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it was shown that the effect of retinoic acid on the migration of neural crest cells was dose-dependant (Tshabalala, unpublished work).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^-5M) inhibited neural crest cell migration and caused disarray in the cytoskeletal elements more than did concentrations of retinoic acid at 10^-6M. 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^-8M 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 $2 \times 10^{-6}$M and $2 \times 10^{-5}$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-\textit{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 \textit{et al.} (1998a) cultured the neural tubes of vitamin A-deficient embryos either in DMEM, DMEM with serum or DMEM with 0.1\(\mu\)mol of all-\textit{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-\textit{trans} retinoic acid to the neural tube cultures increased neurite outgrowth (Maden \textit{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 $2 \times 10^{-7}$ 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 trigeminal 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 lysosomes 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 \textit{in vitro}, the membranes will recover (Webster \textit{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.