THE ROLE OF BASIC FIBROBLAST GROWTH FACTOR IN THE TRANSDIFFERENTIATION OF AVIAN NEURAL CREST-DERIVED SCHWANN CELL PRECURSORS INTO MELANOCYTES

by

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LIST OF ABBREVIATIONS

α-MSH	alpha-melanocyte stimulating hormone
aFGF	acidic fibroblast growth factor
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
CEE	chick embryo extract
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CM	complete medium (see Materials and Methods)
CNTF	ciliary neurotrophic factor
DRG	dorsal root ganglia
EGF	epidermal growth factor
GGF	glial growth factor
HBSS	Hank's balanced salt solution
IGF	insulin-like growth factor
$InsP_6$	inositolhexakisphosphate
NGF	nerve growth factor
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PKC	protein kinase C
PLCγ1	phospholipase Cγ1
rh	recombinant human
TGF-α	transforming growth factor-alpha
TGF-β	transforming growth factor-beta
TPA	12-*O*-tetradecanoyl phorbol-13-acetate

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ABSTRACT

A variety of evidence supports the notion that a subpopulation of neural crest cells are partially restricted to giving rise to either Schwann cells or melanocytes. The fates of these melanocyte/Schwann cell progenitors are presumably influenced by instructive environmental cues which they encounter during their migration. In support of this notion, it has been shown that cultured neural crest-derived cells from embryonic quail dorsal root ganglia (DRG) and peripheral nerves, which do not normally give rise to melanocytes, become committed to melanogenesis following treatment with the phorbol ester drug 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). These data suggest that TPA can reverse the developmental restriction of melanogenesis in cells that normally would have given rise to Schwann cells, since peripheral nerves consist primarily of Schwann cell precursors.

The studies described in this dissertation test the possibility that peptide growth factors found in the embryonic environment might act similarly to TPA to cause this transdifferentiation, which may recapitulate the process by which melanocyte/Schwann cell progenitors undergo commitment *in situ*. Peripheral nerve and DRG explants were cultured in medium supplemented with a variety of growth factors, and then examined for the presence of pigment cells. It was found that basic fibroblast growth factor (bFGF), but not various other growth factors, induced pigmentation in about 20% of these cultures. These effects of bFGF were age-dependent, and could be blocked by addition of a bFGF-neutralizing antibody to the culture medium. To test the possibility that bFGF might play a role in TPA-induced melanogenesis, peripheral nerves were treated with TPA and antisense deoxyoligonucleotides targeted against the bFGF mRNA transcript. Antisense oligonucleotides targeted against two regions of the bFGF mRNA blocked

TPA-induced transdifferentiation of Schwann cell precursors, whereas sense and scrambled antisense controls had no affect in this regard. TPA was also shown to increase bFGF protein expression in these cells in the presence of serum. This expression was detected in the nucleus and cytoplasm of cells in these cultures, but not in their concentrated conditioned media. Furthermore, neither bFGF neutralizing antibodies nor inositolhexakisphosphate affected this TPA-induced melanogenesis, suggesting that bFGF is not released by these cells. Interestingly, another growth factor, transforming growth factor-ß1 (TGF-ß1), was found to inhibit both the TPA- and bFGF-induced pigmentation of DRG cultures. These data indicate that the phenotypes of Schwann cell precursors can be influenced by bFGF and TGF-β1, that bFGF expression by these cells is necessary for the TPA-induced transdifferentiation of Schwann cell precursors into melanocytes, and that bFGF can act in these cells via an intracrine mechanism.

CHAPTER I

Introduction

Tom is now a great man of science . . . and knows everything about everything except why a hen's egg don't turn into a crocodile.

- Charles Kingsley (1863)

Perhaps the most fundamental question in the field of developmental biology is how the apparently homogeneous cells of an early embryo give rise to the tremendous diversity of structures that comprise an adult organism. In 1817, Christian Heinrich Pander (1794-1865), the son of a Latvian banker, completed his medical doctoral thesis and presented one of the first attempts to address this question. Pander characterized what he called the *Keimhaut* (blastoderm) of the embryonic chick, and found that as development proceeded, the *Keimhaut* became organized into three *Keimblätter* (germ-layers), which we know today as the ectoderm, mesoderm, and endoderm. He speculated that these layers each have a different fate, but that "although destined for different purposes", they "interact with one another until each has attained its determined level".

While Pander would make no further contributions to the field of Embryology (nor, apparently, any other field), his observations led others to the notion that homologous structures in the embryos of different animals developed from equivalent germ-layers. Ernst Haeckel (1866), for example, believed that the three germ-layers originate during phylogeny from an ancestral form, the "gastrea", which is recapitulated during ontogeny. This so-called "germ-layer theory" dominated developmental research for nearly a century, and proposals that deviated from it were considered heretical. It was long believed, for example, that all connective tissue, because of where it develops, was

derived from mesoderm even though there was no evidence supporting such a supposition.

Wilhelm His (1831-1904), a Swiss embryologist and Professor of Anatomy and Physiology in Basel, provided one of the earliest refutations of the germ-layer theory. In 1868, His published a treatise on the development of the spinal cord and its associated peripheral structures in the embryonic chick. As was the case for connective tissue, the spinal ganglia were believed to be derived from mesoderm. His, however, discovered a band of cells between the "*Hornblatt*" (epidermal ectoderm) and the neural tube which he called the "*Zwischenstrang*" (intermediate cord). Based on observations of serial sections from numerous early embryos, His determined that the cells of the *Zwischenstrang* migrated to the periphery, where they formed the spinal ganglia and, he believed, the cranial ganglia[1]. His later referred to this band of cells as one of the principal "organ-forming germinal regions" (1874), and, in line with his mechanistic theory on processes of development, suggested that it contained subsets of cells with restricted fates.

Soon after His' discovery, the *Zwischenstrang* became known as the ganglion crest, owing to its presumed derivatives. During the next several decades, however, micro-surgical ablations and transplantation studies (for reviews see Yntema and Hammond, 1947; Hörstadius, 1950) demonstrated that, in addition to the spinal ganglia and portions of the cranial ganglia, numerous other structures and cell types are also derived from this embryonic cell population, including neurosecretory cells, pigment cells, components of the cardiac outflow tract, some skeletal and connective tissues of the head, and dental papillae (see Table I-1). The ganglion crest, therefore, was re-named the neural crest based on the fact that its cells transiently border the dorsal neural tube as a band, then, after migrating to the periphery, give rise to a wide variety of cellular phenotypes.

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[1]His was only partly correct about the origins of the cranial ganglia. The so-called *Zwischenstrang* only contributes the satellite cells and some neurons to the trigeminal ganglion of the Vth cranial nerve, the geniculate ganglion of the VIIth nerve, and proximal portions of the ganglia of the IXth and Xth cranial nerves. The remainder of the neurons in the cranial ganglia are derived from ectodermal placodes (see D'Amico-Martel and Noden, 1983).

TABLE I-1

Derivatives of the Neural Crest*

I. Peripheral Nervous System

NEURONS
- Spinal sensory ganglia
- Sympathetic ganglia and plexuses
- Parasympathetic ganglia and enteric plexuses
- Cranial sensory ganglia (V and VII, proximal portions of IX and X)
- Trigeminal mesencephalic nucleus
- Rohon-Beard neurons

SCHWANN CELLS

NEUROGLIAL CELLS

II. Pigment cells of the integument and iris

III. Endocrine and paraendocrine cells

ADRENAL CHROMAFFIN CELLS

CALCITONIN-SECRETING CELLS

CAROTID BODY TYPE I CELLS

IV. Mesectodermal derivatives

MENINGES
- Pia-arachnoid of prosencephalon and part of mesencephalon

VISCERAL SKELETON

ANTERIOR VENTRAL SKULL BONES

CONNECTIVE TISSUE
- Connective tissue of glands (thymus, thyroid, pituitary, salivary, lacrymal)
- Connective tissue and smooth muscle of aortic arch-derived arteries
- Dermis, smooth muscle, and adipose tissue of skin of head and ventral part of neck
- Corneal endothelium and stroma
- Tooth papillae

*See Le Douarin (1982) and Hall (1988) for reviews.

Once it was realized that the neural crest contains cells with the potential to give rise to progeny that populate all the classical germ layers, the germ-layer theory was, with some reluctance, abandoned (De Beer, 1947). The discovery of the neural crest, however, raised numerous new theoretical problems, including questions about its origins and boundaries, the modes of neural crest-cell migration, and the mechanisms by which this seemingly homogeneous population of cells becomes committed to diverse cell fates. These questions, most of which remain unanswered, have been the focus of neural crest development research for the past century. Some of this research is reviewed below as a prelude to understanding the issues addressed in the chapters that follow, which include

determining how certain environmental cues influence the phenotype of neural crest-derived Schwann cell precursors. Investigations of these and related phenomena, as initiated by Pander nearly two centuries ago, contribute to our understanding of how the cells of an early embryo become committed to their particular, specialized fates.

The neural crest arises between presumptive neural and epidermal ectoderm

The neural crest, neural ectoderm, and epidermal ectoderm are all found within the neural folds during neurulation (see Brun, 1985 and Fig. I-1). The association between these areas, however, arises in the very early blastula stage. In fact, the presumptive neural crest has been traced to the boundary between presumptive neural ectoderm and epidermal ectoderm in amphibian (Raven, 1931; Baker and Graves, 1939) and, more recently, avian (Rosenquist, 1981) embryos. The junction of the neural and epidermal ectoderm is the position where neuralizing and epidermalizing influences meet, and the combination of these influences are believed to generate the neural crest. In support of this notion, Rollhäuser-ter-Horst (1980) showed that future epidermal ectoderm from gastrulating amphibian embryos, when grafted in place of the presumptive neural crest of neurulating embryos, differentiated into neural crest and, later, various classic neural crest derivatives. A more recent study has shown that neural crest can arise from either ventral epidermis or neural plate in axolotl embryos (Moury and Jacobson, 1990). These findings suggest that neural crest cells arise as a result of reciprocal inductive interactions between the neural ectoderm and the developing epidermis.

Neural crest cells migrate along temporally and spatially distinct pathways

As mentioned above, His (1868) was the first to suggest that neural crest cells migrate, based on his observations of serially sectioned early embryos. Since then, a variety of studies have demonstrated that neural crest cells disperse and migrate from the dorsal neural tube immediately following its closure (Fig. I-1d). This migration starts in

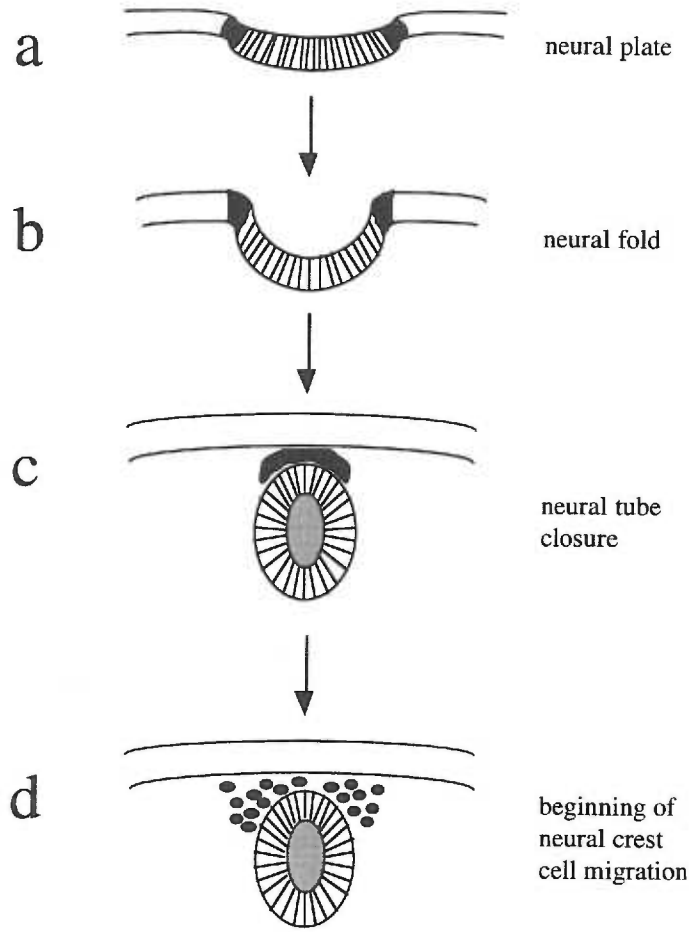

Figure I-1: A representation of the location of the neural crest during neural tube closure in avian embryos. **(a)** The neural crest (black) originates at the junction between neural ectoderm (hatched) and epidermal ectoderm (white) at the early neurula stage. **(b)** As the neural plate begins to fold, the neural crest becomes localized at the dorsal lips of the neural fold. **(c)** With closure of the neural tube, the neural crest is found between the dorsal neural tube and the epidermal ectoderm. **(d)** Immediately following closure of the neural tube, neural crest cells disperse and begin to migrate.

the mid-brain, then extends to progressively more caudal regions of the neural tube, always along particular pathways (see Horstadius, 1950; Le Douarin, 1982; Marusich and Weston, 1991). Johnston (1966) and Weston (1963), for example, determined the fates and pathways traveled by ^{3}H-thymidine-labeled neural crest cells from the cranial and trunk regions of chicken embryos, following grafting into unlabeled embryos. Johnston found that the cranial neural crest cells moved cranial, caudal, and medial to the develop-

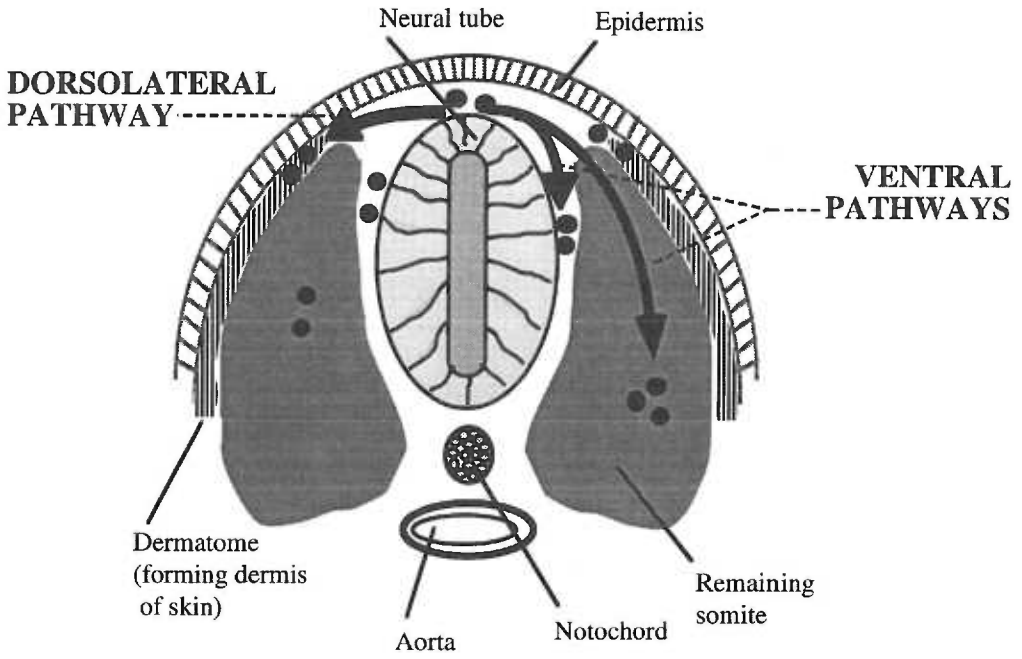

Figure I-2: A representation of the migratory pathways of avian trunk neural crest cells. As the neural folds close to form the neural tube, trunk neural crest cells disperse and migrate from the dorsal neural tube along two distinct pathways. The earliest cells to migrate typically follow a **ventral** pathway, either between the neural tube and the somite or through the rostral half of each somitic sclerotome. These cells form the dorsal root and sympathetic ganglia, and at certain axial levels, the adrenal medulla. After some delay, other crest cells follow a **dorsolateral** pathway, between the dorsal somite and the epidermis. These cells enter the skin and give rise to melanocytes (for a recent review see Marusich and Weston, 1991).

ing eye. These cells have since been shown to differentiate into neuronal derivatives, connective tissue, cartilage and bone of the face and head, dentine, and some of the enteric ganglia of the gut (Le Douarin, 1982; Le Dourain, 1986; Hall, 1988). In the trunk, Weston observed that neural crest cells followed two major migratory pathways. Some cells followed a dorsolateral route, between the dorsal somite and the epidermis, while others migrated along ventral pathways, through the somitic mesenchyme (Fig. I-2). The cells which migrated along the dorsolateral pathway entered the skin and differentiated into melanocytes, while cells migrating along the ventral pathway formed the dorsal root and sympathetic ganglia, and at certain axial levels, the adrenal medulla.

More recent studies have indicated that trunk neural crest cells can travel along more than one ventral pathway. Some crest cells, for example, migrate to the spaces between somites (Newgreen *et al.*, 1990), while others travel between somites and the neural tube (Weston, 1963; Le Douarin, 1973; see Fig. I-2). In the case of amphibians and fish, crest cells traveling along the latter of these pathways migrate within local interstitial spaces (Sadaghiani and Vielkind, 1990), while avian and mammalian crest cells migrate under the dermamyotome, intermingling with cells in the rostral portion of the somitic sclerotome (Serbedzija *et al.*, 1989; Serbedzija *et al.*, 1990). Neural crest cells disperse promptly from the neural tube along these ventral pathways, but delay their lateral dispersal until the dorsolateral pathway opens between the dermamyotome and the ectodermal epithelium (Fig. I-2). In mouse and avian embryos, the dorsolateral pathway does not appear until approximately 24 hours after the initiation of ventral neural crest migration (see Marusich and Weston, 1991).

Subpopulations of neural crest cells are partially restricted to particular fates as they migrate from the neural tube

As mentioned above, understanding how the numerous crest-derived lineages become segregated from multipotent parental cells is one of the principal goals of neural crest development research. One possibility is that following dispersion from the neural tube, crest cells could undergo a series of divisions producing multipotent cells whose fates are influenced by instructive environmental cues encountered during migration. An alternative and equally extreme possibility, however, is that neural crest cells are already committed to distinct lineages or subsets of lineages at the onset of their migration. In this case, crest cells would undergo a series of divisions and further "programmed" restrictions while traveling along migratory pathways that were permissive for their development.

A variety of studies have indicated that some early neural crest cells are likely to be multipotent when they leave the dorsal neural tube. A classic approach to demonstrating multipotentiality is to establish cultures of apparently homogeneous cells at clonal densities, then examine the numbers and types of phenotypes which arise from individual clones. The first such studies of progeny from cloned neural crest cells demonstrated that some clones gave rise to both pigment cells and catecholaminergic neurons (Cohen and Konigsberg, 1975; Sieber-Blum and Cohen, 1980). More recently, however, clones of individual neural crest cells *in vitro* have been shown to give rise to many different combinations of cell types, indicating that their progenitors were not irreversibly restricted to a particular fate (Baroffio *et al.*, 1988; Dupin *et al.*, 1990; Duff *et al.*, 1991; Stemple and Anderson, 1992).

Multipotency of early neural crest cells has also been demonstrated *in vivo*, by tracing the cell lineages of individual cells microinjected with fluorescent dextran. Utilizing this method, the clonal progeny of some labeled cells have been found in all of the different locations to which neural crest cells migrate, including dorsal root and sympathetic ganglia, peripheral nerves, the adrenal glands, and the skin (Bronner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991). Similar findings were obtained by Frank and Sanes (1991), who injected a retrovirus bearing the *E. coli* β-galactosidase (*lac Z*) gene into the neural tubes of early chicken embryos *in ovo*. In this study, some *lac* Z^+ cells, presumably representing clones of an individual early neural crest cell, could be detected which possessed traits of neurons, Schwann cells, and satellite cells. These data support, therefore, the notion that some premigratory neural crest cells are multipotent, or at least, only partially restricted to particular developmental potentials.

There is also strong evidence, however, that phenotypically distinct subpopulations of neural crest cells exist before the onset of migration (for review see Weston, 1991). Numerous studies, for example, have demonstrated that some premigratory and early crest cells *in vitro* express antigens recognized by cell-type specific antibodies (Ciment

and Weston, 1983; Barald, 1982; Ziller *et al.*, 1983; Girdlestone and Weston, 1985; Maxwell *et al.*, 1988). Moreover, various early neural crest derivatives have been isolated and found to give rise to only a limited number of phenotypes in heterospecific grafting experiments (Le Lievre *et al.*, 1980; Nakamura and Ayer-Le Lievre, 1982; Ciment and Weston, 1985; Fontaine-Perus *et al.*, 1988), suggesting that some of these phenotypically distinct neural crest subpopulations are restricted in their developmental potentials. This notion is further supported by the clonal analysis studies described above, in which clones of some neural crest cells both *in vitro* and *in vivo* only gave rise to one or two cell types (Sieber-Blum and Cohen, 1980; Baroffio *et al.*, 1988; Bronner-Fraser and Fraser, 1989; Dupin *et al.*, 1990; Frank and Sanes, 1991). Collectively, these data indicate that the neural crest is a heterogeneous population of cells. Some crest cells have restricted developmental potentials, while others are only partially restricted or multipotent.

Environmental cues may influence the fate of partially restricted neural crest subpopulations

The process by which these multipotent or partially restricted neural crest cells become committed to particular phenotypes is believed to occur by a series of events, rather than by a single determinative step (Wessells, 1977). Presumably during the course of their migration, such progenitor cells become increasingly restricted in their developmental potentials, while simultaneously gaining features of differentiated cell types. It is likely, moreover, that this process is influenced by both environmental cues encountered by crest cells during their migration, and by the repertoire of cell surface receptors they express at particular times. Some of the early evidence supporting this idea comes from the cloning experiments of Sieber-Blum and Cohen (1980) mentioned above, in which extracellular matrix was shown to enhance the proportion of neural crest-derived clones containing catecholaminergic neurons. Similarly, Stemple and Anderson

(1992) recently demonstrated that clones of rat neural crest cells grown on a substrate coated with fibronectin gave rise to Schwann cells, while clones grown on fibronectin plus poly-D-lysine gave rise to Schwann cells and neurons. Various other undefined components of the extracellular matrix and serum have also been shown to selectively influence the development of subpopulations of neural crest-derived cells (Maxwell *et al.*, 1988; Barald, 1989; Morrison-Graham *et al.*, 1990; Maxwell and Forbes, 1990).

Additional support for the notion that environmental cues can affect neural crest cell fate comes from studies of a partially restricted subpopulation whose derivatives include the chromaffin cells of the adrenal medulla and extra-adrenal chromaffin bodies, and the neurons and small intensely fluorescent (SIF) cells of sympathetic ganglia (Anderson and Axel, 1986; Landis and Patterson, 1981; Carnahan and Patterson, 1991). Although each of the derivatives of this so-called sympathoadrenal precursor population express a stable differentiated phenotype in the adult organism, these cell types can be interconverted (see Fig. I-3). For example, individual SIF cells, which share phenotypic characteristics of chromaffin cells and neurons (Eranko, 1975), will transform into sympathetic neurons in the presence of nerve growth factor (NGF), or chromaffin cells in the presence of glucocorticoids (Doupe *et al.*, 1985). Similarly, NGF or basic fibroblast growth factor (bFGF) can cause single chromaffin cells from either embryonic, neonatal, or adult rat adrenal glands to become converted into sympathetic neurons (Unsicker *et al.*, 1978; Doupe *et al.*, 1985; Anderson and Axel, 1986; Stemple *et al.*, 1988; Seidl and Unsicker, 1989). Neuronal differentiation in immortalized sympathoadrenal progenitor cell lines is also influenced by these growth factors (Anderson, 1989).

These observations suggest that chromaffin cell differentiation *in vivo* is the result of sympathoadrenal cells encountering steroids produced by the adrenal cortex either during or following their migration to the adrenal gland. Migration of these progenitors to the sympathetic ganglia, however, would likely result in their exposure to agents that would promote neuronal differentiation, like NGF or bFGF. A variety of studies have

shown that such interconversions can occur *in vivo*. Injections of either glucocorticoids or anti-NGF antibodies into neonatal rats, for example, increase the number of SIF cells in sympathetic ganglia (Päivärinta *et al.*, 1984; Carnahan and Patterson, 1991), while injections of NGF result in the appearance of sympathetic neurons in the adrenal glands (Aloe and Levi-Montalcini, 1979). Furthermore, cells in primordial sympathetic ganglia *in vivo* co-express neuronal and adrenal chromaffin markers at early developmental stages, but stop expressing chromaffin cell markers at later stages (Anderson *et al.*, 1991). These data suggest that environmental cues present in the developing sympathetic ganglia can cause sympathoadrenal progenitors to become restricted to a neuronal phenotype.

The fate of a bipotent melanocyte/Schwann cell progenitor may also be influenced by environmental cues

There is evidence supporting the existence of another neural crest-derived bipotent intermediate which can give rise to either Schwann cells or melanocytes (for review see Ciment, 1990). Melanocytes are branched, migratory cells found in both the iris and integument of most higher vertebrates. These cells form various types of melanins, which scavenge harmful free radicals, shield underlying epithelial cells in the skin from ultra-violet radiation, and, in the eye, absorb scattered light as it passes through the retina (Quevedo *et al.*, 1987; Sarna *et al.*, 1986). Numerous extirpation and grafting experiments (reviewed by Hörstadius, 1950 and Le Douarin, 1982) have indicated that, with the exception of the optic cup-derived pigment cells of the retina, all pigment cells in vertebrates are derived from the neural crest. It is generally accepted that all Schwann cells are also neural crest derivatives (Le Douarin *et al.*, 1991; Jessen and Mirsky, 1992), although a modest number may arise from the ventral neural tube (Rickmann *et al.*, 1985; Lunn *et al.*, 1987). These flattened cells with small nuclei, named after the famous cell biologist Theodor Schwann (1810-1882), enfold all axons in peripheral nerves. Among their other functions, Schwann cells produce the myelin which insulates peripheral axons

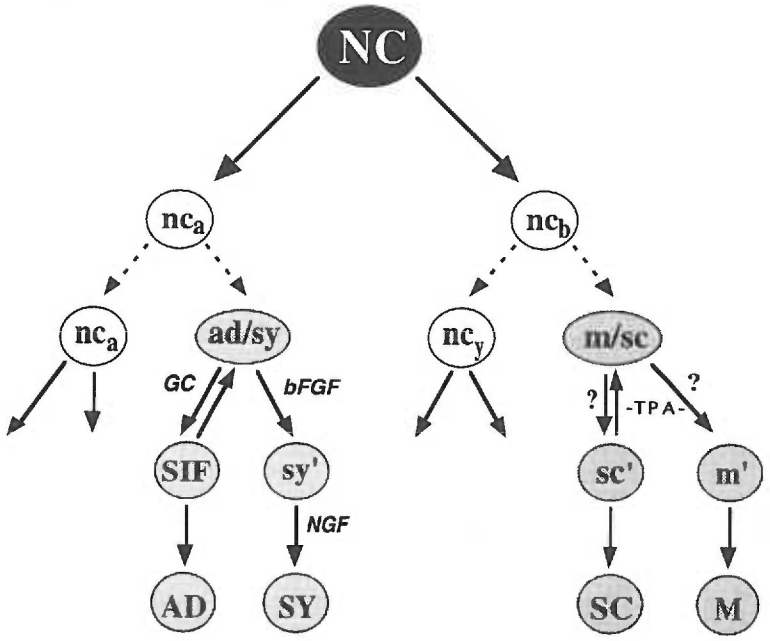

Figure I-3: A model of how the developmental potentialities of some neural crest derivatives may become segregated. In this model, multipotential neural crest cells (**NC**) become increasingly restricted in their developmental potentialities. Some become bipotent, like the sympathoadrenal precursor (**ad/sy**) and the melanocyte/Schwann cell progenitor (**m/sc**), while others may be multipotent or restricted to a single cell fate. These partially restricted derivatives can be influenced by environmental cues. The sympathoadrenal precursor, for example, gives rise to SIF cells and, eventually, adrenal chromaffin cells (**AD**) in the presence of glucocorticoids (**GC**). In the presence of bFGF and NGF, however, the sympathoadrenal cells give rise to neuroblasts (**sy'**) and, later, sympathetic neurons (**SY**). Environmental cues are also believed to play a role in causing the melanocyte/Schwann cell progenitor to become committed to either the Schwann cell (**SC**) or melanocyte (**M**) fate.

and increases their conduction velocities, synthesize all of the basement membrane components of the neurilemma, and are believed to produce neurotrophic agents, such as NGF, during axonal outgrowth and regeneration (Heumann, 1987).

The earliest indication that some Schwann cells and melanocytes might arise from a common progenitor came from the studies of Peterson and Murray (1955), who observed "stellate cells which later produced melanin pigment" in cultured dorsal root ganglia (DRG) from 4-6-day chick embryos. Interestingly, the number of melanocytes in these DRG cultures decreased with increasing embryonic age, and no melanocytes were

observed in explants from embryos older than 6 days. Cowell and Weston (1970) repeated these experiments, and demonstrated that the appearance of pigmented cells in early DRG cultures was sensitive to both actinomycin D (which selectively inhibits RNA synthesis) and cytosine arabinoside (which inhibits DNA synthesis). The conclusions of these studies were that (1) some cells in nascent dorsal root ganglia become pigment cells *in vitro* when they would not normally do so *in situ*, and (2) that cell division and RNA transcription must occur in this culture environment before such altered differentiation can take place. Implicit in these conclusions is the idea that some component of the culture medium used in these studies was acting as a cue to promote developmentally labile cells to undergo melanogenesis. What remained unclear, however, was the normal fate of the cells giving rise to melanocytes.

Dorsal root ganglia consist of several different neural crest derivatives, including sensory neurons, Schwann cells, and satellite cells, and peri- and epineurial fibroblasts derived from mesoderm (Haninec, 1988; Bunge *et al.*, 1989; Halata *et al.*, 1990). As an attempt to ascertain the prospective fate of the cells with melanogenic potential in early DRG, Nichols and Weston (1977) cultured DRG, peripheral nerves (which contain Schwann cells and perineural fibroblasts), and spinal meninges (which, like peri- and epineurium, consist of mesodermally-derived fibroblasts) from 5-day chick embryos. In these studies, melanocytes were only observed in explants of peripheral nerves and DRG grown in a medium containing NGF, fetal bovine serum, and chick embryo extract. One interpretation of this finding is that the cells giving rise to melanocytes in these cultures would have given rise to Schwann cells *in situ*, since they are the only neural crest-derived cell type common to both peripheral nerves and DRG. An alternative explanation, however, is that these cells were "melanoblasts" (i.e., cells committed to the melanocyte fate) that would not have survived *in situ*, but which thrive and differentiate when placed in the culture conditions used in this study.

In 1986, Ciment and co-workers demonstrated that DRG or peripheral nerve explants from 7-9-day Japanese quail embryos, which, like their counterparts in chick embryos do not normally contain pigment cells, gave rise to melanocytes when cultured in the presence of the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). These cells appeared to be *bona fide* melanocytes, moreover, based on their morphology and the observation that they could migrate into the overlying epidermis and contribute to feather pigmentation following grafting into White Leghorn chicken embryos (a species with unpigmented feathers) *in ovo*. This rather surprising observation indicated that cells with latent melanogenic potential were present in these tissues, and ruled-out the possibility that DRG and peripheral nerves contained melanoblasts that would normally die between day 6 and 7 *in situ*. It is unlikely, moreover, that these effects of TPA were due solely to the proliferation of a small population of melanoblasts, since even small numbers of melanocytes are not seen in peripheral nerve or DRG explants from embryos older than 6 days. A more probable explanation is that TPA promoted melanocyte differentiation by reversing the developmental restriction of melanogenesis that somehow occurs in older peripheral nerves and DRG (see Ciment, 1990). Furthermore, since this effect was observed in peripheral nerve explants, it is likely that TPA caused the transdifferentiation of Schwann cell precursors[2] into melanocytes.

These data, in conjunction with a number of clonal analysis studies both *in vitro* and *in vivo*, have led to the suggestion that some Schwann cells and melanocytes are derived from a bipotent neural crest-derived subpopulation of cells (Bronner-Fraser and Fraser, 1989; Dupin *et al.*, 1990; Duff *et al.*, 1991; Ciment, 1990). In the *in vivo* clonal analysis studies of Bronner-Fraser and Fraser (1989), for example, a small number of clones gave rise to both pigment cells and Schwann cells in ventral roots, but not other neural crest derivatives. A variety of indirect evidence also supports the existence of this bipotent

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[2]The term "Schwann cell precursor" is not used here to imply that these cells are committed to becoming Schwann cells, but, rather, to suggest that these cells would have become committed to the Schwann cell lineage *in situ*.

progenitor. Tumors consisting of Schwann-like cells containing melanosomes (the organelle in melanocytes responsible for melanin formation) and pre-melanosomes, for example, can be induced in mouse skin by application of the tumor initiator 9,10-dimethyl-1,2-benzanthracene followed be repeated applications of TPA (Kanno *et al.*, 1987; Garcia and Szabo, 1989). Various human tumors and other lesions have also been described in which Schwann cells appear to transdifferentiate into melanin-forming cells, including pigmented Schwannoma, ganglioneuroblastoma, and certain oral mucosal lesions (Hahn *et al.*, 1976; Mennemeyer *et al.*, 1979; Stefansson *et al.*, 1982; Burns *et al.*, 1983; El-Labban, 1988). Moreover, patients with von Recklinghausen's neurofibromatosis, a disease which influences the development of many neural crest-derived tissues, typically have numerous neurofibromas (Schwann cell tumors) accompanied by multiple patches of hyperpigmentation (café-au-lait spots) and pigmented lesions of the iris (hamartomas), called Lisch nodules (Stumpf *et al.*, 1987; Riccardi and Eichner, 1986). These patients also have a high incidence of pigmented neurofibromas, in which Schwann-like cells contain melanosomes (Morishima *et al.*, 1976). Each of these observations suggest, therefore, that some melanocytes and Schwann cells are derived from a common bipotent progenitor cell type whose fate is influenced by agents that presumably act via mechanisms similar to TPA.

Growth factors may be among the endogenous environmental cues influencing the fate of melanocyte/Schwann cell progenitors

In addition to its effects on melanogenesis in peripheral nerve tissue, TPA has been shown to influence the development of other neural crest subpopulations (Glimelius and Weston, 1981; Sieber-Blum and Sieber, 1981) and to alter the migratory behavior of neural crest-derived cells (Sears and Ciment, 1988). There is strong evidence indicating that TPA is acting on these subpopulations by activating a signal transduction pathway normally utilized by polypeptide growth factors. Some of the activities attributed to TPA

TABLE I-2

Growth factors which influence neural crest cell differentiation, proliferation, and survival

Growth Factor	Neural Crest Derivatives: NEURONS Sensory	NEURONS Cholinergic	NEURONS Sympathetic	Chromaffin Cells	Schwann Cells	Pigment Cells	Connective Tissue	REFERENCES*
NGF	*,+		*,+	-				Bernd, 1988; Black *et al.*, 1990
bFGF	*,+	*	*,+	-	*, x	x	0	Stemple *et al.*, 1988 Kalcheim, 1989 Halaban *et al.*, 1987
EGF			+		0			Herschman *et al.*, 1983
TGF-β					x	-	+	Rogers, *et al.*, 1992 Weston, 1991
PDGF					x	+	+	Weinmaster & Lemke, 1990; Weston, 1991
IGF	+		+					Xue *et al.*, 1988
CNTF	*	*	*,+					Manthorpe *et al.*, 1986; Saadat *et al.*, 1989
BDNF	*	0	0					Kalcheim *et al.*, 1987; Sieber-Blum, 1991
GGF	0	0	0		*, +			Lemke & Brockes, 1984

+ = promotes differentiation; - = prevents differentiation; * = promotes survival; x = promotes proliferation; 0 = demonstrated to have no effect

NGF: nerve growth factor; bFGF: basic fibroblast growth factor; EGF: epidermal growth factor; TGF-β: transforming growth factor-beta; PDGF: platelet-derived growth factor; IGF: insulin-like growth factor; CNTF: ciliary neurotrophic factor; BDNF: brain-derived neurotrophic factor; GGF: glial growth factor.

*See text for further references.

are known, for example, to be the result of its interaction with the calcium-activated, phospholipid-dependent protein kinase C (PKC) family of serine/threonine kinases. The association of several growth factor receptors with their ligands (e.g. EGF, PDGF) leads to the activation of phospholipase Cγ and the subsequent hydrolysis of phosphoinositol-4,5-bisphosphate into inositol triphosphate and diacylglycerol (Cantley *et al.*, 1991). In

cell membranes, small amounts of diacylglycerol then activate PKC by dramatically increasing its affinity for Ca^{2+} and phosphatidylserine (Takai *et al.*, 1979). TPA acts by entering the cell membrane and substituting for diacylglycerol, where it activates PKC at low concentrations, and down-regulates the enzyme at higher concentrations (Nishizuka, 1986). It is possible, therefore, that the effects of TPA on the development of neural crest derivatives reflect the activities of growth factors which the cells encounter during their migration and subsequent differentiation.

As shown in Table I-2, different neural crest-derived subpopulations appear to have specific responses to particular growth factors (for review see Weston, 1991). The expression of many of these growth factors in certain embryonic tissues and interstitial spaces is developmentally regulated, consistent with the notion that they play important roles in neural crest development (e.g. Smith, 1989; Lyons and Hogan, 1990; Wilkinson *et al.*, 1988; Kalcheim and Neufeld, 1990; Nilsen-Hamilton, 1990). Furthermore, many neural crest cells and their derivatives express receptors for these growth factors during the course of their migration and differentiation. NGF, for example, is present in embryos as early as the neural plate stage, and is expressed within the ventral neural crest migratory pathway (Guerra *et al.*, 1989; Mjaatvedt *et al.*, 1991). While early migrating neural crest cells appear to express only low affinity NGF receptors (Bernd, 1985), cells expressing high affinity receptors have been observed during the initial formation of the dorsal root and sympathetic ganglia (Ernfors *et al.*, 1988). These data are consistent with NGF having a role in promoting neuronal commitment in sympathoadrenal precursor cells, as described above.

A limited number of growth factors have been shown to affect both Schwann cells and melanocytes, and could, therefore, influence the fate of melanocyte/Schwann cell progenitors. PDGF, for example, may be involved in melanocyte differentiation (Weston, 1991; Marusich and Weston, 1992), and has been shown to promote Schwann cell survival and proliferation (Weinmaster and Lemke, 1990; Davis and Stroobant,

1990). The evidence supporting a role for PDGF in melanocyte differentiation comes from studies of a mouse mutant, *Patch* (*Ph*), in which pigment cells and other non-neurogenic crest derivatives (cranial ectomesenchyme and septal structures of the cardiac outflow tract) develop abnormally. The mutation appears to be the result of a deletion in the coding region of the α-PDGF receptor (Stephenson *et al.*, 1991), suggesting that PDGF is somehow necessary for normal melanocyte development. The role played by PDGF during melanocyte commitment, however, is unclear, since it does not appear to influence melanocyte growth and differentiation *in vitro* (Halaban, 1988).

Two other growth factors which might affect the fate of melanocyte/Schwann cell progenitors are bFGF and TGF-β. These growth factors have diverse effects on a variety of neural crest derivatives, including melanocytes and Schwann cells (Eccleston *et al.*, 1987; Davis and Stroobant, 1990; Birren and Anderson, 1991; Rogers *et al.*, 1992; Delannet and Duband, 1992; Brill *et al.*, 1992; Shubert, 1992). Furthermore, in certain cell types, bFGF and TGF-β have been shown to have antagonistic interactions with each other (for review see Nilsen-Hamilton, 1990), a characteristic one might expect of growth factors that differentially influence the fate of multipotent or partially restricted sub-populations of cells. The data presented below indicate that bFGF and TGF-β can have such effects on both Schwann cell and melanocyte differentiation, and suggest a means by which the fate of a common, bipotent progenitor of these cells might be influenced.

Transforming Growth Factor-β

The TGF-β family of growth factors influence numerous functions in animal cells from virtually every lineage, including differentiation, adhesion, migration, and extra-cellular matrix production (Nilsen-Hamilton, 1990). Five of these proteins have been cloned and shown to share a relatively high degree of homology (64-82%) at the amino acid level (Roberts and Sporn, 1990). Each TGF-β is synthesized as an inactive pre-cursor, consisting of a latent associated protein and an active mature form which is cleaved from the carboxy terminus of the precursor, yielding biologically active disulfide-

linked homodimers. Mature TGF-βs possess several essential structural features in common, including multiple N-linked glycosylation sites in the precursor peptide, a hydrophobic signal sequence, and conservation of nine cysteine residues.

Several reports have indicated that TGF-β may play a role in early neural crest development. A number of studies have indicated, for example, that TGF-βs are expressed both in neural crest migratory spaces, such as the somitic sclerotome (Heine *et al.*, 1987; Jakowlew *et al.*, 1993), and in primordial neural crest-derived structures, such as peripheral nerves, DRG, and facial mesenchyme (Pelton *et al.*, 1989; Schmid *et al.*, 1991; Millan *et al.*, 1991; Jakowlew *et al.*, 1993). *In vitro*, TGF-β1 and TGF-β2 have been shown to cause premature emigration of neural crest cells from cultured embryonic Japanese quail neural tubes (Delannet and Duband, 1992). TGF-β1 has also been shown to induce fibronectin and integrin expression in neural crest and other cell types (Ignotz and Massague, 1986; Heino *et al.*, 1989; Rogers *et al.*, 1992), and to increase fibronectin-mediated neural crest cell adhesion (Delannet and Duband, 1992). Furthermore, TGF-β1 has been reported to inhibit melanocyte differentiation in avian neural crest cells (Rogers *et al.*, 1992), and to promote neonatal rat Schwann cell proliferation (Eccleston *et al.*, 1989; Schubert, 1992). These data indicate that neural crest cells can respond to TGF-βs, and that different crest-derived subpopulations have distinct responses to this family of growth factors.

Based on binding studies utilizing biotinylated TGF-β1, pre-migratory and migratory neural crest cells appear to express cell surface TGF-β receptors (Delannet and Duband, 1992). The most widely distributed cell surface TGF-β-binding proteins are the type I and type II TGF-β receptors, which bind TGF-β1 and TGF-β3 with high affinities, and TGF-β2 with low affinity. While the primary structure of the type I receptor is unknown, the intracellular portion of the type II receptor includes a serine/threonine kinase domain (Lin *et al.*, 1992; Massagué, 1990). Wrana and co-workers (1992) recently demonstrated that the type II receptor requires both its kinase activity and

association with the type I receptor to elicit its biological activities. Other TGF-β-binding proteins include betaglycan, which is expressed in mesenchymal, neural, and epithelial cells (Wang *et al.*, 1991), and endoglin, which exists as a disulfide-linked homodimer, and is expressed primarily in vascular endothelial cells (Cheifetz *et al.*, 1992). These binding proteins have different affinities for the various TGF-β family members, but neither betaglycan or endoglin possess any obvious signaling motifs, and their functions have not yet been clearly determined.

While the mechanisms by which TGF-β receptors affect cells are largely unknown, there is substantial evidence indicating that some of the actions attributed to TGF-β may result from its influences on the expression and activities of other growth factors (see Nilsen-Hamilton, 1990). The expression of PDGF and interferon-γ, for example, are stimulated in certain cell types by some members of the TGF-β family. TGF-β has also been shown to oppose the activities of certain growth factors, including EGF and bFGF, presumably by interfering with the expression of growth factor receptors or the signal transduction pathways used by these receptors (Assoian, 1985; Massagué, 1985; Roberts *et al*, 1985). It is possible, therefore, that some of the effects of TGF-β on neural crest cells may result from its interference with their responses to other growth factors.

Basic fibroblast growth factor

The FGF family of growth factors is comprised of at least seven distinct, structurally related gene products, each of which are probably derived from a common ancestral gene (see Gospodarowicz, 1990). Basic FGF, one of the best characterized members of this family, is expressed in numerous tissues and acts as a mitogen and differentiation factor for various mesoderm- and neuroectoderm-derived cells, including various neural crest derivatives (for reviews see Haynes, 1988; Rifkin and Moscatelli, 1989; Gospodarowicz, 1990). In addition to promoting neuronal development in sympathoadrenal precursor cells, this growth factor has been shown to be a survival

factor for a subpopulation of non-neuronal crest cells (Kalcheim, 1989), and to promote the differentiation of neurons in early neural crest populations (Brill *et al.*, 1992). Basic FGF is also a potent mitogen for melanocytes (Halaban *et al.*, 1987) and, in certain circumstances, Schwann cells (Krikorian et al., 1982; Eccleston et al., 1987; Ratner et al., 1988; Davis and Stroobant, 1990; Schubert, 1992). Furthermore, Kalcheim and Neufeld (1990) have demonstrated that biologically active bFGF is expressed in the neural tubes of avian embryos both before and during neural crest migration. These studies suggest that bFGF can have a variety of effects on neural crest and neural crest-derived cells at different times during embryonic development.

Multiple molecular weight forms of bFGF are expressed in various mammalian and avian species in a developmentally regulated, tissue specific manner (Brigstock *et al.*, 1990; Sherman *et al.*, 1991; Giordano *et al.*, 1992). In humans, for example, four forms of bFGF with molecular weights of 18, 22, 22.5 and 24 kDa are known to be expressed, and all are derived from a single messenger RNA. Translation of the 18 kDa form

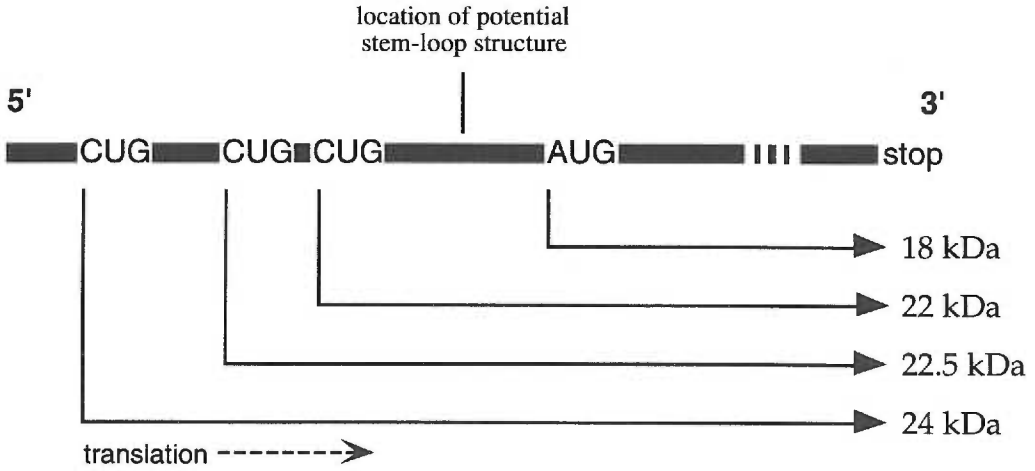

Figure I-4: A representation of how multiple molecular weight forms of bFGF are translated from a single mRNA transcript. In humans, three in-frame CUG codons 5' of the AUG translational start site can initiate translation of high molecular weight protein species of bFGF. A predicted stem-loop structure between the three CUGs and the AUG may be involved in post-transcriptional regulation of the expression of these bFGF forms.

initiates from an AUG codon, while the higher molecular weight forms initiate from CUG codons 5' of the AUG codon (Florkiewicz and Sommer, 1989; Prats *et al*, 1989; see Fig. I-4). Although the function of such amino terminal extended forms of bFGF is unclear, they have been observed exclusively in cell nuclei, and selective expression of each form appears to have distinct effects of cell behavior (Renko *et al.*, 1990; Bugler *et al.*, 1991; Florkiewicz *et al.*, 1991; Couderc *et al.*, 1991; Quarto *et al.*, 1991a). The translocation of these bFGF forms to the nucleus appears, moreover, to be directed by a nuclear localization sequence (Quarto *et al.*, 1991b).

The mechanisms governing the regulation of bFGF gene expression are also poorly understood. The human bFGF gene, however, contains a potential TPA-responsive promoter element (TRE) located 243 bases 5' of the ATG translational start site (Shibata *et al.*, 1991). This consensus core DNA sequence (TGA[C/G]TCA) is the binding site of the nuclear transcription factors *fos* and *jun* (Lee *et al.*, 1987; Angel *et al.*, 1987), whose expression is induced by numerous growth factors and other agents (Kerr *et al.*, 1992). Evidence suggesting that the putative TRE in the bFGF gene is functional include various studies demonstrating that TPA can influence bFGF expression. Up to ten-fold increases in bFGF mRNA levels have been observed, for example, in several cell types, between 3 and 6 hours after treatment with concentrations of TPA that either activate or down-regulate PKC (Murphy *et al.*, 1988a; Bikfalvi *et al.*, 1990; Weich *et al.*, 1991; Lowe *et al.*, 1992). In one study, this increased bFGF mRNA expression persisted for 12 hours before returning to basal levels (Murphy *et al.*, 1988a). It is possible, therefore, that some of the effects of TPA on neural crest-derived cells may be the result of increases in bFGF expression.

There are four known high affinity FGF receptor genes, designated R1-R4, each of which appear to have unique patterns of expression during embryonic development (Houssaint *et al.*, 1990; Johnson *et al.*, 1990; Keegan *et al.*, 1991; Partanen *et al.*, 1991; Patstone *et al.*, 1993). The extracelluar region of these receptors consist of three

immunoglobulin-like domains, the first two of which are separated by a short, highly acidic sequence. Following the transmembrane domain is a long juxtamembrane sequence, which precedes a tyrosine kinase domain that is interrupted by a 14-amino acid insert. The carboxy terminus, the first immunoglobulin domain, the juxtamembrane domain, and the kinase insert are not well conserved among these different receptors, and presumably contribute to differences in binding affinities for the various FGF family members (Pasquale, 1990). Alternatively spliced forms of FGF-R1 and FGF-R2 have also been found which vary in these domains, and which have varying affinities for different FGFs. Basic FGF also binds to heparan sulfate proteoglycans that function as low affinity receptors, which, along with bFGF and the high affinity receptors, form a complex that is required for bFGF activity (Klagsburn and Baird, 1991). While the role played by each of these receptors in neural crest development is unclear, some migrating crest cells transiently express FGF-R1 mRNA (Heuer *et al.*, 1990), the protein product of which binds bFGF, acidic FGF (aFGF) and, in one splice variant, Kaposi-FGF (K-FGF) with similar affinities (Mansukhani *et al.*, 1990).

Although most FGF receptors bind bFGF with high affinities, bFGF lacks a hydrophobic secretory signal sequence (Abraham *et al.*, 1986), and it is unclear how and if it is secreted by some cells. In fact, only a few cell types have been shown to secrete bFGF, presumably by means of a secretory pathway independent of the endoplasmic reticulum-Golgi complex (Vlodavsky *et al.*, 1987; Kandel *et al.*, 1991; Mignatti and Rifkin, 1991; Mignatti *et al.*, 1992; Shain *et al.*, 1992). It has been suggested, therefore, that bFGF may act in some instances via an "intracrine" pathway, serving as an intracellular messenger without being secreted or interacting with extracellular receptors (for review see Logan, 1990). In support of this supposition, bFGF has been localized in the nucleus, nucleolus and cytosol of cells both *in vitro* and *in vivo* (Kardami and Fandrich, 1989; Joseph-Silverstein *et al.*, 1989; Renko *et al.*, 1990; Kalcheim and Neufeld, 1990; Tessler and Neufeld, 1990; Yamamoto *et al.*, 1991; Woodward *et al.*,

1992). Furthermore, various cells transfected with bFGF-expression constructs have demonstrated increased growth characteristics even though little or no bFGF was detected in their conditioned media (Neufeld *et al.*, 1988; Sasada *et al.*, 1988; Halaban *et al.*, 1988). The transformed state of some of these transfected cells was not affected by bFGF-neutralizing antibodies, indicating that any bFGF that was released into their conditioned medium was not required for these cells to proliferate (Neufeld *et al.*, 1988; Halaban *et al.*, 1988).

Additional support for the notion that bFGF can act via an intracrine pathway comes from studies suggesting that intracellular bFGF directly influences gene transcription. In isolated nuclei from bovine aortic endothelial cells, for example, bFGF was shown to stimulate transcription of ribosomal genes and to increase RNA polymerase I activity (Bouche *et al.*, 1987). In nuclear extracts from Ehrlich ascites tumor cells, bFGF influenced the transcription of genes encoding isozymes of phosphoglycerate kinase, which are involved in a transcriptional switch during spermatogenesis from somatic-type to sperm-specific forms of the enzyme (Nakanishi *et al.*, 1992). Furthermore, bFGF, which has been implicated in mesoderm induction (Kimelman and Kirschner, 1987), was recently shown to translocate from the cytosol of cleavage and blastula stage cells of *Xenopus* embryos to cell nuclei during mid-blastula transition (Shiurba *et al.*, 1991). These data suggest that some of the biological activities of bFGF may be mediated by nuclear binding proteins or by the direct interaction of bFGF with DNA, and that there are multiple mechanisms by which bFGF influences cell growth and development.

Antisense deoxyoligonucleotides and neutralizing antibodies can be used to study the mode of growth factor actions

To understand the roles of particular growth factors in the regulation of biological processes, one must be able to interfere specifically with either their expression or their function. Two powerful tools that have been utilized to achieve these goals are neutral-

izing antibodies that selectively bind to particular growth factors, and antisense oligonucleotides, which can prevent growth factor and growth factor-receptor synthesis (for reviews see Pestka, 1992; Morrison *et al.*, 1993). Most neutralizing antibodies act by preventing growth factors from interacting with their receptors either by blocking the receptor-binding site on the ligand, blocking the ligand-binding site on the receptor, or somehow changing the conformation of the ligand or the receptor (e.g. Schweigerer *et al.*, 1987; Sasada *et al.*, 1988). Such antibodies are highly effective at inhibiting the activities of exogenous and paracrine growth factors. Autocrine growth factors, however, may associate with their receptors as soon as they reach the cell surface, or may already be associated with receptors before their secretion. Neutralizing antibodies could, therefore, be less effective at blocking autocrine growth factor activity (e.g. Neufeld *et al.*, 1988; Fleming *et al.*, 1989). To get around this problem, antibodies have been injected into cells with some moderate success (e.g. Halaban *et al.*, 1988). A limitation of this approach, however, is that intracellular proteins may not possess the same epitopes as their extracellular counterparts due to their conformations and associations with other intracellular proteins.

In contrast to neutralizing antibodies, antisense oligonucleotides act by preventing the translation of targeted gene products. These agents are taken up by cells via pinocytosis, then hybridize with cellular RNA. This hybridization, in turn, has been shown in some cases to result in the cleavage of the RNA portion of these DNA/RNA hybrids by RNaseH (Toulmé and Hélène, 1988). While the variables influencing the effectiveness of antisense oligonucleotides are still not entirely clear, a number of studies have indicated that there are certain common features among successful antisense sequences (Scherczinger *et al.*, 1992). The most effective antisense oligonucleotides, for example, are typically those targeted to either regions immediately upstream of and containing the AUG translation initiation codon, or regions incorporating intron sequences, especially splice donor or acceptor sites. In addition, oligonucleotide lengths

between 15-20 bases tend to produce the most consistent results, and certain modifications of oligonucleotide backbones, including phosphorothioate analogs, have been shown to decrease the degradation of these antisense molecules by nucleases.

A final important variable to consider in designing antisense oligonucleotides that influence growth factor activities is the uniqueness of the targeted sequences. Selective inhibition of growth factor expression has been achieved, for example, when the targeted portions of the gene of interest were not common or similar to sequences of other genes, especially genes belonging to the same family. Controls for specificity are, therefore, important, and typically include testing the effects of sense and scrambled antisense oligonucleotides, to control for non-specific effects of molecules with a similar base composition (Morrison *et al.*, 1993).

The chapters that follow describe studies where these and related techniques were used to evaluate a number of growth factors for their abilities to influence the transdifferentiation of Schwann cell precursors from early avian embryonic peripheral nerve tissue into melanocytes. These studies demonstrate that TGF-β1 and bFGF have opposing effects on this phenomenon, and that bFGF acts in these neural crest-derived cells via an intracrine mechanism. The examination of such developmental switches and the environmental cues that regulate them help us to understand how cell type diversity is established during embryogenesis.

CHAPTER II
Materials and methods

Embryos

Fertile quail (*Coturnix coturnix japonica*) and White Leghorn chicken (*Gallus domesticus)* eggs were obtained from the Poultry Sciences Department at Oregon State University (Corvalis, OR). Eggs were incubated in a humidified incubator at 38°C, and the embryos were staged using the criteria of Hamburger and Hamilton (1951).

Reagents

Recombinant human bFGF (rh-bFGF) and NGF were obtained from Collaborative Research (Lexington, MA). Recombinant human aFGF (Merck, Rahway, NJ), PDGF (Zymogenetics, Seattle, WA), and TGF-α (Genentech, San Francisco, CA) were the generous gifts of Drs. Kenneth Thomas, Felix Eckenstein, and Rik Derynck, respectively. TGF-β1, purified from human blood platelets, and EGF were the generous gifts of Dr. Bruce Magun. Insulin, transferrin, selenium, α-MSH, l-glutamine, penicillin, streptomycin, CHAPS, inositolhexakisphosphate ($InsP_6$), and TPA were all purchased from Sigma Chemical Co. (St. Louis, MO).

Antibodies

Rabbit anti-human placental bFGF antiserum was purchased from American Diagnostica Inc. (Greenwich, CT). This antiserum has been shown to neutralize bFGF activity in several assay systems (Sato and Rifkin, 1988; Broadley *et al.,* 1989; Mignatti *et al.,* 1989), and to cross-react with murine, bovine, and chick homologues of bFGF, but not aFGF, at the concentrations used in these studies. Anti-human bFGF monoclonal antibodies DE6 (Reilly *et al.*, 1989), anti-human bFGF monoclonal antibody 148.6.1.1,

and anti-P_0 monoclonal antibody 1E8 (Bhattacharyya *et al.*, 1991), were the generous gifts of Drs. Janet Gross (E. I. Dupont), Charles Hart (Zymogenetics), and Eric Frank (Univ. Pittsburgh), respectively. Conditioned medium containing the neural crest-specific monoclonal antibody, HNK-1 (Bronner-Fraser, 1986), was harvested from HNK-1 hybridoma cells which were obtained from the American Type Culture Collection.

Oligonucleotides

Oligonucleotides corresponding to codons spanning the human bFGF translation initiation site (ATG) and the first splice-donor acceptor site (codon 58), were synthesized by Research Genetics (Huntsville, AL). Oligonucleotides (Table I) were solubilized in sterile isotonic phosphate-buffered saline (PBS) and added directly to peripheral nerve explants soon after dissection. Phosphorothioate-modified oligonucleotides were used at a final concentration of 10 μM, while unmodified oligonucleotides were used at 50 μM, as described previously (Morrison, 1991).

TABLE II-1

Location and structure of bFGF oligonucleotides

Sequence Name	Oligonucleotide	Location	Sequence
AS1	**Antisense**	**Start site**	**5'-GGC-TGC-CAT-GGT-CCC-3'**
PAS1	**Modified Antisense**	**Start site**	**5'-GGC-TGC-CAT-GGT-CCC-3'**
AS2	**Antisense**	**Codons 58-62**	**5'-TAG-CTT-GAT-GTG-AGG-3'**
S2	**Sense**	**Codons 58-62**	**5'-CCT-CAC-ATC-AAG-CTA-3'**
SAS2	**Scrambled Antisense**	**Codons 58-62**	**5'-ATC-GGG-TTG-TGG-TAA-3'**

There are two introns that interrupt the human bFGF coding sequence (Abraham *et al.*, 1986). The sense and antisense oligonucleotides corresponding to codons 58-62 span the first splice-donor acceptor site of the first of these introns. The start site refers to the translation initiation (ATG) site.

Tissue Culture

Peripheral nerves or DRG were dissected from the brachial, thoracic, and lumbosacral levels of quail embryos, cut into halves or fourths, and cultured as explants in either 96-well or 60 mm Primaria plates (Falcon Plastics, Ventura, CA) in a humidified 5% CO_2-95% air atmosphere at 37°C. Tissue explants were used because previous studies had shown that explant cultures produced more reproducible pigmentation than did dissociated cell cultures of DRG and peripheral nerves (unpublished observations). Complete medium ("CM") consisted of HEPES-buffered Ham's F-12 medium (Sigma, St. Louis, MO) supplemented with 0.03% l-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 20% fetal bovine serum (Hyclone, Logan, UT). The cultures described in Chapter III also contained 10% chick embryo extract (CEE) prepared by the method of Loring and co-workers (1982). For the defined medium ("DM"), the embryo extract and serum were replaced with 1 mg/ml bovine serum albumin, 5 ng/ml selenium, 1 µg/ml transferrin, 1 µg/ml insulin, and 100 ng/ml α-MSH. Media were further supplemented with various combinations of growth factors, oligonucleotides, and TPA (made from a 1000x stock solution in ethanol). Culture medium without TPA contained 0.1% ethanol as a vehicle control. Half of the medium was replaced with fresh medium and supplements (except oligonucleotides) after 5 days, unless otherwise indicated.

Assay for melanogenesis

Live cultures were examined at various times for the presence of pigmented cells, using a Nikon TMS inverted microscope with bright-field illumination. Wells were scored as positive when they contained one or more pigmented cells. Care was taken to distinguish melanocyte-like cells (cells with typically dendritic morphology containing dark, ovoid melanosomes) from brownish-yellow degenerating cells, containing irregularly-shaped granulations. "Percent pigmentation" was calculated by dividing the number of positive wells by the total number of wells containing viable DRG or PN explants. Each data point represents the data from 48 wells, unless otherwise noted.

Statistical analysis involved the Newman Keuls multi-comparison test, with $p < 0.05$ defined as statistically significant.

Heterospecific grafting

Peripheral nerves were dissected from 7 day quail embryos as described above and cultured in 35 mm Primaria plates for 4 days in CM supplemented with 10% CEE, in the presence and absence of either 10 ng/ml rh-bFGF or 0.1 μM TPA. Monolayers arising from individual explants were then carefully scraped from culture dishes using a fine tungsten needle, then folded into a ball. The folded monolayers were then washed in HBSS, and grafted into the base of the right wing bud of 4-day White Leghorn chicken embryos *in ovo* as previously described (Ciment and Weston, 1983). The host embryos were allowed to develop for an additional 10 days, then were examined for pigmented feather primordia.

Preparation of cell lysates and conditioned media

Cultures of 80-100 peripheral nerve explants were washed 3 times in ice-cold PBS, then scraped from dishes in 50 μl of 20 mM Tris-HCl pH 7.0 (4° C) containing 2.0 M NaCl, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.2 mM phenylmethylsulfonyl fluoride, and 100 μg/ml of leupeptin, pepstatin, and aprotonin (Boehringer-Mannheim, Indianapolis, IN). The cell suspension was then sonicated for 1 min, clarified by centrifugation at 14,000 x *g* for 2 hours at 4° C, and stored at -80° C. In some experiments, cell lysates and conditioned media were applied to heparin-Affigel columns (Biorad, Richmond, CA). The columns were washed with 0.6 M NaCl, and heparin-bound proteins were eluted with 2.0 M NaCl. Samples were then concentrated approximately 100-fold in a speed vacuum apparatus, and dialyzed against 20 mM Tris-HCl (pH 7.0) for 16 hours. Protein concentrations in cell lysates and column eluates were determined using the method of Bradford (1976).

Gel electrophoresis and bFGF protein immunoblots

Protein bands were resolved by SDS-polyacrylamide (15%) gel electrophoresis using standard methods (Laemmli, 1970). Proteins were then transferred to nitrocellulose by electroblotting, and non-specific binding sites were blocked by treatment in 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20, 1% bovine serum albumin and 10% normal horse serum (TBST) for 30 minutes at room temperature. Blots were then incubated for 1 hour at room temperature in TBST with either a 1:5000 dilution of the anti-bFGF monoclonal antibody DE6 or a 1:2500 dilution of the anti-bFGF monoclonal antibody 148.6.1.1. Blots were then washed three times for 5 minutes each with TBST, incubated for 30 minutes with a 1:2500 dilution of a biotinylated horse anti-mouse immunoglobulin antibody (Vector Laboratories, Burlingame, CA) in TBST, washed again, and then incubated for 30 minutes in strepavidin-HRP. Immunoreactive bands were visualized using an ECL enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). Negative controls involved the exclusion of primary antibodies or preabsorbing anti-bFGF antibodies with excess rh-bFGF prior to the incubation steps. The molecular weights of immunoreactive bands were determined by comparison with pre-stained "low molecular weight" standards (Biorad).

Immunostaining

Peripheral nerve explant cultures were first washed with PBS, fixed in ice cold 90% ethanol:5% glacial acetic acid for 30 minutes, and then washed again with PBS. To block non-specific immunostaining, cultures were incubated at room temperature for 1 hour in 10% serum in "Buffer A," consisting of 100 mM Tris buffer (pH 7.8), containing 150 mM NaCl, 0.5% Triton X-100, 0.05% sodium azide. HNK-1 immunostaining was performed by incubating cultures overnight at 4^{o} C with HNK-1 conditioned medium diluted 1:20 in Buffer A, followed by secondary incubation with fluorescein isothiocyanate-labeled rabbit anti-mouse IgM antisera (1:200 dilution; Cappel, Durham, NC). Fluorescently labeled cultures were then mounted in a 1:1 solution of PBS and glycerol

containing 1 μg/ml p-phenylenediamine. Basic FGF and P_0 immunostaining was performed by incubating cultures overnight at 4° C with either a 1:150 dilution of DE6 or a 1:250 dilution of anti-P_0 antibody 1E8 in Buffer A, and then processing these cultures using a Vectastain kit (Vector Laboratories), following the manufacturer's instructions. Negative control experiments involved either leaving out primary antibody or, in the case of bFGF immunostaining, DE6 was pre-adsorbed with 1 μg/ml of rh-bFGF.

Bromodeoxyuridine Assay

The effects of TPA and bFGF on peripheral nerve cell proliferation were determined using a bromodeoxyuridine (BrdU) cell proliferation kit from Amersham. Briefly, individual peripheral nerve explants were cultured for 47 hours in the presence or absence of either TPA or bFGF. These cultures were then incubated at 37 °C for 1 hour with a 10:1 mixture of 5-bromo-2'-deoxyuridine and 5-fluoro-2'-deoxyuridine (a thymidilate synthetase inhibitor) prior to fixation in 90% ethanol (0° C):5% glacial acetic acid for 30 minutes. Immunostaining for BrdU incorporated into nuclei was performed according to the manufacturer's instructions using cobalt and nickel to intensify the diaminobenzidine reaction product. Cells were then lightly counterstained with 0.1% light-green yellowish for 5 min, and examined under a Nikon TMS inverted microscope with bright-field illumination. The percentage of $BrdU^+$ peripheral nerve cells was determined by dividing the number of darkly stained nuclei by the total number of cells in each culture.

Photomicroscopy

Photomicrographs of live cultures were taken with a Zeiss IM-35 inverted microscope, with bright-field illumination using Kodak Plus-X panchromatic black-and-white film. All other photomicrographs were taken with a Zeiss axioplan microscope equipped with an Olympus photomicroscopy system.

CHAPTER III

Basic FGF and TGF-β1 influence commitment to melanogenesis in neural crest-derived cells of avian embryos[1]

In previous studies, the phorbol ester TPA was shown to induce adventitious pigmentation in a variety of neural crest-derived tissues (Ciment *et al.*, 1986; Sears and Ciment, 1988). That is, TPA induced the appearance of pigmented cells in neural crest derivatives which normally do not give rise to melanocytes. The aims of the studies described in this chapter are to determine whether various peptide growth factors might mimic this effect of TPA on neural crest-derived cells, by screening a number of growth factors for their ability to influence melanogenesis in embryonic quail DRG and peripheral nerves.

Effects of various growth factors on adventitious pigmentation in embryonic quail DRG cultures

DRG from 7 day (stage 32-33) quail embryos were dissected, cultured as explants in CM supplemented with 3.0 nM TPA in the presence or absence of growth factors, and then examined over the course of 10 days for the presence of pigmented cells. This low concentration of TPA was used because it caused an intermediate level of pigmentation, which allowed for the screening of both inhibitory as well as stimulatory effects of the growth factors tested. These growth factors included basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF) (in the presence or absence of 10 mg/ml of heparin), epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), transforming growth factor-beta (TGF-β1), platelet-derived growth factor (PDGF) and

[1] Some of the data presented in this chapter appear in Stocker *et al.*, 1991.

nerve growth factor (NGF). These growth factors were tested, moreover, at various concentrations. Fig. III-1A contains data from a representative experiment and shows that bFGF, but not various other growth factors, augmented the proportion of cultures containing pigmented cells, as compared to cultures treated with TPA alone (broken line). This augmented TPA-induced pigmentation was characterized by both an increased rate of pigmentation, as well as an increase in the final percentage of pigmented cultures.

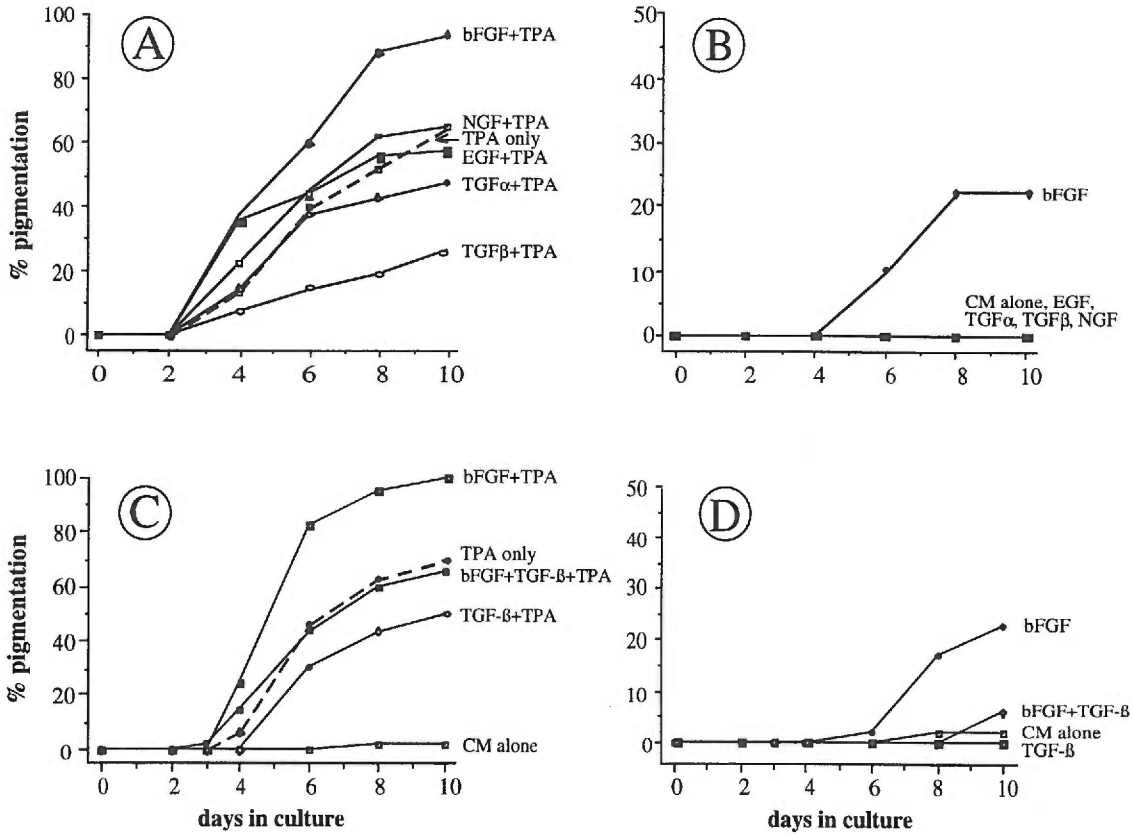

Figure III-1: Effects of growth factors on adventitious pigmentation of E7 DRG cells. (A) DRG explants from stage 32-33 quail embryos were cultured in CM with 3.0 nM TPA and 10 ng/ml of either rh-bFGF, NGF, EGF, TGF-β or TGF-α, and then the cultures were assayed for pigment cells. Note that only bFGF augmented TPA-induced pigmentation. Note also that TGF-β1 inhibited this pigmentation. (B) DRG explants were cultured in the presence of each of the growth factors mentioned in A, but without TPA. Note that only bFGF induced adventitious pigmentation. (C) DRG explants were cultured in CM with 3.0 nM TPA and either 10 ng/ml bFGF, 10 ng/ml TGF-β, or both bFGF and TGF-β1. Note that TGF-β1 blocked bFGF-induced pigmentation. (D) DRG explants were cultured in CM without TPA, but supplemented with either bFGF, TGF-β1, or both bFGF and TGF-β1. Note that TGF-β1 reduced the bFGF-induced adventitious pigmentation in the absence of TPA.

This experiment was performed three times, and although the absolute values varied between experiments, the same qualitative differences were consistently observed.

To determine whether bFGF or other growth factors could influence pigmentation in the absence of TPA, DRG explants were also cultured in medium supplemented only with the growth factors mentioned above. Fig. III-1B shows that bFGF induced pigmentation in 23% of these cultures, but that none of the other growth factors tested induced any pigmentation. The effects of bFGF and TPA appeared to be additive, rather than synergistic, since the sum of pigmented cultures treated with either bFGF alone (panel B) or TPA alone (panel A) did not exceed the number of pigmented cultures treated simultaneously with both bFGF and TPA (panel A).

In contrast to the stimulatory effects of bFGF, TGF-β1 was found to antagonize the TPA-induced pigmentation in these experiments. Fig. III-1A shows that addition of TGF-β1 decreased the extent of pigmentation in TPA-supplemented medium from 67% to 23% of the cultures after 10 days in culture. This inhibition by TGF-β1 was found, moreover, to be dose-dependent, with 10.0 ng/ml TGF-β1 producing a greater extent of inhibition than 1.0 ng/ml (data not shown). To determine whether the stimulatory and inhibitory effects of TGF-β1 and bFGF were subtractive, DRG explants were cultured in the presence of either bFGF alone, TGF-β1 alone, or with both TGF-β1 and bFGF. Fig. III-1D shows that TGF-β1 inhibited the bFGF-induced pigmentation, reducing the proportion of pigmented cultures at day 10 from 27% to 6%. In the presence of TPA, TGF-β1 was also found to antagonize the stimulatory effects of bFGF on E7 DRG cells, decreasing the proportion of pigmented cultures to that of TPA alone (Fig. III-1C). These experiments were performed four times with similar results.

Age-dependent effects of bFGF and TGF-β1 on melanogenesis in DRG cultures

The ability of TPA to induce adventitious pigmentation of DRG cells was previously shown to be age-dependent (Ciment *et al.*, 1986). To determine whether the

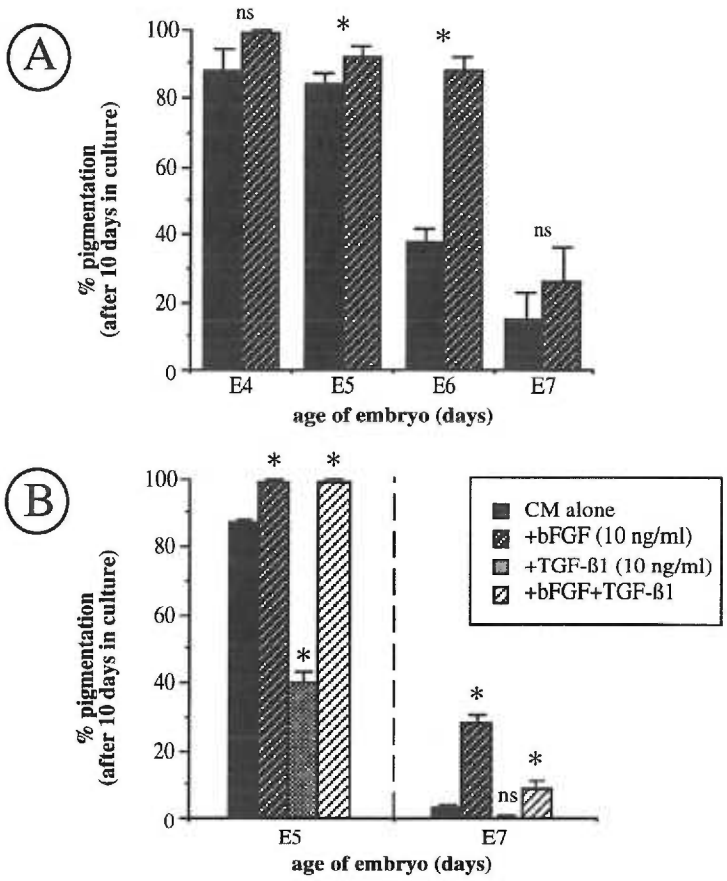

Figure III-2: Age-dependent differences in the abilities of bFGF and TGF-β1 to induce pigmentation in DRG cells. **(A)** DRG explants from E4 (stage 23-24), E5 (stage 26-27), E6 (stage 29-30), and E7 (stage 32-33) quail embryos were cultured for 10 days in CM in the presence or absence of 10 ng/ml bFGF, and then the proportion of pigmented cultures was determined and statistical analysis performed. Data represent the means ± standard errors of the mean for three experiments. Note that there were statistically significant differences in the extent of pigmentation between CM- and CM/bFGF-treated cultures from E5 and E6 embryos. **(B)** DRG explants from E5 and E7 quail embryos were cultured for 10 days in CM or in CM supplemented with either 10 ng/ml bFGF, 10 ng/ml TGF-β1, or both bFGF and TGF-β1, and then the proportion of pigmented cultures was determined and statistical analysis performed. In E5 cultures, note that bFGF-, TGFβ1-, and bFGF/TGFβ1-treated cultures all differed significantly from CM-only cultures. In E7 cultures, note that bFGF- and bFGF/TGFβ1-treated cultures differed significantly from cultures grown in CM alone, and that bFGF- and bFGF/TGFβ1-treated cultures differed significantly from one another. n.s. = not significant; * = $p < 0.05$.

bFGF-induced pigmentation showed a similar age-dependency, DRG from E4 (stage 23-24), E5 (stage 26-27), E6 (stage 29-30), and E7 (stage 32-33) quail embryos were cultured in CM in the presence or absence of bFGF. Fig. III-2A shows that bFGF

extended the developmental stages at which adventitious pigmentation occurred in DRG explants. In one experiment, for example, the addition of exogenous bFGF to the culture medium of E6 DRG explants increased the proportion of pigmented cultures from 32% to 77%. It should be noted that during normal quail development, pigmentation of the skin begins around embryonic day 7 (Padgett and Ivey, 1960), suggesting that the loss of bFGF-responsiveness in these neural crest-derived cells occurs at roughly the same time that pigmentation normally begins *in vivo*. This experiment was performed three times with similar results.

The ability of TGF-β1 to inhibit pigmentation in the absence of TPA was also found to be age-dependent. Fig. III-2B compares the effects of bFGF and TGF-β1 on E5 and E7 quail DRG explants, and shows that TGF-β1 had no significant effect on bFGF-induced pigmentation in E5 embryos (i.e., compare light striped with dark striped bars), but inhibited such pigmentation in E7 DRG explants. This figure also shows that TGF-β1 inhibited the spontaneous pigmentation seen in E5 DRG cultured in the absence of bFGF (i.e., compare black with stippled bars). This experiment was performed three times with similar results. These data indicate that the growth factor-responsiveness of these neural crest-derived cells may change with developmental age.

DRG cultured in defined medium

To determine whether these effects of bFGF and TPA depended on other, unknown factors present in CM, experiments were also performed using a serum-free and embryo extract-free defined medium ("DM"), consisting of F-12 medium supplemented with insulin, transferrin, α-MSH, selenium and bovine serum albumin. DRG explants from 7-day embryonic DRG were cultured in DM, therefore, in the presence of either TPA alone, bFGF alone (at concentrations ranging from 1 to 50 ng/ml), or a combination of these two agents, and then the proportion of pigmented cultures was determined over the course of 10 days. In these experiments, TPA was used at 100 nM, which was determined to be the

concentration which elicited optimal pigmentation in this medium (data not shown). Fig. III-3 shows that neither 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 by day 10, and cultures treated with both bFGF (50 ng/ml) and TPA induced even higher levels of pigmentation. The ability of bFGF to stimulate pigmentation in these cultures was dose-dependent, moreover, with 1 ng/ml inducing pigmentation in 50% of the cultures and 50 ng/ml inducing pigmentation in 86% of the cultures. Note that the onset of pigmentation in these DM cultures is delayed compared to CM cultures (see Fig. III-1A). Similar results were obtained in three separate trials of this experiment. These results suggest that bFGF requires additional factors to induce pigmentation under these conditions.

Bright-field photomicrographs from these studies (Fig. III-4) demonstrate that bFGF may also influence the survival of non-neuronal cells in these cultures. Cultures in DM without bFGF, for example, often contained cells which were poorly attached to the

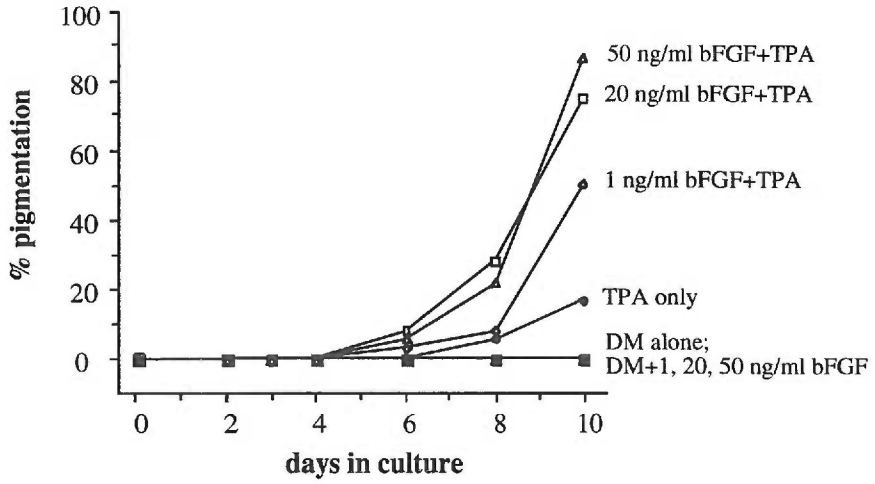

Figure III-3: Basic FGF- and TPA-induced pigmentation of E7 DRG cells cultured in defined medium. DRG explants from E7 quail embryos were cultured in defined medium (DM) supplemented with TPA and/or bFGF at various concentrations, and then cultures were examined at various times and the proportion of pigmented cultures determined. Note that bFGF alone did not induce pigmentation; that TPA alone induced pigmentation in only 17% of the cultures; but that bFGF acted synergistically with TPA to induce pigmentation.

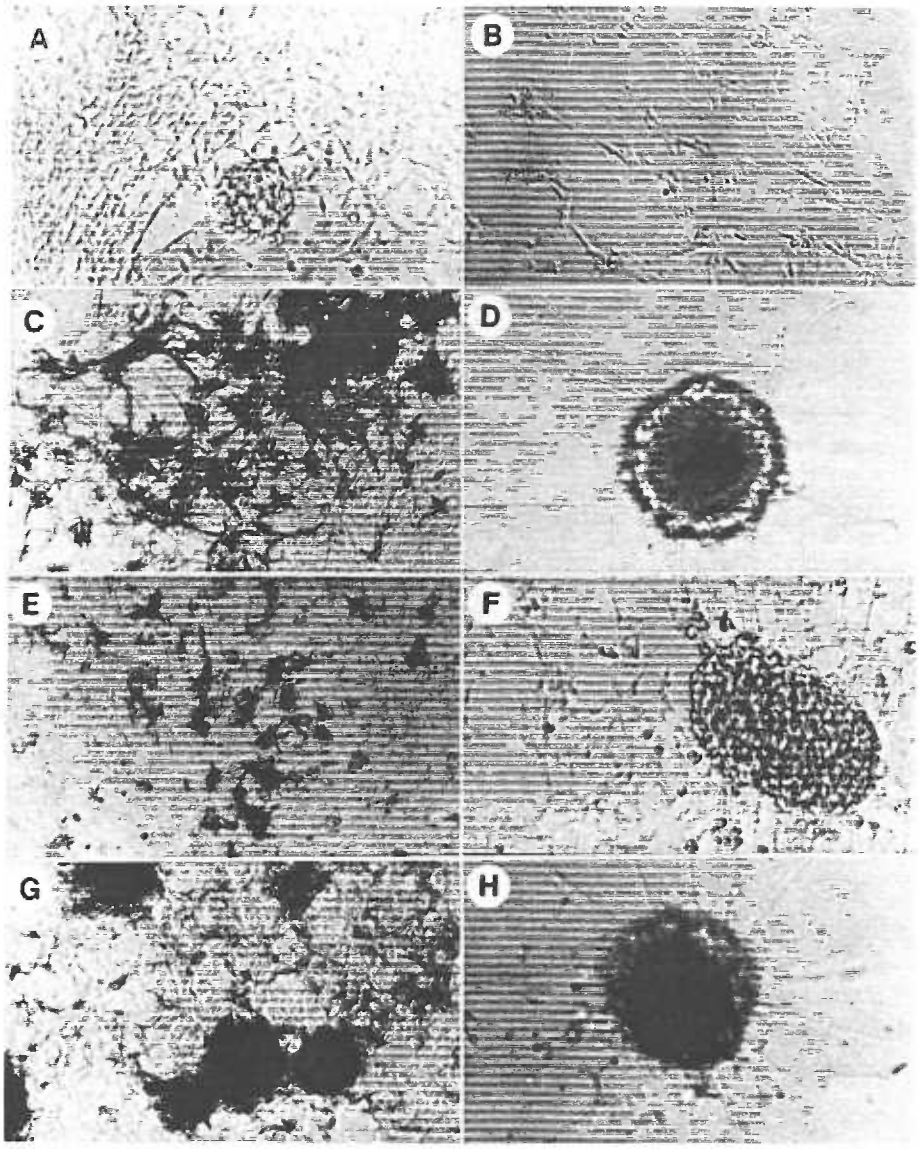

Figure III-4: Bright-field photomicrographs of E7 DRG explants in CM or DM supplemented with bFGF and/or TPA. DRG explants from E7 quail embryos were cultured in either: (**A**) CM alone; (**B**) DM alone; (**C**) CM in the presence of 3.0 nM TPA; (**D**) DM in the presence of 0.1 μM TPA; (**E**) CM supplemented with 10 ng/ml bFGF; (**F**) DM supplemented with 10 ng/ml bFGF; (**G**) CM containing both 3.0 nM TPA and 10 ng/ml bFGF; or (**H**) DM containing both 0.1 μM TPA and 10 ng/ml bFGF. Photographs were taken on the sixth day in culture. Note that in the absence of bFGF (i.e., panels B and D), viability of non-neuronal cells in the DM cultures was generally poor.

substratum and contained vacuoles, suggesting that bFGF is required for the optimal health of these cultures (compare panel B with panel A). In the presence of TPA, DRG cultures in DM still contained a large proportion of non-attached or vacuolated cells (compare panels C and D), although the presence of TPA caused the appearance of many more neurites (compare panel D with panel B). This would suggest that TPA cannot substitute for bFGF in these culture conditions. When DRG were cultured in DM supplemented with bFGF, the cells appeared healthy, and both neurites as well as numerous non-neuronal cells could be seen (compare panel B with panel F). In the presence of both bFGF and TPA, many of these DM cultures also contained pigmented cells (panel H).

In contrast to these studies with DM, DRG cultured in CM plus TPA and/or bFGF contained large numbers of non-neuronal cells and pigmented cells (compare panel A with panels C,E,G). No significant cell death was observed, moreover, in explants cultured in CM alone (compare panel A with panel B). These results suggest, therefore, that bFGF may also promote the survival of non-neuronal cells in DM, but requires the presence of some unknown factor in either serum or chick embryo extract (which can be replaced by TPA) to induce adventitious pigmentation.

The effects of TGF-β1 on DRG explant pigmentation were also tested in DM. In three experiments, however, E7 DRG consistently demonstrated poor cell viability when cultured in DM supplemented with TGF-β1 (at either 1 or 10 ng/ml) both in the presence and absence of bFGF (10 ng/ml) and/or TPA (100 nM), making conclusions about the effects of TGF-β1 on pigmentation in DM difficult.

Effects of basic FGF-neutralizing antibodies on adventitious pigmentation in DRG explants

Since E5 DRG cultures underwent pigmentation in the absence of exogenously-added bFGF, it is possible that CM contains a low level of bFGF, presumably from the

chick embryo extract (e.g., Seed *et al.*, 1988; Kimura *et al.*, 1989). To determine whether this bFGF may have been responsible for pigmentation of some DRG cells from younger embryos, studies were performed utilizing rabbit anti-human bFGF neutralizing antiserum. DRG from 5- and 7-day quail embryos were cultured in the presence or absence of this neutralizing antiserum in either CM, CM plus TPA, or CM plus bFGF. Fig. III-5 shows that E5 DRG explants underwent adventitious pigmentation in CM alone, but that a 1:500 dilution of bFGF-neutralizing antibodies blocked this pigmentation (panel A). In the presence of antiserum and exogenous bFGF (10 ng/ml), however, the proportion of pigmented wells at 10 days increased from 0% to 100% of the cultures (compare panels A and E). When bFGF was added to CM containing a 1:250 dilution of bFGF-neutralizing antiserum, no pigmentation was observed after 10 days in culture (panel G). Addition of excess rh-bFGF (50 ng/ml) to cultures treated with the 1:250 dilution of neutralizing antibodies overcame this inhibition (data not shown). It should be noted, moreover, that E5 cultures containing neutralizing antiserum demonstrated poor cell viability, with most of the surviving cells having fibroblast-like or neuronal morphologies (data not shown). These data indicate, therefore, that bFGF or a closely-related agent present in CM is required for melanogenesis to occur in these cultures, and further support the notion that bFGF can support the survival of non-neuronal DRG cells.

The bFGF neutralizing antiserum also inhibited pigmentation in E7 DRG cultures. As with the E5 explants, the addition of bFGF (10 ng/ml) to E7 DRG blocked the effects of the neutralizing antiserum diluted 1:500 (panel F), but failed to block bFGF-neutralizing activity at a higher concentration of the antiserum (panel H). In contrast to E5 DRG cultures, however, the cell viability in E7 cultures appeared unaffected by the antiserum, suggesting that while bFGF may act as a survival factor for some early (i.e., E5), DRG cells, it is not required for the survival of cells in older DRG cultures.

Interestingly, the bFGF-neutralizing antibody also decreased TPA-induced pigmentation in DRG cultures. With E5 DRG, for example, the proportion of pigmented

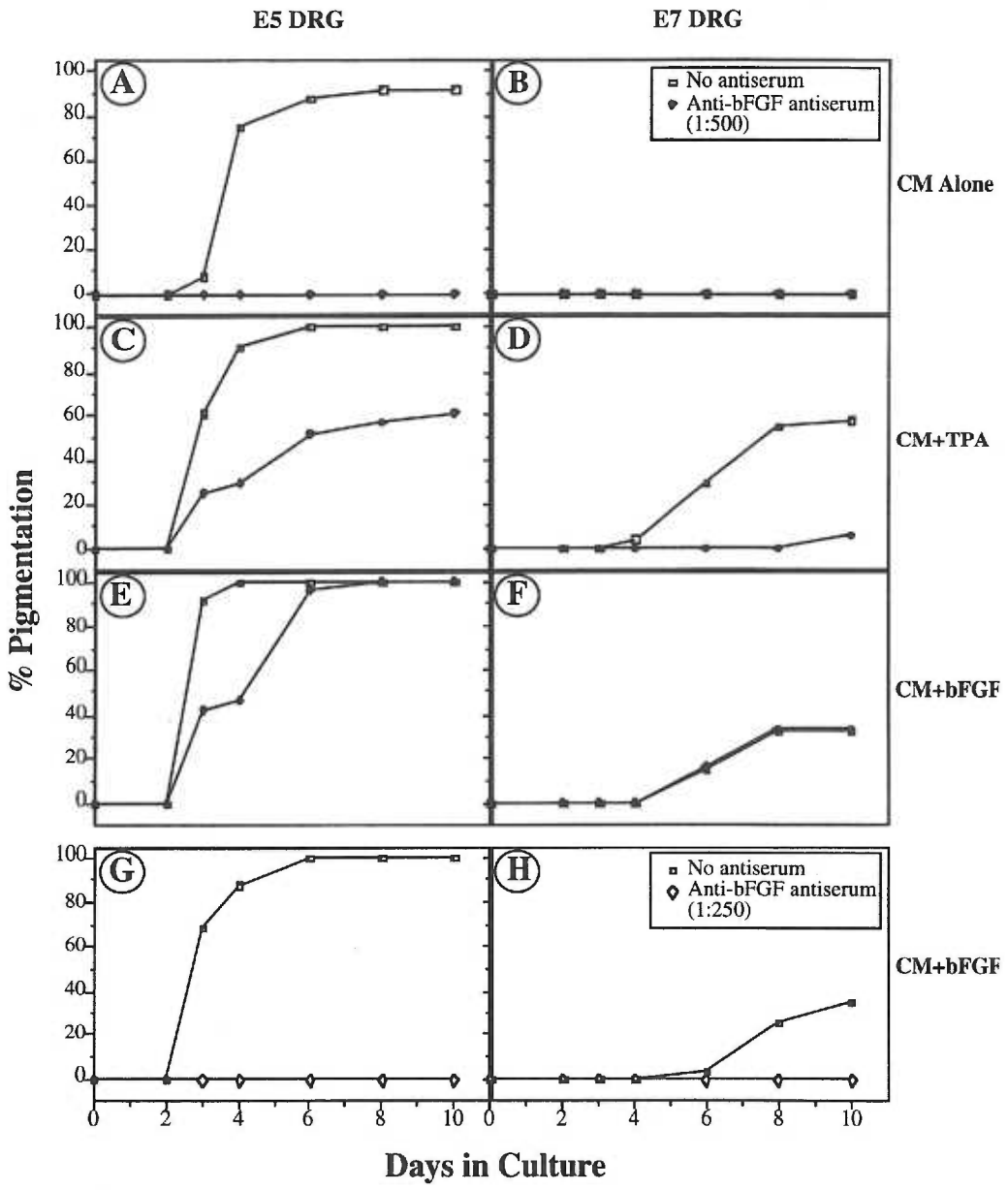

Figure III-5: Effects of bFGF-neutralizing antibodies on pigmentation of E5 and E7 DRG cells cultured in CM. DRG explants from E5 (panels A,C,E,G) and E7 (panels B,D,F,H) quail embryos were cultured in the absence (open squares) or presence (filled diamonds) of a 1:500 dilution of a bFGF-neutralizing antiserum in either: **(A,B)** CM with no supplements; **(C,D)** CM supplemented with 3.0 nM TPA; **(E,F)** CM supplemented with 10 ng/ml bFGF; **(G,H)** CM supplemented with 50 ng/ml bFGF. Each data point represents 24 culture wells. Note that bFGF-neutralizing antibody completely inhibited the spontaneous pigmentation of E5 DRG cells (panel A) and that an excess of bFGF reversed this inhibition (panel E). Note also that bFGF-neutralizing antibody partially inhibited the TPA-induced pigmentation of E5 DRG cells (panel B) whereas with E7 DRG, the proportion dropped from 58% to 5% of the cultures (panel D). These experiments were performed three times with similar results.

cultures decreased from 100% to 61% in the presence of neutralizing antibody (panel C), whereas with E7 DRG, the proportion dropped from 58% to 5% of the cultures (panel D). These experiments were performed three times with similar results.

Effects of bFGF on pigmentation in peripheral nerve cultures

Although DRG provide a relatively abundant source of neural crest-derived cells, these ganglia contain both neuronal and Schwann cell precursors, complicating the interpretation of these data. Previous studies had shown that peripheral nerve explants, which do not contain neuronal precursors, do contain cells which readily undergo adventitious pigmentation in response to TPA (Ciment *et al.,* 1986). To determine, therefore, whether bFGF would induce pigmentation in cells which would normally give rise to Schwann cells, peripheral nerves were dissected from 7 day quail embryos and cultured in CM in the presence of TPA and/or bFGF. These cultures were then examined over the course of 10 days for the appearance of pigmented cells. Fig. III-6 shows that none of the cultures in CM alone underwent adventitious pigmentation, but about 63% of

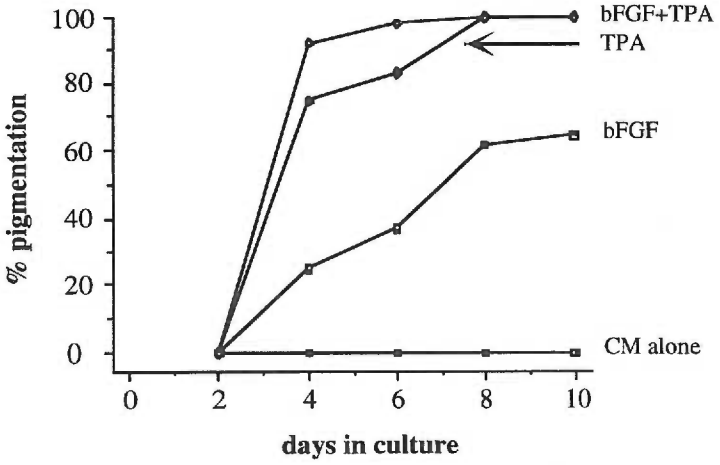

Figure III-6: Peripheral nerve cells also undergo pigmentation in response to bFGF. Distal portions of spinal nerves from the thoracic region of E7 quail embryos were cultured in CM supplemented with either 3.0 nM TPA, 10 ng/ml bFGF, or both bFGF and TPA. Each data point represents the percentage of 24 culture wells. Note that bFGF induced pigmentation in these peripheral nerve cultures.

the bFGF-treated cultures did contain pigmented cells, and in some cultures, 100% of the non-fibroblastic cells contained pigment granules (data not shown). As seen before, TPA induced pigmentation in 100% of the cultures (Ciment *et al.,* 1986). Qualitatively similar results were obtained in three separate trials of this experiment. These studies suggest, therefore, that at least some of the bFGF-responsive cells in these experiments would normally have given rise to Schwann cells.

Effects of bFGF on peripheral nerve cell melanogenesis in vivo

In previous studies, TPA-treated day-7 quail DRG explants were grafted into the wing buds of 4-day White Leghorn chicken embryos (an unpigmented strain) to determine if the pigmented cells in these cultures had the properties of melanocytes *in vivo* (Ciment *et al.*, 1986). Pigmented feather buds were seen at the graft site of many of these host embryos, suggesting that the DRG-derived pigment cells exhibit the behaviors of normal melanocytes (Ciment *et al.*, 1986). To determine whether both TPA- and bFGF-treated peripheral nerve cells would also give rise to melanocytes in feather primordia of host embryos, distal portions of quail peripheral nerve tissue were cultured for 4 days in either CM alone, 0.1 μM TPA or 10 ng/ml of rh-bFGF, then grafted into the right wing bud of 4-day (stage 23-24) White Leghorn chicken embryos. Approximately 63% (5 out of 8) of the host chicken embryos receiving bFGF-treated grafts had extensive pigmentation in the feather primordia and overlying skin of the right wing (see Fig. III-7b), while 88% (7 out of 8) of the hosts receiving TPA-treated grafts had pigmented right wings. No pigmentation was observed in either the unoperated left wing buds of these embryos (data not shown) or in embryos receiving a graft cultured in CM alone (0 out of 5; see Fig. III-7a). These data indicate that, like DRG, the pigmented cells in peripheral nerve explants behave like normal melanocytes *in vivo*, and that these cells, although derived from peripheral nerve tissue, appear to become committed to the melanocyte fate following treatment with either TPA or bFGF.

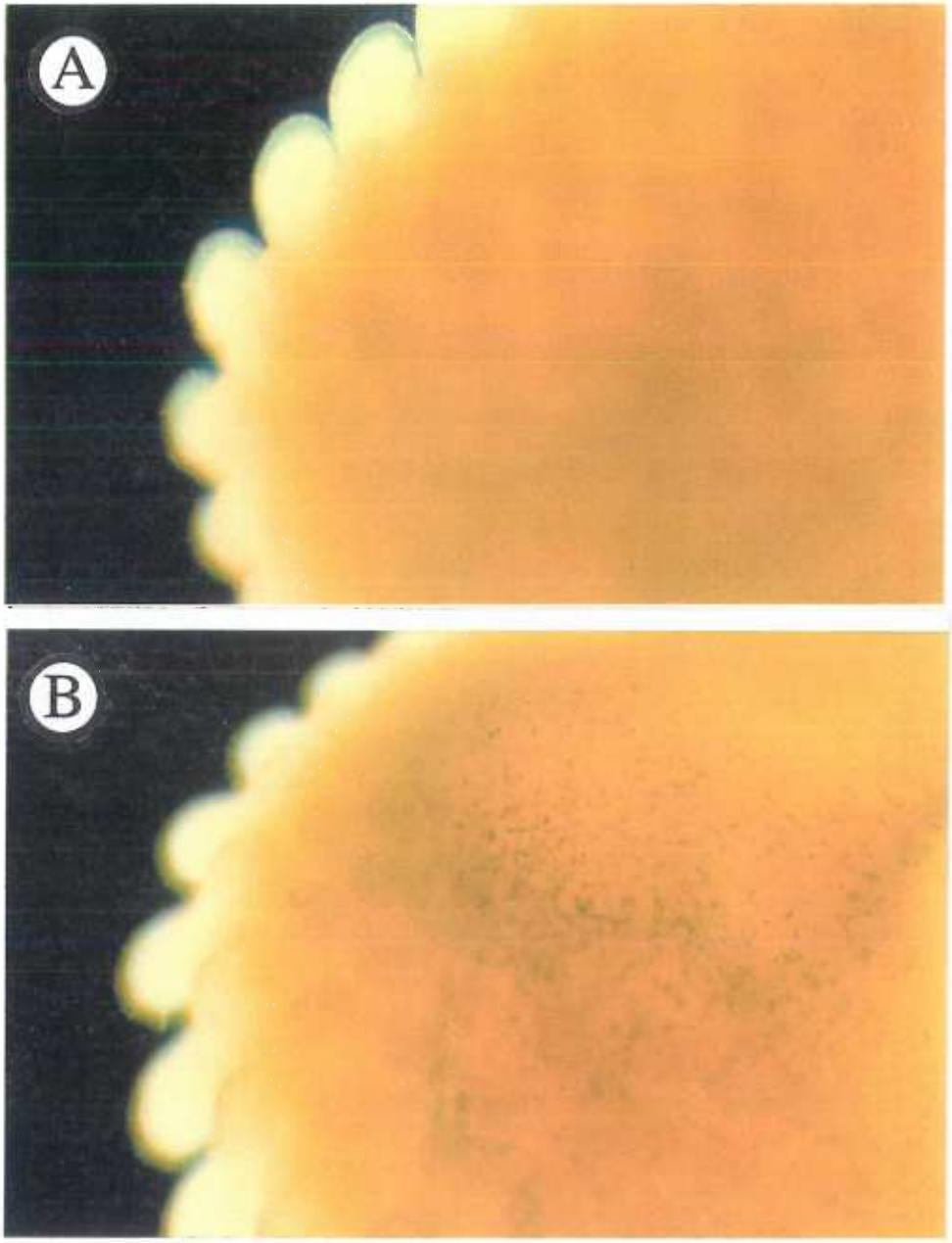

Figure III-7: Melanogenesis in 12-day White Leghorn chicken embryo after grafting 7-day quail peripheral nerve cells into the right wing bud. **(A)** Grafted peripheral nerve cells were cultured in CM alone for 4 days. Note absence of pigmented cells. **(B)** Grafted peripheral nerve cells were cultured in CM supplemented with 10 ng/ml rh-bFGF for 4 days. Note numerous pigmented cells in both skin and feather primordia.

DISCUSSION

Basic FGF induces pigmentation in various neural crest-derivatives, probably acting on Schwann cell precursors

The data presented here indicate that bFGF, but not various other growth factors, induced the appearance of pigmented cells in DRG and peripheral nerve cultures from E7 quail embryos. This effect of bFGF could be blocked by the addition of bFGF-neutralizing antibodies, and the effects of these neutralizing antibodies could be overcome, in turn, by additional bFGF. This last observation suggests that this antibody preparation is not cytotoxic, but instead, produces its effects via binding bFGF. Further evidence for a role of bFGF in this adventitious pigmentation comes from the defined medium experiments, in which bFGF induced pigmentation in E7 DRG explants in a dose-dependent fashion. It may be noteworthy that TPA was also necessary for pigmentation in the DM studies, acting synergistically with bFGF to induce higher levels of pigmentation. In CM, in contrast, the effects of bFGF and TPA seemed to be additive. These observations suggest, therefore, that other factors present in CM may influence melanogenesis.

Pigmentation was also observed, however, in E5 DRG explants cultured in CM in the absence of exogenous bFGF. This spontaneous pigmentation may also have been due to bFGF, probably present in the chick embryo extract which was added to the CM used in the studies presented in this chapter (see Seed *et al.*, 1988; Kimura *et al.*, 1989). This conclusion is based on the observations that neutralizing antibodies against bFGF were able to block the spontaneous pigmentation seen in E5 DRG cultures, and that this inhibition could again be overcome by adding higher concentrations of bFGF.

The observation that peripheral nerve cells underwent bFGF-induced pigmentation in these experiments supports the notion that the bFGF-responsive cells would normally have given rise to Schwann cells, rather than neurons. Schwann cells make up the

majority of the cells within peripheral nerve, although some perineurial fibroblasts are also present. Since these fibroblasts are not of neural crest origin, however, it seems unlikely that this small subpopulation of cells would be responsible for the appearance of melanocytes in either bFGF- or TPA-treated peripheral nerve explants. In some peripheral nerve cultures, moreover, most or all of the non-fibroblastic cells underwent pigmentation in response to bFGF. It is possible, therefore, that Schwann cell precursors found in various neural crest-derived neural tissues retain, at least transiently, the developmental capability to undergo a transdifferentiation into melanocytes in response to bFGF.

Basic FGF may also support the survival of a subpopulation of DRG cells

An alternative explanation for the effects of bFGF on DRG and peripheral nerve pigmentation, however, is that bFGF promotes the survival and/or mitogenesis of melanocyte precursors or other uncommitted neural crest-derived cells (see Halaban *et al.*, 1987; Schubert *et al.*, 1987; Unsicker *et al.*, 1987; Eckenstein *et al.*, 1990). In the current study, for example, the viability of E5 DRG cultures grown in CM in the presence of bFGF-neutralizing antibodies or in DM without bFGF was consistently poor, with the few surviving cells having a fibroblast-like morphology. Kalcheim (1989) has also provided evidence that bFGF may promote the survival of a subpopulation of neural crest-derived cells within embryonic chicken DRG *in vivo*. In these experiments, impermeable barriers implanted between the spinal cord and nascent DRG were found to cause a decrease in the number of HNK-1 immunoreactive cells [i.e., neural crest-derived cells (Bronner-Fraser, 1986)] within the DRG, whereas bFGF-coated barriers did not cause such a decrease. Since the embryonic spinal cord contains high concentrations of bFGF at these developmental stages (Kalcheim and Neufeld, 1990), the interpretation is that bFGF released from the spinal cord supports the survival of a subpopulation of neural crest-derived cells.

It seems unlikely, however, that proliferation of committed melanoblasts can fully explain the bFGF-induced pigmentation seen in this study. Basic FGF induced pigmentation in a large proportion of E7 DRG cells, for example, and yet there was very little apparent cell death in cultures treated with bFGF-neutralizing antibodies. If bFGF acted principally as a survival and/or a mitogenic factor, one might have expected to see significant cell death in such cultures. It seems likely, therefore, that at least some of the effects of bFGF are to reverse the developmental restriction of melanogenesis in these neural crest-derived cells (see Ciment *et al.,* 1986).

TGF-β1 inhibits the adventitious pigmentation of DRG cells

In contrast to the stimulatory effects of bFGF, TGF-β1, but not various other growth factors, decreased the extent of pigmentation in E7 DRG explants cultured in the presence of TPA, and prevented the bFGF-induced increase in pigmentation. TGF-β1 inhibited, moreover, the pigmentation that normally occurs in DRG from E5 quail embryos cultured in unsupplemented CM. TGF-β1 seems to antagonize, therefore, the ability of bFGF to promote adventitious pigmentation of DRG cells. It is intriguing to speculate that TGF-β1 may act to promote commitment of the putative melanocyte/Schwann cell progenitor to the Schwann cell lineage, or at least, to prevent melanocyte development within peripheral ganglia. As noted in Chapter I, TGF-β has been shown to inhibit the appearance of pigment cells in neural crest cultures (Rogers *et al.*, 1992), and immuno-reactivity for various members of the TGF-β gene family is present in the ventral neural crest migratory space where some Schwann cell precursors would travel (Jakolew *et al.*, 1993).

The data presented in this chapter suggest, therefore, that the fate of the putative melanocyte/Schwann cell precursor may be influenced by specific environmental cues. Basic FGF, or some other member of this growth factor family, may support melano-genesis, while TGF-β may support commitment to the Schwann cell lineage, or at least,

antagonize the effects of bFGF. Furthermore, the observations made of peripheral nerve explants suggest that cells which are presumably Schwann cell precursors change their fates, transdifferentiating into melanocytes in the presence of bFGF in culture. The identity of these cells and the mechanisms by which bFGF can influence their phenotype are explored further in the next chapter.

CHAPTER IV

Basic FGF acts intracellularly to cause the transdifferentiation of avian neural crest-derived Schwann cell precursors into melanocytes

As described in Chapter I, the mechanisms governing bFGF expression are poorly understood. The putative human bFGF promoter, however, is known to contain a potential TPA-responsive element (Shibata *et al.,* 1991), and TPA has been shown to induce bFGF mRNA expression in a variety of cell types (Murphy *et al.*, 1988a; Bikfalvi *et al.*, 1990; Weich *et al.*, 1991; Lowe *et al.*, 1992). It is possible, therefore, that bFGF may mediate the effects of TPA on pigmentation in peripheral nerves and other neural crest derivatives. The goals of the studies described in this chapter are to further charactertize the cells in early embryonic peripheral nerve explants and to investigate the role of bFGF in the TPA-induced transdifferentiation of Schwann cell precursors in these cultures into melanocytes.

Characterization of embryonic peripheral nerve explant cultures

Although adult peripheral nerves are known to contain primarily neural crest-derived Schwann cells and some mesoderm-derived perineurial fibroblasts (Bunge *et al.*, 1989), the precise cellular composition of peripheral nerve explant cultures from stage 32-33 (E6.5-E7) quail embryos is unclear. To characterize the cellular composition of this tissue in culture, individual peripheral nerve explants were grown for 4 days, and then fixed and immunostained using either the neural crest-specific antibody HNK-1 (Bronner-Fraser, 1986) or the Schwann cell-specific monoclonal antibody 1E8 (Bhattacharyya *et al.*, 1991). Fig. IV-1A is a phase contrast photomicrograph from one such culture, and shows that the majority of the cells from these explants have a flattened

morphology with the relatively small nucleus characteristic of early Schwann cells (e.g., Jessen and Mirsky, 1991). Fig. IV-1B shows HNK-1 immunostaining in this same microscopic field, indicating that the vast majority of cells in E7 quail peripheral nerve cultures express this neural crest cell trait. The small numbers of HNK-1$^-$ cells in these cultures have relatively larger nuclei than the HNK-1$^+$ cells, and are presumably perineurial fibroblasts. Fig. IV-1C represents a similar culture immunostained for the Schwann cell marker P_0 using peroxidase-labeled secondary antibodies instead of fluorescence antibodies to increase sensitivity, showing that virtually all cells are P_0^+. Quantitative analyses of 12 such cultures shows that nearly all of the cells (> 95%) stained positive for both HNK-1 and P_0, indicating that the vast majority of the cells in these peripheral nerve cultures are neural crest-derived cells of the Schwann cell lineage.

Effects of TPA on bFGF expression

As mentioned above, TPA has been shown to increase bFGF mRNA levels in various cell types. To determine whether TPA influenced bFGF protein expression in peripheral nerve explants, cultures were grown for various periods of time in the presence or absence of 1.0 μM TPA, and then cell lysates and conditioned medium were assayed for bFGF, using protein immunoblot analysis. Fig. IV-2A shows that lysates of peripheral nerve cells cultured in CM alone contained low levels of three molecular weight species of bFGF [approximately 17, 20, and 23 kilodaltons (kDa)] at 24 hours and slightly higher levels at 48 hours (lanes 4 and 6, respectively). TPA had no significant effects on bFGF expression after 24 hours in culture (compare lanes 4 and 5), but caused between 3 to 4-fold increases in the levels of the various bFGF isoforms after 48 hours (compare lanes 6 and 7), as determined by densitometry. In a separate experiment, bFGF protein levels were found to return to basal levels by about 72 hours (not shown). In contrast to these cell lysates, bFGF was never detected in the 100-fold concentrated conditioned medium from these same cultures (Fig. IV-2B; lanes 4-7). These experi-

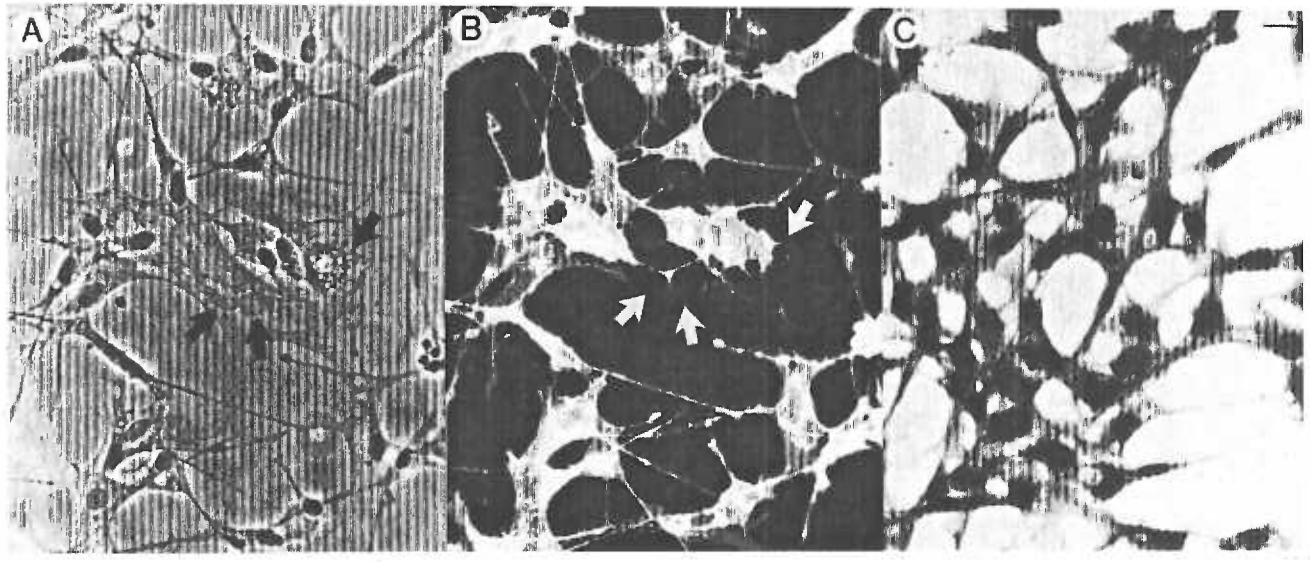

Figure IV-1: Immunostaining studies of the neural crest marker, HNK-1, and the Schwann cell marker, P_0, in explant cultures of E7 quail (stage 32-33) peripheral nerves maintained for 4 days in CM. (A) Phase-contrast photomicrograph of a typical peripheral nerve explant culture. **(B)** Fluorescence photomicrograph of HNK-1 immunostaining in this same culture. Note that the majority of the cells in these cultures are HNK-1$^+$, and that the few HNK-1$^-$ cells (at the arrows) possess large nuclei and a fibroblast-like morphology. **(C)** Bright-field photomicrograph of P_0 immunoperoxidase staining in a similar culture to that seen in panels A and B. Note that all of the cells in this culture are 1E8$^+$, suggesting that the vast majority of the cells in these peripheral nerve cultures are part of the Schwann cell lineage. The bar in the upper right corner represents 20 μm.

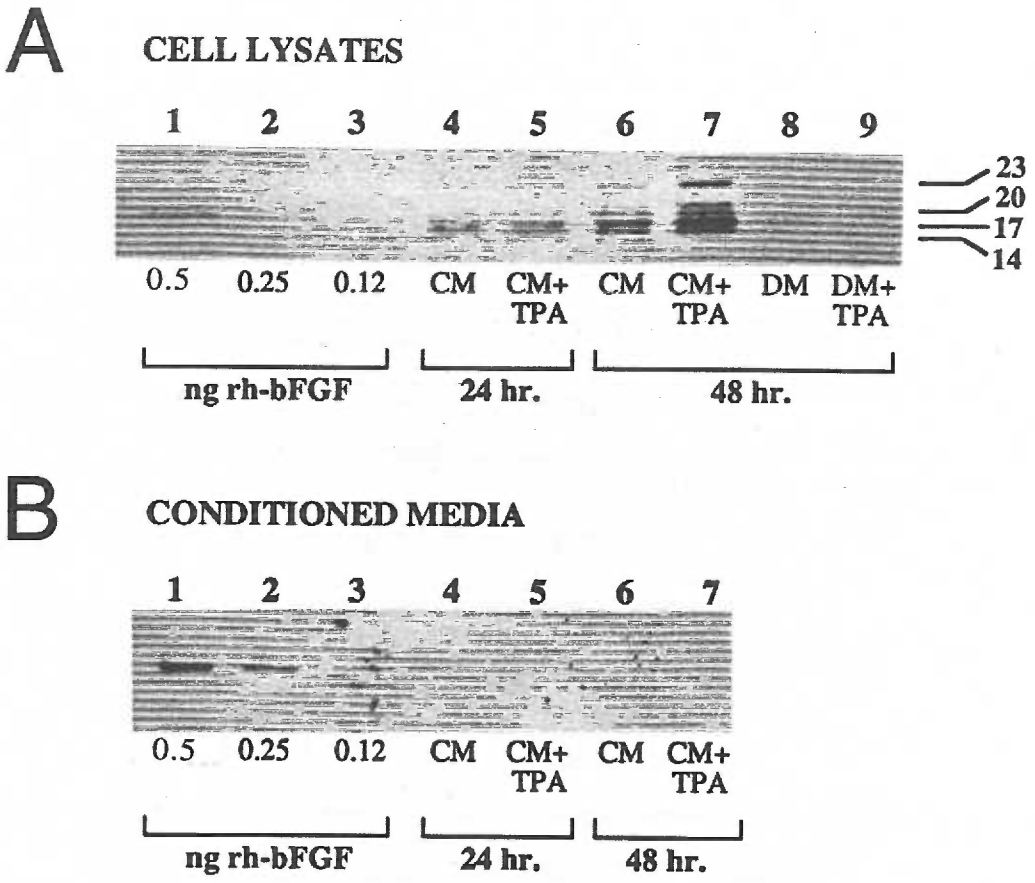

Figure IV-2: Effects of TPA and serum on the levels of the various bFGF isoforms in peripheral nerve explant cultures. Explants of stage 32-33 quail embryo peripheral nerves were cultured in serum-containing ("CM") or serum-free ("DM") medium for either 24 or 48 hours in the presence or absence of TPA (1.0 μM TPA). At these times, the cells and conditioned media were harvested and analyzed for the presence of bFGF protein using immunoblot analysis. **(A)** Basic FGF expression in cell lysates. Note that explants cultured in CM expressed three molecular weight isoforms of bFGF (approximately 17, 20, and 23 kDa), and that the levels of these isoforms, and in particular the 23 kDa form, increased by 48 hours (compare lanes 4 and 6). A smaller molecular weight band at approximately 14 kDa was also present, and may represent degraded bFGF. Also note that TPA augmented about 4-fold the levels of expression of all forms of bFGF at 48 hours (compare lane 7 with lane 6), but were unchanged at 24 hours (compare lane 5 with lane 4). Little or no bFGF expression, however, could be detected in explants cultured in DM alone (lane 8) or in DM+TPA (lane 9). Lanes 1-3 represent serial dilutions of the 18 kDa rh-bFGF used as standards. **(B)** Basic FGF expression in concentrated (about 100-fold) conditioned medium from the same cultures shown in panel A. Note the lack of detectable levels of any of the bFGF isoforms in the conditioned medium from these same cultures. The amount of protein added to these lanes has been adjusted to represent the same numbers of cells as that shown in panel A. Again, lanes 1-3 represent serial dilutions of the 18 kDa rh-bFGF used as standards.

ments were performed a total of five times using either DE6 or 148.6.1.1 antibodies with qualitatively similar results. Control experiments using DE6 antibodies preabsorbed with excess rh-bFGF showed no detectable bands in similar protein immunoblots (not shown).

Serum has also been reported to induce bFGF mRNA expression and may be necessary for bFGF protein expression in some cultured cell types (Murphy *et al.*, 1988b). To determine whether the basal levels of bFGF seen in lanes 4 and 6 of Fig. IV-2A may have been induced by the serum present in CM, peripheral nerve explants were grown in a defined ("DM") serum-free culture medium (see Chapter II) in the presence and absence of 1.0 μM TPA, and then assayed for bFGF immunoreactivity. As shown in lane 8 of Fig. IV-2A, no bFGF could be detected in cell lysates prepared from these cultures, even in the presence of TPA (lane 9), suggesting that some component(s) of fetal bovine serum can contribute to, and is necessary for, the induction of bFGF expression in these cultured peripheral nerve cells.

Effects of bFGF-antisense oligonucleotides on TPA-induced transdifferentiation

To determine if bFGF protein expression was necessary for TPA-induced melanogenesis in peripheral nerve cells, cultures were treated with TPA in the presence and absence of antisense oligonucleotides corresponding to two distinct regions of the bFGF mRNA transcript (Table I), and then examined at various times for the presence of melanocytes and for bFGF expression. Fig. IV-3A represents the time course of pigmentation under these various culture conditions from a representative experiment; Fig. IV-3B presents the statistical analysis at the end of 10 days from four such experiments. As shown in these panels, bFGF antisense oligonucleotides "AS1" and "AS2" delayed the initial appearance of pigment cells by 24-48 hours and caused a statistically significant 40% reduction of TPA-induced pigmentation after 10 days. In contrast, TPA-induced pigmentation was not significantly influenced in cultures incubated with sense ("S2") and scrambled antisense oligonucleotides ("SAS2"). Addition of phosphorothioate-modified

antisense oligonucleotides ("PAS1"), which are presumably less vulnerable to nuclease degradation (Marcus-Sekura *et al.*, 1987), did not change the levels of pigmentation as compared to cultures treated with unmodified oligonucleotides ("AS1").

To confirm that the antisense oligonucleotides inhibited bFGF protein expression, cell lysates from TPA-treated peripheral nerve cultures were assayed for bFGF using protein immunoblotting methods, as described in Chapter II. Fig. IV-3C shows that the antisense oligonucleotides significantly reduced TPA-induced bFGF expression in these cultures (compare lane 4 with 5 and lane 6 with 7). Densitometric analyses of such blots indicate that AS1 blocked the appearance of all TPA-induced isoforms of bFGF protein after 24 hours, and inhibited the appearance of these isoforms by approximately 60-80% after 48 hours. Together, these data indicate that the antisense oligonucleotides used in this study inhibited bFGF expression in peripheral nerve explant cultures, and that this expression is necessary for the TPA-induced transdifferentiation into melanocytes.

The different isoforms of bFGF have been reported to be differentially localized within the nucleus or cytoplasm (Renko *et al.*, 1990; Florkiewicz *et al.*, 1991). To characterize the intracellular location of bFGF-immunoreactivity in Schwann cell precursors and whether antisense oligonucleotide-treatment influenced this localization, peripheral nerve explant cultures were grown for 2 days in CM in the presence or absence of TPA and AS1, and then fixed and stained for bFGF-immunoreactivity. Fig. IV-4 shows that cultures treated with TPA showed significantly higher overall levels of bFGF immunoreactivity than untreated cultures (compare panels B and A), and that this immunoreactivity could be detected in the cytoplasm and, at slightly higher levels, in the nucleus. Cultures treated with both TPA and AS1 oligonucleotides displayed less overall bFGF-immunoreactivity (compare panels D and B), although this immunoreactivity was still present in both the nucleus and cytoplasm. In contrast, bFGF immunoreactivity was very low in negative controls, including TPA-treated cultures incubated without primary antibody (data not shown), and cultures incubated with primary antibody pre-absorbed with rh-bFGF (Fig. IV-4C).

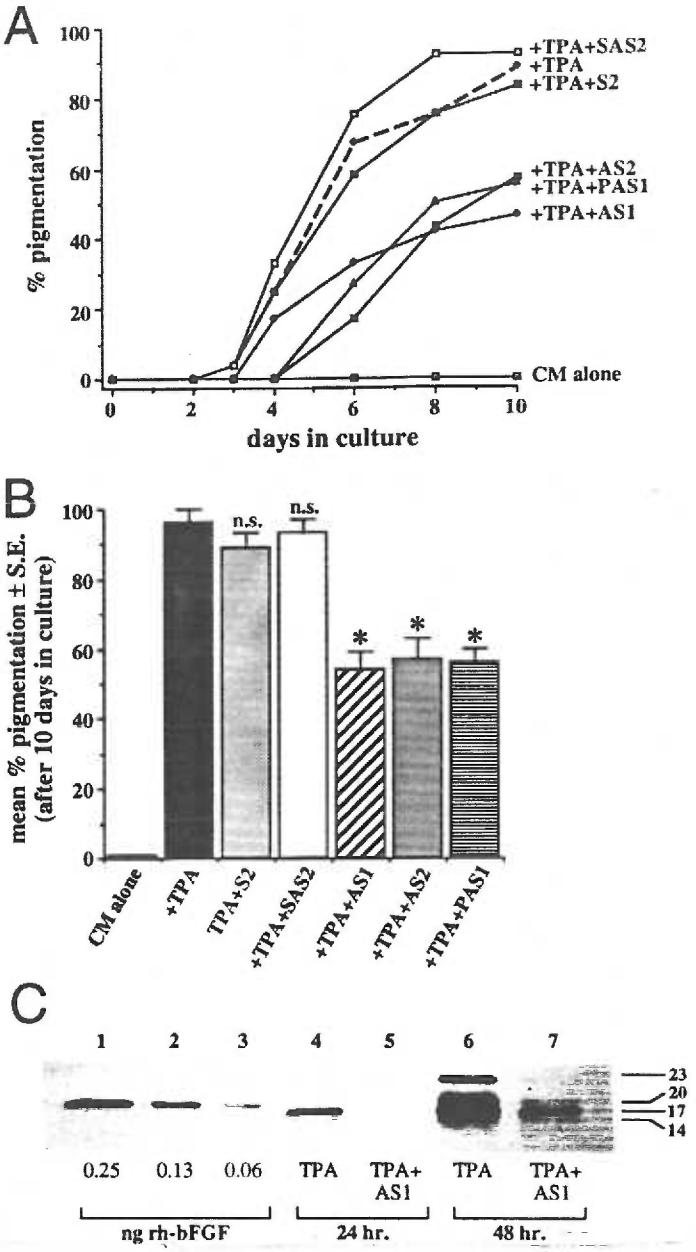

Figure IV-3: Effects of bFGF-antisense oligonucleotides on TPA-induced pigmentation and bFGF expression in peripheral nerve explants. Explants were grown in CM with 1.0 μM TPA plus or minus 50 μM bFGF oligonucleotides (antisense "AS1" or "AS2", scrambled antisense "SAS2," or sense "S2") or 10 μM phosphorothioate-modified bFGF antisense oligonucleotide ("PAS1"). Cultures were then scored for pigment cells or assayed for bFGF expression. **(A)** Time-course of pigmentation in a representative experiment. Note that all of the antisense oligonucleotides delayed the onset of TPA-induced pigmentation and reduced final levels of pigmentation, while control oligonucleotides had no effects in this regard. **(B)** Means of percent pigmentation after 10 days of culture from 4 experiments. Note that there was no significant difference in pigmentation between cultures treated with TPA alone and cultures treated with TPA+S2 or TPA+SAS2. All 3 antisense oligonucleotides, however, significantly inhibited TPA-induced pigmentation. **(C)** Protein immunoblot showing bFGF expression in cell lysates of explants treated with TPA (lanes 4 and 6) or TPA+AS1 (lanes 5 and 7). Lanes 4-7 each contain approximately 20 μg of protein. Note that AS1 blocked bFGF expression at 24 hours, and continued to inhibit up to 80% of bFGF expression by 48 hours. Lanes 1-3 contain serial dilutions of 18 kDa rh-bFGF used as controls. n.s. = not significant; * = $p < 0.05$.

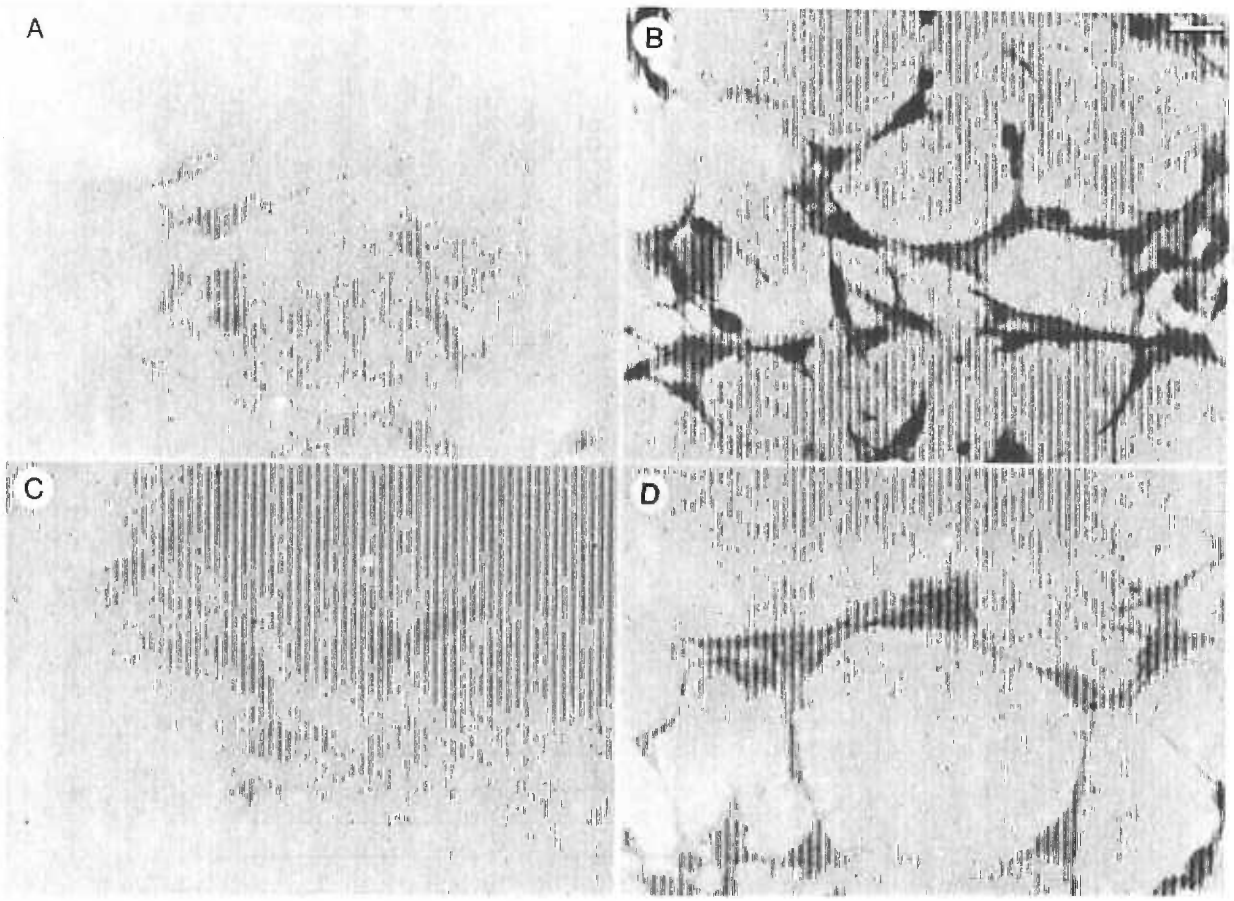

Figure IV-4: Effects of bFGF-antisense oligonucleotides on the immunocytochemical localization of bFGF in cultured peripheral nerve explants. **(A)** Basic FGF immunostaining in cells cultured in CM alone. Note the low level of bFGF immunoreactivity in the cell nuclei and cytoplasm. **(B)** Basic FGF immunostaining in cells cultured in CM+TPA. Note the presence of bFGF immunoreactivity in cell nuclei and slightly lower levels in the cytoplasm. **(C)** Negative control for antibody specificity. These cultures were grown in CM+TPA, but were analyzed using DE6 preabsorbed with an excess of rh-bFGF. Note that staining was reduced to background levels. **(D)** Basic FGF immunostaining in cells cultured in CM+TPA+AS1. Note that the bFGF antisense oligonucleotides reduced the levels of TPA-augmented bFGF expression, but that immunostaining could still be detected in both cell nuclei and cytoplasm. The bar in the upper right corner represents 30 μm.

Effects of bFGF-neutralizing antibodies and $InsP_6$ on TPA-induced transdifferentiation

Since the protein immunoblotting studies described above failed to show detectable levels of bFGF released into the conditioned medium, it is unclear whether bFGF acts intracellularly or whether a small, but biologically important, fraction of bFGF is released and acts extracellularly. To distinguish between these possibilities, peripheral nerve explants were cultured in the presence or absence of TPA with either the DE6 monoclonal antibody, which has been shown to completely block the effects of exogenously added bFGF (Reilly *et al.*, 1989), or inositol hexakisphosphate ($InsP_6$), which blocks the binding of bFGF to its extracellular receptors (Morrison *et al.*, submitted). Fig. IV-5A represents the time course of pigmentation in a single experiment; Fig. IV-5B is the statistical analysis after 10 days of culture from 4 such experiments. These data indicate that both DE6 and $InsP_6$ completely inhibited the bFGF-induced pigmentation in peripheral nerve explants, but that neither of these agents had any effect on TPA-induced pigmentation. These findings suggest that the bFGF expressed by Schwann cell precursors is not secreted, suggesting that at least some bFGF acts intracellularly.

Effects of bFGF-antisense oligonucleotides on bFGF-induced melanogenesis

Although intracellular bFGF expression appears to be necessary for the transdifferentiation of Schwann cell precursors, exogenous bFGF can also induce melanogenesis in these cultures (see Chapter III and Stocker *et al.*, 1991). One possible explanation for this is that extracellular bFGF may itself induce intracellular bFGF expression (Weich *et al.*, 1991). To test the possibility that bFGF expression is necessary for the melanogenesis induced by exogenous bFGF, peripheral nerve explants were grown in the presence of either bFGF or TPA plus or minus the bFGF antisense oligonucleotide AS1, then examined at the end of 10 days for the presence of pigmented cells. Fig. IV-6 shows that AS1 significantly blocked TPA-induced pigmentation, but had no

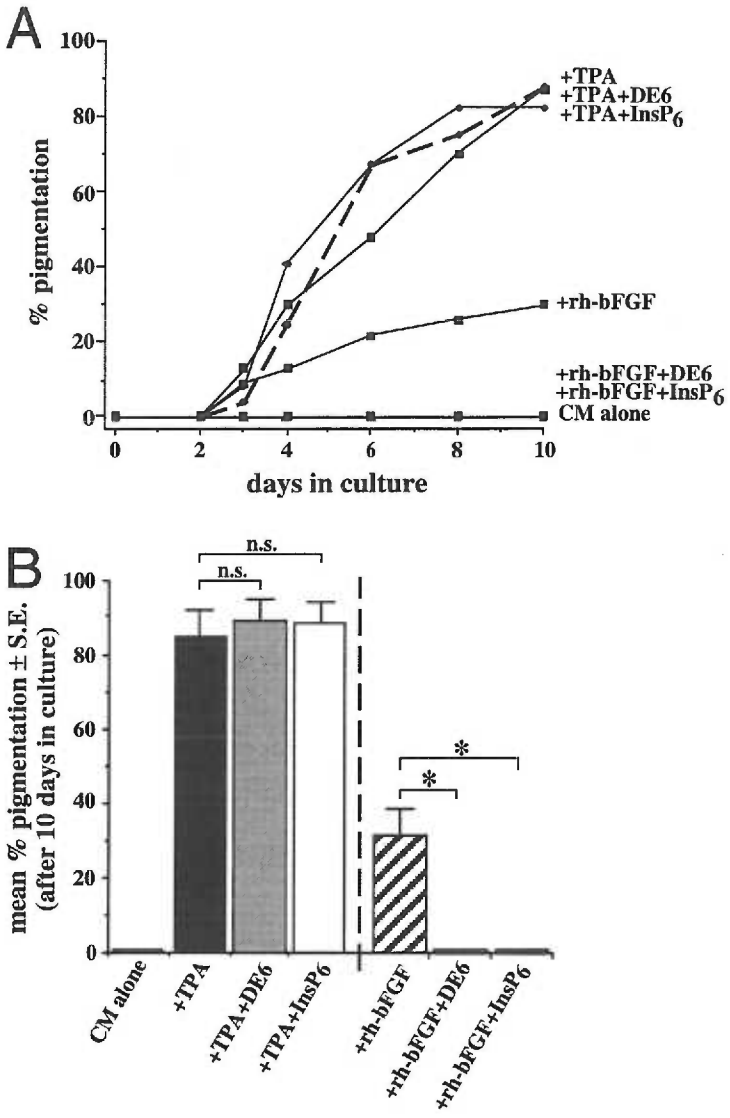

Figure IV-5: Effects of bFGF-neutralizing antibodies and inositol hexakisphosphate ($InsP_6$) on TPA-induced melanogenesis. Peripheral nerve explants from stage 32-33 quail embryos were grown for various periods of time in CM supplemented with either 1.0 μM TPA or 20 ng/ml rh-bFGF in the presence or absence of a 1:250 dilution of monoclonal anti-bFGF antibody DE6 or 25 μM $InsP_6$. Cultures were then scored for the presence of pigment cells. **(A)** Time-course of pigmentation in one representative experiment. Note that neither DE6 nor $InsP_6$ affected the onset or extent of pigmentation as compared to cultures treated with TPA alone (broken line), but that both of these agents blocked completely the rh-bFGF-induced pigmentation. **(B)** Means (± standard errors of the mean) of percent pigmented cultures after 10 days from 4 separate experiments. Note the lack of statistically significant differences in pigmentation between cultures treated with TPA alone and cultures treated with TPA+DE6 or TPA+$InsP_6$. Pigmentation induced by rh-bFGF, however, was totally blocked by both of these agents. n.s. = not significant; * = $p < 0.05$.

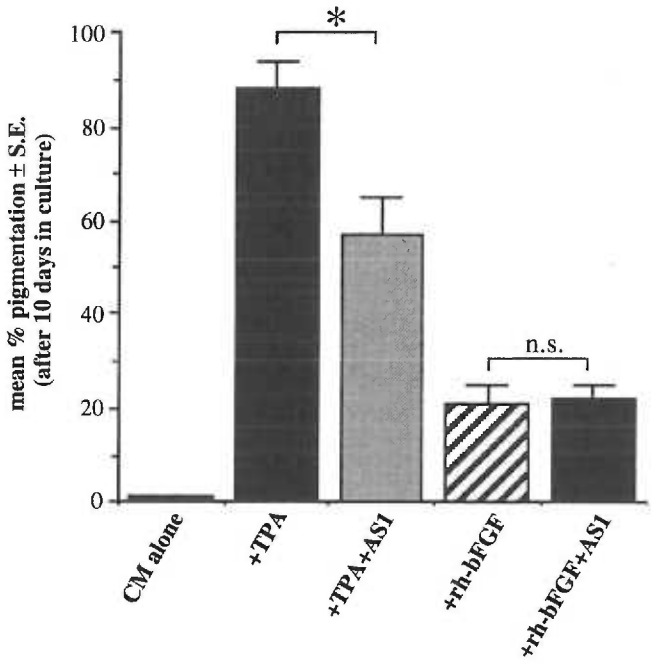

Figure IV-6: Effects of bFGF antisense oligonucleotides on melanogenesis induced by exogenous bFGF. Peripheral nerve explants from stage 32-33 quail embryos were cultured for 10 days in CM supplemented with either 1.0 μM TPA or 20 ng/ml rh-bFGF in the presence and absence of 50 μM AS1, and then assayed for pigment cells. Note that AS1 significantly inhibited TPA-induced pigmentation, but had no significant effect on rh-bFGF-induced pigmentation. Data are plotted as the means ± standard errors of the mean for 3 experiments. n.s. = not significant; * = $p < 0.05$.

significant effects on bFGF-induced pigmentation. This experiment was performed three times with qualitatively similar results, suggesting that induction of intracellular bFGF by exogenously added bFGF does not play a significant role in the transdifferentiation of Schwann cell precursors into melanocytes.

Effects of bFGF and TPA on BrdU incorporation

As shown in Chapter III, TPA caused a greater proportion of peripheral nerve explant cultures to become pigmented than exogenously added bFGF (see also Stocker *et al.*, 1991). TPA and extracellular bFGF may, therefore, have other effects on peripheral nerve cells, such as acting as mitogens. This notion is supported by reports that TPA and/

or bFGF are mitogens for rodent Schwann cells (Krikorian *et al.*, 1982; Eccleston *et al.*, 1987; Ratner *et al.*, 1988; Davis and Stroobant, 1990; Schubert, 1992), human melanocytes (Eisinger and Marko, 1981; Halaban *et al.*, 1987) and avian neural crest cells (Sieber-Blum and Sieber, 1981). To determine whether TPA and bFGF were mitogenic for embryonic quail peripheral nerve cells, explant cultures were grown for 47 hours in the presence or absence of either 1.0 μM TPA or 20 ng/ml rh-bFGF, and then these cultures were assayed for mitogenesis using a BrdU-incorporation assay. Fig. IV-7

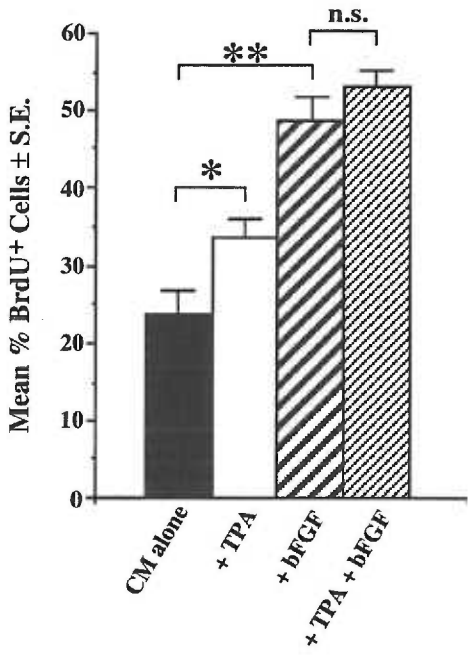

Figure IV-7: Mitogenic effects of TPA and exogenous bFGF on cultured peripheral nerve explants. Individual peripheral nerve explants from stage 32-33 quail embryos were cultured for 47 hours in CM supplemented with either 1.0 μM TPA, 20 ng/ml rh-bFGF, or both TPA and rh-bFGF. The cultures were then labelled for 1 hour with bromo-deoxyuridine (BrdU) and then fixed. Cells incorporating BrdU were then identified using a kit, as described in Materials and Methods. Note that TPA only weakly stimulated mitogenesis in these cultures (34%), while rh-bFGF strongly stimulated mitogenesis (48%). There was no significant difference in the BrdU labeling index between cultures treated with bFGF alone and cultures treated with bFGF+TPA. Each data point is plotted as the mean ± standard error of the mean of 6 separate cultures. n.s. = not significant; * = $p < 0.01$; **$p < 0.005$.

shows that TPA caused a statistically significant increase in the BrdU labeling index (approximately 40%; N = 6; $p < 0.01$). Exogenously added bFGF, however, caused a significantly greater increase in the labeling index (approximately 110%; N = 6; $p < 0.005$). Cultures treated with both TPA and bFGF did not contain significantly more $BrdU^+$ cells than cultures treated with either agent alone. These experiments were performed three times with qualitatively similar results.

DISCUSSION

Expression of bFGF is necessary for the transdifferentiation of Schwann cell precursors into melanocytes

In the previous chapter both bFGF and TPA were shown to induce the adventitious appearance of pigmented cells in cultured embryonic quail peripheral nerve explants, presumably by causing the transdifferentiation of Schwann cell precursors into melanocytes (see also Ciment *et al.*, 1986; Sears and Ciment, 1988; Stocker *et al.*, 1991). The data presented in this chapter show that the cells undergoing TPA-induced pigmentation in these cultures express the P_0 protein, confirming that they are part of the early Schwann cell lineage. Basic FGF expression by these cells was also found to be necessary for this transdifferentiation. This conclusion is based on the observations (i) that TPA augmented the expression of the various bFGF protein isoforms in these cultures; and (ii) that interference with this bFGF expression, either by treatment with specific antisense oligonucleotides or by the use of serum-free culture medium, significantly inhibited the extent of TPA-induced melanogenesis.

Although treatment with the bFGF antisense oligonucleotides produced only a partial inhibition of TPA-induced melanogenesis in these peripheral nerve cultures, there are reasons to believe that these effects were specific and mediated by inhibition of bFGF protein expression. First, two different antisense oligonucleotides, corresponding to non-overlapping regions of the mRNA transcript, produced similar inhibition of melano-genesis in peripheral nerve cells, whereas the corresponding sense oligonucleotides and scrambled antisense oligonucleotides had no significant effects in this regard. Second, antisense oligonucleotides transiently inhibited bFGF protein expression in these cultures, as shown by both protein immunoblotting and immunocytochemical methods. The partial inhibition of pigmentation may have been due to the rate at which the antisense oligonucleotides were taken up into cells, or their rate of degradation (Toulmé and

Hélène, 1988). Phosphorothioate-modified oligonucleotides, which are presumably less susceptible to nuclease degradation (Marcus-Sekura *et al.*, 1987), were found, however, to be no more effective at inhibiting TPA-induced transdifferentiation than non-modified oligonucleotides. The constitutive expression of bFGF protein which was observed in non-TPA-treated Schwann cell precursors may explain why some transdifferentiation occurred in TPA-treated cells cultured in the presence of bFGF antisense oligonucleotides, since these oligonucleotides would not affect the levels of proteins already synthesized. The bFGF protein is highly resistant to degradation, especially in the presence of heparan sulfate proteoglycans (Gospodarowicz and Cheng, 1986), and may persist within cells for relatively long periods of time. It is possible, therefore, that some of the Schwann cell precursors in peripheral nerve explants may have contained sufficient levels of bFGF at the time of culture to facilitate their TPA-induced transdifferentiation into melanocytes.

Only a subpopulation of P_0^+ cells underwent pigmentation in response to TPA, even though all of these cells expressed intracellular bFGF, suggesting that there was some heterogeneity among these Schwann cell precursors. Although the nature of this heterogeneity is not clear, there are at least two possible explanations. First, there may be a differential responsiveness to these agents depending on which phase of the cell cycle a cell is in at the time of exposure. Schwann cells have relatively long cell cycles (e.g., Eccleston *et al.*, 1987) and it is possible that the phase in which initial exposure to TPA or bFGF occurs is a critical factor in determining whether the cell undergoes transdifferentiation. Alternatively, these cultures may consist of Schwann cell precursors at different stages of their committment, having differential responsiveness to exogenous TPA and bFGF. This notion is supported by previous observations that peripheral nerve explant cultures established from progressively older quail embryos demonstrate decreasing levels of TPA-induced pigmentation (Ciment *et al.*, 1986). These data would

suggest, therefore, that the developmental period in which TPA or bFGF can induce transdifferentiation of Schwann cell precursors into melanocytes is rather limited.

The three protein isoforms of bFGF found in cell lysates of quail peripheral nerve cultures were found to be similar in their sizes and relative abundances to those previously found in homogenates of early avian embryos (Sherman *et al.*, 1991). One isoform had a molecular weight of 20 kDa and was weakly expressed, while two isoforms had molecular weights of 17 and 23 kDa and were strongly expressed in these embryonic quail peripheral nerve cells. Although the function of these amino terminal extended forms of bFGF remains unclear, their selective expression may have diverse effects on various cellular behaviors (Couderc *et al.*, 1991; Quarto *et al.*, 1991a). Since all three isoforms were inhibited by the antisense oligonucleotides in these peripheral nerve cultures, however, it is not clear whether all or one of them were responsible for TPA-induced transdifferentiation of peripheral nerve cells. Nonetheless, it is conceivable that the selective expression of one of these bFGF isoforms in Schwann cell precursors of avian embryos may have been sufficient to cause their transdifferentiation into melanocytes.

As mentioned in Chapter I, it is not clear how bFGF expression is regulated by TPA-treatment in avian and other species. In previous studies, however, the time course of TPA-induced pigmentation was consistent with that of PKC down-regulation in avian neural crest-derived cells (Hess *et al.*, 1988), suggesting that the loss of PKC activities somehow participates in the transdifferentiation process. The presence of a TPA-responsive element within the avian bFGF gene might also explain how TPA could augment bFGF expression in these Schwann cell precursors. The notion that such an element exists is supported by the observations that TPA, at concentrations which both activate and down-regulate PKC, induces bFGF mRNA expression in a wide variety of cell types (Murphy *et al.*, 1988a; Bikfalvi *et al.*, 1990; Weich *et al.*, 1991; Lowe *et al.*, 1992).

Basic FGF acts in Schwann cell precursors via an intracrine mechanism

These studies also indicate that the bFGF expressed by Schwann cell precursors is not secreted and is likely not to interact with extracellular receptors. Neither bFGF-neutralizing antibodies nor $InsP_6$, for example, affected the TPA-induced transdifferentiation of these cells into melanocytes, even though the effects of exogenously added bFGF were totally blocked by these agents. Furthermore, bFGF was not detected in the concentrated conditioned medium of cultured peripheral nerve explants, even though it was easily detected in cell lysates from these same cultures. Immunocytochemistry revealed high levels of bFGF expression in both the cytosol and nucleus of these TPA-treated Schwann cell precursors. It is likely, therefore, that bFGF remains within these cells, where it acts directly as an intracellular signal.

While secretion has conventionally been considered a prerequisite of growth factor activity, these and other data support the notion that bFGF can act by means of an intracrine mechanism (Logan, 1990). As described in Chapter I, several studies have shown that bFGF is localized within the nucleus and nucleolus (Kardami and Fandrich, 1989; Joseph-Silverstein *et al.*, 1989; Renko *et al.*, 1990; Kalcheim and Neufeld, 1990; Tessler and Neufeld, 1990; Yamamoto *et al.*, 1991; Woodward *et al.*, 1992), which are not conventional locations for secreted proteins, in addition to its localization within the cytoplasm. Indeed, the amino terminal extension of the higher molecular weight forms of bFGF contains a nuclear localization sequence which has been shown to cause translocation of heterologous gene products into nuclei (Bugler *et al.*, 1991; Quarto *et al.*, 1991b; Florkiewicz *et al.*, 1991). Various cells transfected with bFGF-expression constructs have demonstrated increased growth characteristics, moreover, even though little or no bFGF was detected in their conditioned media (Neufeld *et al.*, 1988; Halaban *et al.*, 1988). Furthermore, there is reason to believe that bFGF may play a direct role in transcriptional control (Bouche *et al.*, 1989; Baldin *et al.*, 1990; Quarto *et al.*, 1991a; Nakanishi *et al.,* 1992), suggesting that some of the biological activities attributed to

bFGF may be mediated by nuclear bFGF binding proteins or by the direct interaction of bFGF with DNA.

Intracellular and extracellular bFGF may have different effects on mitogenesis of Schwann cell precursors

Previous studies have shown that exogenous bFGF can act as a Schwann cell mitogen in various mammalian species, especially when administered with agents that increase intracellular dibutyryl cyclic AMP (Krikorian *et al.*, 1982; Eccleston *et al.*, 1987; Ratner *et al.*, 1988; Davis and Stroobant, 1990) or in the presence of transforming growth factor β (TGF-β) (Schubert, 1992). In the present study, exogenous bFGF was shown to act as a strong mitogen for embryonic quail Schwann cell precursors in the presence of serum. TPA, however, had only weak mitogenic activity in these cultures, and did not influence the mitogenic activity of bFGF. These observations are consistent with the notion that the effects of intracellular bFGF on Schwann cell precursors are distinct from the effects of extracellular bFGF, which presumably interacts with cell surface receptors. Since multiple proteins are derived from each of the four bFGF high-affinity receptor genes by alternative mRNA splicing, and each protein is thought to have different affinities for the various members of the FGF family (Johnson *et al.*, 1991; Eisemann *et al.*, 1991; Miki *et al.*, 1991), it is likely that the number and types of receptors expressed by neural crest-derived cells determines how these cells will respond to extracellular bFGF and FGF family members. Although the localization and specificities of these FGF receptors are still largely unknown, some migrating neural crest cells have been shown to transiently express FGFR1 mRNA (Heuer *et al.*, 1990).

The finding that both exogenously added bFGF and intracellular bFGF induce the transdifferentiation of Schwann cell precursors into melanocytes raises the possibility that exogenous bFGF may have induced its own expression (Weich *et al.*, 1991). Since bFGF antisense oligonucleotides had no significant effects on bFGF-induced pigmentation in

these cultures, however, this possibility is unlikely. On the other hand, it is possible that exogenous bFGF might be internalized by Schwann cell precursors to act intracellularly. Various polypeptide growth factors, including bFGF, are known to be translocated into the nucleus after cell surface binding and internalization (Rakowicz-Szulczynska *et al.*, 1986; Bouche *et al.*, 1987; Baldin *et al.*, 1990; Hawker and Granger, 1992). In one study, bFGF was found to be internalized both via high affinity binding sites, presumably one of the FGF tyrosine kinase receptors, and via low affinity heparin-binding proteoglycans (Gannoun-Zaki *et al.*, 1991). Since heparin protects bFGF from proteolytic degradation (Gospodarowicz and Cheng, 1986), it seems likely that some bFGF-proteoglycan complexes may have remained intact inside cells (Hawker and Granger, 1992). These observations may explain some of the dose-dependent effects of bFGF. In a recent study, for example, BHK-21 cells producing low amounts of bFGF were cocultured with neural crest cells and found to have a moderate effect on neurogenesis, but no effect on the appearance of non-neuronal cells (Brill *et al.*, 1992). BHK-21 cells releasing high levels of bFGF, however, caused an increase in both neuronal and non-neuronal phenotypes in cocultured neural crest cells. It is conceivable, therefore, that Schwann cell progenitors that encounter low extracellular concentrations of bFGF in the embryo would proliferate, while cells encountering higher concentrations of bFGF, or agents which elevate intracellular bFGF, would differentiate into melanocytes.

CHAPTER V
Summary and conclusions

Clues about the mechanisms by which multipotent cells become committed to particular phenotypes may be inferred from observations of various experimentally-induced transdifferentiation events, in which embryonic cells are transformed from one cell type into another (e.g. Aloe and Levi-Montalcini, 1979; Okada, 1980; Doupe *et al.*, 1985; Pittack *et al.*, 1991). Such transdifferentiation events may reveal aspects of the lineage relationship between cells, as well as hints about the molecular mechanisms involved in the determination of their fates. The experiments described in the two previous chapters, for example, demonstrated that bFGF and TGF-β1 can influence the transdifferentiation of Schwann cell precursors into melanocytes. These studies provide insights into the means by which growth factors may affect the fate of melanocyte/Schwann cell progenitors and other neural crest-derived subpopulations during early embryonic development.

Basic FGF can act via two pathways, both inhibited by TGF-β1, to influence the transdifferentiation of Schwann cell precursors into melanocytes

Certain conclusions can be drawn about the activities of bFGF and TGF-β1 in these cultures of avian embryonic Schwann cell precursors. First, the intracellular expression of bFGF is necessary for the TPA-induced transdifferentiation of these cells into melanocytes. Although it cannot be ruled-out that some small quantity of bFGF is secreted by these cells, the bFGF that is required for melanocyte differentiation does not appear to interact with extracellular receptors. Interestingly, the expression of bFGF in these cultures is not induced by TPA alone, but requires the presence of serum. This finding might explain why TPA only rarely causes melanogenesis in early embryonic

DRG explants cultured in a serum-free medium. Together, these observations indicate that agents present in serum are required for the TPA-induced transdifferentiation of Schwann cell precursors into melanocytes, and that this transdifferentiation is dependent on bFGF expression by these cells.

A second major conclusion of these studies, however, is that extracellular bFGF can also promote melanogenesis in cultured Schwann cell precursors. In contrast to TPA-induced melanogenesis, exogenous bFGF appears to cause pigmentation in these cultures independent of intracellular bFGF expression. As indicated above, one possible explanation for this effect is that some bFGF is internalized by cells and is not degraded. If this were the case, then a small quantity of bFGF would be available within Schwann cell precursors to participate in the signal transduction pathways involved in transdifferentiation. It is unclear, however, whether activation of the tyrosine kinase FGF receptors can also participate in the promotion of melanocyte differentiation in these cultures. While this activation apparently results in mitogenesis in some Schwann cell precursors (an effect mimicked only weakly by TPA at concentrations that down-regulate PKC), it cannot be ruled-out from the available data that activation of high-affinity FGF receptors contributes to the mechanisms involved in Schwann cell precursor transdifferentiation. How a cell makes the decision to proliferate versus differentiate in response to extracellular bFGF is also not clear. Such decisions may depend on what stage of the cell cycle a particular cell is in, or it may simply depend on the efficiency by which a cell internalizes and degrades bFGF (see Fig. V-1).

A final conclusion of these studies is that TGF-β1 can inhibit both TPA-induced and exogenous bFGF-induced melanogenesis in cultures of Schwann cell precursors. These cells maintained morphologies characteristic of Schwann cells, suggesting that TGF-β1 may promote Schwann cell differentiation. While it is impossible to determine from these observations how TGF-β1 acts to prevent melanogenesis, one likely possibility is that the signal transduction pathway which is activated by the TGF-β serine/threonine

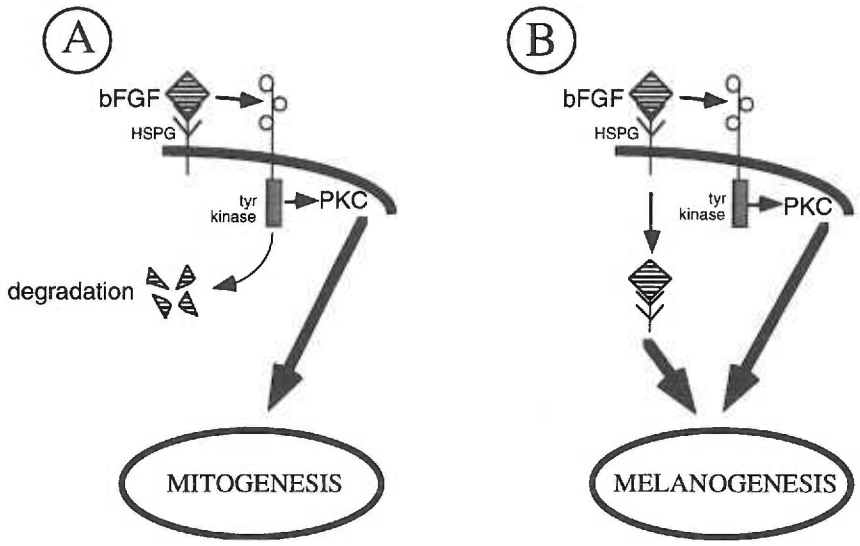

Figure V-1: A model of how exogenous bFGF could influence both mitogenesis and melanogenesis in peripheral nerve explants cultured in the presence of serum. Exogenous bFGF presumably binds to Schwann cell precursors by associating with cell surface low affinity heparin-sulfate proteoglycan receptors (HSPG) and high-affinity receptors possessing intracellular tyrosine kinase domains. **(A)** In some cells, bFGF binding results in activation of PKC and various other agents, followed by receptor-mediated endocytosis and degradation of bFGF. These events cause Schwann cell precursors to divide. **(B)** In other cells, bFGF is protected from degradation by continued association with HSPGs and acts as an intracellular signal. The activities of this internalized bFGF may oppose signals involved in mitogenesis, or may work in concert with them to cause the cells to transdifferentiate into melanocytes.

kinase receptor interferes with the intracellular activities of bFGF or of PKC. This notion is consistent with previous observations indicating that members of the TGF-β family oppose the actions of other growth factors, including bFGF (Roberts *et al.*, 1985).

Intracellular bFGF expression alone may not be sufficient to cause Schwann cell precursors to transdifferentiate into melanocytes

Although intracellular bFGF expression appears to be necessary for melanogenesis to occur in cultured Schwann cell precursors, it is unlikely to be sufficient. As indicated in Chapter IV, for example, Schwann cell precursors cultured in serum do not give rise to melanocytes in the absence of TPA or exogenous bFGF, even though they express

intracellular bFGF. It is possible that elevated intracellular bFGF concentrations could directly promote melanocyte differentiation, as suggested by the finding that TPA augments bFGF expression. This possibility is unlikely, however, because even very high concentrations (50 ng/ml) of exogenous bFGF did not influence melanogenesis in Schwann cell precursors cultured in a defined medium unless TPA was also present. Since TPA does not influence bFGF expression under these conditions, the combined effects of bFGF and TPA are probably not due to raising the intracellular concentrations of bFGF to some threshold level that causes melanocyte differentiation.

The most direct way to determine if intracellular bFGF expression is sufficient to promote melanogenesis in these cultures would be to inject bFGF into individual Schwann cell precursors, then determine if injected cells become melanocytes. In such experiments, cells would be co-injected with fluorescent dextran so that their fates could be followed through a limited number of cell divisions. It would also be necessary to culture these cells in the presence of bFGF-neutralizing antibodies, or other agents which prevent the interaction of bFGF with extracellular receptors (such as $InsP_6$), to ensure that any observed melanogenesis was not due to bFGF that leaked from the cells or was somehow secreted. An alternative way to address this problem would be to infect Schwann cell precursors with a retrovirus containing both a cell marker gene, such as *lac Z*, and the coding sequence for bFGF. Such retroviral constructs have previously been used to determine the effects of particular genes on cell fate (Koo *et al.*, 1992), and have the advantage over dye injections that the marker gene product does not become diluted with successive rounds of cell division. A disadvantage of this technique, however, is that infection efficiencies tend to be relatively low, whereas there are no such limitations with cell injections. Nonetheless, with appropriate controls, either of these techniques would demonstrate to what degree bFGF alone can act to cause melanocyte differentiation in cultures of Schwann cell progenitors.

Other endogenous growth factors may influence the fate of the melanocyte/Schwann cell progenitor

While intracellular bFGF expression may be required for melanocyte/Schwann cell progenitors to become committed to the melanocyte fate, it is unclear whether extracellular bFGF is the instructive growth factor which promotes this process. Compared to TPA, for example, exogenous bFGF only causes a small number of Schwann cell precursors to transdifferentiate into melanocytes. Furthermore, bFGF cannot promote melanogenesis in these cultures unless either serum or TPA are present, suggesting that serum contains at least one other agent that is also required for melanogenesis to occur. Thus, even if extracellular bFGF is involved in promoting melanogenesis, it remains to be determined whether other growth factors or related agents might act as instructive environmental cues which cause melanocyte differentiation.

It is conceivable that other members of the FGF family could play a role in promoting melanocyte commitment. The concentrations of exogenous bFGF which caused pigmentation in the DRG and peripheral nerve explants in these studies were relatively high, and may have resulted in the activation of FGF receptors with low affinities for bFGF, but high affinities for other FGF family members. Although aFGF did not affect Schwann cell precursor transdifferentiation (see Chapter III and Stocker *et al.*, 1991), *int-2*, kFGF, *hst*, and FGF-6 have all been implicated in early developmental events (Wilkinson *et al.*, 1989; Paterno *et al.*, 1989; Niswander and Martin, 1992; Haub and Goldfarb, 1991), and could influence neural crest cell differentiation. It is not presently known, however, if neural crest and crest-derived cells either bind or respond to any of these factors.

As indicated in Chapter I, PDGF is likely to somehow be involved in melanocyte development, based on observations of the murine *Ph* mutant. The abnormal development of melanocytes and various other non-neurogenic crest-derived cells in these mice is believed to be due to their inability to respond to PDGF, since *Ph* mutations

are characterized by deletions in the gene coding for the α-subunit of the PDGF receptor (Stephenson *et al.*, 1991). Although the addition of PDGF to DRG cultures failed to cause melanogenesis (see Chapter III), it is possible that PDGF, while not itself an instructive cue, can act in concert with other agents to cause melanogenesis. Both the serum and chick embryo extract used in these studies probably contained significant quantities of PDGF, making this growth factor a reasonable candidate for the component of serum required for induction of intracellular bFGF expression, and for both TPA- and exogenous bFGF-induced melanocyte differentiation. Since the PDGF receptor is a tyrosine kinase whose activities can be mimicked by TPA (for review see Pawson, 1992), it is also conceivable that, in serum-free cultures of Schwann cell precursors, TPA causes the activation of the signaling pathways normally utilized by PDGF.

Another candidate growth factor which may be involved in instructing melanocyte/ Schwann cell progenitors to become committed to melanogenesis is the Steel factor (SLF), which is the gene product of the murine *Steel* (*Sl*) locus (see Williams *et al.*, 1992). This growth factor is initially synthesized as a precursor protein with cytoplasmic, transmembrane, and extracellular domains. Mature, soluble SLF is then produced by the action of an as yet unknown protease. Mutations in the *Sl* locus are characterized by defects in pigmentation, fertility, and hematopoiesis. The pigmentation abnormalities in these animals range from total lack of pigmentation in homozygotes (black-eyed white), to variable degrees of coat color dilution and white spotting in heterozygotes. These pigmentation abnormalities are known to be due to a lack of pigment cells, suggesting that SLF may be involved in early events in melanocyte differentiation. In support of this notion, it has recently been shown that combinations of SLF and TPA strongly promote melanogenesis in cultures of murine neural crest cells, and that SLF alone can transiently support the survival of neural crest cells which later give rise to melanocytes (Murphy *et al.*, 1992). SLF has also been shown to be a survival factor for cultured human

melanocytes, and to promote melanocyte proliferation in the presence of TPA (Funasaka *et al.*, 1992).

The receptor for SLF is a tyrosine kinase encoded by *c-kit* (Huang *et al.*, 1990), which shares structural homology with a number of other growth factor receptors, including the PDGF receptor (Yarden and Ullrich, 1988). This protooncogene is allelic with the murine dominant-white spotting locus (*W*), mutations of which are essentially identical to *Sl* mutants. Experimental evidence indicating that Kit expression by neural crest cells is crucial for melanocyte development includes the observation that when wild-type crest cells are microinjected *in utero* into neurulating *W* embryos, the crest cells give rise to normal, functional melanocytes (Huszar *et al.*, 1991). Furthermore, injections of monoclonal anti-Kit antibodies into pregnant mice result in coat color abnormalities in offspring (Nishikawa *et al.*, 1991), indicating that the *W* or *Steel* phenotypes can be mimicked in wild-type mice by interfering with SLF-Kit interactions.

The signal transduction pathway utilized by Kit appears to be cell-type specific, partly due, perhaps, to the fact that both SLF and *c-kit* undergo tissue-specific alternate splicing of their mRNAs (Reith *et al.*, 1991; Flanagan *et al.*, 1991; Rottapel *et al.*, 1991; Funasaka *et al.*, 1992). The cascade of tyrosine phosphorylations on various intracellular substrates in mast cells, for example, appear to be different from the substrates phosphorylated in melanocytes (Funasaka *et al.*, 1992; Rottapel *et al.*, 1991). Furthermore, the activities and signal transduction pathways utilized by normal melanocytes *in vitro* appear different from those of melanoma cells, an observation that is consistent with the idea that SLF activities may change with cell differentiation (Funasaka *et al.*, 1992). The association of SLF with the Kit protein typically does, however, cause Kit autophosphorylation on tyrosine, the association of Kit with phosphatidylinositol 3'-kinase and phopholipase Cγ1 (PLCγ1), and activation of $p21^{ras}$ (Rottapel *et al.*, 1991; Duronio *et al.*, 1992). These data suggest that SLF may affect neural crest cell differentiation and survival via mechanisms typical of other growth factors with tyrosine kinase receptors,

and that these mechanisms differ depending on the lineage and developmental stage of cells.

Neurofibromin and ras *may be involved in the signal transduction pathways by which extracellular signals influence the fate of melanocyte/Schwann cell progenitors*

Based on the evidence presented in this dissertation, certain testable predictions can be made about the nature of the intracellular signals induced by environmental cues which influence the fate of melanocyte/Schwann cell progenitors. The interaction, for example, of individual growth factors with their cell-surface receptors is known to elicit a number of non-convergent signals (for review see Kerr *et al.*, 1992). As indicated by the studies described above, the immediate consequence of some of these ligands (i.e. bFGF, PDGF, and SLF) interacting with their receptors is the stimulation of tyrosine kinase activity. This activity, in turn, typically leads to receptor autophosphorylation on tyrosine residues, and association of the receptor with numerous intracellular messenger molecules, including PLCγ1 and GTPase activating protein (GAP). A variety of indirect evidence suggests that each of these signaling molecules and the agents with which they interact are involved in melanocyte/Schwann cell progenitor commitment.

A role for PLCγ1 activity in influencing the fate of melanocyte/Schwann cell progenitors is implied by the finding that TPA, which affects PKC activity, promotes the transdifferentiation of Schwann cell precursors into melanocytes. As indicated in Chapter I, PLCγ1 causes the hydrolysis of phosphoinositol-4,5-bisphosphate into inositol triphosphate (IP_3) and diacylglycerol. The receptors that bind IP_3 have been shown to form tetrameric Ca^{2+} channels, whose activation leads to Ca^{2+} release (Irvine, 1992). Diacylglycerol, meanwhile, dramatically increases the affinity of PKC for Ca^{2+}, which, in turn, activates the enzyme (Cantley *et al.*, 1991). Although the concentrations of TPA used to cause Schwann cell precursors to transdifferentiate into melanocytes have been shown to down-regulate PKC activity (Hess *et al.*, 1988), it is likely that there is an initial

activation of PKC that precedes its down-regulation. This activation is probably necessary for melanocyte differentiation in these cells, moreover, since simply inhibiting PKC activity, using agents such as H7, does not cause melanogenesis in avian embryonic DRG explant cultures (unpublished observations).

The GAP proteins may also be involved in the signaling pathways affecting melanocyte/Schwann cell progenitor commitment. GAP proteins stimulate the GTPase activity of proteins encoded by the *c-ras* gene family (collectively known as $p21^{ras}$), which have been shown to have a wide variety of effects on cell proliferation and differentiation (Hall, 1990; Chardin, 1991). The $p21^{ras}$ proteins are active when they bind GTP, but inactive once they catalyze the conversion of GTP to GDP. The phosphorylation of GAP by some tyrosine kinase receptors has been shown to cause GAP to preferentially associate with the cell membrane, where $p21^{ras}$ and PKC are localized (Kaplan *et al.*, 1990; Molloy *et al.*, 1990). When PKC is active, both GAP activity and expression have been shown to be inhibited in some cell types, resulting in the activation of $p21^{ras}$ (Downward *et al.*, 1990; Lacal *et al.*, 1990; Vaidya *et al.*, 1991).

Another protein which has been implicated in the regulation of $p21^{ras}$ activity is the neurofibromatosis type 1 (*NF1*) gene product, neurofibromin. In humans, the gene encoding this 280 kDa protein is organized into at least eleven exons, spanning more than 130 kb of the q11.2 region of chromosome 17 (Cawthon *et al.*, 1990; Viskochil *et al.*, 1990; Wallace *et al.*, 1990). A portion of neurofibromin shares striking amino acid sequence homology with the *IRA* proteins of yeast (inhibitory regulators of the *ras*-cAMP pathway) and the mammalian GAP proteins (Xu *et al.*, 1990a). Furthermore, it has been demonstrated that this GAP-like domain stimulates both *IRA* and $p21^{ras}$ GTPase activities (Xu *et al.*, 1990b), and cultured malignant Schwannomas from neurofibromatosis patients have been shown to express low levels of neurofibromin and high levels of GTP-bound $p21^{ras}$ (DeClue *et al.*, 1992). It is possible, therefore, that $p21^{ras}$ activity plays an

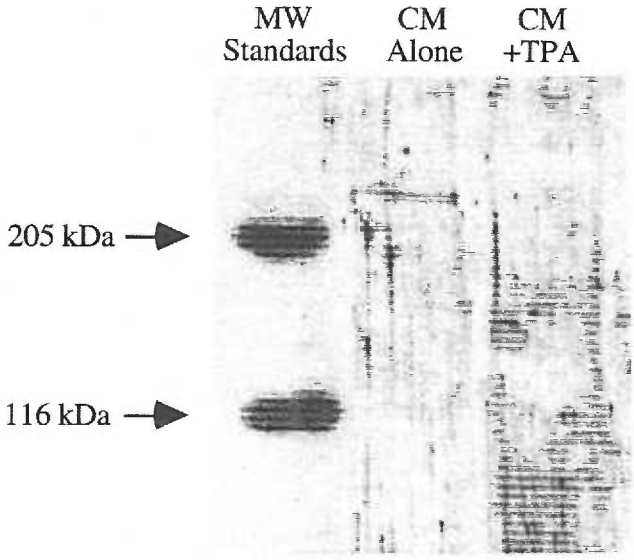

Figure V-2: Effects of TPA on neurofibromin expression in cultuted avian embryonic peripheral nerve explants. Peripheral nerves from stage 32-33 quail embryos were cultured in CM in the presence and absence of 1.0 μM TPA. After 4 days, cells were harvested and washed extensively with HBSS. Soluble protein was extracted in 10 mM Tris-HCl, pH 7.4, with 100 mM NaCl, 1% deoxycholate, 1% Triton X-100, and 0.1% SDS. Approximately 6 μg of total protein from TPA-treated and control cultures was resolved by SDS-PAGE using a 7.5% acrylamide mini-gel, then blotted to nitrocellulose. The blot was then incubated with an antibody directed against the carboxy-terminal 500 amino acids of chicken neurofibromin, using standard methods (see Chapter II). Bound antibody was detected using the Tropix™ "Westen Light" kit. Note that in CM alone, peripheral nerve cells expressed a 250 kDa protein recognized by the chick neurofibromin antibodies. Cultures treated with TPA, however, contained no detectable neurofibromin-immunoreactive material.

important role in Schwann cell proliferation and differentiation, and that neurofibromin regulates this activity.

To test the possibility that neurofibromin expression might somehow be involved in the transdifferentiation of Schwann cell precursors into melanocytes, embryonic quail peripheral nerves were cultured in the presence and absence of TPA, then assayed for neurofibromin via protein immunoblot analysis[1]. As shown in Fig. V-2, TPA down-regulates neurofibromin expression in these cells. This finding is consistent with the notion that PKC can down-regulate neurofibromin activity, and that stimulation of $p21^{ras}$

[1]This experiment was performed in collaboration with L. Baizer amd K. Stocker.

is involved in the transdifferentiation of Schwann cell precursors into melanocytes, and, possibly, the mechanisms by which melanocyte/Schwann cell progenitors undergo commitment.

If $p21^{ras}$ activity is a necessary component of the mechanisms involved in Schwann cell precursor transdifferentiation, then one might expect that increased $p21^{ras}$ activity would correlate with increased bFGF expression in these cells. In support of this notion, Iberg and co-workers (1989) demonstrated that Rat-1 fibroblasts transformed by the H-*ras* oncogene synthesized 2-4 times more bFGF than non-transformed cells, an increase comparable to that seen in TPA-treated Schwann cell precursors. Furthermore, no similar increases could be detected in the levels of aFGF, suggesting that other members of the FGF family were not influenced by $p21^{H\text{-}ras}$.

These studies each suggest that the interaction of one or more growth factors with receptors on the surface of melanocyte/Schwann cell progenitors can lead to a well-defined set of events that culminate in bFGF expression and commitment to either the Schwann cell or melanocyte lineage. Figure V-3 is a model of how the various intracellular signals described above might interact to cause this bFGF expression and at least contribute to melanocyte differentiation in these progenitor cells. As already mentioned, both PDGF and SLF could be involved in initiating this cascade of events, since they have been shown to activate various players in this putative signal transduction pathway, including $p21^{ras}$, and since both have been implicated in promoting melanocyte differentiation. As for the induction of bFGF expression by activation of $p21^{ras}$ proteins, such a mechanism is likely to involve transcriptional activation of the immediate early genes *fos* and *jun*, both of which have been shown to be induced by $p21^{ras}$ (Sassone-Corsi *et al.*, 1989). As indicated in Chapter I, the possibility that the bFGF promoter contains a TRE (Shibata *et al.*, 1991) supports the notion that these nuclear transcription factors are involved in the regulation of bFGF expression.

It is clear that much work remains to be done before we can completely understand

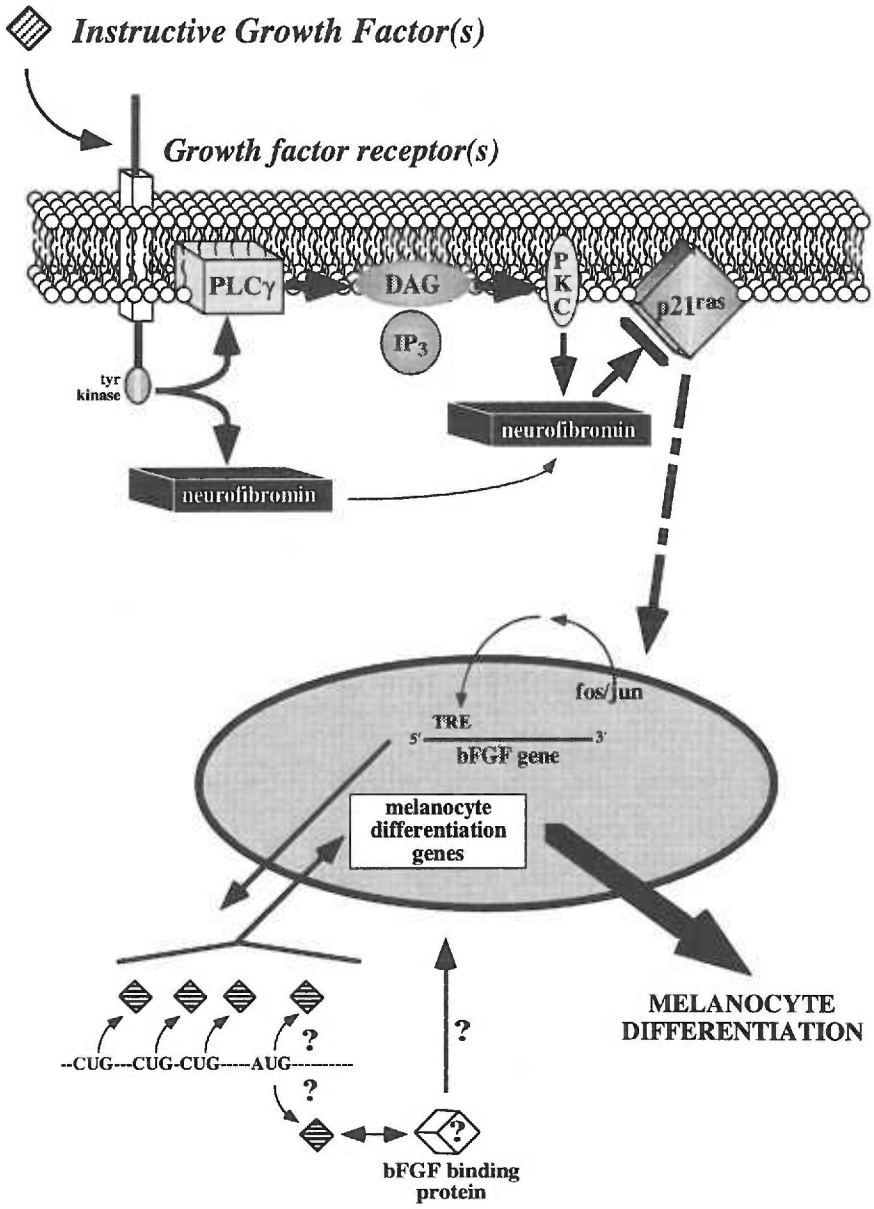

Figure V-3: Possible roles of neurofibromin and *ras* in the signal transduction pathways involved in melanocyte commitment. In this model, a growth factor or group of growth factors bind to tyrosine kinase receptors, which phosphorylate a number of substrates, including PLCγ1 and neurofibromin. Neurofibromin, in turn, translocates to the cell membrane, while PLCγ1 hydrolyzes phosphoinositol-4,5-bisphosphate into IP_3 and diacylglycerol (DAG). The resulting activation of PKC causes the inactivation of neurofibromin, which prevents the hydrolysis of GTP into GDP by $p21^{ras}$. The GTP-bound active $p21^{ras}$ then induces the transcription of *fos* and *jun*, which are translated and associate with the TREs of various genes, including bFGF. The multiple forms of bFGF are then translated and either associate with bFGF-binding proteins or are translocated directly to the nucleus where they influence the transcription of some of the genes involved in melanocyte differentiation.

the functions of both intracellular and extracellular bFGF, and other environmental cues, in neural crest development. Nonetheless, our understanding of the molecular events which lead these multipotent cells to become committed to particular fates is slowly growing, as is our ability to make conclusions about the roles played by various molecules in this process. The continued study of these issues will eventually permit us to answer some of the questions put forth over a century ago by His and his colleagues, about how cells make decisions about their identities.

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