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.