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Migration, Patterning and Regulation of Trunk Neural Crest Cells in the Zebrafish, *Danio rerio*

by

Janet L. Vaglia

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
January 2000

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by Janet Lynn Vaglia

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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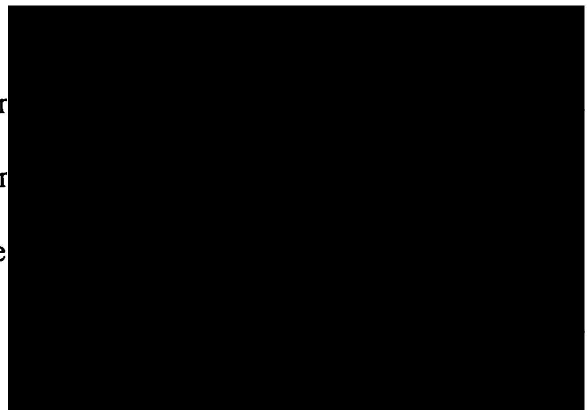

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Abstract

Regulation is the replacement of lost, undifferentiated embryonic cells by neighboring cells in response to environmental signals. Neural crest cells, embryonic cells unique to craniates, are good candidates for studies of regulation because they are pluripotent, and thus might be able to alter their behavior in response to environmental signals. Neural crest cells are classified as cranial, cardiac or trunk according to where they originate along the embryonic axis and the derivatives they form. My study investigated regulation for trunk neural crest (TNC) cells in the zebrafish, *Danio rerio*. The first part of my study asked 'What are the normal patterns of TNC development, migration and differentiation?' I then addressed the hypothesis that there is regulation for loss of TNC, and that regulation would vary with the amount of premigratory neural crest removed, and the position or stage of removal. Lastly, I asked 'what are potential mechanisms of regulation?' DiI labeling, SEM and *snail2* expression in control embryos revealed that TNC cells undergo several successive waves of 'sheet' and 'segmental' migration. Cells labeled at similar axial levels form different derivatives at different stages. Pigment cells served as landmarks for analyzing the effects of TNC extirpation. Regulation occurred on a sliding scale, ranging from complete to incomplete. The amount of TNC removed along the embryonic axis and the position of removal affected regulation to a greater extent than did stage of removal. Defects in development and/or pigmentation occurred if large regions of premigratory TNC were removed, or if neural crest was removed from the cardiac region or the posterior trunk. Otherwise, pigmentation was remarkably normal. Altered cell migration and increased cell division were investigated as mechanisms of regulation. Coincident with epidermal regeneration, neural crest cells consistently migrated into extirpated regions from the posterior edges over a period of 24 hours. Also, more cells were dividing adjacent to the surgery site relative to controls, including TNC migrating along medial and lateral pathways.

Abbreviations

AER	Apical ectodermal ridge
ECM	Extracellular matrix
hpf	Hours post-fertilization
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween
SEM	Scanning electron microscopy
ss	Somite stage(s)
TNC	Trunk neural crest

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Chapter 1

General Introduction

Adult organisms have long been known to compensate for injurious environmental insults. Compensatory growth, wound healing, regeneration and regulation are processes by which organisms heal tissue wounds, regenerate lost body parts such as limbs and tails, or replace lost or damaged cells (Goss, 1964, 1969). The subject of my dissertation is regulation, an embryonic event that has neither been well defined nor distinguished from wound healing or regeneration.

What is regulation?

Regulation has been identified in a variety of organisms from invertebrates to urochordates to craniates. The term 'regulation' historically relates to patterns of early embryonic determination that are classified as either 'autonomous' (mosaic) or 'conditional' (regulative). With autonomous development, embryonic morphogens are distributed early in development, cell fates are fixed, and cells develop independently of their neighbours (see Gilbert, 1997 for an up-to-date summary). In embryos with conditional development, the determination and distribution of morphogens

occur later in development and cell fates are dependent upon environmental cues, usually in the form of cell-cell interactions.

Embryos have classically been categorized as conditional or autonomous developers based on how development proceeds after cells are separated during early cleavage stages. Driesch (1883, summarized in Gilbert, 1997) demonstrated that complete sea urchin embryos (conditional developers) could develop from isolated blastomeres at the 8-cell stage. More dramatically, Bellairs (1971) found that two separate embryos formed when the blastoderm from the lizard *Lacerta vivipara* was divided. In contrast, when half of the cells were removed from frog embryos (autonomous developers) as early as the two- or four-cell stage, 'half-neurulae' rather than entire embryos developed (Roux, 1888, summarized in Gilbert, 1997). The ability of cells to produce a complete organism when isolated from one another early in development came to be known as regulation.

Since the classic experiments by Spemann and Mangold (see Gilbert, 1997) the concept of regulation has been broadened to include the ability of embryonic cells to compensate for cell loss, as might occur during defective proliferation or following delayed onset of migration (Bellairs, 1971). Lehman and Youngs (1952, pp. 420-21) defined regulation as the "ability or inability of a part of the pigment primordium to compensate for an experimentally produced deficiency in the amount of neural crest material present in the embryo." Or simply and more generally, regulation is the ability of an embryo to compensate for actions that would otherwise lead to

abnormal development (Hall and Hörstadius, 1988). Although these definitions appear to set regulation apart from other developmental events, there is considerable overlap between the terms 'regulation' and 'regeneration' in the literature. This brings to question the level at which 'regulation' and 'regeneration' describe similar phenomena. I suggest these terms do represent distinct events; the challenge is to define how they are distinct. To say the difference lies in regulation being a strictly embryonic event is not adequate; replacement of an already formed embryonic chick limb would be considered regeneration, while replacement of a partial limb field or early limb bud would be regulation. Although regeneration loosely describes replacement of lost cells/tissues, it deviates from regulation at the mechanistic level. Regeneration typically involves a cascade of signals that promote dedifferentiation of cells in the wound site, blastema formation, and outgrowth of new tissue (e.g. Mescher, 1996). Regulation involves compensation for deficiencies in undifferentiated embryonic cell populations, rather than differentiated, organized tissues. Moreover, embryonic cells may be replaced by like cells, or by cells from neighbouring tissues. The definition I use for regulation is 'the replacement of lost, undifferentiated embryonic cells by other cells in response to signals received from the environment.'

Cellular requirements for regulation

Cells that are able to regulate must have particular properties. Of utmost significance is the ability to recognize and respond to changes in the

environment, such as occurs with cell-cell signaling. From the moment an egg begins to divide, cell interactions are essential for organizing the embryo, specifying germ layers, tissues, organs and functions, and initiating and maintaining specialized cell states by regulating gene expression. If an important interaction is lost, and if cells are competent to receive alternative signals, they could transform to a different state. The ability of grasshopper ectodermal cells to differentiate into neuroblasts following a change in cell-cell contact, or the transformation of axolotl iris cells into lens cells after the removal of the lens, are examples of regulation through cell fate change (Taghert et al., 1984; Gilbert, 1997).

Relatively little is known about regulative potential beyond the gastrula stage. Neural crest cells, embryonic cells unique to craniates, are good candidates for studies of regulation because they:

- Exhibit broad potentials during development.
- Are essential for normal vertebrate embryonic development.
- Are accessible for extirpation experiments from where they originate at the boundary of epidermal and neural ectoderm.

Furthermore, several alternative cell populations could regulate for neural crest cell loss. What follows in this chapter is a review of how neural crest cells are induced, how neural crest populations migrate, and how they regulate. The studies I discuss have notably contributed to our understanding

of regulation among neural crest cell populations and provide important baseline data for ongoing research on embryonic regulation.

Primary players in the induction of major cell populations in neurula stage embryos

To explore how neural crest cells and neighbouring cell populations (epidermal ectoderm, neural ectoderm, placodes) compensate for loss, we need to understand how neural crest cells are induced. The contribution of neural crest cells to numerous, diverse embryonic tissues (including skeletal, neural and connective) makes them critical for embryonic survival and development. In fact, it has been argued that these cells comprise a fourth craniate germ layer (Hall, 1998b, 1999).

Great strides have been made to refine and expand our understanding of the sequential interactions of gene products required for induction of neural crest and neighbouring cell types. During neural tube formation, progenitor cells acquire dorsal or ventral fates. Axial mesoderm cells of the notochord express the secreted protein product of *Sonic Hedgehog* (*Shh*), which ventralizes the neural tube (Echelard et al., 1993; Krauss et al., 1993; Bronner-Fraser, 1995; Marcelle et al., 1997). Cells of the ventral neural tube subsequently differentiate into floor plate and motor neurons (Fig. 1).

Dorsalization of the neural tube is more complex, involving specialization of dorsally fated cells into epidermal and neural through a

highly coordinated series of inductions and inhibitions. Neural crest cells are subsequently induced at the boundary of epidermal and neural ectoderm (Northcutt and Gans, 1983; Moury and Jacobson, 1989; Bronner-Fraser, 1995; Dickinson et al., 1995; Liem et al., 1995). Bone Morphogenetic Proteins (BMPs, members of the TGFβ gene family of growth factors — reviewed by Kolodziejczyk and Hall, 1996), namely BMP-4 and 7, promote epidermal ectoderm (Liem et al., 1995, 1997; Tanabe and Jesseu, 1996; La Bonne and Bronner-Fraser, 1998; Marchant et al., 1998). A recent model for neural induction postulates that dorsal ectoderm acquires neural fate by being exposed to a gradient of BMP-binding molecules (Marchant et al., 1998), rather than through a series of reciprocal inductions between epidermal and neural ectoderm (Moury and Jacobson, 1989, 1990). According to the gradient model, BMP specifies ectoderm and is then opposed by inhibitory molecules produced by dorsolateral mesoderm, such as Chordin, Follistatin and Noggin (La Bonne and Bronner-Fraser, 1998; Marchant et al., 1998; Nguyen et al., 1998). The inhibitory molecules lower the concentration of BMP, which promotes induction of neural tissue from dorsal ectoderm (reviewed by Tanabe and Jesseu, 1996). Once specified, the dorsal half of the neural tube gives rise to dorsal commissural neurons and roof plate cells and contributes to neural crest cell formation (Fig. 1).

According to Nguyen (1998), the genes *Bmp-2B* and *Swirl* may specify neural crest and placodal cell populations. Placodes are induced in the epidermis adjacent to the neural tube and bordering presumptive neural crest

territory (Northcutt, 1996; Stark et al., 1997; Begbie et al., 1999). Two dorsal neural tube markers, *Pax-3* and *slug*, are thought to distinguish placodes from neural crest cells at later developmental stages because they mark spatially distinct embryonic cell populations and differ in timing of expression (Buxton et al., 1997). *Slug*, a member of the Snail family of zinc-finger transcription factors, is expressed by pre-migratory and migratory neural crest cells, while *Pax-3* appears in more ventrolateral neural tube cells and in ectodermally-derived cells of the ophthalmic trigeminal placode (Nieto et al., 1994; Liem et al., 1995; Buxton et al., 1997; Stark et al., 1997; Sefton et al., 1998). Examples of other factors implicated in neural crest cell induction include genes such as *Dorsalin-1* (TGFβ family, Basler et al., 1993), *Zic2* (Gli superfamily, Brewster et al., 1998), *Wnt-1* and *Wnt-3a* (Wnt family of secreted glycosylated proteins, Ikeya et al., 1997), and growth factors such as bFGF (basic fibroblast growth factor, Kengaku and Okamoto, 1993; Mayor et al., 1997; LaBonne and Bronner-Fraser, 1998).

Neural crest cell populations and migration

Populations of neural crest cells are referred to by most authors as either cranial, cardiac (vagal), or trunk according to where they originate along the embryonic axis and the derivatives they form (Hall and Hörstadius, 1988) (Fig. 2). Some authors also recognize a sacral neural crest cell population. In general, neural crest cells segregate from the neural

epithelium slightly before, during, or following fusion of neural folds, depending on the organism and position along the embryonic axis (Hall, 1999). In bony fishes, neural crest cells segregate from a compact wedge of cells known as the neural keel (Sadaghiani and Vielkind, 1989, 1990; Schmitz et al., 1993; Papan and Campos-Ortega, 1994). In chick, neural crest migration is initiated when cells detach from the neural tube basement membrane (Maclean and Hall, 1987) and is associated with down-regulation of cadherin molecules (e.g. N-cad, cad6B; Nakagawa and Takeichi, 1998). The exact pathways of migration differ along the rostrocaudal axis and among organisms (e.g. Kirby, 1987; Serbedzija et al., 1989, 1992; Lumsden et al., 1991; Epperlein and Löfberg, 1993; Le Douarin and Dupin, 1993). For instance, in teleost fish migration occurs along a medial pathway between the somites and neural keel, and a lateral pathway between the somites and epidermis (Lamers et al., 1981; Raible et al., 1992; Sadaghiani et al., 1994) (Fig. 3).

Epithelial-mesenchymal interactions are required for the differentiation of neural crest cells (Weston, 1983; Maclean and Hall, 1987; Hall, 1999); environmental signals from these interactions may be encountered either during migration or at the site of differentiation (Bronner-Fraser, 1995). For more complete reviews on the origin, migration and developmental potentials of neural crest cells see Le Douarin (1982), Weston (1983), Hall and Hörstadius (1988), Bronner-Fraser (1995) and Hall (1999).

Fate-mapping studies provide evidence for neural crest cell regulation

Results from fate-mapping studies provide a major source of information on regulation. Ironically, fate-mapping studies were conducted to discern the origins and developmental potentials of neural crest cell populations, not to investigate whether cells could compensate for loss (Yntema and Hammond, 1945; Yntema and Hammond, 1954, and references therein; Newth, 1951, 1956; Langille and Hall, 1988a,b; Moury and Jacobson, 1990). Nonetheless, fate-mapping studies exposed regulation as a significant developmental event, presented mechanisms of regulation for further study, and introduced techniques useful for studying regulation. I revisit fate-mapping results in the context of compensation for surgically removed neural crest cells (Chapter three).

Tracing the origins and developmental potentials of neural crest cells often involved surgically removing or electrocauterizing presumptive neural crest territories and observing which structures were missing or malformed after a period of development (Le Douarin, 1982; Hall and Hörstadius, 1988; Serbedzija et al., 1989). In a series of studies extending over a decade, Yntema and Hammond (1954, and references therein) investigated neural crest cell potential in the chick and the amphibian, *Amblystoma punctatum* (=*Ambystoma maculatum*, Wake, 1976), by removing portions of the neural fold, including neural crest cells. These extirpations generated depletions and/or reductions in neural-crest-derived tissues such as visceral arch

cartilage, sheath cells, and ganglia associated with cranial and trunk regions. A major challenge to fate-mapping was determining the length of neural fold tissue extirpation needed to affect cell differentiation without generating severe abnormalities or mortality. In fact, extirpations frequently had to be extended several segments more posterior than originally planned to create neural-crest-derived deficiencies (Newth, 1951, 1956; Yntema and Hammond, 1954; Hammond and Yntema, 1964). Newth (1956) surmised that such extensive removal of neural fold/crest tissue was necessary to prevent regulation by adjacent cells. Fate-mapping of the head skeleton and anterior trunk in the Japanese medaka, *Oryzias latipes* (Langille and Hall, 1988a) and the sea lamprey, *Petromyzon marinus* (Langille and Hall, 1988b) showed that cartilaginous elements of the braincase and branchial arches were missing and/or reduced following removal of neural crest cells. In other cases, neural fold/crest extirpations did not eliminate formation of neural crest cells (Moury and Jacobson 1990, *Ambystoma mexicanum*).

Indeed, because the goal of fate-mapping studies was to identify cellular origins, replacement of cells was a nuisance. Furthermore, neural crest cell contributions vary temporally and spatially along the embryonic axis (see Chapters two, three). Hammond and Yntema (1964) alluded to this when they found that chicks with neural crest removed at later developmental stages had normal cartilages and neural derivatives. The amount of neural tube and number of presumptive neural crest cells removed also greatly influences results. For example, Newth (1956) contended that inconsistencies

in his extirpation experiments reflected differences between surgeries that damaged the spinal cord versus those in which the neural folds were only slightly reduced. Knowing the amount, position and time of neural crest cell removal is imperative to interpreting studies of regulation.

Regulation for cranial neural crest

Morphological evidence for regulation – the craniofacial skeleton

In general, cranial neural crest cells contribute to connective tissue, cartilage and bone of the craniofacial skeleton, odontoblasts of the teeth, and to neurons and glia of cranial ganglia (Hall and Hörstadius, 1988; Bronner-Fraser, 1995) (Fig. 2). As described in the context of fate-mapping, the restoration of normal morphology is evidence of regulation. Although lack of regulation was implied in early studies of the chick where extensive ablations of cranial neural crest produced facial deformities (Yntema and Hammond, 1954; Hammond and Yntema, 1964), more recently, unilateral and bilateral extirpations of migrating chick mesencephalon neural crest (stages 9-11, Hamburger-Hamilton, 1951) were shown to neither disrupt nor delay facial morphogenesis — skeletal, hypobranchial and neural structures (Noden, 1983). Large regions of cranial neural crest also have been ablated or transplanted in creating quail/chick chimeras without generating the extent of deformities in donors previously reported (McKee and Ferguson, 1984; Scherson et al., 1993; Hunt et al., 1995; Couly et al., 1996; Suzuki and Kirby,

1997) (Table 1). Similarly, unilateral removal of portions of the brain and inclusive neural crest cells in *Ambystoma* reveals considerable regulation (e.g. Detwiler, 1944, 1946, 1947; Harrison, 1947; Birge, 1959). Invariably, there are exceptions. Sechrist et al. (1995) ablated mid- and hindbrain neural folds bilaterally in the chick and observed craniofacial delays along with decreases in crest-derived ganglia and the first branchial arch. Additionally, optimal regulation occurred within a narrower time frame — between somite stages two and five — rather than up to seven somites as demonstrated for unilateral ablations in which the intact side may contribute neural crest cells and facilitate regulation (Scherson et al., 1993). Such experiments are extremely important in revealing how gross abnormalities are generated during development (Johnston, 1975; Johnston and Sulik, 1979; Johnston et al., 1985; Müller and O'Rahilly, 1986).

Neural crest and epidermal, neural and placodal ectoderm as sources of regulating cells

Data on the identity of replacement cell populations are lacking in many studies of regulation. While the obvious candidates to replace ablated cranial neural crest would be other neural crest cells, surprisingly little evidence exists for regulation of neural crest by neural crest. For instance, Stark et al. (1997) showed virtually no regulation of the neural-crest-derived component of the avian trigeminal ganglia following mid- and hindbrain ablations of the dorsal neural tube and adjacent ectoderm. Contrary to Stark

et al. (1997), Scherson et al. (1993) reported that mid-hindbrain ablations had no effect on development of the trigeminal ganglia. Furthermore, neural-crest-derived neuronal axons differentiated normally, and there was no significant difference in the average cell number between operated and control treatments after removal of rhombomeres four and five (Diaz and Glover, 1996). Thus, the question of whether cranial neural crest can regulate for its own contribution to cranial ganglia remains unresolved (Table 1).

Tissue transplantation and culture experiments reveal that epidermal ectoderm and neural ectoderm/plate may have the potential to produce some neural crest derivatives, thereby presenting alternatives to regulation for cranial neural crest by only pre-existing cranial neural crest. Moury and Jacobson (1989, 1990) transplanted epidermal ectoderm and neural plate from wild type (pigmented) axolotls (stages 14-16) to cranial and trunk regions of host albino axolotls. The resulting production of spinal ganglia from epidermal ectoderm and of melanophores from neural plate suggests that presumptive neural crest cells are not limited to the neural folds. Selleck and Bronner-Fraser (1995) demonstrated that neural plate cultured from chick embryos of stages six-ten can form pigment cells. Based on the positions of neural crest cells following neural fold injections with DiI, they also interpreted ectoderm as able to produce neural crest. Epidermis cultured alone, however, did not produce neural crest cells. Further study is needed to determine the potential for epidermal and neural ectoderm to form neural crest cells in different organisms during early development (Table 1).

Under specific conditions, placodal cells also may regulate for extirpated neural crest cells (Kirby, 1987, 1988a,b). This is not surprising considering the suggestion that neural crest and placodal cells originated from a common precursor tissue (Northcutt and Gans, 1983; Smith et al., 1994b) (Table 1). Placodes arise in the head as ectodermal thickenings composed of columnar cells; for reviews see Le Douarin et al. (1986) and Webb and Noden (1993). The columnar cells invaginate and later delaminate following transformation from an epithelial to a mesenchymal cell type (in all but the lens placode). Both neural crest and placodal cells contribute to glia and neurons of sensory ganglia, namely in cranial and cardiac regions (Stark et al., 1997). Supporting cells and proximal neurons of these sensory ganglia are derived from neural crest cells; distal neurons are placodal in origin (Hamburger, 1961; D'Amico-Martel and Noden, 1983). Although placodal cells comprise part of the cranial ganglia, there is no evidence that they can regulate for loss of the cranial neural crest-derived portion of cranial ganglia. However, as discussed in the following section, placodal cells can regulate for cardiac neural crest.

Regulation for cardiac neural crest

Cardiac neural crest extends from the mid-otic placode to the caudal limit of the third somite, rendering it transitional between cranial and trunk neural crest (Kirby et al., 1985; Kuratani et al., 1991; Kirby, 1993) (Fig. 2).

Derivatives of cells from this region populate pharyngeal arches three, four and six, providing neuronal and ectomesenchymal components to the heart, aorta and pulmonary arteries (great vessels) (Le Lièvre and Le Douarin, 1975; Bockman et al., 1987; Kirby, 1988a,b; Kuratani and Kirby, 1991).

Morphological evidence for regulation – ectomesenchymal component

Ectomesenchyme cells provide structural support for the great vessels, contribute connective tissue to the thymus, thyroid and parathyroid glands and are necessary for septation of the conotruncal and aorticopulmonary (AP) regions (Kirby, 1988b). Ablation of cardiac neural crest from between the mid-otic placode and third somite, or between the first and fifth somite, frequently generates abnormalities in the pharyngeal arch vessels, aorta, and pulmonary trunk. This reflects how poorly ectomesenchymal-fated cardiac neural crest cells regulate. In the absence of cardiac neural crest, pharyngeal arch arteries become misshapen, lose bilateral symmetry, suffer abnormal structural integrity and subsequently transform into abnormal adult vessels (Waldo et al., 1996). Hood and Rosenquist (1992) observed that extirpation of cardiac neural crest cells causes abnormal deposition of smooth muscle alpha actin, thereby preventing the myocardiac sheath from retracting from surrounding structures and altering the site of origin of the coronary arteries.
Abnormalities of the great vessels and AP septal defects indicate a cumulative loss of cardiac neural crest. In combination with loss or decreases in thymus, parathyroid, and thyroid tissue, many AP defects characterize medical

disorders such as DiGeorge Syndrome (Bockman and Kirby, 1984; Kirby, 1987; Nishibatake et al., 1987; Kirby, 1988b; Kuratani et al., 1991). The high incidence of abnormalities following extirpation of cardiac neural crest cells indicates that proper timing of cell migration is critical to heart development and that regulation of the ectomesenchymal component is limited (Suzuki and Kirby, 1997).

Placodes and neural crest as sources of regulating cells for neuronal and ectomesenchymal components

The neuronal component of cardiac neural crest consists of supporting cells and neurons of cardiac ganglia (Kirby and Stewart, 1983; Kirby, 1988a). Although placodes are defined as head structures, precursor cells from the nodose placode overlap with cardiac neural crest cells. Nodose placodal cells deposit neurons in the vagal (nodose) ganglia of the vagus nerve that provides sensory innervation to the heart and other visceral organs (Kirby, 1993; Harrison et al., 1995). Ablation of cardiac neural crest cells and/or nodose placodal cells results in nearly complete regulation of vagal ganglia by the nodose placode (Kirby et al., 1985; Kirby, 1987; Kirby, 1988a,b). But, similar to results from cranial neural crest cell extirpations (Stark et al., 1997), the neural crest itself does not appear to regulate for ablated cardiac neural crest, specifically with regard to the neural-crest-derived component of cardiac ganglia. The potential for regulation of cardiac neural crest by epidermal and neural ectoderm is unknown (Table 1).

In the absence of cardiac neural crest, cells from the nodose placode provide ectomesenchyme to outflow vessels of the heart; however, placodal cells cannot reestablish a normally functioning AP septa (Kirby, 1988b). The nodose placode also substitutes cells to initiate elastogenesis of the great vessels after cardiac neural crest ablation. Because the cells cannot propagate, formation of the elastic laminae is inhibited (Rosenquiest et al., 1990). It is not known whether regenerated ectomesenchyme cells are not competent because of a biochemical deficiency, or because they arrive at the heart later than the original cells. In either event, it is possible that specificity of the ectomesenchymal component of cardiac neural crest is determined by the time of migration (Kirby, 1988b; 1989).

In contrast to the lack of regulation shown for cardiac neural crest, a recent study by Serbedzija et al. (1998) showed that normal hearts developed following unilateral removal of precursor cardiac mesoderm in the chick. Surrounding cells compensated by migrating into the gap from more cranial regions and functioning as heart, rather than head, mesenchyme. This study complements what is currently known about neural crest cell regulation, and is an example of how regulation continues to provide insight to inherent cell plasticity, the origins of embryonic defects and development of other tissues.

Regulation for trunk neural crest

Trunk neural crest contributes to pigment cells, sensory neurons, glia, sympathoadrenal and Schwann cells (Bronner-Fraser, 1995), as well as to

formation of the dorsal fin of amphibians and teleost fishes (Hall and Hörstadius, 1988; Smith et al., 1994a; Hall, 1999), and to induction of the dorsal fin fold in amphibians such as *Triturus* and *Ambystoma* (Twitty and Bodenstein, 1941; Bodenstein, 1952) and in lampreys (Newth, 1951, 1956) (Fig. 2). Compared to cranial and cardiac neural crest cell populations, fewer derivatives arise from trunk neural crest. Thus, studies of regulation in this cell population have been limited to analyses of pigment cells and dorsal root ganglia.

Raible and Eisen (1996) determined that zebrafish regulate for the loss of an early-migrating population of trunk neural crest cells. Typically, the early-migrating cell population produces both non-neuronal derivatives such as pigment cells and glia, and neuronal derivatives such as dorsal root ganglia. A late-migrating population of cells produces only non-neuronal derivatives (both populations migrate along a medial path between the neural keel and somites). However, if early-migrating cells are ablated, dorsal root ganglia will differentiate from late-migrating trunk neural crest. Interestingly, other studies describe less complete regulation for nerve cells, especially spinal ganglia, than for other derivatives after extirpation of trunk neural fold and crest cells (Detwiler, 1937, 1944; DuShane, 1938; Piatt, 1949; Stefanelli, 1950; Lehman and Youngs, 1952).

Following extensive bilateral extirpations of neural crest from the level of the six most caudal somites in the chick, Yntema and Hammond (1945) observed no regulation of dorsal root ganglia from cranial or caudal spinal

levels. After performing a parallel experiment with quail (18-27 somite stage; removing seven-eight segments), Suzuki and Kirby (1997) did not find regenerated dorsal root ganglia but did see normal pigment and feather patterning. Contrary to the greater regulative ability exhibited by neuronal over ectomesenchymal components in the cardiac region, Chibon (1970) also observed more efficient regulation of pigment cells in the trunk compared to other neuronal derivatives such as Rohon Beard cells (Table 1).

Neural crest cell regulation in mammals

Most studies of mammalian regulation have dealt with the ability of embryos to compensate for cell loss or damage following exposure to a teratogen. Mitomycin C (MMC) is a teratogen known to inhibit cell division in developing mouse embryos; lack of cell division during critical phases of development would be expected to cause death or abnormal development (Hall and Hörstadius, 1988). Interestingly, mouse embryos exposed to MMC through injected females exhibited higher mitotic activity than controls, and a remarkable ability to recover (Snow and Tam, 1979). However, female mice exposed to MMC tended to have smaller litters with many runts, and survivors of a litter frequently exhibited motor defects, poor postnatal development and decreased fertility (Snow and Tam, 1979; Tam and Snow, 1981). Although this study is not specific to neural crest cells, a small percentage of embryos exhibited craniofacial neural-crest-derived

malformations (e.g. microphthalmia), indicating that the neural crest cell population was affected by MMC. Extensive recovery of embryos following MMC treatments suggests that mammalian neural crest cell populations can regulate.

Mechanisms of regulation

Having presented examples of regulation and potential sources of replacement cells, I now discuss how neural crest cells potentially regulate. To regulate, cells must be receptive to signals such as transcription and growth factors in their environment. Four possible mechanisms of regulation are (1) altered cell migration, (2) increased cell division, (3) decreased cell death, and/or (4) change in cell fate. This discussion is limited to the mechanisms of altered migration and increased division, as these potential responses to removal of trunk neural crest cells are explored in Chapter four.

Cell division

From results of amphibian neural crest extirpations, Chibon (1970) suggested that one way cells respond to changes in their environment is by modifying mitotic behavior. This is demonstrated when uni- and bilateral extirpation of neural tube and crest cells is followed by increased proliferation among remaining tissues (e.g. Detwiler, 1944; Källen, 1955; McKee and

Ferguson, 1984; Couly et al., 1996). Such increased division following neural crest cell ablations can be detected with bromodeoxyuridine (BrdU; Scherson et al., 1993). Furthermore, BrdU labeling in zebrafish indicates that cell division is elevated at the cranial and caudal extremities of neural crest-ablated regions (Chapter four).

To understand how altering the cellular environment induces change in mitotic behavior, it is necessary to identify the conditions, genes and molecules which control proliferation. Birge (1959) and Couly et al. (1996) converged on the idea that there is a relationship between cell population density and division rate. This implies that cell division is maintained by signals functioning within the parameters of a specific cell density. An example can be drawn from *Drosophila,* where a single large cell (Tip cell) controls proliferation of neighbouring Malpighian tubule cells. A disruption in signaling to or from the Tip cell could result in uncontrolled cell proliferation (Kerber et al., 1998; Skaer, 1989). Although an equivalent to the *Drosophila* Tip cell has not been found among neural crest populations, epithelial cells stimulate proliferation and/or differentiation of neural crest-derived mesenchyme (Hall and Coffin-Collins, 1990; Clouthier et al., 1998). Removing cells important for initiating and maintaining proliferation and differentiation (e.g. epithelial and neural crest cells) might induce proliferation of neighbouring cells, similar to how decreased cell death follows a decrease in the number of sensory neurons in fields of developing ganglia (Carr, 1984).

We know little about what factors initiate changes in highly regulated patterns of cell division. Several growth factors, such as epidermal and fibroblast growth factors and Neurotrophin-3, are important to cell proliferation and survival (e.g. Hall and Coffin-Collins, 1990; Stemple and Anderson, 1993). TGFβ increases proliferation of some cell populations while decreasing proliferation of others (Rogers et al., 1994). Other growth factors stimulate proliferation after binding to tyrosine kinase receptors (reviewed by Hall and Ekanayake, 1991; Kolodziejczyk and Hall, 1997). Although not directly applicable to newly emerging or migrating neural crest cells, Endothelin-3 and Glial-cell-line-derived neurotrophic factor stimulate proliferation of vagal neural crest cells before they enter the gut (Hearn et al., 1998). Lastly, it has been suggested that members of the Wnt family of secreted factors signal the mitotic activity of select cell populations such as precursor spinal cord (Dickinson et al., 1995) and neural crest cells (Ikeya et al., 1997). If so, genes of the Wnt family would be good candidates for analyzing differential cell division in control and neural crest-ablated embryos.

Cell migration

Regulation would not be complete without cells migrating to replace extirpated cells, whether to locations from which cells were removed, or to locations where they would differentiate. Migration of neural tube and crest cells into 'gaps' – regions devoid of cells – from adjacent locations has been

observed using fluorescent markers (e.g. DiI). However, almost nothing is known about the signals that govern such detours in normal migratory patterns. Movement of cells into gaps may simply reflect passive migration from regions of high to low concentration, an idea referred to as 'contact inhibition' by Weston (1970). This idea has great appeal when considering that cell migration must work in concert with other mechanisms such as increased cell division.

When regions of neural crest are extirpated, replacement cells may migrate from rostral and/or caudal extremities, underlying neural tube or the contralateral neural fold. In all cases, additional neural crest cells would need to be generated for regulation to be complete. Such cells could come from either the neural tube or from pre-existing neural crest cells and would involve different developmental mechanisms. The majority of studies contend that cells migrate from locations rostral, caudal, or contralateral (in the case of unilateral neural fold ablation) to the ablation site (Yntema and Hammond, 1945, 1954; Hammond and Yntema, 1947; Lehman, 1951; Newth, 1951, 1956; Sechrist et al., 1995; Couly et al., 1996; Diaz and Glover, 1996; Saldivar et al., 1997). Few studies have observed neural crest cells migrating from the ventral neural tube following mid- and hindbrain ablation. In those that have, labeled cells in the ventro-lateral neural tube were interpreted as originating ventrally (McKee and Ferguson, 1984; Scherson et al., 1993). However, it is plausible that those cells were either neural crest cells that migrated from anterior or posterior to the ablated regions, or were

mesodermal in origin (Couly et al., 1996). Replacement from the ventral neural tube, which seems to be more the exception than the rule, provides a good scenario to consider why there are differences in regulative ability under virtually identical experimental conditions.

Differences in the origins of replacement cells in seemingly similar studies under similar conditions hint at how position and time of neural crest cell removal affect regulation. Evidence suggests that the location and time of neural crest cell extirpation influence the completeness of regulation and the source(s) of repopulating cells. As discussed, there is little regulation in the cardiac region compared to either the head or trunk, and the extent of trunk neural crest regulation falls short of that for cranial neural crest. Axial variation in regulation may reflect differences in cell proliferation at cranial, cardiac and trunk regions (Scherson et al., 1993), or a differential ability of the notochord to induce neural tube. Differences in regulation between specific neural crest cell derivatives demonstrate how the time/stage of neural crest cell extirpation affects regulation. Following trunk neural crest extirpations, dorsal root ganglia were largely missing from the ablated region, yet melanophores migrated into the void and created normal pigmentation (Suzuki and Kirby, 1997). Thus, because the time/stage of extirpation determines the population of neural crest cells removed, differences in regulative potential may be closely associated with the identity of extirpated cells.

Thesis goals

The ultimate goal of this research is to extend our understanding of regulation, especially with regard to regulation of neural crest cells.
To clarify how widespread regulation is among vertebrates, and to augment our knowledge of the potentials and limitations associated with regulation during early development, I test the hypothesis that teleosts are able to regulate for cell loss. As many fish species are easy to maintain and breed in the laboratory, they are excellent for developmental studies. The zebrafish, *Danio rerio*, was chosen to 'represent' teleosts in this study because the eggs are transparent, the chorion is easily removed, and the embryos develop rapidly – hatching in approximately 72 hours. The specific goals of my research were:

1. Clarify and extend what has previously been described on the origins, migration patterns and derivatives of trunk neural crest (TNC) cells in zebrafish. These data provide the framework needed to address whether embryonic cell populations can compensate for cell loss.
2. Determine whether there is regulation for zebrafish TNC cells, and ascertain what conditions are necessary for regulation. This was accomplished by bilaterally extirpating regions of TNC, including the uppermost layers of the neural keel, and asking whether (a) the amount of cells removed, (b) position along the embryonic axis,

and/or (c) timing of TNC cell extirpation affect regulative ability? Pigment cells will be used as landmarks for analyzing effects of extirpation.

3. Identify antibodies that label TNC-derived neural cells prior to 36 hours post-fertilization. Regulation may not be equal for all TNC derivatives. If a marker were available for visualizing neural cells early in development, such as those that contribute to dorsal root ganglia, regulation for neural derivatives could be compared to regulation for pigment cells.
4. Investigate potential mechanisms of regulation, including enhanced cell division and altered cell migration. The mechanistic aspects of regulation will be explored by extirpating constant lengths of trunk neural crest and (a) detecting mitotically active cells with bromodeoxyuridine (BrdU), (b) tracing cell migration with a vital lipophilic fluorescent dye, DiI. Moreover, DiI labeling will identify sources of replacement (regulating) cells.

Table 1. Evidence for regulation by four cell populations in response to loss of neural crest or placodal cells from cranial, cardiac or trunk regions

	CELL TYPE LOST					
	Cranial		Cardiac		Trunk	
Potential replacement cells	NC	PL	NC	PL	NC	PL
Neural Crest	Yes[1] (SK) ?[2] (G)	No[3]	No	Yes (G)	Yes (G, P)	?
Placode*	No	?	YES (EM, G)	?	?	?
Epi. Ectoderm	?	Yes	?	?	?	?
Neural Ectoderm	?	?	?	?	?	?

Notes: [1]'Yes' indicates regulation has been demonstrated in some form, even if only for specific derivatives (in parentheses). [2]'?' indicates evidence for regulation is questionable, needs further testing, or has not been tested. [3]'No' indicates existing studies have not found evidence for regulation. *Placodes contribute to the lateral line system in the trunk. **Abbreviations:** Epi, Epidermal; EM, ectomesenchyme; G, ganglia; NC, neural crest; P, pigment; PL, placode; SK, skeleton

Fig. 1. Generalized model for induction of ventral and dorsal portions of the neural tube, including differentiation of dorsal ectodermal cells into epidermal ectoderm, neural ectoderm and neural crest – shown as diagrammatic cross sections through neurula stage chick embryos. Arrows (→) indicate the direction of inducing or inhibiting (→ ?) activity. T-shaped lines (–|) represent areas where local molecules block the path of signaling molecules. **A:** As the neural folds elevate, Shh (Sonic Hedgehog, stippled) is expressed by the notochord and ventral neural tube; inducing activity extends dorsally towards the mid-neural tube. BMP (Bone Morphogenetic Protein, hatched lines) is expressed throughout the ectoderm. **B:** During closure of the neural tube, Shh expression is maintained ventrally in the developing neural tube and notochord, while BMP expression is now concentrated in the ectoderm at the contact point between the merging neural folds. Noggin (shading), which is expressed in dorso-medial regions of the somites, is thought to inhibit BMP activity (→ ?) and thereby induce neural tissue from dorsal ectoderm, while concurrently preventing BMP from exerting its role in somite differentiation (–|). **C:** Following closure of the neural folds and separation of epidermal and neural ectoderm, BMP expression is focused in mid-dorsal ectoderm and dorsal neural tube, where it is thought to play a role in inducing differentiation of neural crest cells. EN, endoderm; N, notochord; PL, placode; SO, somite.

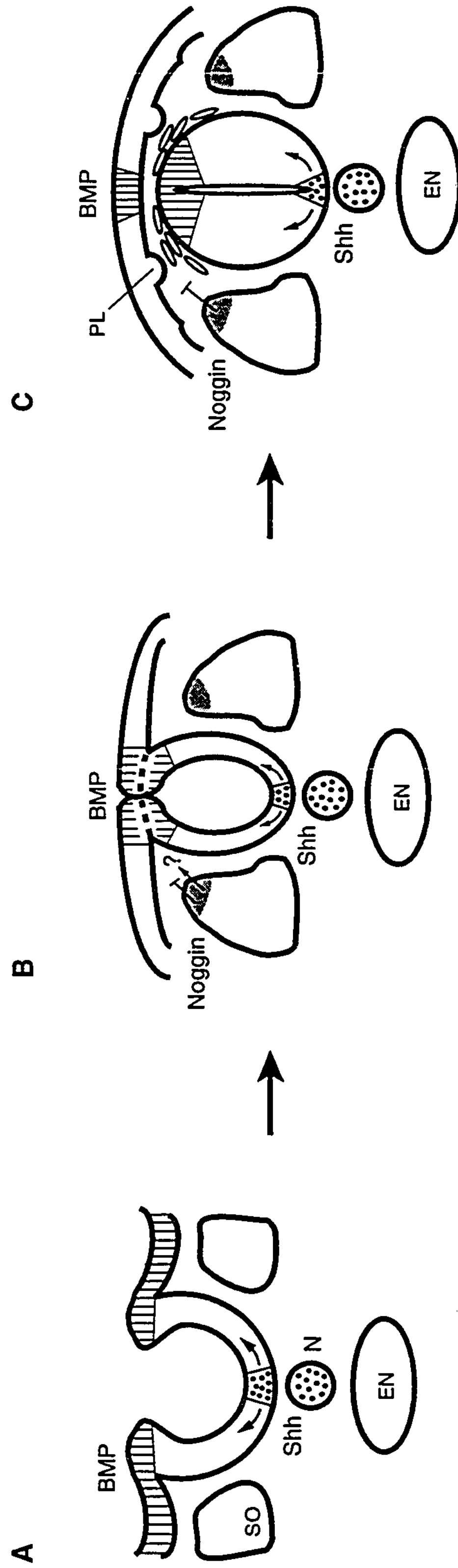


Figure 1

Fig. 2. Locations of cranial, cardiac and trunk neural crest cell populations along the embryonic axis (in this case, in a fish) and examples of corresponding derivatives. Horizontal bars demonstrate the regions which give rise to some of the major neural crest cell derivatives during normal development, but do not encompass all possibilities. For example, under experimental conditions, trunk neural crest cells may be able to produce dentine, or cranial neural crest may have the potential to form fin mesenchyme. A transition between cardiac and trunk neural crest populations is thought to occur between somites three and five; the true boundary remains unclear. C.T., connective tissue; FB, forebrain; MB, midbrain; R1-R7, rhombomeres 1-7; S1-S6, somites 1-6.

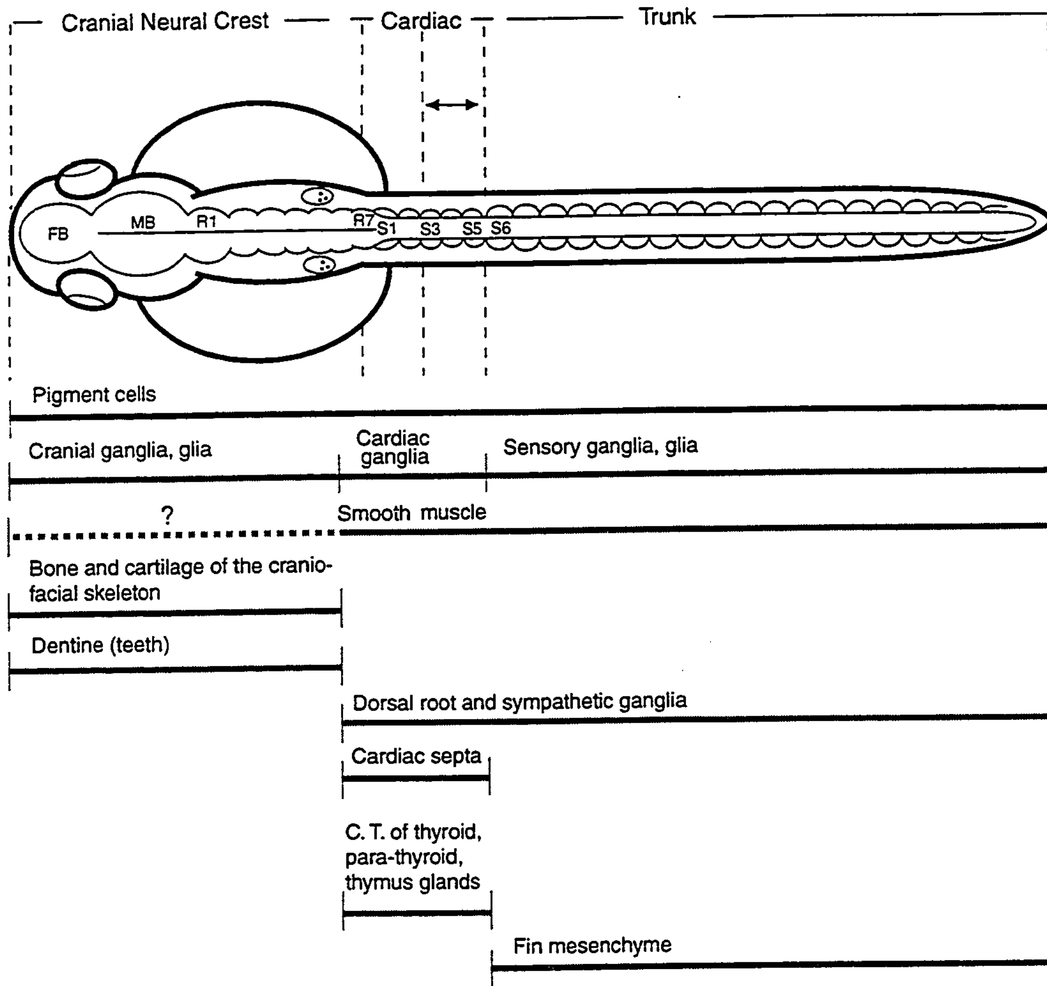


Figure 2

Fig. 3. Migratory pathways of TNC cells in teleost fish. Neural crest cells are known to migrate along two pathways in the trunk of teleost fish – a lateral path between the somites and epidermis (black cells), and a medial path between the somites and neural keel (striped cells). Notochord (n) and somites (so) are labeled for orientation.

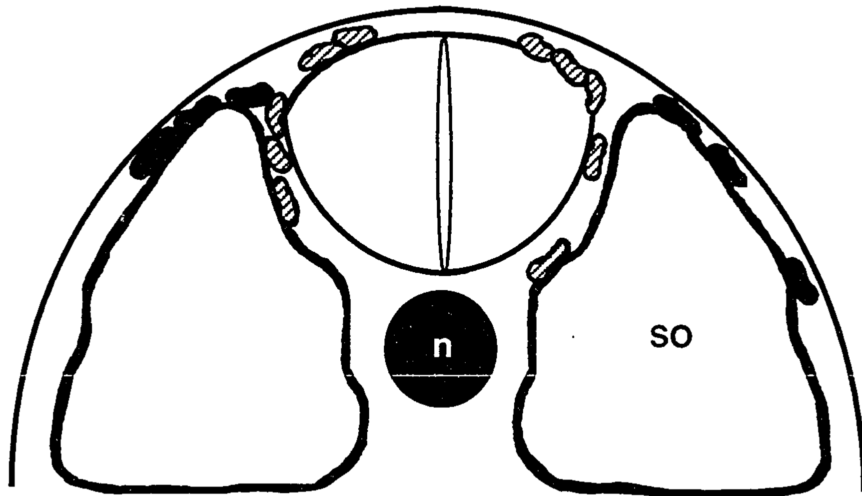


Figure 3

Fig. 4. Diagram of research approach. The encompassing hypothesis of my research is that cells in the trunk of zebrafish embryos regulate for the loss of TNC cells. To address this hypothesis, regions of TNC were extirpated and regulation for loss of TNC-derived pigment cells was assessed (Chapter three). Because cells in zebrafish embryos do exhibit regulation, I then asked 'What are potential mechanisms of regulation?' I hypothesized that increased cell division, altered cell migration, decreased cell death, and change in cell fate could be important for regulation, whether functioning alone or in concert with one another. I chose to investigate increased cell division and altered cell migration as possible mechanisms of regulation (Chapter four). In future studies it would be interesting to ask whether gene expression provides evidence that development is repeated ('recapitulated') during regulation.

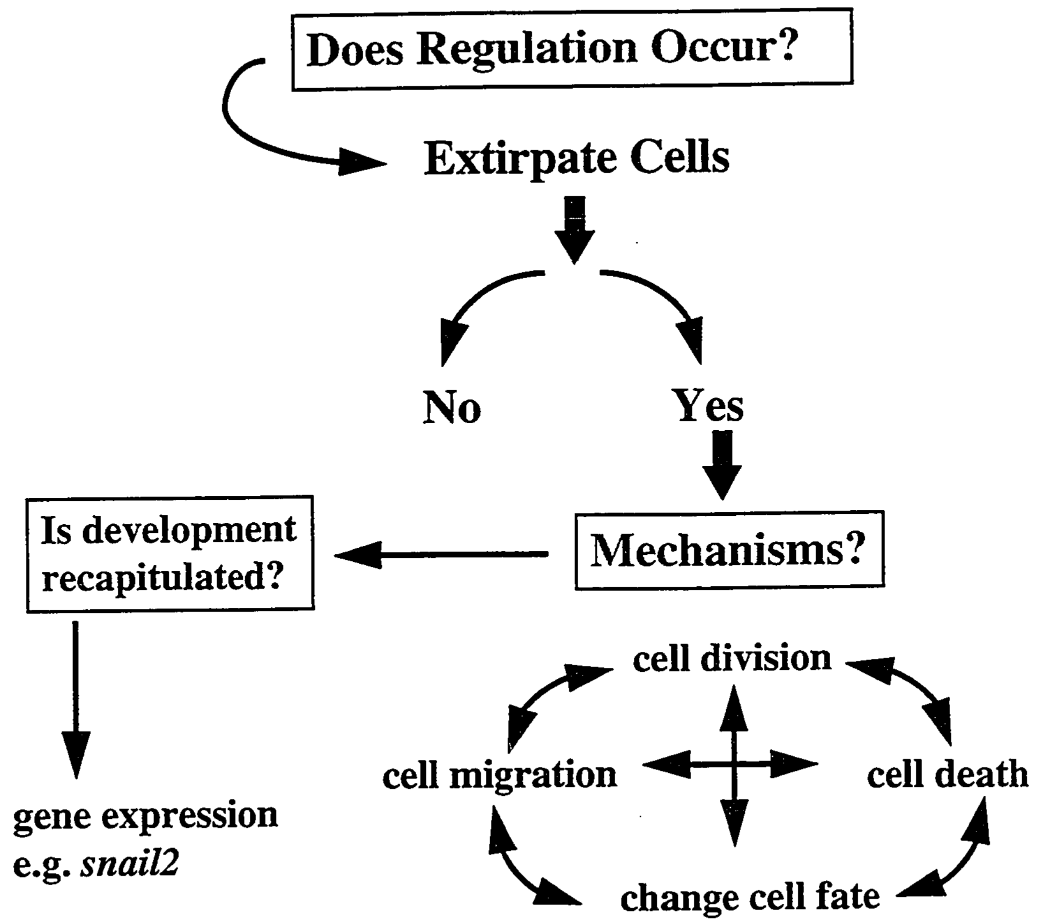


Figure 4

Chapter 2

Migration and Differentiation of Trunk Neural Crest Cells in Zebrafish (*Danio rerio*) Embryos

Introduction

A large diversity of craniate tissues (including skeletal, neural and connective) arise from embryonic populations of neural crest cells. Successful proliferation, migration and differentiation of neural crest cells are vital for embryonic survival and development; thus, it has been argued that these cells comprise a fourth craniate germ layer (Hall, 1998b, 1999). Neural crest cells are commonly referred to as either cranial, cardiac (vagal), or trunk according to where they originate along the embryonic axis and the derivatives they form (Fig. 2, from Vaglia and Hall, 1999). In general, cranial neural crest cells contribute to connective tissue, cartilage and bone of the craniofacial skeleton, odontoblasts of the teeth, and to neurons and glia of cranial ganglia (Hall and Hörstadius, 1988; Bronner-Fraser, 1995; Hall, 1999). Cardiac neural crest cells provide neuronal (supporting cells, autonomic neurons) and ectomesenchymal components to the heart, aorta and pulmonary arteries (Le Lièvre and Le Douarin, 1975; Kirby, 1988a,b). Trunk neural crest (TNC) cells produce pigment cells, sensory neurons, glia, sympathoadrenal and Schwann cells. (Bronner-Fraser, 1995; Hall, 1999).

The timing and patterns of neural crest cell migration are well established for several species that are often used as models of vertebrate developmental, such as the chick (*Gallus domesticus*), mouse (*Mus musculus*), axolotl (urodela; *Ambystoma mexicanum*), and frog (anura, *Xenopus laevis*) (Noden, 1975; Krotoski et al., 1988; Moury and Jacobson, 1990; Serbedzija et al., 1990, 1992; Erickson, 1993). Exploration of neural crest development in fish has been restricted primarily to the Japanese medaka (*Oryzias latipes*), platyfish (*Xiphophorus maculatus*), swordtail (*Xiphophorus helleri*), and zebrafish (*Danio rerio*) (Langille and Hall, 1988a; Sadaghiani and Vielkind, 1989, 1990; Raible et al., 1992; Hirata et al., 1997). Among teleosts, the zebrafish, *Danio rerio*, has become increasingly valuable as a model system for embryology and neurobiology, as well as for neural crest and genetic studies. Zebrafish embryos are easily removed from the chorionic membrane, develop rapidly (72 hours to hatch) and are a prime source of developmental mutants that can be crossed and propagated in the lab.

Many abnormalities generated during the early phases of human development (e.g. otocephaly, Treacher Collins Syndrome) are known to arise from defects in the development, proliferation and migration of neural crest cell populations (Johnston, 1975; Jones, 1990; Johnston and Bronsky, 1995). To understand the role of neural crest cells during embryogenesis, and subsequently, the origins of neural crest-derived defects, it is necessary to identify normal migratory patterns and derivative formation. Details on migration and differentiation of neural crest cells during development are

least resolved for populations of TNC cells. This study is intended to clarify and extend what has previously been described on the origins, migration patterns and derivatives of TNC cells in the zebrafish, *D. rerio*. These data will provide the framework needed to address, in chapters three and four, whether embryonic cell populations can compensate for cell loss, a phenomenon known as regulation.

Scanning electron microscopy, histology, fluorescent DiI labeling and *in situ* hybridization were used to evaluate TNC cell development and migration; DiI also was important for interpreting derivative formation. Numerous genes associated with development of the neural tube have been identified (e.g. *dorsalin-1, BMP-4, Pax-3, Wnt-1, Wnt-3a,* reviewed by Tanabe and Jessell, 1996), but none are specific to the first appearance of neural crest cells. One of the earliest genes known to be expressed by presumptive neural crest cells is *snail2* (*sna2*). *Sna2* is a member of the zinc finger gene family of transcription factors and has been implicated in the formation of mesoderm and neural crest (Thisse et al., 1993, 1995; Sefton et al., 1998). *In situ* hybridization with *sna2* was performed to determine the earliest appearance of neural crest cells in cardiac and trunk regions.

To date, no antibodies have proven reliable for visualizing precursor and migrating neural crest cell populations in zebrafish. The HNK-1 monoclonal antibody recognizes an epitope on the surface of neural crest cells in several vertebrates, including some teleost fishes (Erickson et al., 1989; Sadaghiani Vielkind, 1989, 1990; Hou and Takeuchi, 1994; Sadaghiani et al.,

1994; Hirata et al., 1997). However, in this study trials over a number of embryonic zebrafish stages revealed HNK-1 labeling of only two neural cells types – the neural crest-derived portions of trigeminal ganglia and non-neural crest-derived Rohon-Beard cells (see also Artinger et al., 1999; Cornell and Eisen, 1999).

Trunk neural crest cells in zebrafish migrate along two major pathways – medial and lateral – as previously described for other teleosts. Cell types and tissues formed, including sensory and sympathetic ganglia, Schwann (glial) cells and pigment cells, are consistent with existing descriptions of TNC derivative formation in a variety of vertebrates. Differences in the overall number of TNC cells that develop and temporal patterns of migration are discussed with regard to existing descriptions of zebrafish TNC cells. In addition, I provide the first documentation of a population of early-migrating TNC cells and characterize two major modes of migration that have received little attention in the literature. I use the term 'sheet migration' to describe how neural crest cells spread over the neural keel as an unsegmented, coherent sheet with cells in extensive contact with one another. 'Segmental migration' refers to more localized cell migration in streams over the lateral faces of somites, although cells still contact one another.

Materials and Methods

Zebrafish maintenance and breeding

Adult zebrafish were purchased from either local pet stores in Halifax, Nova Scotia, Canada, or from Boreal Laboratories, Ltd. (Ontario, Canada). Females and males were separated and kept in 10 gallon tanks at a temperature of 27-29° C on a 14 hour light and 10 hour dark cycle. Females tend to be chubby and darker in color, while males are slender and have a yellow hue which intensifies during breeding. Zebrafish were fed a combination of flake food and brine shrimp twice daily. Breeding colonies were established in ratios of one female per one or two males, with the most common arrangement being three females and three-five males. Eggs were collected in the evening and raised at 28.5° C in Hank's solution (Westerfield, 1995). Embryos sampled ranged in age from the 10 to 28 somite stages (ss) (see Appendix I for equivalence in hours of development).

Histology

For both paraffin and plastic sectioning, embryos were fixed in 4% paraformaldehyde. To section using paraffin, embryos were dehydrated in a series of methanol/PBS (Dulbecco's phosphate buffered saline – Freshney, 1987), infiltrated, embedded in low melting point wax at 52° C in a vacuum

oven, and cut at 5 µm. Sections were mounted on poly-L-lysine-coated slides to increase adhesion. Paraffin sections were stained with HBQ (Hall and Brunt's Quadruple Stain; Hall, 1986) or Ehrlich's Hematoxylin (Ehrlich, 1886).

A Leica Historesin Embedding Kit was used for plastic sectioning of embryonic tissue not intended for immunohistochemistry. Tissue was dehydrated in ethanol and infiltrated according to manufacturer's instructions. Specimens were oriented in embedding media in plastic moulds with tops and placed in a desiccator attached to a vacuum pump for at least 18 hours, or until the block hardened sufficiently. The corners of the block commonly remained sticky. Tissue sections intended for immunohistochemistry were embedded using an Immuno-Bed JB-4 mini kit (Polysciences, Inc., PA). For Immuno-Bed, tissue was dehydrated to 100% methanol, infiltrated in a 1:1 methanol/PBS:infiltration media for one hour, followed by 100% infiltration media for two hours with one change. Tissue was kept in infiltration media overnight at 4° C and embedded the next morning. In both cases, blocks were sectioned at 2 µm using glass knives on a retracting microtome. Sections were collected by floating them in water on slides at room temperature. Slides were dried on a slide warmer set to approximately 60° C. Plastic sections, stained with Lee's methylene blue-basic fuchsin (Kier, 1992), were viewed with 25x and 40x oil immersion lenses using either a Leitz Laborlux D compound microscope with a Wild MPS51 camera, or a Leitz Aristoplan fluorescent with a Wild Leitz camera.

SEM

Development and location of trunk neural crest cells were primarily determined using scanning electron microscopy (SEM). To remove the epidermis, dechorionated embryos were incubated in an enzyme solution (0.257 g Trypsin + 0.043 g Pancreatin in 10 mls PBS) at 4° C for 20-30 minutes. Embryos were then transferred to room temperature and covered with a thin layer of 1.0% agarose. A glass-pulled micropipette was used to tease the epidermis away from underlying cells, after which embryos were preserved immediately in Karnovsky's fixative. Following overnight fixation, samples were rinsed in 0.2 M cacodylate buffer at pH 7.4 with 20% sucrose. They were then post-fixed in OsO_4, rinsed a second time in 0.2 M cacodylate with 20% sucrose, dehydrated and critical point dried. Specimens were coated with gold using a 'SAMSPUTTER' prior to viewing. Because of individual variation, and differences associated with processing specimens for SEM, several embryos were used for each stage.

DiI

Fluorescent DiI (SP-$DiIC_{18}$(3)) was obtained from Molecular Probes, Inc. (D-7777) and diluted to a concentration of 2 mg/ml of 92% Dimethyl-formamide (DMF) and 8% H_20. Several forms of DiI are available for cell labeling, but because most function by attaching to the cell membrane, the

fluorescent label is easily lost from tissue during fixation. The DiI chosen for this study is retained by fixed tissue, thus enabling the label to be visualized in histological sections.

Immediately prior to use, DiI aliquots were briefly centrifuged to remove any precipitate that had formed. Thick-walled glass capillaries (OD 1.2 mm, I.D. 0.69 mm with filament) were pulled into micropipettes using a vertical pipette puller. Micropipettes were backfilled with 1.5 µl DiI, followed by 1 µl 0.2 M KCL and stored in glass jars in a 37° C incubator to slow crystallization of dye during injection sessions. At least 10 minutes were allotted for the dye to completely fill the micropipette tip.

For injections, embryos between somite stages 10 and 22 were embedded in 1% agarose. An empty glass-pulled micropipette was used to create an opening in the agarose and pierce the epidermis. The empty needle was replaced by a DiI-filled needle, and DiI was focally injected as a single two millisecond pulse under a Leitz Laborlux D compound microscope using a PV830 pneumatic picopump (World Precision Instruments) attached to a nitrogen air source. Embryos were injected at a constant tank pressure of 100 kPa and ejection pressure of approximately 30 psi. Holding pressure varied slightly according to the size of the micropipette tip opening, but generally ranged between 10 and 20 psi. Specimens were viewed under a Leitz Aristoplan fluorescent microscope (N2.1 filter) at hourly time intervals following injection.

In situ hybridization

Preparation of plasmid DNA

The plasmid pBluescript II SK(+) was provided by Dr. Christine Thisse at the Institut de Genetique et de Biologie Moleculaire et Cellulaire (IGBMC; Illkirch, France). pBluescript II SK(+) encodes a 520 base pair fragment of the *sna2* gene and includes an ampicillin-resistant gene (Fig. 5A). Plasmid DNA was transformed into *E. Coli* with standard transformation protocols (Sambrook et al., 1989) and selected on LB agar plates containing ampicillin. Plasmid DNA was purified using a large-scale DNA purification kit (Quiagen Plasmid Combi Kit, Santa Clarita, CA) following the manufacturer's protocol; sizes of the resulting *sna2* DNA plasmids were verified in a 1% agarose gel. Linearization of pBluescript II S(+) occurred at the xba1 site for antisense mRNA, and the kpn1 site for sense mRNA (control).

Synthesis and purification of RNA probes

Linearized DNA (*sna2* cut at site xba1) was purified using phenol/chisam extraction and verified on a 1% agarose gel with a lambda HindIII marker (Fig. 5B). The antisense mRNA probe was synthesized from linearized DNA with T7 RNA polymerase using an Rneasy total RNA kit from Qiagen Inc. and following instructions of the manufacturer. Presence of *sna2* antisense mRNA was verified on a 1% agarose gel run against a DNA 100 bp ladder (Fig. 5C). The RNA probe mixture contained formamide and

bromophenol blue. For a control, DNA was linearized at the xba1 site, and a sense mRNA probe was synthesized using T3 polymerase in the manner described above.

Results

Migration of neural crest cells

In fishes, neural crest cells segregate from a solid neural keel after the neuroepithelium separates from the epidermis during neurulation (Figs. 6A-C). Following segregation from the neuroepithelium, zebrafish trunk neural crest cells sit tightly wedged between overlying epidermis and underlying neural keel. It can be challenging to distinguish neural crest cells from the surrounding tissues (see also Lamers et al., 1981); moreover, somites are known to produce groups of mesenchymal cells that might be confused with migrating neural crest. In this study, trunk neural crest (TNC) cells were distinguished from overlying epidermis, including the basement membrane, by their shape and presence of filopodia.

Neural crest cell shape varied according to the state of migration. Trunk neural crest cells destined for the medial path between the neural keel and somites took on a bottle shape (Fig. 7B). Cells that migrated laterally over the somites were slightly flattened and round with numerous short filopodia (Fig. 7A). When migrating along the dorsal neural keel, cells were either flattened or elongated antero-posteriorly (Fig. 8D). It was difficult to

distinguish neural crest cells migrating along the lateral path from migratory somitic mesoderm (Horigome et al., 1999; Fig. 7B). Mesodermal cells also were spherical and may overlap the migratory territory of TNC cells.

SEM images revealed striking patterns of extensive TNC cell migration in the cardiac (somites one-five) and trunk regions beginning as early as the 12 somite stage (ss) (Fig. 7A). These patterns support and extend those described by Raible et al. (1992) by revealing:

- the presence of more cells than previously suggested
- the existence of waves of sheet-like (Fig. 7A) as well as segmental (Figs. 7C, 8B, C) migration along much of the trunk, including the yolk (Figs. 7A, 8B)
- migration over larger distances

10-12 ss

Trunk neural crest cells in zebrafish were found to migrate in three or four waves. It has previously been documented that TNC cells first emerge from the neural keel at approximately the 10 ss. According to Jesuthasan (1996), these cells migrate along a medial pathway between the neural keel and somites at the level of somites one-six. DiI injections from this study document the first wave of TNC cells as migrating slightly later, at the 12 ss, along a medial path in the cardiac (somites one-five) and anterior trunk regions (somites six-eight) (Figs. 9A-G). Although migration of neural crest cells from the cranial and cardiac regions was not emphasized in this study,

DiI was injected at the hindbrain/cardiac junction (just anterior to somite one) in several individuals. These injections revealed a population of neural crest cells migrating over the somites and onto the yolk.

A shift in *sna2* expression between the 10-12 ss provided further evidence for the earliest appearance of TNC cells (Figs. 10A, B). At the 10 ss, *sna2* is expressed diffusely in paraxial and lateral mesoderm and neuroepithelilum, consistent with descriptions by Thisse et al. (1993, 1995). *Sna2* expression also extends laterally from the midline to overlie the yolk in both hindbrain and cardiac regions (Fig. 10A). Towards the 12 ss, adaxial cells bordering the notochord strongly express *sna2*, as do cells in the ventral and medial somites (myotome and sclerotome, respectively; Fig. 10B). Non-expressing portions of the somites are visible as clear, block-shaped structures (Fig. 10B). The dorsal neural keel also expresses *sna2*, indicating onset of TNC cell migration (Fig. 10B). *Sna2* expression in the neural keel is maintained through approximately the 22 ss.

13-16 ss

At the 13/14 ss, a second wave of neural crest cells migrates from the head (mid- and hindbrain) and cardiac regions as an expansive sheet. *Sna2* expression at this stage is similar to the previous stage, with *sna2* being newly expressed at the junction where cells from lateral sides of the neural keel meet at the dorsal midline (Fig. 10C). Furthermore, *sna2*-expressing cells are visible over the yolk lateral to the anterior trunk, and migrating anteriorly

across the yolk from the hindbrain (Fig. 10D). The distinction between somite regions that do or do not express *sna-2* is not as obvious at the 14 ss, or at several subsequent stages.

During 'sheet migration,' compact layers of large neural crest cells migrate laterally over the somites and onto the yolk in a continuous dorso-ventral stream (Fig. 7A). As neural crest cells invade areas lateral to the neural keel, furrows between somites are concealed. At any level – cranial, cardiac or trunk – once cells reach the yolk, they engage in 'yolk crawling' by extending numerous lamellar-like processes (filopodia) amidst an extracellular matrix (Figs. 7A, 8B). As mentioned earlier, these cells are so closely associated with one another and surrounding tissues that it is even difficult to distinguish between neural crest, epidermis, basement membrane and somites in histological sections (Figs. 6A-C). Thus, I used cell orientation and layering beneath the epidermis as criteria for recognizing newly emerging neural crest cells in sections.

By the 15 ss, sheet migration is visible in the trunk just posterior to the cardiac region (somites three-seven) and continues to spread caudally during development. Synchronous with ongoing sheet migration in the mid- and posterior trunk, a third wave of neural crest cells emerges in the head and cardiac regions between the 15-16 ss (Fig. 7C). This third wave of neural crest cell migration is characterized by a transformation in how cells associate with one another and their environment – the lateral migratory path – and involves a transition from 'sheet-like' to 'segmental' migration. While

segmental migration proceeds anteriorly, a lull in TNC development is observed more posteriorly as sheet migration gradually ceases and few cells emerge from the neural keel (Fig. 7D).

17-23 ss

The 17 ss marks the beginning of true segmental migration in the trunk, where cells migrate in groups over the lateral face of somites directly beneath the epidermis. During the 17 ss, cells gradually separate into groups between somites three-seven and seven-twelve (Figs. 8B, C). While some neural crest cells continue to bridge clefts between somites, most cells tend to aggregate over individual somites. Also by the 17 ss (Fig. 11B), somites have elevated and lie more ventral than lateral to the neural keel; in younger stages (e.g. 14 ss) somite pairs are distinct from one another and lie more lateral to the neural keel (Figs. 6C, 7D). This morphological change occurs in concert with a raising and narrowing of the neural keel. Posterior to somite 12, cells continue to migrate as a sheet, or remain over the neural keel (Figs. 8D, 11A). The first cells to leave the posterior trunk as new somites are added generally exhibit sheet migration. However, it is difficult to classify migratory patterns in the posterior trunk because neural crest cells from more anterior regions mix with those that have just formed. Cells migrating from more anterior locations tend to overlie and be oriented obliquely to newly emerging or migrating cells.

Concurrent with lateral TNC migration in the mid-trunk at the 18-20 ss, cells continue to penetrate the medial migratory pathway between the neural keel, notochord and somites (Fig. 11B). Cells migrating along the medial path at these later stages may represent a fourth wave of TNC, especially if they are forming derivatives not represented by earlier populations. However, it is equally conceivable that they represent a continuation of the third wave. Classification of this phase of migration awaits further study using clonal cell culture or cell labeling.

The migratory status of neural crest cells along the embryonic axis is most variable between somite stages 19-22. Cells in the posterior trunk exhibit sheet migration, while more anterior cells undergo segmental and/or sheet migration according to their axial location. At all levels, groups of TNC cells migrate posteriorly and/or anteriorly atop the neural keel and potentially add to preexisting populations, or fill in regions where cells do not emerge.

Although lateral migration persists in the trunk beyond the 20 ss, fewer new cells are emerging. While the anterior half of the trunk is devoid of neural crest cells, cells continue to migrate along the lateral path in the posterior trunk and tail as new somites are generated. Migration also is exhibited by differentiated pigment cells. Dorsal expression of *sna2* decreases by the 22 ss; however, expression in notochord adaxial cells and mesoderm persists through the 28 ss. Staining also is visible anterior and posterior to the otic region (Fig. 10G).

24 ss and beyond

By the 24-25 ss the dorsal neural keel is smooth, with no visible neural crest cell migration along the trunk, except towards the tail tip. Because trunk neural crest derivatives are ultimately present along the entire embryonic axis, the posterior trunk might be patterned by a small population of neural crest cells that emerges from the neural keel as the last few somites form, or by cells migrating from more anterior regions (Figs. 12A, B). Beyond the 26 ss, somites are no longer smooth, compact structures. Rather, they appear to be breaking down and rearranging into muscle and connective tissues.

Trunk neural crest patterning and derivative formation

Focal DiI injections confirmed timing of TNC cell migration in the anterior-mid trunk and provided unique data on derivative formation. Injections were replicated several times to compensate for developmental variation. For consistency, cardiac and TNC cells were labeled over the length of two somites between the level of somites four and eleven. No neural crest cells were observed migrating in 10-11 ss embryos after DiI labeling of the dorsal neural keel in that region (Table 2).

12-13 ss

At the 12-13 ss, DiI labeling between somites four-eight revealed TNC cells migrating medially between the neural keel and somites (Figs. 9A-G). These cells migrate rapidly away from the injection site in a ventral direction

and within 15-20 minutes form a 'beaded' segmental array down the medial faces of adjacent somites. Within two hours (16 ss), labeled cells have migrated the length of three somites in the anterior direction and one somite posterior. The cells also appear to be linked as a chain between the ventral notochord and dorsal aorta (Figs. 9A, C, H). It is difficult to definitively characterize these cells without antibodies specific to early trunk neural crest precursors. However, the location and pattern of segmental migration that persists through embryogenesis (at least 36 hours post-fertilization) suggests that these are either glial (Schwann) cells destined to form part of the dorsal root ganglia, cells that provide a migratory tract for TNC cells destined to form more ventral derivatives such as enteric and sympathetic ganglia, or neural precursor cells for the primary sympathetic chain ganglia (Table 2).

When TNC cells in 12 ss embryos were injected quite anteriorly (level of somites four-six), cells were observed migrating over the yolk, confirming the results obtained with SEM. DiI-labeled neural crest from these anterior injections later contributed to enteric and/or sympathetic ganglia, the aortic wall, and/or tail reticular cells – a labyrinth-like network of channels that connect the caudal artery and vein (Westerfield, 1995; Table 2; Figs. 9H, 13A). It is not clear whether cells traversed the yolk and then migrated ventral to the somites to reach their final locations adjacent to the notochord/aorta or gut, or whether a separate population of cells migrated along the medial path. In either case, labeled neural crest cells disappeared from view shortly after injection and were not seen again until the derivatives began to take form

approximately 30 hpf (hours post fertilization). The possibility that the yolk is a migratory bridge for TNC cells destined to contribute to the developing heart was not thoroughly investigated in this study.

Several injections caught the earliest migration of TNC cells along a lateral, or external path between the keel, somites and epidermis. DiI-labeled cells stayed dorsal but migrated two somites posterior to the injection site (e.g from somites five-seven to five-nine) and contributed to xanthophore pigmentation. Precursor dorsal pigment cells, especially xanthophores, tend to congregate over the neural keel and may migrate one to three somites in anterior and/or posterior directions. Cells that migrate along the medial path at this stage exhibit even greater potential for migration. Neural crest cells may migrate up to 18 somites length posterior when injected at the level of somites five-six, and at least eight somites length posterior when injected at the level of somites seven-eight.

14-15 ss

Trunk neural crest cells labeled with DiI between the level of somites four and eight at the 14-15 ss gave rise to glia, dorsal root and enteric ganglia, and pigment cells (Table 2). In approximately half of the embryos, TNC cells were labeled at the beginning of sheet migration Within 20 minutes these cells move rapidly up to two somites posterior and slightly ventral to the injected site. Within two hours the labeled neural crest cells resemble xanthophore and melanophore pigment derivatives (Figs. 14 A-D). When 15

ss embryos were DiI injected more anteriorly (somites four-six), labeled TNC cells differentiated close to the injection site.

Also in half of the injected embryos, DiI-labeled TNC cells were observed migrating along the medial path and forming glial/neuronal derivatives, as in the 12-13 ss. However, TNC cells labeled at the 14-15 ss exhibited less extensive migration than those at the 12-13 ss over the course of 36-48 hours. Medial migration of TNC cells revealed an intricate coordination between the age of an embryo and the somite level of TNC migration. For instance, at the 14 ss DiI injection at the level of somite six resulted in medially stained glial and neural precursors. At the 15.5 ss, DiI-labeled cells were only found along the medial path if the injection occurred posterior to somite six. This exemplifies how migration progresses from anterior to posterior axial levels in a manner that at least partially corresponds with the age of the embryo.

16 ss

In 16 ss embryos neural crest cells were injected at the level of somites five-nine. DiI labeling at this stage, especially when it occurred at the level of somites eight-nine, yielded little data (Table 2). The lack of DiI-labeled TNC cells is confirmed by the lull in migration demonstrated with SEM in the mid trunk. In one embryo DiI labeled a small population of TNC that differentiated as three iridiophores. Labeling was occasionally observed dorsal to the aorta when DiI was injected between somites seven-nine. As in

12-13 ss embryos, these cells exhibited rather extensive migration (up to eight somite lengths posterior).

17-19 ss

Trunk neural crest cells injected with DiI at the level of somites seven-eleven in 17-19 ss embryos became pigment cells – including dorsal melanophores, xanthophores, and in one case, iridiophores – and contributed to dorsal root and sympathetic ganglia, and ectomesenchyme of the aorta (Table 2; Figs. 13B, 15A-H). Labeled cells were visible in the dorsal fin folds of two embryos, but did not correspond to a particular stage or level of injection. DiI injections at the level of somites seven-eight in 18 ss embryos revealed surprisingly extensive migration. In one individual, precursor pigment cells migrated three somites anterior to the site of DiI injection and 25 somites posterior. Trunk neural crest cells labeled at somite stages 18/19 and older consistently traveled further in the posterior and lateral directions compared to younger stages. Extensive dispersion of TNC-derived cells over the course of 36 hpf suggests not only that large numbers of cells develop from the neural keel at specific locations and stages, but also that cells, especially pigment, divide along their migratory paths.

Some TNC cells labeled at the level of somites six-nine in 18-19 ss embryos migrate medial to consecutive somites (Figs. 15B-D, F). This is reminiscent of cells observed migrating medially in younger stages and raises the possibility that two separate populations of TNC contribute to the

development of dorsal root ganglia. DiI-labeled TNC in younger stages may contribute glial cells to developing dorsal root ganglia. In embryos 18 ss and older, it is likely that labeled cells form the neuronal components of dorsal root ganglia, as several studies have documented the first migration of dorsal root ganglia neuroblasts at these later stages (Laudal and Lim, 1993; Raible and Eisen, 1996). Furthermore, medial migration at the 18 ss and beyond is supported by histological sections (Fig. 11B).

20-22 ss

TNC migration and derivative formation in 20-22 ss embryos is similar to stages 18-19. Migration is extensive, especially at stages 19-21, and cells that migrate along a medial path disappear from view for several hours after injection. They are later visualized as pigment cells and as contributing to dorsal root, sympathetic and enteric ganglia, and the aorta. All DiI injections labeled precursor pigment cells in dorsal and/or ventral-lateral locations; approximately 50% of injections labeled derivatives in the dorsal root, sympathetic and posterior enteric ganglia. Cells injected posterior to the level of somite 10 were found in the dorsal fin fold. For example, within one hour, TNC labeled at the level of somites nine-ten in 20 ss embryos migrate two somites anteriorly and posteriorly, as well as ventral-medially and laterally. At 20 hours post-injection, labeled cells contribute to dorsal root ganglia at the level of somites two-five, dorsal melanophores at the level of somites six to 16, lateral melanophores at the level of somites 12 to 20, and enteric ganglia

posterior to somite eight. Tail reticular cells also are labeled. Beyond the 22 ss, cells are primarily differentiating at their final locations, with the exception of pigment precursors that remain mobile through embryogenesis and into the larval period.

Discussion

Migration of neural crest cells

SEM was used to investigate development and migration of zebrafish trunk neural crest cells. Comparison of neural crest cell morphology between vertebrates such as fishes and lampreys versus amniotes reveals distinct and consistent differences in cell size and shape. Namely, neural crest cells in fishes and lampreys are larger and rounder than their amniote counterparts (Löfberg et al., 1980; Sadaghiani and Vielkind, 1989; Horigome et al., 1999).

During neurulation in fishes, the neural ectoderm progressively thickens in an anterior to posterior direction to form a solid keel of cells (Sadaghiani, 1989; Sadaghiani and Vielkind, 1990; Schmitz et al., 1993; Papan and Campos-Ortega, 1994). Neural crest cells segregate from the neural keel (neuroepithelium) after the neuroepithelium separates from non-neural ectoderm. In most other vertebrates, neural crest cells segregate from the neuroepithelium before, during or following fusion of neural folds, depending on the organism and position along the neural axis (Schroeder, 1970; Noden, 1975; Weston, 1983; Meier and Packard, 1984; Lumsden et al.,

1991; Serbedzija et al., 1992; Epperlein and Löfberg, 1993; Erickson, 1993; Morriss-Kay et al., 1993; Sechrist, 1995). Zebrafish neural crest cells have been described as being larger and less numerous (10-12 cells per segment) than those of other vertebrates such as the chick (Raible et al., 1992; Raible and Eisen, 1996). This observation has never made sense to me considering the number and variety of derivatives that arise from teleost neural crest cells. While zebrafish neural crest cells are definitely large, SEM images and DiI injections from this study demonstrate the presence of more cells than previously documented. Development of populations of TNC containing larger numbers of cells more closely corresponds to the extensive migration and derivative formation observed along the length of the trunk.

In several fish species, neural crest cells follow two migratory pathways – a medial path between the neural tube, notochord and somites, and a lateral path between the ectoderm and somites (Lamers et al., 1981; Sadaghiani and Vielkind, 1989, 1990; Raible et al., 1992; Sadaghiani et al., 1994). The medial and lateral migratory routes correspond to the ventral and dorsolateral paths seen in amniotes. However, TNC cells in fishes travel within the space between the neural tube and somites, rather than through the somites, as they do in amniotes (Lamers et al., 1981; Serbedzija et al., 1990; Laudal and Lim, 1993; Erickson, 1993; Smith et al., 1994; Krull et al., 1995). Similar to the platyfish and swordtail, populations of TNC cells in zebrafish migrate in sequential waves – firstly along a medial path, secondly along a lateral path, and thirdly along a medial path.

The first wave of TNC cells migrates along the medial path

A specific pattern and pathway of migration is associated with each major population of TNC cells that leaves the neural keel. In amniotes, the first neural crest cells to leave the neural folds migrate along the ventral path (Loring, 1987; Newgreen, 1989; Serbedzija et al., 1989, 1990). Likewise, in zebrafish, the first cells to segregate from the neural keel migrate along the corresponding medial path, specifically at the level of somites one-six between the 10-14 ss (Raible and Eisen, 1996). Similar results have been obtained for the teleosts *Xiphoporous* and *Oryzias latipes* (Sadaghiani and Vielkind, 1990; Sadaghiani et al., 1994). Interestingly, in axolotls the pattern differs; the initial neural crest cell population migrates laterally rather than medially (Löfberg et al., 1980).

The second wave of TNC cells exhibits 'sheet-like' migration along the lateral path

Raible et al. (1992) described TNC cells at any axial level as entering the lateral path approximately four hours later than first entry onto the medial path. This would suggest, for example, that if cells enter the medial path at the 12 ss, no lateral migration would occur until the 20 ss (Jesuthasan, 1996). No lengthy delay in timing of TNC migration between the medial and lateral paths was observed in this study. Trunk neural crest migrated along the lateral path two-four somite stages after the onset of medial migration. At later stages lateral and medial migration were simultaneous. Trunk neural crest cells also migrated distances equivalent to several somite lengths in the

anterior and posterior directions. Newly emerging TNC cells in the anterior trunk may migrate one-three somites anteriorly and as far posterior as somite 25. Pigment cells exhibit extensive migration en route to their terminal dorsal or ventro-lateral positions. Cells destined to contribute to enteric and sympathetic ganglia or to the aorta first migrate ventrally along the medial pathway. Upon reaching ventral tracts located between the notochord and aorta, or between the aorta and gut, these cells may migrate large distances posteriorly.

Between the 14-15 ss, neural crest cells migrated laterally as a uniform sheet over the somites and yolk in the hindbrain and cardiac regions (Fig. 7A). By the 15 ss, sheet migration had extended to the level of somites three-seven, and proceeded in a rostral-caudal direction during development. Although, as pointed out by Weston (1970), neural crest cells migrate individually, they are often associated with other neural crest and appear as a continuous sheet. Sadaghiani and Vielkind (1989) describe a similar phenomenon of neural crest cells arranged as a sheet between the otic vesicle and the first somite in *Xiphophorus* (swordfish).

On the lateral path, neural crest cells extend numerous filopodia, yet migrate within a relatively sparse extracellular matrix (ECM), compared to the extensive ECM found in amphibians (Löfberg et al., 1980) and other teleosts (Sadaghiani and Vielkind, 1987). Moreover, there is little mention in the literature of extensive sheet-like migration along the entire embryonic axis, or of neural crest cell migration over the yolk (Sadaghiani and Vielkind,

1989). It remains unclear what cell types arise from the sheet of migrating neural crest, but precursor pigment cells and ectomesenchymal cells of the heart and vessels are good candidates (Bockman et al., 1987; Erickson, 1993; Olsson, 1994; Sadaghiani et al., 1994; Kelsh et al., 1996).

A third wave of TNC cells migrates segmentally along the lateral path

Between the 15-17 ss, neural crest cells in the head and cardiac region complete sheet migration and a new wave of neural crest begins to migrate segmentally over the somites (Fig. 7C, 8B). By the 17 ss, neural crest cells are migrating segmentally in the anterior-mid trunk, while sheet migration persists more posteriorly. Segmental migration of neural crest cells in the head is a common pattern among amniotes and teleosts (Sadaghiani and Vielkind, 1989; Lumsden et al., 1991; Bemis and Grande, 1992; Serbedzija et al., 1992), while segmental migration through somites in the trunk has been well documented for birds (Teillet et al., 1987; Serbedzija et al., 1989, 1990; Krull et al., 1995). In teleosts, TNC cells migrate segmentally on the lateral path external rather than internal to the somites (Sadaghiani and Vielkind, 1989). It is thought that factors extrinsic to the neural tube, such as sclerotomal influences, guide segmental migration of TNC cells (Lumsden et al., 1991; Serbedzija et al., 1992).

A potential fourth wave of TNC cells migrates along the medial path

At the 18 ss a new population of TNC begins migrating on the medial path. These cells represent the second group of medially migrating neural

crest detected by Raible et al. (1992) in embryos older than the 14 ss (Fig. 11B). Migration of a second group of neural crest cells along the medial path appears to be simultaneous with ongoing migration on the lateral path.

In situ hybridization with *sna2*

Whole mount *sna2* expression helped pinpoint transitions in neuroepithelial tissue and mesoderm associated with the beginning of TNC cell migration. Many aspects of *sna2* expression agreed with the original descriptions by Thisse et al. (1995). Moreover, analysis of SEM images and DiI extended descriptions regarding localization of *sna2* transcripts at the early stages. For instance, it was thought that migrating neural crest cells do not express *sna2;* at the 10 ss *sna2* expression was characterized as being widely dispersed in the mesoderm, with no evidence of expression by presumptive neural crest cells (Thisse et al., 1995). However, *sna2* expression is visible over the yolk lateral to the hindbrain and cardiac regions. Because SEM and DiI data show that hindbrain/cardiac neural crest cells migrate as a sheet over the yolk at the 10 ss, it seems likely that migrating neural crest express *sna2* at the onset, and perhaps during early phases of migration.

Expression of *sna2* by migrating neural crest cells is further supported by changes in *sna2* expression lateral to the neural keel. Beginning at the 12 ss, *sna2* is expressed preferentially by the posterior borders of somites (myotome and sclerotome). Non-expressing regions are clear and block-shaped. Interestingly, beginning with 14-15 ss embryos, *sna2* appears to be

expressed by entire somites. Portions of somites that do not normally express *sna2* may be masked by *sna2*-expressing TNC cells that migrate laterally over the somites and onto the yolk (Fig. 10E, F).

Sna2 is expressed diffusely in the dorsal neural keel starting with somite stage 10 and continuing through stages 22-23. This expression corresponds to the length of time neural crest cells develop in the trunk. Although whole mount *sna2* expression was a good indicator of basic neural crest patterning, details could be better resolved with histological sections.

Differentiation of TNC cells

To confirm SEM data on the timing of TNC development and migration, regions of pre-migratory crest cells were labeled with fluorescent DiI. More importantly, DiI-labeled cells could be traced through at least 60 hpf to investigate derivative formation. DiI was injected the length of two somites between the levels of somites four-12. Injections were replicated several times in different embryos to ensure the specificity of the injection – that only neural crest or dorsal neural keel were labeled, and not adjacent mesoderm. Repeated injections also helped account for developmental variation in the time of onset of migration at different axial levels. Single cell injections simply do not provide complete information to elucidate patterns in migration and differentiation of TNC populations.

Derivative identification was largely based on whole mount morphology and histological sections over a 36-48 hour time interval following DiI injections. As early as the 12 ss, TNC cells were observed migrating along the medial path between neural keel and somites. Several studies using chick embryos have traced the origins of early migrating neural crest cells to specific locations along the embryonic axis. Although axial levels in chick are not equivalent to zebrafish, the chick system allows for comparison of derivatives that arise from particular regions in a rostral-caudal sequence. For instance, in chick, parasympathetic neurons, enteric neurons of the entire gut, and glia arise from the vagal level (somites one-seven) (Le Douarin, 1982; Purves and Lichtman, 1985; Jacobson, 1991; Hearn et al., 1998). DiI labeling of neural crest cells from this level in zebrafish similarly revealed contributions to at least sensory ganglia, the aorta and tail vascularization. Labeling of sympathetic and enteric ganglia and of the aorta also has been described for the rat (Pisana and Birren, 1999).

Chick neural crest cells from the level of somites 8-27 form the sympathetic neural chain (Jacobson, 1991) and most enteric neurons of the foregut (Hearn et al., 1998). Cells from the level of somites 18-24 colonize adrenal medulla, while cells from posterior to the level of somite 27 form enteric neurons of the caudal midgut and hindgut (Jacobson, 1991; Hearn et al, 1998). Zebrafish embryos were not injected posterior to somite 11, but injections anterior to somite 11 extensively labeled sympathetic ganglia, especially in 17 ss or older embryos. Labeled cells were not found in the

adrenal medulla, which might be expected since neural crest cells were not injected at the level appropriate for differentiation of adrenergic neurons.

One frustration in identifying cells that migrate at early stages is lack of antibodies specific to zebrafish TNC derivatives, especially neural (e.g. Erickson and Reedy, 1998). This was primarily an issue with regard to DiI-labeled cells that migrated ventrally along the medial segmental path as early as the 12 ss (Fig. 9A-G). While labeled cells were in the correct position and arrangement to be precursor dorsal root ganglia cells, studies have shown that dorsal root ganglia cells do not normally migrate until the 18 ss in zebrafish (Raible et al., 1992; Raible and Eisen, 1996). This was confirmed by the presence of precursor dorsal root ganglia cells that migrated later on the medial path (Figs. 15A-G). One possibility is that the labeled cells are glial (e.g. Schwann) cells that will surround peripheral nerve fibers. Alternatively, these cells could be early-migrating neurons or glia that provide a substrate for extending growth cones and later migration of neurons, thus creating a scaffold to be followed by other cells (Jacobs and Goodman, 1989; Korzh et al., 1993). Purves and Lichtman (1985) found that glia in *Drosophila* may `guide' longitudinal and commissural axon tracts, although glial cells ultimately ensheath and support neuronal processes. It is also possible that these cells eventually form portions of the primary sympathetic chain ganglia.

I can claim with certainty that neither Rohon-Beard cells nor their associated axons are labeled. Rohon-Beard cells are transitory sensory neurons located on either side of the dorsal edges of the neural keel. They

have central axons that migrate ventrally and peripheral neurites that innervate the skin, neither of which are aligned segmentally with adjacent somites (Taylor and Robert, 1983; Canger et al., 1998). Moreover, the dorsal cell bodies of Rohon-Beard cells were not DiI-labeled at any stage, and labeling would be expected if cells other than neural crest were injected. The labeled cells also were not motoneurons. Motoneurons arise from the ventral neural keel (opposite the middle myotomes) rather than dorsally, and axons then extend ventrally around the notochord (Weiss, 1968; Beattie and Eisen, 1997; Zeller and Granato, 1999).

With the onset of lateral migration by a few cells in 14-15 ss embryos, and with many more cells at the 17-19 ss, precursor pigment cells are dispersed dorsally, laterally, and ventrally throughout the embryo in a pattern that is common among vertebrates (Bagnara, 1983; Epperlein and Löfberg, 1990; Erickson, 1993; Raible and Eisen, 1994; Parichy, 1996). The final TNC cell derivatives are distributed via the medial path in 18 ss and older embryos. Some overlap was found in the derivatives formed by neural crest cells migrating medially at later stages versus those that migrated earlier along the medial path. DiI-labeled cells that migrated along the medial path in later stage embryos contributed to dorsal root and sympathetic ganglia, aorta and tail reticular cells. Sympathetic ganglia form a chain along the dorso-lateral surface of the aorta and later extend axons dorsally. The pattern of DiI labeling that resulted from injections performed between the 12 and 19 ss

closely resembles descriptions of neural crest-derived structures in the cyprinid *Barbus conchonius* (Lamers et al., 1981).

Potential contribution of neural crest cells to the dorsal fin fold

The caudal and pectoral fin folds in zebrafish develop from a structure that is similar to the ectodermal portion (the apical ectodermal ridge, AER) of tetrapod limb buds in both morphology and gene expression (Dane and Tucker, 1985; Van Eeden et al., 1996). Soon after AER formation in the fin bud, the ectoderm begins to elevate and bend back on itself by pinching inwards (Wood, 1982; Thorogood and Wood, 1987; Wood and Thorogood, 1987; Thorogood, 1991). The resulting fin fold – seen in larval fishes and amphibians – is composed of epithelial and connective tissues and supported by a core of mesenchyme cells thought to be neural crest in origin (Terentiev, 1941; Bodenstein, 1952; Balinsky, 1975; Thorogood and Wood, 1987; Krotoski et al., 1988; Löfberg et al., 1990; Smith et al., 1994a; Graveson et al., 1995).

Experimental work in urodele amphibians provides evidence that induction of the median fin fold is dependent on TNC cells. For instance, fin folds develop when TNC cells are transplanted under the epidermis in other parts of the embryo, such as the head or yolk sac. Unlike trunk neural crest cells, cranial neural crest cells are not able to induce fin fold development, even in the trunk where fin folds are normally induced (Du Shane, 1935; Terentiev, 1941; Twitty and Bodenstein, 1941; Bodenstein, 1952; Balinsky, 1975). Extirpations of the neural folds/TNC cells have produced a diversity of

fin fold morphologies, from total fin fold absence in extirpated regions to incomplete (smaller) and complete, normal fin folds (Du Shane, 1935; Bodenstein, 1952). Despite the effect of TNC cell removal on fin fold development in urodeles, Newth (1956) only 'provisionally' concluded that fin mesenchyme is of neural crest origin following extirpation of TNC cells in the lamprey.

More recently, Smith et al. (1994a) complemented experimental work in amphibians and lampreys by tracing migration of TNC cells in zebrafish using DiI labeling. Their results suggest that TNC cells contribute to the dorsal, median, and caudal fins in teleosts. HNK-1 antibody labeling of neural crest cells in the swordtail and lamprey also supports a neural crest contribution to the fin fold (Hirata et al., 1997). In my study, DiI injections between the levels of somites four to eight only sporadically produced labeled cells in the middle core or dorsal margin of the fin fold. If TNC cells were injected at the level of somites underlying the future site of the dorsal fin fold, labeling appeared more frequently in the fin.

This is not conclusive evidence that TNC cells contribute directly to fin fold development. In some instances DiI injection disturbed the epidermis, so that the fin fold was lower than normal, or developed with a ragged edge. What the data do suggest is that contact between epidermis and underlying TNC cells is important for fin fold induction. If neural crest cells do contribute to the fin fold structure, it is difficult to pinpoint when and where these cells arise; injections at various stages result in DiI-labeled fin folds.

Neural crest cells may disperse from the neural keel early in development and wait over the neural keel for a cue to form fin. Alternatively, if only mesodermal cells contribute mesenchyme to fin fold development, they might be labeled inadvertently through contact with DiI crystals stuck to the ventral side of the epidermis.

Concluding Remarks

This study has described patterns of normal migration and differentiation of TNC cells in zebrafish. In concert with existing descriptions, the new data provide a more complete understanding of the development of this critical embryonic cell population. They also provide baseline data for studies on regulation ('regeneration') of neural crest cell populations, the subject of chapters three and four.

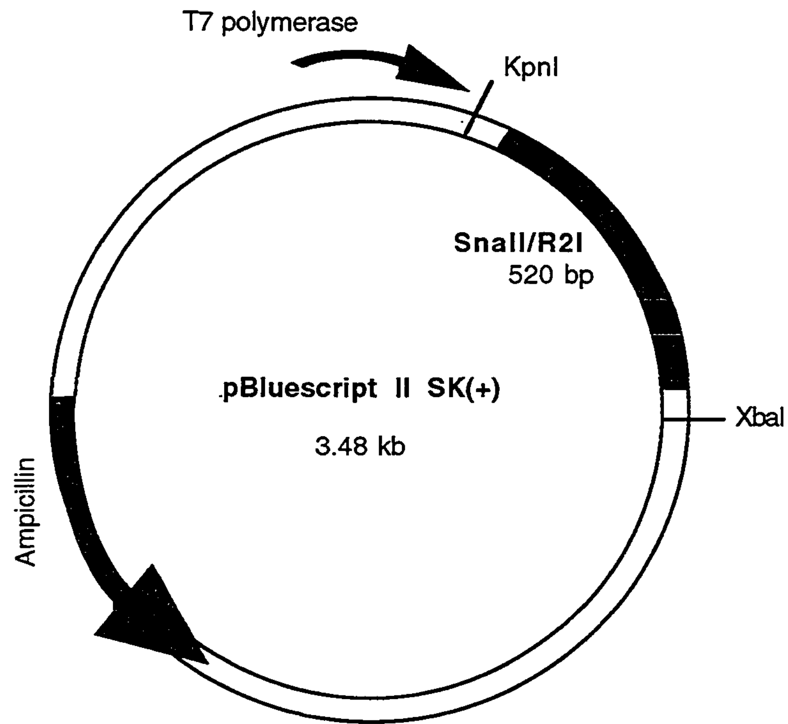
Table 2. Migration and derivative formation of DiI-labeled cells in *D. rerio* embryos

					Derivatives[2]					
Stage[1]	level[1] injected	distance[1] migrated	n	un-[2] labeled	Pig.[3]	Glial/ Drg	Symp. ganglia	Enteric ganglia	Aorta	Fin
10-11	5-8	-----	5	100	0	0	0	0	0	0
12-13	4-8	18	13	23	23 [dor; X][3]	54	38	54	23	0
14-15	4-9	5	12	0	50 [dor; M]	50	25	0	0	0
16	5-9	2	8	0	12 [dor; I]	25	0	0	0	12
17-19	7-11	12	14	14	36 [dor; M, I]	50	64	0	64	14
20-22	7-12	tail tip	10	10	90 [dor, v/l; M, I]	50	50	50	20	40

Notes: [1] Represented by number of somites. Distance migrated is the maximum observed for each stage. Because DiI-labeled cells migrated few somites anteriorly, distance is in somites migrated posteriorly.
[2]Numbers represent the percentage of embryos with/without DiI labeling in various TNC derivatives.
[3] [ ] Describes location of pigment differentiation and cell types labeled (dor, dorsal; v/l, ventro-lateral, X, xanthophores; M, melanophores; I, iridiohores). Drg, dorsal root ganglia; Symp, sympathetic

Fig. 5. pBluescript II plasmid containing a fragment of the *sna2* gene, and agarose gels verifying the presence of the linearized *sna2* plasmid and antisense mRNA probe. A: Diagram of the pBluescript II SK(+) plasmid containing a 520 base pair fragment of the *sna2* gene. **B:** Linearized pBluescript II SK (+) plasmid. Lane 1: lambda HindIII marker; Lane 2: linearized plasmid containing the *sna2* gene fragment. **C:** Agarose gel verifying the presence of the *sna2* mRNA probe. Lane 1: DNA 100 bp ladder; Lane 2: antisense *sna2* mRNA probe.

A



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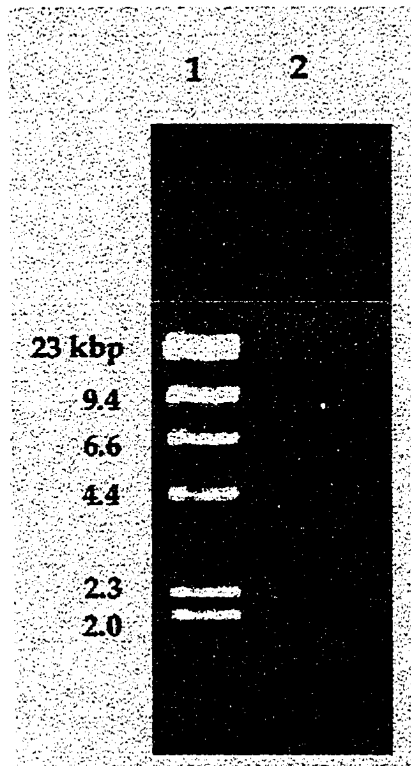

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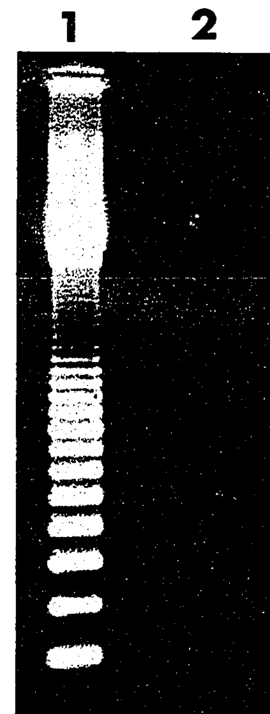


Figure 5

Fig. 6. Histological sections taken through the hindbrain and trunk regions of a 15 ss embryo. A-C: Cross sections through the hindbrain, boundary of head and trunk, and anterior trunk, respectively. In all sections neural crest cells are emerging between the neuroepithelium and epidermis, after which they lie dorsal to the neural keel (arrows). n, notochord; nk, neural keel; oc, placodal primordium of the otic capsule; so, somite. Bar = 50 μm

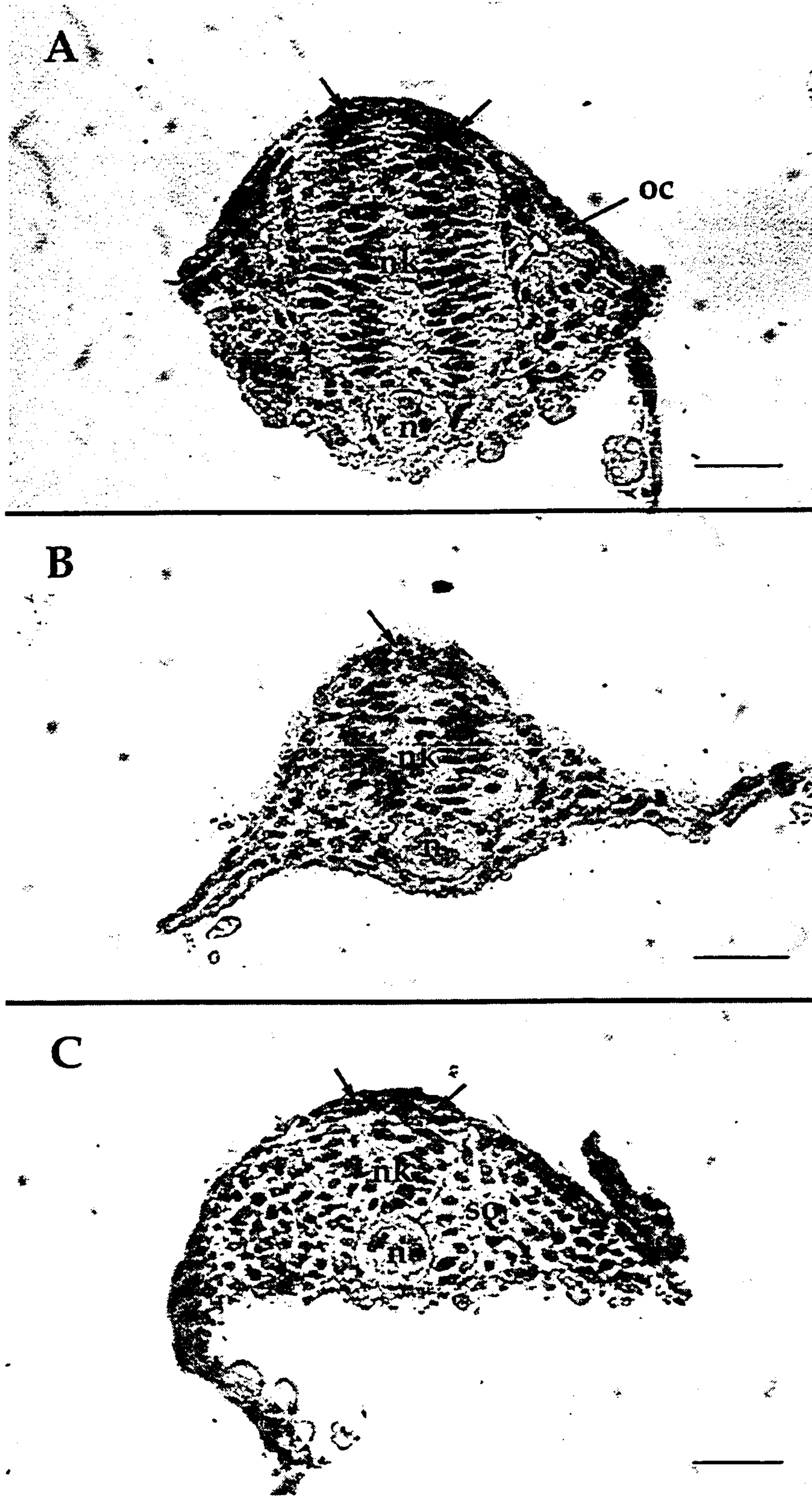


Figure 6

Fig. 7. SEM images in the head and trunk of zebrafish embryos. Head (anterior) is to the right in all images. **A:** In the cardiac/anterior trunk region, neural crest cells (arrows) are migrating as a continuous sheet over somites and yolk (y) within a relatively sparse extracellular matrix (13 ss). e, epidermis. **B:** TNC cells assume a 'bottle' shape as they migrate towards the medial path between the neural keel and somites (arrows). Groups of cells overlying somites (arrowheads) are thought to be TNC cells undergoing lateral sheet-like migration. However, it is possible that these are somite-derived mesenchymal cells (14 ss). **C:** Streams of migrating neural crest cells (arrows) in the hindbrain and cardiac regions (16 ss). e, epidermis.
D: TNC cells destined to migrate segmentally along the lateral path have not yet emerged from the neural keel in the posterior trunk (level of somites 11-16; 16 ss).

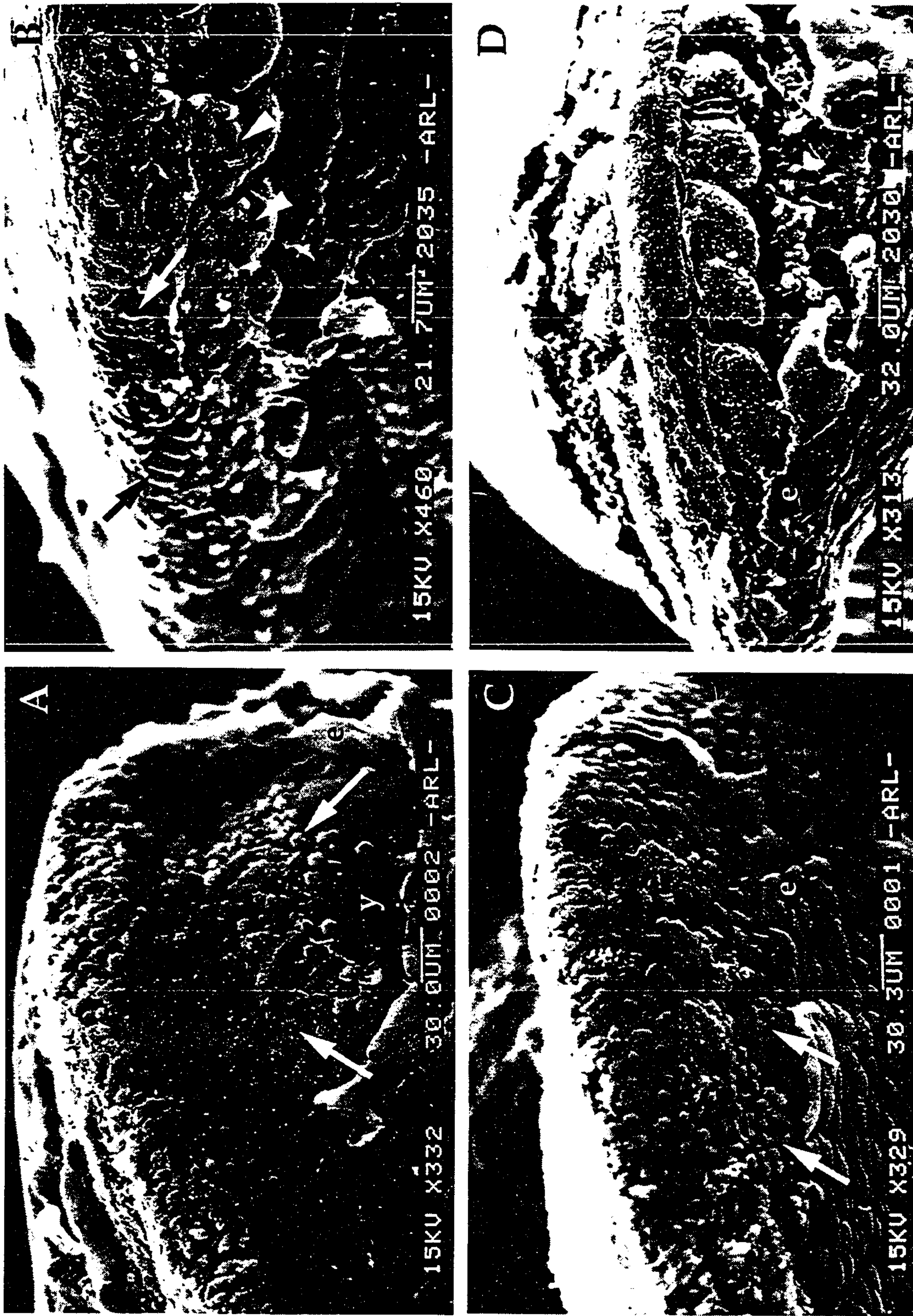


Figure 7

Fig. 8. SEM images in the head and trunk of zebrafish embryos. Head (anterior) is to the right in all images. **A:** TNC cells (arrows) are layered over the neural keel prior to and during segmental migration along lateral and medial pathways at the level of somites one-six (17 ss). **B:** At the level of somites four-eight, TNC cells are separating into streams (between lines; arrows). The area between the two parallel vertical lines is magnified in **C** (17 ss). **A, B** – e, epidermis. **C:** Somites four-eight magnified from **B**. Arrows indicate groups of TNC cells migrating segmentally (in streams) over the somites. **D:** TNC cells overlying the neural keel in the posterior trunk are elongated antero-posteriorly (arrows) and migrate along the dorsal neural keel prior to migrating laterally over the somites (18 ss).

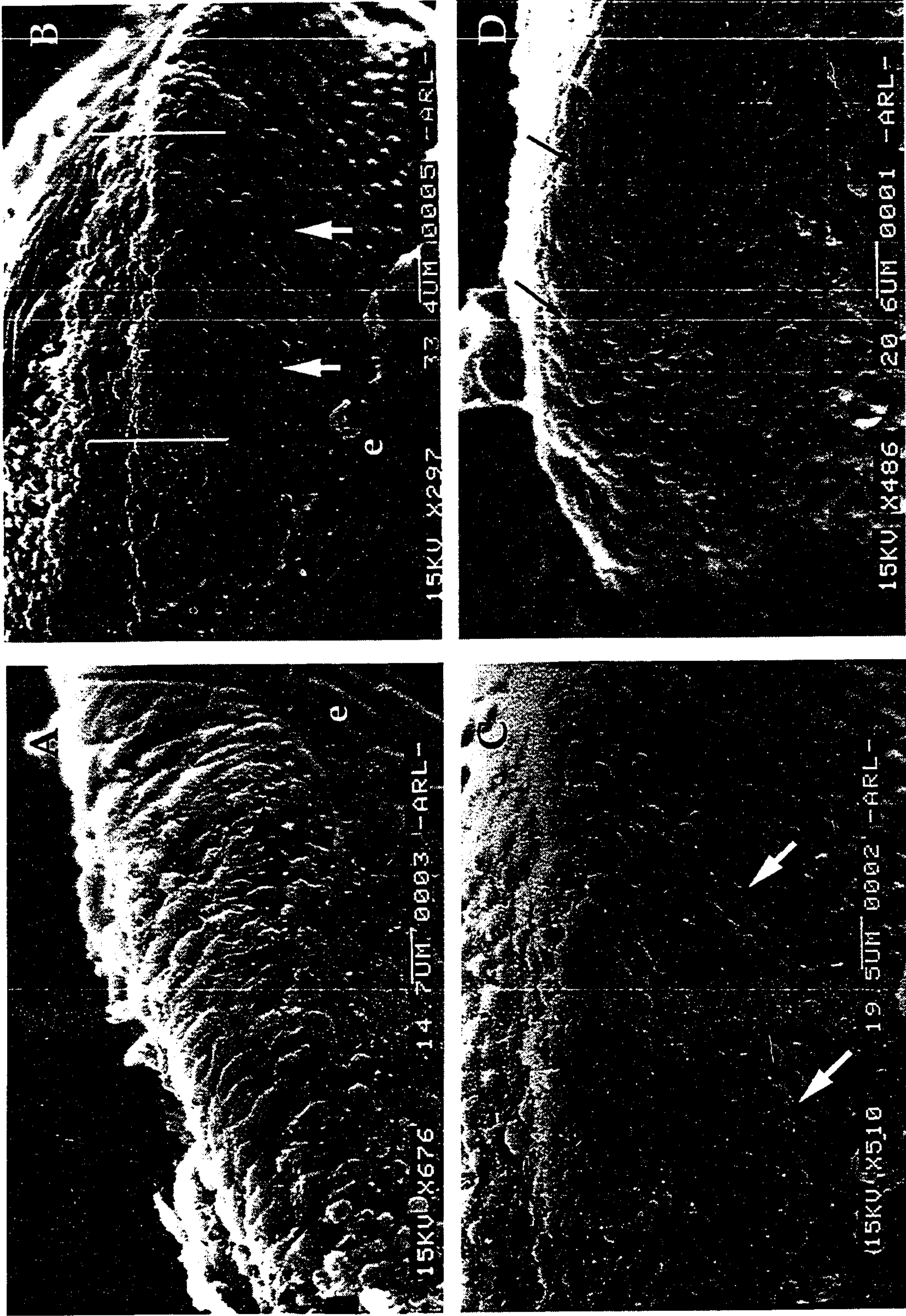


Figure 8

Fig. 9. DiI labeling of TNC cells in a 12 ss zebrafish embryo, shown in whole mount and sections and traced in this single individual through 36 hours post fertilization (hpf). Head (anterior) is to the left in images A-C, H. Parallel vertical white lines in A-C refer to the original site of injection. **A:** Ten minutes after DiI injection, TNC cells labeled with DiI at the level of somites (so) six-seven in a 12 ss embryo began to migrate anteriorly and ventrally along the medial path. **B:** One and a half hours after injection (16 ss), labeled TNC cells had migrated two somites anteriorly and one somite posteriorly (level of somites four-eight), and were differentiating in a segmental manner along the medial path (arrow). **C:** TNC cell migration and differentiation were unchanged 2.5 hours after DiI injection (18 ss). **D-G:** Transverse histological sections close to the level of somites 14-16 confirm the DiI-labeled derivatives represented in **H**. UV-illuminated sections **(D, F)** are shown for orienting the DiI label to embryonic tissues. g, gut; n, notochord. Bar = 50 µm. **E:** At 36 hpf DiI labeling is seen in enteric ganglia (lower arrow) and in patches of xanthophore cells dorso-lateral to the neural keel (upper arrow). **G:** Dorsal root ganglia located along the medial path between the neural keel and somites also are labeled (arrows). **H:** More detail can be seen in the 36 hpf whole mount, such as the location of the unknown cell type (arrow), sympathetic ganglia (arrowheads) and tail reticular cells (asterisk) that are part of a TNC-derived vascular network surrounding the aorta. From this point on, see Appendix II for actual sizes of whole mount embryos depicted in the figures.

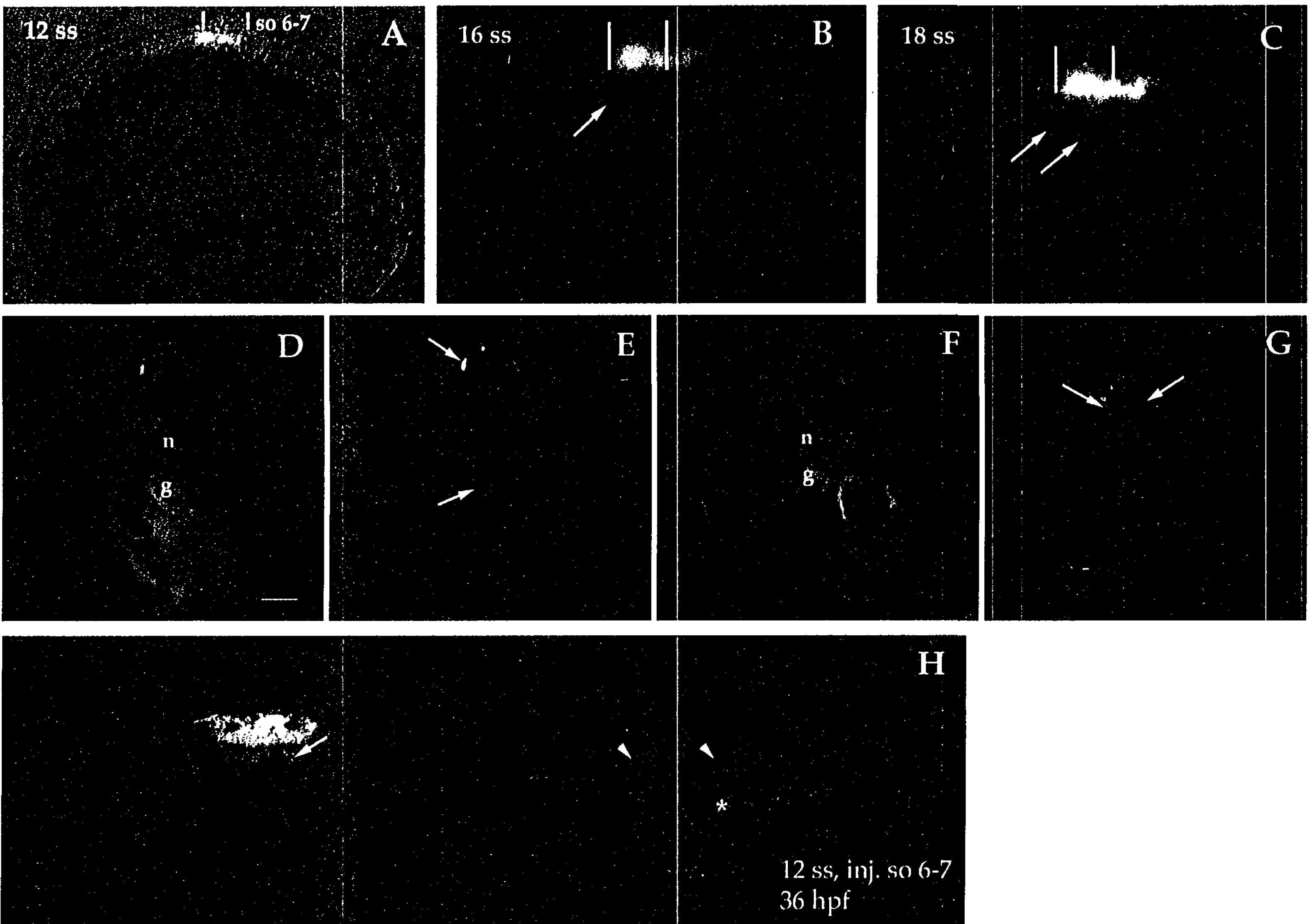


Figure 9

Fig. 10. Whole mount *Sna2* expression in 10-22 ss control zebrafish embryos. Head (anterior) is to the left, or upper left-hand corner in all images except **A, D**, where the head is in the lower right-hand corner. **A:** At the 10 ss *sna2* is expressed diffusely in mesoderm and neuroepithelium, extending onto the yolk in the hindbrain and cardiac regions (arrowheads). **B:** From the 12 ss onwards, *sna2* is expressed along the dorsal neural keel. Notochord adaxial cells (arrows) and mesoderm also express *sna2* – non-expressing portions of somites appear as clear 'blocks' (arrowheads) **C:** At the 14 ss, a new region of *sna2* expression is visible at the mid-dorsal neural keel (easier to visualize in E, arrow), and *sna2*-expressing cells are seen over the yolk in the trunk (asterisk). **D:** *Sna-2* expressing cells migrate laterally and anteriorly across the yolk (arrows) from the hindbrain in a 14 ss embryo. **E:** *Sna2* expression at the 16 ss resembles earlier stages. *Sna2* is still seen at the dorsal midline (arrow), in adaxial cells, and mesoderm (arrowheads). **F:** At the 18 ss, *sna2* expression is strong lateral to the head in the region where branchial arches will form (arrows), as well as in the somites – visible as segmental staining (arrowheads) **G:** By the 22 ss, dorsal expression of *sna2* has decreased. Note *sna2*-expressing cells in the otic region (arrow).

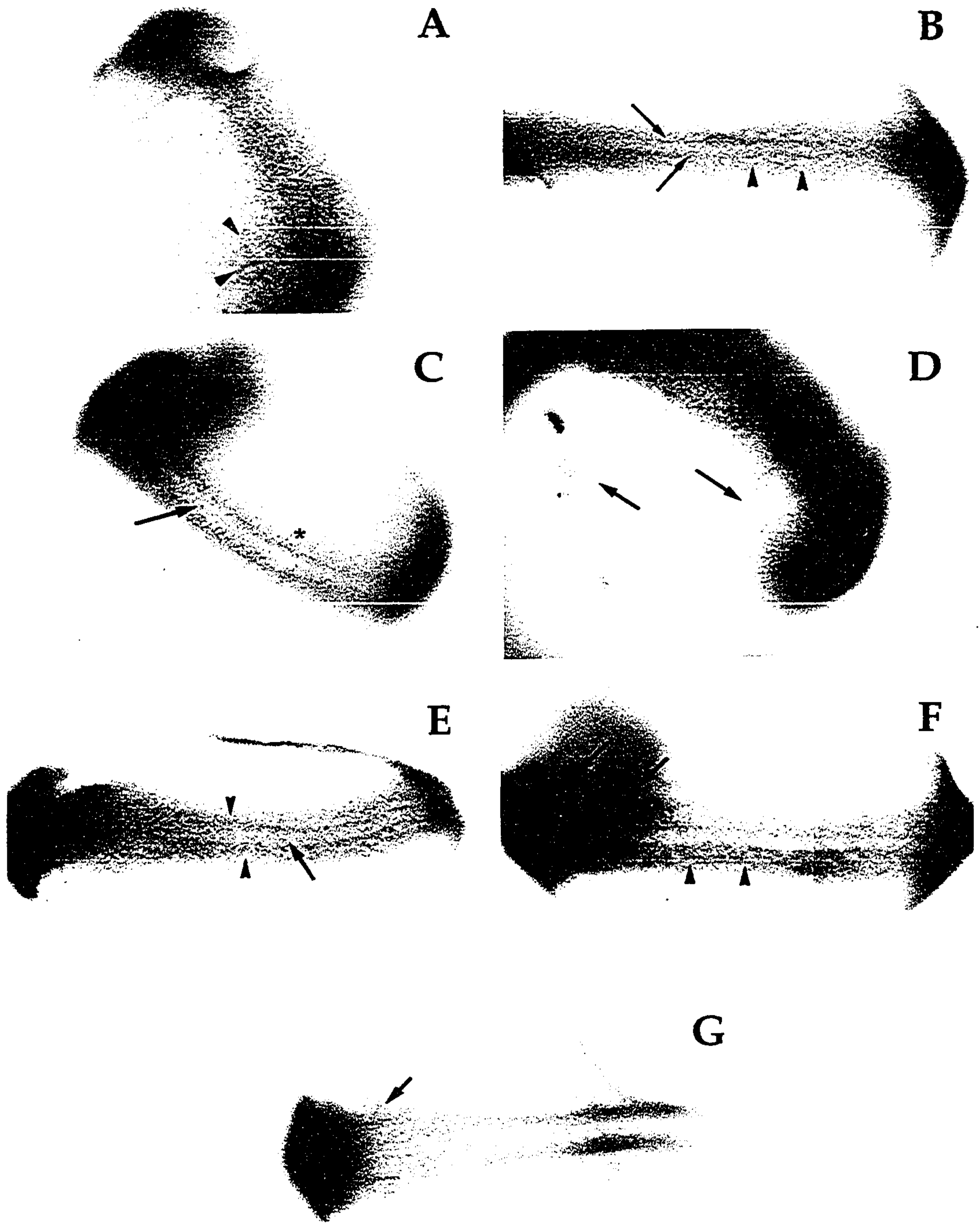


Figure 10

Fig. 11. Histological sections through the trunk of an 18 ss embryo. A: In this longitudinal section taken through the trunk posterior to somite 12, TNC cells can be seen migrating along the dorsal neural keel (arrows). **B:** By the 18 ss the somites (arrowheads) have elevated and lie more ventral than lateral to the neural keel. TNC cells are penetrating the medial and lateral pathways at the level of the mid-trunk (arrows). Bar = 50 µm

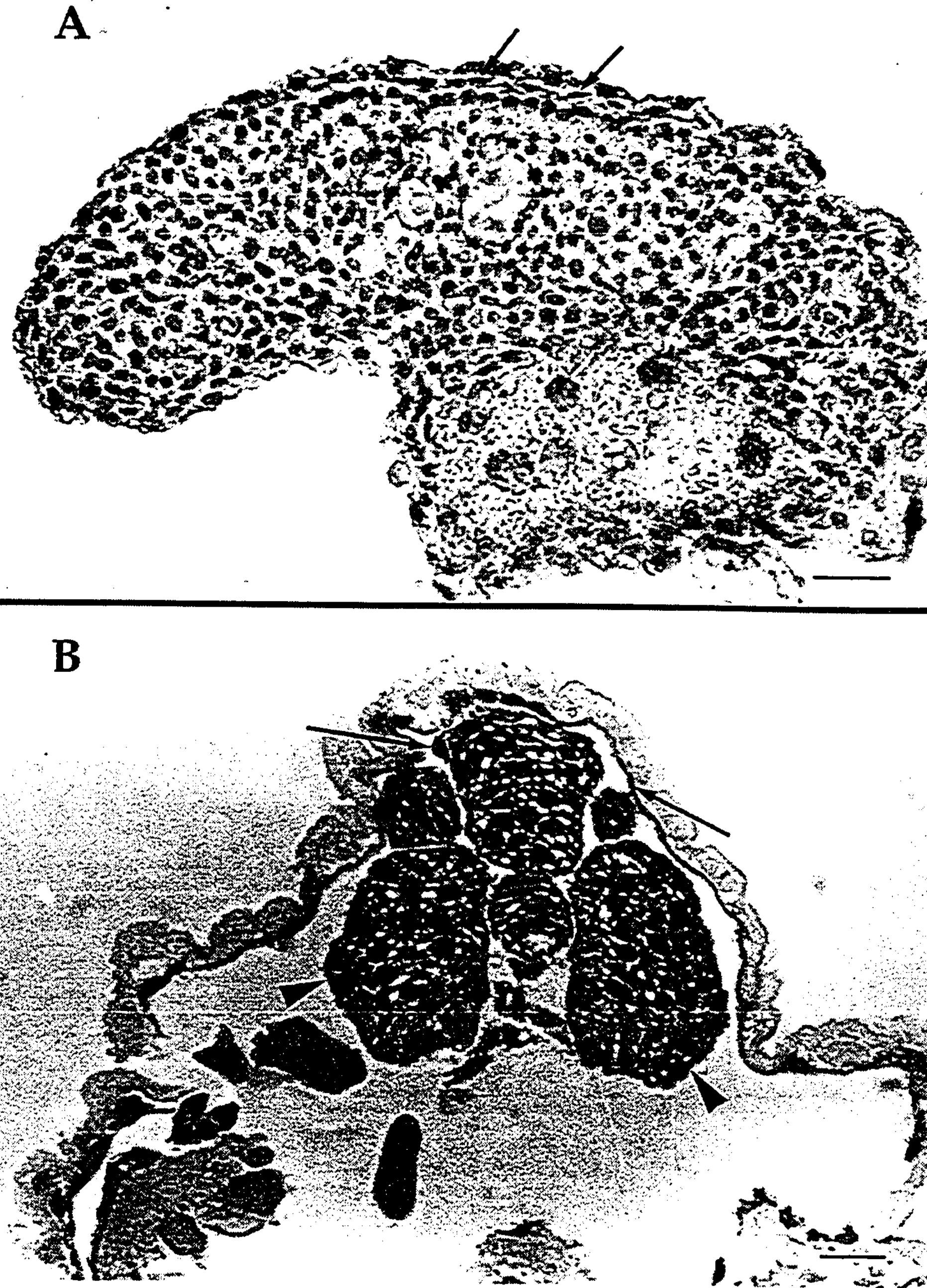


Figure 11

Fig. 12. SEM images of the tail tip in zebrafish embryos. A: Neural crest cells (arrow) are migrating posteriorly along the dorsal neural keel and laterally over the somites in the tail tip of a 21 ss embryo. 692x. **B:** At the 24 ss few neural crest cells are emerging from the tail tip. 492 x.

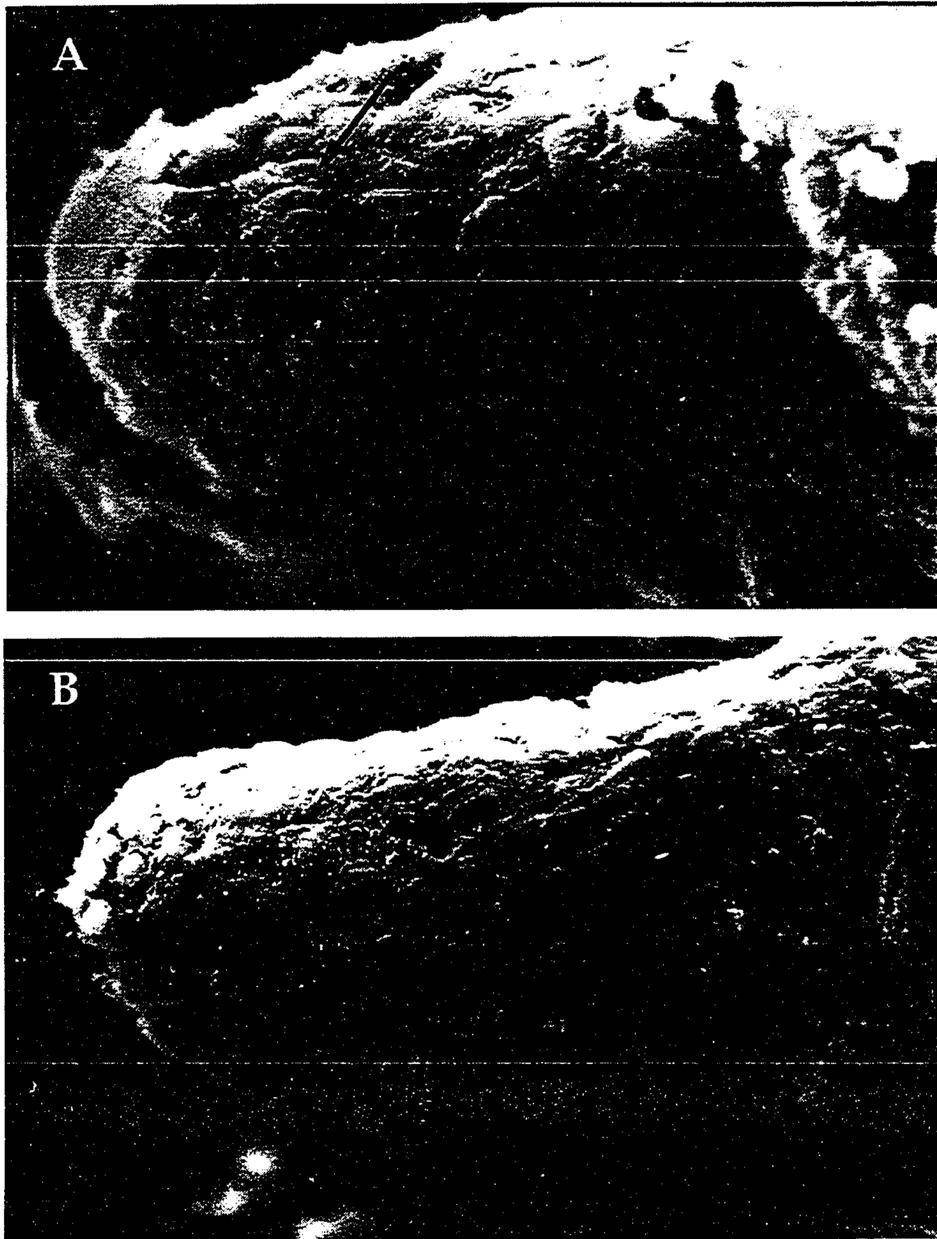
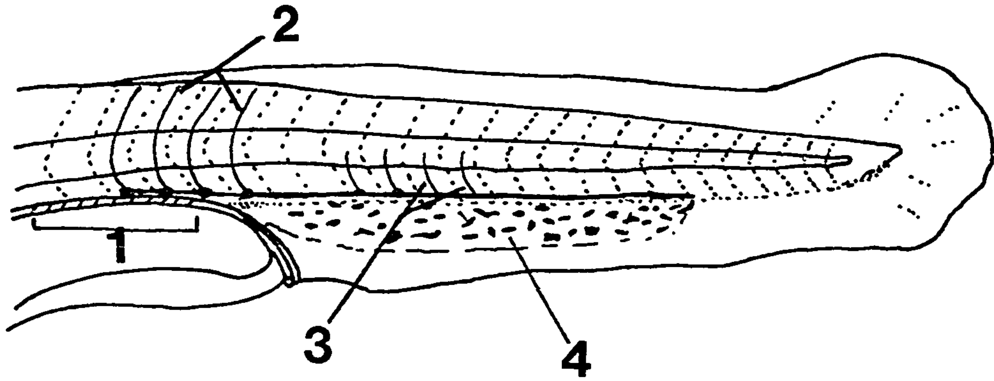


Figure 12

Fig. 13. Diagram of TNC cell derivatives observed at 36 hpf in embryos labeled with DiI at two different stages. A: TNC cells were labeled with DiI at the level of somites six-seven in a 12 ss embryo. At 36 hpf the labeled cells had formed: (1) enteric neurons, (2) Schwann (glial) cells or dorsal root ganglia precursors or pre-sympathetic neuroblasts, (3) sympathetic ganglia and (4) tail reticular cells and/or other elements contributing to the vascular complex in the tail. **B:** TNC cells were labeled with DiI at the level of somites seven-eight in a 19 ss embryo. The labeled cells often formed or contributed to: (1) dorsal pigmentation – especially melanophores and xanthophores, (2) dorsal fin fold, (3) primary and (4) secondary chains of sympathetic ganglia and (5) tail reticular cells.

A



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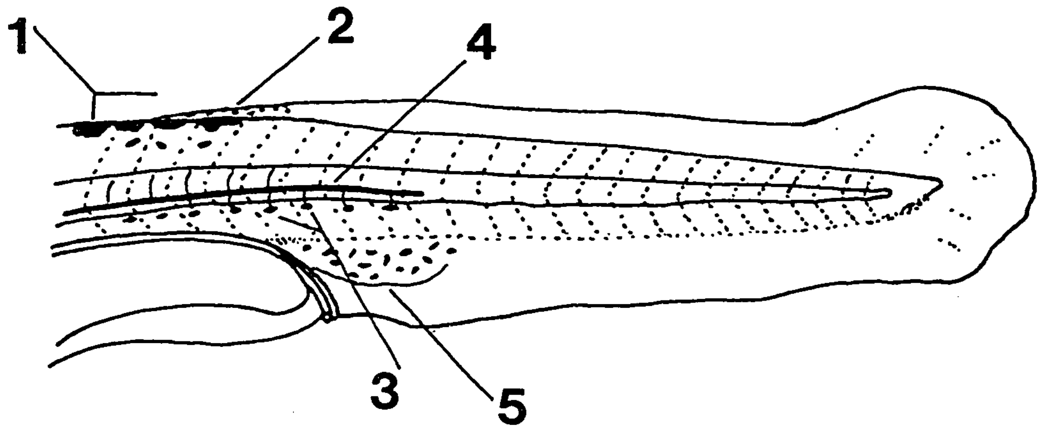


Figure 13

Fig. 14. DiI labeling of TNC cells in a 14 ss embryo and traced through 36 hpf. Head (anterior) is to the left in all images. White arrows indicate the site of the original injection. **A:** Within 20 minutes of DiI injection at the level of somite six (s6), labeled cells had begun to migrate away from the injection site. **B:** Two hours after the injection (18 ss), cells have migrated a short distance equivalent to two somite lengths posteriorly and slightly ventral to somite six. **C:** Four hours after injection (22 ss), labeled cells are differentiating into xanthophores and remain fairly close to the initial injection site. **D:** Labeled pigment cells can be seen more clearly in this dorsal view at 21 hpf.

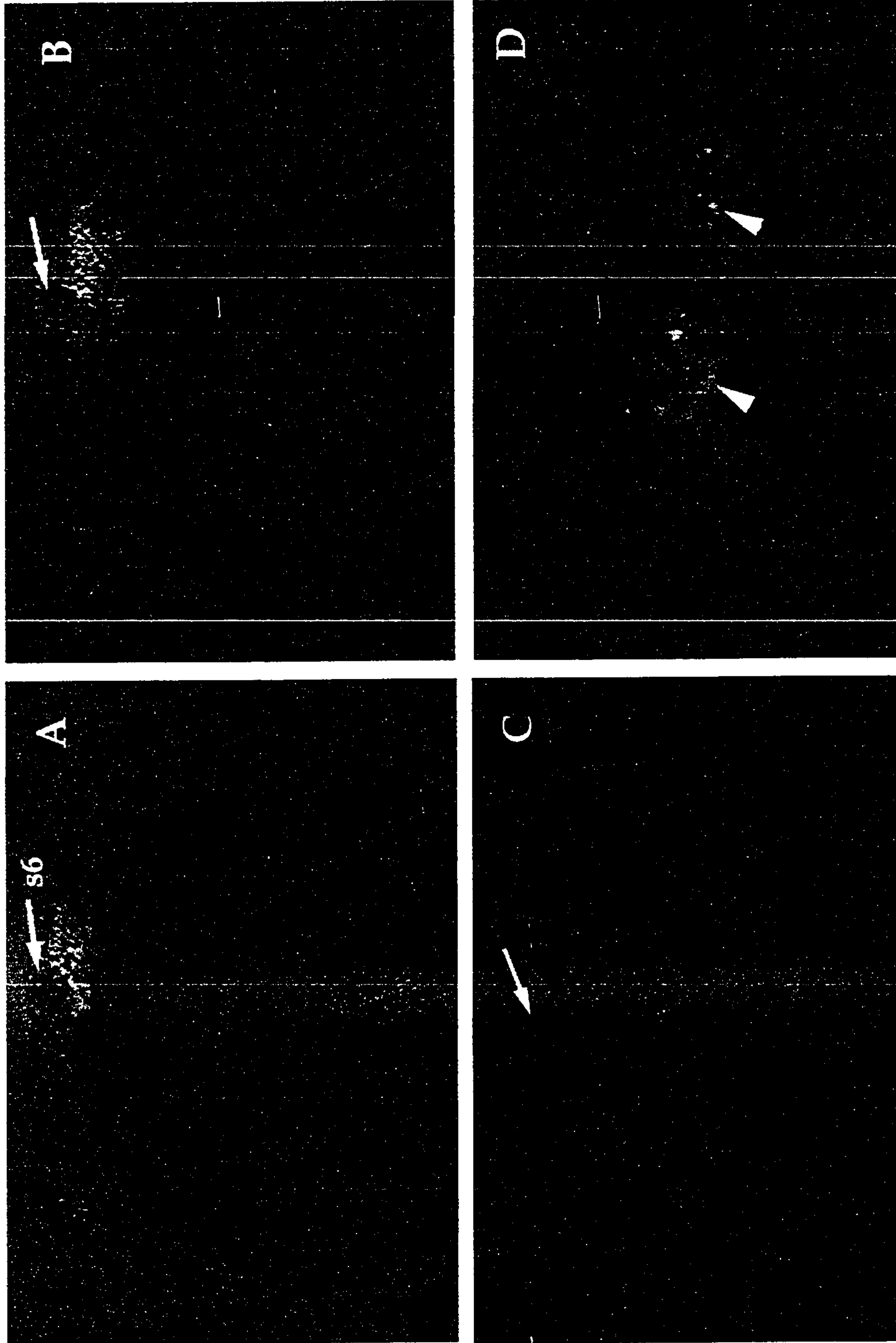


Figure 14

Fig. 15. DiI labeling of TNC cells in a 19 ss embryo, shown in whole mount and in histological sections and traced through 64 hpf. Histological sections confirm the DiI-labeled derivatives represented in **H**; UV-illuminated sections **(A, E)** are shown for orienting the DiI label to embryonic tissues. a, aorta; g, gut; n, notochord. **A-C:** These histological sections represent the anterior trunk close to the level of somites seven-eight where DiI was initially injected in a 19 ss embryo (white line, **H**). Many TNC cells migrated extensively along the medial path (not just ventrally – also anteriorly and posteriorly) and formed portions of the sympathetic ganglia chain (**B, C,** short arrows) and dorsal root ganglia (**B, C,** long arrows). **D-G:** Histological sections through the posterior extremity of the yolk extension (white line, **H**). DiI label is found in dorsal root ganglia (**D, F**; arrows), the vascular system (aorta and/or tail reticular cells; **D, F**; arrowheads) and cell bodies and axon extensions of sympathetic ganglia (**G**; arrowheads). **H:** Whole mount view of a 64 hpf embryo showing DiI labeling of the derivatives mentioned above, namely dorsal root ganglia (short arrow), sympathetic ganglia (long arrow) and the vascular complex (asterisk). Bars in A, D = 50 μm.

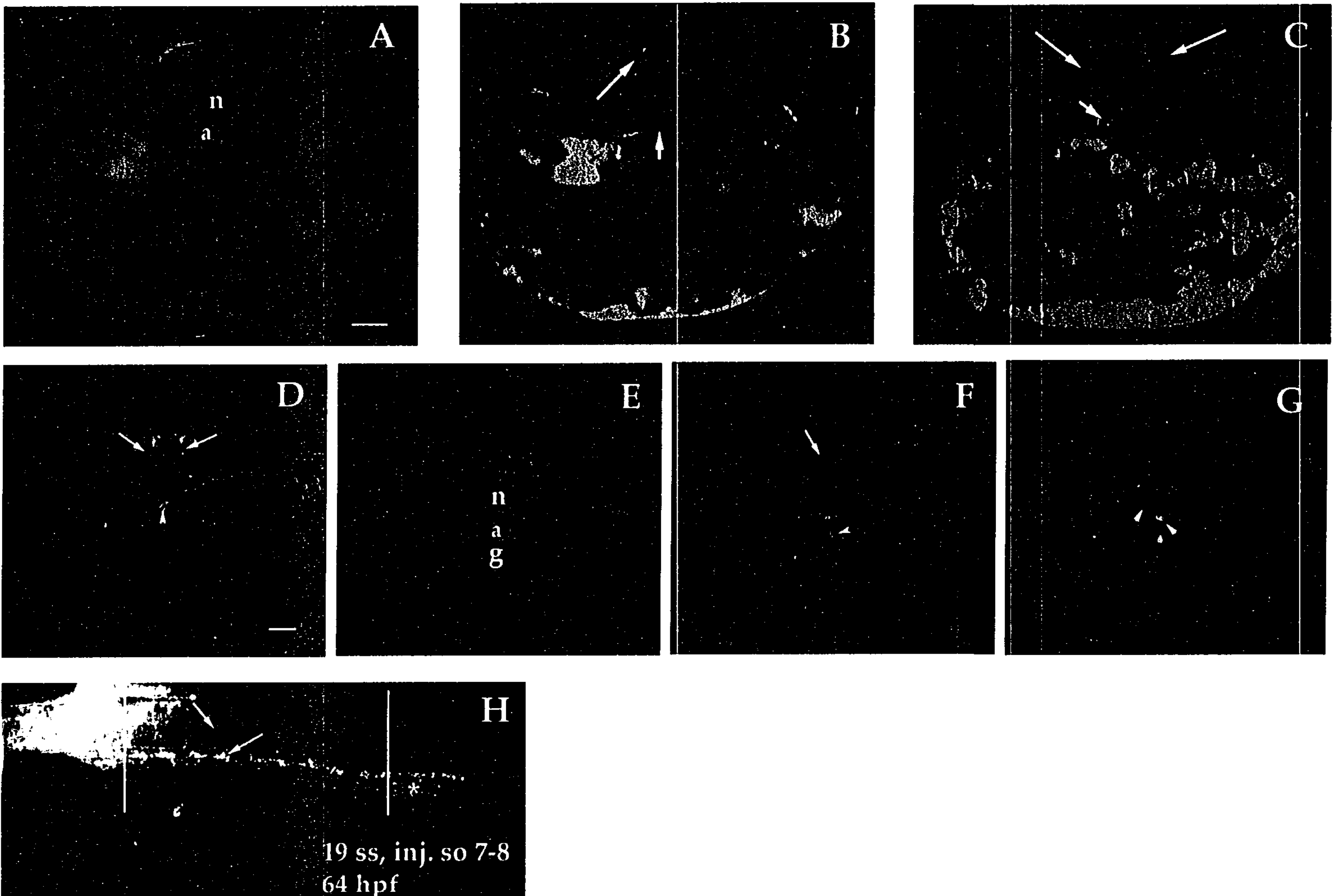


Figure 15

Chapter 3

Regulation of Trunk Neural Crest Cells in Zebrafish

Introduction

Successful proliferation and migration of cell populations are critical to shaping embryos. If errors occur during development, such as through delayed cell migration or defective proliferation, effects on the embryo can be profound. For instance, deficient neural crest cell migration to the human distal mandibular arch can result in otocephaly (absence of lower jaws, ear defects, heart anomalies) or Treacher Collins syndrome (open eyelid, ear defects, cleft palate; Johnston, 1975; Jones, 1990; Hall, 1999). The finding that some populations of embryonic cells can compensate for cell loss, or for actions that would otherwise lead to abnormal development is termed regulation (Bellairs, 1971; Hall and Hörstadius, 1988). More specifically, I define regulation as the replacement of lost, undifferentiated embryonic cells by other cells in response to environmental signals.

Research on regulative potential in embryos is scattered among invertebrates and vertebrates and only alludes to underlying mechanisms, which include cells taking on alternative fates in response to cell loss or trauma during development (Taghert et al., 1984; Ettensohn and McClay, 1988). Neural crest cells have been the subject of many studies of regulation.

After segregation from the neural tube or neural keel, neural crest cells migrate along comparable pathways in all vertebrates (between the somites and epidermis and between somites and neural tube/keel, see Chapter two) and differentiate into a diversity of tissues and cell types, including cartilage, bone, connective tissues, odontoblasts, neurons and glia of the autonomic nervous system and pigment cells (Le Lièvre and Le Douarin, 1975; Hall and Hörstadius, 1988; Kirby, 1988a,b; Bronner-Fraser, 1995). This study uses pigment derivatives to assess the regulative response of populations of trunk neural crest (TNC) cells.

Regulation of neural crest cells has been shown in lampreys (Newth, 1951, 1956; Langille and Hall, 1988b), teleost fish (Langille and Hall, 1988a; Raible and Eisen, 1996), amphibians (e.g. Chibon, 1970; Moury and Jacobson, 1990), birds (e.g. Kirby et al., 1983; Scherson et al., 1993; Couly et al., 1996) and mice (Snow and Tam, 1979) (also reviewed in Vaglia and Hall, 1999). The limitations and potentials associated with regulation of cranial (e.g. Yntema and Hammond, 1954 and references therein; McKee and Ferguson, 1984; Couly et al., 1996; Hunt et al., 1995; Sechrist et al., 1995) and cardiac (vagal) (e.g. Bockman and Kirby, 1984; Bockman et al., 1987; Kirby, 1987; Nishibatake et al., 1987; Kuratani et al., 1991; Waldo et al., 1996; Suzuki and Kirby, 1997) neural crest cells have been well established, especially in chicks. Regulation of TNC cells is less resolved, studies being limited to analyses of pigment cells and dorsal root ganglia in amphibians (Lehman and Youngs, 1952) and dorsal root ganglia derivatives of early-migrating versus late-migrating populations

of TNC cells in zebrafish (Raible and Eisen, 1996). Although early-migrating TNC cells typically produce dorsal root ganglia (DRG), Raible and Eisen (1996) found that DRG still form after ablation of early-migrating cells. From this result the authors suggest that late-migrating TNC cells can regulate for loss of the early migrating cells by changing fate. Late-migrating TNC cells may be bi- or multipotent, but interactions with early-migrating cells prevent them from forming DRG under normal circumstances.

In light of the single study by Raible and Eisen (1996), regulation of TNC cells needs to be thoroughly investigated in species of fishes. For studies of regulation TNC cells have the added advantages of forming a few specific derivatives and being accessible to experimentation, as they sit wedged between epidermis and neural keel prior to migrating. This study asks whether TNC cells can regulate. Regions of TNC were extirpated from embryos of the zebrafish, *Danio rerio,* to determine whether the amount of TNC removed, position along the embryonic axis, and/or timing of TNC cell extirpation affect regulative ability.

Pigment cells (melanophores, iridiophores, xanthophores) served as landmarks for analyzing the effects of extirpation in live embryos throughout embryogenesis and during the larval period. Ideally, regulation of pigment cells would be compared with regulation of other TNC derivatives such as dorsal root ganglia, the sensory components of the peripheral spinal nerve. Dorsal root ganglia accumulate between the 16-19 somite stages (ss) as segmentally arranged clusters of neurons and glia at the mid-somite where

the ventral root exits (Laudal and Lim, 1993; Raible and Eisen, 1996). To determine whether dorsal root ganglia can be identified within the first 24-36 hours of development, embryos were labeled with one of five antibodies:

1. Anti-acetylated-α-tubulin (6-11B-1, anti-tub). Acetylated-α-tubulin is found in sea urchin sperm flagella (Piperno and Fuller, 1985). The anti-tub antibodies raised against this form of tubulin have been described as labeling most axons in the developing embryonic nervous system of vertebrates (Karlstrom et al., 1996).
2. Anti-Hu (16A11). Anti-Hu recognizes the Hu protein expressed by the central regions of some neurogenic cells such as dorsal root and primary sympathetic ganglia prior to or during their last round of DNA synthesis (Marusich et al., 1993).

3, 4. HNK-1 and zn-12. Both HNK-1 and zn-12 label non neural crest-derived Rohon Beard cells (Canger et al., 1998) and neural crest-derived tissues such as dorsal root ganglia (Laudal and Lim, 1993; Raible and Eisen, 1996).

5. Zn-5. Zn-5 labels cell bodies and axons of secondary motor neurons, as well as dorsal root ganglia in zebrafish embryos that are 48 hours or older (Brand et al., 1996; Morin-Kensicki and Eisen, 1997).

None of the antibodies tested in zebrafish embryos labeled dorsal root ganglia prior to 48 hpf. Although the presence of dorsal root ganglia in a neural crest-ablated region at 48 hpf could be indicative of regulation, neural precursor cells may have migrated into the region prior to ablation, or cells surrounding the extirpated site may have been pre-programmed to form dorsal root ganglia in that area. Because the first 24 hours after TNC cell extirpation are important for monitoring development and patterning of neural derivatives, 48 hours was considered too late to use dorsal root ganglia as indicators of regulation in this study.

As first demonstrated by Raible and Eisen (1996), I show that TNC cells in zebrafish can be replaced by regulation. Pigment patterns revealed that regulation varied spatially along the embryonic axis and temporally during development. The results strongly suggest that regulation for TNC cells is by TNC cells, rather than by an alternative cell population such as placodes or epidermal ectoderm.

Materials and Methods

Zebrafish maintenance and breeding

Adult zebrafish (*Danio rerio*) were purchased from either local pet stores in Halifax, Nova Scotia, Canada or from Boreal Laboratories, Ltd. (Ontario, Canada). Females and males were separated and kept in 10 gallon

tanks at a temperature of 27-29° C on a 14 hour light and 10 hour dark cycle. Breeding colonies were established with three females and three-five males. Eggs were collected in the evening and raised at 28.5° C in Hank's solution (Westerfield, 1995).

Removal of trunk neural crest cells

To investigate regulation of embryonic cells, trunk neural crest (TNC) and neural keel cells were removed from regions equivalent to the length of five or more somites along the anterio-posterior axis. Zebrafish embryos ranged in stages from 12 to 25 pairs of somites (Appendix I). After removing the chorion with #5 watchmaker forceps, embryos were positioned on a slide in 1.2% low melting point agarose and viewed using a Zeiss inverted compound microscope. Under magnifications of 25 or 40x, cells were removed using a pulled glass micropipette attached to a micromanipulator (Fig. 16). Sham-operated control embryos had only the overlying epidermis removed. Following surgery, embryos recovered in Hank's solution containing 2% antibiotic-antimycotics (GIBCO). This solution was replaced completely once a day for two days after surgery. Embryos were subsequently kept in Hank's without antibiotics. Embryos hatch approximately 72 hours (three days) after fertilization; three days after hatching, larvae were fed cultured *Parameciam* (Boreal Laboratories, Ltd.) and raised until seven or eight days post-hatching.

Live embryos

Pigment development and patterning were traced through embryogenesis and into the larval period in individual control and experimental embryos. To document changes in pigmentation, embryos and larvae were anesthetized using a 0.4% solution of MS-222 (Tricaine) and stabilized on slides using 3% methyl cellulose. Live embryos or larvae were photographed under a Tessavar dissecting microscope at magnifications of 10x or 16x. After removing the animals from methyl cellulose, recovery was rapid and they developed as usual in Hank's solution.

Histology

The depth of neural keel extirpation and completeness of trunk neural crest removal were verified in sectioned embryos (Fig. 17). For paraffin sectioning, embryos were fixed in 4% paraformaldehyde, dehydrated in a series of methanol/PBS (Dulbecco's phosphate buffered saline – Freshney, 1987), infiltrated, embedded in low melting point wax at 52° C in a vacuum oven, and cut at 5 µm. Sections were mounted on Poly-L-Lysine-coated slides to increase adhesion. Sections were stained with HBQ (Hall and Brunt's Quadruple Stain, Hall, 1986) or Ehrlich's Hematoxylin (Ehrlich, 1886).

Antibody staining

For whole-mount antibody staining, embryos were fixed overnight in 4% paraformaldehyde at 4° C followed by several rinses in 0.1 M PBS. Embryos were incubated for one hour in blocking solution (PBS with 0.1% tween, 1% BSA and 20% normal goat serum for HNK-1; 20% normal sheep serum for all other antibodies), then incubated overnight in primary antibody at 4° C (6-11B-1, anti-acetylated-∝-tubulin: 1:1000; 16A11 (anti-Hu): 1:150; HNK-1: 1:50; zn-5: 1:50; zn-12: 1:10). Following a one hour rinse with several changes in 0.1M PBS with 0.1% tween, HNK-1-labeled embryos were incubated in fluorescent CY3-conjugated goat anti-mouse secondary antibody (1:500) for two hours at room temperature in the dark. Embryos labeled with other antibodies were incubated in horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (1:250) for two-three hours at room temperature or overnight at 4° C. HNK-1-labeled embryos were then rinsed extensively over one-three hours with several changes in ddH_20 and viewed under a Leitz Aritstoplan fluorescent microscope using an N2.1 filter. Embryos labeled with other antibodies were rinsed for one hour with several changes in PBS with tween, washed for 10 minutes in TRIS (pH 7.6), and then incubated in 1.0 mg/2.0 ml diaminobenzidine (DAB) dissolved in 20 µl DMSO (dimethylsulfoxide) and brought to a final volume of 20 ml with TRIS buffer.

One µl H_2O_2 was added to the final solution to develop the HRP (POD) reaction product.

Results

Results of trunk neural crest (TNC) extirpations are based on somite stages (ss) that relate to patterns of TNC cell migration described in Chapter two, with embryos between the 10 and 12 somite stages representing early neural crest cell migration along the medial path, and embryos older than the 23 somite stage representing near completion of cell migration. While some data analyses include results from extirpations that overlapped with the cardiac (vagal) region (somites one-three/five), emphasis is placed on regulation of TNC cells.

Of the 107 zebrafish embryos from which cardiac and trunk neural crest cells were removed, a mere 13% died prior to one week post-hatching. Fifty-seven percent of the total mortality occurred when cells were removed from regions that included all or part of the first five somites in 14-15 ss embryos. The remaining 43% died following removal of large amounts of neural crest, which may or may not be associated with the fact that these extirpations included cells from the region of somites one-five (Table 3).

The presence and patterning of pigment cells (melanophores, iridiophores, xanthophores) were the characteristics used to assess regulation of TNC cells. Because the absence of xanthophores created regions that had

very pale (almost albino-like) coloration, xanthophores were classified simply as present (N=normal) or absent (A=absent) following extirpations.

Normal pigmentation

Pigment patterning in larval zebrafish is complete by six days post-hatching. It is easily studied by viewing the three naturally labeled pigment cell types – melanophores, iridiophores, and xanthophores – under a dissecting microscope. Melanophores are black, melanin-producing cells that form dorsal, ventral, lateral, and yolk sac stripes in the larvae. Beginning close to the level of the first somite, melanophores are arranged in pairs or trios along the dorsal midline. They vary from being more circular in shape to having dendritic-like extensions. Over the braincase individual melanophores merge into a 'V' that is closed-over between the eyes (Figs. 18A, C). On each side of the body, melanophores form mid-lateral interrupted stripes, beginning at approximately the sixth somite (just posterior to the swim bladder) and extending to somite 22. The ventral stripe begins at the anterior end of the yolk sac, extending from above the swim bladder to the tail (Figs. 18B, D).

Iridiophores are reflecting pigment cells that appear as iridescent gold spots in association with the dorsal, ventral and yolk sac melanophore stripes. In some regions such as over the braincase, swim bladder and ventral yolk sac, iridiophores merge to cover larger areas (Fig. 18C). In older juveniles,

iridiophores along the dorsal midline merge into a single stripe.

Xanthophores produce pteridine and/or cartenoid pigments that appear as a fairly uniform greenish yellow color. These pigment cells are most prominent dorsally, extending from the head to tail and wrapping slightly over the lateral sides (Fig. 18D). They also cover the ventral yolk and extend slightly posterior. Xanthophore data are presented, but these cells were rarely affected by extirpations.

Sham-operated control embryos

Two control (sham-operated) embryos per stage had only the epidermis removed from the level of somites seven-11. At four days post-hatch, pigmentation was normal in all but one of the controls in which the melanophore patterning was severely disrupted, most likely because the epidermis did not heal properly over the wound (Figs. 23A-D). As discussed in the following sections, regulation for the pigment derivatives of TNC cells was incredibly complete at most stages analyzed 48 hpf (36 hours after extirpation). Forty-eight hpf was chosen as the first time interval to assess pigment morphology because all three types of pigment cells were visible, and patterning was underway. Regulation was even more complete by four days post-hatch.

Does the amount of neural crest removed affect pigmentation?

In a series of preliminary experiments, regions of neural crest cells equivalent to the length of three somites (e.g., somites one-three, four-six) were extirpated from embryos in stage classes that correspond to patterns of TNC cell migration discussed in Chapter two (ss 10-12, 13-16, 17-23, > 23). Initial 'disruptions' in pigmentation were not evident by seven days post-hatching, except when cells were removed from between somites one and five. From these data, it was concluded that zebrafish embryos regulate completely for removal of small amounts of TNC cells. In order to push the limits under which regulation may occur, the amount of neural crest removed was increased to regions five somites or greater in length.

Removing larger amounts of neural crest cells (five or more somite lengths) increased mortality and generated defects ranging from missing to increased densities or disruptions of pigment cells (Table 3). These types of defects in pigment pattern represent various extremes of incomplete regulation. Post-hatch mortality occurred only when cells were removed from the cardiac region (somites one-three/five). The severity of defects in pigmentation and development increased slightly with removal of greater amounts of neural crest, depending on the location and stage of extirpation. Embryos at the 12 ss survived extirpations up to seven somites in length, even when extirpation overlapped the cardiac region. In these embryos, patches of missing melanophores and/or iridiophores were the only evidence

that cells had been removed (Fig. 19A). On the other hand, few 13-15 ss embryos survived removal of five or less somite lengths of neural crest cells if extirpations overlapped the cardiac region. Survivors were underdeveloped and exhibited different degrees of body curvature and pericardial deformaties (Fig. 19B). At the other extreme, 21 ss or older embryos frequently exhibited patches of missing or disrupted pigmentation, especially when TNC cells were removed over the length of ten or more somites (Figs. 19D, 20B-D). This seemingly lesser ability to regulate at older stages may reflect a loss of plasticity among cells that could regulate for pigment derivatives.

Similar to the 12 ss, embryos between the 18-19 ss were not as sensitive to extirpations that included the first five somites (Fig. 19C). Thus, 18-19 ss embryos were analyzed to more accurately address how removing different lengths of TNC affects pigmentation (Table 3). No correlation was found between the amount of TNC cells extirpated and number of pigment cell types affected. In other words, absence of more than one cell type (e.g., melanophores and iridiophores) was just as likely to occur whether five or ten somite lengths of TNC cells were removed (Table 3; Figs. 19C, 20A).

Generally, embryos that had more than 10 somite lengths of TNC cells extirpated died within 48 hours (Table 3). Loss or disruption of melanophores was frequently accompanied by loss or disruption of iridiophores. While melanophores were often missing from the most anterior aspect of the extirpated region (e.g., somite two; Fig. 19C), iridiophores tended to be absent

from the mid- or posterior levels of extirpated regions. Excessive concentrations of melanin, or disruptions in melanophore or iridiophore patterning frequently occurred posterior to where cells were removed (Fig. 19C, 20A). Xanthophores were always present in patterns that appeared unchanged from controls (19C).

Does the position of neural crest removal affect pigmentation?

From experiments that involved removing increasingly larger amounts of neural crest cells beginning at the level of somite one, it became apparent that there was decreased regulation for cells removed from the cardiac region. Removing a constant amount of TNC from different regions of similarly staged embryos confirmed the observation that regulation was poor in embryos where cells were extirpated from the level of somites one-five (Fig. 19B). These experiments also revealed poor regulation following removal of TNC from the posterior extremity of the trunk (Table 4; Fig. 19D). Moreover, variation in cell migration and differentiation at different axial positions must be considered relative to the stage of the embryo. Embryos of 18-19 ss were used to test how the position from which neural crest cells are removed affects pigmentation. Embryos at these stages are fairly resilient to neural crest cell extirpations (there was no post-hatch mortality), and many cells are emerging from the neural keel along much of the trunk.

The highest percentage of missing or disrupted pigment cells in 18-19 ss embryos corresponded to TNC removed from the most anterior and posterior regions of the trunk (Table 4). Pigmentation was normal in all embryos when TNC cells were removed from a region just posterior to somite five (somites five-nine) (Table 4). Some embryos with TNC cells removed from posterior to somite nine were missing patches of melanophores and iridiophores that closely matched regions from which cells were removed. Even xanthophores developed abnormally when TNC cells were removed from the posterior trunk/tail. In one embryo in which TNC cells were removed from the posterior extremity of the trunk, abnormal pigmentation was accompanied by delayed development, a degenerating tail (Fig. 19D) and spastic muscle twitching. Regardless of the axial level, melanophore disruptions most frequently occurred within and anterior to operated regions; iridiophore disruptions more frequently occurred posterior to operated regions.

Does the stage of neural crest removal affect pigmentation?

To determine whether the stage when neural crest cells are removed affects regulation, TNC cells were removed from the level of somites seven-11 between the 16-20 ss. A comparison of regulation across several stages is significant for determining whether regulation does not occur or is limited during specific times of development (Table 5). Bearing in mind that neural crest cells are not equally distributed over the neural keel through

development, this comparison also indicates how TNC cells regulate at various stages of development.

16 ss

Neural crest cells in the head and cardiac regions of 16 ss embryos are in transition from migrating as a sheet along the lateral path between the epidermis and somites to migrating segmentally in smaller numbers along the same path (see Chapter two). In the trunk, sheet migration of neural crest cells is nearly complete and a lull in TNC development is observed. Of the stages examined, the 16 ss exhibited relatively less regulation for pigment cells derivatives. Under these conditions, the word 'relatively' is used liberally because it is not practical to have sample sizes large enough for true statistical comparisons. While 'gaps' in melanophore pigmentation were typically 'filled-in' by four days post-hatch for most stages, it took relatively longer for these cells to be replaced when extirpations occurred at the 16 ss (Table 5; Figs. 21A-D). Minor disruptions in melanophore and iridiophore patterning were observed towards the anterior end of extirpated regions. Xanthophores were unaffected at this and subsequent stages.

17 ss

Although development of normal pigmentation, especially laterally positioned melanophores, often was delayed in some embryos following surgery, pigmentation was normal within 48 hours after TNC cell extirpations (Table 5; Figs. 22A, B). Disruptions in melanophore or

iridiophore patterning occurred within the anterior segments of the surgery sites or anterior to where cells were removed.

18 ss

TNC extirpations at the 18 ss created disruptions in melanophore patterning such as abnormally spaced cells or increased densities of melanin in half of the embryos within the first 48 hours. These disruptions always occurred anteriorly, whether anterior to the surgery site or anteriorly within the surgery site. More embryos were lacking iridiophores, or had slight abnormalities in patterning at four days post-hatch than at previous stages (Table 5).

19/20 ss

Regulation at the 19/20 ss most resembled regulation at the 17 ss. No loss of pigmentation was observed at 48 hours; minor disruptions in melanophores and iridiophore patterning were not evident by four days post-hatch. In one individual, iridiophores were randomly merged in some areas and distantly spaced in others. Even for this more dramatic disruption in iridiophore patterning, the pigment pattern was restored by four days post-hatch.

To summarize regulation as assessed by pigment cells, the stage when TNC cells are removed from the mid-trunk does not impact regulation for pigment cells to the same extent as the amount removed and the location from which they are removed. Embryonic survival was poor when neural

crest cells were removed from the cardiac region. Embryos that made it through hatching exhibited abnormal development, pericardial deformities and/or absence of pigmentation. On the other hand, trunk neural crest cells (posterior to somite five) exhibited extensive regulation, especially when fewer than nine somite lengths were removed. Experiments demonstrated that regulation was often complete when TNC cells were removed from the level of somites seven-11 at various stages. At this axial level, the absence and disruption of pigmentation were indicators of incomplete regulation. Generally, absence of melanophores correlated with greater densities and disruptions of surrounding melanophores at all stages, regardless of the amount of neural crest cells removed. This pattern was not shared by iridiophores which tended to be missing in localized patches. Whereas iridiophores were missing alone or in conjunction with melanophores at later stages (Table 5; Figs. 21A, C), melanophores alone were more frequently missing after TNC extirpations in early stage embryos (Fig. 20A). In most embryos, initial disturbances in pigment patterning were resolved by four days post-hatching. Xanthophores were the least affected by extirpations.

Neuronal antibody labeling trials in 10 ss to 36 hpf embryos

Embryos ranging from the 10 ss to 36 hpf were immunostained with one of five antibodies: anti-acetylated-α-tubulin (6-11B-1), 16A11 (anti-Hu), HNK-1, zn-5 or zn-12. None of these antibodies, with the possible exception

of anti-Hu, consistently identified developing dorsal root ganglia neurons in the stages indicated.

HNK-1 and zn-12 reactivity were almost identical. In 10-12 ss embryos, HNK-1 labeled trigeminal ganglia and paired aggregates of Rohon-Beard neurons regularly spaced along both sides of the dorsal neural keel in the trunk. Rohon-Beard cells are transitory sensory neurons positioned in pairs along the dorsal neural keel (Taylor and Robert, 1983). By the 20 ss, HNK-l label appeared in the large trigeminal ganglia, and in the dorsal longitudinal fasciculus (dlf) that runs parallel on both sides of the dorsal neural keel and links Rohon-Beard cells as bilateral chains (Figs. 24A, B), as well as in Rohon-Beard axons that have extended ventrally beneath the epidermis (Figs. 24A-D) (Canger et al. 1998).

Anti-Hu was expressed in 14-15 ss embryos by neurons located lateral to the neural keel along the trunk axis. Between the 16-18 ss anti-Hu labeling was negative. In 20 ss to 24 hpf embryos, neuronal deposits located lateral to the neural keel were labeled in a regular pattern along the dorsal-lateral axis, similar to earlier stages. However, anti-Hu labeling was highly variable in older embryos (especially at 22-24 hpf) and frequently appeared only in the most caudal neurons. The central regions of dorsal root ganglia were labeled in embryos older than 48 hpf.

Identical to previous experiments (e.g., Brand et al., 1996; Morin-Kensicki and Eisen, 1997), the zn-5 antibody labeled the axons and cell bodies of secondary motor neurons. As demonstrated for the anti-Hu antibody,

dorsal root ganglia were labeled beginning at 48 hpf. Peripheral nerves to axial muscles also were labeled at this stage.

Although anti-tub has been described as labeling most axons in the developing embryonic nervous system (Karlstrom et al., 1996), my results suggest that anti-tub more selectively labels neural cells. Between the 13 and 14 somite stages, the ventral somite/dorsal yolk interface (identity unknown) was weakly labeled with anti-tub. Anti-tub label also appeared at the dorsal midline of the neural keel. Little can be inferred from anti-tub labeling between the 15 and 25 somite stages. Beginning with the 26 ss, anti-tub labeled Rohon-Beard cell bodies; by 24 hpf Rohon-Beard axons were labeled along with some peripheral nerve fibers. Neither Rohon-Beard axons nor peripheral nerve fibers exhibited a consistent segmental organization (Figs. 24E, F), although the position and patterning of these axons give them the appearance of being part of the dorsal root ganglia.

Discussion

Regulation

Much of our insight on regulation has been indirectly harnessed from neural crest 'fate-mapping' studies. The goal of such studies was to remove cells and subsequently infer missing or reduced structures as being derived from those cells. In order to prevent regulation from occurring, and to effectively deplete structures, it was necessary to extirpate large regions of

neural crest (e.g., Newth, 1951; Hammond and Yntema, 1964). In addition to 'fate-mapping' studies, more direct tests of regulation have tended to be specific to a particular population, or even derivative of neural crest cells. In this study, the development and patterning of TNC-derived pigment cells were used as measures of regulation in zebrafish. The experimental procedure involved extirpating regions of TNC and varying the amount removed, position and stage of removal. Regulation occurred on a sliding scale, ranging from complete to incomplete.

A combination of the amount of neural crest removed, and the position and stage of removal affects regulation

Based on the early fate-mapping studies by Yntema and Hammond (1964) and Langille and Hall (1984), I predicted that a sizable amount of TNC must be extirpated to produce defects in pigmentation. In experiments where the amount of neural crest cells removed spanned regions five somites or greater in length, defects in the amount and/or patterning of pigmentation were generated. Almost no pigment defects were observed when smaller regions of neural crest cells were extirpated, unless extirpations overlapped the cardiac region (somites one-three/five). The nature and extent of the defects also correlated with the position from which cells were removed. Regulation was most affected when TNC cells were removed from anterior (cardiac) and posterior (tail) extremities of the trunk at various stages (Table 4; Figs. 19A-D). Embryos younger than the 16 ss rarely survived manipulations in the cardiac region. Those that did survive were underdeveloped, had

minor pericardial defects and/or spinal column deformities, and were missing pigment cells (Fig. 19B).

Although heart development was not investigated following neural crest cell extirpations, the results suggest that cardiac neural crest cells have limited regulative ability. This was no surprise; several studies have shown that precursor cardiac neural crest cells in the chick do not regulate (Bockman and Kirby, 1984; Kuratani et al., 1991; Waldo et al. 1996; Suzuki and Kirby, 1997). Cardiac neural crest cells provide both ectomesenchymal and neuronal components to the heart and great vessels, and connective tissue to thymus, thyroid and parathyroid glands (Le Lièvre and Le Douarin, 1975; Bockman et al., 1997; Kirby, 1988b; Kuratani and Kirby, 1991). The lack of regulation for cardiac neural crest cells suggests that they are specified early in development, and that timing of migration is critical for normal differentiation. Thus, any opportunity for these cells to regulate would likely fall within a narrow window of time. Because cardiac neural crest precursors emerge and migrate as a large population of tightly layered cells (Chapter two; Fig. 7A), extirpations may target so many cells that the embryo cannot adjust for the loss.

Regulation also was poor when TNC cells were removed from the posterior trunk/tail. In zebrafish, tail and trunk tissue contribute neural crest cells as far anteriorly as somites 11-12 (Kanki and Ho, 1997). Because cellular movements and differentiation in the tail are less well studied than in the

anterior-mid trunk, interpreting how regulation functions for cells extirpated from the tail will need to be the topic of another study.

Extirpations of TNC cells at different stages affect populations with different migratory patterns, from cells that are prevalent at the 14-16 ss and destined to migrate laterally as a uniform sheet, to cells that migrate segmentally on the medial path from the 10 ss onward, or on the lateral path from the 18 ss onward. Moreover, neural crest cells are not uniformly present along the entire length of the dorsal neural keel, and therefore, may not be uniformly present within the surgery site. Bearing these factors in mind, regulation for zebrafish TNC cells did not appear to vary significantly across stages (with the exception of somite stage 16). In studies of the chick, regulation for hindbrain neural crest cells changes during development, yet there is some contention as to whether optimal regulation occurs early (prior to the four ss; Saldivar et al., 1997), or later in development (approximately the 10 ss; Diaz and Glover, 1996).

Large amounts of zebrafish TNC cells emerge from the neural keel between the 17-21 ss. When constant lengths of TNC cells were removed from the level of somites seven-11 between the 16 and 20 ss, pigmentation was normal in most embryos examined at four days post-hatch. In other individuals, pigment cells appeared to be missing or disrupted at 48 hours, but had recovered in those same individuals by four days post-hatch. Embryos at the 16 ss were the most likely to be missing pigment cells, namely melanophores and iriodiophores, at four days post-hatch. Whereas segmental

migration of TNC is occurring along the lateral path in 18-21 ss embryos, fewer cells are emerging at the 16 ss during the transition between cells that migrated as sheets and those that will migrate segmentally.

The nature of pigment cells and the role of extracellular matrix

Trunk neural crest cells may acquire different regulative abilities over development, but my data suggest (with the exception of the cardiac region and tail) that regulation is most efficient when large numbers of TNC cells are migrating from the neural keel over a length of fewer than six consecutive somites. This would allow greater opportunity for relatively 'plastic' (undifferentiated) cells to compensate for the loss, compared to a stage where few cells are migrating, or cells still have to migrate. In a situation where cells still have to migrate, extirpation of TNC and neural keel cells would affect the development, migration and patterning of the next wave of cells (Weston, 1970; Nishida and Satoh, 1989; Patterson, 1990; Thibaudeau and Holder, 1998). At the other extreme, regulation is less, or at least delayed, when cells are removed from later stages (e.g. 21 ss and older; Figs. 20B-D). Cells that have differentiated or are beginning to differentiate might be less able to regulate – early migratory neural crest are heterogenous and may be more responsive to environmental signals (e.g. Frank and Sanes, 1991; Ito and Sieber-Blum, 1993; Le Douarin et al., 1994; Thibaudeau and Holder, 1998). At later stages of development, we would need to consider whether cell

replacement in larvae or among differentiated cells is regulation, or a different form of wound healing.

Evidence of decreased regulation in older embryos does not imply that compensation for cells removed during the embryonic period ends with a specific stage. Following TNC cell extirpations, pigmentation was typically monitored for up to seven days post-hatching. If abnormalities in pigmentation were observed between four and seven days post-hatching, the morphology was viewed as 'permanent' for the individual. I later found that the absence of pigmentation in one-week old larvae was not necessarily indicative of the final juvenile/adult pigment pattern. Extirpation of TNC cells at the level of somites seven-11 in a 16 ss embryo resulted in a patch of missing melanophores and iridiophores that was still evident at four days post-hatch. By 10 days post-hatching, pigmentation was perfectly normal (Figs. 21A-E). Thus, pigment cells are able to replace themselves, or to be replaced, well into the larval period (Johnson et al., 1995; Parichy et al., 1999). Furthermore, normal pigmentation in species of the salamander genus *Triturus* arises in part from a secondary invasion of pigment cells later in development (Niu, 1954).

Defects in pigmentation ranged from missing melanophores, iridiophores and/or xanthophores to increased densities and/or disrupted patterning of these cells. Disruptions in melanophore patterning were most commonly associated with iridiophores. Abnormalities in xanthophores were least common, but when they occurred, other pigment cells were

similarly affected. The absence of more than one pigment cell type after extirpations supports the suggestion that all three types originate from a common stem cell precursor (Epperlein and Löfberg, 1990).

In general, regions lacking pigmentation corresponded to locations from which TNC cells were removed. Patches of dense and/or disrupted pigmentation frequently occurred anterior to, and occasionally posterior to pigment-free surgery areas. In other cases, an excess of pigment cells, namely melanophores, was produced along the length of extirpated regions and persisted through at least one week post-hatching. Extirpations of both cranial and trunk neural folds in salamanders also have resulted in larvae with regions of increased pigmentation (Twitty, 1944; Lehman and Youngs, 1952) versus pigment free areas (Niu, 1947). Such uncharacteristically dense regions of pigmentation give the appearance of 'over regulation' (i.e. excess cell division) for cell loss. Conversely, isolated patches of dense or disrupted pigmentation may represent cells that failed to migrate as a result of severely disrupted extracellular matrix (ECM).

Several studies have demonstrated that the ECM is both structurally and molecularly important for the migration and differentiation of neural crest cells (Löfberg et al., 1980; Erickson et al., 1992; Erickson, 1993; reviewed by Parichy, 1996). The importance of ECM to neural crest cell migration is exemplified in the white axolotl mutant that lacks pigmentation because the ECM is a defective substrate for migrating precursor pigment cells (Löfberg et al., 1989). If TNC cells in zebrafish lack a scaffolding/matrix upon which to

migrate in either the anterior or posterior direction (Kanki and Ho, 1997), a "traffic jam" could occur at the borders of the surgery region where there is no matrix. Similarly, disrupted pigment cells could reflect an altered ECM that prevented cells from migrating along their normal routes.

Role of the epidermis in regulation

In these experiments regulation was accompanied by healing of the overlying epidermis. Interaction between non-neural ectoderm (epidermis) and neuro-epithelium (neural keel) is essential for neural crest cell development (Hall, 1999). In teleost fishes, the first neural crest cells appear after neural and epidermal ectoderm separate from one another (Schmitz et al., 1993; Papan and Campos-Ortega, 1994). In sham-operated control embryos where only the epidermis was removed, pigmentation was normal, although occasionally delayed. Delays in establishing normal pigment patterning could be a function of the time it takes for epidermis to heal. Studies on regeneration of fins and limbs have demonstrated that the epidermis heals rather quickly, within several hours up to 24 hours after surgery (Martin, 1996; Mescher, 1996; Ruiz et al., 1996). When neural crest cells are removed, the initial absence of epidermis might prevent induction of a new population of potentially regulating cells from the underlying neural keel. In addition, the epidermis is not immediately available as a substrate for cells to migrate into the neural crest-ablated area from surrounding regions. Because epithelium is an essential positioning signal for embryonic cells (Ho and

Weisblat, 1987), some instances of incomplete regulation may result from delays in epidermal closure.

Speculation on how neural elements may have been affected by extirpations

Assessment of regulation for TNC cells is not complete without corresponding neural data. A variety of neural cells in the trunk are known to be neural crest-derived, including those that form adrenergic, cholinergic (enteric), dorsal root and sympathetic ganglia (Le Douarin, 1982; Hall, 1999). To investigate whether precursor neural cells are replaced by regulation, markers are needed that can identify these cell types at embryonic stages closely following surgery. Korzh et al. (1993) comment that few markers are available for the earliest stages of commitment and differentiation of neurons in fishes. A marker that reveals neurons two-three days after extirpation might show the presence or absence of structures, but will not be an accurate indication of whether regulation occurred shortly after cell extirpation (i.e. was development delayed?). This is not exactly the case with pigment cells where presence or absence can be assessed within several hours of surgery, even though patterning is not complete.

In this study no antibodies were found to label dorsal root ganglia within 24 hours after fertilization. The response of embryos at the same stage and labeled with the same antibody also was quite variable. Several antibodies ultimately labeled the same neural derivatives, but at different times during development. For example, HNK-1 and zn-12 labeled Rohon-

Beard cells at all stages, beginning early in development (10-12 ss), while anti-tubulin did not reveal these cells until close to the 26 ss. Because mortality was low and few embryos exhibited problems such as decreased tactile sensitivity, upturned tail, and spastic ('aphasic') motor activity that might be associated with neural deficiencies following TNC extirpations, (pers. obs.; see also Grunwald et al., 1988), I predict that there is some regulation for neural derivatives. Until more is understood about early development of neural elements, the question of regulation for these cells is unresolved.

Potential sources of replacement cells – a summary

Many studies have discussed possible sources of cells that could replace extirpated neural crest. They include placodes, neural and epidermal ectoderm and neural crest itself (Kirby, 1987; 1988a,b; Kirby et al., 1989). Placodal cells are precursors to sensory receptors and cranial ganglia. They arise in the head as ectodermal thickenings that invaginate and further delaminate following transformation from an epithelial to a mesenchymal cell type in all but the lens placode. In this study, for placode cells to be considered a source of replacement cells for cardiac and TNC cells, they would either need to be in close proximity to extirpated regions, or migrate a considerable distance. Moreover, epidermis is not expected to be a source of replacement cells because it is necessary as an inducing factor for neural crest cells to be generated from the neural keel.

I propose that neural crest cells most commonly regulate for other neural crest cells, especially with regard to pigment derivatives in the trunk. Many studies (see Chapters one) have shown that neural crest cells regulate, and that regulative potential varies with cranial versus cardiac versus trunk crest populations. As alluded to in Chapter one in the context of fate-mapping studies, cranial neural crest cells regulate quite proficiently in organisms ranging from lampreys to chicks (e.g. McKee and Ferguson, 1984; Langille and Hall, 1988a,b; Moury and Jacobson, 1990; Scherson et al., 1993; Hunt et al., 1995; Couly et al., 1995; Diaz and Glover, 1996; Suzuki and Kirby, 1997). Cardiac neural crest, on the other hand, has little potential for regulation (e.g. Waldo et al., 1996; Suzuki and Kirby, 1997). Bearing in mind that regulation varies with the amount and position of removal, my study has shown there is regulation for TNC-derived pigment cells in zebrafish. The capacity for regulation of TNC-derived ganglia is unknown. Having demonstrated in zebrafish that at least a single cell lineage derived from the the neural crest is compensated for, I then investigated the source of replacement cells and the mechanism(s) of replacement (see Chapter four).

Table 3. Effects of extirpating different lengths of TNC cells at the 18, 19 ss on the presence and patterning of pigment cells in zebrafish embryos as assessed one week post-hatching.

Stage (ss)	Neural crest Removed (somites)[1]	n	Melanophores[2] N	Melanophores[2] A	Melanophores[2] D	Iridiophores[2] N	Iridiophores[2] A	Iridiophores[2] D	Xanthophores[2] N	Xanthophores[2] A	Mortality
19	1-5	5	0	40 (1-2)[3]	60 (4-6)	40	40 (7-11)	20 (6-9)	100	0	-----
18	1-7	6	0	33.3 (hb-2)	33.3 (hb-4)	16.7	50 (5-9)	0	100	0	33.3% died
18	1-9	5	0	60 (hb-2)	40 (hb-11)	0	60 (5-9)	40 (3-4)	100	0	-----
18	1-12	4		-----			-----		-----		All died

Notes: [1]Refers to the level (prs. somites) from which TNC cells were removed. [2]Numbers under pigment headings indicate percentages of the total sample size for each stage. [3]Numbers in parentheses refer to the somite levels from which cells were missing or patterning was disturbed. In some cases, the anterior extremity is the hindbrain (hb). **Abbrevreviations:** A: absence of pigment cells; D: pigment cell patterning is disturbed; N: normal pigment morphology in both amount and patterning; n: sample size.

Table 4. Effects of extirpating different regions of TNC cells at the 18, 19 ss on the presence and patterning of pigment cells in zebrafish embryos as assessed one week post-hatching.

Stage (ss)	Neural crest Removed (somites)[1]	n	Melanophores[2]			Iridiophores[2]			Xanthophores[2]		Other
			N	A	D	N	A	D	N	A	
19	1-5	5	0	60 (1-2)[3]	40 (1-4)	60	40 (7-11)	0	100	0	-----
18	5-9	7	100	0	0	100	0	0	100	0	-----
18	10-14	6	16.7	33.3 (9-12)	50 (2-9)	50	33.3 (9-12)	16.7 (7-8)	100	0	33.3 % short fin
18	14-18	5	20	60 (15-18)	20 (13-17)	40	40 (<14)	20 (<15)	40	60 (>11)	-----

Notes: [1]Refers to the level (prs. somites) from which TNC cells were removed. [2]Numbers under pigment headings indicate percentages of the total sample size for each stage. [3]Numbers in parentheses refer to the somite level from which cells were missing or patterning was disturbed (<, anterior to; >, posterior to). **Abbreviations:** A: absence of pigment cells; D: pigment cell patterning is disturbed; N: normal pigment morphology in both amount and patterning; n: sample size.

Table 5. Effects of removing a constant length of TNC (somites 7-11) on presence and patterning of pigment cells. Cells were removed from 16-20 ss embryos, and pigmentation analyzed at 48 hpf and 4 days post-hatching.

	Stages (ss)[1]											
	16			17			18			19/20		
	N	A	D	N	A	D	N	A	D	N	A	D
Melanophores												
48 hpf	5	2 (5; 6-8)[2]	1 (7-9)	6	0	2 (5/7-9)	4	0	4 (5/7-8/9)	7	0	1 (9-11)
4 dph	6	2 (5; 6)	0	8	0	0	8	0	0	8	0	0
Iridiophores												
48 hpf	6	1 (5-6)	1 (7-9)	7	0	1 (7-9)	5	2 (7-8)	1 (7-9)	6	0	2 (9-11; 2-9)
4 dph	7	1 (6)	0	8	0	0	6	1 (7-8)	1 (7-9)	8	0	0
Xanthophores												
48 hpf	8	0	na	8	0	na	8	0	na	8	0	na
4 dph	8	0	na	8	0	na	8	0	na	8	0	na

Notes: [1]Sample size is n=8 for each stage from 16-19/20. Numbers (no parentheses) under each stage category are individuals per sample size. [2]Numbers in parentheses refer to the somite level from which cells were missing or patterning was disturbed. **Abbreviations:** A: absence of pigment cells; D: pigment cell patterning is disturbed; N: normal pigment morphology in both amount and patterning; na: not applicable – xanthophores were analyzed only for presence or absence.

Fig. 16. TNC extirpation. Lateral view of an 18 ss embryo demonstrating surgical extirpation of TNC cells (arrows) using a glass-pulled micropipette that has been inserted just beneath the epidermis at the level of somites eight-eleven. Somites are visible as chevron-shaped blocks; the notochord is visible behind the somites. Rostral is to the left.

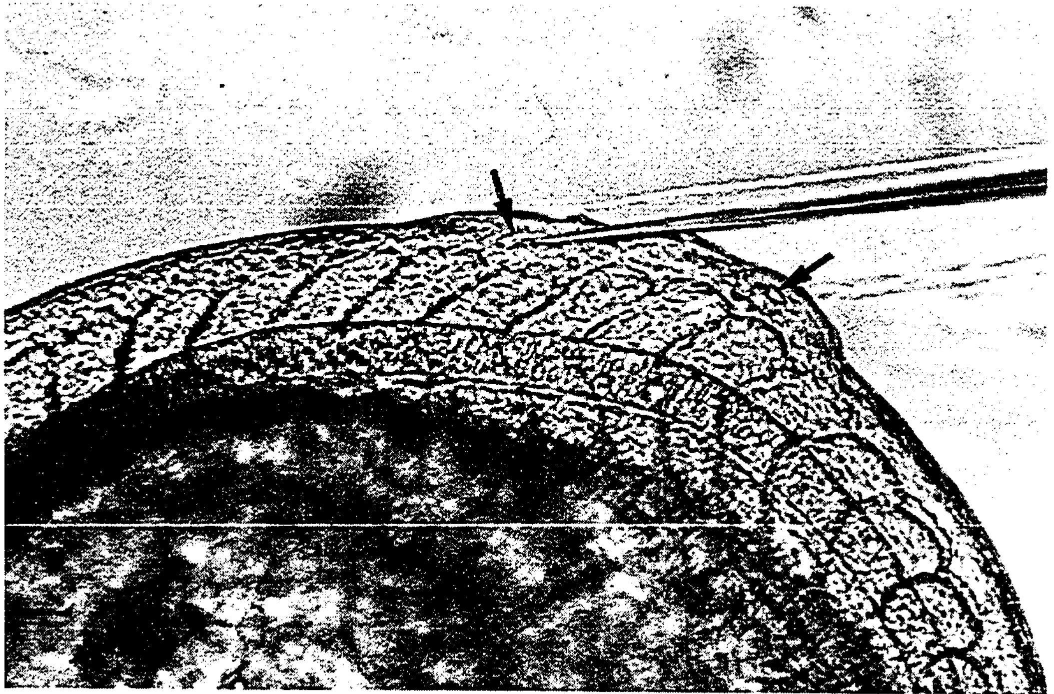


Figure 16

Fig. 17. Histological section through the mid-trunk of an operated 17 ss embryo. The epidermis and uppermost cell layers of the neural keel have been surgically extirpated (arrow). The intact epidermis is visible lateral to both sides of the neural keel (arrowheads). Bar = 50 μm.

Figure 17

Fig. 18. Pigmentation in controls. Dorsal and lateral views of pigmentation in the same individual at 48 hours (**A, B**) and four days post-hatch (**C, D**). **A:** Melanophores form a dorsal stripe that opens into a 'v' over the braincase. **B:** Laterally, melanophores form a mid-lateral 'dashed' stripe (arrows), as well as ventral and yolk sac stripes (arrowheads). **C:** Iridiophores (arrows) are associated with melanophores in regions such as the head, trunk and yolk sac. **D:** Xanthophores (arrowheads) produce a yellowish-colored pigment that can be seen dorso-laterally from head to tail.

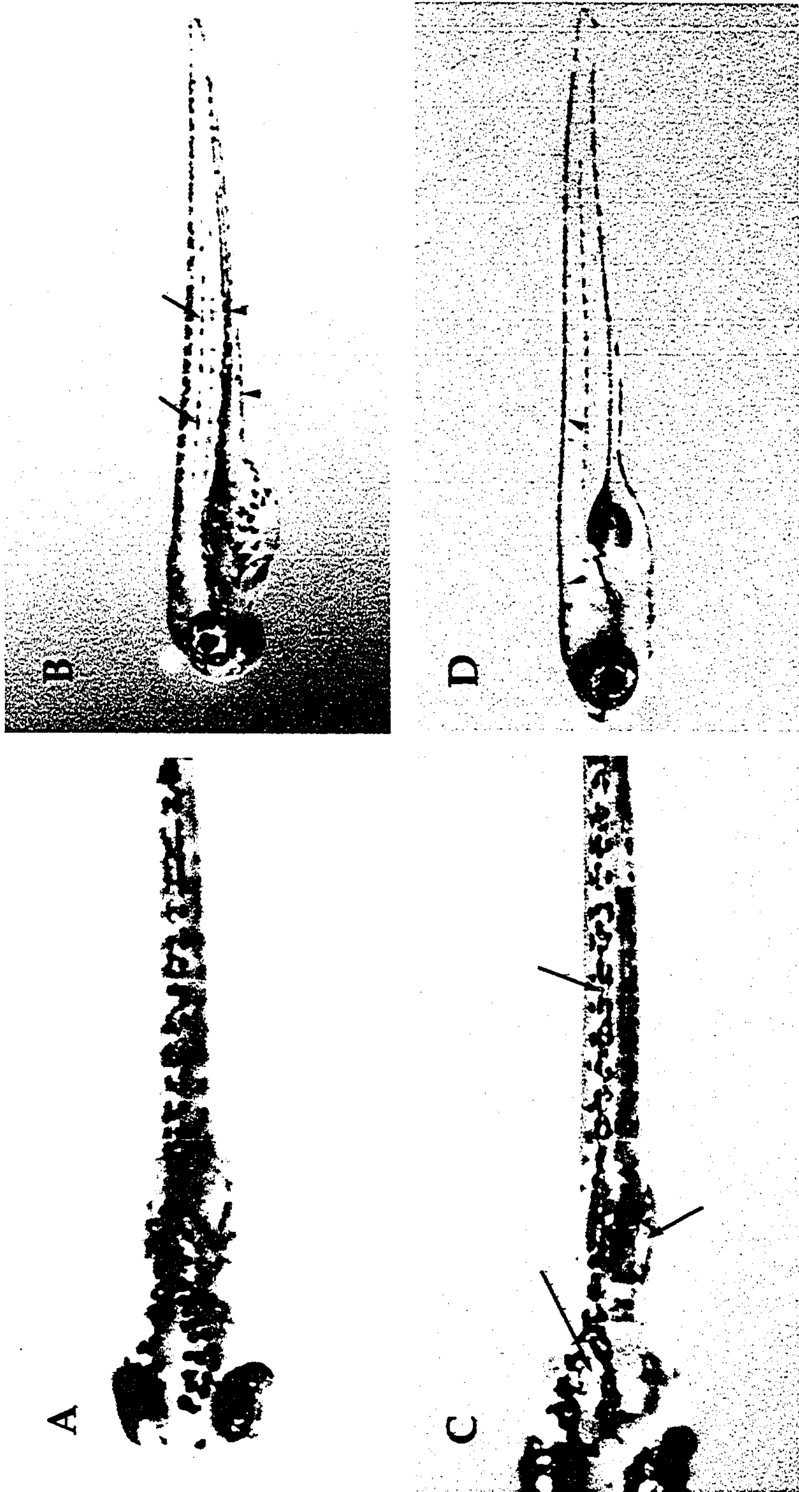


Figure 18

Fig. 19. Pigment and developmental abnormalities in larvae after extirpation of TNC cells. Indicated for each image are the stage of TNC extirpation (ss) and the somite level from which cells were removed (parentheses). All larvae are one-week post-hatching except for **(D)** which is 60 hours post-fertilization (hatching occurs at 72 hours). **A:** Melanophores, iridiophores and xanthophores are missing dorsally over the hindbrain and at the level of the first two somites (arrow). Abnormally dense patches of melanophores are present anterior and posterior to the region of missing cells (arrowheads). **B:** This embryo is underdeveloped with an enlarged pericardial cavity (arrow) and enlarged yolk sac. Abnormal pigmentation included loss of dorsal melanophores over first three somites within the operated region, and loss of dorsal iridiophores within and posterior to the same area. **C:** Melanophores are missing over the posterior hindbrain and at the level of the first somite (arrow), while iridiophores have merged at the level of the third somite (arrowhead). **D:** Prior to hatching this embryo was underdeveloped with a degenerating tail. In addition, melanophore patterning was severely disrupted, and iridiophores and xanthophores were absent.

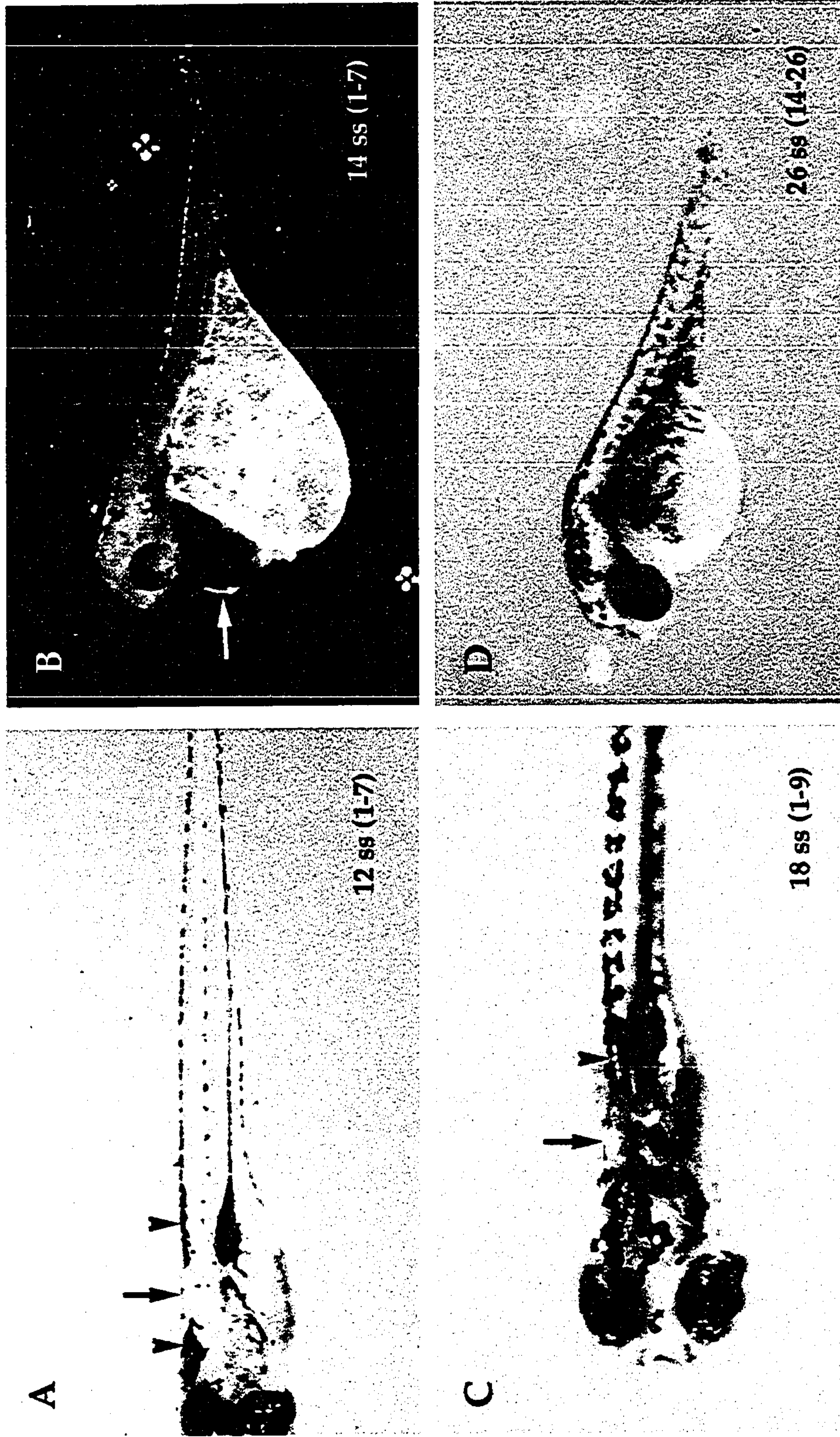


Figure 19

Fig. 20. Pigment morphology in larvae after extirpation of TNC cells.
Indicated for each image are the stage of TNC extirpation (ss) and the somite level from which cells were removed (parentheses). All larvae are one-week post-hatching. Vertical lines indicate the regions from which TNC cells were extirpated. **A:** There is little disruption of melanophores; however, iridiophores are missing within and posterior to the operated region (between lines). **B:** Melanophores are disrupted and abnormally dense anterior to and within the operated region (between lines). Iridiophores are absent from the same region, and xanthophores are sparse. Although not visible in this image, patterning of the mid-lateral melanophore stripe also is disrupted. **C:** Melanophores are missing from the hindbrain (arrow), and both melanophores and iridiophores are absent the length of somites 6-10 (arrow, between lines) and disrupted posterior to somite 10. **D:** The lateral view of **(C)** shows the dorsal fin fold missing or reduced along the length of somites six-10 (arrow, between lines).

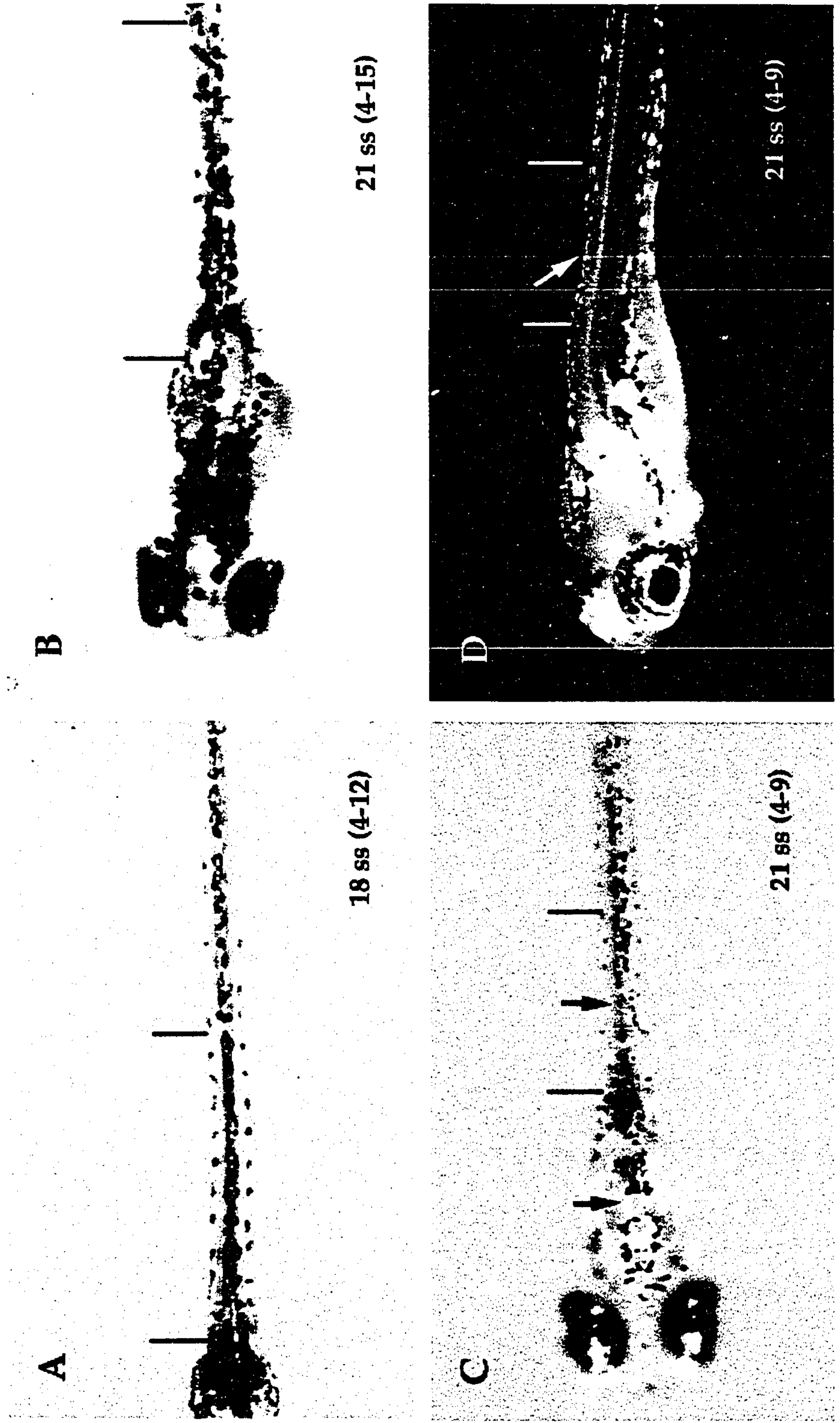


Figure 20

Fig. 21. Pigmentation in larvae after extirpation of TNC cells at the 16 ss. Dorsal and lateral views of pigmentation in the same individual at 48 hours (**A, B**), four days post-hatch (**C, D**), and a dorsal view at 10 days post-hatching (**E**). TNC cells were removed from the level of somites seven-11, as indicated by the parallel vertical lines. **A-B:** Melanophores and iridiophores are absent at the level of somite seven (**A**, arrow), and melanophores are disrupted anterior to and within the operated region. **C-D:** Melanophores and iridiophores are absent from the same location as in A-B (arrow).
E: Interestingly, pigmentation in this embryo is completely normal 10 days after hatching. Note how melanophores have filled in the 'gap' observed at the younger stages.

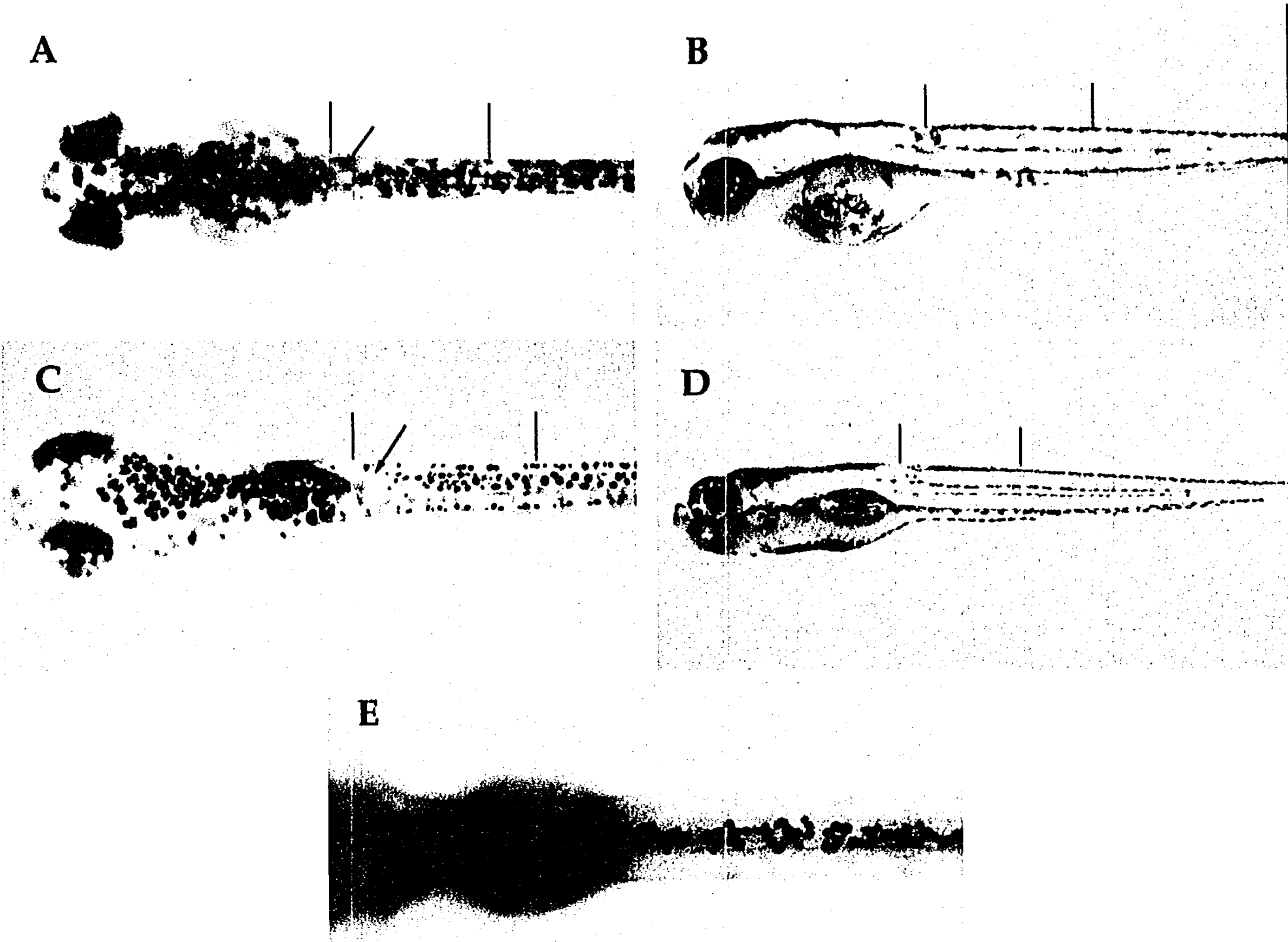


Figure 21

Fig. 22. Pigmentation in larvae after extirpation of TNC cells at the 17 ss. Dorsal and lateral views of pigmentation in the same individual at 48 hours (**A, B**) and four days post-hatch (**C, D**). TNC cells were removed from the level of somites seven-11, as indicated by the parallel vertical lines.

A-B: Development of pigmentation is slightly delayed and disorganized, especially among lateral-stripe and yolk sac melanophores (**B**, arrowheads). By four days post-hatching, pigmentation is normal **(C-D)**.

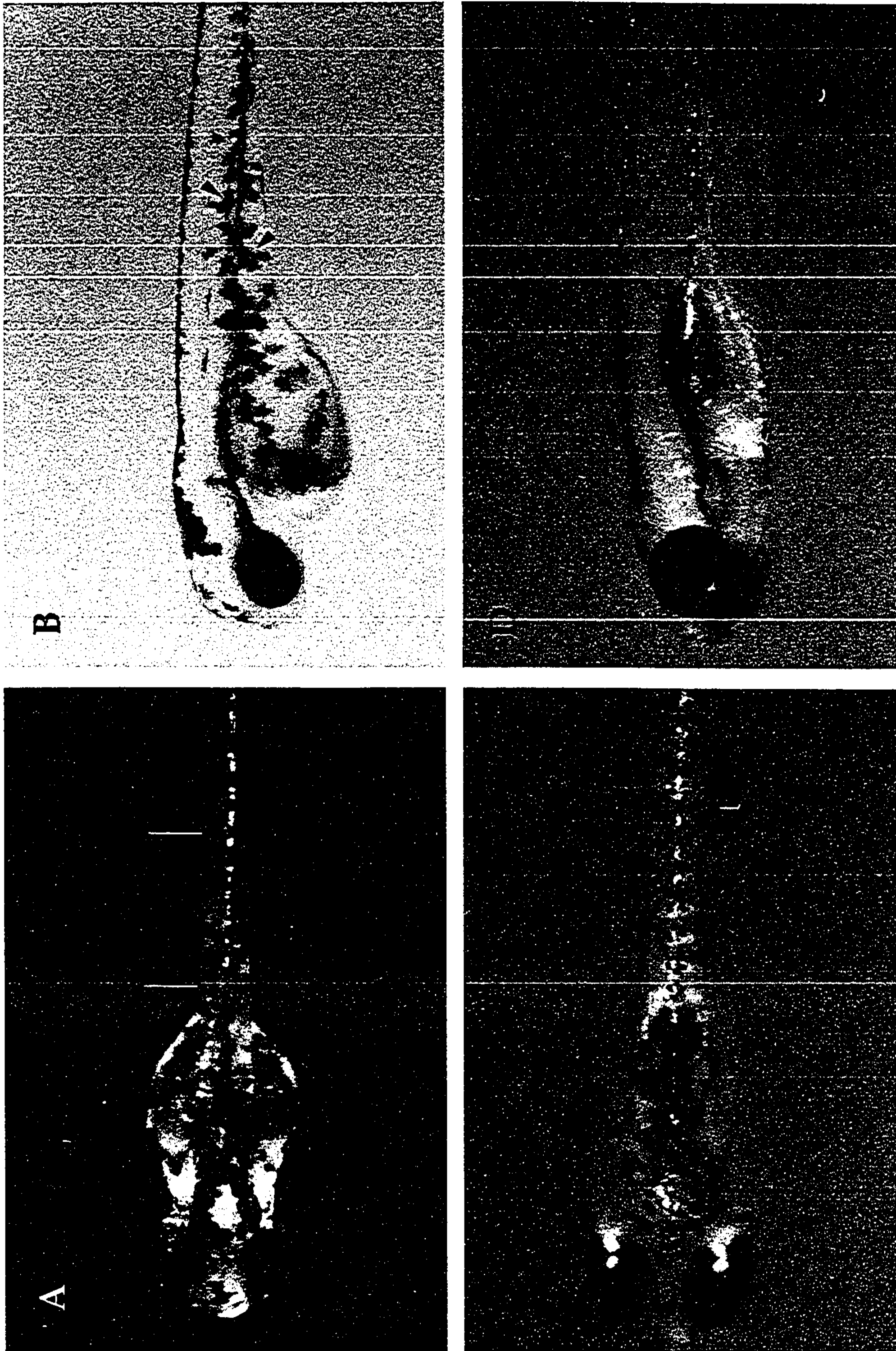


Figure 22

Fig. 23 Pigmentation in larvae after extirpation of epidermis only (sham-operated controls), or of TNC cells (including epidermis) at the 19 ss. Dorsal and lateral views of pigmentation in two different individuals at two days (**A-B; E-F**) and four days post-hatch (**C-D; G-H**). In one individual, epidermis alone was removed (**A-D**). In the other individual, TNC cells (including epidermis) were removed (**E-H**). In both cases, cells were removed from the level of somites seven-11, as indicated by the parallel vertical lines. **A-D:** No loss or disruption of pigmentation or dorsal fin fold was observed in sham-operated control embryos from which only the overlying epidermis was removed. **E-H:** Likewise, pigmentation was normal in embryos from which TNC cells were removed, even in those as young as 48 hpf. Lateral melanophore stripes (**F**, arrows) and dorsal iridiophore and melanophore pigmentation (**G**, arrows) were strikingly well developed and organized.

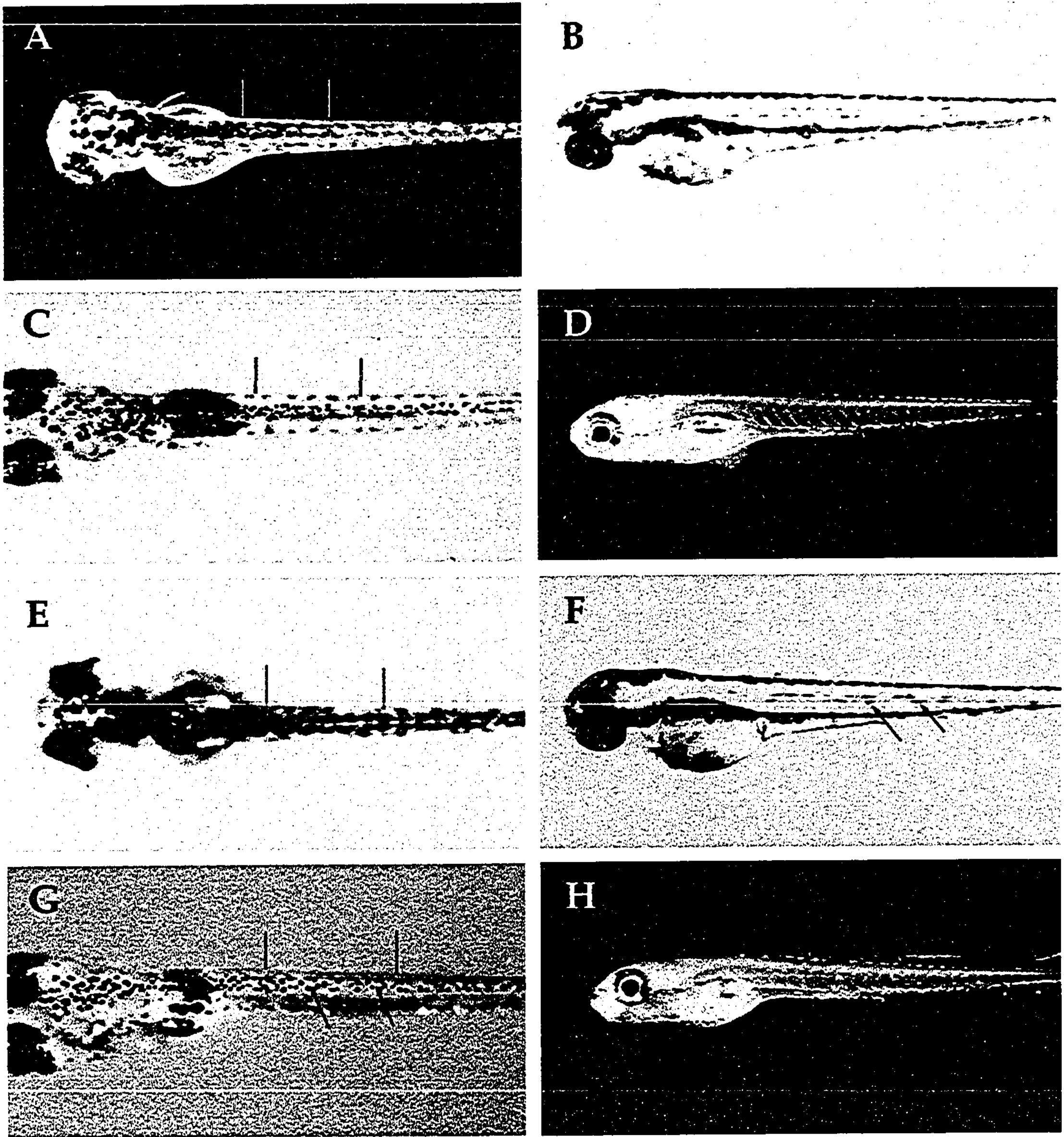


Figure 23

Fig. 24. Whole mount neuronal antibody labeling in zebrafish embryos. Head (anterior) is to the left in all images. **A-D:** HNK-1 labeling in 21 ss embryos. Unlike many vertebrates, TNC cells in zebrafish embryos were not labeled by the HNK-1 antibody. Cells that were labeled included the neural crest-derived trigeminal ganglia in the head (**A**, arrow), Rohon-Beard cell bodies (**B**, arrowheads) and the dorsal longitudinal fasciculus (dlf; **C**, arrow). The boxed region in **C** is enlarged (**D**) for a closer view of Rohon-Beard cells (arrowheads) and the dlf (arrow). **E, F:** Anti-acetylated-∝-tubulin (anti-tub) labeling in 24 hpf embryos. As with HNK-1, anti-tub labels Rohon-Beard cells (**E**, arrowheads) and the dlf (**E**, arrow). The boxed region in **E** is enlarged (**F**) for a closer view of superficial axons thought to be peripheral nerve fibers (arrows).

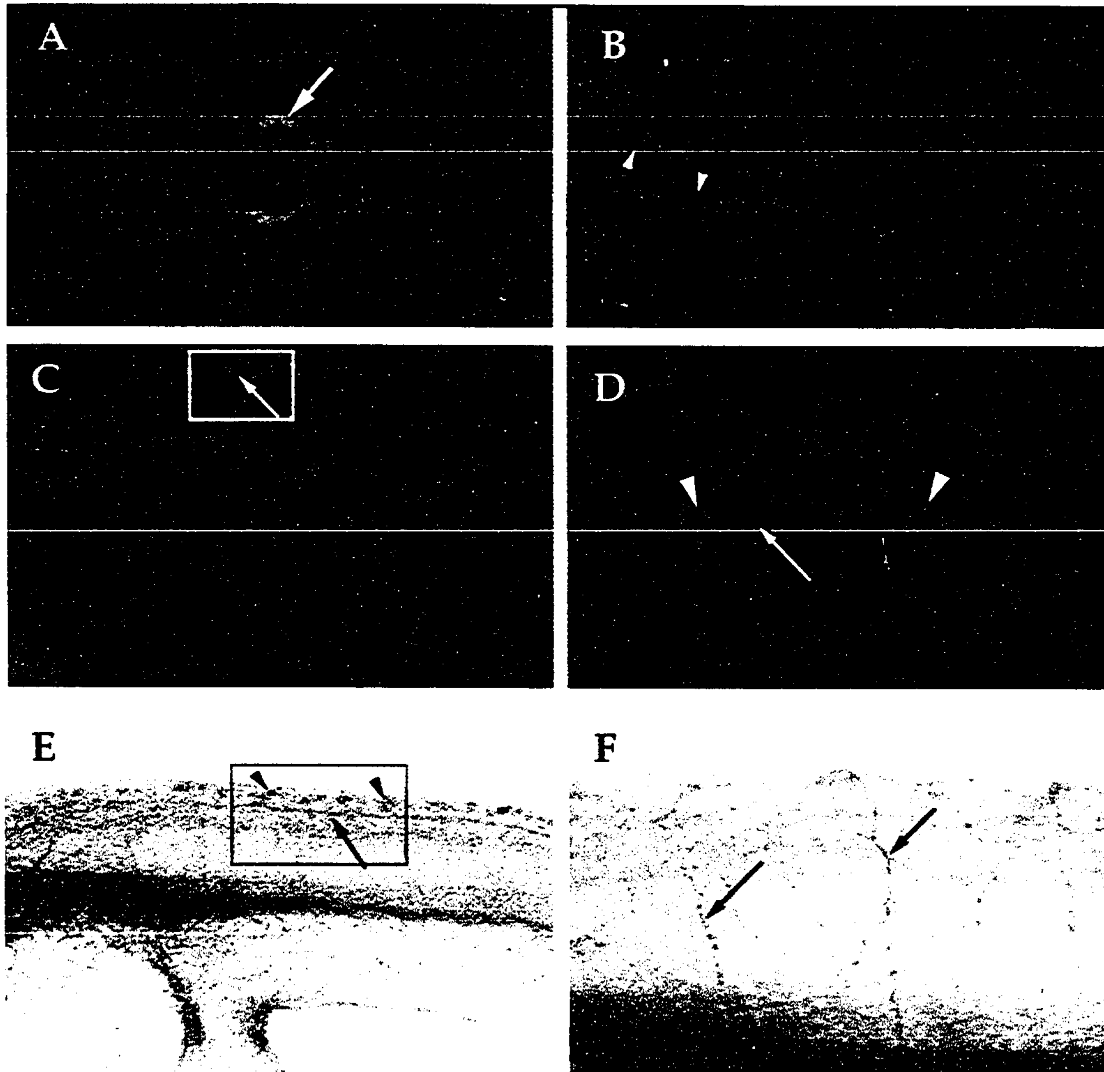


Figure 24

Chapter 4

Mechanisms of Regulation for Trunk Neural Crest Cells in Zebrafish

Introduction

Wound healing and regeneration are commonly associated with post-embryonic cell and tissue repair in vertebrates. But what if cells are damaged, defective, or lost during embryogensis? Regulation is the phenomenon by which embryos compensate for actions that would otherwise lead to abnormal development, such as defective cell proliferation or migration (Bellairs, 1971; Hall and Hörstadius, 1988). I have more specifically defined regulation as the replacement of lost, undifferentiated embryonic cells by other cells in response to signals received from the surrounding environment.

Neural crest cell populations are good subjects for studies of regulation because they exhibit broad potentials and are accessible to experimental manipulation and observation. In the head, cranial neural crest cells become connective tissue, cartilage, bone, odontoblasts and ganglia. Cardiac neural crest cells contribute neuronal and ectomesencymal components to the heart. Trunk neural crest (TNC) cells differentiate as pigment, Schwann and

sympathoadrenal cells, glia, and sensory ganglia (Hall, 1999). In humans it has been shown that mistakes in neural crest cell migration or proliferation can result in a variety of abnormalities from cleft palate and cardiac pulmonary septation defects to neuroblastoma (Jones, 1990; Hall, 1999).

Regulation of neural crest cell populations has been demonstrated in a variety of vertebrates, especially the chick, and primarily in the cranial region. Studies have shown that extirpation of cranial neural crest cells results in normal morphology of skeletal, hypobranchial and neuronal elements in the head (e.g., Noden, 1983; McKee and Ferguson, 1984; Scherson et al., 1993; Hunt et al., 1995; Couly et al., 1996; Suzuki and Kirby, 1997) (Chapter one). In contrast, cardiac neural crest ablation frequently generates abnormalities of the heart, aorta and pulmonary arteries (Nishibatake et al., 1987; Waldo et al., 1996; Suzuki and Kirby, 1997) (Chapters one, three). Least is known about regulation for populations of trunk neural crest cells; studies have described less compete regulation for TNC derivatives such as spinal ganglia than for pigment cells (Yntema and Hammond, 1945; Lehman and Youngs, 1952; Chibon, 1970; Suzuki and Kirby, 1997) (Chapters one, three).

Because most studies of regulation have focused on the chick, research on regulative potential in other craniates is needed to elucidate the evolution of this little understood phenomenon. In Chapter three I demonstrated that there is regulation for trunk neural crest-derived pigment cells in zebrafish. Having established that regulation occurs, the question becomes how do these cells regulate? Four potential mechanisms of regulation are (1) altered cell

migration, (2) increased cell division, (3) change in cell fate and (4) decreased cell death.

In this chapter data are presented for altered cell migration and increased cell division as possible cellular mechanisms of regulation in zebrafish embryos. Fluorescent DiI labeling of neural crest cells in chicks revealed that cells migrate from locations adjacent to extirpation sites (e.g., Sechrist et al., 1995; Couley et al., 1996; Diaz and Glover, 1996; Saldivar et al., 1997). Furthermore, increased cell division has been demonstrated in many studies of the chick through the detection of mitotically active cells with bromodeoxyuridine (BrdU; McKee and Ferguson, 1984; Diaz and Glover, 1996; Couly et al., 1997). The potential for cells to change fate is another important aspect of regulation that will be addressed in the context of other studies in the discussion of this chapter.

Because I found evidence of regulation for extirpated TNC cells (Chapter three), possible sources of replacement cells were investigated by injecting DiI at the anterior and posterior margins of TNC-extirpated regions, as well into the underlying neural keel. TNC cells were observed migrating into ablated regions from adjacent locations, with migration occurring preferentially from posterior to the surgery site. In some cases, labeled cells formed derivatives that matched those formed at the same stage and in similar locations in controls. Additionally, BrdU labeling for cell division revealed that increased division is a rapid response to extirpation, namely

among groups of TNC and neural keel cells in close proximity to extirpated regions.

Materials and Methods

Zebrafish maintenance and breeding

Adult zebrafish (*Danio rerio*) were purchased from either local pet stores in Halifax, Nova Scotia, Canada, or from Boreal Laboratories, Ltd. (Ontario, Canada). Females and males were separated and kept in 10 gallon tanks at a temperature of 27-29° C on a 14 hour light and 10 hour dark cycle. Breeding colonies were established with three females and three-five males. Eggs were collected in the evening and raised in Hank's solution (Westerfield, 1995) in a water bath set to 28.5° C.

Removal of trunk neural crest cells

To investigate how cells in the trunk region of zebrafish embryos regulate for the loss of neural crest, trunk neural crest (TNC) and dorsal neural keel cells were removed from the level of somites seven to 11 (approximately five somite lengths). Zebrafish embryos ranged in stages from 12 to 20 pairs of somites (Appendix I). After removing the chorion with #5 watchmaker forceps, embryos were positioned on a slide in 1.2% low melting point agarose and viewed using a Zeiss inverted compound microscope.

Under magnifications of 25 or 40x cells were removed using a pulled glass micropipette attached to a micromanipulator. Following surgery, embryos were either immediately injected with DiI, or recovered in Hank's solution containing 2% antibiotic-antimyotics (GIBCO) prior to treatment with BrdU.

DiI

Fluorescent DiI (SP-DiIC_{18}(3)) was obtained from Molecular Probes, Inc. (D-7777) and diluted to a 2 mg/ml solution consisting of 92% Dimethylformamide (DMF) and 8% H_20. Several forms of DiI are available for cell labeling, but because most function by attaching to the cell membrane, the fluorescent label is easily lost from the tissue during fixation. The DiI chosen for this study is retained by fixed tissue, thus enabling the label to be visualized in histological sections or in whole mounts fixed in 4% paraformaldehyde and kept at 4° C in the dark.

Immediately prior to use, DiI aliquots were briefly centrifuged to removed any precipitate. Thick-walled glass capillaries (OD 1.2 mm, I.D. 0.69 mm with filament) were pulled into micropipettes using a vertical pipette puller. Micropipettes were backfilled with 1.5 µl DiI, followed by 1 µl 0.2M KCL and stored in glass jars in a 37° C incubator to slow crystallization of dye during injection sessions. At least 10 minutes were allotted for the dye to completely fill the micropipette tip.

Operated embryos were kept on slides in 1.2 % agarose and transferred from the inverted compound microscope to a Leitz Laborlux D compound microscope. DiI was focally injected beneath the epidermis two-three somite lengths anterior or posterior to the surgery site, or into the neural keel within the surgery site (Fig. 25). Injections consisted of a single two millisecond pulse using a PV830 pneumatic picopump (World Precision Instruments) attached to a nitrogen air source. The tank pressure was held constant at 100 kPa; ejection pressure was approximately 30 psi. Holding pressure varied slightly according to the size of the micropipette tip opening, but generally ranged between 10 and 20 psi. Specimens were viewed under a Leitz Aristoplan fluorescent microscope (N2.1 filter) at regular time intervals for up to 36 hours following injection.

BrdU (5′-bromo-2′-deoxyuridine)

Labeling dividing cells

Bromodeoxyuridine (BrdU) is a thymidine analogue that labels the DNA of dividing cells and can then be detected using a monoclonal antibody against BrdU. Dividing cells were labeled by soaking dechorionated embryos in a 10 mM solution of BrdU (BrdU powder dissolved in 15% DMSO and brought to the final volume with Hank's solution). Embryos from which TNC cells were removed at the 12-20 somite stages (ss) were transferred to Hank's solution containing 2% antibiotic-antimyotics (GIBCO) and allowed to

develop for an additional 30 minutes, one hour, two hours, or more than three hours in order to provide a 'snapshot' of cell division at different time intervals following exptirpation. Embryos were then soaked for 20 minutes in chilled BrdU solution, rinsed several times in Hank's solution, left to develop for 10 minutes in fresh Hank's at 28.5°, and fixed in 4% paraformaldehyde overnight at 4° C. Control embryos were processed exactly as operated embryos, with the stages matching the stages of operated embryos as they were placed in BrdU solution.

Antibody labeling of BrdU

Preserved embryos were rinsed several times in 0.1 M PBS (Dulbecco's phosphate buffered saline, Freshney, 1987), followed by two brief washes in PBS containing 0.1% tween (PBST). Embryos were soaked in 2 N HCl at room temperature for 1.5 hours, rinsed with PBST, incubated in 0.1 M Borax at room temperature for 10 minutes, and rinsed with PBST, followed by incubation in blocking solution (20% normal goat serum, 1% BSA, PBST) for three hours at room temperature. Embryos were then incubated overnight at 4° C in primary anti-BrdU antibody (Mouse IgG – supernatant, Hybridoma Bank, dilution 1:10). Following a one hour rinse with several changes in PBST, embryos were incubated in a secondary IgG-fluorescent CY3-conjugate (1:600) for two hours at room temperature in the dark, followed by extensive

rinsing with several changes of ddH_20 for several hours at room temperature and/or overnight at 4° C – again, in the dark.

Confocal microscopy

Labeled embryos were gradually processed to 100% glycerol (10% glycerol:dd_H20; 30% glycerol:ddH_20 etc.) and flat-mounted on slides with number zero coverslips for confocal microscopy. Embryos were viewed with a 10x or 25x objective lens and F set 15 Rhodamine filter on a Zeiss LSM 510 microscope. Embryos were optically scanned with a laser and images of the scanned sections were captured and transferred to Adobe Photoshop.

Results

Cells migrate into extirpated regions

In Chapter three I showed that there is regulation for pigment derivatives in zebrafish following extirpation of trunk neural crest cells. The possibility that surrounding TNC or neural keel cells regulate for loss by migrating into trunk neural crest-ablated regions was investigated by focally injecting operated embryos with DiI. Trunk neural crest cells were removed from the level of somites seven-11 in embryos ranging from the 12 to 20 ss. DiI was injected beneath the epidermis two-three somite lengths anterior or posterior to the extirpated site, or into the underlying neural keel to determine potential sources of regulating cells. Because experiments

discussed in Chapter four demonstrated that the stage when TNC cells are removed does not impact regulation to the same extent as the amount removed and location from which they are removed, differences in how cells regulate across developmental stages might not be expected. However, injecting a range of stages could reveal whether the efficiency and completeness of regulation varies with the population of cells extirpated. For instance, is regulation more complete and/or efficient (faster) if TNC cells destined to migrate along the medial path in 12-13 ss embryos are extirpated, or if TNC destined to migrate segmentally along the lateral path at the 17-18 ss are extirpated (Chapter Two).

Data from DiI injections are organized relative to five different stage groups. The stages correspond with different modes and pathways of TNC cell migration that would be observed in the anterior-mid trunk in control embryos. Moreover, DiI injections in control embryos have provided a good sense of the cell types derived from this region at different stages (Chapter Two).

12-13 ss

Cells labeled at the anterior and posterior margins of TNC-ablated regions in 12-13 ss embryos migrated across a portion of the surgery site. Trunk neural crest cells injected anterior to the extirpated region migrated up to three somites anteriorly (away from the surgery site) and two somites posteriorly. Cells injected posterior to the extirpated region migrated a

maximum of one-two somites anteriorly (into the surgery site), and one-two somites posteriorly (Table 6). Thus, cells at the anterior and posterior margins were unable to completely traverse the 'gap'. Regardless of whether DiI was injected anteriorly or posteriorly, labeled cells differentiated in dorsal positions as xanthophores. TNC cells injected at the posterior margin also contributed to the dorsal fin fold. Differentiation of TNC cells labeled anterior to the extirpated region (anterior to somite seven) differed from that of TNC cells labeled at the same level in controls. In addition to forming pigment, cells labeled in controls would typically differentiate into dorsal root and sympathetic ganglia, and in some instances, enteric ganglia and ectomesenchymal cells of the aorta. This discrepancy may reflect developmental variation in the timing of neural crest cell migration, or in the size of injections. Alternatively, if TNC cells normally migrate some distance posteriorly before migrating along the medial path at the 12-13 ss, extirpated regions might have prevented the normal migration and patterning of cells situated more anteriorly.

14-15 ss

Trunk neural crest cells migrated the entire length of the surgery site in approximately half of the 14-15 ss embryos with cells injected posterior to extirpated regions. In the other half of injected embryos, migration across the extirpated region was not complete (Figs. 26E-H). In contrast, TNC cells labeled at the anterior margin did not migrate more than two-three somites

posteriorly (towards the surgery site), and similar to the 12-13 ss, a maximum of two-three somites anteriorly (Figs. 26A-D). As with anteriorly labeled cells, cells from the neural keel did not traverse the entire surgery site, although some migration was observed. By 36 hpf DiI labeling was found in glial (e.g., Schwann) cells, or dorsal root ganglia, as has been demonstrated for control embryos injected at the 14-15 ss (Figs. 26C,D; G,H). The dorsal fin also was labeled, but there appeared to be no preferential contribution from cells injected anterior to, posterior to or within the extirpated region (Fig. 26H).

16 ss

In all but one individual, labeled cells did not migrate the length of the extirpated region. In fact, the epidermis did not heal properly in several embryos, and subsequently affected the migration of surrounding cells. In embryos where the posterior margin of the extirpated region was labeled, DiI stuck to the underside of the epidermis and was pulled along as it healed, but no TNC cells were observed migrating. TNC cells labeled at the anterior margin of the extirpated region migrated up to two somites anteriorly, similar to labeling in this position at other stages. It is not surprising that fewer TNC cells were labeled, and that labeled cells migrated shorter distances in 16 ss embryos compared to other stages. In Chapter two it was demonstrated that almost no TNC cells migrate from the mid-trunk at the 16 ss.

Typically, neural keel cells labeled within the extirpated region migrated a maximum of three somites anteriorly. Neural keel labeling in

one individual resulted in complete migration of cells across the ablated area, and differentiation of labeled cells into a variety of cells/tissues located anterior to, posterior to and within the extirpated region (Figs. 27A-D). Derivatives formed included pigment cells, dorsal root and sympathetic ganglia, and dorsal fin fold. It is possible that such extensive migration and differentiation occurred in one embryo because neural keel cells were in a phase of rapid proliferation at the time of extirpation and DiI labeling.

17-18 ss

Trunk neural crest cells labeled anterior to the extirpated region did not completely fill the ablated area in 17-18 ss embryos. Anteriorly labeled cells migrated a maximum of one somite posteriorly and four somites anteriorly, where they differentiated as pigment cells, dorsal root ganglia and fin fold (Figs. 28A-C). At the opposite extreme, in six of seven individuals, TNC cells labeled posterior to the extirpated region migrated the length of the surgery site and beyond (Figs. 28D-G). Extensive differentiation of the labeled cells closely resembled cell types that would be expected to migrate from levels anterior to somite eleven in control 17-18 ss embryos. Labeling was found in pigment cells, primarily melanophores, dorsal root and sympathetic ganglia, dorsal fin fold and ectomesenchymal cells of the aorta (Figs. 28F,G). Moreover, cell migration towards and within extirpated regions occurred rapidly, within three to five hours, in comparison to other stages where cells continued to migrate for up to 10 hours after injections.

Labeled neural keel cells completely traversed the extirpated region in half of the individuals injected. These cells differentiated as pigment and dorsal root ganglia, and contributed to the fin fold, but differentiation was less consistent and not as extensive as from cells labeled at the posterior margin. Labeled cells may be less able to differentiate if the neural keel is utilizing cellular resources to regenerate. This also would generate a time lag in subsequent TNC development and migration. It is not known if labeled keel cells change their fates to become TNC prior to differentiation.

19-20 ss

Unlike previous stages where complete migration into extirpated regions was limited to cells injected posteriorly, at the 19-20 ss cells injected both anteriorly and posteriorly migrated across the surgery site. As with the 17-18 ss, patterning and differentiation were most complete when cells at the edges of the 'gap' migrated shortly after TNC extirpation. TNC cells began to differentiate within eight hours of DiI labeling (20 hours post-fertilization), and by 36 hours post-fertilization, labeling was found in pigment cells (melanophores and iridiophores), dorsal root ganglia, dorsal fin fold and the aorta. In some cases cells labeled anterior to somite seven also contributed to the tail reticular system – a labyrinth-like network of channels that connect the caudal artery and vein (Chapter Two). It was difficult to discern why cells surrounding extirpated regions did not migrate throughout the surgery site in all embryos at this stage. Differences in the timing of TNC development and

migration that result from developmental variation might explain why it is nearly impossible to view identical events in all embryos specific to one experiment.

DiI labeling experiments demonstrated that TNC cells do migrate into extirpated regions from surrounding locations. This migration appears to be coincident with healing of the epidermis over the wound. It is possible that the epidermis closing over the wound pulls underlying cells along, thereby facilitating migration into the extirpated region. Although there were instances when DiI-labeled cells from the anterior margin or from the neural keel migrated the length of extirpated regions, cells migrating from the posterior margin most commonly filled in the 'gap.' Moreover, migration into extirpated regions was most complete when TNC cells were removed from 17-20 ss embryos.

The time frame for when cells migrated into TNC-ablated regions varied slightly with stage. To some degree, this variation could reflect differences in the depth of extirpation and cellular disruption. However, overall migration of DiI-labeled cells in embryos between the 12-16 ss was slower than migration in older embryos, regardless of whether cells migrated towards or away from the surgery site. In embryos between the 17 -20 ss labeled cells had migrated several somite lengths within three hours (20 hpf) of TNC extirpation. For cells labeled posterior to the extirpated region, this rapid migration occurred in the direction of the surgery site. Within seven hours of injection (24 hpf) labeled cells were differentiating into cell types

corresponding to those revealed by DiI labeling in control embryos. For instance, cells labeled posterior to the ablated region (somites 12-13/14) at the 17-18 ss ultimately formed pigment, dorsal root ganglia, sympathetic ganglia and contributed to development of the vascular system (aorta, tail reticular cells), as do cells labeled at that level in controls.

Differentiation and migration were observed through 36, and even 48 hpf. In some individuals DiI-labeled cells appeared to 'stack up' at the anterior and posterior edges of the surgery site. This behavior might signify that cells are prevented from migrating into the ablated region because the extracellular matrix (ECM) is disrupted, or because the epidermis has not healed sufficiently to supply the necessary signals for migration. Cells that appeared to be 'stalled' or 'stuck' eventually began to disperse short distances (one-two somite lengths) within seven-10 hours of DiI labeling (at least 24 hpf). These cells also began to differentiate, and by 48 hpf derivative formation in operated embryos looked remarkably normal. It is possible that neither exact timing of migration nor migration into extirpated regions are necessary for TNC cells to form the proper complement of derivatives required for normal development. The 'stacking' phenomenon also is indicative of another potential mechanism of regulation – that of increased cell division.

Cell division is elevated adjacent to extirpated regions

The idea that increased cell division is an important mechanism of regulation was tested by labeling cells with BrdU. Two embryonic stages were chosen for BrdU treatments based on the timing and pattern of trunk neural crest cell migration. The 15 ss represents a developmental period when TNC cells are completing early migration along the medial path (between the neural keel and somites) and beginning 'sheet-like' migration laterally between the somites and epidermis in the anterior-mid trunk. At the 18 ss neural crest cells begin to migrate in large numbers segmentally along the lateral path. Moreover, results from cell migration experiments demonstrated that TNC cells at this stage migrate quite proficiently throughout extirpated regions. Migration into the surgery site was not exhibited by most cells in the 15 ss. Comparison of cell division in these two stages might reveal whether the mode of regulation differs in younger versus older embryos.

Unfortunately, the BrdU technique does not allow for continuous observation of embryos through development. Patterns of cell division at the level of somites seven-11 were analyzed as 'snapshots' in time for different time intervals: 30 minutes, one hour, two hours, or more than three hours at the 15 and 18 ss. Cell division in operated and control embryos was compared through confocal images taken as optical longitudinal slices through identical levels of different embryos. In these experiments the epidermis, neural keel

and surrounding TNC cells were emphasized as potential sources of regulating cells.

Controls

Dividing cells in control embryos were labeled at the 17, 19 and 24 ss to correspond to equivalent stages in operated embryos. In all stages some cell division was observed in the endoderm. At the level of somites seven-11 in 17 ss embryos, somitic (sclerotomal) cells and some TNC cells divided in a regular, segmental pattern between the somites and neural keel and between the somites and epidermis (Fig. 29A, B). Some proliferation was observed in cells of the neural keel, while division was prevalent in cells of the floor plate and notochord (Fig. 29C). At the 19 ss TNC cells appeared to be dividing as they emerged from the neural keel and migrated laterally between the somites and epidermis (Figs. 29D-E). Epidermis and notochord also were dividing; little division was seen in neural keel cells (Fig. 29F). At the 24 ss, sclerotome, epidermis and notochord were dividing (Figs. 29G-I).

15 ss operated embryos

Trunk neural crest cells were removed from the level of somites seven-11 in 15 ss embryos. After extirpations embryos were allowed to develop for one, two or five hours and were then treated with BrdU. One hour after removal of TNC cells (17 ss), division was elevated in the underlying neural keel and in TNC cells located anterior, posterior and lateral to the surgery site (Figs. 30A-C; 31A,B). The population of TNC cells

undergoing division along the lateral path between the epidermis and somites might create replacements for extirpated cells. Two hours after ablation (19 ss), TNC cells overlying the dorso-lateral neural keel, and neural keel cells were dividing throughout the extirpated region (Figs. 30D-F; 31C,D). At the midline of the embryo, division is elevated posterior to and within the surgery site (Fig. 30E). Cell division five hours after TNC extirpation (25 ss) more closely approximates that of controls (Figs. 30G-I; 31E,F).

18 ss operated embryos

Following TNC cell extirpations at the 18 ss, embryos developed for 30 minutes, two, or four hours prior to treatment with BrdU. Within 30 minutes of surgery (19 ss) TNC cells migrating along the lateral path between somites and epidermis were dividing extensively in the vicinity of the ablated region (Figs. 32A-C). Neural keel cells located more posteriorly within the surgery site, and TNC cells located more anteriorly also showed greater division than cells at similar levels in controls (Figs. 29D-F). Cell division in embryos two and four hours after extirpation (22 and 26 ss) was specific to the epidermis and patches of somitic cells and could not be distinguished from patterns of division observed in controls (Figs. 32D-I; 29G-I).

In summary, cell division is elevated among TNC and neural keel cells within or adjacent to extirpated regions compared to cell division in controls. The most striking differences are observed within 30 minutes to one hour following TNC extirpations. While in controls TNC and keel cells divide in a

more regular, segmental pattern at the level of somites seven-11, patches of those cells show increased division in and around the surgery site in operated embryos. These results suggest that cell division is an important mechanism of regulation, and that alteration to the normal pattern of cell division is a rapid response to injury (TNC cell removal). Cell division remains elevated and can be distinguished from controls for a longer period of development when TNC cells are extirpated at younger stages (15 ss) versus slightly later (18 ss). However, in either case cell division resembles that of controls within four-five hours of TNC extirpation if the embryo has healed properly and acquired no infections.

Discussion

In this study, altered cell migration and increased cell division were investigated as two possible mechanisms of regulation. Cells must be able to detect and respond to environmental signals such as transcription and growth factors in order to compensate for loss or damage of neighboring cells. While changes in cell migration and division are not the only potential mechanisms of regulation, studies have shown that cells do modify migratory and mitotic behavior in response to changes in the environment (Fig. 33) (Chibon, 1970; Weston, 1970; Couly et al., 1996; Suzuki and Kirby, 1997). Moreover, because embryos developed normally, even after extirpations delayed differentiation of adjacent and regulating TNC cells, the

timing of neural crest cell migration in the trunk might not be as critical to normal development as in the cardiac region.

During development TNC cells migrate along specific pathways – medially between the neural keel and somites, and laterally between the somites and epidermis. It is not precisely known what factors govern migration. Some data indicate that migration along the 'correct' paths is intrinsic to the emerging neural crest cells, while others suggest the path/direction of migration is imposed by the environment (Hall, 1999). Surrounding TNC and neural keel cells were emphasized as potential sources of replacement cells following TNC extirpations at the level of somites seven-11. Tissue transplantation and culture experiments in the axolotl (Moury and Jacobson, 1989, 1990) and chick (Selleck and Bronner-Fraser, 1995) have suggested that epidermis (epidermal ectoderm) can produce neural crest cells, but I did not observe DiI-labeled epidermal cells migrating or differentiating in the trunk region of zebrafish. Placodal cells also may regulate for extirpated neural crest, but more likely in the cardiac region or the head than in the trunk (Kirby et al., 1985; Kirby, 1987; 1988a, b). Placodes arise as ectodermal thickenings in the head prior to transforming to a mesenchymal cell type (see reviews by Le Douarin et al., 1986; Webb and Noden, 1993). These cells were not good candidates for regulation, as they would need to migrate long distances to effectively replace cells removed from the anterior-mid trunk. Moreover, placodal cells might be too differentiated (specified) to regulate by the time trunk neural crest cells emerge from the neural keel.

Cells regulate by migrating into extirpated regions

When regions of neural crest are extirpated, replacement cells may migrate from locations rostral, caudal, contralateral (in the case of unilateral neural fold ablation), or ventral (underlying neural tube/keel) to the ablation site (Fig. 33, Yntema and Hammond, 1945, 1954; Hammond and Yntema, 1947; Lehman, 1951; Newth, 1951; 1956; Sechrist et al., 1995; Couly et al., 1996; Diaz and Glover, 1996; Saldivar et al., 1997). Following trunk neural crest extirpations in zebrafish, DiI labeling revealed TNC cells migrating into the surgery site from surrounding locations, especially the posterior margin. Although TNC cells situated anteriorly did migrate towards and into extirpated regions in some embryos, these cells rarely filled the region devoid of neural crest. More often, cells at the anterior margin migrated several somites anteriorly (away from) the extirpated region and then dispersed along medial or lateral paths to differentiate. Cells labeled with DiI at similar levels in controls (somites four-six; 12-14) frequently migrated several somites in the anterior and/or posterior directions prior to dispersing along the medial or lateral paths. Thus, it is not surprising that TNC cells located posterior to the extirpated region would migrate anteriorly. But, cells from posterior locations consistently migrating five or more somite lengths in one direction suggests that these cells are changing their migratory behavior in response to cues from the environment. Having more recently emerged from the neural keel, it is possible that TNC cells at the posterior extremities of extirpated

regions are slightly more 'plastic' than cells located anteriorly. If this were true, posterior TNC cells may be more receptive to signals from the environment. Alternatively, migration of posteriorly located TNC cells into extirpated regions could be linked to the healing epidermis. Removal of TNC cells disrupts the extracellular matrix and detaches the epidermis from its contact with the neural keel, both of which are important for migrating neural crest cells (Löfberg et al., 1980, 1989; Erickson et al., 1992). As the epidermis regrows following removal of TNC cells, the posterior margin advances faster than anterior (pers. obs.). This also might facilitate the migration of posteriorly located cells into extirpated regions.

Few studies have observed neural crest cells migrating from the ventral neural tube following mid- and/or hindbrain ablation (McKee and Ferguson, 1984; Scherson et al., 1993), and this source of regulating cells seems to be more the exception than the rule. In this study both TNC cells and the upper cell layers of the neural keel were removed. Labeled neural keel cells (somites eight-nine) migrated anteriorly and posteriorly to occupy the entire surgery site after TNC/keel extirpation in some 17-18 ss embryos. This observation suggests that neural keel (and neural tube) cells are able to regulate if they are not exposed to ventral signals from the notochord (Bronner-Fraser, 1995; Marcelle et al., 1997). Because the neural keel contributes to TNC formation through interactions with dorsal ectoderm, it seems logical that the neural keel (or neural tube) could contribute cells to replace those that are lost. It is not known whether labeled neural keel cells

have changed fate to migrate and differentiate as TNC, or if labeled cells have been induced to become TNC after the keel regains contact with epidermis. If neural keel cells first replace themselves, the time it takes for a new population of TNC cells to be generated may render them unresponsive to signals from the environment. At the 12-13 ss no neural keel cells were labeled, and no cells migrated into the extirpated region from the anterior or posterior margins. Additional DiI labeling is needed to determine whether the neural keel is an important source of regulating cells in younger embryos.

Trunk neural crest cells that had begun to migrate along medial or lateral paths at the time of DiI injections dorsal to the neural keel could not be accurately labeled. However, these cells should not be discounted as potential regulators; signals in the environment might cause them to deviate from their normal migratory paths, and almost nothing is known about the signals that govern such detours in normal migratory patterns. Movement of cells into gaps – regions devoid of cells – may simply reflect passive migration from regions of high to low concentration, an idea referred to as 'contact inhibition' by Weston (1970). This idea has great appeal when considering that cell migration must work in concert with other mechanisms such as increased cell division. If cells leave one area to differentiate in a different location, a new 'gap' is created. For regulation to be complete more cells must be generated through cell division.

Cells regulate through increased division

BrdU labeling in control and operated embryos suggests that cell division is elevated at the cranial and caudal extremes of ablated regions, in the underlying neural keel and among TNC cells migrating on the medial or lateral pathways. Unlike the preferential migration of cells located posterior to extirpated regions, cell division was equally increased among populations located anterior and posterior to the surgery site. Such increased division has been demonstrated following uni- and bilateral extirpations of neural tube and crest cells (e.g., Detwiler, 1944; Källen, 1955; McKee and Ferguson, 1984; Couly et al., 1996). The next challenge is to understand how altering the cellular environment induces changes in mitotic behavior.

Epithelial cells stimulate proliferation and/or differentiation of neural crest-derived mesenchyme (Hall and Coffin-Collins, 1990; Clouthier et al., 1998). Removing cells important for initiating and maintaining division and differentiation (e.g. epithelial and neural crest cells) might induce proliferation of neighboring cells, similar to how decreased cell death follows a decrease in the number of sensory neurons in fields of developing ganglia (Carr, 1984). Birge (1959) and Couly et al. (1996) converged on the idea that there is a relationship between cell population density and division rate. Extirpating a region of TNC cells may disrupt signaling that is maintained by a specific cell density. One result of this disruption could be increased division among neighboring cells. This notion is supported by occasional

observations of increased densities of melanophores following TNC cell extirpations in zebrafish.

While controls exhibited a more regular, segmental pattern of division among TNC and neural keel cells, additional division was observed among small groups of those cells underlying and adjacent to surgery sites within one hour after extirpation. Most interesting was the large amount of division exhibited by cells migrating along the lateral and medial paths ventral to where cells were extirpated. Rather than migrating into extirpated regions, these cells may regulate on site by generating cells to replace those that would have migrated along the same paths. It also was found that more neural keel cells were dividing ventral to extirpated regions than in controls, suggesting these are another important source of regulating cells, as alluded to earlier in the context of cell migration. To learn more about what conditions initiate changes in highly regulated patterns of cell division, it will be necessary to investigate factors important to cell proliferation and survival, such as epidermal and fibroblast growth factors, Neurotrophin-3 and TGF-ß (Hall and Coffin-Collins, 1990; Stemple and Anderson, 1993; Rogers et al., 1994; Kolodziejczyk and Hall, 1996).

In conclusion, this study has emphasized two potential mechanisms of regulation – altered cell migration and increased cell division. Results have provided evidence that both mechanisms contribute to regulation, yet there are additional mechanisms to be tested. One of the more difficult mechanisms to study, and also one of the more significant, is change in cell

fate. Migrating neural crest cells are heterogenous, consisting of cells that are pluripotent, have restricted potential, or are already committed to a particular fate (Weston and Butler, 1966; Sieber-Blum and Cohen, 1980; Ito et al., 1993; Le Douarin et al., 1994; Raible and Eisen, 1994; Henion and Weston, 1997; Bronner-Fraser, 1998). As with cell division and migration, differentiation is controlled by environmental cues (Weston, 1970; Anderson, 1989; Collazo et al., 1993; Gershon, 1993; Le Douarin and Dupin, 1993; Bronner-Fraser, 1995; Darland and Le Blanc, 1996; Groves and Anderson, 1996; Baker et al., 1997). Because the early neural crest population is heterogenous, and because virtually all studies of regulation involve pre-migratory or newly migrating cells, environmental signals may influence neural crest cells that are not terminally differentiated to adopt an alternative fate. Moreover, *in vitro* cell culture has revealed that some neural crest derivatives can 'trans-differentiate,' meaning to switch from one terminally differentiated cell state to an alternate cell state (Nice, 1954; Patterson, 1990; Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991; Thibaudeau and Holder, 1998). In either case, the potential for neural crest cells to change fate in response to the environment is an aspect of regulation that should be tested.

Lastly, it would be interesting to compare gene expression of corresponding regions in control and neural crest-ablated embryos to interpret whether regulation repeats ('recapitulates') original developmental patterns. Are the genes expressed by neural crest cells and along the neural axis during initial development and migration re-expressed in a similar

manner following neural crest cell extirpations? Because it has been well established that *Hox* genes pattern rhombencephalic neural crest derivatives (Hunt et al., 1991, 1995; Grapin-Botton et al., 1995; Couly et al., 1996), members of this gene family are prime candidates for such studies. Genes that are expressed by pre-migratory and migratory neural crest such as *Krox-20* (Nieto et al., 1995; Saldivar et al., 1997) and *sna2* (Thisse et al., 1993, 1995; Sefton et al., 1998) also would be useful markers of regulation.

Table 6. Migration and derivative formation of DiI-labeled cells in *D. rerio* embryos from which trunk neural crest cells were removed at the level of somites seven to eleven.

				Derivatives[4]				
Stage[1]	DiI[2] Label	n	distance[3] migrated	Pigment	Glial/ Drg	Symp. ganglia	Aorta	Fin
12-13	Ant.	4	4 IC [3 ant., 2 post.]	4				
	Post.	4	4 IC [2 ant., 2 post.]	4				4
14-15	Ant.	4	4 IC [1 ant., 2 post.]		4			4
	Post.	4	2 C, 2 IC [1 ant., 2 post.]					2
	Keel	4	4 IC [2 ant.]		3			
16	Ant.	3	3 IC [2 ant.]					1
	Post.	3	3 IC [no migration]					
	Keel	5	1 C, 4 IC [3 ant.]	3	1	1		5
17-18	Ant.	4	4 IC [4 ant., 1 post.]	4	2			4
	Post.	7	6 C, 1 IC [1 ant.]	6	4	6	6	2
	Keel	4	2 C, 2 IC [2 ant.]	2	1			2
19-20	Ant.	5	3 C; 2 IC [2 ant.. 3 post.]	5	2		1	2
	Post.	5	4 C, 1 IC [2 ant., 3 post.]	4	2			1

Notes: [1]Represented by number of somites. [2]Cells were labeled two somite lengths anterior to (ant.), posterior to (post.), or at the neural keel of the surgery site. [3]Number of individuals from the total sample size that had migrating cells fill the surgery site (C-complete) or not (IC=incomplete). Notation in brackets refers to the maximum distance in somite lengths that cells migrated anterior and posterior to the injected sites for IC individuals. [4]The number of individuals showing DiI-labeling in specific derivatives. Drg, dorsal root ganglia; Symp., Sympathetic

Fig. 25. DiI labeling in a 17 ss embryo following extirpation of TNC cells.

Head is at the bottom right. Trunk neural crest cells were removed from the level of somites seven-11 (vertical lines). The glass micropipette was used to inject fluorescent DiI dorsally just beneath the epidermis and onto TNC cells posterior to the extirpated region (So, somite 13). Magnification 40x.

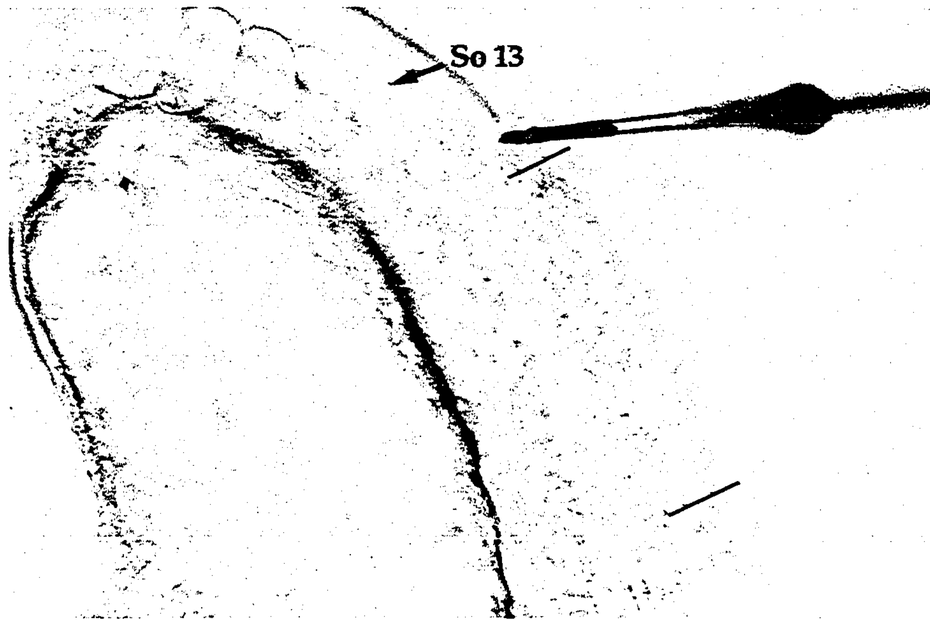


Figure 25

Fig. 26. DiI labeling of TNC cells in 14 ss embryos following extirpation of TNC cells, and traced through 36 hours following injection. Head (anterior) is to the left in all images. TNC cells were removed from the level of somites seven-11 (between parallel vertical lines). Arrows in all panels show the initial site of DiI injection either anterior (**A-D**) or posterior (**E-H**) to the extirpated region. **A:** Ten minutes following DiI injection. No cells have migrated from the initial injection site at the level of somites five-six. **B:** Five hours after DiI injection. Labeled cells have migrated the length of somites five-nine. **C, D:** Twenty-six and 36 hours, respectively, after DiI injection. Cells have migrated the length of somites four-nine, only one somite further anterior than observed five hours following injection. DiI labeling is found in dorsal pigment cells, glial (e.g., Schwann) cells or dorsal root ganglia (**C**, arrowhead) and dorsal fin fold (**D**, arrowhead). **E:** Ten minutes following DiI injection. No cells have migrated from the initial injection site at the level of somites 12-13. **F:** Five hours after DiI injection. Labeled TNC cells have begun to migrate the length of one somite anteriorly (towards the extirpated region). **G, H:** Twenty-six and 36 hours, respectively, after DiI injection. Labeled cells have migrated the length of somites 10-14. Labeling is found in pigment and dorsal fin fold (**H**, arrowhead).

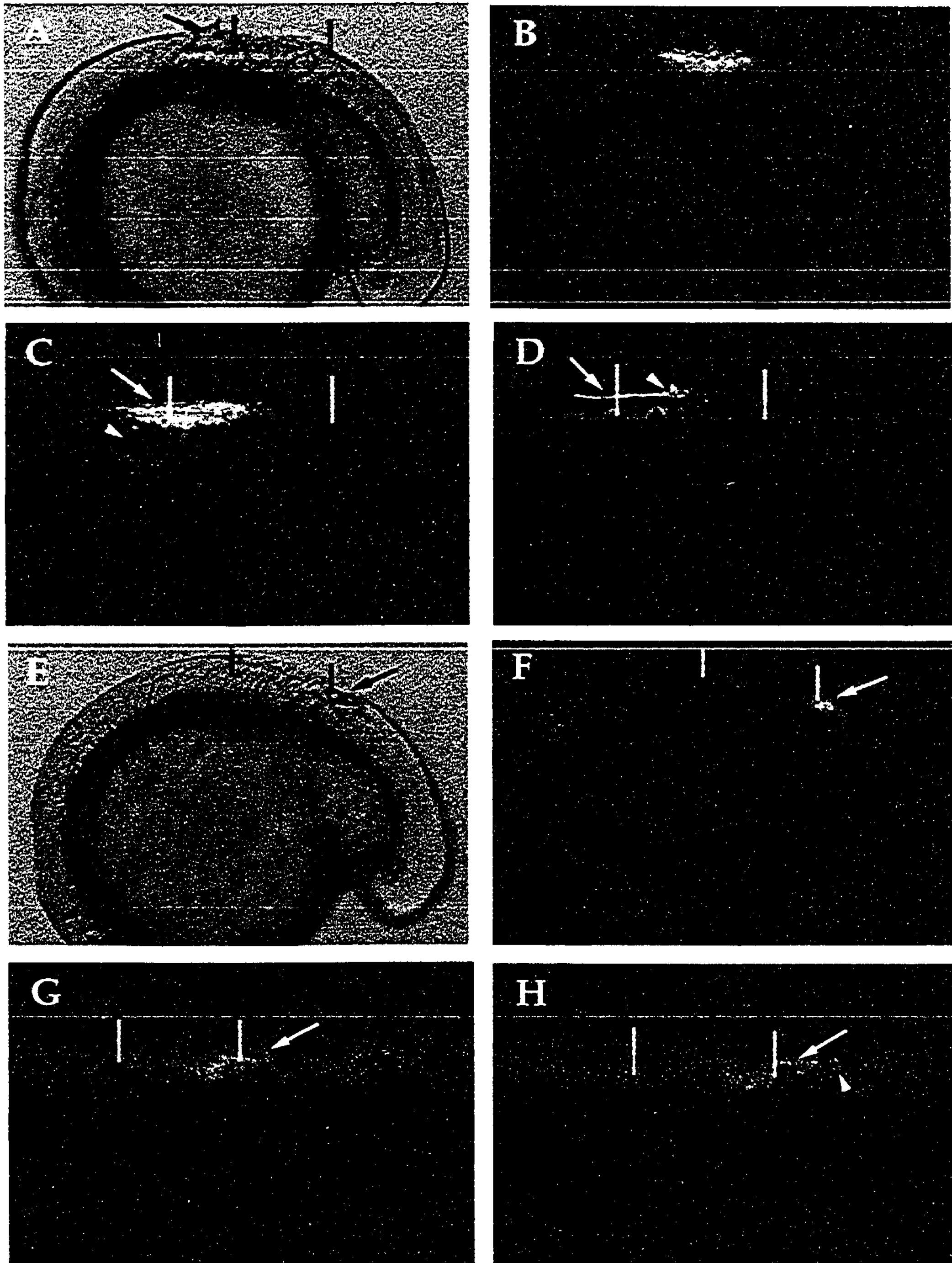


Figure 26

Fig. 27. DiI labeling of TNC cells in a 16 ss embryo following extirpation of TNC cells, and traced through 48 hours following injection. Head (anterior) is to the left in all images. TNC cells were removed from the level of somites seven-11 (between parallel vertical lines). Arrows in **A, B** show the initial site of DiI injection at the neural keel. **A:** Ten minutes following DiI injection. No movement of cells labeled at the level of somites eight-nine in the underlying neural keel is observed. **B:** Five hours after DiI injection. Labeled cells have migrated slightly posterior to the level of somite 10.
C, D: Twenty-six and 48 hours, respectively, after DiI injection. Labeled cells have migrated extensively and are differentiating at the level of somites five-12. Derivatives observed include dorsal pigment, dorsal root ganglia (**C**, arrows), sympathetic ganglia (**C**, arrowheads) and dorsal fin fold (**D**, arrow).

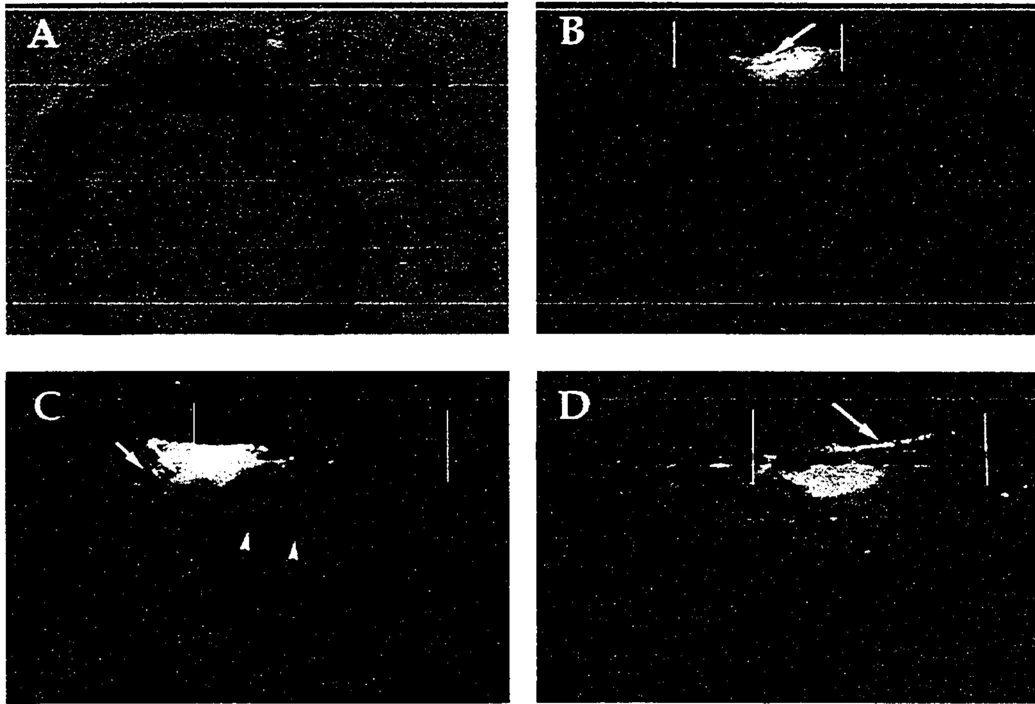


Figure 27

Fig. 28. DiI labeling of TNC cells in 17 ss embryos following extirpation of TNC cells, and traced through 36 hours following injection. Head (anterior) is to the left in all images. TNC cells were removed from the level of somites seven-eleven (between parallel vertical lines). Arrows show the initial site of DiI injection either anterior (**A-C**) or posterior (**D**) to the extirpated region. **A:** Ten minutes following DiI injection. No movement of cells labeled at the level of somites five-six is observed. **B:** Five hours after DiI injection. Labeled cells have migrated slightly anterior to the level of somite four. **C:** Thirty-six hours after DiI injection. Cells have migrated the length of somites three-seven. Some differentiation of cells into pigment and dorsal root ganglia (arrowhead) is observed. **D:** Ten minutes following DiI injection. Cells labeled at the level of somite 12 (a smaller than usual injection) have begun to migrate anteriorly towards the extirpated region (level of somite 11). **E:** Two hours after DiI injection. As the epidermis heals, cells have migrated towards the level of somite 10, but have also moved away from the original site of DiI injection. **F, G:** Thirty-six hours after DiI injection. Labeled cells have migrated the length of somites eight-25. TNC cells almost completely traversed the surgery site, but more significantly, these cells differentiated in disparate locations dorsally and ventrally along the trunk. Derivatives observed include pigment cells, especially melanophores, dorsal root ganglia (**F**, short arrows), sympathetic ganglia (**F**, arrowheads), dorsal fin fold (**F**, long arrow), ectomesenchymal cells of the aorta and tail reticular cells (**G**, arrowhead).

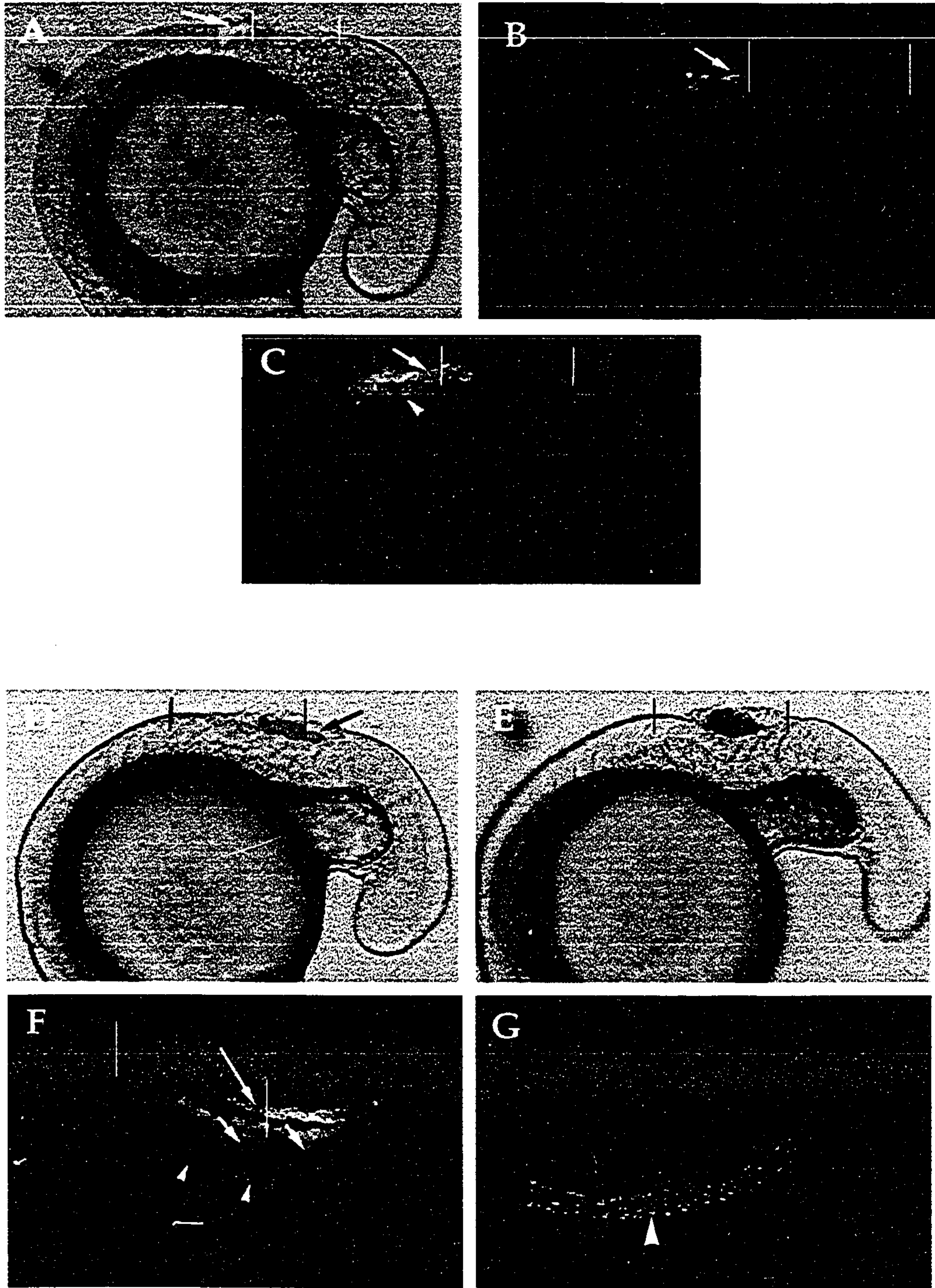


Figure 28

Fig. 29. Confocal images of BrdU labeling in control embryos. Images are shown as longitudinal slices through the trunk at approximately the level of somites six-ten (**A-C**) and five-thirteen (**D-I**). In each series the three images (**A-C; D-F; G-I**) are arranged from more lateral to the neural keel and notochord (**A, D, G**) to medially (**C, F, I**). Head (anterior) is to the left in images **A-F**, and to the right in **G-I**; dorsal is up. **A-C:** 17 ss. TNC and somitic cells are dividing in a segmental pattern between the somites and epidermis (**A**, arrows) and between the somites and neural keel (**B**, arrows). Neural keel cells also are dividing, along with cells of the floor plate (**C**, arrowheads) and notochord (**C**, arrows). **D-F:** 19 ss. The majority of cell divisions are observed in somitic (**D**, arrows), epidermal (**F**, arrowheads), and notochordal cells. **G-I:** 24 ss. Fewer somitic (sclerotomal) cells are dividing (**G**, arrows), although division is prevalent among epidermal cells (**I**, arrows). Green bar (**A**) = 50 μm for A-C; white bar (**D**) = 50 μm for D-I.

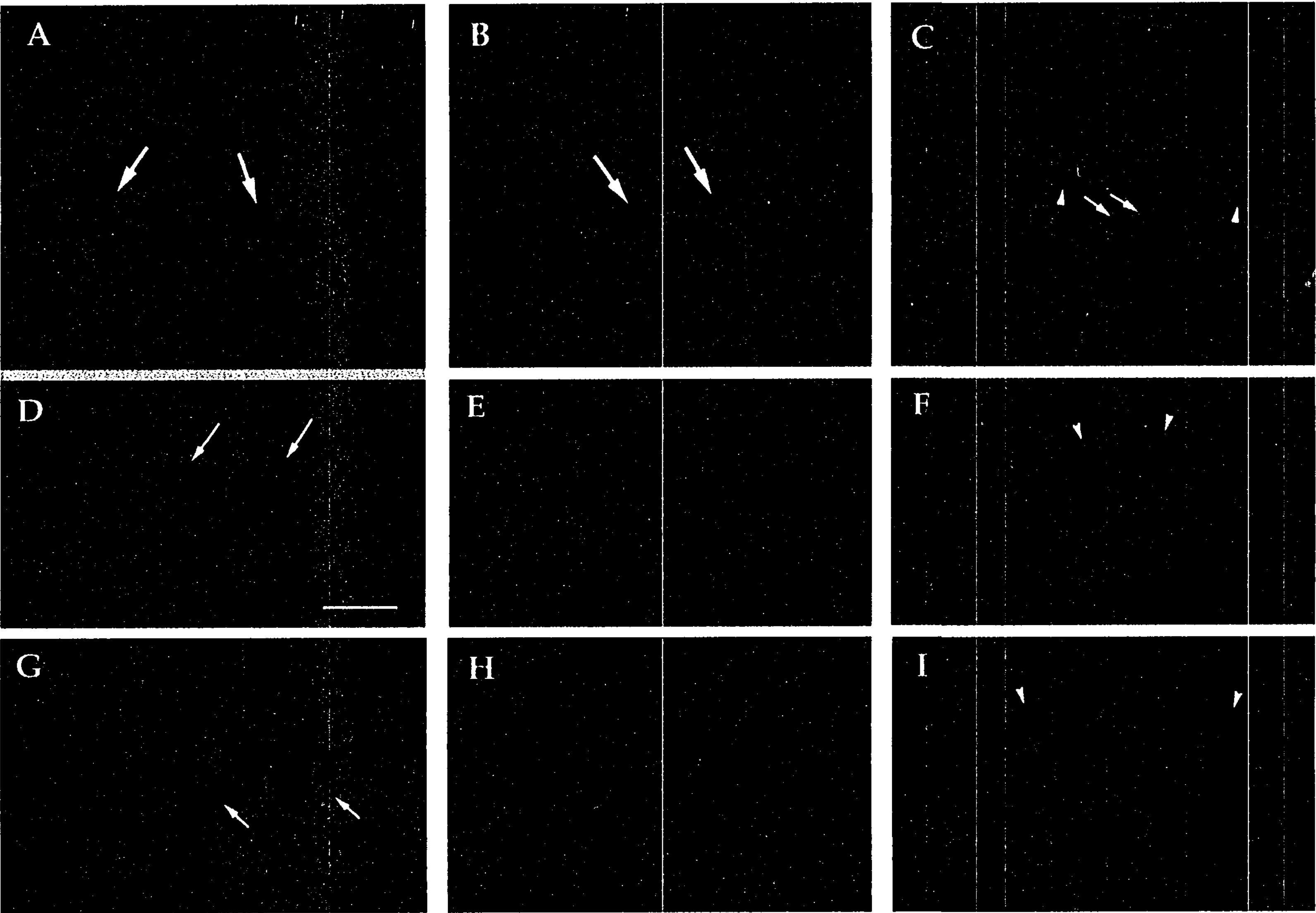


Figure 29

Fig. 30. Confocal images of BrdU labeling in embryos following extirpation of TNC cells at the 15 ss. Images are shown as longitudinal slices through the trunk at approximately the level of somites five-thirteen. In each series the three images (**A-C**; **D-F**; **G-I**) are arranged from more lateral to the neural keel and notochord (**A, D, G**) to medially (**C, F, I**). Head (anterior) is to the right; dorsal is up. **A-C:** 17 ss. One hour after TNC cell removal. Cell division is elevated in the underlying neural keel and among TNC cells anterior, posterior and lateral to the surgery site (arrows). **D-F:** 19 ss. Two hours after TNC cell removal. Division of TNC and neural keel cells is elevated dorso-lateral to and within the surgery site (arrowheads). **G-I:** Five hours after TNC cell removal. Division is still slightly higher among TNC cells in the proximity of the extirpated regions, but overall, the pattern closely resembles controls (arrowheads). Bar (**A**) = 50 μm for all panels.

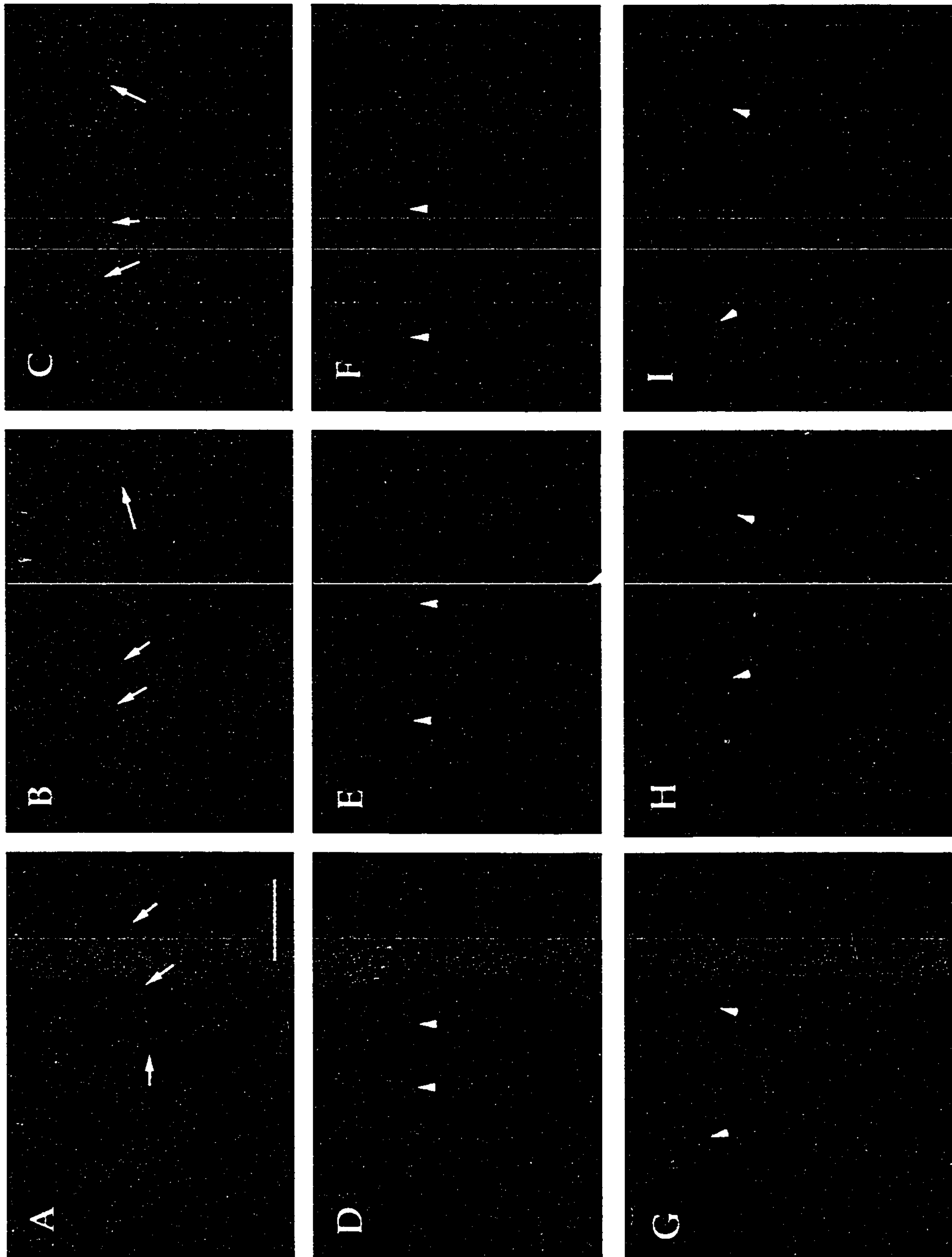


Figure 30

Fig. 31. A comparison of BrdU confocal images in control and experimental embryos. Images are shown as longitudinal slices through the trunk at approximately the level of somites seven-11. Head (anterior) is to the right; dorsal is up. Controls are the left-hand column (**A, C, E**); experimental embryos are on the right (**B, D, F**). **A, B:** 17 ss. One hour after TNC cell removal. Patches of dividing TNC cells can be seen anterior, ventral and posterior to the extirpated region (**B**, arrows), compared to the control where mainly neural keel cells are dividing. **C, D:** 19 ss. Two hours after TNC cell removal. Epidermal (**D**, arrow) and TNC cells (**D**, arrowheads) are dividing in the vicinity of the surgery site. Epidermis is also dividing in controls (**C**, arrowheads), but fewer TNC cells are proliferating. **E, F:** 25 ss. Five hours after TNC cell removal. Division in control and experimental embryos is similar, although more TNC cells are dividing at the dorso-lateral neural keel in the region where cells were removed (**F**, arrowheads). Note the labeled structures that resemble dorsal root ganglia axons in appearance and patterning (**F**, arrows). Bar (**A**) = 50 μm for all panels.

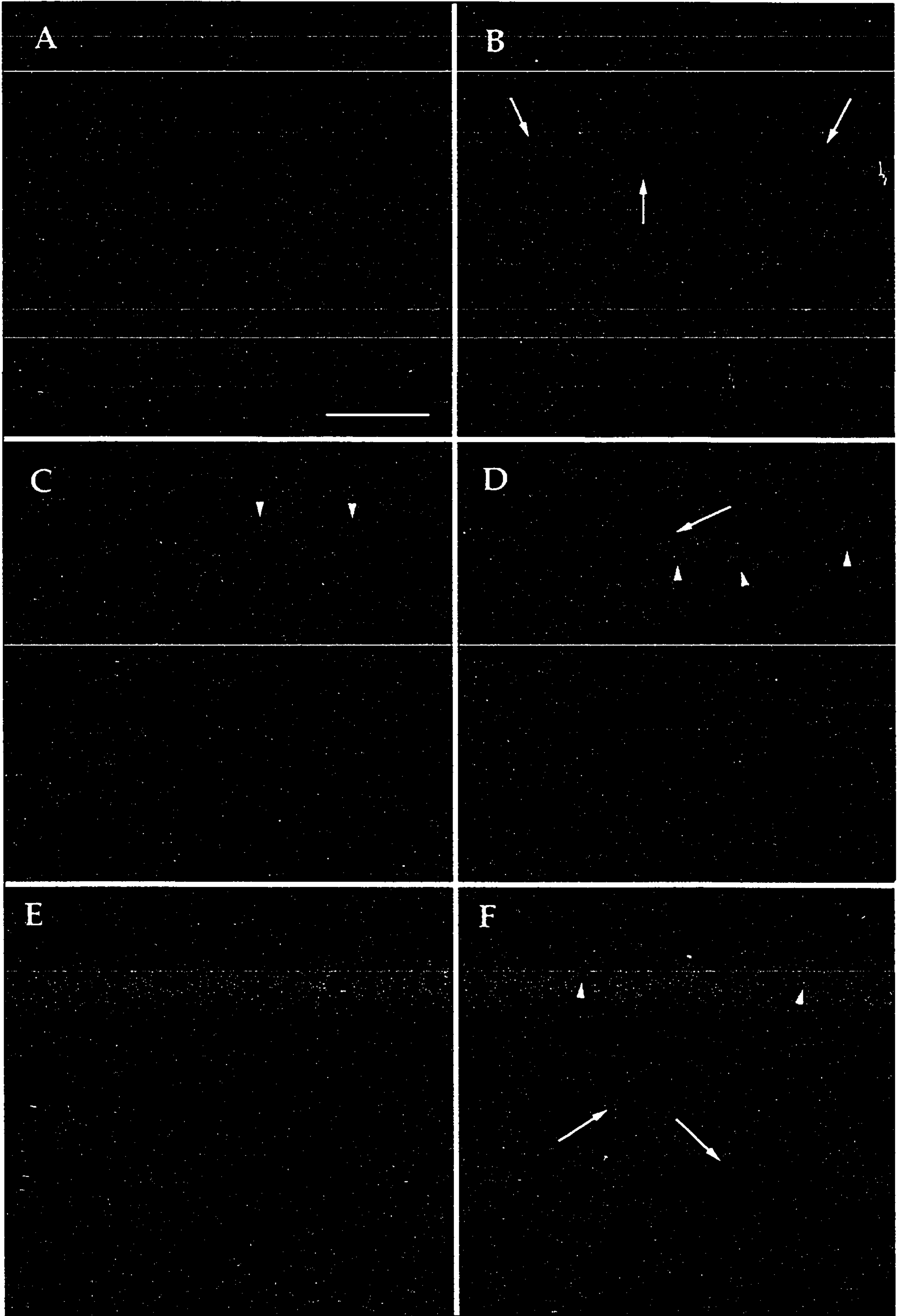


Figure 31

Fig. 32. Confocal images of BrdU labeling in embryos following extirpation of TNC cells at the 18 ss. Images are shown as longitudinal slices through the trunk at approximately the level of somites five-thirteen. In each series the three images (**A-C**; **D-F**; **G-I**) are arranged from more lateral to the neural keel and notochord (**A, D, G**) to medially (**C, F, I**). Head (anterior) is to the right; dorsal is up. **A-C:** 19 ss. Thirty minutes following TNC cell removal. Elevated division is seen in TNC cells migrating lateral between the somites and epidermis of the ablated region, as well as just anterior and posterior to where cells were removed (arrows). **D-F:** 22 ss. Two hours after TNC cell removal primarily epidermal cells are seen dividing (**E, F**, arrowheads). **G-I**: 26 ss. Four hours after TNC cell removal, division is limited to the epidermis (**H, I**, arrowheads) and patches of somitic cells and is not distinguishable from controls. Note again the axon-like structures (**H**, arrows). Bar (**A**) = 50 μm for all panels.

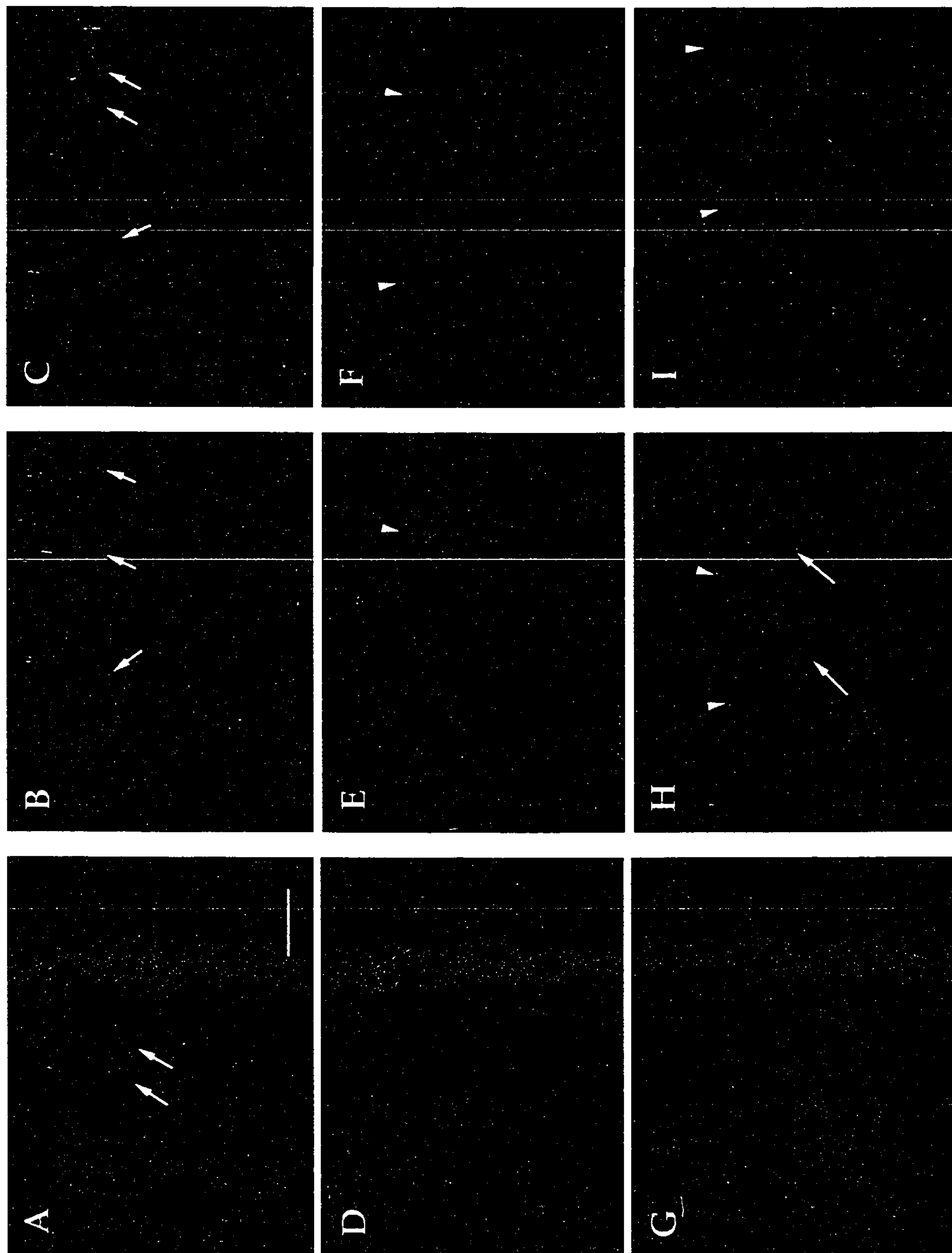


Figure 32

Fig. 33. Potential sources of regulating cells and suggested mechanisms of regulation. A: A three-dimensional diagram through the trunk of a fish embryo. After removal of TNC/neural keel cells (bluish-gray arrow), replacement cells may migrate into the extirpated region from anterior, posterior, ventral or lateral locations (black arrows). These replacement cells may be epidermal or neural, including neighboring neural keel and neural crest cells. Neural crest cells-dark red; neural keel-light blue; notochord-yellow; somites-pale red. **B:** During normal development neural crest (NC) progenitor cells are gradually restricted in potential – shown as restricted NC progenitors #1 and #2. (1) Signaling between closely associated neural crest cell populations both induces and maintains cell differentiation. (2) If a group of undifferentiated neural crest cells is removed, signaling is disrupted, and regulation is initiated. (3) Hypothesized mechanisms of regulation (not mutually inclusive) include: (a) change in fate of closely associated cells – shown as the gray cells that have changed fate from the intact population (yellow), (b) migration of cells into the ablated regions, (c) increased cell division and/or decreased death (gray) within the population from which cells were removed, or within an alternative (but potentially contributing) population.

A

B

Normal Development

NC progenitor

Restricted NC progenitor #1

Restricted progenitors #2

① Cell-cell signaling

Remove cells

Signaling restored

Regulation

② **Cell signaling disrupted**

③ **Cells replaced by:**

a. Changing fate

b. Migration into ablated region from adjacent population(s)

c. Increased division/Decreased Death

Figure 33

Chapter 5

Conclusions

Regulation is a significant developmental event because successful cell proliferation and migration are critical to shaping young embryos. Regulation — the replacement of undifferentiated embryonic cells by other cells in response to signals received from the environment — is distinct from wound healing and regeneration. Investigations on regulation of neural crest cells span all vertebrates and have revealed that regulative ability varies among classes, and even species. The results of my studies on trunk neural crest (TNC) cell migration, differentiation and regulation in zebrafish are summarized below.

1. Zebrafish TNC cells are large and numerous. The observation that populations of TNC contain large numbers of cells differs from previous studies, but it more closely corresponds to the extensive migration and derivative formation occurring along the length of the trunk.

2. TNC cells undergo several successive waves of 'sheet' and 'segmental' migration during development. During 'sheet migration,' compact layers of neural crest cells spread over the neural keel as an unsegmented, coherent sheet with cells in extensive contact with one another. 'Segmental' migration refers to more localized cell migration in streams over the lateral faces of somites, although cells still contact one another.

3. Migration of TNC cells begins along the medial path as early as the 10-12 ss; migration begins along the lateral path two-four somites stages later. At the 14-15 ss, TNC cells migrate laterally as a uniform sheet over the somites and yolk in hindbrain and cardiac regions. Sheet migration continues to extend posteriorly during development. A transition from sheet to segmental migration of TNC along the lateral path occurs in the anterior trunk between the 16-17 ss. An additional population of medially migrating TNC cells emerges at approximately the 18 ss. At these later stages lateral and medial migration are simultaneous and persist through at least the 22 ss. *Snail2* expression in pre-migratory and newly migrating TNC is consistent with SEM and DiI labeling of migrating cell populations. Such detailed descriptions on the timing and patterns of TNC cell migration in zebrafish have not been previously available.

4. DiI labeling confirmed SEM data on the timing of TNC development and migration and traced derivative formation (differentiation). In control embryos TNC cells migrated several somite lengths anteriorly and posteriorly along the trunk axis. At younger stages (e.g., 12-13 ss), migrating TNC cells differentiated as glial (e.g., Schwann) or dorsal root ganglia cells, pigment, enteric and sympathetic ganglia, ectomesenchyme of the aorta and tail reticular cells. At later stages (e.g., 17-22 ss), the majority of DiI-labeled cells included pigment, glia, dorsal root and sympathetic ganglia, ectomesenchyme of the aorta and tail reticular cells. Data on TNC cell migration, patterning and differentiation were essential for designing TNC extirpation experiments

and determining whether the migration of cells surrounding TNC-extirpated regions deviates from normal.

5. Pigment cell patterning provides evidence that regulation occurs on a sliding scale, ranging from complete to incomplete. The amount of TNC removed and the position of removal affected regulation to a greater extent than stage of removal.

6. Defects in developing zebrafish occurred if large regions of TNC cells were removed. When regions of TNC less than or equivalent to the length of three somites were removed, regulation was consistently complete, with no effect on pigmentation. Removal of TNC spanning regions five somites or greater in length frequently generated defects ranging from lack of pigmentation to increased densities or disruptions of pigment cells.

7. Defects in zebrafish occurred if cells were removed from anterior (cardiac) and posterior (tail) extremities of the trunk at various stages. Young embryos (e.g., 12-15 ss) had severe heart anomalies and rarely survived to hatching when cells were removed anterior to the fifth somite. Embryos that did survive exhibited minor pericardial defects and missing pigment cells. Extirpation of TNC posterior to somite five created a range of phenotypes, from perfectly normal embryos to those with nonlethal pigment irregularities.

8. Regulation was least affected by the stage of TNC removal. When constant lengths of TNC were removed at the 16-20 ss, pigmentation was normal in most embryos examined at 48 hpf. In some 16 ss embryos pigment

cells, especially melanophores and iridiophores, were missing or disrupted at 48 hpf, but pigmentation was normal by four days post-hatch.

9. Melanophores were the cell type most visibly affected by TNC cell extirpations; iridiophores were sometimes missing or disrupted along with melanophores. Xanthophore cells were rarely disrupted.

10. I proposed that the completeness and efficiency of regulation largely depends upon healing of the overlying epidermis. Presence of the epidermis is required to provide a substrate for the migration of regulating cells.

11. Zebrafish embryos were immunostained with one of five antibodies: anti-acetylated-α-tubulin, anti-Hu, HNK-1, Zn-5 and Zn-12. None labeled dorsal root ganglia at 24 or 36 hpf.

12. Regulation for pigment cells persisted through embryogenesis and into the larval period. Cells migrated and divided extensively to compensate for cell loss. TNC and neural keel cells surrounding extirpated regions were emphasized as sources of replacement cells.

13. DiI labeling showed that as the epidermis regenerates, TNC cells migrate into the extirpated site from cranial and caudal locations over a period of at least 24 hours. Underlying neural keel cells also migrated throughout the surgery site in some embryos (e.g. 17-18 ss). Interestingly, cells that migrated into 'the gap' most frequently originated from posterior to the extirpated region, suggesting these cells may be more 'plastic'. TNC labeled

anterior to the extirpated region tended to migrate away from rather than towards the surgery site.

14. BrdU labeling of dividing cells revealed that division is elevated cranial and caudal to the surgery site within one hour after TNC extirpation. Moreover, there appeared to be a high turnover of cells, especially in the dorsal neural keel. Greater numbers of cells adjacent to the surgery site, namely TNC migrating along the medial and lateral pathways, were dividing relative to controls.

A major premise underlying regulation is that cell potential is greater than cell fate. This concept was born from studies which demonstrated that some cells were able to express alternative fates if transplanted to a new environment. Although change in cell fate was not explored in my study, the ability for cells to take on a new identity may be the driving factor in regulation of embryonic cells. Once reliable mechanisms for assessing cell potential are established, we may find that fates are commonly altered in response to environmental signals.

My research has demonstrated that there is regulation for loss of TNC-derived pigment cells in the trunk region of zebrafish embryos. I also have shown that increased cell division and altered cell migration are cellular responses to changes in the environment. The question of *how* cells are able to 'detect' changes in their environment or their developmental program and alter their fates accordingly remains to be answered. For instance, what

signals trigger compensation? and what signals announce that compensation is complete?

Regulation is significant both as a basic developmental mechanism and as a mechanism for evolutionary change. The more labile the fate of embryonic cells, the more potential there is for maintaining existing characters and for generating new ones. According to Ettensohn (1992, p. 50), further analysis of such systems might "shed light both on the way evolutionary processes act to modify ontogenetic programs and on the cellular and molecular mechanisms of cell interactions during development". With regard to the neural crest, studies on regulation of this vital population of cells provide insight into the origin of the neural crest, embryonic repair, and the source of many craniofacial malformations, heart and other embryonic defects.

On a broader scale, few studies have approached investigations of neural crest deficiencies by looking at both short term compensation and long term developmental and morphogenetic affects of neural crest cell extirpation. In future studies it will be important to compare regulation for similar cell populations among several vertebrate groups. A comparative approach would build a framework for understanding the evolution of an important, but insufficiently understood, property of vertebrate embryos and the evolution of neural crest-derived structures.

Appendix I

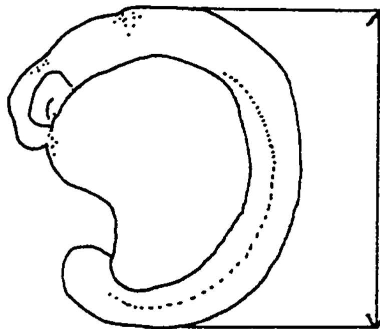
Stage (somites)	Age (hrs. post fertilization)
10	14
12	15
14	16
16	17
18	18
20	19
22	20
24	21
26	22

Beyond the 30 somite stage (approximately 24 hrs.), embryos are staged according to hours of development post-fertilization.

Appendix II

Stage	Length (mm)
10-12 ss	0.75-0.81
13-15 ss	0.83-0.90
16-18 ss	0.91-0.98
19-21 ss	10.0-11.0
22-24 ss	11.5-12.5
25 ss	13.0
24 hrs.	20.0
36 hrs.	23.0
48 hrs	28.0
60 hrs.	30.0
2 days post-hatch	37.0
7 days post-hatch	1.0-1.1 cm
10 days post-hatch	1.4-1.5 cm

Notes: Length measurements refer to the distance between the highest point on the head to the lowest point on the tail, as shown below.



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