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THE EFFECT OF ALL-*TRANS* RETINOIC ACID ON THE MIGRATION OF AVIAN NEURAL CREST CELLS *IN VITRO* AND *IN VIVO*

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Abstract

Retinoic acid, the active metabolite of Vitamin A is known to play a major role in embryonic growth and differentiation during development. It has been shown that either excess or deficiency of retinoic acid during embryogenesis can be teratogenic. In order to study the teratogenic effects of retinoic acid, the aim of the present study was therefore to investigate the effect of all-*trans* retinoic acid on the migration and fate of neural crest cells *in vitro* and *in vivo*. In addition, the study investigated the effect of retinoic acid on the cytoskeletal elements of neural crest cells and on Rac and Rho, two members of the Rho family of GTPases. The neural tubes containing neural crest cells of quail embryos were removed at cranial levels and cultured on fibronectin as a substrate. The neural tubes were cultured in either Dulbecco's minimal essential medium (DMEM) or in DMEM+Dimethylsulphoxide (DMSO) as controls. In order to test the effect of retinoic acid, the neural tubes were cultured in 10^{-5}M all-*trans* retinoic acid (RA) which was reconstituted in DMSO. The distance of migration of the cultured quail neural crest cells was measured and compared between the controls and the experimentals. To study the effect of RA on the cell actin cytoskeleton *in vitro*, cultured neural crest cells were stained with rhodamine phalloidin. In addition, following 24 hours of culture, the quail neural crest cells were brought into suspension and micro-injected into 36 hour-old chick hosts. While the migration of neural crest cells was extensive in the control cultures *in vitro*, migration was inhibited in the retinoic acid-treated neural crest cells. In addition, retinoic-acid treated neural crest cells showed pigmentation and neuronal processes earlier than did the control neural crest cells. Retinoic acid-treated neural crest cells showed a disarray of the cytoskeletal elements as they were devoid of stress fibres and focal adhesions. In addition, retinoic acid appears to decrease the expression of Rac and Rho of cultured quail neural crest cells. Following micro-injection of cultured control and RA-treated quail neural crest into the cranial region of chick hosts, the control cells populated the beak area, whereas the retinoic acid-treated quail neural crest cells migrated to the retina of the eye, a region they normally do not populate. These results suggest that retinoic acid disturbs the migration of neural crest cells. It appears to do this by affecting the cytoskeletal elements of neural crest cells and the genes that are involved in forming these elements.

List of abbreviations

BMP-	Bone morphogenetic protein
BSA-	Bovine Serum Albumin
CRABP1-	Cellular retinoic acid binding protein 1
CRBP1-	Cytoplasmic retinol binding protein 1
DAB-	Diaminobenzine
DMEM-	Dulbecco's minimal essential medium
DMSO-	Dimethylsulphoxide
FGF-	Fibroblast growth factor
GAGs-	Glycosaminoglycans
JNK-	c-Jun N-terminal kinase
MLCK-	Myosin light chain kinase
NT-	Neural Tube
PBS-	Phosphate Buffered Saline
PIP2-	Phosphoinositol bisphosphate
RA-	Retinoic acid
RALDH2-	Retinaldehyde dehydrogenase
RAR-	Retinoic acid receptor
ROCK-	Rho-kinase
RT-PCR-	Reverse Transcriptase Polymerase chain reaction
RXR-	Retinoic X receptor
TBE-	Tris-Borate EDTA
TGF-	Transforming growth factor
ZPA-	Zone of polarizing activity
MAP-	Mitogen-activated protein
GEFs-	Guanine-nucleotide exchange factors
GAPs-	GTPase-activating proteins
GDIs-	Guanine nucleotide dissociation inhibitors
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

INTRODUCTION

Numerous studies have been carried out in order to understand the growth and patterning of craniofacial structures in humans (Ferguson 1988; Noden 1988; Williams *et al*., 2004). Most of these structures, which are located in the ventral head and neck, are formed by neural crest cells. This includes the facial mesenchyme (Bronner-Fraser, 1994; Gilbert, 1994; Bronner-Fraser, 1995). Neural crest cells coordinate various visceral functions through the peripheral nervous system and enteric nervous system. In addition, neural crest cells give rise to most of the brain, spinal cord, and the two branches of the nervous system (Le Douarin *et al*., 2004).

The Neural Crest

During neurulation, the dorsal ectodermal cells in the prospective midline of the embryo thicken, invaginate and close thereby forming a cylindrical neural tube that extends along the rostrocaudal axis of the embryo and which differentiates into all the elements of the central nervous system (Noden 1988; Bronner-Fraser, 1995; Shimamura *et al.,* 1995). In the head, the neural tube expands to form the brain, while in the more caudal regions it will form the spinal cord (Le Douarin *et al*., 2004). The surface ectoderm reconstitutes itself above the neural tube and will eventually differentiate into the epidermis. In the cranial region, neural crest cells migrate from the crests of the neural folds shortly after closure of the tube and undergo extensive migration in amphibians (Selleck and Bronner-Fraser, 1995). In mammalian embryos, cranial neural crest cells migrate out of the neural folds before the neural tube fuses (Kalthoff, 1996).

Within the neural tube, prior to neural crest cells migration, there is no obvious morphological difference between prospective neural tube cells (neuroepithelium) and prospective neural crest cells. According to Bronner-Fraser (1996), it is possible that all neural tube cells have the ability to form neural crest cells, but only those close to the dorsal midline have the potential to migrate. Another explanation is that the neural crest may be a segregated population within the neural folds, with a separate and distinct developmental potential (Bronner-Fraser, 1995; Bronner-Fraser, 1996). Once the early migrating neural crest cells have migrated out of the neural tube, they appear morphologically similar to each other and take on the appearance of mesenchymal cells. Thus, they are often termed "ectomesenchymal" cells in order to link their origin to ectoderm. Despite their similarity in morphology, neural crest cells migrate along distinct pathways and differentiate into a variety of cell types (Erickson, 1988; Tucker *et al.,* 1988; Kalthoff, 1996).

The migratory neural crest cells form a variety of derivatives, including most of the peripheral nervous system, as well as cranial, sensory, sympathetic and parasympathetic ganglia (Le Douarin *et al.,* 1995; Selleck and Bronner-Fraser, 1995; Barrio and Nieto, 2002). Neural crest cells also form chromaffin cells of the suprarenal medulla, pigment cells of most of the body (but not the retina) and connective tissue of the face (Erickson, 1988; Dupin and Le Douarin, 1994).

Cranial neural crest progenitors will form the supporting tissues of the head, such as cartilage and bone. Interestingly, the ability to form skeletal tissue is not uniformly

distributed within the cranial neural crest population (Le Douarin *et al.,* 1995; Selleck and Bronner-Fraser, 1995). The rostral region of the neural crest, which extends from the mid-diancephalon level to rhombomere 2, is the part of the neural crest which participates in forming the facial skeleton and the skull. Neural crest cells arising from trunk levels of the embryo form pigment cells but are unable to form cartilage (Selleck and Bronner-Fraser, 1995).

It has been demonstrated that the facial and visceral skeleton, including the hyoid cartilages, as well as the frontal, parietal and squamous part of the temporal bones, are derived from the neural crest (Bronner-Fraser, 1993; Bronner-Fraser, 1994; Kirby and Waldo, 1995). Only the basi-occipital bone and part of the otic regions of the skull are of mesodermal origin (Kirby and Waldo, 1995). In addition, much of the dermis, all of the connective tissue components of the facial musculature and the walls of blood vessels (but not the endothelium) of the face and forebrain are derived from the neural crest (Bronner-Fraser, 1996). Cranial neural crest cells also contribute to the leptomeninges of the forebrain (Bronner-Fraser, 1996).

The Neural Crest and Hox Genes

A key source of patterning information in the developing head is the vertebrate hindbrain, which exerts a profound influence on craniofacial morphogenesis, through its ability to generate cranial neural crest (Wendling *et al.,* 2000). During early embryonic development, the hindbrain is transiently sub-divided into seven segments called rhombomeres (Maden *et al*., 1998a). Each rhombomere has a unique identity based on

segment-restricted domains of Hox gene expression which are ordered and partially overlapping, and gives rise to a well-defined region of the adult brain (Maden *et al.,* 1998a; Barrow *et al*., 2000). This segmental organization is critical for establishing the proper spatial organization of the cranial ganglia, branchiomotor nerves and the pathways of cranial neural crest migration (Gale *et al*., 1996).

The expression patterns of various Hox and other genes which are expressed in one or several rhombomeres makes it possible to individually identify each rhombomere in the embryo (Gale *et al.,* 1996). Krox-20 is expressed in rhombomeres 3 and 5, Fgf-3 is expressed in rhombomeres 4, 5, and 6, Hoxa-2 is expressed in rhombomeres 2 and 7 in levels of varying intensities, and Hoxb-1 is expressed in rhombomeres 4 and 7 to rhombomere 2. It was first established in the mouse by Hunt *et al*. (1991) and later in the chick (Couly *et al*., 1996) that the caudal domain of the neural crest expresses Hox genes of the first four paralogous groups, whereas in the rostral domain, which yields the facial skeleton, these Hox genes are not expressed. Membrane bones arise only from Hox-negative skeletogenic neural crest cells, whereas cartilage originates from both Hox-positive and Hox-negative neural crest (Le Douarin *et al*., 2004).

Neural crest cells and differentiation

Neural crest cells are initially multipotent and become progressively commited to their fates as they migrate to, or after they reach their destinations (Jiang *et al.*, 1998). Evidence comes from experiments in the trunk of chick embryos where individual neural crest cell precursors were shown to contribute to multiple neural crest derivatives

(Bronner-Fraser and Fraser, 1988). In addition, single cranial neural crest cells can form derivatives as diverse as neurons and melanocytes (Jiang *et al.,* 1998). It appears that the fate of such multipotent precursors can be influenced by a variety of growth factors including BMPs and neuregulins, which induce adrenergic and glial derivatives (La Bonne and Bronner-Fraser, 1999; Thompson *et al.,* 2003). Thus, neural crest cells in the head may differentiate into cartilage, because the developing head contains cartilage-inducing signals not present at other levels of the developing embryo (La Bonne and Bronner-Fraser, 1999). Neural crest cells destined for different branchial arches arise from specific rostrocaudal levels of the neural folds. Using heterotopic grafting of head and trunk neural fold fragments, the fate of different neural crest cell populations was investigated. It was found that trunk neural crest grafted into the head fails to migrate in an orderly fashion and was not observed to form cartilage (Hall and Horstadius, 1988). Tissue grafts and ablation experiments have demonstrated that some aspects of neural crest cell migration and differentiation are plastic, whereas others are fixed (Le Douarin *et al.,* 2004). For example, removal of the neural crest by the ablation of a small portion of the dorsal neural tube at the mesencephalic, occipital or cervical levels results in a normal embryo (Le Douarin *et al.,* 2004).

Molecular signals affecting the neural crest

Much has been learned about the migratory pathways followed by neural crest cells and the signals which may trigger their differentiation. Neural induction is initiated when the ectoderm is exposed to signals coming from Spemann's organizer in amphibians, or its functional equivalent, the node, in amniotes (Le Douarin

et al., 2004). Studies have revealed a number of secreted molecules which are expressed in the organizer. These factors include noggin (Mayor *et al.,* 1997; Lee *et al.,* 2001), chordin (Sasai *et al.,* 1994) and follistatin. These molecules mediate neural induction via similar mechanisms. They bind to and inhibit the activity of a subset of bone morphogenetic proteins (BMP2, 4 and 7), which are members of the transforming growth factor-β (TGF-β) superfamily (Hemmati-Brivanlou *et al.,* 1994). If BMP-2 or BMP-4 expressing cells are injected at the neural plate border at the primitive streak stage of embryos in avians, the neural plate is narrowed. In contrast, if cells expressing chordin, a BMP antagonist are injected, the neural plate is expanded (Lyons *et al.,* 1995). *Wnts* can also induce the expression of neural crest markers in ectodermal explants in conjunction with BMP inhibition (Hadebal *et al*., 1998). In addition, *Snail* and *slug,* members of the *Snail* family are also expressed by the neural crest (Jiang *et al*., 1998). In *Xenopus* and chick, *Snail* and *Slug* are expressed by pre-migratory and migratory neural crest cells (La Bonne and Bronner-Fraser, 1999). The inhibition of *Slug* expression has been reported to prevent neural crest emigration from the neural tube. Over-expression of *Slug* promotes the delamination of neural crest cells from the neural tube (La Bonne and Bronner-Fraser, 1999).

It remains unknown what factors cause dorsal neural tube cells to emigrate from the neural tube and what prevents the remaining cells from emigrating as well. Recent evidence has implicated both cell adhesion molecules (Kimura *et al*., 1995) and Rho-family GTPases (Wozniak *et al.,* 2004) in this process.

The delamination of neural crest cells from the dorsal neural tube is accompanied by cytoskeletal changes, as well as changes in the expression of cadherin molecules at the cell surface (Kimura *et al.,* 1995). Neural crest cells in the dorsal neural tube express cadherin molecules throughout sites of cell-cell contact before their delamination from the neural folds (Kalthoff, 1996; Braga *et al*., 1999). In particular, two members of this family, N-cadherin and cadherin-6B, are expressed in the neural folds and dorsal neural tube of avian embryos prior to neural crest emigration (La Bonne and Bronner-Fraser, 1999). Once neural crest cells have emigrated from the neural tube, expression of these cadherins is downregulated (Kalthoff, 1996; Webb *et al.,* 2003). However, neural crest cells express other cadherins. These include cadherin 7 and cadherin 11 (Kimura *et al*., 1995). It has been suggested that this cadherin type switching plays a role in neural crest emigration from the neural tube and further neural crest migration (Kalthoff, 1996).

Cell Migration

Cell migration is a multi-step process involving changes in the cytoskeleton, cell-substrate adhesions and the extracellular matrix (Ridley, 2001). Most cell types such as neural crest cells migrate individually, whereas epithelial cells and endothelial cells often move as sheets or group of cells (Ridley, 2001; Bronner-Fraser, 1993; Kalthoff, 1996). Cell migration is initiated in response to extracellular cues such as diffusible factors, signals from neighbouring cells and signals from the extracellular matrix (Ridley *et al*., 2003). Many different signaling molecules have been implicated in the migration of cells. These include GTPases, cadherins, mitogen-activated protein kinase (MAP Kinase) and protein kinases (Ridley, 2001; Worthylake and Burridge, 2003). The hypothesis that the

Rho family of GTPases could regulate cell migration comes from the fact that they mediate the formation of specific actin containing structures (Fukata *et al*., 2003). Rho proteins regulate cell substrate adhesion, cell-cell adhesion, protein secretion, vesicle trafficking and transcription, and these are processes which are relevant to cell migration. (Ridley, 2001; Fukata *et al*., 2003).

Rho proteins usually cycle between an active, GTP-bound form and an inactive GDP bound state (Ridley, *et al*., 2003; Webb *et al*., 2003). In the GTP-bound form, they interact with downstream target proteins in order to induce cellular responses. Rho proteins can exchange nucleotide and hydrolyze GTP. These reactions are catalyzed by guanine-nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively (Fukata *et al*., 2003). In addition, Rho proteins can bind to proteins known as guanine-nucleotide-dissociation inhibitors (GDIs), which prevent their interaction with the plasma membrane but not necessarily with downstream targets (Ridley, 2001; Fukata *et al*., 2003).

Cell migration can be divided into four processes, namely, lamellipodia extension, formation of new adhesions, cell body contraction, and tail detachment (Lauffenburger and Horwitz, 1996).

Polarization is the keystone of cell migration (Lauffenburger and Horwitz, 1996; Ridley, 2001). For cells to migrate, they must acquire a special asymmetry which will enable them to turn intracellularly generated forces into net cell body translocation. This means a

migrating cell must have a clear distinction between cell front and rear (Lauffenburger and Horwitz, 1996; Ridley, 2001; Horwitz and Webb, 2003). A vital consequence of polarization is that extension of both lamellipodia and filapodia takes place primarily around the cell front, so that directional turning is gradually accomplished (Alberts *et al*., 1994). The extension of lamellipodia requires actin polymerization. It has been hypothesized that lamellipodia consist of branching filament networks formed through the actin-nucleating activity of the Arp2/3 complex (Ridley, 2001). Rac is required for the extension of lamellipodia. When Rac is inhibited, cells do not migrate (Alberts *et al*., 1994; Ridley, 2001). It has been shown that Rac co-ordinates lamellipodia extension by involving some Rac targets (Ridley *et al*., 2003). Rac stimulates new actin polymerization, by stimulating the Arp2/3 complex, which in turn initiates the formation of new actin filaments on the sides of existing filaments to form a branching actin filament network (Ridley, 2001; Ridley *et al*., 2003). In addition to activating the Arp2/3 complex, Rac can also stimulate actin polymerization by promoting the uncapping of actin filaments at the plasma membrane (Ridley, 2001).

Small focal complex structures are localized in the lamellipodia of most migrating cells. These structures are believed to be important in mediating the attachment of the extending lamellipodia to the extracellular matrix (Alberts *et al*., 1994; Webb *et al*., 2003). Rac is involved in the formation of the focal complex assembly. Rac and cdc42 are activated by cell adhesion to the extracellular matrix. As an example, if cells are cultured on fibronectin, Rac and cdc42 activation are induced. This is required for cell spreading (Horwitz and Webb, 2003). Consequently, it is possible that continuous

formation of new interactions between integrins and the extracellular matrix at the leading edge of cells maintains Rac activity. This could provide a positive feedback loop allowing cells to carry on migrating even when receptor signaling is downregulated (Ridley, 2001). The speed of cell migration is dependant on the composition of the substrate. The relative levels of Rho, Rac and cdc42 activation vary with extracellular matrix composition (Van Wetering *et al*., 2002; Horwitz and Webb, 2003).

The activation of Rac or cdc42 is a key regulatory event that stimulates actin polymerization at the leading edge of lamellipodia or filapodia, respectively (Horwitz and Webb, 2003). Actin polymerization helps to push the membrane forward resulting in the extension of a protrusion in the direction of migration. Regulators of actin dynamics are localized at or near the leading edge. In the lamellipodia, these regulators include phosphatidyl inositol biphosphate (PIP_2), WASP, Scar and Arp2/3, which control the formation of new actin filament branches on existing filaments (Lauffenburger and Horwitz, 1996). The polymerization itself is regulated by proteins that serve to cap growing filaments, sever older portions of existing filaments and control the availability of activated actin monomers (Lauffenburger and Horwitz, 1996).

Migrating cells move by acquiring two distinct types of force that are generated independently (Ridley, 2001). The protrusive force is involved in extending membrane processes such as lamellipodia or filapodia. Actin polymerization can provide such a force independent of myosin motor activity. To propel the cell forward, the second type of force is needed. This is called the contractile force. This force depends on active

myosin-based motors and may involve separate mechanisms of force generation within the anterior and posterior regions of the cell (Takaishi *et al.,* 1997; Ridley, 2001).

Cell body contraction is dependant on actomyosin contractility, and can be regulated by Rho (Hall, 1998). For example, migrating cells continue to extend processes, but the cell body does not translocate significantly when Rho is inactive. Rho acts via Rho-kinases (ROCKs) to affect MLC phosphorylation by inhibiting MLC kinase (MLCK) (Fukata *et al.,* 2003). The effect of the inhibition of Rho on the cell migration rate depends on the cell type, and this probably reflects the basal levels of stress fibres and focal adhesions in cells (Kalthoff, 1996). The high level of substrate adhesion through stress-fibre-associated focal adhesions inhibits cell migration. A decrease in the activity of Rho has two opposing effects: it increases migration by lowering adhesion, but decreases cell migration by inhibiting cell body contraction.

Tail detachment is the rate-limiting step of cell migration (Ridley *et al*., 2003). The mechanisms regulating tail detachment depend on the type of cell and the strength of adhesion to the extracellular matrix. Rapid migration requires efficient mechanisms to release adhesions at the rear of the cell (Ridley *et al*., 2003). The process of tail detachment depends on the action of the protease calpain, which degrades focal adhesion components at the rear of the cell (Nobes and Hall, 1995). A reduction in the activity of Rho could inhibit tail detachment through decreased actomyosin contractility (Nobes and Hall, 1995).

Actin filaments generate a myosin force at the leading edge, and this serves to pull the cell body toward the protrusion. Release of adhesion connections in the rear of the cell and retraction of the tail, completes the cycle of cell migration. Spatial and temporal regulation of Rho GTPases controls these processes through effectors such as ROCK which regulates actomyosin contractility. ROCK has been implicated in the release of adhesions at the rear of the cell via regulation of myosin II (Worthylake and Burridge, 2003).

The role of Rac and Rho in the formation of stress fibres and focal adhesions during cell migration

Focal adhesions provide attachment for stress fibres, which are formed by bundles of actin filaments inside the cell (Darnel *et al.,* 1990; Alberts *et al.,* 1994; Hall, 1998). Therefore, focal adhesions are sites of structural linkage between the extracellular matrix on the outside and the cytoskeleton on the inside of the cell (Taylor *et al.*, 1999). It has been shown that focal adhesions are also regions of signal transduction (Amano *et al.,* 1998; Albertinzzi *et al.*, 1999; Braga *et al.*, 1999). Within fibroblasts as well as many other cells in culture, tyrosine phosphorylation is involved in multiple signal transduction pathways in focal adhesions (Magdalena and Burridge, 1996). Paxillin and tyrosine kinase-focal adhesion kinase (FAK) - have been implicated as the most prominently tyrosine-phosphorylated proteins (Machesky and Hall, 1997). Tyrosine phosphorylation is stimulated under conditions of focal adhesion assembly when cells are cultured on an extracellular matrix substrate. Agents that induce the formation of focal adhesions and stress fibres act via GTP-binding protein Rho A (Machesky and Hall, 1997; Nobes and

Hall, 1999; Takaishi *et al.*, 1997). Microinjection of activated Rho into fibroblasts stimulates the assembly of stress fibres and focal adhesions (Alberts *et al.*, 1994; Machesky and Hall, 1997). The assembly of these structures is blocked when Rho is inactivated via ADP-ribosylation (Machesky and Hall, 1997). In addition, there is an increase in focal adhesion kinase activity in Rho stimulated cells (Horwitz and Parsons, 2001). In contrast, the activation of Rac, another member of the Rho family of GTPases, causes extension of lamellipodia (Clark *et al.*, 1998). A third member of the family, cdc42 regulates the formation of peripheral filapodial extensions (Clark *et al*., 1998). It has been assumed that the activation of Rac and Rho leads to actin polymerization. According to Machesky and Hall (1997), focal complexes induced by Rac activation are relatively smaller than focal adhesions induced by Rho. However, these complexes are thought to be composed of the same proteins, namely, vinculin, talin and paxillin (Alberts *et al*., 1994).

Recent studies (Williams *et al*., 2004) have shown that the migration of neural crest cells can be influenced by growth factors and signals that may affect their migratory pathways and differentiation. Some of these regulatory factors, which include fibroblast growth factor-8 (La Bonne and Bronner-Fraser, 1999), promote migration of neural crest cells. Other factors, could be inhibitory and teratogenic to the differentiation of these cells, and thus produce permanent malformations such as cleft lip and palate in the regions to which these cells are migrating (Smith *et al.,* 1998). One such factor which is known to cause abnormalities when utilized during pregnancy is retinoic acid.

Retinoic acid

The importance of retinoic acid

Retinoic acid plays a major role in cellular development, growth and differentiation during embryogenesis (Brickell and Thorogood, 1997; White *et al.,* 1998; Malpel *et al*., 2000; Ross *et al*., 2000; Marklund *et al.,* 2004). Teratogenic effects due to Vitamin A deficiency were first shown by Hale in 1933. In his experiments, Hale fed a vitamin A-deficient diet to pregnant pigs, which resulted in piglets being born without eyeballs. In another of his experiments, Hale (1933) fed rats vitamin A deficient diets before and during gestation. These rats showed defects in the eyes, urogenital tract, diaphragm, lungs and heart.

The importance of retinoids in normal embryogenesis was shown by Kalter and Warkany (1959) who described specific alterations in ocular, cardiac and urogenital morphogenesis during gestational vitamin A deficiency. The dependence of cardiogenesis on retinoids was first shown in vitamin A-deficient rats, which displayed specific aortic arch, ventricular septal and myocardial deficits. Retinoid-deficient quail embryos fail to connect the cardiac and extraembryonic vasculature and exhibit *situs inversus and cardia bifida*. Thompson *et al.,* (1969) have shown that retinoids are essential to embryonic development in the domestic fowl. In the absence of retinoids, the vasculature of the embryo fails to develop and the embryo eventually disintegrates. In addition, recent studies have shown that retinoic acid is essential in the development of the central nervous system, organogenesis, cellular growth and differentiation (Altaba and Jessel,

1991, Blumberg *et al.,* 1997; Maden *et al*., 1998a; Chazaud *et al*., 1999; Escriva *et al.,* 2002; Halilagic *et al.,* 2003; Zile 2004).

Retinoic acid has been shown to act as a morphogenic substance in the formation of the digit pattern in the chick limb bud (De Luca, 1991). The posterior region of the limb bud which contains the zone of polarizing activity (ZPA) of the bud, when transplanted to the anterior portion of a second embryo, causes digit pattern duplication in the mirror image of the posterior digits normally expressed in the bud. Retinoic acid mimics the action of the ZPA, causing digit pattern duplication (De Luca, 1991). It has been shown that either excess or defiency of retinoic acid in mammalian models may be equally teratogenic (Maden *et al*., 1998b).

Retinoic acid teratology

Vitamin A and its active metabolite, retinoic acid are teratogenic when administered at high concentrations to pregnant women (Hall and Horstadius, 1988; Dickman *et al.,* 1997 Helms *et al.,* 1997).

Cohlan (1954) was the first to determine that vitamin A administered at high levels to pregnant rats from gestational day 2-4 until day 16 was teratogenic. The most commonly produced defects were exencephaly, cleft palate, spina bifida, eye defects, hydrocephaly, and shortening of the mandible and maxilla.

Vitamin A produces abnormalities that simulate mammalian craniofacial defects which can also arise through mutations (Moore *et al*., 1988; Poswillo, 1988; Zile, 1998; Mulder *et al.*, 2000). Administration of vitamin A to embryonic chicks has been shown to prevent the migration of neural crest cells, an effect that can lead to craniofacial defects (Keith, 1977; Yip *et al.*, 1980; Hall and Horstadius, 1988; Li *et al.,* 2001). Vitamin A has specific actions on the facial processes in developing chick embryos, affecting the outgrowth of the facial skeleton by acting specifically on the neural crest-derived mesenchyme of these processes. The administration of excess vitamin A to pregnant mice results in the failure of development of elements of the craniofacial skeleton, or their development in abnormal or ectopic positions (Hall and Horstadius, 1988).

By injecting fertilized eggs via the yolk sac with single 50 microliter doses of 1.5 micrograms, 15 micrograms, or 150 micrograms of 13-*cis*-retinoic acid on varying days of incubation, Hart *et al.* (1990) found that the effects of retinoic acid on mortality and total malformations were both dose- and developmental-stage responsive. The defects caused by retinoic acid occurred in mesenchymal tissues derived in part from the cranial neural crest. Hart *et al.* (1990) also found that the craniofacial and cardiovascular malformations produced in the chick were analogous to those seen in animal models of retinoid teratogenesis and in human foetuses exposed to 13-*cis*-retinoic acid during maternal therapy for cystic acne. Following 13-*cis*-retinoic acid treatment, craniofacial and specific cardiovascular malformations were increased significantly. The greatest number of malformations occurred when 13-*cis*-retinoic acid was administered after cranial neural crest cell migration was complete.

According to Holland and Holland, (1996), excess all-*trans* retinoic acid causes severe craniofacial malformations in vertebrate embryos. When administered in excess, all-*trans* retinoic acid causes the fusion or absence of the pharyngeal arches. An increase in Hoxb-1 expression in the hindbrain shows that anterior rhombomeres are re-specified to a more posterior identity. Holland and Holland (1996) administered all-*trans* retinoic acid to developing amphioxus. The nerve cord in normal amphioxus has only a slight anterior swelling, the cerebral vesicle, and lacks migratory neural crest. For both amphioxus and mouse genes, excess retinoic acid causes either continuous expression throughout the preoptic hindbrain (mouse) and from the level of somite 7 to the anterior end of the nerve cord or discontinuous expression with a gap in rhombomere 3, and a gap at the posterior end of the cerebral vesicle (amphioxus).

In order to determine the pattern and mechanism of retinoic acid-induced effects on early cephalic development, Balbas *et al.,* (1993) treated chick embryos at stages 9-10 (Hamburger-Hamilton, 1951) with all-*trans* retinoic acid (0.5 micrograms, 1.5 micrograms, and 2.5 micrograms). Balbas *et al.,* (1993) found that while 0.5 micrograms of retinoic acid did not produce any significant malformations, 2.5 micrograms of retinoic acid produced a variety of malformations of both cephalic and trunk regions. However, 1.5 micrograms of retinoic acid produced specific alterations at the cephalic level. These changes consisted of morphological alterations, changes in neural crest cell migration and extracellular matrix composition. Changes in morphology included hypoplasia of the first three pharyngeal arches, swelling of either the anterior cardinal veins or dorsal aortae,

and atrophy of pharyngeal arch arteries. Changes in glycosaminoglycans (GAGs) concentrations were shown in the extracellular matrix of the retinoic acid-treated embryos as compared with controls. That is, there was an increase in the non-sulphated GAG's.

Li *et al.*, (2001) examined the relationship between retinoic acid and mitogen-activated protein kinase signaling in neural crest cells. In their experiment, Li *et al.*, (2001) demonstrated that c-Jun N-terminal kinase (JNK) activation is prevented by the presence of retinoic acid. Retinoic acid disturbed the migration and proliferation of primary cultures of mouse neural crest cells treated *in vitro,* as well as neural crest cells from animals treated *in vivo*. The activation of JNK in neural crest cultures was reduced. The stimulation of neural crest cell outgrowth as well as the phosphorylation of JNK by platelet-derived growth factor AA, which promotes outgrowth but not proliferation of neural crest cultures, was completely suppressed by retinoic acid.

Vitamin A-induced craniofacial defects have been attributed to abnormal migration of neural crest cells (Le Douarin, 1982). Neural crest cells either fail to migrate, or migrate to abnormal positions within embryos which had been treated with retinoic acid (Hall and Horstadius, 1988). That retinoic acid produces its effects by altering migration of neural crest cells is based on the fact that the administration of the vitamin at the time of known neural crest cell migration but not earlier or later, evokes particular syndromes. Neural crest cells can be seen to accumulate in abnormal locations in the embryo after administration of vitamin A. The migration of neural crest cells is inhibited when they are

maintained *in vitro* in the presence of vitamin A (Pratt and Goulding, 1987, in Hall and Horstadius 1988). In previous studies, vitamin A was shown to inhibit the interaction of neural crest cells with extracellular matrix products, an interaction that is required for normal migration to take place (Hall and Horstadius, 1988). Vitamin A enhances apoptosis, causes "blebbing" of neural crest cells and slows the secretion of the products of the extracellular matrix such as hyaluronic acid. Hall and Horstadius (1988) suggested that retinoic acid impairs the filapodial extensions of neural crest cells and thus inhibits their migration.

Retinoic acid and dose-response

Collins and Mao (1999) emphasize that there should be a dose response relationship associated with the administration of retinoic acid, and this has been repeatedly demonstrated in animal experiments. Many retinoids are teratogenic when administered orally but are not teratogenic when administered dermally (Collins and Mao, 1999). Studies performed in a number of species show that the blood levels of retinoid following dermal exposure were reduced compared to blood level following an oral exposure (Sonderlund *et al.,* 2005). Another aspect of this principle is whether compounds have a threshold for teratogenesis. For vitamin A, the question is complicated by the fact that it is an essential nutrient and therefore produces the classic U-shaped dose-response curve. However, it is generally accepted that embryonic hypervitaminosis A has a threshold (Sonderlund *et al.,* 2005).

In a study which suggests a specific vitamin A threshold, Rothman *et al.* (1995) found a daily dose of 0.3µg of retinol to be the threshold. Rothman *et al.* (1995) hypothesized that the slope of the dose-response curve was higher if the vitamin A was administered as a supplement as opposed to as a dietary component. It was then concluded that retinoids are believed to have a threshold for teratogenecity and generally follow a dose-response relationship.

Mitogenesis and Retinoic acid

Salvarezza and Rovasio (1997) determined whether exogenous retinoic acid is a potential modulator of the mitotic rate of neural crest cells. Salvarezza and Rovasio (1997) explored the hypothesis of an inhibitory effect exerted by retinoic acid on the proliferative behavior of neural crest cells *in vivo* and *in vitro*. A diminution of DNA synthesis was shown by homogenates of retinoic acid-treated chick embryos. Contrary to the findings of Hall and Horstadius (1988), the length of the phases of the cell cycle of neural crest cells was similar in both the controls and the experimentals, except for the G1 phase. The G1 phase was significantly longer in the neural crest cells of retinoic acid-treated embryos than in controls. In another study, Li *et al.* (2001) showed that retinoic acid blocks DNA synthesis and lengthens the duration by which proliferation is achieved in neural crest cells both in early chick embryos and *in vitro*.

Stage specific response and sensitivity to retinoic acid

According to Collins and Mao (1999), the early embryo during cleavage, blastocyst, and early germ-layer stages is relatively insensitive to teratogens. Organogenesis-stage

embryos, in contrast, are highly sensitive to teratogenesis (Collins and Mao, 1999). Teratogenic sensitivity decreases gradually as the foetal period progresses (Maden *et al.*, 1998a). An investigation on the sensitivity of the hamster to retinoic acid-induced teratogenesis and embryolethality showed that small changes in gestational timing could cause relatively major shifts in sensitivity to embryolethality and malformations. However, pre-organogenesis periods were insensitive to teratogenesis, and each of the observed malformations had a specific critical period (Collins and Mao, 1999).

Human malformations caused by retinoids appear to be induced in two cellular populations (Brickell and Thorogood, 1997). These are the cranial neural crest cells and an unidentified population of central nervous system cells. The cranial neural crest cells are primarily responsible for malformations which are observed in the craniofacial, thymic and cardiovascular systems, although different populations of cranial neural crest cells are differentially sensitive to retinoids (Brickell and Thorogood, 1997). In contrast, the central nervous system population may be responsible for defects which are observed in the central nervous system as well as postnatal behavioral effects (Le Douarin *et al*, 1995).

The foetal developmental period shows a reduction in sensitivity to anatomical defects. However, it is a period of sensitivity for neuron formation in the central nervous system (Collins and Mao, 1999). Disturbances at this stage of development reportedly can cause functional disorders or behavioral teratogenesis (Collins and Mao, 1999).

The administration of vitamin A to pregnant mice during the foetal period caused a decrease in the quantity and differentiation of neuroblasts, and the mice displayed spasticity, tremors and hyperactivity. When mouse embryos were exposed to retinoids before organogenesis, egg-cylinder stage mouse embryos which were administered doses of all-*trans* retinoic acid were found to form supernumerary limbs, most frequently caudally and ventrally to the hindlimbs. Thus, it appeared that the early embryonic, or preorganogenesis, periods are susceptible to retinoid teratogenesis (Collins and Mao, 1999).

Retinoic acid receptors

Retinoic acid is regulated by transcription factors called Retinoic Acid Receptors (RAR's) (Denker *et al.*, 1990). These proteins function as transcription factors in the nucleus. In addition to these proteins, there are cytoplasmic proteins, whose main function is to bind retinoids. Cellular retinol-binding protein 1 (CRBP1), which is found in the yolk sac, binds retinol from the maternal circulation (Denker *et al,* 1990). CRBP1 transports retinol to tissues where the retinol can be converted into retinoic acid. Retinoic acid can enter the nucleus and bind to RAR, once it has entered the cytoplasm. At the same time retinoic acid can also bind to cellular retinoic acid-binding proteins (CRABPs). CRABP1 binds to the retinoic acid and thus prevents it from entering the nucleus. However, retinoic acid can enter the nucleus and bind to an RAR, in the absence of CRABP1 (Ruberte *et al*., 1991). (See Fig.1).

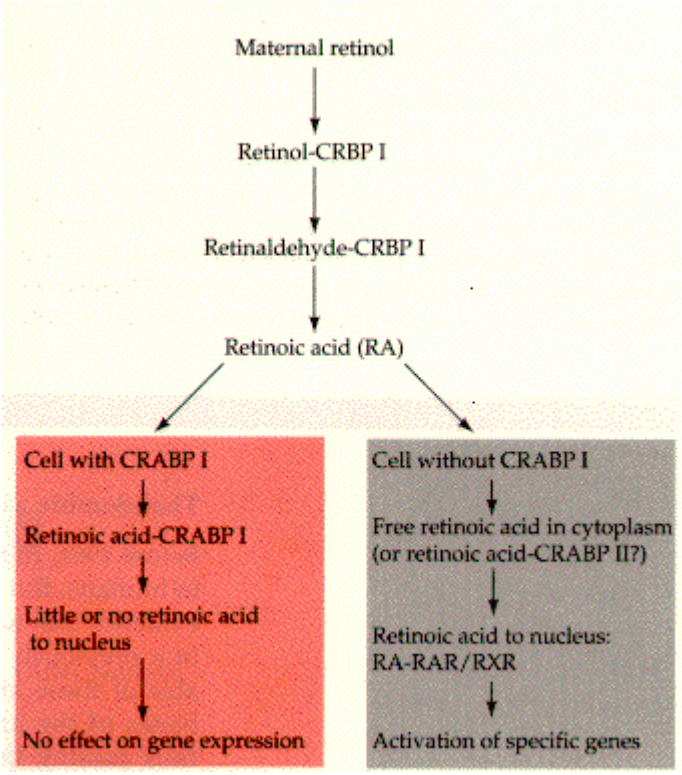


Fig.1. Summary of the relationships between retinoic acid synthesis, retinoic acid binding proteins, and receptors in the mammalian embryo. Retinoic acid would be able to enter the nucleus, bind to its receptors and change the normal pattern of gene expression, if CRABP were to be saturated by external supplies of retinoic acid (Denker *et al.*, 1990).

Exogenous retinoic acid acts as a teratogen in tissues that have both RAR and the CRABP1 protein i.e. cranial neural crest cells and hindbrain (Denker *et al.,* 1990; Chambon, 1996). This exogenous retinoic acid circumvents the protective blockade of the CRABP in these tissues, and in doing so, is able to bind to the nuclear RAR. Two modes of action have been identified in the retinoic acid bound RAR (Figure 2) (Denker *et al.,* 1990; Desbois *et al.,* 1991). First, they can bind to their DNA enhancer sequences and activate particular genes that are not usually activated in these cells, like homeotic genes that specify the antero-posterior position along the body axis. This means that they

can cause homeotic transformations, which convert anterior structures into more posterior structures. Secondly, they can inhibit those genes that are activated by another enhancer-binding transcription factor called AP-1 (Desbois *et al.,* 1991). AP-1 plays a role in activating cell division, and in this way retinoic acid may be able to inhibit normal cell division by preventing the activity of AP1 (Desbois *et al.,* 1991).

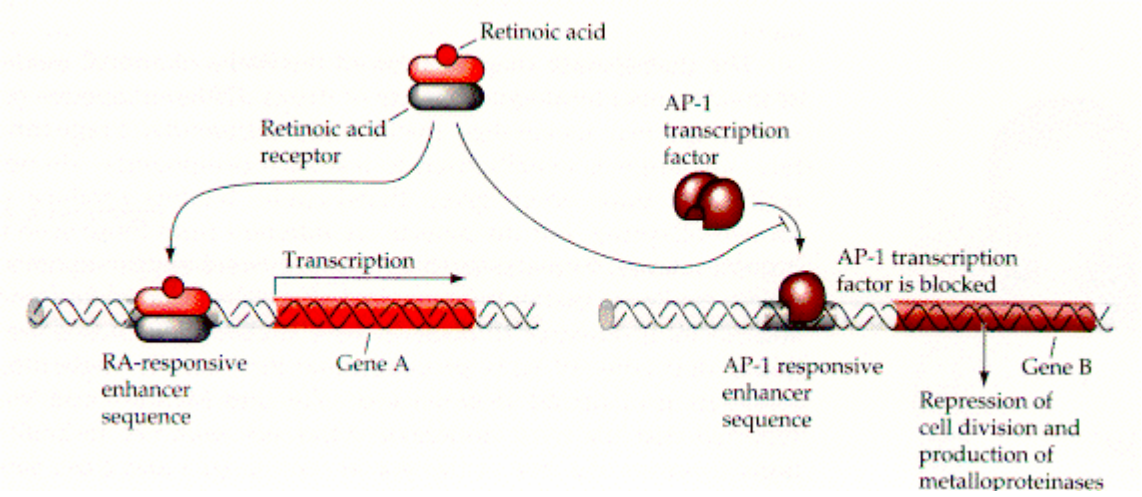


Fig.2. The dual action of retinoic acid-bound RARs model. They activate the transcription of genes whose enhancers enable the RARs to bind (Desbois *et al.,* 1991).

The ability of retinoic acid to influence development is made possible by a series of enzymes controlling a two-step metabolic pathway in which retinol is oxidized to produce retinal followed by further oxidation of retinal to produce retinoic acid (Mic *et al.,* 2002). Retinoic acid serves as a ligand for nuclear receptors that function in transcriptional regulation (Morris-Kay, 1993; Mic *et al.,* 2002).

Enzymes regulating retinoic acid synthesis

The analysis of enzymes regulating retinoic acid synthesis show that retinol is oxidized to retinal by alcohol dehydrogenase (Jiang *et al.,* 1994; Kurlandsky *et al.,* 1994; Abu-Abed *et al*., 2001; Wagner *et al.,* 2002). Retinoic acid is not produced by all cells of the body at all stages of development, but is instead generated in a spatiotemporal pattern (Jiang *et al.,* 1994; Ang *et al*., 1996). Metabolism of retinol to retinoic acid occurs at relatively low levels, but retinoic acid has been detected in embryos using sensitive reporter assays (Duester, 2000; Fan *et al.,* 2003). It has been shown that retinoic acid is undetectable in mouse embryos at embryonic day 6.5. Retinoic acid becomes detectable in the mouse embryo at embryonic day 7.5 onwards (Mic *et al.,* 2002). The observation that endogenous retinoic acid synthesis initiates in the mouse at embryonic day 7.5 is supported by studies indicating that retinaldehyde dehydrogenase 2 (RALDH2), capable of oxidizing retinal to retinoic acid, is first expressed at embryonic day 7.5 in trunk mesoderm, with additional expression by embryonic day 8.5 in the optic vesicles. The disruption of RALDH2 results in embryonic teratology with failure to develop beyond embryonic day 8.75, indicating that this enzyme is essential for development (Fan *et al*., 2003). Embryos that lack this enzyme, lack all retinoic acid detection, except for a reduced level in the eye field. Thus, an additional retinoic acid-generating enzyme must be at work in the eye (Mic *et al.,* 2002).

Retinoic acid, as opposed to retinol, is rapidly cleared from the maternal system, therefore allowing analysis of effects for narrow time periods in development. Shenefelt

(1972) conducted a study on hamster embryos using all-*trans*-retinoic acid. In his studies he illustrated critical periods for a wide variety of malformations, including limb malformations and cleft palate. Sulik *et al* (unpublished results) showed that low doses of retinoic acid given early at embryonic day 7 result in the same type of malformations as observed with ethanol exposure at this time.

Aim of the present study

In a pilot study which was conducted using quail embryos, all-*trans* retinoic acid-treated neural crest cells exhibited altered cytoskeletal elements and inhibition of migration. These cells lacked stress fibres and focal adhesions (Tshabalala, unpublished results). Hence, the aim of the present study is to investigate the effect of all-*trans* retinoic acid on the migration and the cytoskeletal elements of neural crest cells *in vitro* and the ability of these cells to migrate along a normal pathway *in vivo* following treatment.

The study also investigated the effect of retinoic acid on the activities of both Rac and Rho in cultured neural crest cells using the Reverse Transcriptase-Polymerase Reaction (RT- PCR).

MATERIAL AND METHODS

All experimentation was carried out in a laminar flow hood under aseptic conditions. Fertile Japanese quail (*Cortunix Cortunix japonica*) and White Leghorn chicken (*Gallus domesticus*) eggs were used. The eggs were obtained from the Central Animal Unit, University of the Witwatersrand (Animal Ethics Clearance Number 2003/64/1). The quail and chicken eggs were incubated for 48 hours and 36 hours respectively at 37˚C in a humidified incubator. Twenty-eight dozen quail and twenty-two dozen chicken eggs were used in various parts of the experiment.

PREPARATION OF EQUIPMENT

All glassware and solutions were sterilized. Glassware was dry-heat sterilized at 180˚C for 2 hours. Solutions were autoclaved at 121˚C at 100 kPa for 30 minutes. Rubber teats and sponges were boiled for 30 minutes in distilled water. Black wax dishes were flamed using a Bunsen burner. Sharp instruments were immersed in 70% alcohol, whereas blunt instruments were immersed in 95% alcohol.

Four-well Nunc culture multidishes (Nunc) were layered with fibronectin (Sigma) made up in distilled water (25µl/ml; 30µl per well). The dishes containing fibronectin were incubated for one hour at 37˚C in a humidified incubator. After incubation, excess fibronectin was removed using a fine pipette. 30µl of DMEM (Highveld Biological) was added to each well, and the dishes were incubated for a further one hour at 37˚C until use.

EXPERIMENTAL PROCEDURES

Neural tube cultures

The shell of each quail egg was wiped with cotton wool dipped in 70% alcohol. The egg was cut open, and emptied into an oval dish containing chick Ringer's solution (see Appendix A). The blastoderm was removed from the underlying yolk and transferred into a sterile black wax dish containing chick Ringer's solution. The blastoderm was pinned out firmly on the black wax dish. The embryos were staged according to Hamburger and Hamilton's (1951) table of normal chick development. Embryos ranging between stages 10 and 14 were used as neural crest cells are just beginning to migrate out of the folds in the cranial region at this stage (Le Douarin, 1969 cited in Le Douarin *et al*., 2004). The neural tube, together with adjacent tissue, e.g. somites, was dissected out at the level between the mid-otic placode and the third somite for cranial neural crest cells (FigA). The tissue was placed in collagenase (Appendix A, Sigma) in Tyrode's solution (Appendix A) for 20 minutes at 4˚C. The tissue was then removed from the collagenase solution and returned to a black wax dish containing Ringer's solution.

The neural tube, including neural crest, was separated from the surrounding tissues by microdissection and pipetted on to the fibronectin-coated well of a Nunc culture multidish. The explant was then incubated for 30 minutes at 37˚C, after which 1 ml of either DMEM containing 15% chick embryo extract (see Appendix A) and 10% horse serum (Bronner-Fraser, 1996) (control) or DMEM+all *trans*-retinoic acid (made in DMSO) was added to each well. The concentration of retinoic acid (Sigma) was 10^{-5}M. This concentration was determined due to altered migration of neural crest in a pilot

study (Tshabalala, unpublished results). In addition, as a further control, neural crest cells were cultured in the same concentration of DMEM (with horse serum and chick embryo extract) + DMSO, as DMSO was the vehicle in which the retinoic acid was diluted. The cultures were then placed in an incubator at 37˚C in a humidified atmosphere. The cultures were viewed after eight and twenty-four hours and photographed at 24 hours on an Olympus inverted phase contrast microscope. Photography was not carried out at 8 hours as the cells were only beginning to migrate from the neural tubes. The neural tubes and migrating neural crest cells were photographed on days one and two of culture at specific magnifications.

The distance of migration of the neural crest cells was calculated on the negative film using Coreldraw image analysis software. A total of 182 cultures were used: 52 DMEM, 59 DMEM+DMSO, and 71 Retinoic acid + DMEM. Negatives were scanned on Photoshop software. The distance of migration of the neural crest cells was calculated using CorelDraw. The distance between the edge of the neural tube and the leading migrated neural crest cell was measured at zero degrees. An F test and a Student "t"-test, including One-way Anova test were carried out to investigate if there was a significant difference in migration between the neural crest cells of the controls and the retinoic acid-treated cultures.

Suspension of neural crest cells to obtain cells for micro-injection and RT-PCR

Migrating quail neural crest cells which had been cultured on fibronectin for 24 hours were brought into suspension as follows using 2.5% trypsin. Both the culture medium

and the neural tube were removed from each well, and the neural crest cells were washed with pre-warmed PBS. 150µl of trypsin (Highveld Biological, Gauteng) was added to each well and the adhering neural crest cells were then incubated at 37°C for 3-5 minutes. The trypsin was used neat. The action of trypsin was stopped by adding 500µl of DMEM to each well. The suspended cells were transferred into a sterile centrifuge tube and spun for 5 minutes at 1500rpm. The supernatant was poured off and the pelleted cells were either used for micro-injection into the cranial regions of chick embryos or for analysis of actin GTPases using RT-PCR. To test for cell viability, neural crest cells were re-plated onto a clean fibronectin-coated four-well Nunc culture multidish. The cells were viewed hourly to determine whether they re-plated, and, how long it took for them to re-adhere to the fibronectin.

Accessing the chick embryo in ovo

In order to attempt micro-injections of quail cranial neural crest cells into the cranial region of chick hosts, fertile chick eggs were incubated for 36 hours at 37°C in a humidified incubator. The chick-quail chimaera was used as the large quail nucleoli are easily distinguished from the nucleoli of chick cells and hence any neural crest cell which had migrated from the quail donor could be identified.

Each chick egg (stages 9-10) was wiped with 70% alcohol and placed horizontally onto an egg holder. The blunt end of the egg was punctured using a hack-saw blade to release air from the air sac. Following this, 1.5ml of albumen was removed by penetrating the pointed end of the egg, slightly below the equator of the shell with the needle and a

syringe. The needle was pointed downward, almost vertically, as it is passed into the shell to avoid damage to the yolk. To avoid leakage of albumen, the entry point was sealed with clear tape. A window was then cut in the shell, overlying the position of the embryo at the highest point of the egg when lying transversely, and the shell membrane removed. The embryo was then visible through the window. To keep the blastoderm moist, a few drops of chick Ringer's solution (Appendix A) with antibiotics (1µl penicillin and streptomyosin, Sigma) was placed on to the chorioallantoic membrane of the embryo.

In order to visualize the different regions of the embryo, 100 µl of 1% Pelican India ink was injected below the blastoderm. The cultured, suspended quail neural crest cells were then backfilled into a sterile-pulled thin glass needle of unknown diameter. The needle was connected to an aspirator tube. The tip of the needle was then inserted into the desired region of the embryo. The quail neural crest cells (of unknown quantity) were then micro-injected under the surface ectoderm into the mesenchyme adjacent to the cranial neural tube (fig A). This procedure was carried out with both the non-retinoic acid-treated (both controls) and the retinoic acid-treated (experimental) neural crest cells. Following micro-injection of the neural crest cells, the egg was sealed with clear cellophane tape and returned to the humidified incubator at 37ºC for three, four, five, six or twelve days, respectively. Of the 84 chick hosts twenty one hosts received neural crest cells cultured in DMEM, twenty one hosts received neural crest cells cultured in DMEM+DMSO, and forty-two hosts were injected with retinoic acid-treated neural crest cells. The embryos were removed from the eggs, fixed in 10% formalin, processed in an automatic processor (Shandon Citadel 1000) or by hand, and embedded in paraffin wax.

In the case of the twelve day old embryos, the head was removed from the body prior to fixation. Embryos of days three to six were sectioned as a whole. The tissue/embryos were serially sectioned on a Leica microtome at 5μm and stained with the Feulgen-Rossenbeck method (appendix) in order to identify the large nucleoli present in quail cells. To avoid loss of tissue during processing, silane-coated slides were used. Light microscopy was used to view the tissues.

Rationale for the use of chick-quail chimaeras

The present study investigated whether retinoic acid-treated quail neural crest cells will reach their designated destinations when introduced into the head of a chick embryo. This will involve micro-injecting cultured retinoic acid-treated quail cranial neural crest cells into the cranial region of a chick embryo, *in vivo*. The technique involves the production of chick-quail chimaeras.

The quail-chick chimaera system was first used to establish a fate map of neural crest derivatives along the anteroposterior neural axis. The system was devised by Nicole Le Douarin, who noticed that the interface nuclei of all embryonic and adult cells in the Japanese quail (*Coturnix coturnix japonica*) contained a large amount of heterochromatin (Le Douarin, 1969 cited in Le Douarin *et al.,* 2004). This is rare, as heterochromatin is usually evenly distributed within the nucleoplasm of animal cells, particularly in the chick. Thus, this feature of quail cells allowed them to be distinguished from chick embryonic cells in tissues grafts performed *in ovo*. This system was used to determine the origin of neural crest derivatives, first by ablating a particular region of the neural tube or

neural fold before the onset of neural crest migration in a chick or quail embryo. The region was then replaced by an equivalent region from a stage-matched quail or chick embryo. Quail cells were identified by the Feulgen reaction or by species-specific monoclonal antibodies (Le Douarin and Kalcheim 1999).

Immunolocalization of quail cells

Sections of the quail-chick chimaeras were rehydrated through a graded series of alcohols, rinsed in PBS, and placed in 10% rabbit serum (Dako) for 45 minutes. The sections were then incubated with a quail cell nuclear marker QCPN mAB (Sigma) overnight at 4°C. The antibody was used neat, as per manufacturer's specification. After thorough rinsing in PBS, the bound antibody was labeled with rabbit anti-mouse horseradish peroxidase (Dako), diluted 1:50 in PBS for 1 hour. The antibody was visualized with diaminobenzidine (DAB, Sigma). The sections were dehydrated, cleared in xylene and mounted in entellan (Merck).

Confocal staining for actin

Neural crest cells which were cultured for 48 hours *in vitro* with or without retinoic acid were washed with pre-warmed phosphate-buffered saline at pH 7.4. After rinsing, the cells were fixed in 3.7% formaldehyde solution in PBS for 10 minutes at room temperature. After washing extensively in PBS, the cells were layered with 0.1% triton-X in PBS for 5 minutes, and washed in PBS thereafter.

To reduce non-specific background, the fixed cells were pre-incubated with PBS containing 1% BSA for 25 minutes prior to adding the staining solution (2.5% Rhodamine Phalloidin, Sigma) to stain for actin. The cells were covered with a solution of 5µl rhodamine phalloidin in 200µl PBS for 20 minutes at room temperature. After extensive rinsing in PBS, the cells were viewed using a Zeiss Confocal microscope.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for Rac and Rho primer detection.

Total mRNA extraction

Quail neural crest cells cultured either with or without retinoic acid for 24 hours were placed in suspension. The RNA of these neural crest cells was extracted using an RNeasy Mini Kit (Quaigen). The extraction procedures were performed by following the protocol in the RNeasy Mini handbook. The cells were lysed using RLT buffer and RNA was bound to RNeasy MinElute Spin Columns. The extraction procedure was carried out at room temperature. Following extraction, a spectrophotometer reading was carried out to quantify the total amount of RNA extracted from each sample.

RT-PCR

RT-PCR was performed according to the Access RT-PCR system protocol (Roche). Indicated volumes (appendix) of nuclease free water, AMV/Tfl5 X reaction buffer, dnTP mix, Rho, Rac, and tubulin (as a control) upstream and downstream primers, and 25mM $MgSO_4$ were combined in thin-walled 0.5 ml reaction tubes on ice. The sequences for the primers were as follows:

Rac forward: GATCGGTACCCAGGCCATCAAGTGTGTGGT,

Rac reverse: GATCGAATTCTTACAAACAGCAGGCATTTTCTC,

Rho forward: GATCGGTACCGCTGCCATCCGGAAGAAACT,

Rho reverse: GATCGAATTCTCACAAGACAAGGCAACCAG.

Tubulin forward: GGGCTGCAGGGTACCATGGCTGCCATCCGGAAG,

Tubulin reverse: TGCTGATCGTCTTCAGCAAGGACCAGTTC.

Each reaction mix was mixed by gentle pipetting. AMV Reverse Transcriptase and Tfl DNA Polymerase were added and vortexed to mix.

The reaction was started by adding the template, and the reactions were overlayed with 10µl of mineral oil. All work was carried out under RNAse free conditions. RT-PCR was performed using the PCR machine. See appendix for the reverse transcription and PCR cycling conditions. The PCR products were analyzed by agarose gel electrophoresis.

Agarose gel electrophoresis

100 ml of 1.6% agarose solution was prepared in a conical flask. The gel was made in Tris-Borate EDTA (TBE) buffer. The mixture was placed in the microwave until the agarose was dissolved and the solution was clear. The solution was allowed to cool to 55°C before pouring into a gel tray. The gel tray was prepared by sealing the ends with a tape.

The comb (which is used to make wells in the gel) was placed in the gel tray about 2cm from one end of the tray. The comb was placed vertically such that the teeth were about

1-2mm above the surface of the tray. The gel solution was poured into the tray to a depth of 5mm and allowed to solidify at room temperature. The comb was gently removed. The tray was submerged in an electrophoresis chamber until the wells in the agar were just below the electrophoresis buffer. 25µl of ethidium bromide was added to the tank which contained TBE buffer. Ethidium bromide is used for identifying and visualizing nucleic acid bands in electrophoresis.

For sample preparation, 2µl of the loading dye was mixed with the PCR products. The same amount of the loading dye was needed for the DNA ladder (100bp, Roche). The PCR products were then loaded simultaneously with the DNA ladder into the wells. The DNA ladder indicates the molecular weight of a particular primer on the gel. Electrophoresis was carried out at 200 volts until the dye markers had migrated the entire length of the gel. The gel was viewed and photographed on a UV-transilluminator.

SDS-PAGE

In an attempt to investigate the effect of retinoic acid on the membrane proteins of the retinoic acid-treated and non-retinoic acid-treated neural crest cells, SDS-PAGE was performed. A total of 36 cultures were used for this part of the study: 12 DMEM, 12 DMEM+DMSO, and 12 DMEM+Retinoic acid-treated neural crest cells

Protein extraction

Following trypsinization, the cells were suspended in 1ml of Tris-Mannitol, and left for 40 minutes at 4°C. The suspension was centrifuged at 20 000rpm for 45 minutes, and the

pellet was re-dissolved in 20 volumes of cold homogenization buffer. The cells were homogenized using a dance homogenizer and slowly stirred for 60 minutes at 4°C. The cell suspension was re-homogenized and centrifuged at 5000 rpm for 30 minutes. The supernatant was collected and centrifuged at 45 000 rpm for 60 minutes at 4°C. The pellet was solubilised in an equal volume of solubilisation buffer and slowly stirred for 60 minutes at 4°C. After centrifugation at 45 000 rpm for 60 minutes, the supernatant was collected and the protein content adjusted to 20 mg/ml to enable a semi-quantitative visual comparison of the protein bands on the gel. The concentrations of the samples were calculated by reading off the absorbance at 260 and 280nm using a spectrophotometer. SDS-PAGE was carried out on the protein samples.

Running and stacking gel solutions were prepared (see Appendix A). The running gel solution was poured into a gel casting form. A region of about 2cm was left at the bottom of the comb to create space for the introduction of the stacking gel. A region was marked below the comb for the height of the stacking gel required. In order to remove bubbles at the top of the gel, the gel surface was layered with water saturated butanol. The gel was allowed to polymerize for 30 minutes. The stacking gel was poured on top of the running gel, after which the comb was inserted and the stacking gel was allowed to polymerize for 45 minutes. The protein samples were prepared by mixing with sample buffer (ratio 4:1). The protein-buffer solution was then boiled for 5 minutes. The gel was clamped, and the buffer chambers were filled with gel running buffer. Five microgram of each protein sample was introduced into the gel using a pipette. The power leads were attached and the gel was run at 250V for 30 minutes. The gel was removed from the power supply and visualized using silver stain.

RESULTS

Migration of neural crest cells *in vitro*

Migration of neural crest cells from the explanted quail cranial neural tubes *in vitro* started after approximately eight hours. However, onset of migration of neural crest cells started at different times in different neural tubes. This was independent of whether the cell cultures were controls or retinoic acid-treated. However, after 24 hours, a statistically significant difference in the distance of migration of neural crest cells from the neural tube was noted between the control cultures (i.e. neural tubes cultured in DMEM alone or DMEM containing DMSO) and neural tubes cultured in DMEM containing retinoic acid.

As shown with Student's "t" test and the F-test (Appendix B), together with the box plot (Fig3), the retinoic acid-treated neural crest cells did not migrate as extensively as did neural crest cells of the control cultures. The vertical extensions on the box plot represent the quartiles which show the minimum and maximum value of distance of migration of neural crest cells. There was no statistically significant difference in the distance of migration between those neural crest cells cultured in DMEM alone and those cultured in DMEM with DMSO. Migration of neural crest cells cultured in DMEM was extensive with numerous neural crest cells migrating away from the neural tube (Fig.4). Similarly, neural crest cells cultured in DMEM+DMSO showed extensive migration (Fig. 5). In contrast, the retinoic acid-treated neural crest cells (Figure 6) did not migrate as extensively as did the neural crest cells in the two controls. In addition, these cultures showed clumping of neural crest cells. In the retinoic acid-treated neural tubes, the migration of the neural crest cells was either not as extensive as in the control cultures or

the cells failed to migrate at all (figure 7). Eighty eight percent of the neural tubes that were cultured in DMEM showed migration of neural crest cells. Similarly, 91% neural tubes which were cultured in DMEM+DMSO produced migrating neural crest cells. In contrast, only 63% of the neural tubes that were cultured in DMEM with retinoic acid showed migrating neural crest cells, and migration of these neural crest cells was reduced in extent. All stages of embryos used (stages 10 to 14) produced migrating neural crest cells. In less than 1% of the neural crest cell cultures, the medium changed to a yellow colour. This was probably evidence of bacterial contamination. As a result, these cultures were immediately discarded.

Pigmentation of neural crest cells in culture

Neural crest cells which were cultured in DMEM alone or DMEM + DMSO, pigmented later than did the retinoic acid-treated neural crest cells. Neural crest cells of both control cultures showed pigmentation after 48 hours whereas retinoic acid-treated neural crest cells pigmented earlier i.e. after 24 hours (Fig. 8 and Fig. 9). Eighty three percent of the retinoic acid-treated neural crest cell cultures showed early pigmentation. The other 17% of these cultures pigmented after 48 hours. In contrast, 48% of neural crest cultures which were cultured in DMEM alone showed pigmentation after 48 hours, while the other 52% of these cultures showed pigmentation after 72 hours. Similarly, about 42 % of neural crest cells which were cultured in DMEM+DMSO showed pigmentation after 48 hours. Fifty-eight percent of these neural crest cells showed pigmentation after 72 hours. The pigment granules were equally distributed within the body of the cells. Pigmentation was more intense within the cell bodies compared to the processes. There was no difference in

the pigment distribution within the cells between the retinoic acid-treated neural crest cells and the controls.

Neurite formation of neural crest cells in culture

Certain of the neural crest cells *in vitro* developed long processes resembling the processes of neurons. This “neurite” formation occurred after 48 hours in 78% of the two control cell cultures. However, neurite formation occurred 24 hours earlier (24 hours) in some neural crest cells treated with retinoic acid (Figure 10). Neurite outgrowth was evident on more than 90% of retinoic acid-treated neural crest cells.

Shape and size of neural crest cells in culture

Neural crest cells of the two control cultures exhibited a polygonal shape with filapodia extending from every cell, while most of the retinoic acid-treated cells were more rounded in shape. Because the number of neural crest cells in the control cultures were in abundance and tended to “pile on top” of each other, some of the cell membranes appeared wavy (Fig 11), whereas some cell membranes showed more continuity (more straight) (Fig 12).There was no apparent difference in size between neural crest cells of the control cultures and the retinoic acid-treated neural crest cells.

Actin cytoskeleton of cultured neural crest cells

The neural crest cells of the two control cultures showed formation of stress fibers and focal adhesions (Figs. 11, 12 and Table 1.). In contrast, the retinoic acid-treated neural

crest cells were either devoid of, or showed less of these trans-membrane proteins and stress fibres (Fig. 13).

Actin fibres were regularly arranged in the cytoplasm of the neural crest cells of the two types of control cultures, whereas in the retinoic acid- treated cells, disarray of the cytoskeletal elements occurred (Fig.13 and Table1). These cells do not show actin filaments in the core of the body of the cell, but generally at the periphery. Due to the disarray of the cytoskeleton, the retinoic acid-treated neural crest cells appeared to be more rounded and showed fewer and shorter filapodial extensions than those of the controls (Fig. 13). The control cultures exhibited normal polygonal shapes with extensive filapodia. Although there was no apparent differences in size between the retinoic acid-treated neural crest cells and control cultures with phase contrast microscopy, the retinoic acid-treated cells appeared to be unusually small using the confocal technique. In contrast, neural crest cells which were cultured in DMEM and in DMEM + DMSO appeared to be larger. Table 1 summarizes the differences shown between the retinoic acid treated cells and the controls.

Table 1. The cytoskeletal differences between quail cranial neural crest cells cultured in DMEM or DMEM + DMSO and those cultured in retinoic acid

Feature	DMEM	DMSO	DMEM + 10^{-5}M RA
Cell Size	Normal	Normal	Unusually small at times
Stress fibres	Present	Present	Few or absent
Focal adhesions	Present	Present	Few or absent
Arrangement of actin fibres	Regularly arranged	Regularly arranged	Irregularly arranged
Actin distribution	Even	Even	Disarrayed

Localization of micro-injected quail neural crest cells

Neural crest cell viability

Neural crest cells which had been trypsinized off the fibronectin-coated Nunc wells, centrifuged and re-plated to the freshly prepared fibronectin-coated wells adhered to the fibronectin after approximately four hours. This duration was regardless of whether the cells were controls or retinoic acid-treated. This showed that the cultured neural crest cells were still viable following trypsinization and centrifugation (Figs 14, 15).

Following the injection of India ink below the chick blastoderm, the survival rate of host chick embryo was usually below 50 per cent as most of the embryos died from the ink or puncturing the yolk. Of the 84 chick hosts used only 25 embryos survived. Fourteen out

of forty-two DMEM (six) and DMEM+DMSO (eight) hosts survived. In addition, only 11 out of 42 hosts injected with retinoic acid-treated neural crest cells survived.

Only a few quail cells could be detected in the chick embryos of 6 and 12 days following micro-injection of either the retinoic acid-treated or the control (both DMEM and DMEM + DMSO) quail cranial neural crest cells. No micro-injected quail neural crest cells were observed in embryos after 3, 4 or 5 days. The Feulgen-Rossenbeck technique demonstrated the large quail nucleoli well, whereas the QCPN antibody immunolocalized the perinuclear region of the cells, and only showed the nucleoli faintly. The control cells (DMEM and DMEM+DMSO) populated the chick beak area. Quail cells of control cultures could be seen populating the beak area of the chick embryo with the Feulgen-Rossenbeck technique (Fig. 16). In contrast, retinoic acid-treated cranial neural crest cells were located in the retina of the eye, which they normally do not populate (Figs. 17 and 18). Figures 19 and 20 show sections through quail embryos stained with Feulgen-Rossenbeck and QCPN methods, respectively. These sections were used as positive controls.

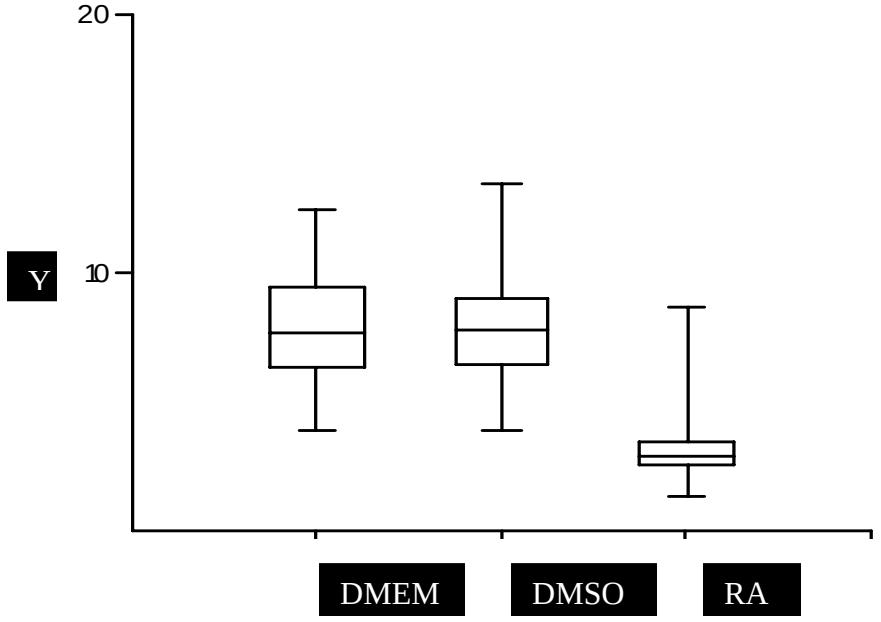
RT-PCR

Rac and Rho were intensely expressed in the RNA of neural crest cells that were cultured in DMEM alone and those that were cultured in DMEM+DMSO (Figs. 21 and 22). RT-PCR showed a decrease in the expression of Rac and Rho in the retinoic acid-treated neural crest cells. The bands for the RNA extracted from retinoic acid-treated neural crest cultures were less intense when compared to both controls. While differences were

observed, they could not be quantified. The negative and water controls did not show any bands, showing that there was no amplification of cDNA. Tubulin, used as a positive control was also intensely expressed.

SDS-PAGE

No bands could be detected with the SDS-PAGE



Key		**Y-axis-**	distance of migration (in nm)
	1	**DMEM-**	control medium 1
	2	**DMSO-**	DMEM + DMSO, control medium 2
	3	**RA-**	retinoic acid

Fig. 3 Mean distance of migration of retinoic acid-treated and non-treated quail neural crest cells *in vitro*

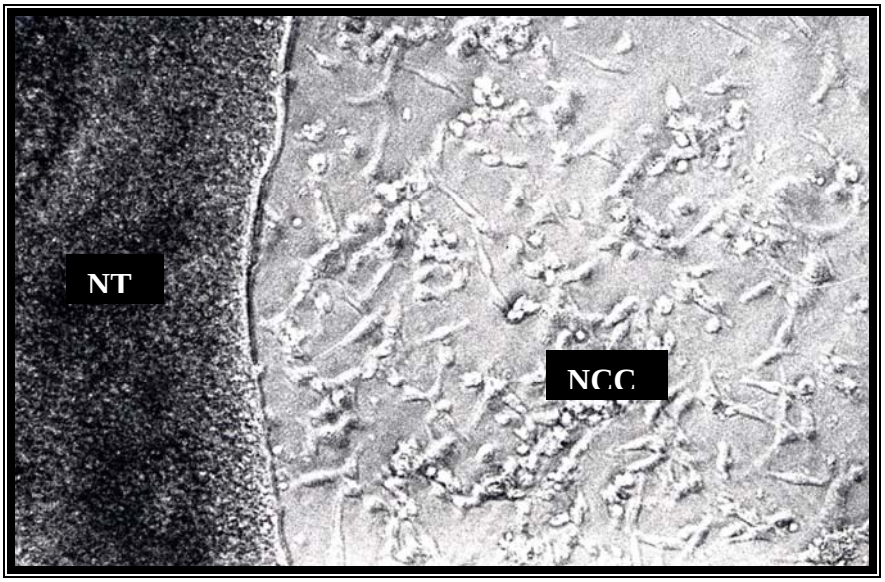


Figure 4. Representative photomicrograph showing a stage 11 quail neural tube (NT) and migrating neural crest cells (NCC) following 24 hours of culture in DMEM. Phase contrast 100X

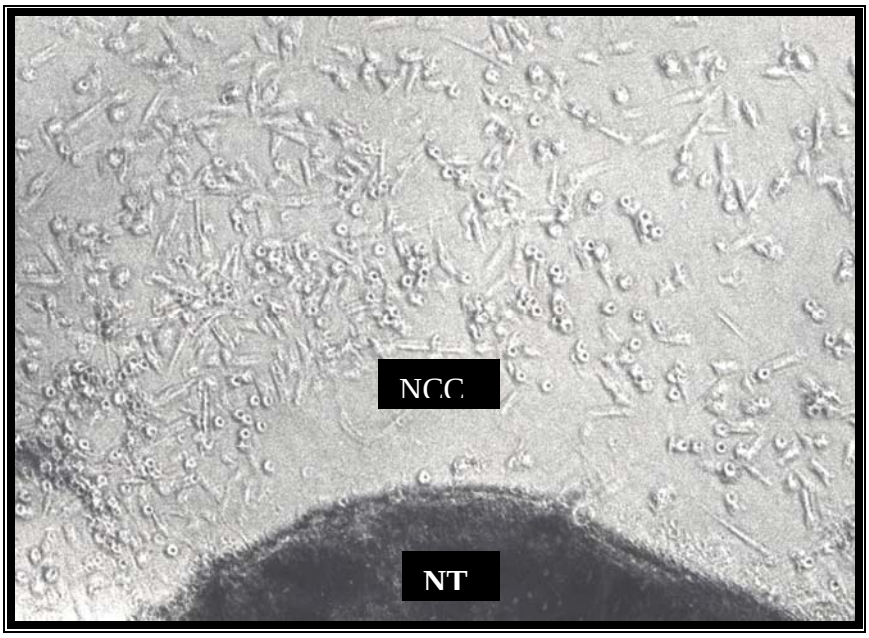


Figure 5. Representative photomicrograph showing a stage 11 neural tube (NT) and migrating neural crest cells following 24 hours of culture in DMEM + DMSO. Phase contrast 100X

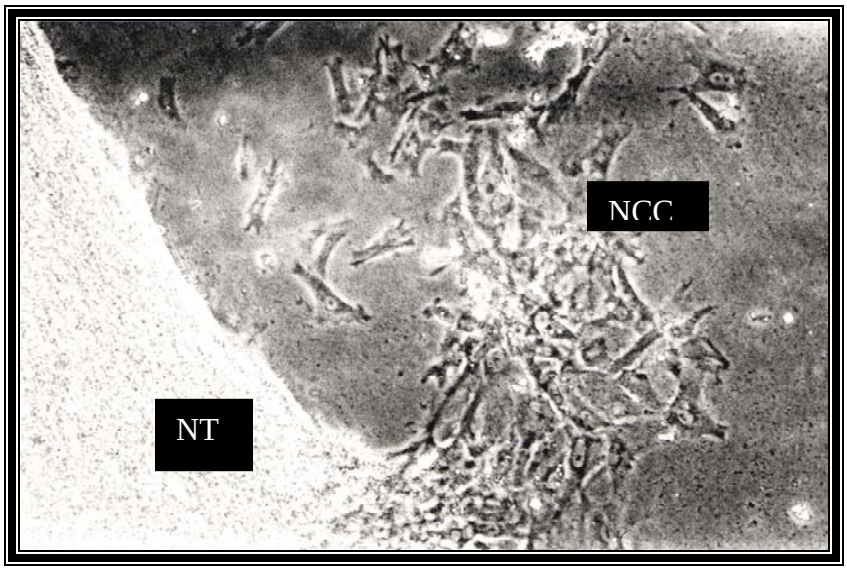


Figure 6. Representative photomicrograph showing a neural tube and migrating neural crest cells following 24 hours of culture in DMEM + retinoic acid. Neural crest cells are fewer than in both control cultures and migrate less extensively. Phase contrast 100X.

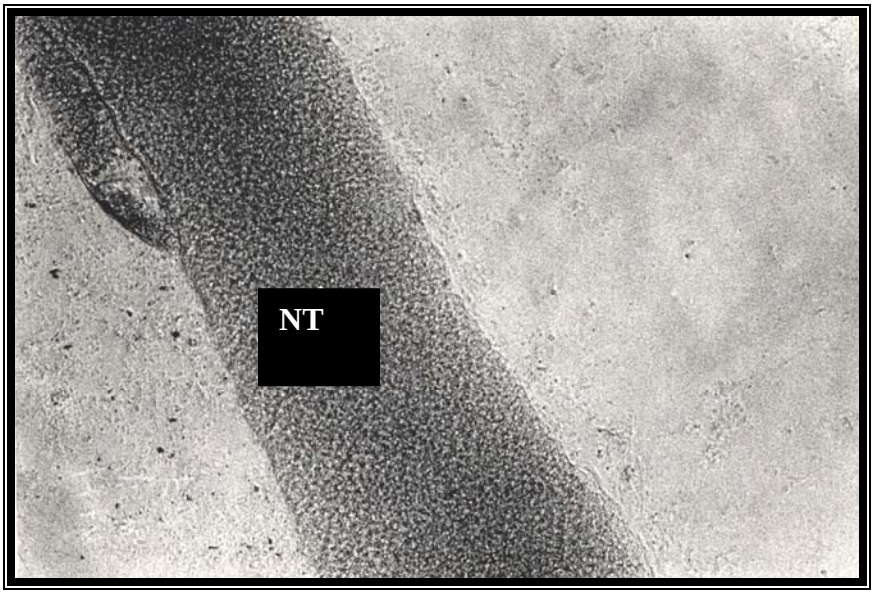


Figure 7. Representative photomicrograph showing a neural tube (NT) following 24 hours of culture in DMEM + retinoic acid. No migrating neural crest cells are present in this culture. Phase contrast 100X.



Figure 8. Representative photomicrograph showing pigmentation in migrating quail neural crest cells cultured in retinoic acid after 24 hours. These cells show early pigmentation compared to neural crest cells in the control cultures. Phase contrast 1000X

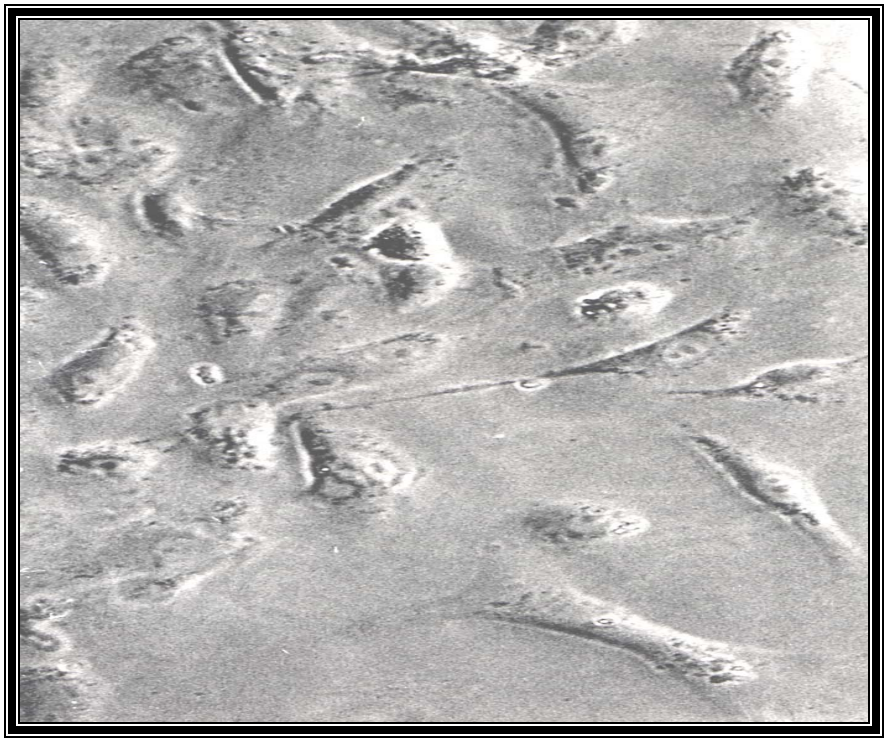


Figure 9. Representative photomicrograph showing migrating quail neural crest cells cultured in DMEM after 24 hours. These cells do not show pigmentation yet. Phase contrast 1000X

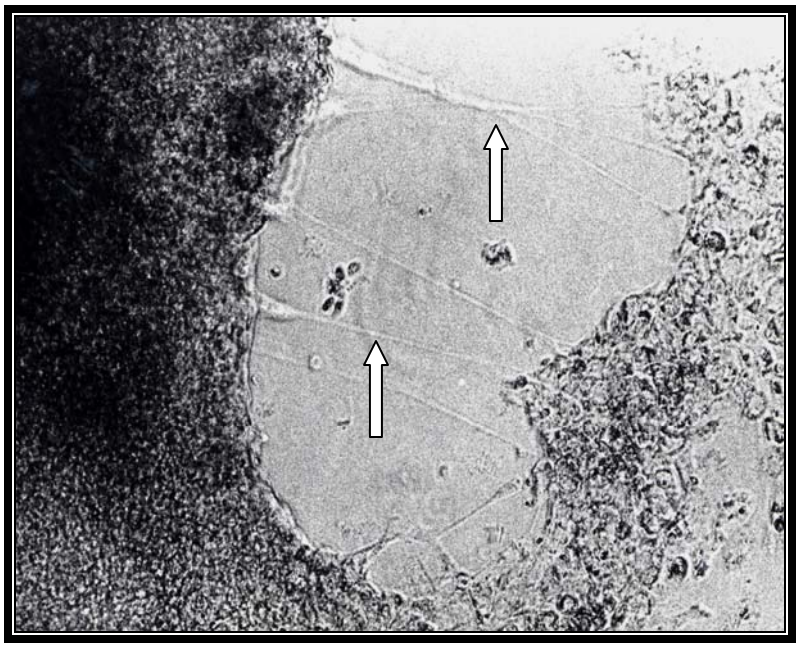


Figure 10. Representative photomicrograph showing neurite formation (arrows) in migrating quail neural crest cells following 24 hours of culture in retinoic acid. These cells showed neurite outgrowth earlier than did neural crest cells cultured either in DMEM alone or DMEM + DMSO. Phase contrast 100X.

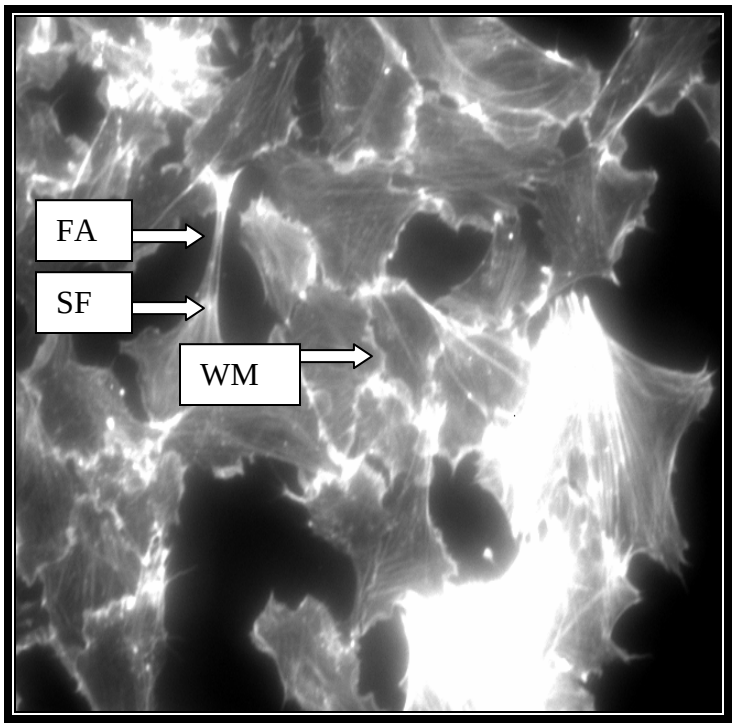


Figure 11. Representative photomicrograph showing the formation of stress fibres (SF) and focal adhesions (FA) in quail neural crest cells cultured in DMEM. The cell membranes appear wavy (WM). Confocal 1000X.

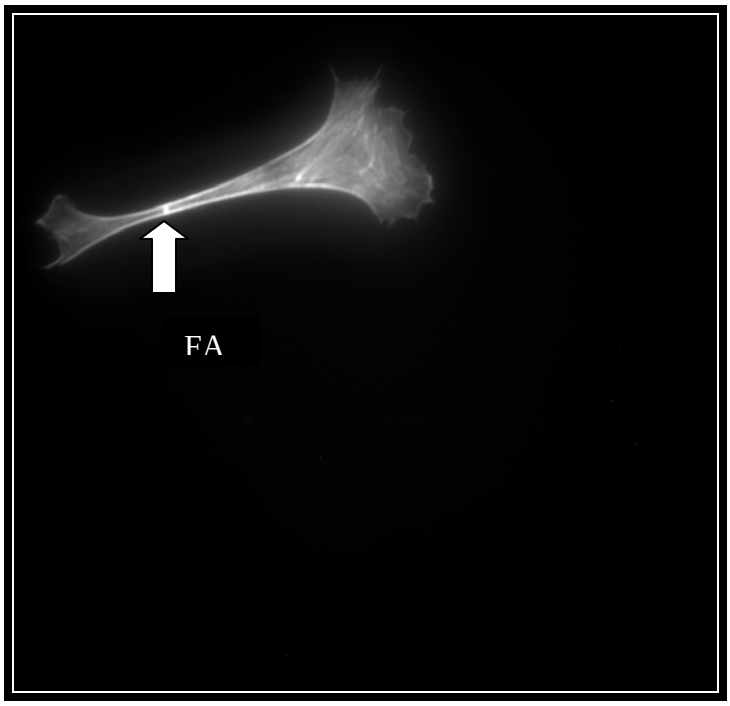


Figure 12. Representative photomicrograph showing a focal adhesion (arrow) of a migrating quail neural crest cell following 24 hours of culture in DMEM. Confocal 1000X.

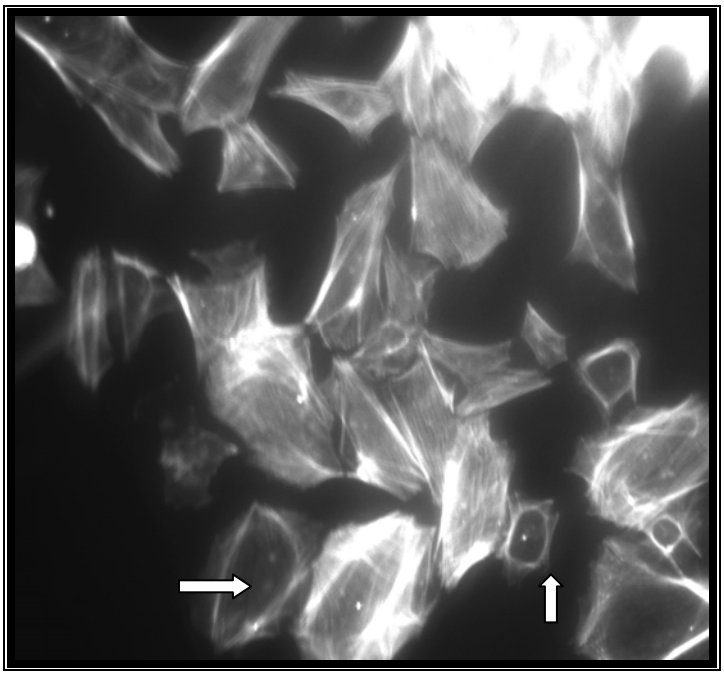


Figure 13. Representative photomicrograph showing neural crest cells cultured in DMEM + retinoic acid. Neural crest cells are more rounded (arrows) than in both controls, and show no filapodial extensions. Confocal 1000X.



Figure 14. Representative photomicrograph showing re-plated quail retinoic acid-treated neural crest cells tested for viability. Neural crest cells were still viable following trypsinization and centrifugation after approximately four hours. Phase contrast 100X.



Figure 15. Representative photomicrograph showing re-plated quail neural crest cells cultured in DMEM alone tested for viability. Neural crest cells were still viable following trypsinization and centrifugation after approximately four hours. The crystal-like particles on the background show changes in the fibronectin. Phase contrast 200X.

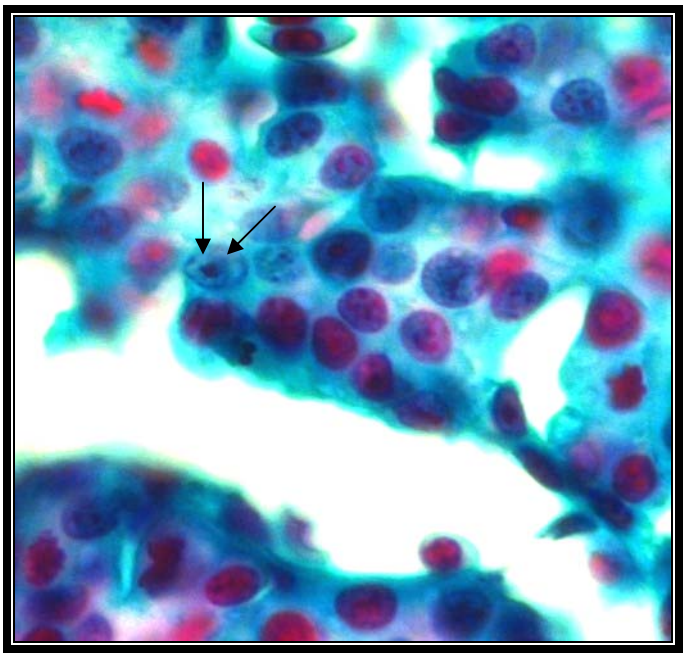


Figure 16. Representative photomicrograph showing a micro-injected quail neural crest cell (arrows) in the beak of the chick embryo. Only a few cells migrated to this area. Light microscopy, 1000X.

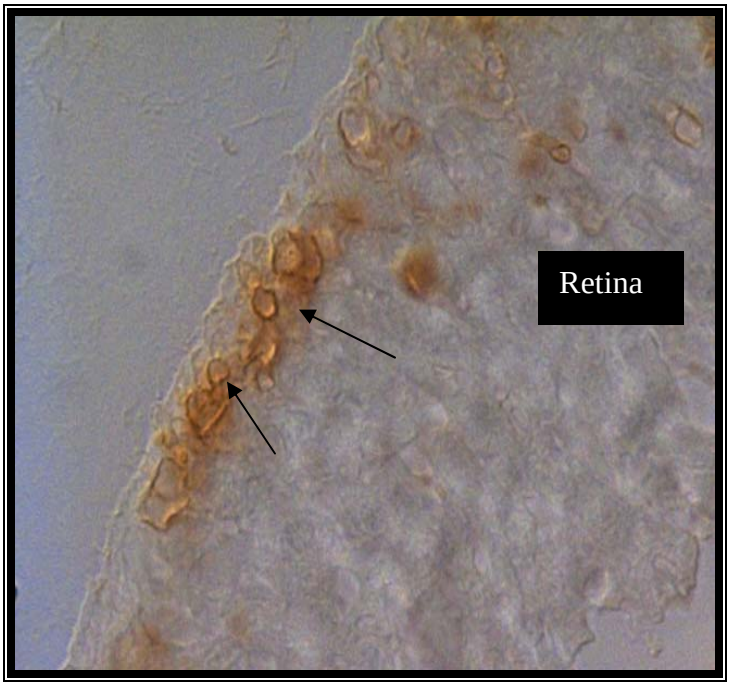


Figure 17. Representative micrograph of immunolocalization of micro-injected retinoic acid-treated quail neural crest cells (arrows) in the retina of the chick embryo using QPCN. Neural crest cells do not normally migrate to this region. (Differential interference contrast, 1000X)



Figure 18. Representative micrograph of immunolocalization of micro-injected retinoic acid-treated quail neural crest cells (arrows) in the retina of the chick embryo using QPCN. Neural crest cells do not normally migrate to this region. Differential interference contrast, 1000X

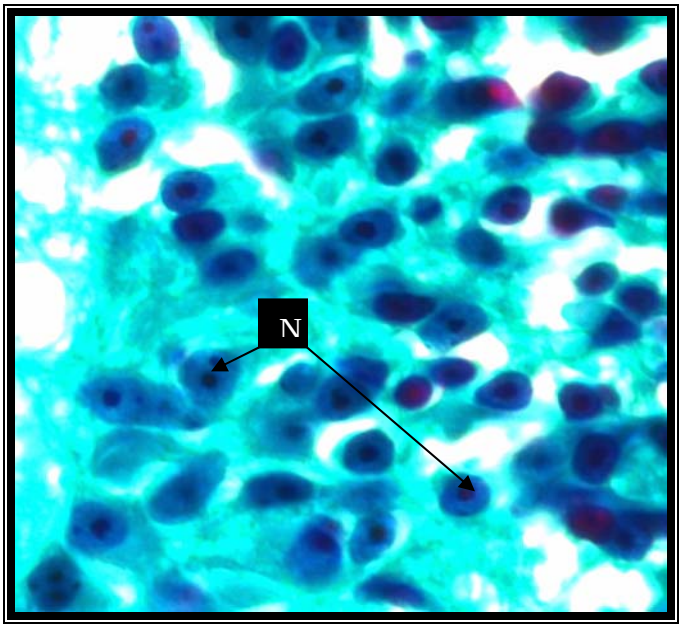


Figure 19. Representative photomicrograph showing a section through a quail embryo. The Feulgen-Rossenbeck method shows the large quail nucleoli (N) present in many of the cells. Light microscopy 1000X

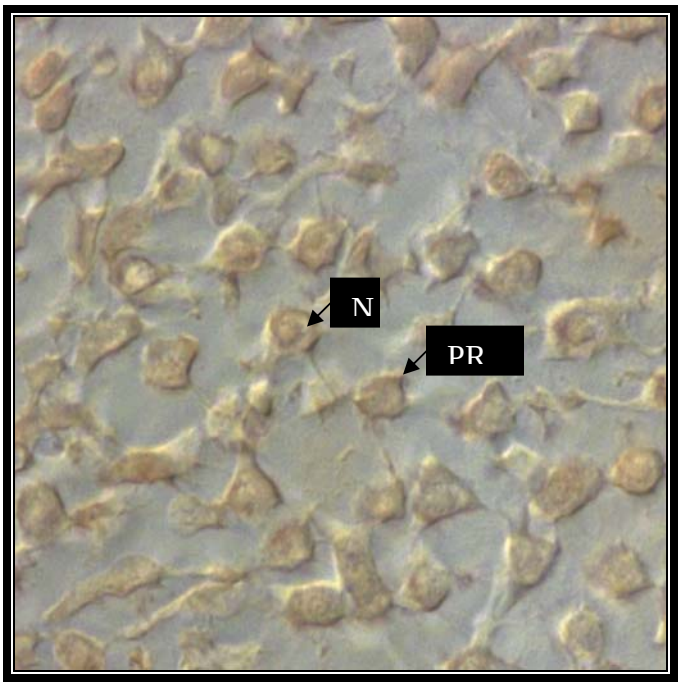


Figure 20. Representative photomicrograph showing a section through a quail embryo. The QPCN method shows perinuclear ring (PR) and the large quail nucleoli (N). Differential interference contrast 1000X.

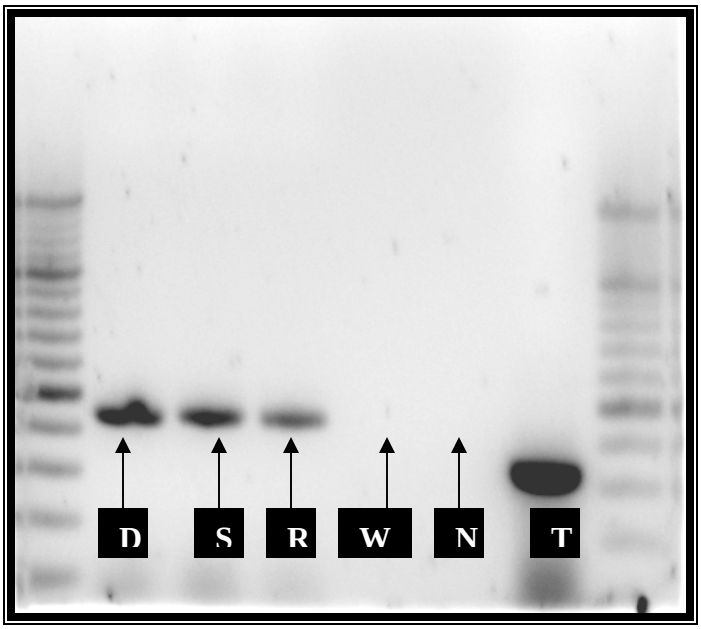


Figure 21. Representative photomicrograph showing the expression of Rac in DMEM- (D), DMSO- (S) and retinoic acid (R)-treated neural crest cells. W and N are water and negative controls, respectively. Tubulin (T) is the positive control.

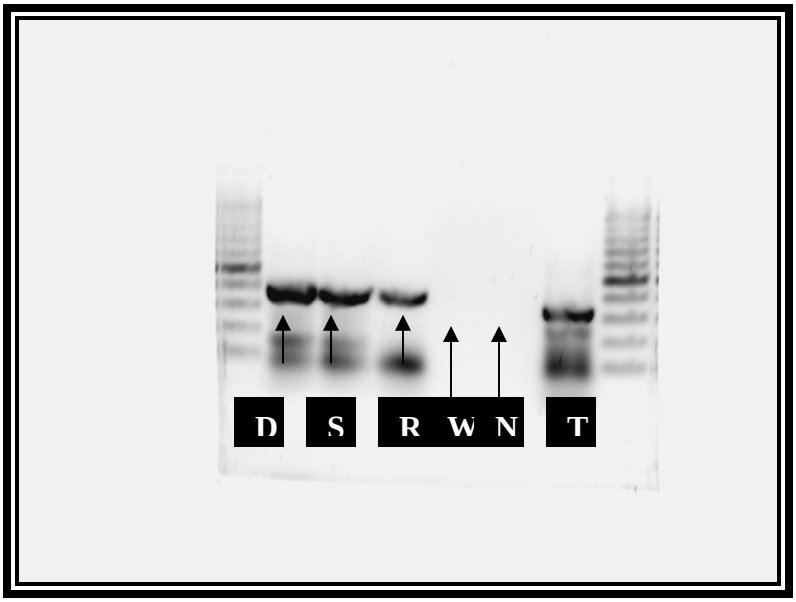


Figure 22. Representative photomicrograph showing the expression of Rho in DMEM- (D), DMSO- (S) and retinoic acid- (R) treated neural crest cells. W and N are water and negative controls, respectively. Tubulin (T) is the positive control.

Discussion

Migration of neural crest cells

In a previous pilot study, it was shown that the effect of retinoic acid on the migration of neural crest cells was dose-dependant (Tshabalala, unpublished work). Higher concentrations of retinoic acid (10^{-5}M) inhibited neural crest cell migration and caused disarray in the cytoskeletal elements more than did concentrations of retinoic acid at 10^{-6}M. In the present study, utilizing the high dose of 10^{-5} M of all *trans* retinoic acid, some of the retinoic acid-treated neural crest cells failed to migrate at all, whereas the distance of migration of most of the neural crest cells was reduced compared to those in both control cultures. The latter is an interesting observation as it suggests that the action of retinoic acid on the neural crest may begin before the onset of migration.

Li *et al.* (2001) showed that the inhibition of migration of neural crest cells was dose-dependant. A concentration of 10^{-8}M retinoic acid yielded no effects on the distance of migration of neural crest cells. In contrast, higher levels of retinoic acid produced inhibition of neural crest cell migration. The results of the present study are consistent with these results, as high concentrations of retinoic acid inhibited the migration of neural crest cells.

Neural crest cells which were cultured either in DMEM alone or in DMEM + DMSO in the present study, migrated a greater distance than did retinoic acid-treated neural crest cells. As there was no statistically significant difference between the migration of neural crest cells treated with DMEM alone or with DMEM plus DMSO, it was concluded that

the inhibition of migration in retinoic acid-treated cultures was due to the retinoic acid itself.

The results show that all-*trans* retinoic acid exerts specific, previously identified effects on the migration of neural crest cells. These results are consistent with other vitamin A related teratology studies, which have shown that retinoids inhibit the migration of neural crest cells when administered at low and high concentrations (Thorogood *et al.,* 1982; Pratt *et al.,* 1987; Maden *et al.,* 1998a; Lee *et al.,* 1995; Williams *et al*., 2004; Wang *et al*., 2005).

Pratt *et al.* (1987) examined the migration of mouse neural crest cells in the presence of retinoic acid. Whole mouse embryos were cultured in the presence of 13-*cis* retinoic acid at $2X10^{-6}M$ and $2X10^{-5}M$ concentrations. While control cranial neural crest cells in the mid-brain region migrated to the region of the first and second visceral arches after 6 hours in culture, the retinoic acid-treated cranial neural crest cells did not leave the neuroepithelium, or they did not migrate away from the neuroepithelium. The results of the present study are consistent with these results, as the retinoic acid-treated neural crest either failed to produce migration of the neural crest cells or the neural crest cells failed to migrate as far as did cells in control cultures. Thorogood *et al*. (1982) investigated the effect of retinol on the migration, morphology, and locomotory behavior of avian neural crest cells. Concentrations of $3.5 \times 10^{-5}M$ and $3.5 \times 10^{-6}M$ were used. The retinol inhibited the migration of neural crest cells. It was shown that this inhibition was due to

the disruption of cell-extracellular matrix adhesion. Retinol-treated neural crest cells adhere to the extracellular matrix less, and are unable to extend or maintain lamellipodia.

In their study, Thorogood *et al.* (1982) exposed neural crest cells to retinol in three different series of experiments. In the first of the experiments, neural crest cells which had been cultured in medium for 24 hours were exposed to retinol for a further 72 hours. In the second series, neural crest cells were exposed to retinol at 0 hours of culture. Interestingly, the inhibitory effect of retinol on the neural crest cells was the same in both cases. This shows that retinoic acid has an effect on pre-migratory neural crest cells, and those which are already migrating. In the present study retinoic acid was always administered at 0 hours. In the third series carried out by Thorogood *et al.* (1982) it was shown that the effects of 24 hour exposure to retinol could be reversed. Retinol was replaced with plain medium in neural crest cultures which had been cultured for 24 hours. Morphology and distance of migration of these cells was the same as in controls, in spite of having been initially abnormal following exposure to retinol.

According to Smith *et al*. (1998), the abnormalities in the development of ectomesenchymal derivatives due to the inhibition of neural crest migration may occur at every stage from initial to final differentiation of the neural crest. The craniofacial abnormalities resulting from neural crest development may result in diminished size or absence of embryonic facial processes leading to the defects of the face and/or palate, which are among the most commonly encountered malformations in humans (Hall and Horstadius, 1988; Lohnes *et al*., 1994). In Treacher Collins Syndrome for example, the

failure of normal development of the lower facial region, such as mandibular deficiency, may also result from abnormalities in the migration of the neural crest (Gale *et al*., 1996). The deleterious effects on craniofacial development appear to be due to a direct effect of the retinoic acid on the cranial neural crest cells which gives rise to the facial mesenchyme (Mic *et al*., 2002).

During the first 24 hours of culture, all-*trans* retinoic acid did not alter the size and shape of the neural crest cells qualitatively as seen by phase contrast microscopy. The differences in size and shape of the neural crest cells only became apparent after 48 hours. In contrast to this observation, confocal microscopy revealed a change in the shape of these cells at 24 hours. These observations show that changes to the size and shape of neural crest cells happens after 24 hours. These changes are more apparent when viewed with a confocal microscope. This is because the confocal microscope shows the formation of actin filaments and their contribution to the shape of the cell. As a result, any small alteration which happens to either actin filaments or cell shape becomes apparent. The phase contrast microscope does not show these details. In contrast to the present study, Thorogood *et al*. (1982) showed that neural crest cells which were exposed to lower concentrations of retinol were slightly larger than the control cells. Contrary to the findings of the present study, with higher retinol concentrations, the sizes of neural crest cells were twice those of control cells. In the present study, the size of neural crest cells was reduced when treated with elevated levels of retinoic acid.

Differentiation of neural crest cells in culture

The present study showed that retinoic acid-treated neural crest cell cultures showed early pigmentation. In addition, the retinoic acid-treated neural crest cell cultures showed neurite formation earlier than did the control cultures. This supports the findings that normal levels of retinoic acid promote cell differentiation and growth (Dupin and Le Douarin, 1994; Wang *et al*., 2005). These results are consistent with studies by Dupin and Le Douarin (1994) who showed that 10μM of all-*trans* retinoic acid promotes the differentiation of quail melanocytes and adrenergic cells. Melanocytes were highly pigmented in the presence of all-*trans* retinoic acid. In addition, a larger number of melanocytes were produced in neural crest cells which were exposed to retinoic acid than in non-retinoic acid treated-cultures.

Wang *et al*. (2005) cultured mouse neural crest cells and treated them with 10^{-6}M retinoic acid. Wang *et al*. (2005) then conducted a time-course microarray analysis of the retinoic acid-treated neural crest cells. In this study, the expression of cell adhesion genes was decreased. In contrast, the expression of genes regulating the cell cycle (cyclin-dependent kinase inhibitors, cdkn1a) was increased. Cyclin-dependent kinases regulate the G1 to S phase transitions. This is vital in differentiation, proliferation and apoptosis (Stein *et al.,* 1999; Wang *et al.,* 2005). This shows that elevated levels of retinoic acid could inhibit neural crest proliferation and genes which regulate growth. The present study suggests that retinoic acid plays a role in the differentiation of pigment cells and neuronal processes.

According to Kalter and Warkany (1959), the early formation of neurites in the retinoic acid-treated neural crest cells may be linked to some congenital defects of the central nervous system. *In vitro* studies by Cohlan (1954) showed vital stimulatory effects of vitamin A on neurite outgrowth in cultures of various types of neuronal cells and also on neuronal differentiation in embryonic carcinoma cells. All-*trans* retinoic acid, which is the active metabolite of retinoic acid, induces embryonic carcinoma cells to differentiate into a range of cell types, including neural cells, depending on the concentration of retinoic acid applied. In cells that are already neuronal, retinoic acid induces either neurite extension where there was none before, or longer neurites, if neurites were already present. This was revealed by Maden and Holder (1992) in dissociated cultures or explanted tissue using embryonic dorsal root ganglia, spinal cord or sympathetic ganglia from chick, mouse, rat and human embryos. The results of the present study, where the retinoic acid-treated neural crest cells showed early neurite formation are consistent with the work of Maden and Holder (1992).

In a study similar to the present study, Maden *et al.* (1998a) cultured the neural tubes of vitamin A-deficient embryos either in DMEM, DMEM with serum or DMEM with 0.1µmol of *all- trans* retinoic acid. Explants of the neural tube from vitamin A-deficient embryos extended few neurites into the medium. The cells were, however, deemed to be healthy, as flat cells migrated out from the explant, and one of the neural tubes produced one neurite. The addition of all- *trans* retinoic acid to the neural tube cultures increased neurite outgrowth (Maden *et al*., 1998a). This shows that retinoic acid can rescue vitamin A-deficient embryos when administered at desirable concentrations. In addition, the

addition of fetal calf serum, which contains high levels of retinoids as well as other nutrients, also increased/stimulated neurite outgrowth from the vitamin A deficient embryos. Contrary to the present study, Maden *et al.* (1998a) showed that all-*trans* retinoic acid is not only teratogenic but can also rescue cells when administered at lower concentrations.

Other abnormalities of neural crest cell migration have been revealed in the central nervous system. According to Leonard *et al*. (1995), when excess all-*trans* retinoic acid is administered in both mouse and zebrafish embryos at gastrulation, a segment of the anterior hindbrain/posterior midbrain does not form. If the dose of retinoic acid is minimized, a segment of the anterior hindbrain is respecified into another, more posterior segment. In these experiments rhombomere 2 takes some of the characteristics of rhombomere 4. In the zebrafish experiments, the difference in doses between deletion and respecification was between 0.15μmol and 0.1μmol, a remarkably small difference for such a significant difference in results.

Actin cytoskeleton

In the present study, retinoic acid appears to have a deleterious effect on the cytoskeleton of neural crest cells. This was shown by a lack of formation of the transmembrane proteins linking the actin cytoskeleton to the extracellular matrix, namely stress fibres and focal adhesions. The cells also showed disarray in the cytoskeleton as they appeared to be more rounded than the neural crest cells which were cultured in DMEM alone or in DMEM +DMSO.

Wang *et al.* (2005) studied the architecture of the neural crest cell cytoskeleton by staining both the control and retinoic acid-treated neural crest cells with alpha actin. Stress fibres were distributed around the periphery and throughout the inside of the control neural crest cells. The number of actin filaments and the intensity of staining were reduced in the interior of the retinoic acid-treated neural crest cells. The results of the present study which shows disarray in the actin cytoskeleton of neural crest cells are consistent with the work of Wang *et al.* (2005). However, the present study could not quantify the amount of actin present in the neural crest cells. As a result, it is not clear whether the change in the architecture of the actin cytoskeleton of neural crest cells is due to a change in the quantity of actin or not.

Thorogood *et al.* (1982) showed that neural crest cells which have been exposed to retinol have a diffuse actin distribution. In addition these cells are devoid of fibrillar organization. The organization of actin was recovered by the addition of plain medium. The rate of recovery was dependant on the concentration of retinol used.

Integrins are transmembrane proteins mediating the interactions between the cytoskeleton and the extracellular matrix which are required for the cell to attach the matrix (Alberts *et al.*, 1994). Most integrins connect to bundles of actin filaments. If the integrins are disrupted, the cells fail to attach to the substrate, and they become rounded (Alberts *et al.*, 1994). If cells cannot attach to the substrate, failure of migration results. This may explain why some of the retinoic acid-treated cultured neural crest cells were rounded

and failed to migrate. Failure to form stress fibres and focal adhesions by neural crest cells causes an inhibition in migration.

After the binding of a typical integrin to its ligand in the matrix, the cytoplasmic tail of the integrin β chain binds to both talin and alpha actinin and thus initiates the assembly of a complex of intracellular attachments proteins that link the integrin to actin filaments in the cell cortex. This is thought to be how focal adhesions form between cells and the extracellular matrix. It is believed that retinoic acid interferes with this binding and thus disrupts the integrins. If the cytoplasmic domain of the beta chain is deleted, the mutant integrins still bind to their ligands but no longer mediate robust cell adhesion at focal contacts. In order to bind cells to the matrix, the cells must interact with the cytoskeleton. A transmembrane attachment to the cytoskeleton appears to be an important general requirement for both cell-matrix and cell-cell adhesions (Alberts *et al*., 1994).

The cell cytoskeleton can be influenced by the organization of the matrix. For example, transformed cells make less fibronectin than normal cultured cells and behave differently (Lodish *et al.*, 1990). They adhere poorly to the substrate and become more rounded. They fail to flatten and do not develop stress fibres (This may contribute to the tendency of cancer cells to break away from the primary tumor and spread to the other parts of the body) (Darnell, *et al.,* 1990; Lodish *et al*., 1990; Alberts *et al*., 1994). If the cells are grown on the matrix of organized fibronectin filaments, they flatten out and assemble intracellular stress fibres that are aligned with the extracellular fibronectin filaments.

Observations with the confocal microscope in the present study show that the retinoic acid-treated cultures resemble transformed cells in that they are more rounded and lack stress fibres, which are important for cell-matrix adhesion. The lack of stress fibres indicates that cells cannot adhere to the extracellular matrix as they cannot form focal adhesions, and thus they become rounded. This concurs with the hypothesis by Hall and Horstadius (1988) that retinoic acid inhibits the association between the extracellular matrix and the cell. In addition, the retinoic acid-treated cells exhibit disrupted positioning of actin filaments. This could contribute to the changes in shapes and size of the neural crest cells in the retinoic acid-treated cultures. In support, Rac and Rho expression in the retinoic acid-treated neural crest cells appeared to be less than in the controls following electrophoresis.

Rho activation stimulates the assembly of stress fibres and focal adhesions (Alberts *et al*., 1994; Machesky and Hall, 1997), and the assembly of these structures is blocked when Rho is inactivated via ADP-ribosylation (Machesky and Hall, 1997). In contrast, the activation of Rac causes extension of lamellipodia (Clark *et al*., 1998). A third member of the family, cdc42 regulates the formation of peripheral filapodial extensions (Clark et al., 1998). It has been assumed that the activation of Rac and Rho leads to actin polymerization. According to observations from the present study, retinoic acid plays a role in the decreased expression of Rac and Rho. The absence of stress fibres, filapodial extensions and lamellipodia, and thus inhibition of migration, could result from the deactivation of the two members of the Rho family of GTPases.

Micro-injections of quail neural crest cells into chick embryos

Following trypsinization and centrifugation, control and retinoic acid-treated neural crest cells re-adhered to the culture plates. This showed that the cells were still viable and that they still had migratory ability.

In order to visualize the chick host embryo, India ink was injected below the blastoderm. The recommended concentration of the ink is 10% (Bronner-Fraser, 1996). Only 1% of Pelican India ink was used in this study, as the survival rate of chick host embryos was low following introduction of the ink.

Following micro-injections of the retinoic acid-treated quail neural crest cells and non-treated neural crest cells into chick embryos, the neural crest cells populated the chick retina and the beak area, respectively. Neural crest cells do not normally populate the retina. This finding of neural crest cells migrating to the retina is consistent with the study by Hall and Horstadius (1988) and Lee *et al*. (1995) in which they show that the administration of vitamin A to pregnant mice results in the failure of the development of neural crest derivatives or their development in ectopic positions.

Numbers of neural crest cells were low and could not be quantified due to loss of cells and contamination during centrifugation. Low numbers of neural crest cells could have affected the study in that only few cells migrated to reach their destinations

Administration of 2X 10^{-7} M all-*trans* retinoic acid to rat cranial neural crest cells induced neural crest malformations (Lee *et al.,* 1995). Retinoic acid exposure induced

ectopic migration of anterior hindbrain neural crest cells. The neural crest cells ectopically migrated to the second branchial arch and the acousticofacial ganglion. They also migrated to the first arch and trigerminal ganglion (Lee *et al.,* 1995).

Although alteration of neural crest migration has been proposed as an explanation for retinoic acid-induced craniofacial defects, not many *in vivo* studies have been done to examine neural crest behavior after exposure to retinoic acid. Some of the *in vivo* studies reported inhibition of migration (Pratt *et al*., 1987; Thorogood et al., 1982; Webster *et al*., 1986), while Lee *et al*. (1995) showed a mis-migration of the neural crest cells into ectopic positions. Both the *in vitro* and *in vivo* studies in the present study show that the retinoic acid-treated neural crest cells show both inhibition and mis-migration as they populate ectopic positions in the retina of the eye.

The survival rate of chick host embryos was low, as it took time to learn the technique of micro-injecting. The embryos died from either the India ink or if too large a hole was made in the blastoderm during the injection of the India ink. In the latter instance, yolk would leak out and the embryo would die. In most of the surviving chick hosts, quail neural crest cells were not identified. This is probably due to low numbers of neural crest cells injected. In order to follow the path which neural crest cells travel, some researchers use different techniques which are equivalent to those used in the present study. These techniques include using neural crest cells markers such as HNK-1 and DiI (Bronner-Fraser, 1996).

Retinoic acid and cell membranes (SDS-PAGE)

No results were obtained using the SDS-PAGE technique. Unfortunately insufficient protein was extracted from the cultured neural crest cells to make the technique viable. A disarray of the cell membranes was expected, as studies show that retinoic acid has a deleterious effect on neural crest cell membranes. These effects could not be seen in our cultures using the confocal and phase contrast techniques.

In 1971, Schweichel showed that vitamin A expanded and strengthened interdigital necrotic zones in the rat. Schweichel (1971) hypothesized that this could be the result of the labilization of lysososmes due to its detergent effect on membranes. Lysosomal enzymes were believed to cause cell death. They appeared to do this by being released into the cytoplasm of the cells of origin as well as into the intercellular spaces. Lysosomal membranes of all cells do not lyse after treatment with retinoic acid (Marks *et al.,* 1983). Marks *et al.* (1983) suggested that only those membranes at a particular stage of differentiation or which have been perturbed in some other way react. The destruction of membranes by retinoic acid could interfere with many functions carried out by cells. Morris (1973) noted the blebbing of the plasma membranes during mesodermal gastrulation. In addition, Thorogood *et al*. (1982) and Webster *et al*. (1986) showed blebbing in neural crest cells membranes after retinoic acid administration. This might be expected to interfere with the migratory ability of the neural crest cells. If retinoic acid is removed *in vitro*, the membranes will recover (Webster *et al*, 1986). The results of the present study could add to these observations as some of the control neural crest cell

cultures showed wavy cell membranes when compared to the retinoic acid-treated neural crest cells.

Conclusion

This study has provided evidence that excess retinoic acid has an effect on the migration, differentiation, actin cytoskeleton, and pathway of neural crest cells. Retinoic acid inhibits the migration of neural crest cells by having a negative effect on Rac and Rho, two members of the Rho family of GTPases. The deactivation of Rac and Rho prevents the formation of stress fibres, focal adhesions, and lamellipodia respectively, structures which play an important role in neural crest cell migration. In conclusion, correct levels of vitamin A are required during embryogenesis, and excess or deficiency of this vitamin is equally harmful to the embryo.

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Appendix A

Instruments

1 fine forceps

1 fine scalpel

Insect pins

1 spoon

Coarse scissors

Other equipment

1 bottle 70% alcohol

1 bottle 90% alcohol

Gill soap

Towel, plastic sheeting

Alcohol burner + matches

Solutions

Chick Ringer's solution

8.5g sodium chloride

0.42g potassium chloride

0.25g calcium chloride

1000 ml distilled water

100µl pen-strep antibiotic

Tyrode's solution

Solution A

500 ml distilled water

2.0g sodium chloride

0.05 potassium chloride

0.012g $N_2H_2PO_4H_2O$

Solution B

480 ml distilled water

0.025g $NAHCO_3$

1g glucose was mixed in 20 ml distilled water in a 100 ml bottle and placed in the fridge. Solutions A and B were autoclaved separately, and the swinnexed glucose mixture was added to the solutions. 100 µl pen-strep antibiotic was added to the final solution.

Collagenase

0.0025g collagenase + 6 ml Tyrode's solution

The mixture was refrigerated before use

Culture medium

75 % Dulbecco's minimal essential medium (DMEM)

15 % Embryo extract

10 % Horse serum

Retinoic acid

All *trans* retinoic acid was dissolved in DMSO (concentration 10^{-5}M)

DMSO

DMSO was dissolved in DMEM (concentration 10^{-5}M)

Rhodamine Phalloidin

5µl rhodamine phalloidin + 300µl PBS

Fibronectin

25µl in 1ml sterile distilled water

Solutions for protein extraction

Homogenization buffer, pH 7.4

To make up 500 ml of the solution

10.00g tween 20

0.12g$MgSO_4.7H_2O$

0.15g $CaCl_2.2H_2O$

4.38g Nacl

1.51g Tris

Solubilization buffer pH 8.0

To make 250 ml of the solution

5.0g deoxycholate

0.3g Tris

Tris buffer, pH 7.1

To make 50 ml of the solution

0.01g Tris

0.30g Mannitol1x Running Gel Solution

	7%	**10%**	**12%**	**15%**
H_2O	15.3 ml	12.3 ml	10.2 ml	7.2 ml
1.5 M Tris-HCl, pH 8.8	7.5 ml	7.5 ml	7.5 ml	7.5 ml
20% (w/v) SDS	0.15 ml	0.15 ml	0.15 ml	0.15 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	6.9 ml	9.9 ml	12.0 ml	15.0 ml
10% (w/v) ammonium persulfate (APS)	0.15 ml	0.15 ml	0.15 ml	0.15 ml
TEMED	0.02 ml	0.02 ml	0.02 ml	0.02 ml

Stacking Gel Solution (4% Acrylamide):

H_2O	3.075 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
20% (w/v) SDS	0.025 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (APS)	0.025 ml
TEMED	0.005 ml

Silane dipped slides

Soak slides in 10 % Contrad or Super 10 overnight

Rinse in hot running water-minimum-2 hours

Dry in oven at 60°C

Dip in acetone

Dip in 2% silane in acetone for 30 minutes (6ml silane + 294 ml acetone)

Wash in two changes of acetone

Wash briefly in distilled water

Dry in 42°C incubator overnight.

Tris-HCl stock solution

100 ml of 1M Tris

76.8 ml of 1M HCl

Mix together and adjust pH to7.6

Make up to 2 litres with distilled water

Tris Saline

42.75 g NaCl

4.5l distilled water

Add 500 ml of Tris-HCl stock solution

Diaminobezidene (DAB)

0.01 g DAB dissolved in 2 ml of Tris-HCl stock solution. Add 20µl freshly prepared 1% hydrogen peroxide

Phosphate Buffered Saline (PBS)

8g NaCl

0.2g KCl

1.15g Na_2HPO_4

0.2g KH_2PO_4

<u>Hand processing schedule for embryos</u>

Fix at least 3 hours at 4°C

Wash in phosphate buffer for 30 minutes

Place in 50% alcohol for 20 minutes

Place in 70% alcohol for 30 minutes

Place in 95% alcohol for 15 minutes

Place in 95% alcohol for 15 minutes

Place in absolute alcohol for 15 minutes

Place in absolute alcohol for 30 minutes

Place in Xylene for 10 minutes

Place in Xylene for 10 minutes

Place in wax for 15 minutes twice and for a further 30 minutes

Embed.

Embryo extract preparation

Glassware and solutions were sterilized the preceding day. Eleven-day-old chick eggs were cleaned with 70% alcohol, and broken into a glass dish containing a small amount of chick ringer's solution. The head was cut off, and both the head and body were placed into another sterile glass dish, and washed. Four embryos were prepared in this manner. The prepared embryos were inserted into a 20ml syringe. Five ml was then expressed into a sterile graduated centrifuge tube. Five ml Ringer's solution was added, and the contents were stirred with a glass rod. The contents were covered and left to stand at room temperature for one hour. Following this, the suspension was spun for 20 minutes at 2000g. After spinning, the supernatant was poured into sterile bijou bottle and frozen until use.

India ink

1ml in 99mls Chick Ringer's solution

Feulgen reaction (Feulgen and Rossenbeck, 1924)

The paraffin sections of the chick heads or whole embryos were brought to distilled water. The sections were briefly rinsed in cold NHCL and transferred to NHCL at 60°C

for 8 minutes. As controls, similar sections of each embryo were placed in distilled water at 60°C for the same period of time. After washing in distilled water, the sections were transferred to Schiff's reagent for 60 minutes. The sections were rinsed in three changes of sulphite rinse solution (see appendix), and then in water. The sections were counterstained in 1% aqueous light green for 1 minute, dehydrated, cleared and mounted in entellan. As a positive control, for the Feulgen-Rossenbeck method, six day old quail embryos were used.

Titan One Tube RT-PCR system

Contents

Enzyme mix
RT-PCR buffer
$MgCl_2$ stock solution
DTT solution

Preparation of Master mix 1

Thaw the components listed below and place them on ice
Vortex briefly and centrifuge all reagents before setting up the reactions
Set up the reaction components for Master mix 1 in a separate, nuclease-free microfuge tube placed on ice.

	Sterile distilled water
1μl	dNTP mix
0.4μM	Downstream primer
0.4μM	Upstream primer
1μG-1pg	Template RNA
5Mm	DTT Solution

5-10 U Protector RNase inhibitor

Total Volume 25µl

Preparation of Master Mix 2

Thaw the components listed below and place them on ice.

Vortex briefly and centrifuge all reagents before setting up the reactions

Set up the reaction components for Master mix 2 in a separate, nuclease-free microfuge tube placed on ice.

	Sterile double distilled water
1.5mM	5X RT-PCR buffer with Mg^{2+}
1µL	Enzyme mix

Appendix B

Statistical analysis of the distance of neural crest cells migration

<u>At 0 degrees</u>

DMEM		**RA**	
N	52	**N**	71
Mean	7.84	**Mean**	3.365
Variance	5.6299	**Variance**	2.6782

F: 2.1021	p(same variance):	0.114	
t: 6.9432	p(same mean):	2.9359E-8	
Uneq. Var. t: 6.9432	p(same mean)	5.4966E-8	
Permutation t test	p(same mean)	0	

DMEM		**DMSO**	
N	52	**N**	59
Mean	7.785	**Mean**	7.84
Variance	5.2224	**Variance**	5.6299

F: 1.078	p(same variance):	0.87163
t: -0.074665	p(same mean):	0.94087
uneq. var. t: -0.074665	p(same mean):	0.94087
permutation t test	p(same mean)	

<u>One way Anova</u>

	Sum of squares	**df**	**Mean square**	**P(same)**	**F**
Between groups	263.767	2	131.884	1.823E-9	29.94
Within groups	257.079	57	4.51016		
Total	520.846	59			

RT-PCR cycling conditions

First strand cDNA synthesis	1 cycle	48ºC for 45 minutes	reverse transcription
	1 cycle	94ºC for 2 minutes	AMV RT inactivation and RNA/CDNA/primer denaturation
Second strand cDNA synthesis and PCR amplification	40 cycles	94ºC for 30 seconds	denaturation
		60ºC for 1 minute	annealing
		68ºC for 2 minutes	extension
	1 cycle	68ºC for 7 minutes	final extension
	1 cycle	4ºC	soak

Analysis

Analyze the PCR products by agarose gel electrophoresis of 5% of the total reaction
Store the reaction products at -20ºC until needed.

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