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).

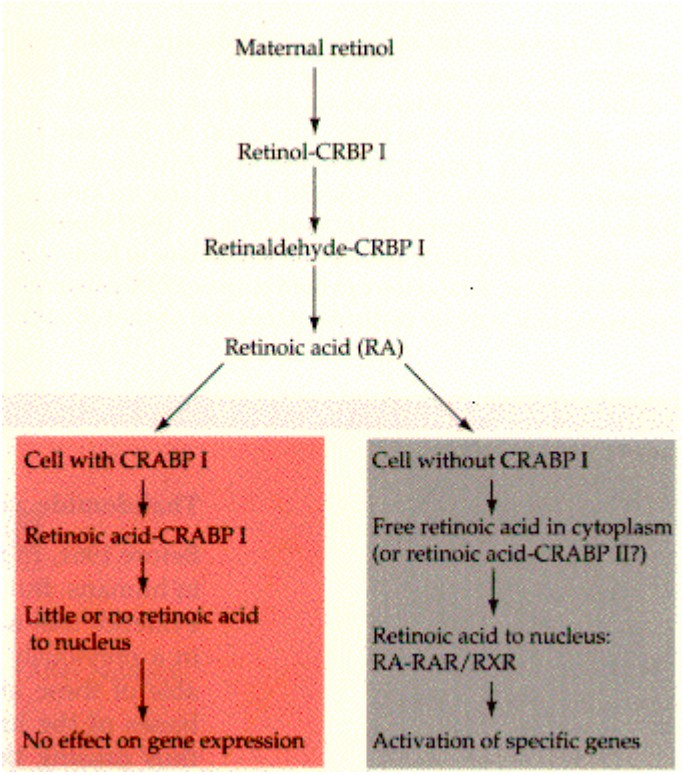

Fig.1. Summary of the relationships between retinoic acid synthesis, retinoic acid binding proteins, and receptors in the mammalian embryo. Retinoic acid would be able to enter the nucleus, bind to its receptors and change the normal pattern of gene expression, if CRABP were to be saturated by external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

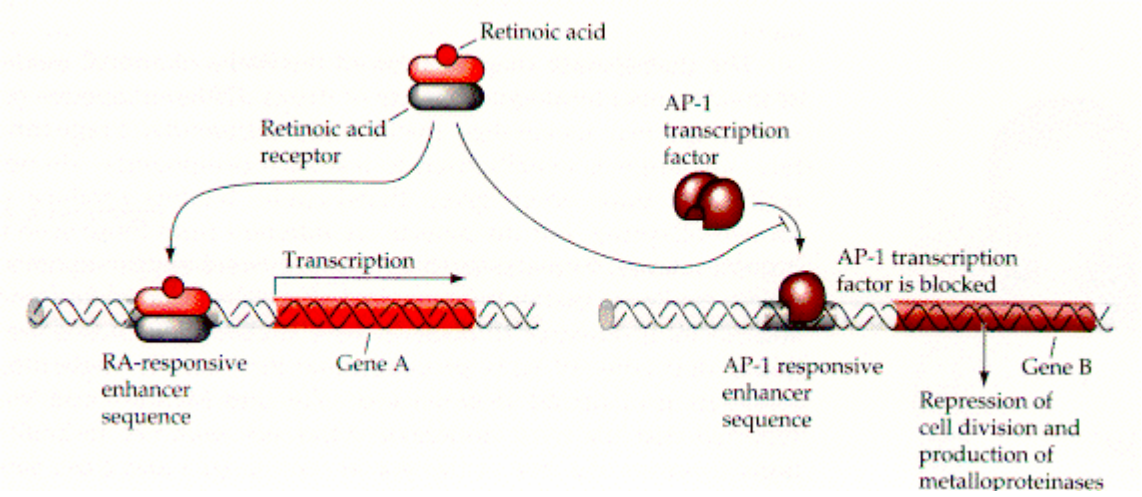

Fig.2. The dual action of retinoic acid-bound RARs model. They activate the transcription of genes whose enhancers enable the RARs to bind (Desbois *et al.,* 1991).

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).