 0.1µg of ligated pGem-3 according to Sambrook et al (1989) and plated on bacterial plates (Appendix II). A colony lift of the bacterial plate was probed with a radiolabeled chick *TYRP2* 1.279 kb probe (created and hybridised by C. April) to locate clones of interest. Mini-lysates from selected clones corresponding to radiolabeled regions were subjected to a series of diagnostic digests to confirm the presence of the 1.279 kb insert. A maxi-preparation of the plasmid DNA was carried out according to Davis et al (1986). Subclones were stored in liquid nitrogen and at -80°C.

2.6 Preparation of Riboprobe

2.6.1 Synthesis of riboprobe

Riboprobes were generated according to standard protocols (Boehringer Mannheim, 1992). All solutions were treated with 0.01% diethyl pyrocarbonate (DEPC) (Appendix III) before use and gloves were worn throughout.

Each reaction mix contained:

1µg linear template DNA

1µl rATP (10mM)

1µl rCTP (10mM)

1µl rGTP (10mM)

1µl rUTP mix (0.65µl rUTP + 0.35µl digoxigenin-11-UTP) (Boehringer Mannheim)

1µl RNAsin (25 Units)

1µl DTT (100mM)

4µl 5X transcription buffer

The reaction mixture was made to a final volume of 20µl by adding DEPC-treated water, mixing gently, and then adding 15-20 Units of T7, T3 or SP6 RNA polymerase (Boehringer Mannheim). When using T7 and T3 RNA polymerase, the reaction mixture was incubated in a 37°C waterbath for 2 hours. Since SP6 RNA polymerase is reported to work optimally at a higher temperature than other polymerases, the reaction mixture containing SP6 RNA polymerase was incubated at 40°C for 2 hours (Sambrook et al, 1989). The reaction was then stopped with 2µl 0.2M EDTA. Riboprobe was precipitated overnight at -20°C with 3µl 4M LiCl to remove unincorporated label, 100µl absolute ethanol and 10µg tRNA, then pelleted by centrifugation and resuspended in 50µl

DEPC-treated water. Aliquots were stored at -20°C and -80°C until required. Repeated freezing and thawing of the probe was avoided.

In order to estimate incorporation of the dig-labeled-UTP and to determine the size of the riboprobe, 4μl of the riboprobe sample was electrophoresed on a 1.3% denaturing formaldehyde gel with bacterial rRNA markers (Appendix II). Northern transfers were carried out and the presence of dig-labeled transcripts determined by immunocytochemical detection of digoxigenin.

2.7 Transfer of nucleic acids onto membranes

2.7.1 Northern transfers

RNA was electrophoresed on denaturing formaldehyde gels as described in Appendix II and the gel washed twice in 10 X SSC for 20 minutes. The RNA was then transferred onto a nylon membrane (Hybond N^+, Amersham) by capillary action using 10 X SSC as a blotting buffer. The RNA was fixed onto the membrane by baking at 80°C for 2 hours. The blot was then ready for detection or stored at room temperature until required.

2.7.2 Southern transfers

DNA was electrophoresed on agarose gels and the gels then denatured and neutralised by washing twice in solution A (1.5M NaCl, 0.5M NaOH) for 20 minutes and twice in solution B (1M NaOAc, 20mM NaOH) for 20 minutes (Davis et al, 1986). The DNA was transferred onto a nylon membrane (Hybond N^+, Amersham) by capillary action using solution B as blotting buffer. The gel was blotted overnight and the DNA immobilised by baking at 80°C for 2 hours. The membrane was then ready for hybridisation or stored at room temperature until required.

2.8 Hybridisation with digoxigenin-labeled riboprobes

Hybridisation of membranes to dig-labeled riboprobes was carried out according to Boehringer Mannheim (1994). The membranes were prehybridised at 60°C for 2 hours (3.7 X SSC, 3.6 X Denhardt's solution, 0.36% sodium dodecyl sulphate (SDS), 500μg salmon sperm DNA). Riboprobe was diluted 1:400 in 2ml of fresh prehybridisation solution, and the membranes hybridised at 60°C for 16 hours in bottles fitted in a rotator in a hybridisation (Hybaid) oven. After hybridisation, the membranes were removed from the bottles and standard detection of the digoxigenin label was immediately carried out.

2.9 Detection of digoxigenin

The membrane was blocked for 1 hour in blocking solution containing 3% milk powder, 2% normal sheep serum, 0.05% Tween. Blocking was followed by incubation in a 1:5000 dilution of the anti-digoxigenin-alkaline phosphatase antibody (Boehringer Mannheim) for 1 hour. The membrane was washed twice in Tris buffered saline (TBS, pH 7.5) for 15 minutes to remove unbound antibody and then equilibrated in a high pH buffer (0.1M Tris, 0.1M NaCl, 0.05M $MgCl_2.6H_20$, pH 9.5) for 2 minutes before detection. The detection solution contained 75 mg/ml nitroblue tetrazolium (NBT) and 50 mg/ml bromochloroindolyl phosphate (BCIP) in the above high pH buffer and was made just before use. The membrane was incubated with this solution (without agitation) in the dark for 5-60 minutes until a suitable signal was obtained. The detection reaction was then stopped by washing the membrane in TE buffer (10mM Tris pH 7.6, 0.1mM EDTA pH 8.0) for 5 minutes and the blot photographed before drying.

2.10 *In situ* hybridisation on sectioned tissue

Sections were cleared in xylol twice for 10 minutes and rehydrated in a series of alcohols to sterile, DEPC treated water. Sections were then permeabilised in 0.03% pepsin (Sigma) solution (in 0.2M HCl) at 37°C for 20 minutes to allow penetration of the riboprobe. Slides were then post-fixed in 4% paraformaldehyde (in PBS) for 15 minutes at room temperature, washed and then acetylated to reduce background. Slides were placed in a 0.1M triethanolamine solution for 2 minutes with agitation. Acetic anhydride was added to a final concentration of 0.25%, and the solution stirred for a further 8 minutes. Acetylation was followed by dehydration through a graded series of alcohols and hybridisation.

Riboprobe was diluted in the hybridisation solution 1:500 for *TYRP2* and 1:125 for *tyrosinase*. [1ml hybridisation mix contains 0.1g dextran sulphate (Sigma), 1x Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrolidone, 0.2% bovine serum albumin), 0.5 X SSC, 2mM EDTA, 50% deionised formamide and 500μg salmon sperm DNA.] Hybridisation was carried out overnight at 60°C in a chamber kept moist with a petri dish containing a 50% formamide/2 X SSC solution (Boehringer Mannheim, 1992). Slides were then washed twice in 2 X SSC at 55°C for 10 minutes and then three times in a 60% formamide/0.2 X SSC solution at 42°C for 20 minutes. Slides were then placed in 2 X SSC for a final wash (10 minutes) before immunocytochemical detection.

Normal sheep serum (2%) was used as a blocking solution and slides were blocked at 37°C for 40 minutes. Slides were then incubated in a moist chamber for 1 hour at 37°C with a 1:500 dilution of the anti-digoxigenin-alkaline phosphatase antibody (Boehringer Mannheim). Unbound

antibody was removed by washing twice in TBS for 5 minutes after which the slides were equilibrated in a high pH buffer for 2 minutes, and placed in detection solution containing 1ml high pH buffer, 0.00034g NBT in 4.5ml dimethyl formamide, and 3.5ml BCIP (Boehringer Mannheim). Detection was carried out in the dark for 5-20 hours and monitored every half hour. The reaction was stopped by washing in TE buffer, after which the slides were rinsed and mounted in veronal buffered glycerol (0.5% sodium veronal barbiturate, 0.3% NaCl pH 8.6, 50% glycerol).

Controls: Negative controls were included in every ISH reaction carried out and were treated identically to the experimental sections except that no riboprobe was added to the hybridisation mix.

2.11 Neural crest cell cultures

Fertile eggs were incubated for 48-55 hours to yield stage 13-14 embryos (Hamburger and Hamilton, 1951). Embryos were removed from the eggs and rinsed in chick saline with penicillin-streptomycin antibiotic 1:100 dilution (Appendix IV). Truncal segments (from the fifth somite to the last somite) were isolated from the rest of the body and treated with 0.05% collagenase in Dulbecco's PBS (Appendix IV) for 10 minutes. This digestion assisted the separation of the neural tube from the adjacent somites and surrounding tissues using dissecting pins. The excised neural tubes were then transferred into 10mm, 24-well tissue culture dishes (Greiner) containing defined medium (DM) and incubated at 37°C.

DM contains Ham's F-12 culture medium (Highveld Biological) supplemented with 1mg/ml bovine serum albumin, 5ng/ml selenium, 1μg/ml transferrin, 1μg/ml insulin, 100ng/ml αMSH and 20ng/ml bFGF (Stocker et al, 1991). Initially, crest cells were also cultured in complete medium, containing Ham's-F12 culture medium supplemented with 20% foetal calf serum, 200mM l-glutamine and gentamycin sulphate (0.1mg/ml), as a positive control. Throughout the present study, all neural crest culture experiments were carried out in duplicate with each experiment containing at least 2, sometimes 3 to 4 neural tubes. Cultures were observed daily with a Leitz phase contrast inverted light microscope. Subjective assessments were made according to three criteria: cell number, cell morphology and pigment granule content.

2.12 Skin extract preparation

Embryos were removed from the eggs and rinsed in chick saline with pen-strep antibiotic 1:100 dilution (Appendix IV). Dorsal and ventral skin was removed using a blade and watchmaker's

forceps, and passed through a sterile, 18-gauge syringe. The resultant homogenate was mixed with an equal volume of chick saline, and allowed to stand for 30 minutes at room temperature. This was followed by centrifugation at 2000xg, at room temperature for 20 minutes. The supernatant (containing 50% skin extract) was first filtered through a 0.45 millipore filter followed by filtration through a 0.22mm filter to ensure sterility. Skin extract was then aliquoted into sterile tubes and stored at -20°C for a maximum period of 6 weeks. To improve its "shelf-life", extract was stored at -80°C.

3. Results

Despite a number of studies aimed at determining whether the white chick is amelanotic due to premature death of melanocytes, or due to a failure of melanocytes to migrate, this issue is still not resolved. The first aim of the present study was to re-examine the question of whether melanocytes are present in the skin and feathers in normal numbers (as in the black control breed), using new molecular tools including melanocyte-specific antibodies and cDNA probes.

3.1 Can immunocytochemistry (ICC) be used to determine whether melanocytes are present in 8- to 13- day white skin in normal numbers?

3.1.1 MelEM antibody tested on quail frozen sections

A melanocyte-specific monoclonal antibody, MelEM, has been reported to recognise quail melanocytes (Nataf et al, 1993). A sample of the antibody was obtained and tested on frozen sections of 10-day quail skin. The presence of specifically bound antibody was detected by immunofluorescence using a FITC-coupled secondary antibody. Light microscopic examination of 10-day quail skin showed extended feather buds consisting of a central core of mesenchyme. In cross-section, feather buds have a layer of epidermal cells on the periphery surrounding a central core of dermal pulp (Fig. 3.1). The intermediate cell layer of the epidermis (between the germinative and the superficial layers) was arranged into 10 to 11 groups which will eventually form the barbs (Fig. 3.1). Numerous melanocytes, located in the dermis and basal layer of the epidermis and in the developing barb ridges of feather follicles, could be distinguished from other epidermal cells by the presence of pigment (not shown). At higher magnification, aggregates of dark cytoplasmic pigment granules were visible in the cell bodies and dendrites of these melanocytes (not shown). When examined for MelEM-specific immunofluorescence, it was noted that fluorescence was restricted to the cytoplasm of the pigment-containing melanocytes (Fig. 3.1, arrows). These results confirmed that MelEM is indeed melanocyte-specific in quail tissues.

3.1.2 MelEM antibody cannot be used to detect chick melanocytes

To determine whether the same antibody could be used to locate chicken melanocytes in frozen sections of 10-day black chicken skin, ICC was carried out in the same way. The results consistently showed fluoresence in unpigmented dermal cells as well as in cells in the connective tissue around all the internal organs, indicating non-specific immunoreactivity of MelEM to a

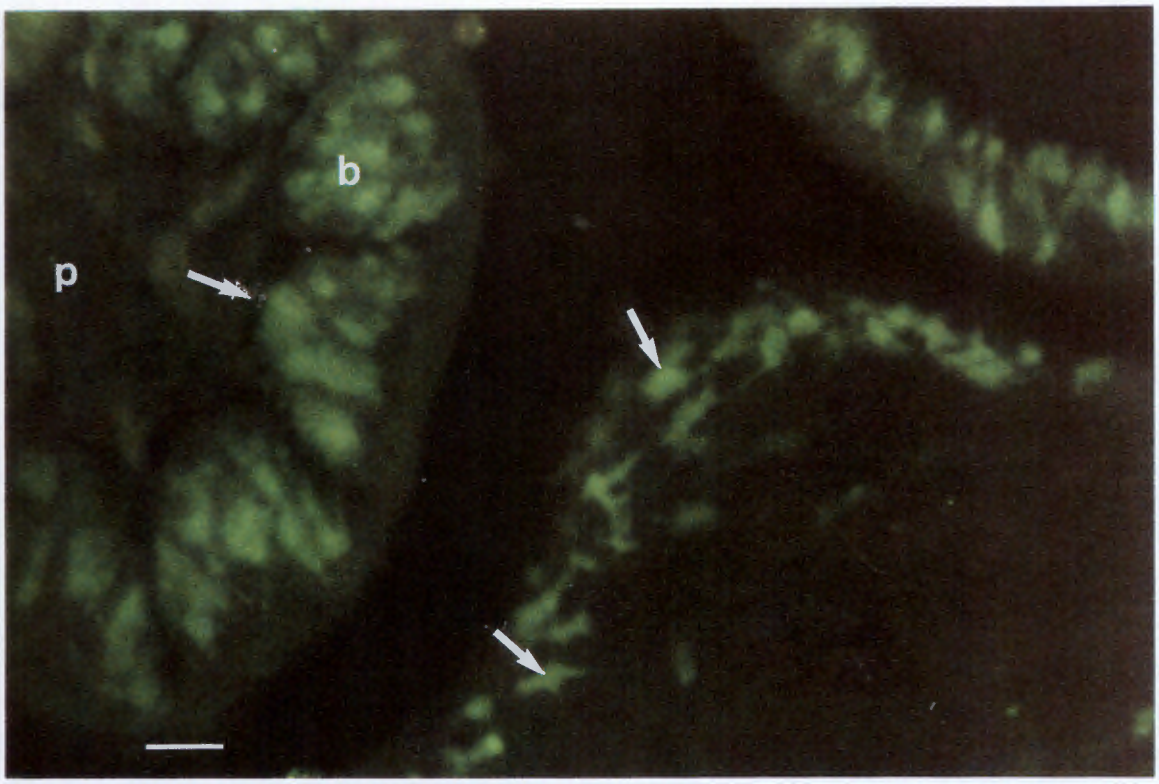


Figure 3.1: Frozen section of 10-day quail skin immunostained with MelEM.
Melanocytes can be seen in the epidermis and barb ridges of the feather follicles (arrows). p, pulp. b, barb ridge.
Bar represents 20μm.

variety of cell types (not shown). No fluorescence was visible in the epidermis (where melanin-containing cells could be seen) indicating that melanocytes were probably not being labeled with the antibody. A variety of conditions were tested to improve the specificity of the antibody, but all attempts proved unsuccessful. Thus, MelEM could not be used to locate chicken melanocytes and alternative labeling procedures were therefore explored.

3.2 Can melanocytes in white chick skin be detected with *in situ* hybridisation?

Since immunocytochemistry could not be used to locate melanocytes in chicken skin and feather tissues, *in situ* hybridisation (ISH) using melanocyte-specific probes was next explored. To determine whether melanocytes are present in the skin and feather follicles of white chicken embryos in normal numbers, non-radioactive, digoxigenin (dig)-labeled antisense chick *TYRP2* and *tyrosinase* riboprobes, previously cloned and sequenced by April et al (1996, 1998), were used in all the studies described below.

3.2.1 Sub-cloning, preparation of template DNA and synthesis of riboprobes

3.2.1.1 Preparation of *TYRP2* riboprobe

The first ISH reactions were carried out with probe synthesised from the untranslated region of a chick *TYRP2* cDNA (April et al, 1998). However, hybridisation results using this probe showed cross-hybridisation to many tissues in the embryo. Subsequent analysis of this cDNA sequence by April et al (1998) revealed a stretch of nucleotides within the untranslated region which shares 87% sequence identity with a region downstream of the chicken *T-cell receptor alpha chain constant region* and upstream of the *defender against cell death 1* genes (Wang et al, 1997). This sequence appears to be a dispersed repetitive transposable element frequently found in the avian genome and could account for the cross-hybridisation to many tissues in the embryo. Since this probe was not suitable for ISH studies, it was therefore necessary to subclone a cDNA encompassing the coding region to provide a suitable template for probe synthesis. Details of the cloning are provided in Materials and Methods, section 2.4.2.

A 1.279 kb Bam HI fragment consisting of coding region of *TYRP2* cDNA was cloned into pGEM-3 (2.87 kb). Ava I and Kpn I digests of two clones revealed the insert in a 3′-5′ direction (Fig. 3.2). Clone 1 was subsequently used for riboprobe synthesis. Antisense and sense, digoxigenin-labeled riboprobes were prepared from Eco RI and Hind III digested DNA, respectively.

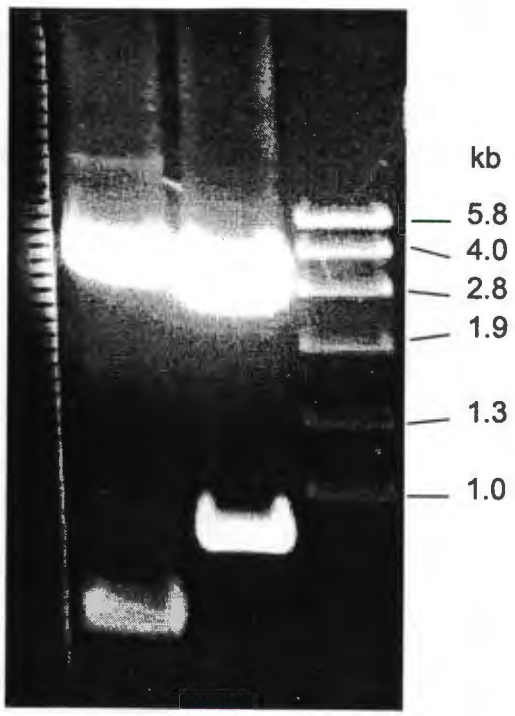


Figure 3.2: Restriction enzyme digest of a clone of a *TYRP2* cDNA with Kpn I (lane 1) and Ava 1 (lane 2) to determine the orientation of the 1.279 kb Bam HI insert. Lane 3, molecular weight marker.
The 1.279 kb coding region of clone 197 (see Figure 2.1 for gene construct) was cloned into pGEM-3 as described in section 2.5 pg 2.4. The insert was present in a 3′-5′ direction, as determined by the predicted digests: 528 bp and 3.6 kb with Kpn I; and 801bp and 3.3 kb with Ava I.

3.2.2 In order to establish and standardise *in situ* hybridisation protocols for chicken *TYRP2*, *in situ* hybridisation was initially carried out on wax sections of the optic cup of three day old chickens

Initially, ISH procedures resulted in high background staining with no positive signal. Background staining was only visible in sections to which riboprobe had been added, and not in control slides (where riboprobe was omitted). This indicated that the background was due to non-specific binding of the riboprobe and not due to binding of the anti-digoxigenin antibody. To reduce this background and increase signal, changes in the hybridisation protocol were carried out until satisfactory results were obtained. Changes included adjustments in salt (0.1 X - 4 X SSC), formamide (30 - 50%), and probe (1:500 dilution) concentrations, and modification of the hybridisation temperature (50 - 60°C) until optimal conditions were established (see Materials and Methods section 2.10).

Once modifications to the protocol were made and hybridisation conditions were suitable, *TYRP2* expression was detected in the RPE as can be seen by the very strong black/blue signal within this region (Fig. 3.3). Signal was strongest at the back of the eye (see long arrows) and lower in the prospective iris and ciliary body (see short arrows). No hybridisation signal was seen in the prospective neural retina, lens or periocular mesenchyme, except for one or two labeled cells thought to be iridial or choroidal melanocytes (see arrowheads). No signal was detected in the negative control samples without probe (not shown).

3.2.3 Can *in situ* hybridisation be used to detect *TYRP2*- expressing cells in the skin and feather follicles ?

To determine whether the above hybridisation conditions are suitable for the detection of *TYRP2* - expressing melanocytes in the skin and feather follicles, *in situ* hybridisation was carried out on wax sections of 10-day black chick skin. Light microscopic examination of skin sections showed elongated feather buds with a central core of mesenchyme. The epidermis between two adjacent feather buds was usually three cell layers thick, while the bud epidermis normally consisted of more than three layers. In cross-section, a feather bud has epidermal cell layers on the periphery surrounding a central dermal pulp (Fig. 3.4 B). Melanocytes could be distinguished from other epidermal cells by the presence of dark cytoplasmic pigment granules in their cell bodies and dendrites (Fig. 3.4 B). In the developing barb ridges, melanocytes were located in longitudinal rows within each ridge with their cell bodies at the apex (nearest the pulp) and their processes extended along the ridge margin towards the periphery of the follicle (Fig. 3.4 B). Sections treated with riboprobe clearly and routinely showed specific blue signal within the cell bodies of these melanocytes (Fig. 3.4 A, C). No signal was detected in the negative control samples without probe (Fig. 3.4 B).

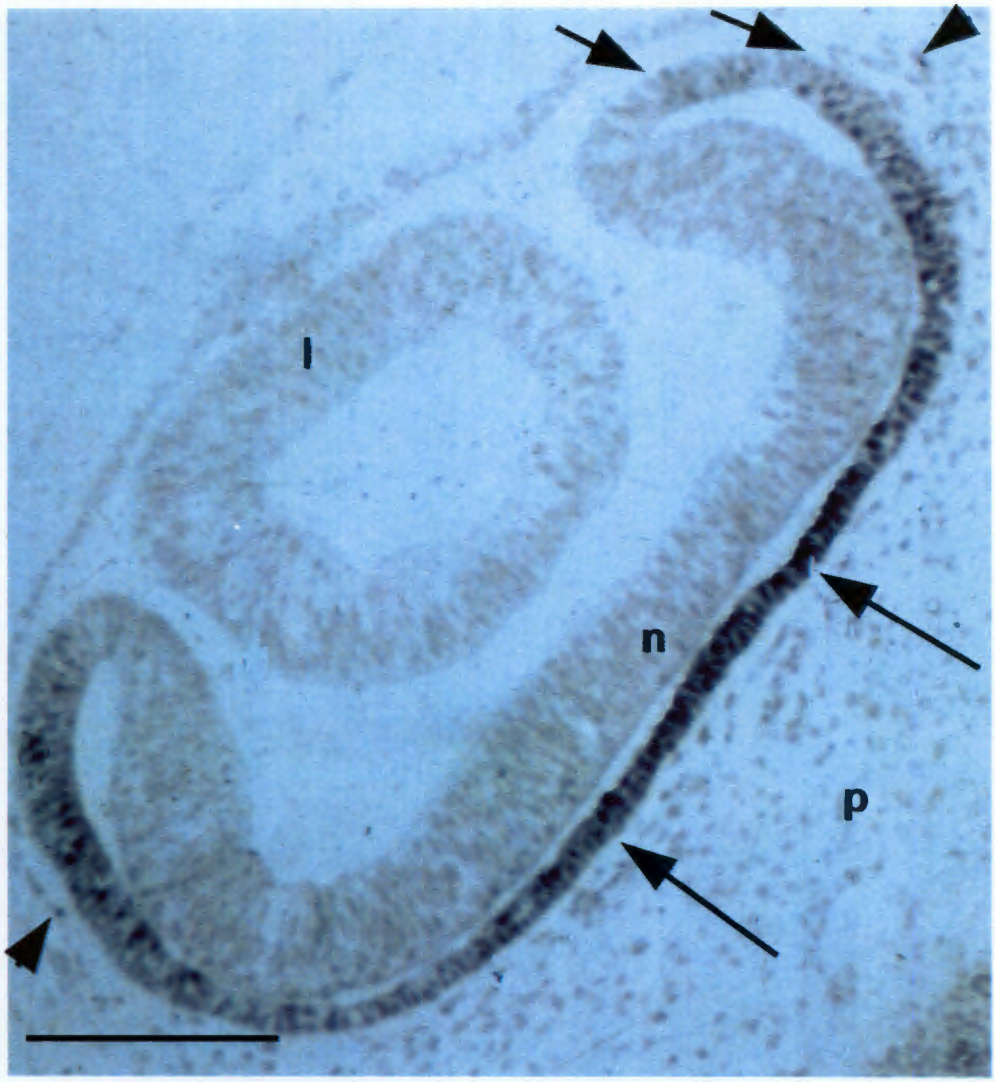


Figure 3.3: *TYRP2* expression in the retinal pigment epithelium of a 3-day black chick embryonic eye cross-section (arrows).
l, lens. p, periocular mesenchyme. n, neural retina. arrowheads, prospective iridial and choroidal melanocytes. Long arrows indicate strong signal at the back of the eye. Short arrows indicate low signal in the prospective iris and ciliary body.
Bar represents 100μm.

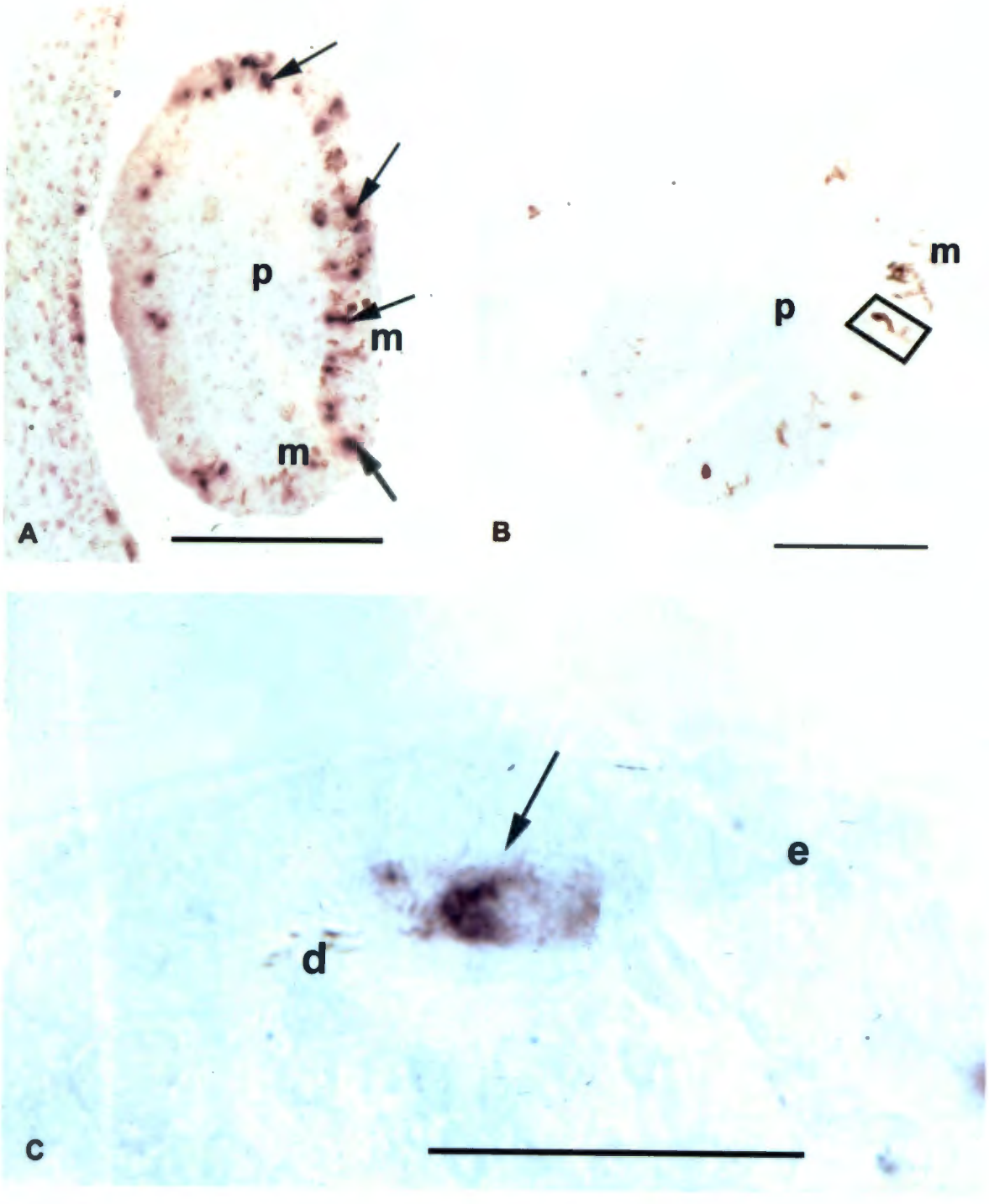


Figure 3.4: (a) 10-day black feather follicle showing *TYRP2* -expressing melanocytes (arrows). *m*, melanin. *p*, pulp.
(b) Negative control of (a) showing melanin (m) but no dig-labeling. Box represents barb ridge with melanocyte.
(c) Expression of *TYRP2* in a 10-day black skin melanocyte (arrow). e, epidermis. d, dendrite containing melanin.
Bar represents 100μm for (a), (b), 20μm for (c).

Having established that the above ISH protocol is suitable for localisation of *TYRP2* -expressing melanocytes in black chick skin, ISH was carried out on sections of 10-day white chicken skin to determine whether melanocytes are present in normal numbers (compared with the control black breed). With light microscopic examination, it was immediately apparent that there is no melanin in the dermis, epidermis or feather follicles of white skin (Fig. 3.5 C). ISH results showed that, as with feather follicles from black chicks, melanocytes were indeed present in the developing barb ridges of white follicles (Fig. 3.5 A, B, arrows). Furthermore, at first glance it was obvious that these *TYRP2* -expressing melanocytes were present in similar numbers to that in black follicles. *TYRP2* -expressing melanocytes could also be seen in the dermis and basal layer of the epidermis (Fig. 3.5 B, arrows). No signal was detected in the negative control samples without probe (Fig. 3.5 C). To obtain a more accurate measure of whether there are differences in *TYRP2* expression between black and white skin, random feather follicle sections from various levels of the follicle (including sections through the barb ridges and collar) were chosen from both black and white skin sections and the number of dig-labeled cells per follicle estimated; each value was corrected for 10000μm^2 area of follicle. An average value of dig-labeled cells per 10000μm^2 was calculated for each breed (Table 1). The results showed that in the developing barb ridges of 10-day white follicles, melanocytes were present in normal, if not greater numbers than in black follicles.

Table 3.1: Results of *in situ* hybridisation using *TYRP2* antisense riboprobe applied to black and white chick feather follicles

Age (days)	Tissue[2]	No. of follicles counted[3]	Dig-labeled cells per 10000μm^2 (mean ± SD)
10 (n[1]=3)	black	16	4.0 ± 1.8
10 (n=3)	white	22	7.0 ± 2.7
12 (n=1)	black	9	7.8 ± 2.7
12 (n=1)	white	10	3.2 ± 1.1
13 (n=3)	black	17	7.8 ± 1.7
13 (n=3)	white	19	1.7 ± 1.0

[1] n = number of embryos examined
[2] black = Black Australorp, white = White Plymouth x Pile Game
[3] See text for details

To determine whether melanocytes are present in white skin at later stages of development, ISH was carried out on sections of feather follicles from 12- and 13-day black and white skin. Within these developing barbs, which are now more distinctive, feather follicles from black chickens showed abundant dig-labeled cells (Fig. 3.6 A, arrows). In contrast, white follicles revealed fewer dig-labeled cells than in black follicles (Fig. 3.6 B, arrows). No signal was detected in the

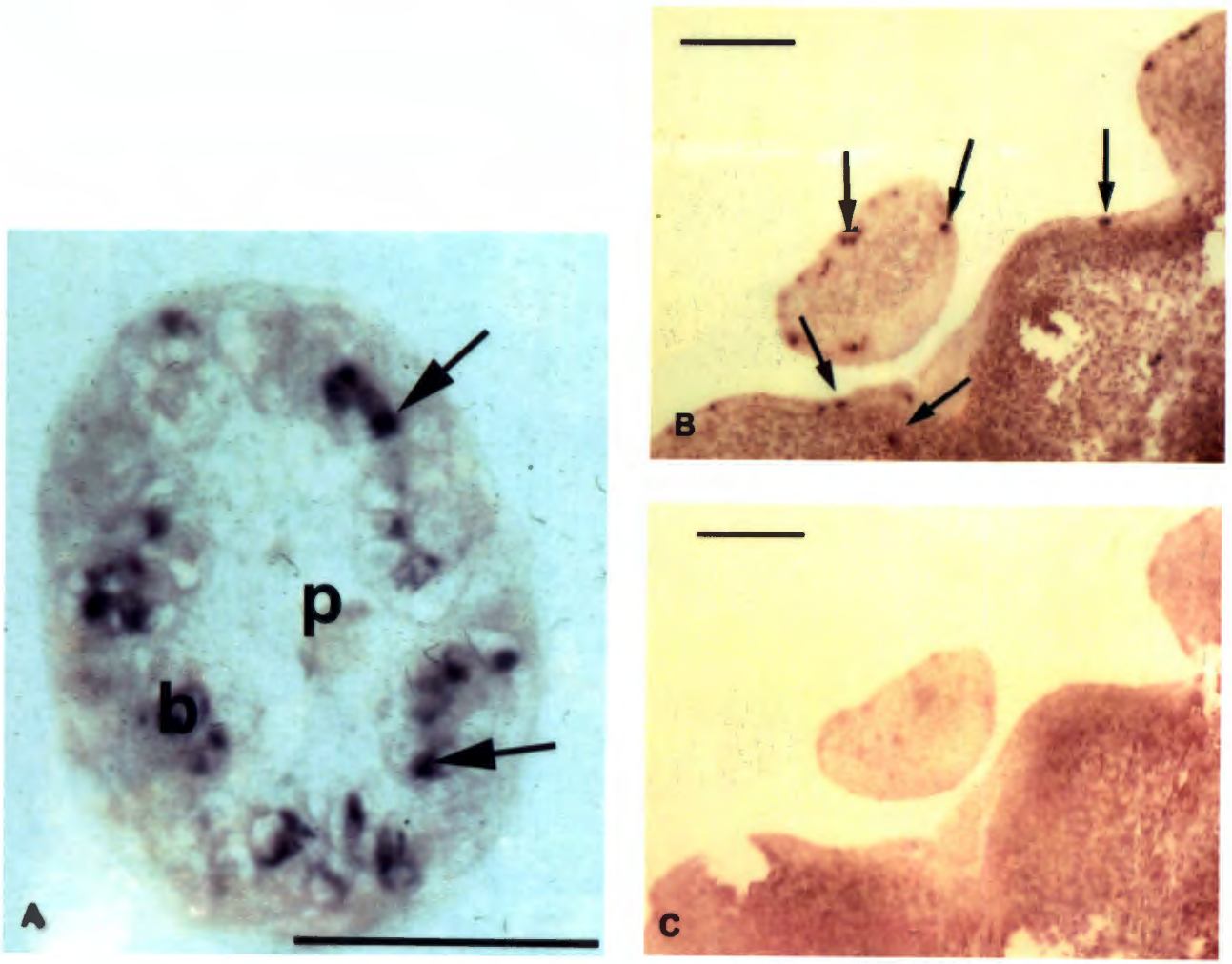


Figure 3.5: 10-day white skin wax sections showing *TYRP2* expresssing melanocytes (arrows) in feather follicles (a), (b) and skin (b). p, pulp; b, barb.
Melanocytes are present in normal numbers in both black (see Fig. 3.4) and white feather follicles. (c) Negative control of (b) showing no dig-labeling.
Bar represents 75μm for (a), 100μm for (b) and (c).

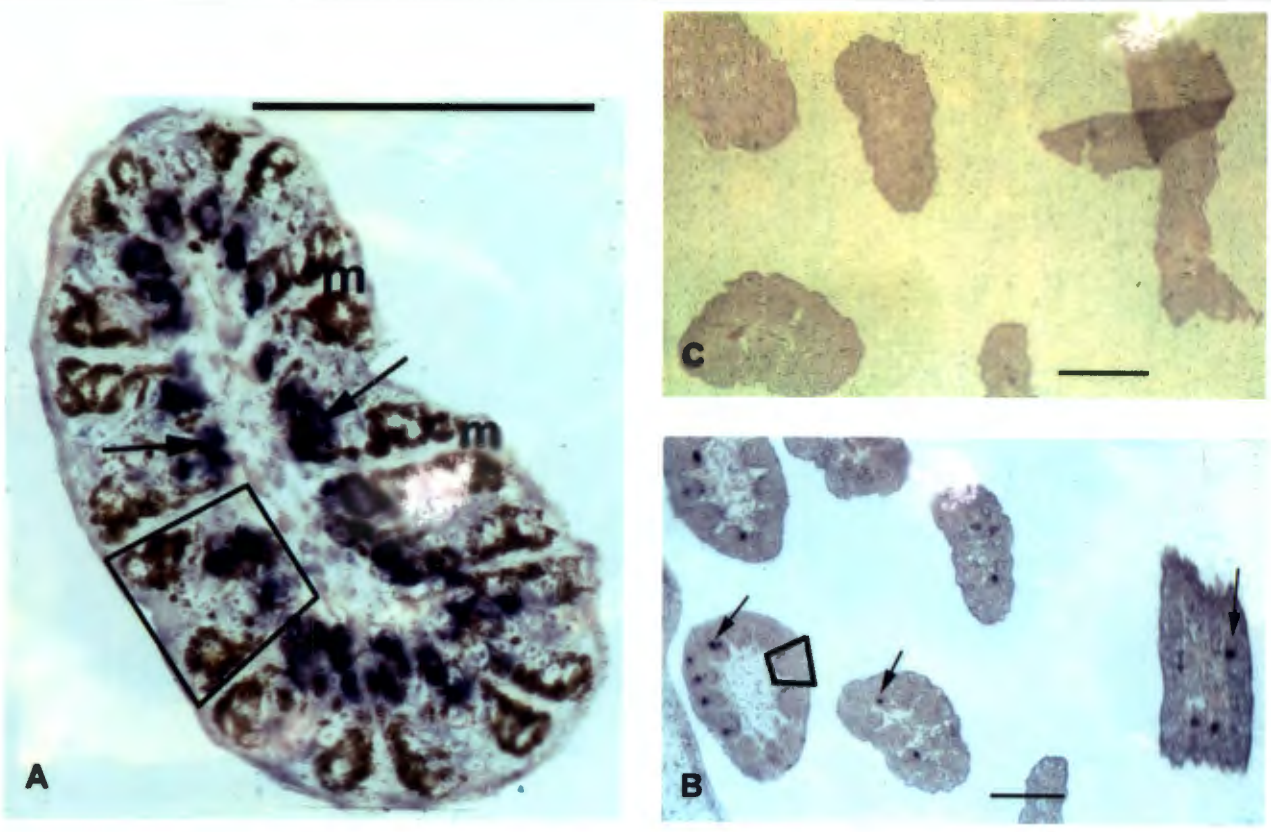


Figure 3.6: Expression of *TYRP2* in 13-day black and white feather follicles (arrows). m, melanin. Black feather follicles (a) contained approximately 4.6 times more *TYRP2* - expressing melanocytes than white feather follicles (b). Box represents barb ridge. (c) Negative control of (b).
Bar represents 100μm.

negative control samples without probe (Fig. 3.6 C). Furthermore, in white skin, the number of dig-labeled cells tended to decrease in the follicles with increasing age, compared with the black breed (Table 1). Examination of Table 1 clearly shows that at 10-days, white follicles contained 7 *TYRP2* -expressing melanocytes per 10000μm^2. Yet, instead of increasing with developmental age as in black follicles, melanocytes in white follicles decreased to 3.2 per 10000μm^2 at 12-days, and 1.7 melanocytes per 10000μm^2 at 13-days. In 12-day embryos, black feather follicles contained approximately 2.4 times more *TYRP2* -expressing melanocytes than white follicles. In 13-day embryos, black feather follicles contained approximately 4.6 times more *TYRP2* -expressing melanocytes than white follicles (Fig. 3.6).

In an attempt to determine whether melanocytes are present at later stages of development, ISH with the *TYRP2* riboprobe was carried out on 17-day black and white chick skin sections. Regions of various levels of the feather were examined. Neither black nor white follicles showed dig-staining along the length of the follicle (not shown). When looking at the dermal papillae located at the base of each follicle, *TYRP2* -expressing cells could be seen in black follicles, yet in white follicles, no *TYRP2* -expressing melanocytes were present (not shown). This confirms the above results which show a decline in the number of *TYRP2* -expressing cells with increasing age of white follicles compared with black follicles. In other words, this trend continued to the extent that no more *TYRP2* -expressing melanocytes could be seen in the older white chicks.

3.2.4 *In situ* hybridisation on black and white chick skin wax sections to detect *tyrosinase*-expressing melanocytes

To determine numbers of *tyrosinase* -expressing melanocytes in black and white chick skin, and to compare these numbers to *TYRP2* -expressing cells, ISH was carried out with a *tyrosinase* riboprobe on 10-day black and 10-day white skin sections. *Tyrosinase* -expressing cells were seen in the developing barb ridges of the feather follicles in both black and white skin (Fig. 3.7 A, C). Furthermore, melanocytes appeared to be present in normal numbers in white follicles. No signal was detected in the negative control samples without probe (Fig. 3.7 B, D). In order to obtain a more accurate measure of whether there are differences in *tyrosinase* expression between black and white skin, random feather follicles were chosen from black and white skin sections and the number of dig-labeled melanocytes per 10000μm^2 estimated, as in *TYRP2* runs above (Table 2). These results clearly indicate the presence of normal numbers of *tyrosinase* -expressing melanocytes in white chick skin at this stage.

Table 3.2: Results of *in situ* hybridisation using *tyrosinase* antisense riboprobe applied to black and white chick feather follicles

Age (days)	Tissue[2]	No. of follicles counted[3]	Dig-labeled cells per 10000μm² (mean ± SD)
10 (n[1]=2)	black	4	6.0 ± 2.2
10 (n=3)	white	14	5.5 ± 1.5
13 (n=2)	black	8	8.7 ± 2.7
13 (n=2)	white	16	0.8 ± 0.5

[1] n = number of embryos examined
[2] black = Black Australorp, white = White Plymouth x Pile Game
[3] See text for details

The above results again confirm that at this stage of development, amelanosis is not due to a reduction in the number of melanocytes in the skin regions of the white chick. Furthermore, amelanosis is not due to a block in *tyrosinase* expression. To determine the expression pattern of *tyrosinase* later in development, ISH was carried out on 13-day black and 13-day white skin sections. Black follicles showed abundant dig-labeled cells (Fig. 3.8 A). In contrast, white follicles revealed fewer dig-labeled cells (Fig. 3.8 C). No signal was detected in the negative control samples without probe (Fig. 3.8 B, D). Furthermore, in white skin, the number of dig-labeled cells decreased in the feather follicles with increasing age compared with the black breed. Examination of Table 2 shows that at 10-days, white follicles contained 5.5 melanocytes per 10000μm². Yet, at 13-days, instead of increasing with developmental age, the number of *tyrosinase* -expressing melanocytes decreased to 0.8 cells per 10000μm². Comparing black with white skin, black feather follicles contained approximately 10.9 times more melanocytes that expressed *tyrosinase* than was the case in white feather follicles (Table 2). These results indicate that melanocytes arrive in the white chick skin in normal numbers, and although they may be dying at later stages, they still do not synthesise melanin at the stage when black skin melanocytes are melanised.

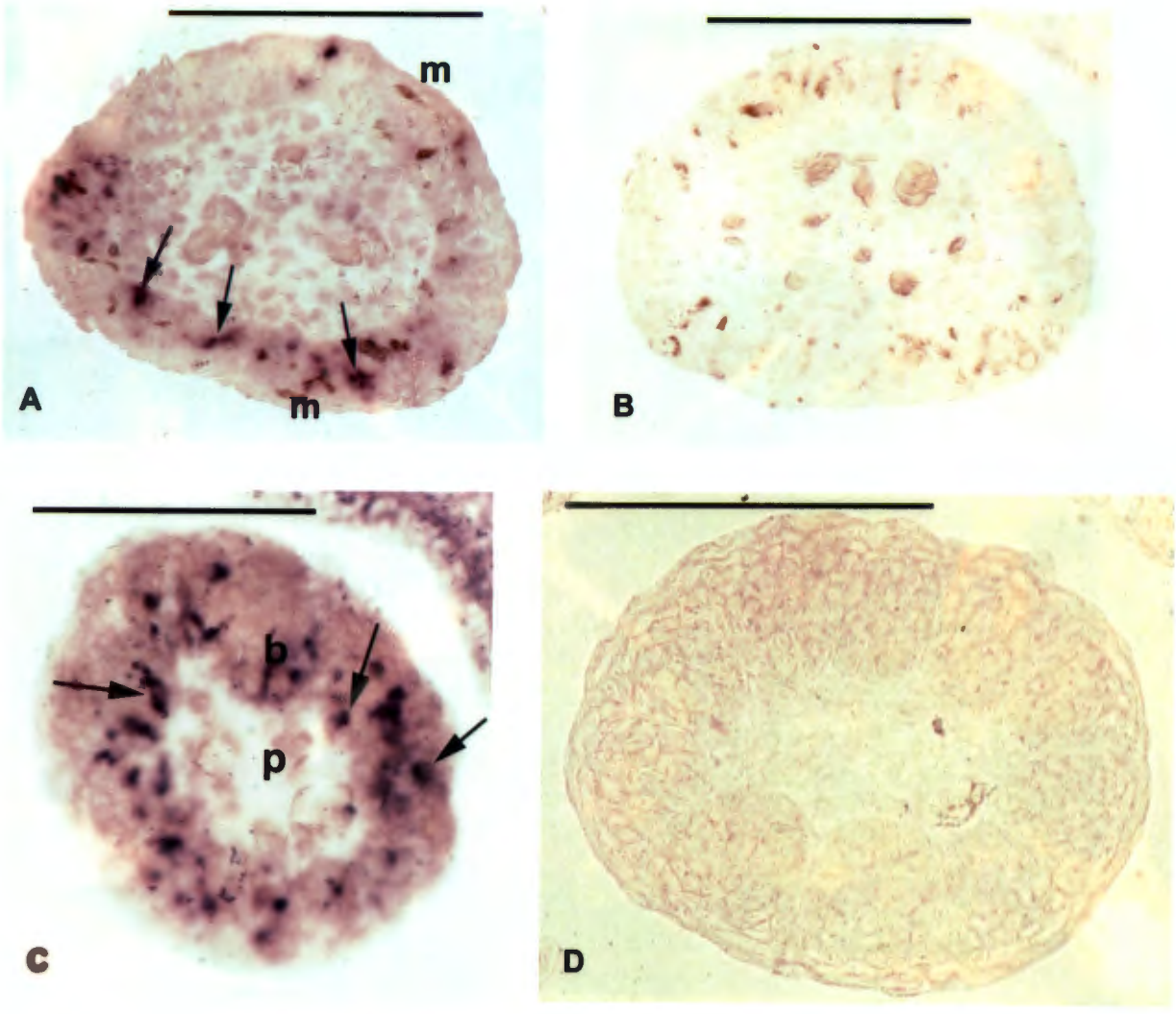


Figure 3.7: *Tyrosinase* expression in 10-day black and white feather bud melanocytes (arrows). m, melanin. p, pulp. b, barb. Melanocytes are present in normal numbers in both black (a) and white feather buds (c).
(b) Negative control of (a) showing melanin but no dig-labeling.
(d) Negative control of (c) showing no dig-labeling.
Bar represents 100μm.

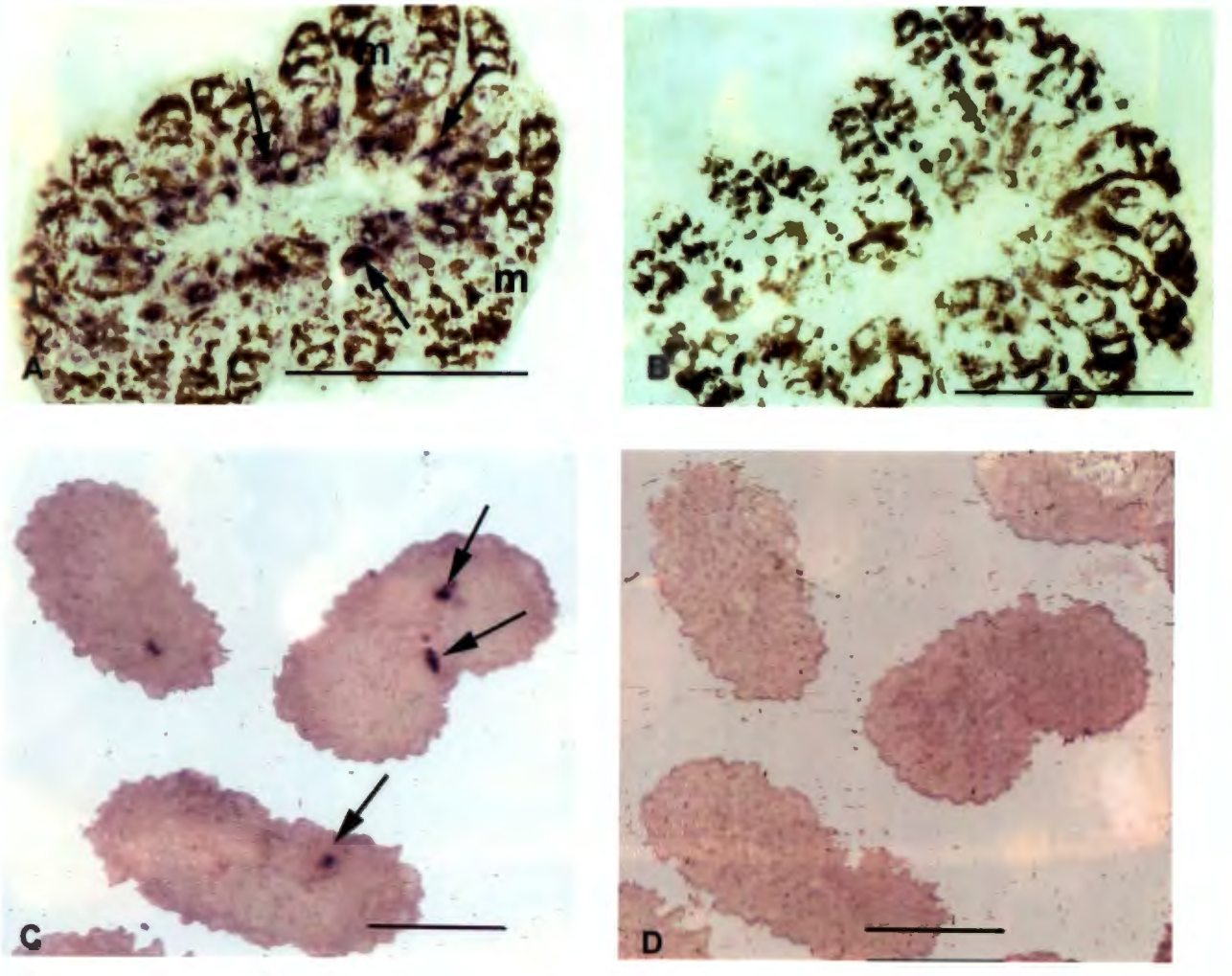


Figure 3.8: *Tyrosinase* expression in 13-day black and white feather follicles as shown by dig-labeled melanocytes (arrows). m, melanin.
Black feather follicles (a) contained approximately 10.9 times more melanocytes expressing *Tyrosinase* than white feather follicles (c). (b) Negative control of (a) showing melanin but no dig-labeling. (d) Negative control of (c) showing no dig-labeling. Bar represents 100μm.

3.3 Neural Crest-Derived Melanocytes In Culture

The second part of this study addressed the question of why white chick melanocytes never synthesise melanin *in vivo*, even though they arrive and occupy appropriate locations in the barb ridges of the feather follicle. Previous studies have demonstrated that neural crest cells from WL and WPR X PG chick embryos pigment in culture medium containing foetal calf serum and a combination of growth factors (Hamilton, 1940; Marco, 1994). This somewhat paradoxical result indicates that the cells are not intrinsically defective in their ability to synthesise melanin and raises the possibility that some factor necessary for differentiation is missing or defective in the white skin. To address this issue, a series of experiments was designed to investigate directly whether there are differences in the black and white skin environment which are able to cause changes in melanisation. Serum-containing medium could not be used for this purpose because it is an undefined mixture of unknown growth factors sufficient for the differentiation of crest cells into melanocytes. It was therefore necessary to establish conditions for the culture of neural crest cells in a defined medium (DM). Neural crest cells were also cultured in serum-containing medium to compare the growth and survival of these cells to those grown in DM.

3.3.1 Can white chick skin support melanocyte survival and differentiation?

3.3.1.1 Neural crest cells cultured in defined- and serum-containing media

Neural crest cells from black chicks were explanted into a defined medium (DM) previously shown to be sufficient for the survival and proliferation of chick neural crest cells, but not their differentiation into melanocytes (Stocker et al, 1991). DM contains both bFGF and αMSH, of which bFGF has been shown to be essential for the survival of neural crest cells. Cells cultured without bFGF contained vacuoles and attached poorly to the culture dish (Stocker et al, 1991). This survival effect of bFGF was also observed in the present study. Neural crest cells were also cultured in Ham's F12 culture medium supplemented with 20% foetal calf serum. Cultures were observed daily with an inverted light microscope and subjective assessments made according to three criteria: cell number, cell morphology and pigment granule content.

In both DM and serum-supplemented medium, within 24 hours after explant, stellate neural crest cells had migrated away from the neural tube and surrounded the explant in a radially symmetric fashion (not shown). These were commonly visible as a monolayer on the plastic substrate but, at times, also in aggregates near the neural tube explant. In DM without bFGF, cell survival was severely compromised and the majority of the cells rounded up and lifted (not shown). By two

days in culture, in bFGF-containing medium, individual neural crest cells had proliferated so that thousands of cells had dispersed further from the neural tube (Fig. 3.9 A). There were no differences in morphology between cells in DM (Fig. 3.9 A) and in serum-containing medium (Fig. 3.10). After three days in culture, the neural tube was removed from the culture dish to prevent the spread of neuroepithelium from the ends of the tube explants. In serum-containing medium, about 30% of neural crest cells started to become dendritic, with a few polygonal and bipolar types (not shown). Fine pigment granules were visible within their cell bodies and dendrites. These pigmented cells became darker in colour with subsequent days in culture (Fig. 3.11, Table 3). In striking contrast, neural crest cells in DM remained stellate, never became dendritic and never contained pigment granules (Fig. 3.9 B, Table 3). In both DM and serum-containing cultures, cells rarely survived beyond 10-days.

These results indicate that DM containing bFGF allows for the proliferation and survival of neural crest cells but is not sufficient for their differentiation into melanocytes. Having established suitable serum-free culture conditions, it became possible to investigate whether chick skin extract contained essential factors which, when added to DM, would allow for the differentiation of crest cells into melanocytes.

Table 3: Effects of black and white skin extract on neural crest cells

Tissue[1]	**20 % Skin extract**	**medium**[3]	**onset of melano-genesis (days)**	**pigment granules**	**Average period of survival (days)**
black (n[2] = 5)	-	CM[3]	3	++	9
black (n = 5)	-	DM	-	-	9
black (n = 4)	black	DM	1	+	8
black (n = 4)	white	DM	1	+	8
white (n = 3)	black	DM	1	+	8
white (n = 3)	white	DM	1	+	8
white (n = 3)	black	DM-αMSH	1	+	6
white (n = 3)	white	DM-αMSH	1	+	6

[1] white = WPR x PG neural crest cells (ncc), black = BA ncc
[2] n = number of times experiment repeated (each experiment was conducted in duplicate, with each experimental well containing two to three, sometimes four neural tubes)
[3]CM = complete medium (Ham's F12 + 20% foetal calf serum), DM = defined medium, DM-αMSH = defined medium without αMSH. + = degree of pigmentation.

3.3.1.2 Effects of black- and white skin extract on black chick neural crest cells

In these experiments, black neural crest cells were grown in DM as described above. On day 3, black skin extract was added to the medium, and the cells were examined with phase contrast and brightfield microscopy. Within 24 hours, almost all neural crest cells converted from

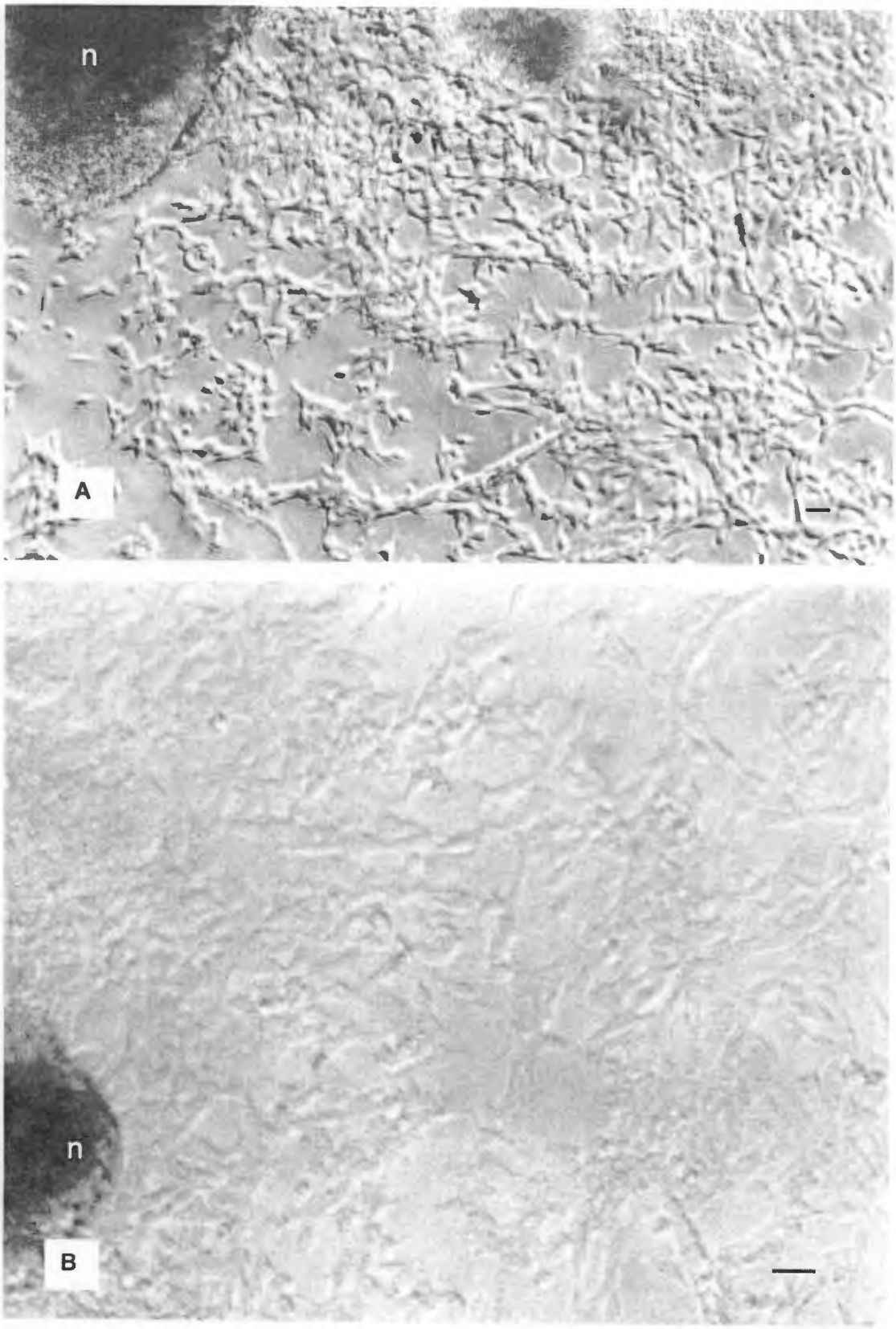


Figure 3.9: Black chick neural crest cells grown in DM containing bFGF and αMSH. (A) Phase contrast and (B) Brightfield light micrographs showing stellate, undifferentiated (unpigmented) cells around the neural tube explant, (n).
Bar represents 20μm.

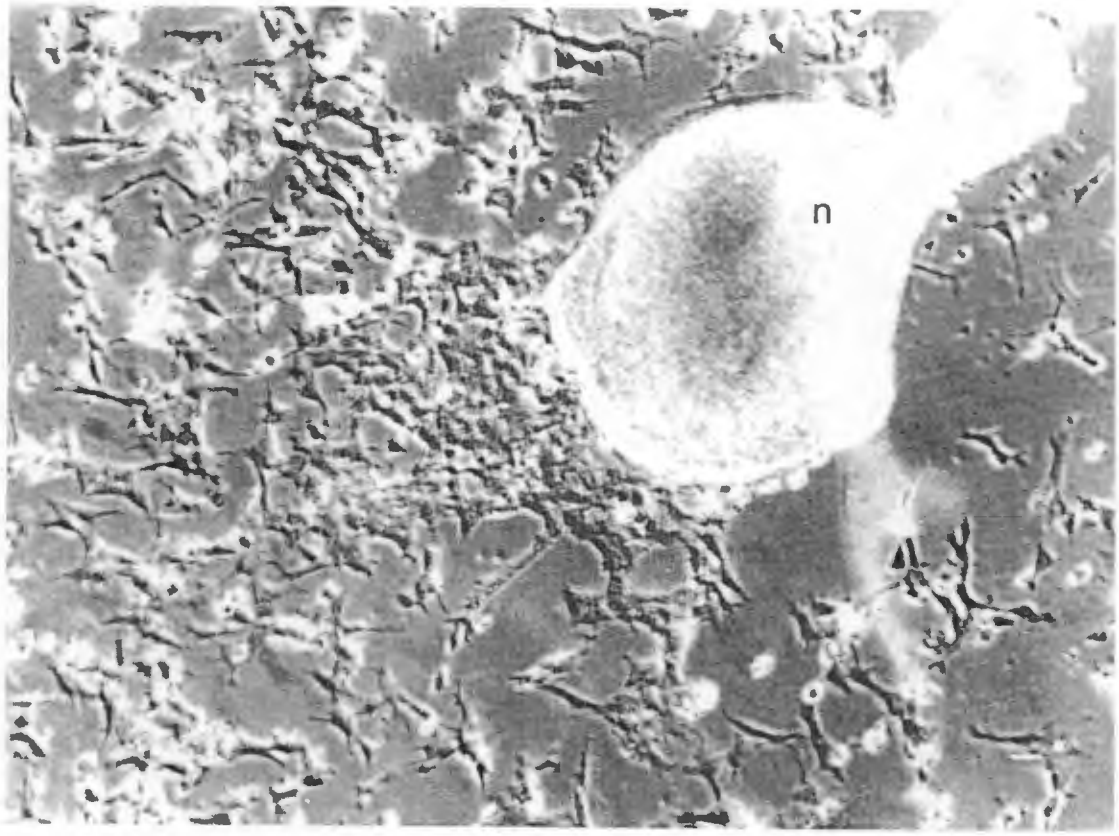


Figure 3.10: Light micrograph of black chick neural crest cells grown in serum-containing medium. n, neural tube explant.
Bar represents 20μm.

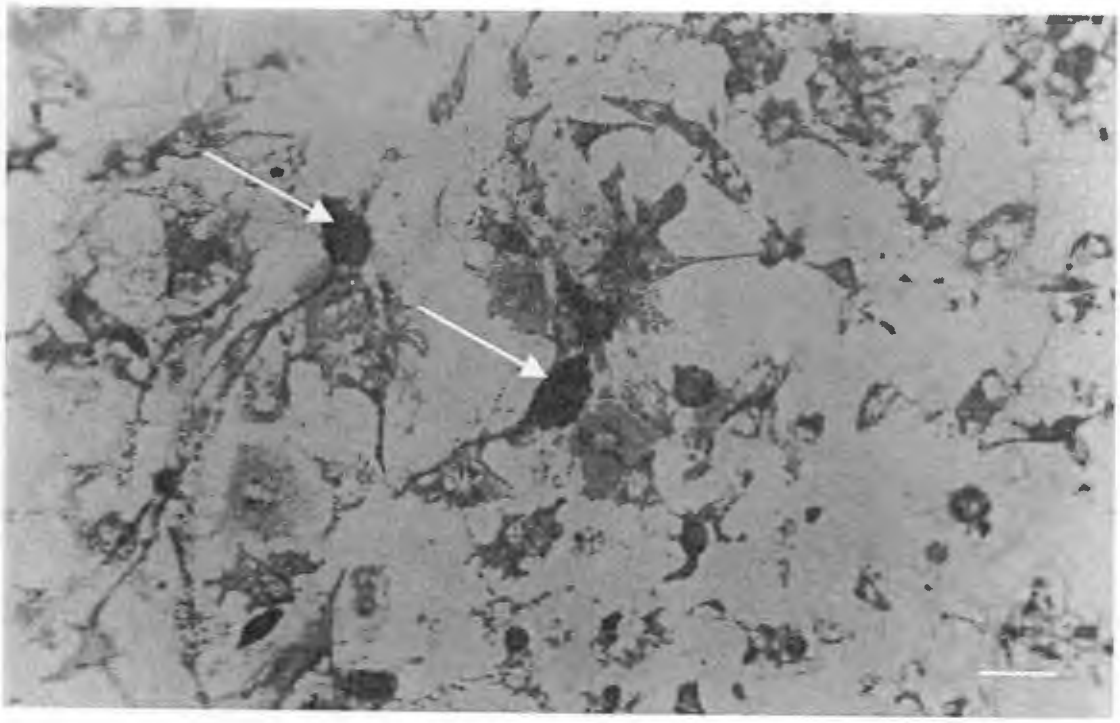


Figure 3.11: Black chick neural crest-derived melanocytes grown in serum-containing medium containing dark pigment (arrows), modified from April et al (1996).
Bar represents 20μm.

a stellate to a dendritic morphology typical of melanocytes (Fig. 3.12), similar to those seen in serum-containing cultures (Fig. 3.11). In addition, these cells contained fine, light brown cytoplasmic pigment granules in their cell bodies (perinuclear) as well as in their dendrites (Fig. 3.12, Table 3). This clearly shows that black skin extract is sufficient for melanocyte differentiation. However, compared with cells in serum-containing medium, the cells never became as darkly pigmented. Cultures in DM alone never became pigmented (not shown).

The next question was to ask whether white skin extract is able to elicit a similar response. Experiments were carried out in the same way and it was found that, as with cultures treated with black skin extract, cells became dendritic and pigmented (Fig. 3.13) within 24 hours. This indicates that white skin extract is sufficient for the survival and differentiation of black neural crest cells into melanocytes. By day 5, the majority of cells in all cultures supplied with skin extract-supplemented medium formed large, shiny (phase-bright) vacuoles which obscured the fine pigment granules, and after two to three days in culture rounded up and lifted (not shown). No consistent differences in survival were observed between cultures treated with black or white skin extract.

3.3.2 Is amelanosis due to an intrinsic melanocyte defect?

It has already been established that white neural crest cells in serum-containing medium differentiate into melanocytes. The next question addressed was whether white neural crest cells grown in serum-free conditions were able to differentiate when cultured in extract of skin. For this purpose, neural crest cells from white chicks were cultured in a defined medium in the presence of black skin extract, as described above. As before, within one day of adding black skin extract, white chick neural crest cells became dendritic and contained fine, light brown pigment granules in their cell bodies (perinuclear) and in their dendrites (Fig. 3.14, Table 3). There were no obvious differences in cell morphology, pigment granule density and colouration of neural crest cells cultured in black skin extract, regardless of their origin (Compare Fig. 3.12 and 3.14). Thus, white neural crest cells are capable of differentiating into melanocytes in the presence of factors contained in black skin extract.

3.3.3 Is amelanosis due to the combination of an intrinsic melanocyte defect and an environmental defect?

Since white skin melanocytes do not melanise *in vivo*, the next step was to investigate whether white skin extract is able to induce differentiation of white neural crest cells into melanocytes. Experiments were carried out in the same way as described above. Surprisingly, within one day of adding white skin extract, crest cells showed a dendritic morphology and contained fine, light brown pigment granules (Fig. 3.15, Table 3). This experiment was repeated three times in

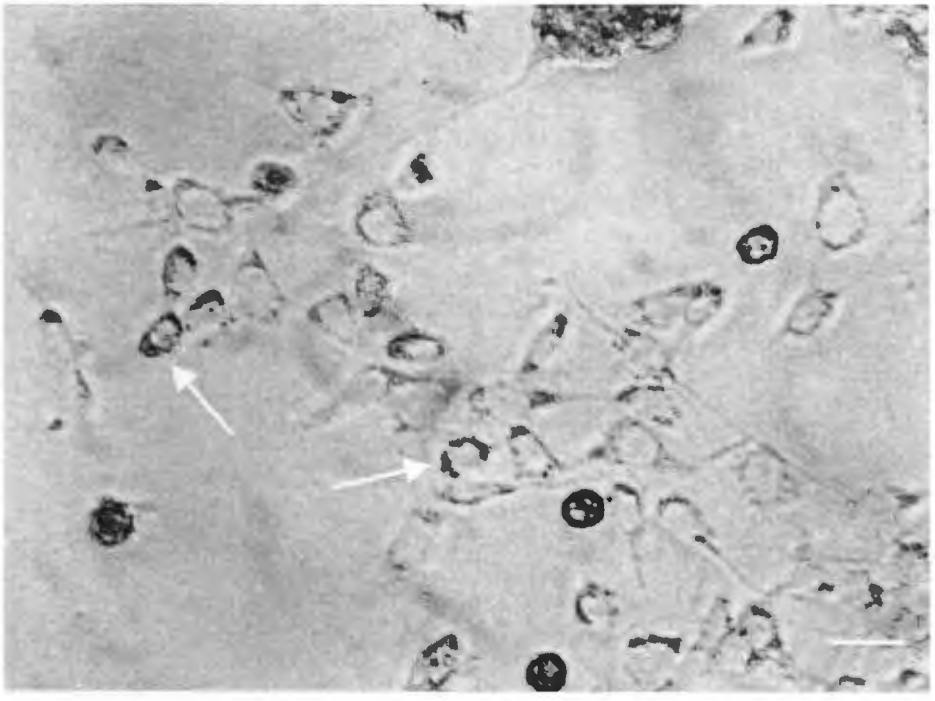


Figure 3.12: Light micrograph of black chick neural crest-derived melanocytes in DM supplemented with 20% black skin extract. The results show dendritic melanocytes with perinuclear, cytoplasmic pigment granules (arrows).
Bar represents 20μm.

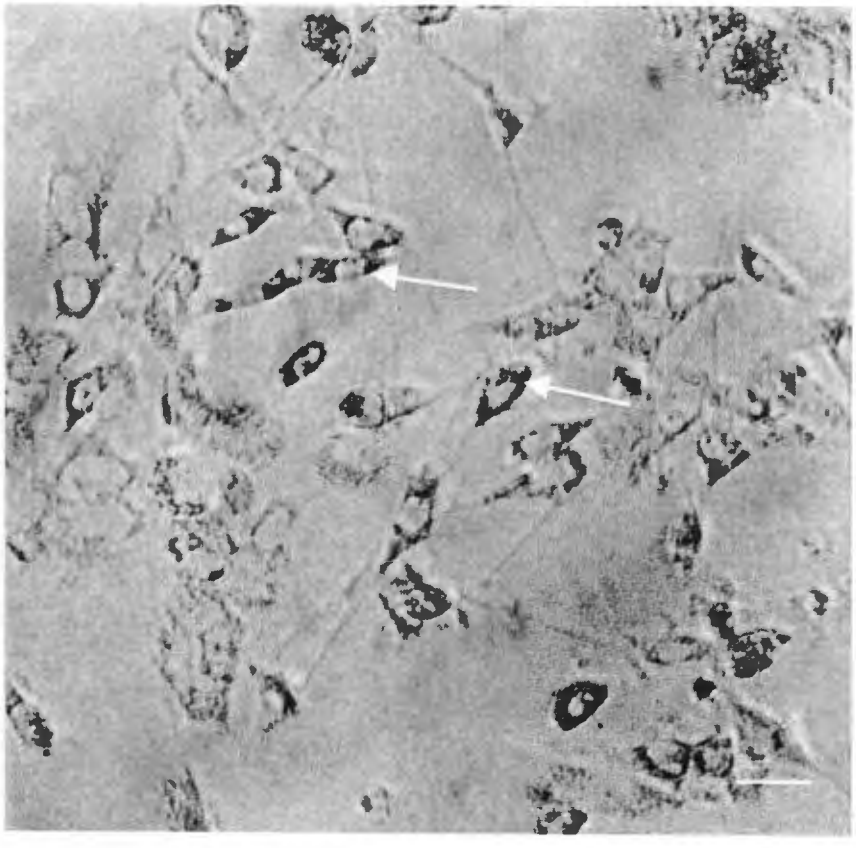


Figure 3.13: Light micrograph of black chick neural crest-derived melanocytes in DM supplemented with 20% white skin extract. The results show dendritic melanocytes with perinuclear, cytoplasmic pigment granules (arrows).
Bar represents 20μm.

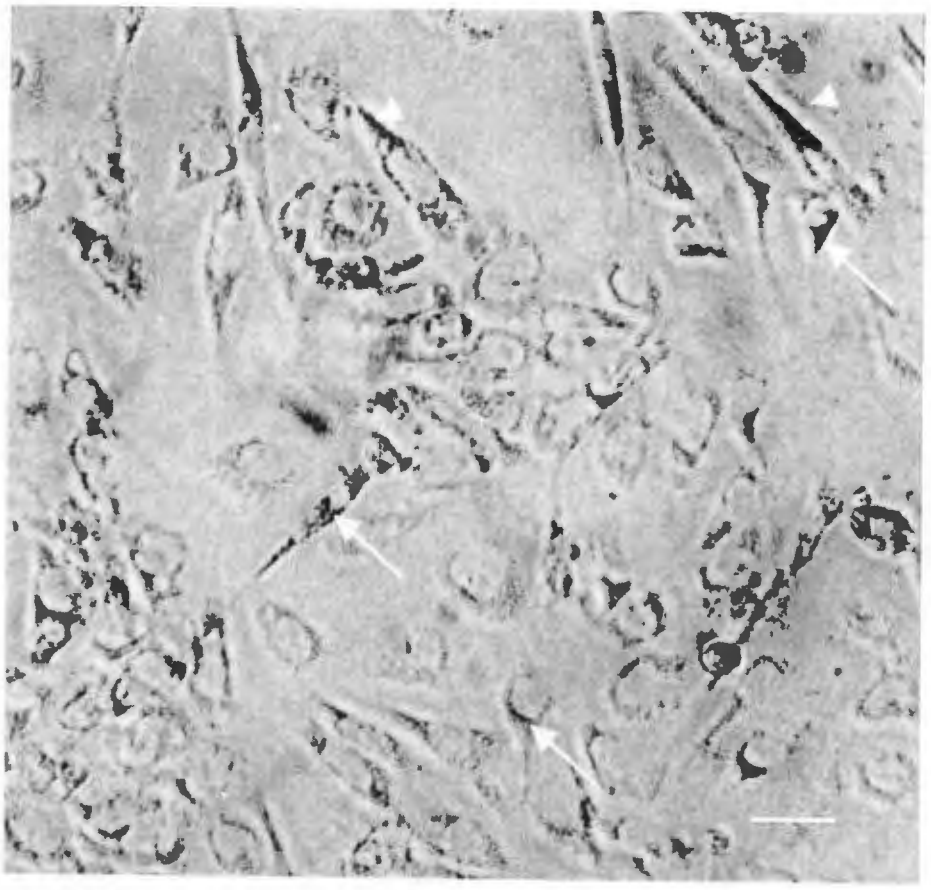


Figure 3.14: Light micrograph showing white chick neural crest-derived melanocytes in DM supplemented with 20% black skin extract. The results show dendritic melanocytes (arrowheads) containing perinuclear pigment granules (arrows).
Bar represents 20μm.

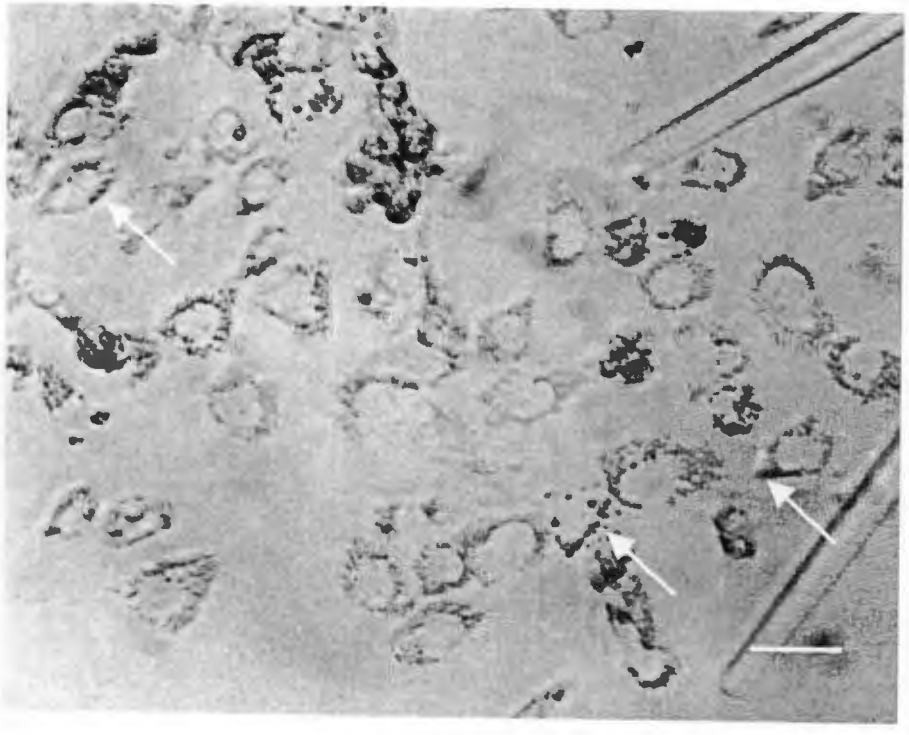


Figure 3.15: Light micrograph showing white chick neural crest-derived melanocytes in DM supplemented with 20% white skin extract. The results show dendritic melanocytes containing perinuclear pigment granules (arrows).
Bar represents 20μm.

4. Discussion

"Dominant white", a dominant mutation of the *I* gene, affects melanin production in neural crest-derived melanocytes, resulting in the characteristic white plumage of many white chicken breeds. Despite a number of informative investigations, the molecular mechanisms underlying the *Dominant white* phenotype remains unclear. Previous investigators have presented evidence to suggest that in *Dominant white* chickens, there is a defect in melanocyte migration through the subepidermal mesenchyme (Hamilton, 1940; Willier and Rawles, 1940). Later studies provided evidence to suggest that *Dominant white* melanocytes die, not during migration, but at later stages of their development. In particular, Bowers et al (1992) reported that melanocytes degenerate in the collar region of WL follicles (homozygous (*I/I*) for *Dominant white*), suggesting that melanocytes die before they migrate into the developing barb ridges.

One of the major limitations of the above studies was that melanin or DOPA histochemistry was utilised as a marker for tracking melanocytes. This approach would not have been suitable to locate melanocytes that are unable to synthesise melanin. Another experimental limitation was that ultrastructural investigations of skin are not appropriate for determining melanocyte numbers. In addition, at the time when these studies were carried out, there were no molecular markers of melanocyte differentiation (other than DOPA histochemistry). It was therefore not possible to determine exactly when, where and if melanocytes do in fact die.

To clarify and overcome the above limitations, the present study made use of *tyrosinase* and *TYRP2* as cDNA molecular markers of melanocyte differentiation. The results showed that *tyrosinase* - and *TYRP2* -expressing melanocytes are indeed present in normal, if not greater numbers, in the skin and feather follicles of 10-day white chick embryos (compared with black follicles of the control breed). This indicates that there is no depletion of melanocyte precursors as they migrate into the developing feather. This is consistent with the work of Jimbow et al (1974) who utilised the DOPA reaction on 9-day WL *(Dominant white)* skin and showed the presence of normal numbers of melanocytes in the feather follicles compared with the wild-type pigmented breed. These results are in contrast to those of Lecoin et al (1995) who reported fewer *c-kit* -positive melanocytes in the epidermis of 9- and 11-day WL embryos compared with wild-type.

To further explore the theory of Bowers et al (1992) that melanocytes die in the collar region, *in situ* hybridisation (ISH) was carried out on sections through different levels of feather follicles.

Melanocytes were found to be present in both the collar region and in the developing barb ridges of both the white and black follicles. This indicates that in WPR x PG chickens, melanocytes do in fact enter the barbs and occupy their normal position within these structures.

To determine the expression pattern of *tyrosinase* and *TYRP2* at later stages of development, ISH was carried out on 12- and 13-day black and white skin. The results again showed the presence of melanocytes in the developing barbs. However, when the *tyrosinase* and *TYRP2* gene expression patterns of black and white follicles were compared, the results showed significant differences between black and white follicles of the same age. With increasing developmental age, there was a decline in the number of *tyrosinase* - and *TYRP2* -expressing melanocytes in white feather follicles compared with their black counterparts. This suggests that melanocytes in the barb ridges of white feather follicles are either 1) dying, as suggested by Jimbow et al (1974) who showed that in 14- and 15-day WL (*l/l*), skin melanocytes begin to disappear; or 2) downregulate *tyrosinase* and *TYRP2* gene expression. These results are also consistent with Lecoin et al (1995) who reported that in 14-day WL epidermis, there were virtually no *c-kit* -positive melanocytes present.

The observed decline in *tyrosinase* and *TYRP2* gene expression between 10- and 13-days is also consistent with Marco's work (1994), who determined the tyrosinase activity of tissue homogenates of 9- and 13-day black and white chick embryos by means of a sensitive radiochemical assay (see Fig. 1.4). In black skin, tyrosinase activity increased markedly between 9- and 13-days of incubation, whereas, in white skin, there was no increase in tyrosinase activity with an increase in age. At 9-days, black skin had twice the amount of tyrosinase activity as did 9-day white skin, and at 13-days, this had increased to an 8.5-fold difference. Since WPR x PG are reported to be heterozygous for *recessive white (C/c)*, melanocytes produce half the normal number of active tyrosinase molecules. This would account for the difference in tyrosinase activity between black and white skin at day 9.

To determine *TYRP2* gene expression at later stages of development, ISH was carried out on 17-day black and white chick skin. The results showed an absence of *TYRP2* gene expression in both the black and the white follicles. This lack of gene expression is probably a consequence of the normal process of feather development. As described in the Introduction (see section 1.1.2), at this late stage of development, feather filaments are already keratinised and consist of dead epidermal cells and melanocytes (Lucas and Stettenheim, 1972). This is consistent with the findings of Lecoin et al (1995) who showed that *c-kit* and *steel* expression was extinguished for the same reason at similar stages of feather morphogenesis.

As the process of feather development continues, after keratinisation of the feather filament, the feather with its resident melanocytes is then shed, and new feathers develop. At the onset of feather regeneration, new melanocytes appear from the dermal papilla, and migrate into the developing feather and transfer their pigment granules into the epithelial cells (Lucas and Stettenheim, 1972). To determine whether melanoblasts in this dermal pool, which contribute to subsequent feather colouration, express *TYRP2,* ISH was carried out on sections of the dermal papilla from 17-day black chick skin. The results showed *TYRP2* -expressing melanoblasts in black skin indicating that the *TYRP2* probe not only detects mature melanocytes in the follicles, but is indeed a suitable marker for melanocyte precursors too. To examine whether a dermal pool of *TYRP2* -expressing melanoblasts is present in the dermal papilla of white chick skin, ISH was carried out on sections through the base of 17-day white follicles. In contrast to black skin, no *TYRP2* -expressing melanoblasts could be detected. This suggests that either there are no stem cells in the dermal papilla or that the melanoblasts are not expressing *TYRP2*. In support of the theory that melanocytes remain viable in the dermal papilla but downregulate gene expression, Bowers and Gatlin (1985) showed that regenerating feather material from WLs can be used as a source of melanocytes for tissue culture.

In summary of the results presented above, there is evidence to suggest that melanocytes are indeed present in 10-day WPR x PG skin and feather follicles in normal, if not greater numbers than in skin and feather buds of black chicks. However, even though they arrive in the skin in normal numbers and occupy appropriate locations in the barb ridges, white skin melanocytes never synthesise melanin at stages when melanocytes of black skin are already melanised.

4.1 Is amelanosis due to an extrinsic or intrinsic defect?

The second part of the present study addresses the question of why WPR x PG melanocytes never synthesise melanin even though they arrive in the skin in normal numbers compared with melanocytes in black chicks. In particular, the following question was addressed. Is amelanosis *in vivo* due to an intrinsic defect in the melanogenic machinery as suggested by Jimbow et al (1974), or due to an extrinsic defect in which a signal from the environment fails to promote melanocyte differentiation? In order to understand these issues in greater detail, some of the important earlier experiments will be summarised.

Although Hamilton (1940) reported that WL melanocytes have a "much lower viability and a higher sensitivity" to adverse environmental conditions than melanocytes from pigmented breeds, they were clearly capable of synthesising melanin in culture. These results indicate that, when

grown in an appropriate environment, *Dominant white* melanocytes are able to synthesise melanin. This suggests that the underlying defect may not be intrinsic to the melanocyte but due to the absence of an environmental signal, as proposed by Bowers et al (1994). This is supported by the study of Marco (1994) where WPR X PG melanocytes, cultured in serum-containing medium, not only produced pigment, but survived as long as wild-type melanocytes.

In order to test directly whether there are differences in the black and white environment that could account for changes in melanisation, a series of *in vitro* investigations was carried out. It was first necessary to establish serum-free conditions that promote the differentiation of melanocytes. In the first experiment, black chick neural crest cells were cultured in a defined medium in black skin extract. The results showed dendritic cells containing fine, light brown pigment granules indicating that skin extract is sufficient to induce melanogenesis. The next step was to determine whether white skin extract is able to elicit a similar response: in this experiment, neural crest cells from black chicks were cultured in defined medium in the presence of white skin extract. The results showed no consistent differences in the survival and differentiation of black chick neural crest cells in extracts of either black or white skin, and demonstrates that white skin is able to promote melanogenesis. This is consistent with grafting experiments by Willier and Rawles (1940) and others who showed that skin melanocytes from black donors are able to survive and differentiate in WL skin.

Although Bowers et al (1988) reported that WL melanocytes survived "quite well" in tissue culture, a more recent investigation showed that, in order for induced premature cell death to be avoided, the culture medium had to be changed regularly and the addition of antioxidants was necessary (Bowers et al, 1994). To determine whether WPR x PG melanocytes behave similarly in serum-free conditions, neural crest cells from white chick embryos were grown in defined medium in the presence of black skin extract. This resulted in dendritic cells containing light brown pigment granules, indicating that white crest cells are able to respond to signals in black skin extract. This is in agreement with Marco (1994) who showed that white neural crest cells grown in serum-containing medium survive and differentiate into fully melanised melanocytes.

The above results present an interesting contradiction as to the underlying cause of amelanosis. On the one hand, there is evidence to suggest that the melanocyte is not intrinsically defective. On the other hand, the findings show an extrinsic environment that is supportive of melanogenesis. This inconsistency may be explained if these defects are not mutually exclusive. In other words, since white melanocytes *in vivo* do not melanise, it was therefore postulated that amelanosis in WPR x PG chickens may result from minor defects in the melanocyte environment and within the melanocyte itself, as proposed by Bowers et al (1994). On their own, these defects may not be severe enough to affect melanogenesis, but together they may exert an additive effect that inhibits melanin synthesis. To investigate whether this is in fact so, the next step was to culture white neural crest cells in serum-free conditions in the presence of white skin extract. To

duplicate (Table 3) and similar results were obtained on each occasion. Comparing the effects of black and white skin extract, there were no visible differences in cell survival and morphology, nor in the degree of pigmentation (Fig. 3.14, 3.15). This striking result clearly indicates that white skin extract is able to induce differentiation of white neural crest cells into melanocytes, and it fails to explain why white cells *in vivo* do not melanise. One possibility in these experiments is that bFGF or αMSH, present in the DM, is involved in adventitious melanogenic induction.

3.3.4 Is αMSH necessary for the differentiation of neural crest cells into melanocytes?

The final experiment in this series was to determine whether neural crest cells from white chicks, cultured in white skin extract, are able to differentiate into mature melanocytes in the absence of αMSH. For this purpose, neural tubes were explanted into DM without αMSH. After three days, the neural tube was picked off and the cells in culture were treated with 20% skin extract, either from black or white chicks. Within one day of adding extract, cells in both black and white skin extract showed a dendritic morphology and displayed fine, light brown pigment granules (not shown, Table 3). There were no consistent differences in the degree of pigmentation in cells treated with either black or white skin extract (not shown). By day five, the majority of the cells formed large, shiny vacuoles which obscured the fine pigment granules. In contrast to cultures in DM with αMSH, the cells rounded up and lifted a day or two earlier. These results indicate that αMSH is not essential for the differentiation of neural crest cells into melanocytes. However, DM containing αMSH tends to prolong survival of crest cells by a day or two. These results still do not explain why melanocytes do not pigment *in vivo*, and are explored in the discussion.

investigate whether this is in fact so, the next step was to culture white neural crest cells in serum-free conditions in the presence of white skin extract. To our great surprise, the results consistently showed that even under these conditions, white cells were still able to differentiate into melanocytes. The experiment was repeated three times in duplicate, and no consistent differences were observed in the survival or differentiation of white crest cells treated with black or white skin extract. It is not clear why white neural crest cells are able to survive and melanise *in vitro* but not *in vivo*. This paradox might be explained by the following possibilities.

Firstly, the physical preparation of the skin extract, where skin pieces are passed through a syringe needle and homogenised, may release factors from the ruptured cells which may not be available to cells *in vivo*. For instance, membrane-bound steel growth factor has been shown to play a role in the survival of proliferating precursor melanocytes in the dermis of the mouse embryo (Wehrle-Haller and Weston, 1995). In avians, *steel* expression is reported in the epidermis, not in the dermis (Lecoin et al, 1995). If *steel* in the epidermis of chickens is also in a membrane-bound form, it is possible that homogenisation of skin extract releases the membrane-bound factor, making it more accessible to cells in culture.

Secondly, skin extract added to the medium may provide a source of growth factors many-fold more concentrated than the available concentration *in vivo*, potentially masking or correcting a real white skin defect. The white environment may provide low levels or even be lacking in growth factors such as steel factor, which is reported to be involved in the survival and differentiation of avian neural crest cells (Lahav et al, 1994; Guo et al, 1997). In the mouse, *steel null* and *steel-dickie* mutants lack the soluble and the membrane-bound form of steel factor respectively, and thus lack coat colouration (Wehrle-Haller and Weston, 1995). In addition, endothelin (EDN3) or αMSH, also found to stimulate melanogenesis, may be defective or missing in the white environment in chickens (Lahav et al, 1996; Bowers et al, 1997). In the mouse, *lethal spotting (ls)* mutants carry mutations in EDN3 and have severely reduced coat colouration (Greenstein Baynash et al, 1994). To investigate whether the white environment *in vivo* is defective, black neural crest cells would have to be cultured in different doses of black and white skin extract to see if there is a crucial point at which they behave differently. Culture conditions could then be manipulated to encourage melanogenesis, and in this way gain insight into what factors are mutated or missing.

Thirdly, the defined medium (DM) itself may contain components present in quantities greater than that produced by white skin keratinocytes *in vivo*, or may even contain factors not necessarily produced in white skin at all. Furthermore, these components may act in combination with factors present in the skin extract to induce adventitious melanogenesis observed in these

cultures. *In vitro* studies by Stocker et al (1991) showed that melanogenesis in neural crest-derived cells was augmented when the DM contained bFGF together with the phorbol ester drug, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). TPA mimics some growth factor present in serum, and endothelin is believed to be a potential candidate (Reid et al, 1996). This factor, possibly also present in skin extract, may act together with bFGF, present in the DM used in this study, to evoke these observed melanogenic effects.

Attempts were made to explore whether any particular component of the DM could promote adventitious melanogenesis in the neural crest cells. Cells were cultured in DM without bFGF, but never survived long enough for the effects of skin extract to be determined. This is consistent with Stocker et al (1991) and Kalcheim (1989), who reported that bFGF influences the survival of neural crest cells. Cultures in DM without bFGF often contained cells that were poorly attached to the substratum and contained vacuoles (Stocker et al, 1991). In an attempt to test another component of the DM used in this study, αMSH was omitted from the medium. However, cells still continued to pigment in the presence of white skin extract. This contrasts the results of Satoh and Ide (1987) who showed that αMSH accelerates melanogenic differentiation of cultured neural crest cells.

To investigate whether the DM contains factors which induce melanogenic commitment of neural crest cells, melanocyte-specific gene expression of these cells would have to be investigated. Lineage tracing studies have demonstrated that avian melanocyte precursors are already specified prior to the onset of neural crest migration, and express melanocyte-specific genes. One of these genes is *microphthalmia (mitf),* which encodes a transcription factor essential for melanocyte differentiation (Wakamatsu et al, 1998). Furthermore, in human melanocytes, microphthalmia binds to a regulatory *cis*-element (M-box) present in promoters of the *TRP* gene family members and upregulates the expression of *tyrosinase* and *TYRP1* (Bentley et al, 1994). *Microphthalmia* as well as *tyrosinase* and *TYRP1* gene expression in neural crest cells grown in DM would indicate that cells are specified as melanocytes even before the addition of skin extract.

To explain further the conflicting results obtained in this study, white skin melanocytes may in fact be intrinsically defective: not however defective in their melanin synthesis machinery, but somehow in their ability to respond to environmental signals that instruct them to complete melanogenesis. In mice, rats and humans, mutations in *c-kit*, the receptor molecule for steel factor, produces animals lacking pigment (Williams et al, 1992). Similarly, in avians, where steel factor is thought to perform a role in differentiation and survival (at later stages of development), a mutation in *c-kit* would also render the cells unresponsive to these signals and result in

amelanosis (Lahav et al, 1994; Guo et al, 1997). Furthermore, *Dominant white* in the pig has been shown to be due to a mutation in *c-kit* (Moller et al, 1996).

Dominant white melanocytes in chickens may be heterozygous for a dominant negative mutation in *c-kit*, similarly to the c-*kit*$^{W\text{-}v}$ mutation in the mouse (MacKenzie et al, 1997), and as a result adopt a dose-sensitive response to growth factors. In response to ligand binding, dimerisation of both normal and defective subunits may occur, of which the mutated catalytic domain abrogates the function of the entire dimer. In an overly favourable tissue culture environment (like serum-containing medium or large quantities of skin extract), where concentrations of growth factors are high, cells with normal receptor subunits may not need to compete for growth factor molecules but receive generously the necessary signals to melanise and survive.

The hypothesis presented above may be used to explain why white chick melanocytes never synthesise melanin *in vivo*, and why at later stages of development (12-,13- and 17-days), melanocytes appear to downregulate gene expression. As discussed in detail in the Introduction (see section 1.3), in contrast to *steel* expression in the mouse, *steel* expression in avians (including WLs) was noted to be restricted only to the epidermis throughout development. The dermis was not found to be *steel* -positive in any of the breeds of chicken considered (Lecoin et al, 1995). Because the sites of *steel* expression during early stages of development are different from those where melanoblasts first appear (Lecoin et al, 1995), Guo et al (1997) explored and suggested other sources of steel factor in the avian embryo. Steel is reported to act in an autocrine or paracrine fashion thought to influence melanogenesis in nearby cells, suggesting that neural crest cell populations play a more active role in the determination of their own cell fate (Guo et al, 1997).

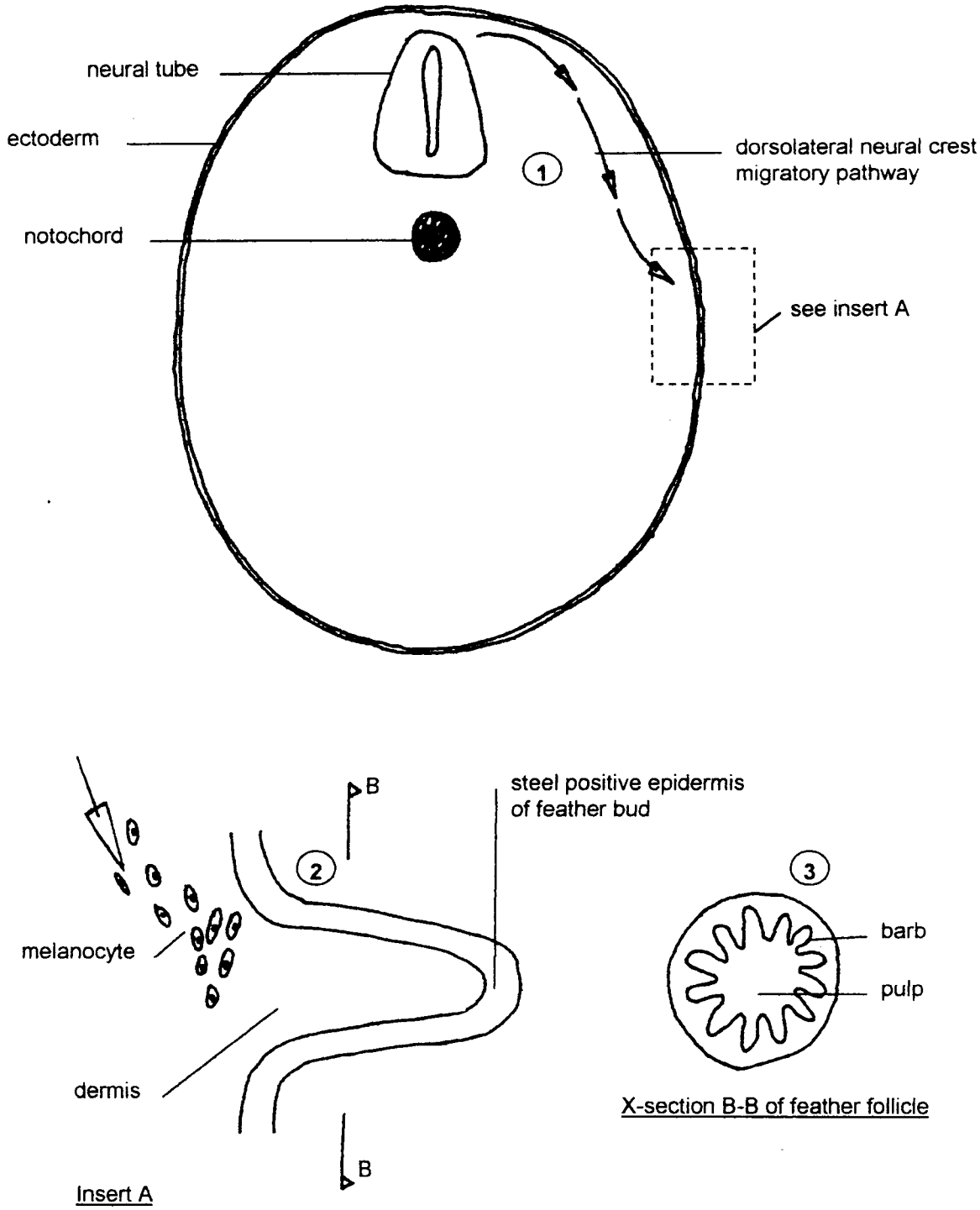
From the conclusions presented above regarding the involvement of steel/c-kit signalling in melanocyte development, the following model is proposed to explain why melanocytes *in vivo* never melanise and why at later stages (12-,13- and 17-days), melanocyte-specific gene expression is extinguished (see Fig. 4.1). It is possible that presumptive melanocytes are not dependent on steel during early stages of migration as suggested by Guo et al, 1997. Or, that the small amounts of endogenous steel produced (as shown by Guo et al, 1997) as well as the presence of EDN3 (Lahav et al, 1996; Nataf et al, 1996) during their dermal migration is sufficient for their survival as well as the onset of their differentiation (as shown by Lahav et al, 1994) into mature melanocytes (with a few able to melanise). Once they reach the epidermis, unmelanised melanocytes become dependent on steel for their terminal differentiation (as suggested by Lecoin et al, 1995; Lahav et al, 1994 and Guo et al, 1997) and possibly for their subsequent survival as shown by Lahav et al (1994). A dominant negative mutation in *c-kit* would render WPR x PG

melanocytes unresponsive to steel once localised in the epidermis. This would explain why melanocytes express *TYRP2* and *tyrosinase* but never terminally differentiate into melanin-producing melanocytes. Furthermore, it is possible that without steel/c-kit signalling, melanocytes cannot maintain melanocyte-specific gene expression. This would explain the observed decline in *TYRP2* and *tyrosinase* gene expression at later stages in the present study, and the decline in *c-kit* -positive melanocytes reported by Lecoin et al (1995) in 14-day WL embryos. This would also explain the absence of *TYRP2* gene expression in stem cells in the dermal papilla of 17-day white skin. Furthermore, in support of the theory that melanocytes remain viable in the dermal papilla but downregulate gene expression, Bowers and Gatlin (1985) showed that regenerating feather material from WLs can be used as a source of melanocytes for tissue culture experiments.

4.2 Concluding comments

Chicken *c-kit* cDNA has been isolated from avian brain by Sasaki et al (1993). Analysis of the amino acid sequence of the chicken c-kit showed a 63% identity to the mouse protein, as well as similar structural and functional features. Interestingly, *c-kit* cDNA was isolated from a White Leghorn chicken brain. The amino acid sequence of WL c-kit would therefore need to be compared to c-kit protein from a pigmented chicken first in order to assess whether the protein is in fact functional. Furthermore, southern analysis of both WL and wild-type *c-kit* cDNA would have to be compared to determine if there are any gross differences between the two breeds. Ultimately, sequencing of *c-kit* cDNA would also have to be carried out.

To explore directly whether white and black neural crest cells in culture respond differently to steel, serum-free cultures (as described in section 2.11) would have to be carried out with varying concentrations of steel. This, combined with sensitive markers of melanocyte differentiation, such as RT-PCR of *microphthalmia* and *TYRP2* (Pinder et al, manuscript in preparation), can be used to assess the role of steel in melanocyte differentiation and survival.



Key:

1) Steel independent migration of melanocyte precursors to the developing feather follicles. During this stage of development, precursor melanocytes are probably dependent on EDN3 for their proliferation, survival and the onset of their differentiation into melanocytes.
2) Melanocytes from the dermis enter the epidermis and become dependent on steel for their terminal differentiation and/or survival.
3) Melanocytes enter the barb ridge, and transfer their melanin granules into the surrounding epithelial cells.

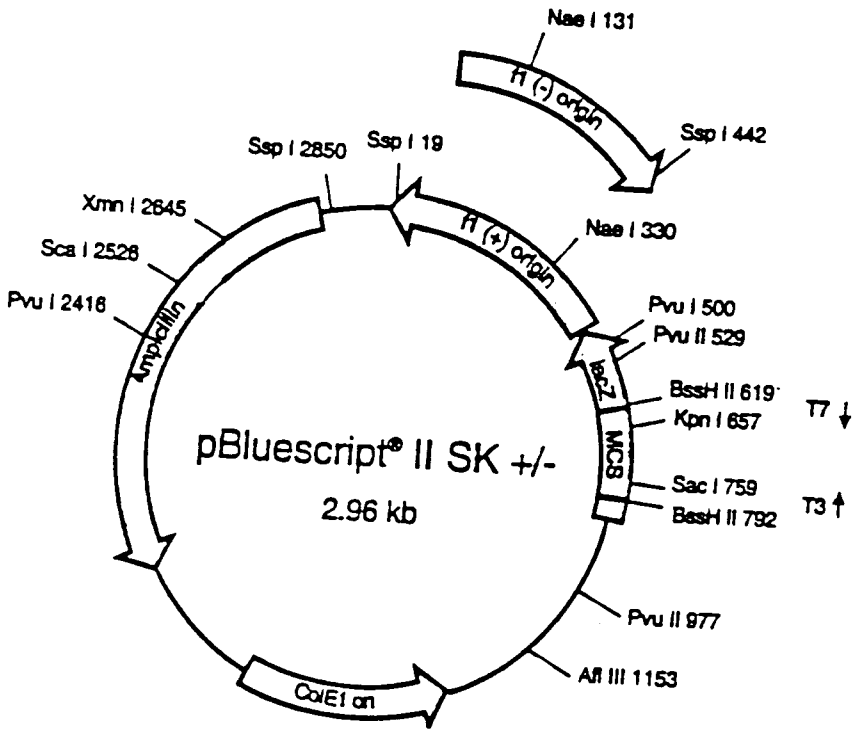
Figure 4.1: Schematic diagram illustrating melanocyte migration and stages of steel dependence and independence for differentiation and/or survival.

5. Appendices

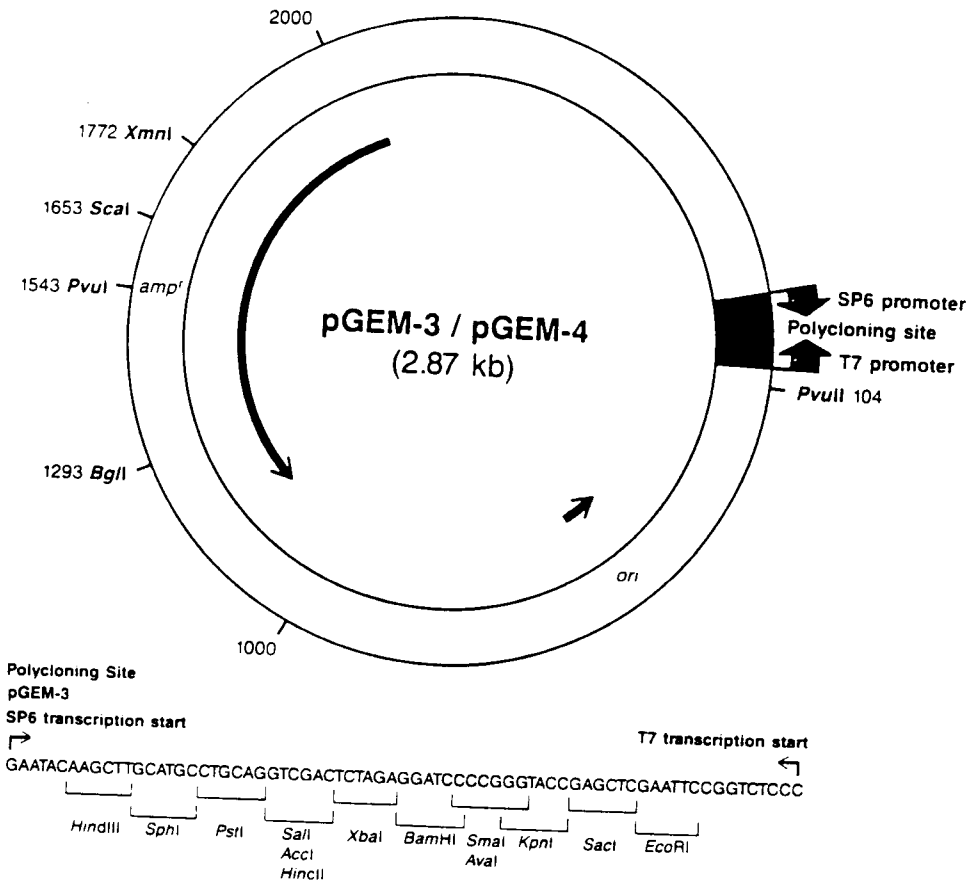
5.1 Appendix 1: Cloning vectors used in the present study

The maps of A, pBluescript II S/K (+/-) (2.96kb) and B, pGem-3 (2.87kb).

A



B



5.2 Appendix II: Additional Methods

Phenol/chloroform extraction of DNA

The DNA sample was made up to a volume of 400µl with sterile water. An equal volume of Tris-saturated phenol was added, the sample vortexed, and then centrifuged at 10 000xg for 2 minutes. After the phenol layer (containing contaminating proteins) was removed, 200µl phenol and 200µl chloroform:isoamyl alcohol (24:1) was then added, the sample vortexed, centrifuged at 10 000xg and the phenol layer removed. The number of phenol extractions performed depended on the presence of proteins at the interface. This was followed by a chloroform:isoamyl alcohol extraction after which the aqueous phase (containing DNA) was transferred to a sterile eppendorf.

Precipitation of DNA

DNA was precipitated by adding 40µl 3M sodium acetate pH 5.2 (1/10th of total sample volume) and 1ml absolute alcohol (2.5x total volume). The sample was then vortexed and placed at -20°C overnight or at -80°C for 30 minutes. The precipitated DNA was collected by centrifugation, the pellet washed in 70% ethanol to remove salts, centrifuged and vacuum or air dried. The pellet was resuspended in a known volume of sterile water and stored at -20 °C.

Standard electrophoresis

DNA and RNA gels were prepared according to methods by Sambrook et al (1989). DNA samples were electrophoresed on 1% agarose gels in 0.5xTBE buffer (45mM Tris-borate, 1mM EDTA). For RNA samples, 1.3% denaturing formaldehyde agarose gels were prepared: 15ml contains 1.5ml 10x MOPS (0.2M MOPS, 0.05M sodium acetate, 0.01M EDTA pH 7, filtered); 0.17g agarose (Sigma); 0.75ml 40% formaldehyde. 1x MOPS was used as a running buffer. Voltages used for electrophoresis were as recommended by Sambrook et al (1989).

Nucleic acid markers were used in each electrophoretic run. Pox DNA digested with Eco RI yields seven bands of known sizes: 5.8 kb, 4.0 kb, 2.8 kb, 1.9 kb, 1.3 kb, 1.0 kb, 0.75 kb. Bacterial rRNA runs as two distinct bands (23S 3.7 kb; 16S 1.7 kb) and was used as a RNA

marker. After electrophoresis, the gels were stained with ethidium bromide (0.01%), viewed with ultra-violet light and photographed.

Preparation of ampicillin/agar plates

950ml distilled water, 10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl was mixed in a sterile bottle. pH was adjusted to 7.4 and 15g bacto-agar added. The resultant mix was then topped up to 1 litre with distilled water and autoclaved. Once the solution had cooled, ampicillin was added at 100μg/ml, and poured onto plates. 1000ml makes approximately 40 plates.

5.3 Appendix III: Preparation of tissue, slides and solutions for immunocytochemistry and *in situ* hybridisation

Processing for wax embedding

After fixation, tissue samples were dehydrated in a series of graded alcohols to molten paraffin wax as follows:

50% alcohol for 30 minutes

70% alcohol for 60 minutes

90% alcohol for 45 minutes

100% alcohol for 3x60 minutes

xylol for 2x30 minutes

molten paraffin wax for 30 minutes

The tissue was then orientated and embedded in paraffin wax on an embedding machine. The blocks were set and stored at 4^0C until required.

Aminopropyltriethoxysaline (APTES)-coated slides

Slides were prewashed in ethanol, followed by rinsing in 10% extran overnight. Slides were then washed in 60°C tap water for 2 hours, and dried at 160° C for a further 2 hours. Once the slides had cooled down, they were coated in 2% 3-aminopropyltriethoxylsaline (APTES) in acetone for 10 seconds, dipped twice into acetone and once in sterile water. Slides were allowed to dry at 42° C overnight, and DEPC treated as described below.

Diethyl pyrocarbonate (DEPC) treatment of slides and solutions

Solutions were DEPC treated adding diethyl pyrocarbonate at a final concentration of 0.01% and shaken for 4-6 hours, after which the solutions were autoclaved. Similarly, slides were allowed to shake in DEPC-treated (0.01%) sterile water for 4-6 hours, and then autoclaved and stored in sealed bags until required.

5.4 Appendix IV: Tissue culture solutions

Chick Saline

pH 7.45; 1 litre:

7.25g NaCl

0.37g KCl

0.18g $CaCl_2.2H_2O$

Autoclave for 30 minutes.

Before use, add 10 ml pen-strep antibiotic with a sterile pipette.

Store at 4° C.

Dulbecco's Phosphate Buffered Solution (Calcium- and Magnesium-free):

pH 7.4; 1 litre:

8.00g NaCl

0.20g KCl

1.15g Na_2HPO_4

0.20g KH_2PO_4

Add distilled water to make up volume.

pH to 7.4

Autoclave.

6. References

April C.S., I.J. Jackson and S.H. Kidson (1998) Molecular Cloning and Sequence Analysis of a Chicken cDNA Encoding Tyrosinase-related protein-2/DOPAchrome Tautomerase. Gene, 219:45-53.

April C.S., T.A. Franz and S.H. Kidson (1996) The Cloning and Characterisation of Chick Tyrosinase from a Novel Embryonic cDNA Library. Exp. Cell Res., 224:372-378.

Balinsky B. (1970) Gastrulation and the Formation of Primary Organ Rudiments. In: An Introduction to Embryology. WB Sanders Company, Philadelphia.

Bentley N.J., T. Elsen and C.R. Goding (1994) Melanocyte-specific Expression of the Human Tyrosinase Promoter: Activation by the Microphthalmia Gene Product and Role of the Initiator. Mol. Cell Biol., 14:7996-8006.

Bitgood J. and J. Smyth (1991) Albinism in White Leghorn Chickens. Poultry Sci., 70:1861-1863.

Boehringer Mannheim (1994) Dig System's Users Guide for Filter Hybridisation. Mannheim GmbH, Biochemica.

Boehringer Mannheim (1992) Non-radioactive ISH Applications Manual. Boehringer Mannheim GmbH, Biochemica.

Bowers R.R., A. Bibosa and O. Chavez (1997) The Role of Alpha-MSH, its Agonists, and C-AMP in *In vitro* Avian Melanocytes. Pig. Cell Res., 10:41-45.

Bowers R.R., J. Lujan, A. Biboso, S. Kridel and C. Varkey (1994) Premature Avian Melanocyte Death Due to Low Antioxidant Levels of Protection: Fowl Model for Vitiligo. Pig. Cell Res., 7:409-418.

Bowers R.R., J. Harmon, S. Prescott, J. Asano and S. Wynne (1992) Fowl Model for Vitiligo: Genetic Regulation on the Fate of the Melanocytes. PCR. Suppl. 2:242-248.

Bowers R.R. (1988) The Melanocyte of the Chicken: A Review. Advances in Pig. Cell Res., 49-63.

Bowers R.R. and J.E. Gatlin (1985) A Simple Method for the Establishment of Tissue Culture Melanocytes from Regenerating Fowl Feathers. *In vitro* Cell Dev. Biol., 21:39-44.

Bronner-Fraser M. (1986) Analysis of the Early Stages of Trunk Neural Crest Migration in Avian Embryos Using Monoclonal Antibody HNK-1. Dev. Biol., 115:44-55.

Brumbaugh J., T.W. Bargar and W.S. Oetting (1983) A "New" Allele at the C Pigment Locus in the Fowl. J. Hered., 74:331-336.

Brumbaugh J.A. and K.W. Lee (1975) The Gene Action and Function of Two Dopa Oxidase Positive Melanocyte Mutants of the Fowl. Genetics, 81:333-347.

Brumbaugh J.A. (1971) The Ultrastructural Effects of the *I* and *S* Loci Upon Black-red Melanin Differentiation in the Fowl. Dev. Biol., 24:392-412.

Davis L.G., M.D. Dibner and J.F. Battey (1986) Basic Methods in Molecular Biology. Elsevier Science Publishing Co., New York.

Del Marmol V. and F. Beerman (1996) Tyrosinase and Related proteins in Mammalian Pigmentation. FEBS Lett., 381: 165-168.

Di Marco E., P.C. Marchisio, S. Bondanza, T. Franzi, R. Cancedda and M. De Luca (1991) Growth-regulated Synthesis and Secretion of Biologically Active Nerve Growth Factor by Human Keratinocytes. J. Biol. Chem., 266:21718-21722.

Erickson C.A. and T.L. Goins (1995) Avian Neural Crest Cells Can Migrate in the Dorsolateral Pathway if they are Specified as Melanocytes. Dev., 121:915-924.

Fitzpatrick T.B., Y. Hori, K. Toda and M. Seiji (1969) Melanin 1969: Some Definitions and Problems. Japanese J. of Dermatol., Series B 79:278-282.

Gilbert S. (1991) Early Vertebrate Development: Neurulation and Ectoderm. In: Developmental Biology. Sinauer Associates Inc, Sunderland, Massachusetts.

Greenstein Baynash A., K. Hosoda, A. Giaid, J.A. Richardson, N. Emoto, R.E. Hammer and M. Yanagisawa (1994) Interaction of Endothelin-3 with Endothelin-B Receptor is Essential for Development of Epidermal Melanocytes and Enteric Neurons. Cell 79:1277-1285.

Guo S.G., B. Wehrle-Haller, J. Rossi and G. Ciment (1997) Autocrine Regulation of Neural Crest Cell Development by Steel Factor. Dev. Biol., 184: 61-69.

Hadley M.E. (1992) Endocrinology, 3rd Edition. 65-97 and 179-207. Prentice Hall, Inc, Engelwood Cliffs, N.J.

Hamburger V. and H.L. Hamilton (1951) A Series of Normal Stages in the Development of the Chick Embryo. J. Morphol., 88:49-92.

Hamilton H.L. (1940) A Study of the Physiological Properties of Melanophores with Special Reference to their Role in Feather Colouration. Anat. Rec., 78:525-547.

Hearing V.J. (1993) Unravelling the Melanocyte (Editorial). Americ. J. Hum. Genet., 52(1):1-7.

Heuer J.G., S. Fatemie-Nainie, E.F. Wheeler and M. Bothwell (1990) Structure and Developmental Expression of the Chicken NGF Receptor. Dev. Biol., 137:287-304.

Hirobe T. (1992) Control of Melanocyte Proliferation and Differentiation in the Mouse Epidermis. Pig. Cell Res., 5:1-11.

Hulley P., C. Stander, S.H. Kidson (1991) Terminal Migration and Early Differentiation of Melanocytes in Embryonic Chick Skin. Dev. Biol., 145:182-194.

Hurst C.C. (1905) Experiments with Poultry. Repts. Evol. Comm. Roy. Soc., 2:131-154.

Hutt F.B. (1949) Variations in the Colour of the Skin; and Variations in the Colour of the Plumage. In: Genetics of the Fowl. 149-161 and 162-221, respect. McGraw-Hill Book Company, Inc. New York.

Jimbow K., W.C. Quevedo, T. Fitzpatrick, and G. Szabo (1976) Some Aspects of Melanin Biology: 1950-1975. J. Invest. Dermatol., 67:72-89.

Jimbow K., G. Szabo and T.B. Fitzpatrick (1974) Ultrastructural Investigation of Autophagocytosis of Melanosomes and Programmed Cell Death of Melanocytes in White Leghorn Feathers: A Study of Morphogenetic Events leading to Hypomelanosis. Dev. Biol., 36:8-23.

Kalcheim C. (1989) Basic Fibroblast Growth Factor Stimulates Survival of Nonneuronal Cells Developing from Trunk Neural Crest. Dev. Biol., 134(1):1-10.

Koubovec D.J. (1996) Hypopigmentation in the White Chick. BSc (Med)(Hons) Project, Department of Anatomy and Cell Biology, UCT, Cape Town, South Africa.

Langtimm-Sedlak C.J., B. Schroeder, J.L. Saskowski, J.F. Carnahan and M. Sieber-Blum (1996) Multiple Actions of Stem Cell Factor in Neural Crest Cell Differentiation *in vitro*. Dev. Biol., 174:345-359.

Lahav R., C. Ziller, E. Dupin, N. Le Douarin (1996) Endothelin 3 Promotes Neural Crest Cell Proliferation and Mediates a Vast Increase in Melanocyte Number in Culture. Proc. Natl. Acad. Sci. USA., 93:3892-3897.

Lahav R., L. Lecoin, C. Ziller, V. Nataf, J. Carnahan, F. Martin, N. Le Douarin (1994) Effect of the Steel Gene Product on Melanogenesis in Avian Neural Crest Cell Cultures. Differentiation, 58:133-139.

Lecoin L., R. Lahav, F.H. Martin, M-A. Teillet, and N.M. Le Douarin (1995) Steel and C-kit in the Development of Avian Melanocytes: A Study of Normally Pigmented Birds and of the Hyperpigmented Mutant Silky Fowl. Dev. Dyn., 203:106-118.

Lucas A.M. and P.R. Stettenheim (1972) Growth of Follicles and Feathers. Colour of Feathers and Integument. In: Avian Anatomy Integument, Part II. Ch 7, 341-419. U.S. Government Printing Office, Washington DC.

MacKenzie M., S. Jordan, P. Budd and I. Jackson (1997) Activation of the Receptor Tyrosine Kinase Kit is Required for the Proliferation of Melanoblasts in the Mouse Embryo. Dev. Biol., 192:99-107.

Marco H. (1994) Hypomelanosis in Chickens. MSc Thesis, Department of Anatomy and Cell Biology, UCT, Cape Town, South Africa.

Maul G. and J. Brumbaugh (1971) On the Possible Function of Coated Vesicles in Melanogenesis of the Regenerating Fowl Feather. J. Cell Biol., 48:41-48.

Moller M.J., R. Chaudhary, E. Hellman, B. Hoyheim, B. Chowdhary, L. Andersson (1996) Pigs with the Dominant White Coat Colour Phenotype Carry a Duplication of the *KIT* Gene encoding the Mast/stem Cell Growth Factor Receptor. Mamm. Genome 7:822-830.

Nakagawa H., A. Rhodes, T. Fitzpatrick, Y. Hori (1984) Acid Phosphatase in Melanosome Formation: A Cytochemical Study in Normal Human Melanocytes. J. Invest. Dermatol., 83:140-144.

Nataf V., L. Lecoin, A. Eichmann, N. Le Douarin (1996) Endothelin B Receptor is Expressed by Neural Crest Cells in the Avian Embryo. Proc. Natl. Acad. Sci. USA., 93:9645-9650.

Nataf V., P. Mercier, C. Ziller, N. Le Douarin (1993) Novel Markers of Melanocyte Differentiation in the Avian Embryo. Exp. Cell Res., 207:171-182.

National Geographic (1998) Antarctic Accidental- an All White Penguin. 193(1).

Nichols D.H. and J.A. Weston (1977) Melanogenesis in Cultures of Peripheral Nervous Tissue. I. The Origin and Prospective Fate of Cells Giving Rise to Melanocytes. Dev. Biol., 60:217-225.

Nishikawa S., M. Kusakabe, K. Yoshinaga, M. Ogawa, S. Hayashi, T. Kunisada, T. Sakakura, SI. Nishikawa (1991) In *Utero* Manipulation of Coat Colour Formation By a Monoclonal Anti-c-kit Antibody: Two Distinct Waves of C-kit-dependency During Melanocyte Development. EMBO J., 10:2111-2118.

Nocka K., J.C Tan, E. Chiu, T.Y. Chu, P. Ray, P. Traktman, P. Besmer (1990) Molecular Basis of Dominant Negative and Loss of Function Mutations at the Murine *c-kit/white* spotting locus: W^{37}, W^{v}, W^{41} and W,. EMBO J., 9:1805-1819.

Oetting W.S., A.M. Churilla, H. Yamamoto, and J.A. Brumbaugh (1985a) *C* Pigment Locus Mutants of the Fowl Produce Enzymatically Inactive Tyrosinase-like Molecules. J. Exp. Zool., 235:237-245.

Oetting W., K. Langner and J.A. Brumbaugh (1985b) Detection of Melanogenic Proteins in Cultured Chick-embryo Melanocytes. Diff., 30:40-46.

Price E.R., M.A. Horstman, A.G. Wells, K.N. Weilbaecher, C.M. Takemoto, M.W. Landis and D.E. Fisher (1998) α-Melanocyte-stimulating Hormone Signaling Regulates Expression of *Microphthalmia*, a Gene Deficient in Waardenberg Syndrome. J. Biol. Chem., 273, 42:88042-88047.

Puri N. and M.H. Brilliant (1998) The Function of the *Pink-eyed* dilution protein. PCR, 11(3)174.

Reid K., A.M. Turnley, G.D. Maxwell, Y. Kurihara, H. Kurihara, P.F. Bartlett and M. Murphy (1996) Multiple Roles for Endothelin in Melanocyte Development: Regulation of Progenitor Number and Stimulation of Differentiation. Dev., 122:3911-3919.

Rinchik E.M., S.J. Bultman, B. Horsthemke, S.T. Lee, K.M. Strunk, R.A. Spritz, K.M. Avidano, M.T. Jong and R.D Nicholls (1993) A Gene for the Mouse Pink-Eyed Dilution Locus and for Human Type II Oculocutaneous Albinism. Nature, 361:72-76.

Rogers SL., P.J. Gegick, S.M. Alexander and P.G. McGuire (1992) Transforming Growth Factor-β Alters Differentiation in Cultures of Avian Neural Crest-Derived Cells: Effects on Cell Morphology, Proliferation, Fibronectin Expression, and Melanogenesis. Dev. Biol., 151:192-203.

Ruyter-Spira C.P., Z. Gu, J.J. Van der Poel and M. Groenen (1997) Bulked Segregant Analysis Using Microsatellites: Mapping of the Dominant White Locus in the Chicken. Poultry Sci., 76:386-391.

Sambrook J., E.F. Fritsch and T. Maniatis (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour, New York.

Sasaki E., H. Okamura, T. Chikamune, Y. Kanai, M. Watanabe, M. Naito and M. Sakurai (1993) Cloning and Expression of the Chicken C-kit Proto-oncogene. Gene 128:257-261.

Satoh M. and H. Ide (1987) Melanocyte-stimulating Hormone Affects Melanogenic Differentiation of Quail Neural Crest Cells *in vitro*. Dev. Biol., 119:579-586.

Smyth J. Jr. (1990) Genetics of Plumage, Skin and Eye Pigmentation in Chickens. In: Poultry Breeding and Genetics (Crawford R.D., Ed.), 109-167. Elsevier, Amsterdam.

Smyth J., N. Ring and J.A. Brumbaugh (1986) A Fourth Allele at the C-locus of the Chicken. Poultry Sci., 65:129.

Stocker K.M., L. Sherman, S. Rees and G. Ciment (1991) Basic FGF and TGF-b1 Influence Commitment to Melanogeneis in Neural Crest-derived Cells of avian Embryos. Dev., 111:635-645.

Ting-Berreth S. and C. Chuong (1996) *Sonic Hedgehog* in Feather Morphogenesis: Induction of Mesenchymal Condensation and Association with Cell Death. Dev. Dyn., 207:157-170.

Vogel K.S. and J.A. Weston (1988) A Subpopulation of Cultured Avian Neural Crest Cells has Transient Neurogenic Potential. Neuron, 1:569-577.

Wakamatsu Y., M. Mochii, K.S. Vogel and J. Weston (1998) Avian Neural Crest Derived Neurogenic Precursors Undergo Apoptosis on the Lateral Migration Pathway. Dev., 125:4205-4213.

Wang K., L. Gan, C.L.. Kuo and L. Hood (1997) A Highly Conserved Apoptotic Suppressor Gene is Located Near the Chicken T-cell Receptor Alpha Chain Constant Region. Immunogenetics 46:376-382.

Wehrle-Haller B. and Weston J. (1995) Soluble and Cell-bound Forms of Steel Factor Activity Play Distinct Roles in Melanocyte Precursor Dispersal and Survival on the Lateral Neural Crest Migration Pathway. Dev., 121: 731-742.

Williams D.E., P. de Vries, A.E. Namen, M.B. Widmer and S.D. Lyman (1992) The *Steel* Factor. Dev. Biol., 151:368-376.

Willier B.H. and M.E. Rawles (1940) The Control of Feather Colour Pattern by Melanophores Grafted From One Embryo to Another of a Different Breed of Fowl. Physiol. Zool., 13:177-199.

Yaar M., K. Grossman, M. Eller and B.A. Gilchrest (1991) Evidence for Nerve Growth Factor-mediated Paracrine Effects in Human Epidermis. J. Cell Biol., 115:821-828.

Yoshida H., T. Kunisada, M. Kusakabe, S. Nishikawa, SI. Nishikawa (1996) Distinct Stages of Melanocyte Differentiation Revealed by Analysis of Non-uniform Pigmentation Patterns. Dev., 122: 1207-1214.