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What insights into vertebrate pigmentation has the axolotl model system provided?

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ABSTRACT Amphibians have been judiciously exploited by developmental biologists for many years for studying basic developmental mechanisms in vertebrates. In this review, the contributions that have been made by urodeles, in particular the axolotl *(Ambystoma mexicanum)*, to the study of pigment cell biology are elaborated. Pigment cell differentiation is described, and the wild-type pigment phenotype is contrasted to pigment mutants such as albino, axanthic, melanoid, and white. Methods used for studying pigmentation, including recently developed molecular biological tools, are included to illustrate the significance of the axolotl as a model system for studying vertebrate pigmentation.

KEY WORDS: *pigmentation, axolotls, melanophores, xanthophores, iridophores*

Introduction

To developmental biologists, especially those who study vertebrate development, pigment cells and pigmentation have provided interesting and useful markers (see Bagnara and Hadley, 1973; Le Douarin, 1982; Frost, 1990; Frost-Mason *et al.*, 1994 for reviews). Pigment cells derive from the embryonic neural crest. The crest also gives rise to nerve cells, secretory cells, and other types of cells, all of which must migrate to virtually all parts of the developing organism. Consequently, the neural crest continues to be studied intensively, and interest in how these cells are directed in their migration and differentiation remains strong. In mature animals, pigment cells are of interest because of the presumed protection they offer to harmful ultraviolet irradiation, and, in some species, because of the patterns and colors that comprise the adult pigment pattern.

Pigment cells in amphibians offer developmental biologists the following: *(1) easily accessible and identifiable marker cells; (2) three distinct types of cells derived from a common stem cell source; and (3) elaborate pigment patterns that change dramatically during metamorphosis.* The axolotl is not the ideal amphibian for studying adult pigmentation because it fails, under normal circumstances, to undergo metamorphosis. Nevertheless, the axolotl develops three distinct pigment cell types like other amphibians, has embryos that are amenable to manipulation of the neural crest like other amphibians, and has a number of mutant genes that are known to affect pigmentation *unlike* most amphibians.

In the pages that follow we present a summary of research on the pigmentary system of the axolotl. The wild-type phenotype is described, and four pigment mutants, melanoid *(m)*, albino *(a)*, axanthic *(ax)* and white *(d)*, are discussed. Strategies and methods for studying pigmentation in the axolotl are outlined. And finally, how this research has contributed to the broader field of pigment cell biology and what this system has to offer for the future are also discussed.

Which pigment cells occur in axolotls?

The three types of pigment cells found in axolotl skin are melanophores, xanthophores, and iridophores. In the embryo, melanophores develop first, at approximately stage 35 (Bordzilovskaya *et al.*, 1989), followed a short time later (stages 38-39) by xanthophores. Eventually, iridophores develop in axolotl skin, but often not until much later in larval life.

In the axolotl, pigment cells are located in the dermis, beneath the basement membrane of the skin (Fig. 1). In some amphibians, chromatophores may be located both dermally and epidermally. In the red-spotted newt, for example, there are epidermal chromatophores that contribute to the spotting pattern of these animals (Forbes *et al.*, 1973). The base, or background, color of urodeles, as is true for most animals, is due to the organization of chromatophores within the dermis.

Of additional interest to pigment cell biologists is the observation that all three pigment cells also develop in the iris of the urodele eye (Fig. 2), often regardless of which pigment cells appear in skin (e.g., the iris depicted in Fig. 2 is from a white mutant axolotl). Little is

Abbreviations used in this paper: MSH, melanocyte-stimulating hormone; PCR, polymerase chain reaction; TRP-1, tyrosinase-related protein-1; XDH, xanthine dehydrogenase.

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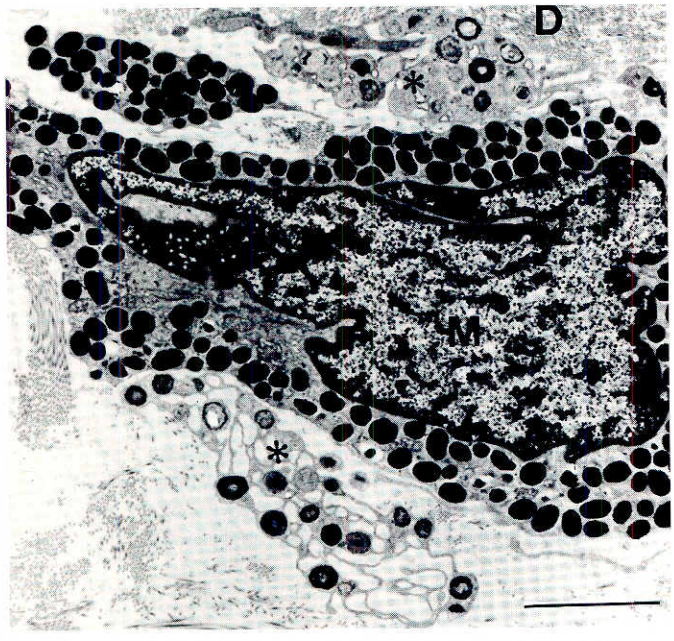

Fig. 1. Transmission electron micrograph of a melanophore (M) from axolotl skin. *Note xanthophore processes (*) and dermis (D). Bar, 3 μm.*

known about when eye pigment cells develop and whether they follow the same developmental progression as is seen in the skin. It is apparent that even before hatching (stage 41), all three pigment cell types are present in the eye. Indeed, the presence of xanthophores and iridophores in the eye is a visible means of sorting out wild-type and melanoid axolotls in mixed spawnings even before the skin pigment pattern phenotype is certain (see The mutants).

Melanophores

Melanophores remain as *the most prominent of the skin pigment cells throughout the development of the axolotl.* Visibly, these cells are black or very dark brown (Fig. 3). The color is due directly to the deposition of the pigment melanin in organelles within melanophores known as melanosomes.

The development of the melanosome has been well documented by transmission electron microscopy. There are two ways in which melanosomes are known to develop, and both of these mechanisms have been observed to occur in axolotl pigment cells. Both pathways to the mature melanosome begin with the appearance of empty vesicles presumed to arise from the endoplasmic reticulum. One option is for Golgi-derived material to be added to these "primordial vesicles" to form a fibrillar matrix-type (see Frost-Mason *et al.,* 1994) premelanosome. Pigment is subsequently deposited upon the matrix resulting in an electron-dense, uniformly pigmented organelle. The other option is for the primordial vesicle to acquire numerous smaller vesicles, forming a multivesicular-type premelanosome. Pigment is thus deposited within the smaller vesicles and the result is again an electron-dense, uniformly pigmented organelle. Melanosomes that form from a matrix-type premelanosome are oval or ellipsoid in shape; those that form from multivesicular-type organelles are round.

Morphologically, melanophores, like all pigment cells, are highly dendritic (Fig. 3). Melanosome-containing processes extend throughout the dermis, producing a web-like network of pigment that contributes to the observed pigmentation pattern. Depending on the physiological state of the animal and the responsiveness of the pigment cells themselves, melanosomes may be either spread throughout the cell body and processes of a melanophore (Fig. 3) or aggregated around the nucleus within the cell body (Fig. 4).

In lower vertebrates, hormones such as MSH (melanocyte-stimulating hormone) are known to induce dispersion of melanosomes, whereas melatonin, a well-known skin-lightening agent, induces melanosome aggregation (Bagnara and Hadley, 1973). Pigment cells in axolotl skin appear to respond to hormones and growth factors just as one would predict based on extensive studies of such agents on frog (Hadley and Bagnara, 1969), fish (Pickford and Kosto, 1957), and reptilian (Taylor and Hadley, 1970) skin (reviewed in Bagnara and Hadley, 1973).

The pigment produced in axolotl melanophores appears to be eumelanin, a black/dark brown pigment, exclusively (Prota, 1992). Although birds and mammals can elaborate only one type of pigment cell (the melanocyte) in contrast to the three types in lower vertebrates, the higher vertebrates are capable of synthesizing a variety of melanins, including eumelanins and pheomelanins. Pheomelanins are responsible for red and blonde shades of hair, feathers, and fur.

Attempts to "force" axolotls to synthesize pheomelanin by loading pigment cells with cysteine (a primary substrate for pheomelanin synthesis) have produced interesting results. Axolotl larvae fed high doses of L-cysteine darken in skin color significantly (J. Muyskens, K. Mason, and S. Frost-Mason, unpubl.). If neural crest cells are cultured in the presence of L-cysteine, they also darken considerably compared to untreated cultures, and more melanophores appear to develop in treated cultures when compared to untreated controls. Furthermore, a substantial number of xanthophores develop in cysteine-treated neural crest cultures, which is not the case in control cultures (Fig. 5). So, although pheomelanin does not appear to be produced, melanin production and the pattern of differentiation may be altered by this treatment.

Normally, when the neural crest is explanted from an axolotl embryo and placed in culture, within 2-3 days a number of cells migrate from the explant and differentiate into melanophores producing obvious pigment (Dean and Frost-Mason, 1991). By 5 (or more) days in culture, an occasional xanthophore may be observed in such cultures. Xanthophores are few in number, and most often they do not attach well to the culture vessel, remaining tenuously attached (at best) to the substrate and/or surrounding cells. However, within 3-5 days following placement of the explant in cultures with cysteine-containing medium, substantial numbers of melanophores and xanthophores develop (Fig. 5), and the xanthophores assume a flattened, dendritic morphology identical to that of melanophores (Fig. 5). Why cysteine should promote these changes in cell behavior is not known, and experiments are currently underway to better understand what has happened to these cells.

Xanthophores

Xanthophores are the second pigment cell type to develop in the skin of axolotls. Within 24-48 h after the appearance of the first melanophores, xanthophores begin to be visible within the embryo. Initially, xanthophores develop where there are no melanophores. Early on, distinct black and yellow vertical stripes appear in late stage embryos and young larvae. This is indicative of the apparent "repulsion" that these two pigment cells appear to

have for each other. Eventually, there may be extensive overlap of cellular processes between these cell types, but there is no overlap of pigment cell bodies in axolotl skin.

Although this organization of pigment cells is typical for many larval amphibians, it is generally not the case in adult frogs and salamanders. *During metamorphosis, amphibian skin, like many other organs and tissues, is completely restructured.* Existing cells begin to reposition themselves within the dermis and new pigment cells appear. The end result is that pigment cells will often "stack" within the dermis, usually with melanophores underlying iridophores, which in turn underlie xanthophores. This arrangement has been termed a "dermal chromatophore unit," and, depending on the arrangement of chromatophores and the pigments within the chromatophores, an array of colors and shades may be produced (Bagnara *et al.,* 1968). Amphibian skin that is intensely black is likely to contain many layers of melanophores stacked upon each other. Intensely red, orange, or yellow spots are likely due to stacking of xanthophores, which may contain either dietarily acquired carotenoids and/or self-synthesized pteridines (Bagnara and Hadley, 1973).

In axolotls, xanthophores are bright yellow in color due most likely to pteridine pigments that are synthesized within these cells. Seven pteridines have been identified from axolotl skin, five of which are white or colorless and two of which are yellow-sepiapterin, which is an intense bright yellow, and xanthopterin, which is a pale yellow color (Frost *et al.,* 1984b). Riboflavin is also present in axolotl skin extracts, and it too is yellow. However, whether riboflavin is solely a component of xanthophores, and thus can contribute to the yellow color of these cells, or whether it is more ubiquitously deposited in cells throughout the skin is not clear. The questionable nature of riboflavin's function as a xanthophore pigment is compounded by the observation of Obika (1976) that riboflavin will bind to both pteridines and melanin *in vitro.*

Visibly, xanthophores are readily distinguishable from other pigment cell types on the basis of color. However, there are a number of features shared by melanophores and xanthophores. For example, the beginning stages of organellogenesis are similar in these two chromatophores. The pigment-containing organelles of xanthophores, pterinosomes, develop initially via empty vesicles derived from the endoplasmic reticulum. From this point forward, however, there is divergence in how pigment organelles develop. Smaller vesicles are subsequently added to prepterinosomes, resulting in the rapid appearance of flocculations within the maturing pterinosome. Over time, many pterinosomes acquire varying amounts of electron-dense, concentrically arranged fibers of presumed pigment (see Fig. 7 in Frost-Mason *et al.,* 1994). Indeed, although there has been no direct way to correlate pteridine pigment deposition with accumulation of fibrous material in pterinosomes, the more pigment that is present in axolotl skin, the more dense is the fibrous material that appears within pterinosomes (Frost *et al.,* 1986b).

The shared features and origins of pigment cells have been exploited in the axolotl with the intent of exploring the plasticity of differentiation. Ide and Hama (1976) were the first to demonstrate that amphibian pigment cells *in vitro* could be induced to transdifferentiate by varying culture media components. Subsequently, the axolotl was used by Frost and coworkers (Thorsteinsdottir and Frost, 1986; Frost *et al.,* 1987, 1989) to demonstrate that transdifferentiation could occur *in vivo* as well as *in vitro.* For example, allopurinol is a suicide-substrate for the

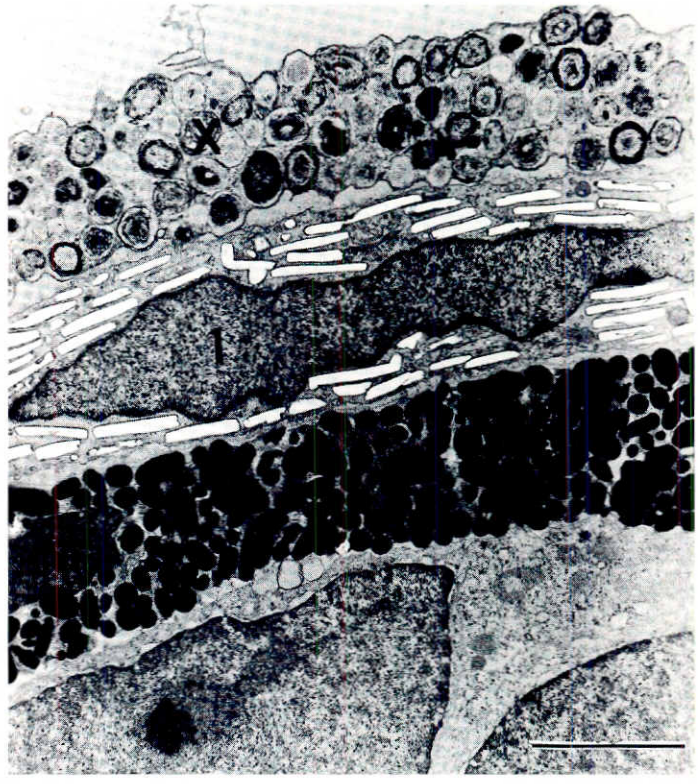

Fig. 2. Transmission electron micrograph of the iris from a white axolotl. *Note the three pigment cell types: xanthophore (X), iridophore (I), and melanophore (M). Bar, 3 μm.*

enzyme xanthine dehydrogenase (XDH), which is involved in several steps of pteridine biosynthesis. If larval wild-type axolotls are fed allopurinol, then xanthophore differentiation is suppressed and melanophore differentiation is enhanced (Frost *et al.,* 1989). Animals fed allopurinol are substantially darker than normal. If skin from allopurinol-treated animals is subjected to TEM, there is clear evidence of mosaic cells, i.e., cells that can be identified as predominantly xanthophores or melanophores, but that contain both melanosomes and pterinosomes, suggesting that they may be in transition. Furthermore, fewer than normal xanthophores appear in skin, and those that do gradually disappear. This probably happens by reprogramming of cells that would have become xanthophores to melanophores and by the transdifferentiation of existing xanthophores to become melanophores as has been observed in axolotls *in vivo* (Frost *et al.,* 1989). Of additional interest is the observation that animals treated with allopurinol become phenocopies of the melanoid mutant (Fig. 6).

In similar experiments, when guanosine is fed to larval axolotls, melanophores are greatly reduced in number and xanthophores are greatly enhanced. Such animals acquire an intense yellow pigmentation and iridophores develop prematurely in the skin (Fig. 6). Guanosine is a presumed substrate for both pteridine and purine (iridophore) pigments and appears to promote the differentiation of xanthophores and iridophores at the expense of melanophores (Frost *et al.,* 1987). Similar results are obtained when axolotl neural crest explants are treated with guanosine — in fact, in these cultures, cells that were once visibly melanophores can be observed to convert to xanthophores (A. Dean and S. Frost-Mason, unpubl.).

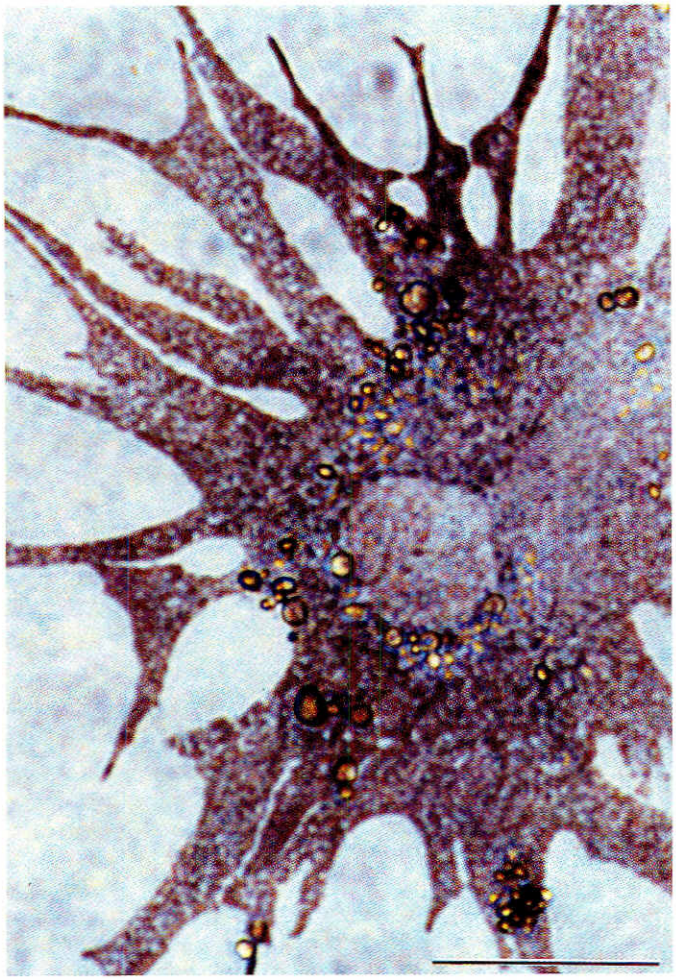

Fig. 3. Melanophore from an axolotl neural crest cell explant. *Bar, 25 μm.*

Although xanthophores are found in all vertebrates except birds and mammals, there are interesting evolutionary implications that can be inferred based on what is known about these cells and pteridine pigments. For example, the enzymatic machinery for synthesizing pteridines, many of which function as essential cofactors for important metabolic reactions, can be found in all vertebrates. Some of these reactions appear to be critical for normal growth and development in vertebrates (see Ayling *et al.*, 1993). Lower vertebrates have evolved ways to use pteridines as pigments. This ability to use pteridines as pigments has been lost in birds and mammals even though they still make pteridines that function as cofactors.

Iridophores

The third and final type of pigment cell that develops in amphibian skin is the iridophore. In axolotls, iridophores are not visible in embryos or very young larvae. They begin developing in skin sometime during early larval life, but precisely when this occurs is difficult to pinpoint and seems to vary especially among genetic strains of animals. For example, *a/a* (albino) animals, that are not also homozygous for other pigment genes, develop an intensely bright yellow color. There are two reasons for this intensely bright color: (1) Albinos develop large numbers of heavily pigmented xanthophores. This excess of xanthophores may be a result of the inability of albino animals to synthesize melanin and thus produce melanophores. (2) Albinos develop prominent and numerous iridophores beginning early in development, and these intensify the color of the xanthophores presumably by acting as bright mirror-like reflectors.

Iridophores are apparent in animal skin by their reflective properties, which contribute to the bright colors, especially blue colors, in some animals' skin. Iridophores are characterized generally by the presence of organelles known as reflecting platelets within the cytoplasm. The pigments within reflecting platelets are presumed to be crystalline purines or possibly pteridines. In many species, reflecting platelets are precisely organized into stacks, 10 or more platelets deep, often oriented parallel to the surface of the skin. When organized in this fashion, the platelets function as a thin-film interference reflector, and the Land equation can be used to calculate the wavelength of light that is being reflected by these cells (Morrison, 1994). Thus, the bright colors produced by xanthophores and underlying iridophores that reflect visible light can combine to produce a wide variety of colors of varying intensity. Indeed, the green color that is typical of many amphibians is produced precisely by layering yellow xanthophores on top of iridophores that reflect blue light (Morrison, 1994).

Because iridophores develop so late in axolotls, the axolotl has contributed little to our understanding of these cells. How reflecting platelets develop has been studied in other amphibians, however. In fact, TEM studies of developing frog *(Bombina orientalis)* skin suggest that reflecting platelets, like the other types of pigment organelles, begin as an ER-derived vesicle to which more vesicles are subsequently added, which presumably contain Golgi-derived materials necessary for the construction of the pigment crystals (see Fig. 9 in Frost-Mason *et al.*, 1994).

A thorough summary of pigment biosynthesis and organellogenesis can be found in Frost-Mason *et al.* (1994). There are common features shared by each of these organelles, and there are also points along the pigment biosynthetic pathways where control can presumably be regulated such that these may be important events in the actual determination of pigment cell type. Unfortunately, as of this moment, we know little about the precise points of regulation that can and do occur during pigment cell development.

What axolotl pigment mutants are available?

The oldest and most studied of the pigment mutants in the axolotl is the white mutant. White is also not a "true" pigment mutant, as it does not seem to be a defect that is exclusively intrinsic to the neural crest (Frost and Malacinski, 1980; Frost *et al.*, 1984a). The white phenotype is characterized by adult animals that have white skin and darkly pigmented eyes. White embryos are initially similar in appearance to the wild type. Pigment cells begin to differentiate and migrate in embryonic white skin only to disappear gradually during early larval development. Although the underlying cause of the white defect is still unknown, it has long been thought that this defect resides within the skin, rather than within the neural crest cells.

Early work on the white mutant indicated that when white neural crest cells were placed in a wild-type environment they would behave "normally" by migrating to appropriate locations within the skin and differentiating into pigment cells (Dalton, 1950a,b). The

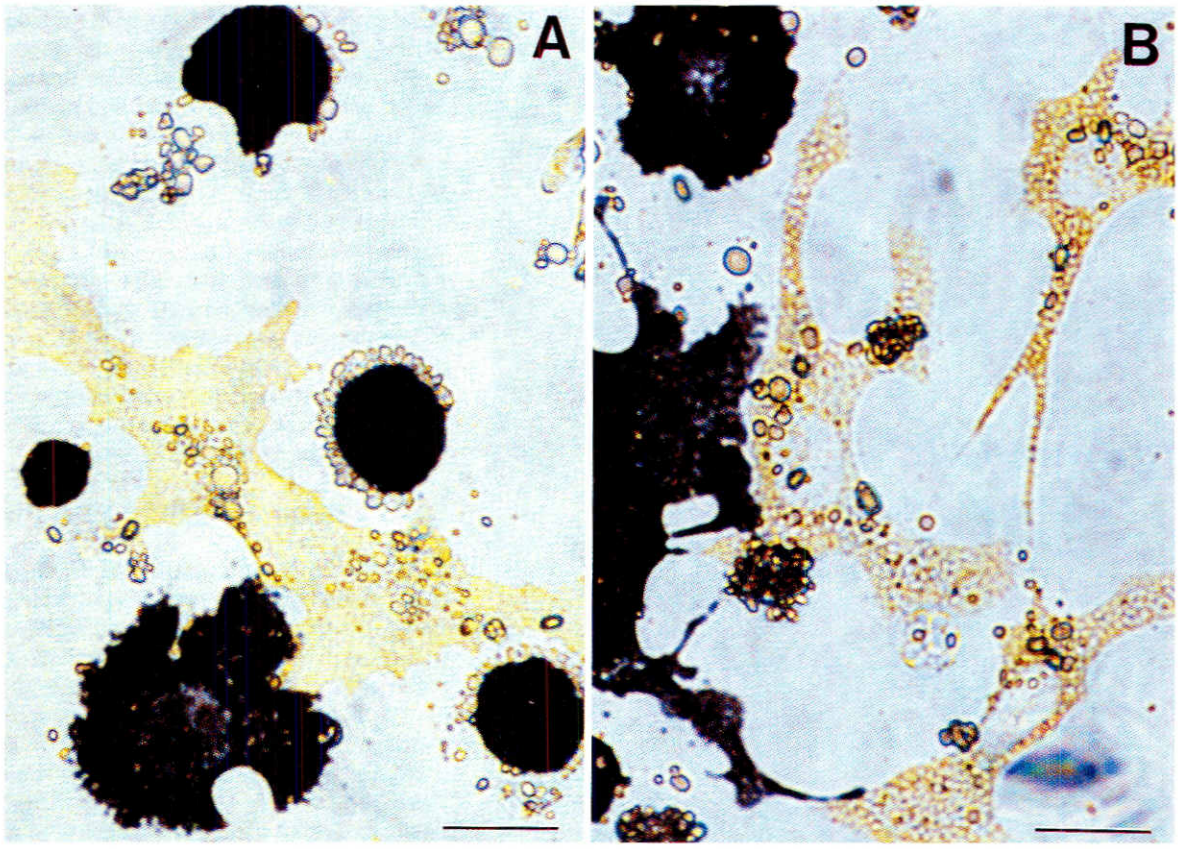

Fig. 4. Axolotl pigment cells *in vitro*. **(A)** *Punctate melanophores and dispersed xanthophore from the axolotl.* **(B)** *Melanophores and xanthophores in axolotl neural crest explant cultures. Bars, 25 μm.*

reciprocal experiment, when wild-type crest cells are transplanted into a white embryo, results in failure of the transplanted neural crest cells to migrate and differentiate normally. Subsequent attempts to identify differences between the white and wild-type tissue environment have not elucidated the nature of the white defect (Perris *et al.*, 1990). Recent studies indicate that the white defect may be far more complex than initially believed. There appears to be a substance in white skin that can actively inhibit differentiation and migration of pigment cells (Thibaudeau and Frost-Mason, 1992). Additionally, white neural crest cells, explanted into minimal culture medium without serum supplements, differentiate significantly fewer melanophores than wild-type crest cells grown in the same conditions (Dean and Frost-Mason, 1991). So, while there is unquestionably a problem in the white tissue environment, the crest cells themselves also seem to possess a unique sensitivity, especially to substances that normally promote pigment cell differentiation. Indeed, when white neural crest is explanted into culture medium containing fetal calf serum and/or combinations of growth factors such as MSH, pigment cells tend to differentiate more rapidly and in greater numbers than from explants of wild-type crest. Consequently, the ultimate eventual explanation for the white defect must take all of these observations into consideration.

"White" animals are common among vertebrates. White tigers, white rhinos, white mice, white chickens and the like are all variants that bear similarity to the white defect in axolotls. Aside from the apparent recessive nature of these genetic defects, there is little known about their biochemistry or cell biology.

In an attempt to understand why some otherwise darkly pigmented amphibians have white belly skin, Fukuzawa and Ide (1988) extracted a factor from frog skin that seems to inhibit the movement of pigment cells. Similar factors seem to be present in fish and mammalian skin as well. How or whether this inhibitory factor has any bearing on inherited white defects remains to be demonstrated.

Given the range of white defects that have been observed and the natural tendency for many animals to have white stripes or ventrums, there are likely to be a number of mechanisms in animals that result in absence of pigmentation and/or pigment cells from skin.

Often confused with white mutants are albino axolotls. Albinism, like the white defect, is common in animals. By strict definition, albinism is characterized by an absence of melanin pigmentation. An albino axolotl is bright yellow. These animals have melanophores in the dermis complete with premelanosomes that fail to pigment. Over time, finding evidence of unpigmented melanophores in albino skin, which can be done only by TEM, becomes increasingly difficult. Instead, xanthophores and iridophores are common, and xanthophores in particular are intensely yellow (Frost *et al.*, 1986b). Thus, while albinism in axolotls is characterized by an absence of melanin pigment, these mutants are also uncharacteristically more brightly pigmented because of more than the normal numbers of xanthophores and iridophores.

Why absence of one pigment cell type should lead to proliferation of the others is not well understood but may be a more common phenomenon than not. For example, the melanoid mutant in axolotls, also a simple Mendelian recessive gene, is characterized by abnormally large numbers of melanophores, an absence of iridophores, and gradually dwindling numbers of xanthophores (Frost *et al.*, 1984c).

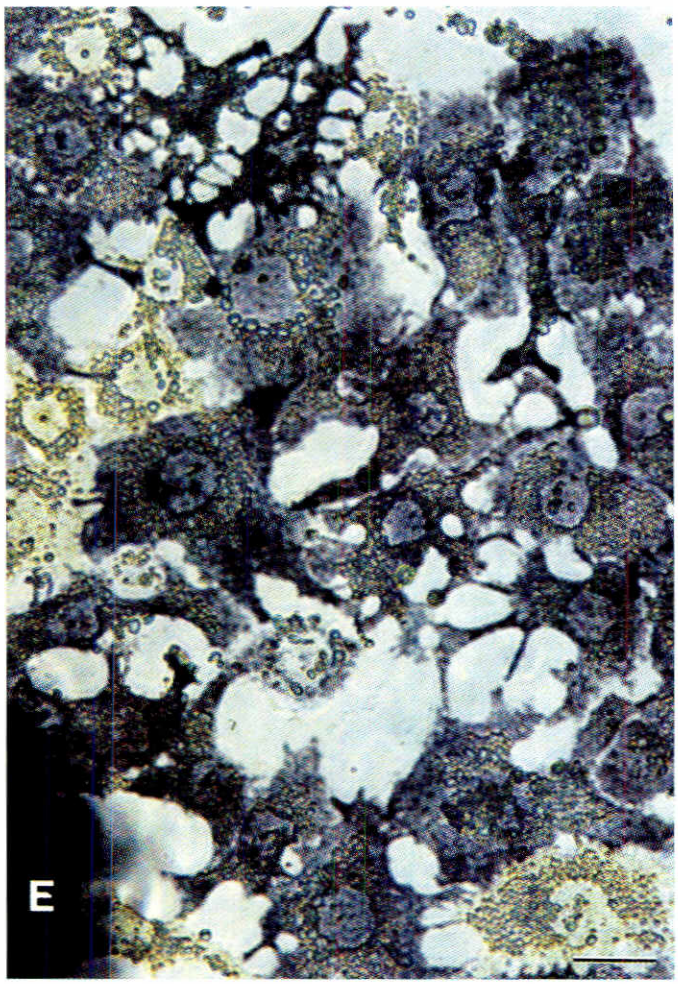

Fig. 5. Examples of pigment cells (melanophores and xanthophores) that differentiate in axolotl neural crest cell explant (E) cultures grown in the presence of L-cysteine. *Bar, 25 μm.*

The melanoid defect is not well understood, but melanoid phenocopies result when axolotl larvae are fed allopurinol. Given that allopurinol is an inhibitor of XDH and XDH levels are greatly reduced in melanoid tissues, the melanoid defect may affect the gene for XDH or a gene that can modulate the activity of XDH. Efforts to isolate and characterize the gene for XDH in axolotls are currently underway in this laboratory.

Genetic melanism is also common in animals. Black panthers and melanistic insects, reptiles, fish, and humans are all examples of melanism. Precisely how genetics predisposes individuals toward production of more melanin and more pigment cells is not well understood. In animals such as the axolotl, melanism appears to be a single-gene recessive trait. Melanism in other animals may be far more complex than this.

Axanthic axolotls, like the melanoid mutant, lack both xanthophores and iridophores (Frost *et al.*, 1986c). Phenotypically, however, axanthics are distinguishable from melanoids because they do not overproliferate melanophores as melanoids do and are thus considerably lighter in color than a melanoid. Another significant phenotypic difference between melanoids and axanthics is that xanthophores in axanthics never appear, whereas in melanoid axolotls there are xanthophores in the skin of very young animals, which gradually disappear over developmental time.

Axanthic, melanoid, and albino are all defects intrinsic to the neural crest stem cells (Frost and Malacinski, 1980; Frost *et al.*, 1984a). It is interesting that several of these simple Mendelian recessive traits, melanoid and albino, produce pleiotropic effects on chromatophores. In the absence of melanin pigment (albino), more yellow pigment appears in the skin. This is coincident with an uncharacteristically low number of unpigmented melanophores in the skin and abnormally high numbers of xanthophores and iridophores. In the presence of an excess of melanin pigment and melanophores (melanoid), there is a coincident absence of iridophores and a dramatic decrease in xanthophore pigment and numbers. Our eventual understanding of these defects will be predicated upon understanding what role intrinsic elements play in the determination of cell fate.

How can pigment cells be most easily studied?

There are a great many ways that pigment cells and thus pigmentation can be studied. The axolotl and other amphibians have provided researchers with a number of opportunities for both *in vivo* and *in vitro* experimentation. Some of the methods we commonly employ in these studied are discussed below.

Embryo manipulation

Axolotl and other urodele embryos are routinely maintained as described in detail in Armstrong *et al.* (1989) in dilute Holtfreter's solution. Because the neural crest is readily accessible in these embryos (see Fig. 1 in Frost, 1990), the embryonic source of pigment cells can be surgically excised from embryos, transplanted between embryos (Frost and Malacinski, 1980), or explanted into a culture dish and observed *in vitro* (Dean and Frost-Mason, 1991). The facility with which such experiments can be conducted has provided many opportunities for experimentation, especially with regard to the study of pigment mutants and pigment cell differentiation. Students of development should be encouraged to examine some of the earliest and most elegant studies of neural crest cell migration and chromatophore differentiation, such as those conducted by Niu and Twitty (Niu, 1947; Twitty, 1936, 1944, 1945, 1953; Twitty and Niu, 1948, 1954).

Cell culture

We continue to capitalize on the fact that amphibians, in general, have large and easy-to-manipulate embryos. Much recent information has been obtained by explanting neural crest cells from urodele embryos and growing/maintaining those cells in cultures (Dean and Frost-Mason, 1991). Figures 3-5 are excellent illustrations of cells grown in this way. These cells were removed from stage 23 axolotl embryos. The procedure is as follows: approximately 12–24 h before use, embryos are dejellied (manually) and placed in sterile 50% Holtfreter's solution containing penicillin and streptomycin. Sterile Petri dishes with a thin layer of 2% agar lining the bottom are prepared with sterile Steinberg's (1957) solution. A layer of Steinberg's solution is then pipetted on top of the agar and the embryo is placed in the dish, resting on the agar. Then, using very sharp tungsten needles, the epidermis over the neural tube is removed, exposing the neural crest, neural tube, and nearby somites. The needles are used to cut the top of the neural tube away from the embryo, and this tissue is then cut into smaller

Fig. 6. All three of these axolotls are siblings from a wild-type spawning. *The topmost animal is a normal wild-type animal. The middle animal has been fed allopurinol for several months and is substantially darker than normal. The bottom animal has been fed guanosine for several months and is considerably more yellow than normal.*

pieces. Individual pieces are transferred to culture dishes containing 70% L-15, 100 units penicillin, 100 μg streptomycin, with or without 5% fetal calf serum and with or without 1% fibronectin.

Pigment cells will adhere better to a fibronectin-coated substrate than to either plastic or glass alone, and the addition of serum to the culture medium promotes rapid differentiation of pigment cells, melanophores in particular (Dean and Frost-Mason, 1991). Neural crest explants will differentiate pigment cells even in the absence of serum, but they do so more slowly and less prolifically than with serum.

There are numerous substances that can affect the overall profile of pigment cell differentiation *in vitro*, and if the goal of an experiment is to assess how single factors affect the differentiation process, it is best to eliminate serum from the culture medium, because the precise nature of the differentiation-enhancing effects of serum on pigment cells is unknown.

Pigment cell analyses

Although it is possible in most cases to identify pigment cells visibly by their color, shape and location, this is not always the case. For example, albino axolotls produce melanophores, but such cells lack pigment and thus are not visible to the naked eye or even under a good light microscope. For unequivocal identification of pigment cells, especially if you wish to distinguish between pigment cells types, the best method is still to employ TEM. The organelles, and pre-organellar structures, of chromatophores are distinctive, and even an amelanistic albino melanophore will contain premelanosomes, which are ready identifiers of this cell type.

For a complete description of how to isolate and analyze pigments from pigment cells, and how to prepare tissues for TEM, the reader is referred to Frost *et al.* (1984b, 1986a).

How can molecular biology facilitate progress?

Molecular biology in urodeles is a growing field, but one still in its infancy. For many years the large genome size was seen as an insurmountable obstacle to productive molecular biology in the axolotl. This has proved not to be the case; it is possible to clone cDNAs from the axolotl by conventional techniques. Genomic libraries for the axolotl have been slowly constructed and as the need for such a libraries grows, more will likely be constructed.

The easiest way to achieve results in such a formative system is to use genes and tools that have been characterized in other systems. In the case of pigmentation, this means looking toward mammalian and avian systems primarily. The sorts of tools that can be found are genes that can be used as heterologous probes, and sequences of genes that can be used to design degenerate primers for the polymerase chain reaction (PCR). We have used degenerate PCR primers that were originally designed by Ian Jackson (Jackson *et al.*, 1994) to isolate members of the tyrosinase gene family (Mason and Mason, 1995). Once isolated, these genes can be characterized by sequencing and identified by comparison to their mammalian homologs using either simple identity or phylogenetic techniques (Morrison *et al.*, 1994). Genes that have been identified and characterized can be used to probe the patterns of expression of these genes on both a temporal and spatial level. This will allow us to begin to analyze the steps that are occurring as a neural crest cell becomes a specific pigment cell. We have targeted genes involved in melanogenesis both because they have been well characterized in the mouse and because there are definite differences in melanogenesis between mammals and urodeles.

These probes will also allow us to analyze the nature of the defects of a number of the mutants that have been discussed

earlier. For example, we have found that in newly-hatched white animals that still have melanophores, there appear to be wild-type levels of tyrosinase-related protein-1 (TRP-1) in animals up to 2 weeks post-hatching. Even more interesting, albino animals at a similar stage also make near wild-type levels of TRP-1 even though they have no visible melanin (K. Mason, unpubl.). As mentioned previously, albino axolotls exhibit an excess of xanthophores, which may occur by transdifferentiation of melanophores. Which cell type in albino axolotls is responsible for TRP-1 transcript production is currently under investigation.

Genes of relevance to pigmentation from other systems that can/are being targeted for study in urodeles include a wide variety of enzymatic activities, from some that are known to be involved in signalling activities to others that may be involved in the transport of pigment precursors into cells. The tyrosinase family genes, which are critical to melanogenesis, and genes for enzymes involved in purine (reflecting platelet pigments) and pteridine (pterinosome pigments) synthesis are also being examined. The mouse has provided us with an opportunity to examine genes that may affect signalling pathways involving pigment cells, including homologs for the *steel, dominant white spotting, agouti*, and melanocortin receptor family (Jackson *et al.*, 1994). The *agouti* locus is especially interesting as its gene product has been shown to interact with the MSH receptor directly (Lu *et al.*, 1994), controlling a switch in the type of melanin that is produced within a melanophore—from eumelanin to pheomelanin. Because urodeles are not known to synthesize pheomelanin, it will be of interest to know what such a signalling molecule might be doing in a urodele. With the availability of cloned genes from the axolotl and other amphibians, it should become possible to disrupt specific elements of the pigmentary system to determine causation for differentiation events. This can be done by constructing antisense probes to cloned genes or by producing antibodies to gene products of interest and applying these to differentiating neural crest cells *in vitro* or whole embryos in an attempt to assess what loss of function of such genes might do to pigment phenotype.

It will be important to develop a suitable expression system for examining genes as they are cloned. This is likely to require production of transgenic animals in the long run. Although transgenics are not available in urodeles at the present time, this is not an impossibility and should become a future priority. Even now, however, examining expression of pigment genes should be possible with the culture systems that have been developed. To do this would allow for (1) possible identification of mutant pigment genes by complementation, (2) analysis of gene regulation in specific cell types of interest, and (3) *in vitro* mutagenesis of cloned genes and possible construction of dominant negative pigment mutants where the mutant gene product interferes with wild-type products to produce a phenotype.

Thus, as more genes are cloned from urodeles, it will be possible to examine, both *in vitro* and *in vivo*, the hierarchical events involved in pigment cell differentiation in these animals. There is much basic information that can be gained beginning with the fundamental elements of the differentiation process and eventually culminating in significant insight into the evolution of the pigmentary system.

References

ARMSTRONG, J.B., DUHON, S.T. and MALACINSKI, G.M. (1989). Raising the axolotl in captivity. In *Developmental Biology of the Axolotl* (Eds. J.B. Armstrong and G.M. Malacinski). Oxford University Press, Oxford, pp. 220-227.

AYLING, J., GOPAL NAIR, M. and BAUGH, C.M. (Eds.) (1993). *Chemistry and Biology of Pteridines and Folates*. Plenum Press, New York.

BAGNARA, J.T. and HADLEY, M.E. (1973). *Chromatophores and Color Change. The Comparative Physiology of Animal Pigmentation*. Prentice-Hall, Englewood Cliffs (NJ).

BAGNARA, J.T., TAYLOR, J.D. and HADLEY, M.E. (1968). The dermal chromatophore unit. *J. Cell Biol. 38:* 67-79.

BORDZILOVSKAYA, N.P., DETTLAFF, T.A., DUHON, S.T. and MALACINSKI, G.M. (1989). Developmental-stage series of axolotl embryos. In *Developmental Biology of the Axolotl* (Eds. J.B. Armstrong and G.M. Malacinski). Oxford University Press, Oxford, pp. 201-219.

DALTON, H.C. (1950a). Comparison of white and black axolotl chromatophores *in vitro*. *J. Exp. Zool. 115:* 17-33.

DALTON, H.C. (1950b). Inhibition of chromatoblast migration as a factor in the development of genetic differences in pigmentation in white and black axolotls. *J. Exp. Zool. 115:* 151-173.

DEAN, A.D. and FROST-MASON, S.K. (1991). The effects of fetal calf serum and serum-free conditions on white and dark axolotl neural crest explants. *In vitro Cell Dev. Biol. 27A:* 402-408.

FORBES, M.S., ZACCARIA, R.A. and DENT, J.A. (1973). Developmental cytology of chromatophores in the red-spotted newt. *Am. J. Anat. 138:* 37-72.

FROST, S.K. (1990). Pattern formation: the differentiation of pigment cells from the embryonic neural crest. *Adv. Cell Biol. 3:* 201-219.

FROST, S.K. and MALACINSKI, G.M. (1980). Developmental genetics of pigment mutants in the Mexican axolotl. *Dev. Genet. 1:* 271-294.

FROST, S.K., BORCHERT, M.E. and CARSON, M.K. (1989). Drug-induced and genetic hypermelanism: effects on pigment cell differentiation. *Pigment Cell Res. 2:* 182-190.

FROST, S.K., BORCHERT, M.E. and THORSTEINSDOTTIR, S. (1986a). A rapid and sensitive TLC assay procedure for measuring xanthine dehydrogenase activity from tissue extracts. *J. Chromatogr. 382:* 314-320.

FROST, S.K., BRIGGS, F. and MALACINSKI, G.M. (1984a). A color atlas of pigment mutants in the axolotl. *Differentiation 26:* 182-188.

FROST, S.K., EPP, L.G. and ROBINSON, S.J. (1984b). The pigmentary system of developing axolotls. I. A biochemical and structural analysis of chromatophores in wild type axolotls. *J. Embryol. Exp. Morphol. 81:* 105-125.

FROST, S.K., EPP, L.G. and ROBINSON, S.J. (1984c). The pigmentary system of developing axolotls. II. An analysis of the melanoid phenotype. *J. Embryol. Exp. Morphol. 81:* 127-142.

FROST, S.K., EPP, L.G. and ROBINSON, S.J. (1986b). The pigmentary system of developing axolotls. III. An analysis of the albino phenotype. *J. Embryol. Exp. Morphol. 92:* 255-268.

FROST, S.K., EPP, L.G. and ROBINSON, S.J. (1986c). The pigmentary system of developing axolotls. IV. An analysis of the axanthic phenotype. *J. Embryol. Exp. Morphol. 95:* 117-130.

FROST, S.K., ROBINSON, S.J., CARSON, M.K., THORSTEINSDOTTIR, S. and GIESLER, J. (1987). The effects of exogenous guanosine on chromatophore differentiation in the axolotl. *Pigment Cell Res. 1:* 37-43.

FROST-MASON, S.K., MORRISON, R. and MASON, K.A. (1994). Pigmentation. In *Amphibian Biology, Vol. 2, The Integument* (Eds. H. Heatwole and G. Barthalmus). Surrey Beatty & Sons, Sydney, pp. 64-97.

FUKUZAWA, T. and IDE, H. (1988). A ventrally localized inhibitor of melanization in *Xenopus laevis* skin. *Dev. Biol. 129:* 25-36.

HADLEY, M.E. and BAGNARA, J.T. (1969). Integrated nature of chromatophore responses in the *in vitro* frog skin bioassay. *Endocrinology 84:* 69-82.

IDE, H. and HAMA, T. (1976). Transformation of amphibian iridophores into melanophores in clonal culture. *Dev. Biol. 53:* 297-302.

JACKSON, I.J., BUDD, P., HORN, M., JOHNSON, R., RAYMOND, S. and STEEL, K. (1994). Genetics and molecular biology of mouse pigmentation. *Pigment Cell Res. 7:* 73-80.

LE DOUARIN, N.M. (1982). *The Neural Crest*. Cambridge University Press, Cambridge.

LU, D., WILLARD, D., PATEL, I.R., KADWELL, OVERTON, L., KOST, T., LUTHER, M., CHEN, W., WOYCHIK, R.P., WILKISON, W.O. and CONE, R.P. (1994).

Agouti protein is an antagonist of the melanocyte-stimulating hormone receptor. *Nature 371:* 799-802.

MASON, K.A. and MASON, S.K. (1995). Identification and partial cloning by PCR of the gene for tyrosinase-related protein 1 from the axolotl. *Pigment Cell Res. 8:* 46-52.

MORRISON, R. (1994). A transmission electron microscopic (TEM) method for determining structural colors reflected by lizard iridophores. *Pigment Cell Res. 8:* 28-36.

MORRISON, R.L., MASON, K.A. and FROST-MASON, S.K. (1994). A cladistic analysis of the evolutionary relationships of the members of the tyrosinase gene family using sequence data. *Pigment Cell Res. 7:* 388-393.

NIU, M.C. (1947). The axial organization of the neural crest, studied with particular reference to its pigmentary component. *J. Exp. Zool. 105:* 79-113.

OBIKA, M. (1976). Reversible binding of riboflavin and pteridines to melanin *in vitro*. *Comp. Biochem. Physiol. 53B:* 521-523.

PERRIS, R., LOFBERG, J., FALLSTROM, C., VONBOXBERG, Y., OLSSON, L. and NEWGREEN, D.F. (1990). Structural and compositional divergencies in the extracellular matrix encountered by neural crest cells in the white mutant axolotl embryo. *Development 109:* 533-551.

PICKFORD, G.E. and KOSTO, B. (1957). Hormonal induction of melanogenesis in hypophysectomized killifish *(Fundulus heteroclitus). Endocrinology 61:*177-196.

PROTA, G. (1992). *Melanins and Melanogenesis.* Academic Press, New York.

STEINBERG, M. (1957). A non-nutrient culture medium for amphibian embryonic tissues. *Carnegie Inst. Wash. Yearb. 56:* 347-348.

TAYLOR, J.D. and HADLEY, M.E. (1970). Chromatophores and color change in the lizard, *Anolis carolinensis. Z. Zellforsch. 104:* 282-294.

THIBAUDEAU, G. and FROST-MASON, S.K. (1992). Inhibition of neural crest cell differentiation by embryo ectodermal extract. *J. Exp. Zool. 261:* 431-440.

THORSTEINSDOTTIR, S. and FROST, S.K. (1986). Pigment cell differentiation and pterin biosynthesis in wild-type, melanoid, and allopurinol-treated axolotls. *Cell Differ. 19:* 161-172.

TWITTY, V.C. (1936). Correlated genetic and embryological experiments on *Triturus*. I and II. *J. Exp. Zool. 74:* 239-302.

TWITTY, V.C. (1944). Chromatophore migration as a response to mutual influences of the developing pigment cells. *J. Exp. Zool. 95:* 259-290.

TWITTY, V.C. (1945). The developmental analysis of specific pigment patterns. *J. Exp. Zool. 100:* 141-178.

TWITTY, V.C. (1953). Intercellular relations in the development of amphibian pigmentation. *J. Embryol. Exp. Morphol. 1:* 263-268.

TWITTY, V.C. and NIU, M.C. (1948). Causal analysis of chromatophore migration. *J. Exp. Zool. 108:* 405-437.

TWITTY, V.C. and NIU, M.C. (1954). The motivation of cell migration, studies by isolation of embryonic pigment cells singly and in small groups *in vitro*. *J. Exp. Zool. 125:* 541-574.