2009

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In embryonic development, many cells migrate in order to correctly form new structures. One way that guidance of this migration occurs is via a repulsion mechanism involving the Eph family of receptors. Signaling through these receptors activates the repulsive mechanism that limits which tissues can interact with each other. The repulsion mechanism is not well understood, but involves the reorganization of the actin cytoskeleton and a loss of cell-cell adhesion. Activation of the EphA4 signaling pathway in *Xenopus laevis* embryos has been shown to cause the repulsion mechanism. The Rho family of GTPases consisting of Cdc42, Rho, and Rac, has been linked to the reorganization of the actin cytoskeleton and Rho has been demonstrated to be part of the EphA4 pathway. The present study tested the hypothesis that a related GTPase, Rac, is also part of EphA4 signaling. This was done by expressing mutant forms of Rac in *Xenopus laevis* embryos and assessing whether they mimicked the effects of EphA4 signaling. As the concentration of RNA coding for constitutively active Rac increased, the degree of cellular dissociation also increased indicating possible EphA4 activity. However, when embryos were injected with the dominant negative form of Rac RNA to prevent cellular dissociation caused by the receptor-ligand, EPP+TGFα, no rescue was observed.

Degree Type
Open Access Senior Honors Thesis

Department
Biology

Keywords
Cell interaction, Cell migration

DOES RAC GTPase PLAY A ROLE IN EPHA4 SIGNALING IN *XENOPUS* EMBRYOS?

By

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An Honor's Thesis presented in partial fulfillment

of the requirements for Departmental Honors in

Biology

College of Arts and Sciences

Eastern Michigan University

April 24, 2009

Abstract

In embryonic development, many cells migrate in order to correctly form new structures. One way that guidance of this migration occurs is via a repulsion mechanism involving the Eph family of receptors. Signaling through these receptors activates the repulsive mechanism that limits which tissues can interact with each other. The repulsion mechanism is not well understood, but involves the reorganization of the actin cytoskeleton and a loss of cell-cell adhesion. Activation of the EphA4 signaling pathway in *Xenopus laevis* embryos has been shown to cause the repulsion mechanism. The Rho family of GTPases consisting of Cdc42, Rho, and Rac, has been linked to the reorganization of the actin cytoskeleton and Rho has been demonstrated to be part of the EphA4 pathway. The present study tested the hypothesis that a related GTPase, Rac, is also part of EphA4 signaling. This was done by expressing mutant forms of Rac in *Xenopus laevis* embryos and assessing whether they mimicked the effects of EphA4 signaling. As the concentration of RNA coding for constitutively active Rac increased, the degree of cellular dissociation also increased indicating possible EphA4 activity. However, when embryos were injected with the dominant negative form of Rac RNA to prevent cellular dissociation caused by the receptor-ligand, EPP+TGFα, no rescue was observed.

Introduction

In the development of vertebrates, tissue formation involves careful regulation of cellular interactions. Some embryonic cells must migrate in order to correctly form new structures. Guidance of this migration occurs via a repulsion mechanism that limits

which tissues can interact with each other. One way this is done is through Eph receptors and ephrins.

The Eph family of receptors (classified as either EphA or EphB by structure) is composed of three domains: an extracellular ligand-binding domain, a transmembrane domain, and an intracellular kinase domain (Zhou, 1998). An Eph receptor can bind to a cell-surface ligand, called ephrin, stimulating kinase activity. Eph/ephrin signaling is bidirectional and regulates the actin cytoskeleton which interacts with adhesion molecules of the cell (reviewed in Tepass et. al., 2002). Eph signaling is thought to control several important processes during embryogenesis including tissue segmentation, guiding axon growth, cell migration, vasculogenesis, and potentially limb development (Holder and Klein, 1999). Eph receptors play a role in establishing neuronal connectivity by guiding axons to the appropriate target and regulating synaptic connections. It has been shown in mice that Eph systems regulate the neuronal connections of the hippocampus, an area of the brain that is important for learning and memory and therefore must be continually remodeled in response to environmental changes. Eph signaling in colorectal, breast, prostate, and skin cancer cells has shown tumor suppressor activity and yet there is also research that suggests tumor-promoting effects depending on the tumor type and context. Additionally, Eph receptors and ephrins are expressed in tumor vasculature where they promote tumor angiogenesis (reviewed in Pasquale, E.B., 2008).

Activation of the EphA4 signaling pathway, a member of the Eph family, has been shown to cause a cellular dissociation and a loss of cell-cell adhesion in *Xenopus laevis* embryos, called the "Eph" phenotype (Winning et. al., 2001). An observed loss of

adhesion was shown to be consistent with a disruption of cortical actin, which means that the reorganization of the actin cytoskeleton is an effect of Eph signaling (Bisson et. al., 2007). EphA4 has been studied in *Xenopus laevis* embryos using a chimeric form of the receptor, EPP, to avoid potential promiscuity in ligand-receptor interactions. EPP consists of an extracellular domain, Epidermal Growth Factor Receptor (EGFR) to bind the ligand, which is fused to the transmembrane and intracellular domains of EphA4 (originally called Pagliaccio) for the catalytic reactions to occur (Winning, et. al., 1996). EPP is therefore activated by the ligands of EGFR, Epidermal Growth Factor (EGF) and Transforming Growth Factor α (TGFα). Co-injecting with RNA encoding EPP and TGFα has been shown to activate the EphA4 signaling pathway (Winning, et. al., 1996).

The Rho family of GTPases, consisting of Cdc42, Rho, and Rac, has been linked to the reorganization of the actin cytoskeleton (Winning et. al., 2002). GTPases are GTP-binding proteins that regulate enzyme function by acting like molecular switches that are either active (GTP-bound) or inactive (GDP-bound) (Hens et. al., 2002). The cycle between the active and inactive form of Rho GTPase is regulated by Guanine Nucleotide Exchange Factors (GEFs) which facilitate the exchange of GDP for GTP (reviewed in Bishop and Hall, 2000). The inhibition of Rho has been shown to be a part of the activated EphA4 signaling pathway. Injecting embryos with Rho inhibitors, C3 Transferase or Toxin A, results in the "Eph" phenotype at the blastula stage (Winning et. al., 2002).

In embryonic development, high activity of Rho has been shown to coincide with low activity of Rac, and vice versa (Noren and Pasquale, 2004). This relationship has led to the hypothesis that the activation of Rac plays a role in the EphA4 signaling pathway

and will cause the "Eph" phenotype. This was tested by expressing mutant forms of Rac in *Xenopus laevis* embryos and assessing whether they mimicked the effects of EphA4 signaling. Constitutively active Rac (caRac) RNA encodes Rac that is GTP-bound. Constitutively active Rac has been generated by amino acid substitutions that prevent GTP hydrolysis. Dominant negative Rac (dnRac) RNA encodes Rac that is GDP-bound; containing an amino acid substitution that renders the protein inactive in its downstream effects but still able to compete with the endogenous GTPase for binding to GEFs. When overexpressed, dnRac is able to outcompete endogenous Rac, greatly reducing Rac activity in cells (reviewed in Bishop and Hall, 2000).

Methods

In vitro transcription of Rac

EPP and TGFα RNA was acquired from stock quantities, synthesis is described elsewhere (Winning et. al., 2002). Constructs for myc-tagged constitutively active Rac1 and dominant negative Rac were kindly provided by Dr. Tim Gomez, University of Wisconsin-Madison. It was prepared by subcloning caRac or dnRac into the EcoR1 site of the pCS2+ expression vector. The plasmids were linearized by XhoI digestion and the linear DNA was purified using a GENECLEAN® kit. RNA was transcribed *in vitro* using an Ambion mMessage mMachine SP6 transcription kit. RNA was purified by Lithium Chloride precipitation. The quantity and integrity of purified RNAs was measured by UV spectroscopy and gel electrophoresis.

Embryo Manipulation and Microinjection

Adult *Xenopus laevis* were purchased from Nasco (Fort Atkinson, WI) and Xenopus Express (Brooksville, FL). Female frogs were injected with human chorionic

gonadotropin hormone (Sigma, St. Louis, MO) 12-15 hours prior to fertilization to induce ovulation. The females were squeezed to release eggs that were then kept in a 0.3 × MMR solution (1 × MMR = 100 mM NaCl, 2.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM HEPES). The male was anesthetized with a 5% Tricaine solution and the testes were removed and stored in 1 × MMR on ice. Testes were macerated in 400 µl of 1 × MMR to release sperm, and then pipetted evenly over the eggs. The dish containing sperm and eggs was placed on an orbital shaker for 20 minutes to allow for fertilization. Embryos were de-jellied in a 2% Cysteine solution, pH 7.8-8.1. Embryos were rinsed and placed in 0.1 × MMR and kept cool (16°C) to prevent rapid mitosis. Fertilized embryos were placed in 5% Ficoll in 1 × MMR and injected at the one-cell stage using a Drummond NanoinjectII microinjector. The RNAs injected consisted of 100 pg, 250 pg, and 500 pg of RNA encoding constitutively active Rac (caRac) for the first experiment. For the rescue experiment the RNAs injected consisted of 0.5ng TGFα + EPP, 8ng of RNA encoding dominant negative Rac (dnRac), 4ng dnRac with 0.5ng TGFα + EPP, and 8ng dnRac with 0.5ng TGFα + EPP. The microinjector released 9.2 µl of solution per injection. After injection, embryos were keep at 24°C for further development. At approximately halfway between stage 1 to 6, embryos that were developing properly were transferred to a solution containing 1 × MMR without Ficoll. Embryos were allowed to develop to the blastula stage before being transferred to microtubes and fixed in 10% Glutaraldehyde in 1 × PBS overnight at 4°C.

Light and Scanning Electron Microscopy

Loss of cell adhesion and cellular dissociation can be evaluated easily during the blastula stage of embryonic development. During this stage a cavity essential to

development, called a blastocoel, forms in the animal pole of the embryo. Evaluation is done at blastula stage because endogenous EphA4 is not expressed until gastrulation, so there is no endogenous signaling to interfere with the assay. If dissociation and a loss of cell adhesion is occurring as seen with activation of EphA4 , the blastocoel may be abnormal or even nonexistent. In preparation for bisection and evaluation, embryos were washed 5 times for 10 minutes each with 1 × PBS. Specimens were cut into halves with a scalpel blade and examined under a dissecting scope. The embryos were graded as intact, abnormal, or nonexistent based on the morphology of the blastocoel.

After bisection and evaluation, the embryos were fixed with 10% Glutaraldehyde in 1 × PBS overnight at 4°C. In preparation for viewing under a scanning electron microscope, embryos were washed twice in 1 × PBS for 10 minutes each and postfixed in 1% osmium tetroxide for 30 minutes. Embryos were washed twice for 10 minutes in distilled water. Embryos were then taken through the following dehydration steps in order: 10 minutes in 50% ethanol, 10 minutes in 70% ethanol, 10 minutes in 95% ethanol, and 2 times for 5 minutes in hexamethyldisilazane (HMDS). After the final wash, the remaining HMDS was removed and the specimens were left overnight to allow for evaporation and further drying. Specimens were then placed under a vacuum for 2 hours before being mounted using hair tweezers onto double-stick, carbon-permeated tape on polished stubs. Colloidal graphite was used to enhance adhesion of the specimens to the stubs. Specimens were sputter coated with gold and viewed on an AMRay 1820I scanning electron microscope.

Results

The purpose of the experiments is to determine if activation of Rac is part of EphA4 signaling. By expressing mutant forms of Rac, either constitutively active (GTP-bound) or dominant negative (GDP-bound) in *Xenopus laevis* embryos, it is possible to assess whether they mimicked the effects of EphA4 signaling. If the hypothesis that Rac activation is involved in EphA4 signaling is correct, we would expect to see the "Eph" phenotype when embryos are injected with caRac. An opposite effect would be expected for embryos injected with dnRac, including proper cellular adhesion and an intact blastocoel at blastula stage. Furthermore, after activating EphA4 we would expect dnRac to rescue embryos from the "Eph" phenotype by maintaining cell adhesion.

The embryos injected with RNA coding for caRac exhibited external lesions on the pigmented animal pole. This experiment was repeated 3 times; a representative experiment is presented by figure 1 and 2. The broken pigmentation and lesions increased in severity as the concentration of injected caRac RNA increased from 100 pg to 500 pg (Fig. 1).

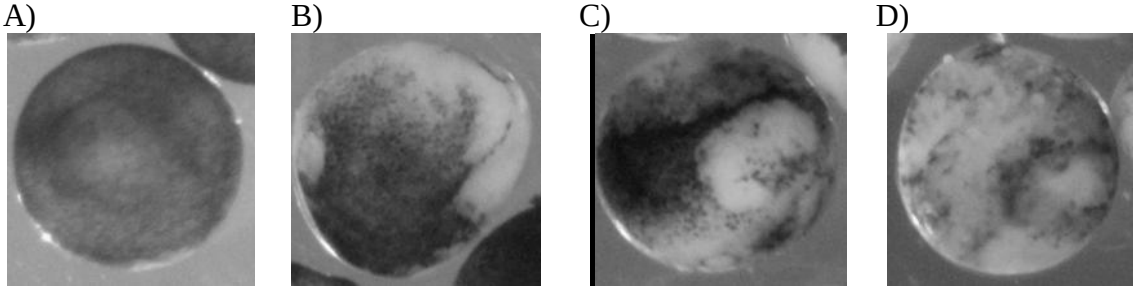
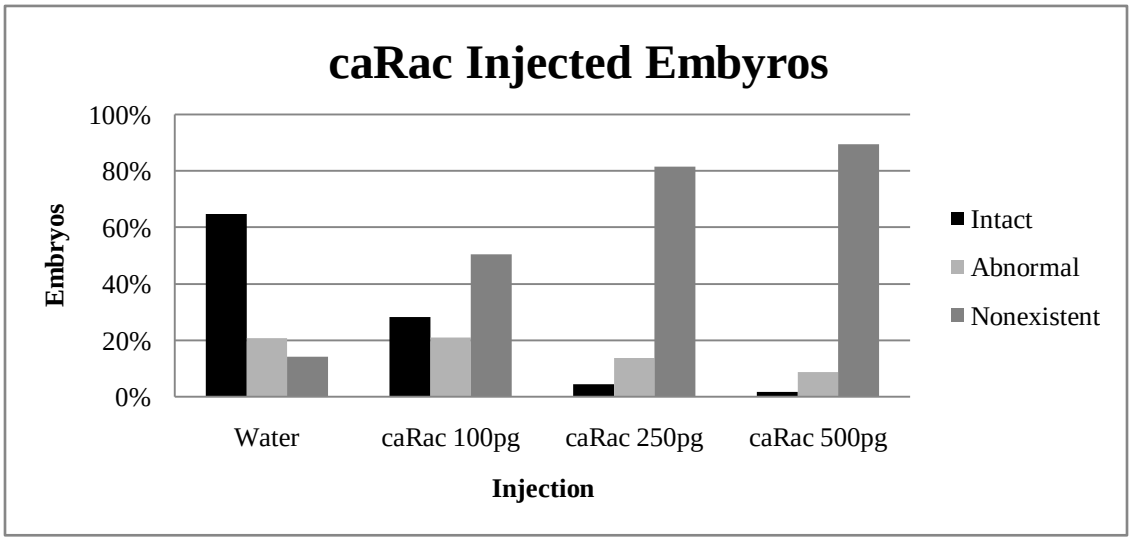


Figure 1. An external view of the animal pole using light microscopy. **A.** Control sample: embryo injected with water. **B.** Embryo injected with 100 pg caRac, exhibiting minor lesions. **C.** Embryo injected with 250 pg caRac. **D.** Embryo injected with 500 pg caRac, exhibiting severe lesions and broken pigmentation.

The control sample of water-injected embryos had no disruption of external pigment and had intact blastocoels (Fig. 1A, 2). The caRac injected embryos showed that as the concentration of RNA coding for caRac increased from 100 pg, to 250 pg, to 500 pg, the number of embryos with an intact blastocoel decreased, while the number of embryos with an abnormal or nonexistent blastocoel increased (Fig. 2A,B). The roof of the blastocoel was assessed to determine the extent of cellular adhesion and dissociation (Fig. 2C). The roof of the blastocoel of water injected embryos was composed of angular cells that fit tightly together. In contrast, the roof of the blastocoel in caRac injected embryos exhibited rounded cells (Fig. 2C).

A)



B)

	Quality of Blastocoel		
Sample	**Intact**	**Abnormal**	**Nonexistent**
Water	64.8% (59)	20.9% (19)	14.3% (13)
100 pg caRac	28.4% (23)	21.0% (17)	50.6% (41)
250 pg caRac	4.6% (3)	13.8% (9)	81.5% (53)
500 pg caRac	1.8% (1)	8.8% (5)	89.5% (51)

C)

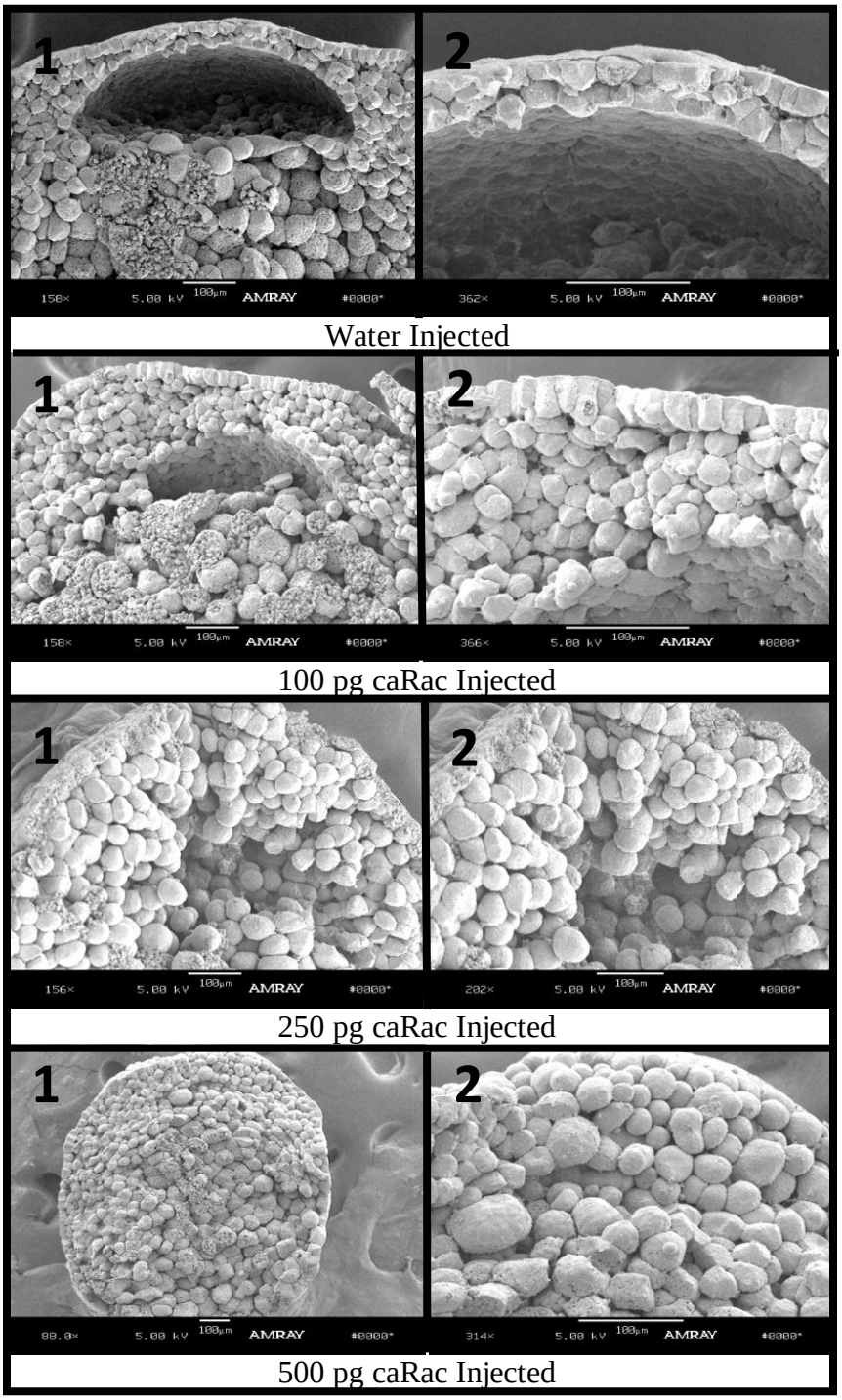


Figure 2. Various representations of data gathered from the internal morphology of injected embryos. **A.** Graph displays the percentage of embryos vs. the graded quality of the embryos for each injection sample. **B.** Table presents the percentage and the quantity

(in parenthesis) of embryos from each injection sample. **C.** Scanning electron microscopy. 1) Image of the bisected internal animal pole. 2) Image of cells making up the roof of the blastocoel.

Following the caRac experiment, a "rescue" experiment was conducted to investigate the effects of dnRac. Embryos were injected with water and 0.5 ng EPP + TGFα to serve as the experimental controls. Additionally, embryos were injected with 8ng of dnRac to observe dnRac alone, as well as two samples of 4 ng and 8 ng of dnRac with EPP + TGFα to study the possible "rescue" effect of dnRac. This experiment was repeated 2 times; a representative experiment is presented by figure 3 and 4.

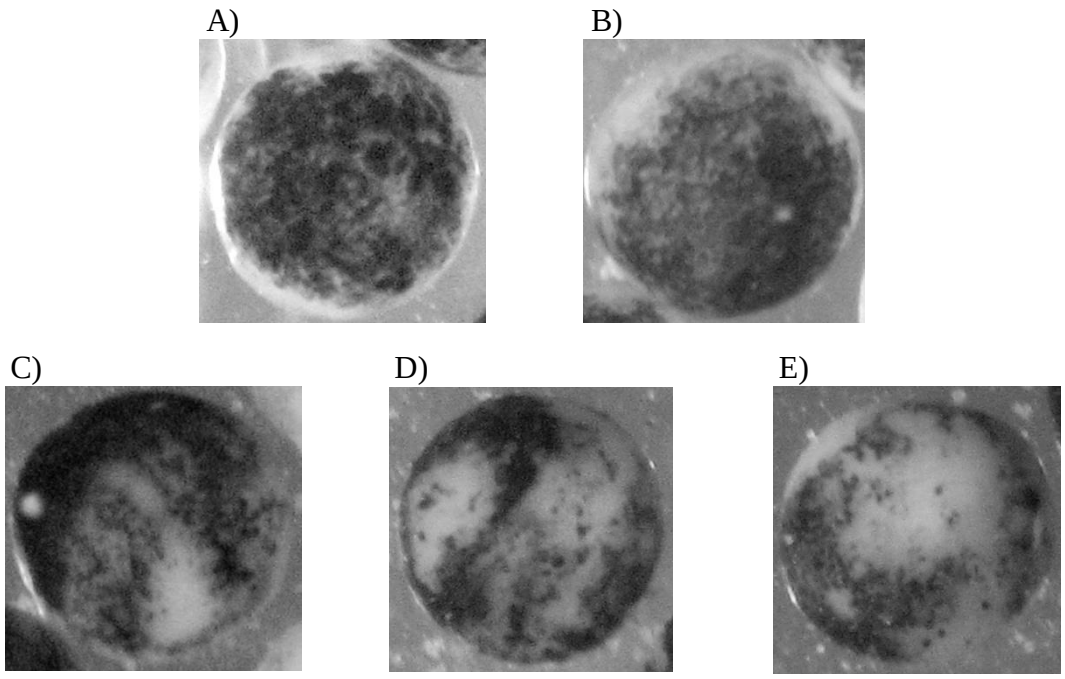
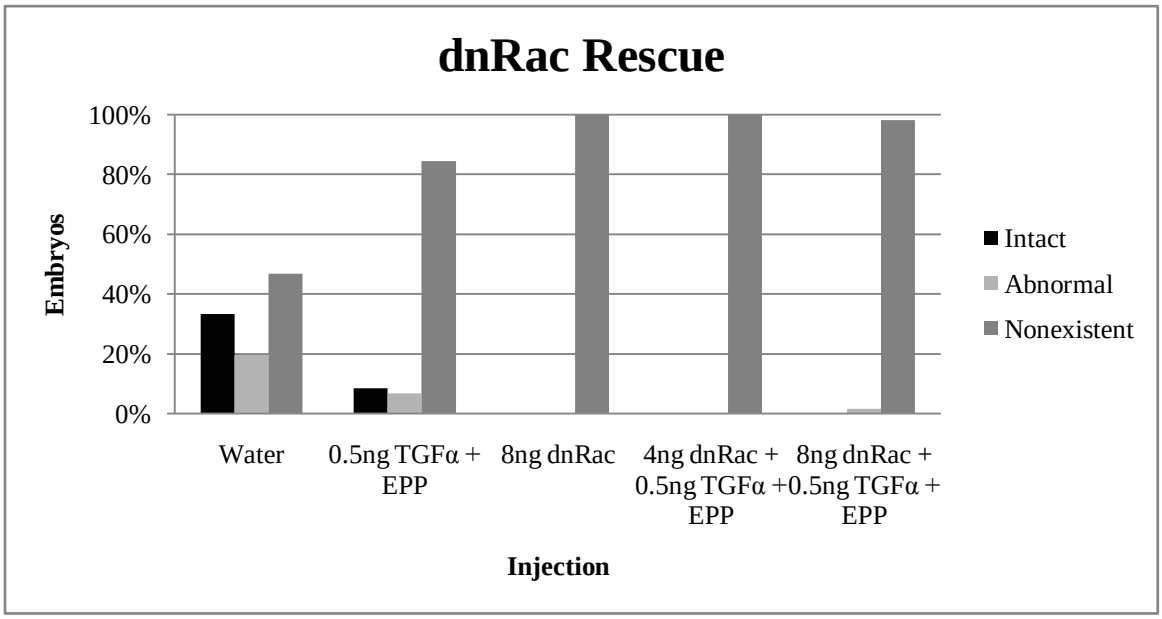


Figure 3. An external view of the animal pole using light microscopy. **A.** Control sample: embryo injected with water. **B.** Control sample: embryo injected with 0.5 ng TGFα + EPP. **C.** Embryo injected with 8ng dnRac, exhibiting minor lesions. **D.** Embryo injected with 4 ng dnRac + 0.5 ng TGFα + EPP. **E.** Embryo injected with 8 ng dnRac + 0.5 ng TGFα + EPP, exhibiting severe lesions and broken pigmentation.

Many embryos of the water-injected control sample had no disruption of external pigmentation and had intact blastocoels (Fig. 3A, 4). The 0.5 ng TGFα + EPP injection

sample did not show external lesions but did show some degree of cellular dissociation as 84.5% had no blastocoel (Fig. 3B, 4). The embryos injected with RNA coding for dnRac (8 ng dnRac, 4 ng dnRac + 0.5 ng TGFα + EPP, and 8 ng dnRac + 0.5 ng TGFα + EPP) all exhibited external lesions and broken pigmentation of the animal pole (Fig. 3C,D,E). Additionally, the quality of the blastocoel was nonexistent in 98-100% of the dnRac injected embryos (Fig. 4A,B).

A)



B)

	Quality of Blastocoel		
Sample	**Intact**	**Abnormal**	**Nonexistent**
Water	33.3% (22)	19.7% (13)	47.0% (31)
0.5 ng TGFα + EPP	8.6% (5)	6.9% (4)	84.5% (49)
8 ng dnRac	0.0% (0)	0.0% (0)	100.0% (55)
4 ng dnRac + 0.5 ng TGFα+EPP	0.0% (0)	0.0% (0)	100.0% (48)
8 ng dnRac + 0.5 ng TGFα+EPP	0.0% (0)	1.7% (1)	98.3% (57)

C)

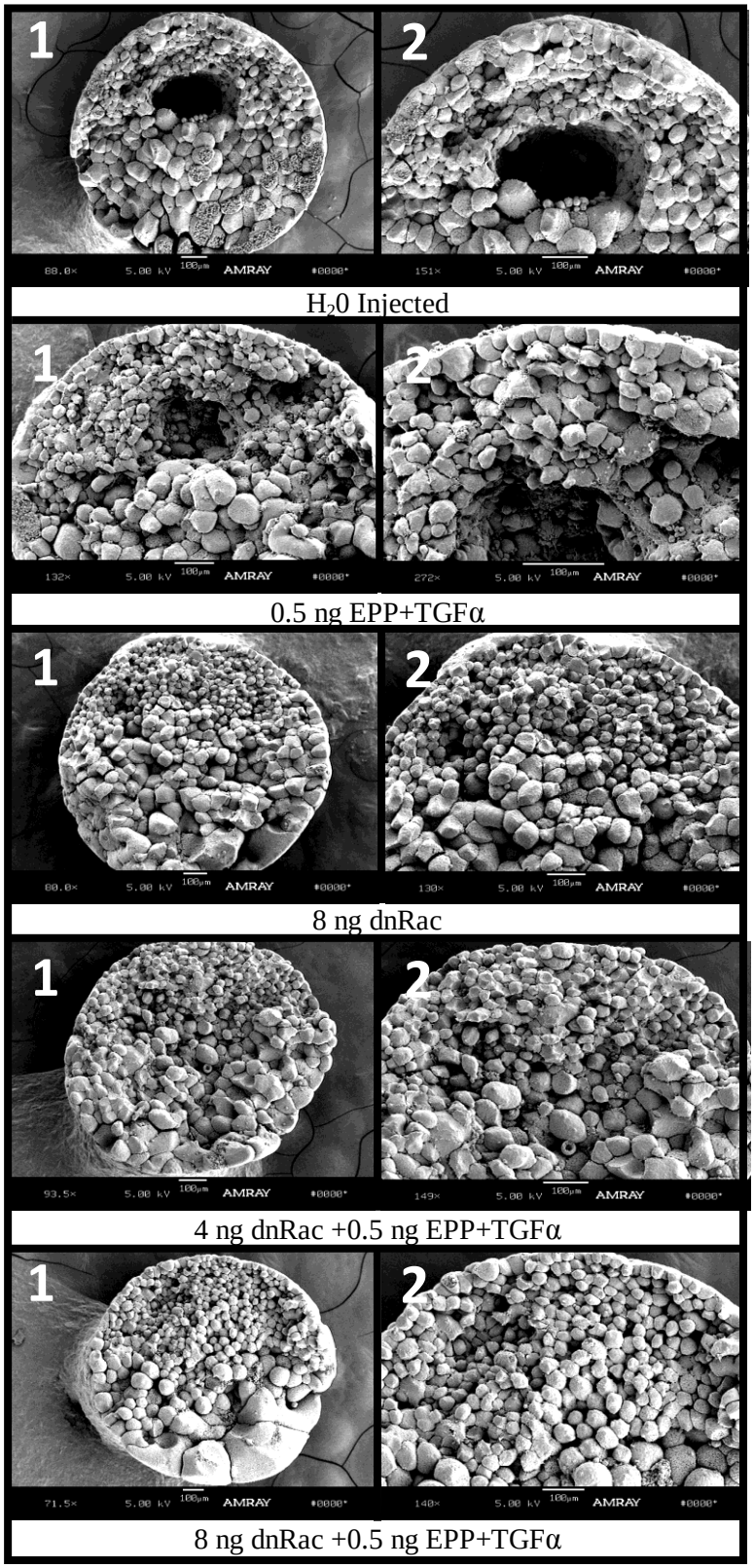

1
2
88.0× 5.00 kV 100µm AMRAY ♦0000♦
151× 5.00 kV 100µm AMRAY ♦0000♦
H20 Injected
1
2
132× 5.00 kV 100µm AMRAY ♦0000♦
272× 5.00 kV 100µm AMRAY ♦0000♦
0.5 ng EPP+TGFα
1
2
88.0× 5.00 kV 100µm AMRAY ♦0000♦
130× 5.00 kV 100µm AMRAY ♦0000♦
8 ng dnRac
1
2
93.5× 5.00 kV 100µm AMRAY ♦0000♦
149× 5.00 kV 100µm AMRAY ♦0000♦
4 ng dnRac +0.5 ng EPP+TGFα
1
2
71.5× 5.00 kV 100µm AMRAY ♦0000♦
140× 5.00 kV 100µm AMRAY ♦0000♦
8 ng dnRac +0.5 ng EPP+TGFα

Figure 4. Various representations of data gathered from the internal morphology of injected embryos. **A.** Graph displays the percentage of embryos vs. the graded quality of the embryos for each injection sample. **B.** Table presents the percentage and the quantity (in parenthesis) of embryos from each injection sample. **C.** Scanning electron microscopy. 1) Image of the bisected internal animal pole. 2) Image of cells making up the roof of the blastocoel.

Discussion

It is possible that Rac activation is part of the EphA4 signaling pathway. Increasing concentrations of caRac caused an increasing loss of cell adhesion indicated by the lesions and broken pigmentation apparent on the external animal pole of the embryos. Loss of cell adhesion is associated with the reorganization of the actin cytoskeleton exhibited by the activation of the EphA4 signaling pathway. Following the caRac experiment, the EphA4 pathway was activated with EPP + TGFα and the embryos were injected with the dnRac in an attempt to rescue from the "Eph" phenotype. dnRac was unable to rescue the embryos, in fact it caused severe abnormalities in the 8 ng dnRac, 4 ng dnRac + 0.5ng TGFα + EPP, and 8 ng dnRac + 0.5 ng TGFα + EPP samples. dnRac is having an unknown effect on development. It is possible that a careful balance of Rac activity is required for proper development. The EphA4 pathway may not be straight forward but rather complex with multiple branches. Future experimentation may include co-injections with RNAs encoding mutant versions of other proteins involved in the EphA4 pathway, such as ROCK and Rho, to establish the order of signaling within the pathway and further investigate the role Rac plays.

A study by Hens, et al. in 2002, agreed that Rac signaling is important for cell adhesion. They suggested that both inactive and active Rac in *Xenopus* embryos caused some degree of cellular dissociation. Inactive Rac injected cells were far less adherent to

one another than active Rac injected cells. The similar results obtained from the expression of both the Rac mutants were explained as a possible Rac activity gradient that is required for proper cellular effects to occur.

The study of Eph receptors has seen a number of advances when it comes to understanding the role of GTPases in guiding cell migration and regulating cell shape. However, much is still needed to fully comprehend the details of Eph/ephrin signaling pathways and morphogenesis.

Acknowledgements

I would like to thank to Dr. Robert S. Winning for his mentorship and laboratory guidance. Additionally, I would like to thank Dr. Glenn K. Walker for his support and his assistance with electron microscopy along with Rae Labadie.

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