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Disruption of Ephrin-A5 in Developing *Xenopus Laevis* Embryos by ShRNA

Sarah C. Engmark
Eastern Michigan University, sengmark@emich.edu

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First Advisor
Robert S. Winning, PhD

Second Advisor
James L. Vanden Bosch, PhD

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DISRUPTION OF EPHRIN-A5 IN DEVELOPING *XENOPUS LAEVIS* EMBRYOS BY SHRNA

By

Sarah Engmark

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Abstract (174)

Eph receptors are the largest class of receptor tyrosine kinases (RTKs) that participate in bidirectional cell signaling with their cell surface ephrin ligands during the development of vertebrate embryos. Ephrin-A5 is one ligand that interacts with the EphA4 receptor, but is present much earlier than EphA4 in developing *Xenopus laevis* embryos. This raises the question why ephrin-A5 is expressed so early and suggests that it may have a role in embryonic development and tissue differentiation independent of EphA4. This project attempted to study ephrin-A5's function using a short hairpin RNA (shRNA) knockdown approach. A vector encoding shRNA of the ephrin-A5 mRNA under the control of a strong eukaryotic promoter was injected into early *Xenopus laevis* embryos in the attempt to knock down ephrin-A5 expression and determine its function. A protein gel visualized the presence of viable proteins and Western Blots visualized the knockdown attempts. The shRNA vector used (pCAX+571) was determined not to have knocked down the expression of ephrin-A5; thus ephrin-A5's function in embryonic development has yet to be determined.

Introduction

Cells rely on signaling pathways for many different functions during all stages of life. One of the most significant is the Eph-ephrin signaling pathway. The cell-bound ephrin ligands activate the largest family of receptor tyrosine kinases (RTKs), the Eph receptors (Sikkema et al., 2012; Matsuo and Otaki, 2012). Both Ephs and ephrins are divided into an A and B subclasses. Eph-A receptors generally interact with ephrin-A ligands and Eph-B receptors generally interact with ephrin-B ligands. However, there are exceptions to this interaction pattern, such as Eph-A4, which can bind to ephrin-B2, ephrin-B3, and ephrin-As; and ephrin-A5, which binds to Eph-A's and Eph-B2 (Matsuo and Otaki, 2012; Pasquale, 2004).

Eprhins are cell-anchored signals that transduce and receive signals to and from corresponding Eph receptors through cell-cell contact, called bidirectional signaling (Cowan and Henkemeyer, 2002; Pasquale, 2004; Winning and Krull, 2011). A-subclass ephrin ligands attach to the outer leaflet of the plasma membrane by glycosylphosphatidylinositol (GPI) linkage and transduce a signal by binding Eph-A receptors (Cowan and Henkemeyer, 2002; Fu et al., 2012). B-subclass ephrin ligands have both transmembrane and cytoplasmic domains (Fu et al., 2012). They use conserved tyrosines in their cytoplasmic tails, which are phosphorylated upon contact with Eph-B receptors to transduce a signal to their corresponding Eph receptors. However, Eph receptors transduce signals by activating their intracellular catalytic tyrosine kinase domain, causing a RTK cascade (Cowan and Henkemeyer, 2002). The signal transduced after cell-cell contact between Eph and ephrin

expressing cells has been shown to cause repulsion between the cells (Winning and Krull, 2011).

The Eph-ephrin bidirectional signaling and repulsion characteristics have been shown to dramatically impact various diseases and developmental processes. Both forward and reverse signaling have been linked to various malignant cancers (Sikkema et al. 2012). The presence of Eph receptors and ephrins have been found to be upregulated in cancer cells and their signaling has been shown to enhance cell migration and proliferation throughout the body, which enhances cancer-promoting processes (Pasquale, 2008; Sikkema et al., 2012). Intriguingly, Eph-ephrin signaling has also been linked to tumor-suppressing effects (Pasquale, 2008; Sikkema et al., 2012). In the various cancers, Ephs and ephrins have been found to play different roles. In breast cancer, for example, Eph-A2 and Eph-B4 are the most studied receptors. Though there is still much that is not understood, it has been shown that both receptors are extensively expressed; however, they do not seem to need ephrin interaction to induce activation. In fact, the overexpression of Eph-A2 has been shown to induce oncogenic transformation in human breast cancer (Pasquale, 2008).

Interestingly, Eph-ephrin signaling has a significant role in embryonic development. Previous studies have found that Eph signaling is essential in neural development, mediating cytoskeleton dynamics, guided migrations, and angiogenesis. Ephrin signaling has also been linked to neural development, in addition to axon guidance (Sikkema et al., 2012). Eph-ephrin signaling has also been shown to induce cell migration, boundary formation, cell position and shape by way of repulsion (Fu et al., 2012). Furthermore, Eph-ephrin signaling

is essential in human placentation and has been shown to aid in attachment between blastocysts and the endometrium in swine (Fu et al., 2012).

One important formation during embryonic development is the formation of somites. Somites are derived from one of the three germ layers, the mesoderm, which will eventually go on to form vertebrae, ribs, muscle, connective tissues, and the sclerotome. During this formation, interaction between Eph-A4 and ephrins is essential in the somites' boundary formation. Furthermore, it has been shown, through a knockdown, that in chickens only Eph-A4 is necessary for somatic boundary formation (Matsuo and Otaki, 2012). Although, in this case, only Eph-A4 was necessary to allow somite formation, Eph-ephrin signaling plays an important role in boundary formations (Fu et al., 2012).

Even though new information about Eph-ephrin signaling is continually being elucidated, within the ephrin ligands ephrin-A5 remains a mystery, with some exceptions. One exception is that it has been shown to be essential in retinocollicular mapping (Feldheim et al., 2000). However, the specific functions ephrin-A5 plays in embryonic development have yet to be determined. From a previous study we know that Eph-A4, a receptor that interacts with ephrin-A5, is first present in *Xenopus laevis* at the gastrula stage (stages 10-12) (Winning and Sargent, 1994). However, previous unpublished research from the Winning lab found that ephrin-A5 is present at the zygote stage (stage 1; Ali and Winning, unpublished results). This finding raises the question, why is the signal present before the receptor? The fact that ephrin-A5 is present at such an early stage suggests that ephrin-A5 plays a significant role in embryonic development. The purpose of this study is to test this hypothesis

using shRNA to knockdown the expression of ephrin-A5 in *Xenopus laevis*, thus allowing the determination of ephrin-A5's function.

Materials and Methods

Bacterial Transformation

Escherichia coli DH5α transformation with the pCAX plasmids encoding ephrin-A5 shRNA beginning at the 571 position (pCAX + 571) and 571 M shRNA (the mutated version of 571 shRNA, pCAX + 571M) was accomplished by means of One Shot Chemically Competent DH5α (Invitrogen), according to the manufacturer's instructions. The DH5α solution was incubated on ice for 30 minutes, then heat-shocked at 42^{O} C for 30 seconds. One milliliter of S.O.C. medium was added and the culture was incubated at 37^{O} C for one hour. One hundred microliters was spread on a LB plate with Ampicillin (50μg/mL) and incubated at 37^{O} C overnight.

Plasmid DNA Isolation

DH5α with the pCAX plasmids were inoculated into 10 mL of super broth containing 100 μg/ml ampicillin and incubated at 37°C overnight prior to plasmid purification using the Wizard *Plus* Midipreps DNA Purification System. Ten milliliters of the bacterial culture was pelleted in a fixed rotor centrifuge for 10 minutes at 10,000 x g at 4^{O} C. Pellets were suspended in 6.0 mL of Cell Resuspension Solution and 6.0 mL of Cell Lysis Solution was added. The tubes were mixed by inversion and incubated at room temperature for 5 minutes. Six milliliters of Neutralization Solution was added and the tubes were inverted. The tubes were centrifuged in a fixed rotor for 15 minutes at 14,000 x g at 4^{O} C. Ten milliliters of resin

was added to the DNA supernatant and placed in the Midicolumn/syringe assembly. Columns were placed on a vacuum until all liquid passed through, then 15 mL of Column Wash Solution was added and left on the vacuum until all liquid had passed. The wash was repeated and left on the vacuum to dry for 30 seconds. The columns were centrifuged for 2 minutes at 10,000 x g. The plasmid DNA was eluted from the column with 300 μL of nuclease free water. The water was left to sit for 1 minute and then the columns were centrifuged in a fixed rotor for 5 minutes at 10,000 x g. The eluate was transferred to new tubes and the plasmid DNA was stored at -20^{O} C.

Microinjections

Xenopus laevis females were injected with 600-800 units of chorionic gonadotropin 12-15 hours before fertilization to stimulate ovulation. Eggs were collected and placed in 0.3X Mark's Modified Ringers (MMR) solution. The male was anaesthetized with 0.5% (w/v) tricaine 3-aminobenzoic acid in water and the testes were extracted and placed in 1X MMR solution. Half of a testis was homogenized and used to fertilize the eggs. Embryos were pipetted in 1% (w/v) cysteine solution with a pH between 7.8-8.1 for 5 minutes and then rinsed with water and placed in 0.1X MMR + 5% Ficoll solution. Three batches of 50-75 embryos were injected in a 16.5^{O} C temperature-controlled room with water (control), pCAX + 571, and pCAX + 571M (control). The first set of microinjections used a plasmid concentration of 50 pg/nl and the second set of injections had 100 pg/nl. Each embryo was injected with 10 nl of plasmid, so for the first set of injections, the total DNA dose was 500 pg per embryo, and for the second set of injections the total DNA dose was 1 ng per embryo. All microinjections occurred before the first cellular cleavage. Microinjected embryos were

stored at room temperature in 0.1X MMR + 5% Ficoll solution. Samples of 12-15 embryos were taken 1 hour, 24 hours, and 48 hours after microinjection and stored at -80^{O} C.

Protein Extraction

Protein extracts were prepared to give a final concentration of 2 embryo equivalents of protein per 10 µl of sample. Embryos were homogenized on ice in homogenization buffer with a volume of 2/3 of the calculated final sample volume, based on the number of embryos per sample. The homogenization buffer was comprised of 50 mM HEPES, 150mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% NP-40, at a pH of 7.5. Samples were extracted with an equal volume of Freon, then centrifuged in a fixed-angle Eppendorf Centrifuge 5415 C rotor at 14,000 rpm for 15 minutes at 4^{0} C. Supernatants were transferred to fresh tubes, to which 0.5 volume of 3X SDS sample buffer (NEB) and 1/29 volume 1.25 M DTT (New England *BioLabs*) were added. Samples were boiled at 100°C for 5 minutes and stored at -20^{0} C.

Protein Assay

Protein concentrations were obtained through a BCA protein assay. A master mix of reagents A and B, from the Pierce BCA Protein Assay Reagent kit, consisted of 98.0% reagent A and 2.0% reagent B. The samples of homogenized embryonic samples were added to the aliquots of master mix in a 1:20 concentration. The samples were then read on a Nano-drop using BCA Protein protocol.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were electrophoresed through a 10% separating gel composed of 0.38M TRIS.HCl pH 8.8, 10% (w/v) acrylamide/bis, 0.1% SDS, 0.05% ammonium persulfate (APS), and 6.67 M TEMED; and a 4% stacking gel composed of 0.05 M TRIS.HCl pH 6.8, 4% (v/v) acrylamide/bis, 0.1% SDS, 0.05% APS, and 2.67 M TEMED. The electrophoresis was conducted at 200V for 30 minutes in a Bio-Rad Mini Trans-blot electrophoresis unit and the gel was stained for 30 minutes with Coomassie blue R-250 (0.1% Coomassie blue R-250, 40% methanol, 10% acetic acid). The gel soaked overnight in destain consisting of 10% methanol and 10% acetic acid. The gel was visualized with a Coomassie blue protocol on the *BioRad ChemiDoc XRS+ gel documentation system running Image Lab Software*.

Western Blot

Proteins were electrophoresed on a 12% separating gel composed of 0.375 M TRIS.HCl pH 8.8, 12% (w/v) acrylamide/bis, 0.1% SDS, 0.05% ammonium persulfate (APS), and 6.67 M TEMED; and a 4% stacking gel, see *Protein Gel.* The electrophoresis was at 200V for 30 minutes and the resultant gel soaked in transfer buffer for 30 minutes. The transfer buffer was at a pH of 8.3 and consisted of 25mM TRIS, 192 mM glycine, and 20% (v/v) methanol. The nitrocellulose was soaked in transfer buffer for 15 minutes prior to trans-blotting. Trans-blotting was performed at 150mA for 1 hour at 4^0 C. After transfer, the nitrocellulose was incubated overnight in a blocking solution, 5% (w/v) bovine serum albumin (BSA) in TRIS buffered saline (20mM TRIS and 500mM NaCl at a pH of 7.5) with 0.1% (v/v) Tween 20 (1X TBST). The nitrocellulose was placed in ephrin-A5 primary

antibody (Novus Biologicals) diluted 1:500 in TBST and left to incubate overnight at 4°C. The filter was washed on a shaker table with TBST six times for 5 minutes each. It was then incubated overnight at 4 °C in a 1:2000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP; sc-2004, Santa Cruz Biotechnology, INC.). Nitrocellulose was washed on a shaker table in TBST six times for 5 minutes each and then placed in a 1:1 Clarity™ Western ECL Substrate for 5 minutes. Since the ladder was removed during the TBST washes, alignment marks were made on the nitrocellulose and the nitrocellulose was imaged prior to antibody probing. Imaging was accomplished with a BioRad ChemiDoc XRS+ gel documentation system running Image Lab Software and using white light to form an image of the protein markers. The image could then be measured and used to determine molecular weights of the proteins on the probed nitrocellulose. Antibody-bound protein bands were visualized with a chemiluminol imaging system.

Nitrocellulose Antibody Stripping

To remove bound antibodies for reprobing of the nitrocellulose, it was placed in a 0.1 M glycine (pH 2.5) solution for 30 minutes at room temperature. The nitrocellulose was then ready to proceed with a new Western blot, starting at the blocking solution.

Protein Concentrations by Reprobing

Nitrocellulose was stripped of its original antibodies and reprobed with antibodies against housekeeping proteins to ensure even loading of the gel. The filter from the first experiment, with 50 pg/nL of pCAX+571 and pCAX+571M injected samples, was reprobed with purified mouse anti-p190 monoclonal antibody (diluted 1:1000) and anti-Mouse IgG

(H+L) secondary antibody. The second blot, with 100 pg/nL of pCAX+571 and pCAX+571M injected samples, was reprobed with a 1:1000 dilution of JLA20 anti-actin primary antibody (Invitrogen) and 1:2000 sc-2064 Goat anti-mouse IgM-HRP secondary antibody.

Results

In order to assess the quality of protein extracts from the embryonic samples, SDS-PAGE was conducted (Figure 1). The figures' labels can be read as the time-sample, thus 1 corresponds to 1 hour after injection, 24 corresponds to 24 hours after injection, and 48 corresponds to 48 hours after injections. The H_2O corresponds to the water control injection sample, the 571 corresponds to the pCAX+571 variable injection sample, and the 571M corresponds to the pCAX+571M control injection sample. The SDS-PAGE showed the protein samples were intact and thus in good condition to move forward with the Western blot. The protein samples did appear to decrease in concentration as the embryos matured, which can be attributed to episodes of cell death within the sample. Although the embryos appeared to be in good condition when sampled for protein extractions, as development progresses it is inevitable there will be cell death, which could lead to sampling dying embryos.

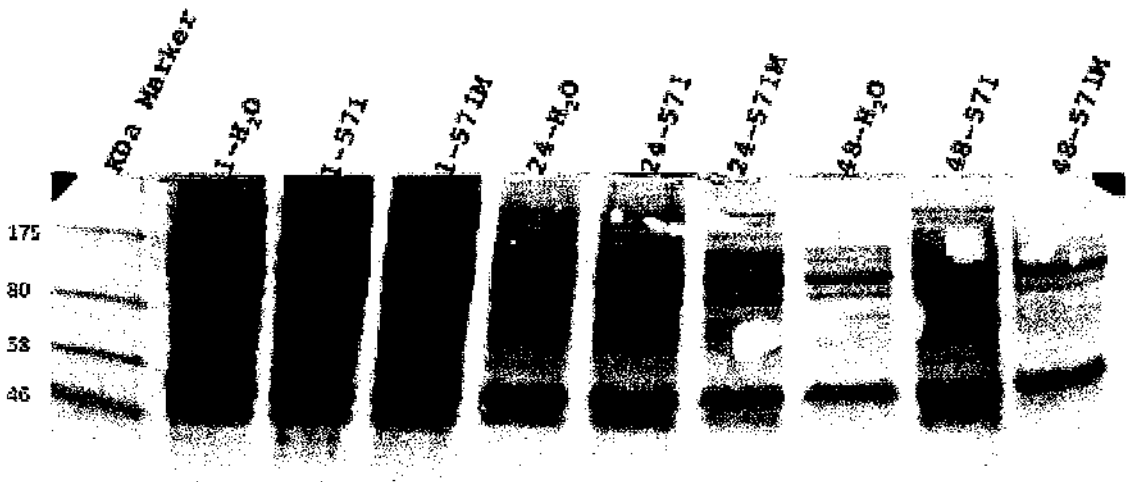

Figure 1. *SDS-PAGE of proteins extracted from embryos injected with 500 pg of either pCAX+571 or pCAX+571M. The lanes, from left to right, are samples taken 1 hour, 24 hours, and 48 hours after injections from the water, pCAX+571, and pCAX+571M samples. The gel was visualized with a BioRad ChemiDoc XRS+ gel documentation system running Image Lab Software, using the Coomassie blue protocol. The gel shows that the protein samples followed the expected pattern for an in vivo extraction. The proteins decreased in intensity with time due to cellular death and expanded the range of normal in vivo proteins This allowed for the conclusion that the method of protein extraction was successful and yielded intact proteins to continue to use. The samples appear to be less concentrated as the embryos develop, which is most likely due to the embryonic samples containing embryos with cell death.*

A Western blot, using the samples from the embryos injected with 500 pg of plasmid, was performed to visualize any knockdown of ephrin-A5 expression the pCAX+571 plasmid may have induced. The proteins were detected with an anti-ephrin-A5 antibody. Figure 2 shows this Western blot at a two-minute exposure and suggests that no knockdown of ephrin-A5 expression occurred. The band that represents ephrin-A5, at 21 kDa, appears stronger in each of the pCAX+571 samples compared to the controls in the same sample times. The

nitrocellulose also includes bands representing proteins larger than ephrin-A5, which were unidentifiable, but can be assumed to be proteins that cross-reacted with the antibodies used (Figures 2 and 4).

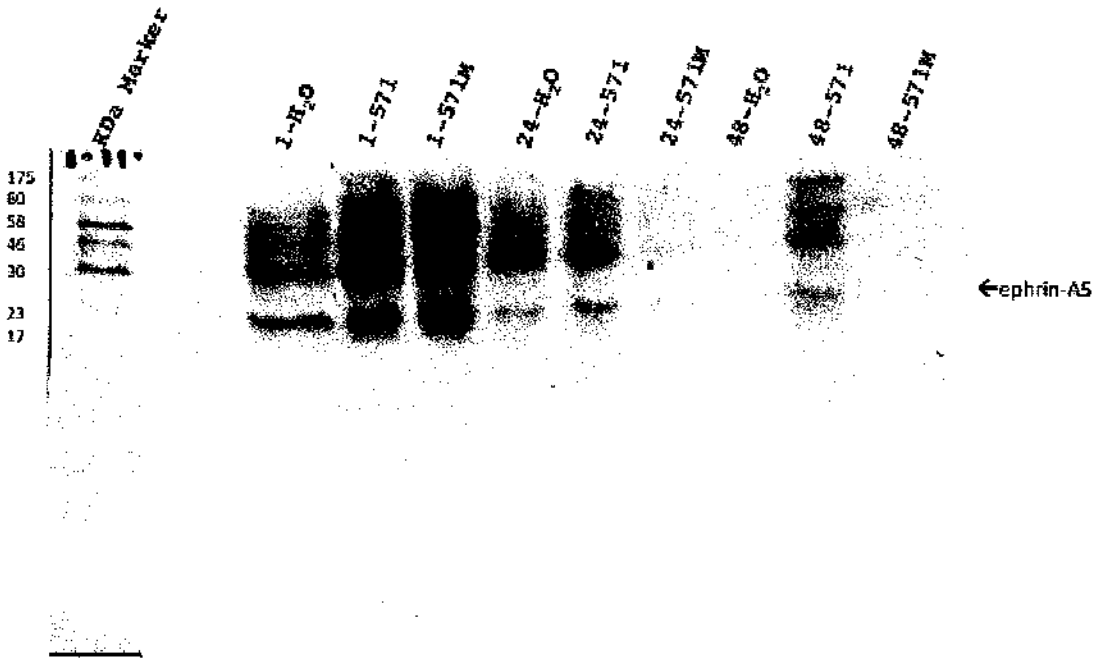

Figure 2. *Western blot of proteins extracted from embryos injected with 500 pg of either pCAX+571 or pCAX+571M. The lanes, from left to right, are samples taken 1 hour, 24 hours, and 48 hours after injections from the water, pCAX+571, and pCAX+571M samples. The protein marker was added separately as a picture taken of the nitrocellulose with white light because the ladder disappeared during the washing process. Antibody-bound protein bands were visualized using a BioRad ChemiDoc XRS+ gel documentation system running Image Lab Software, using the chemiluminescence protocol. The bands larger than ephrin-A5 can be attributed to proteins with similar epitopes to ephrin-A5 binding to the ephrin-A5 antibody and proteins that may interact solely with the secondary antibody. The nitrocellulose seems to show that 500 pg of pCAX+571 was unable to knockdown ephrin-A5 expression.*

To determine the relative protein concentrations in each sample, the original Western blot, shown in Figure 2, was stripped of its antibody and reprobed with anti-p190, which should be present at all embryonic stages in equal amounts between 185kDa and 210kDa. The newly blotted nitrocellulose (Figure 3) shows that among the samples taken 1 hour after

injections, the pCAX+571 and pCAX+571M samples have relatively equal amounts of protein. However, the water-injected sample had significantly less protein. Among the samples taken 24 hours after injections all three samples seem to have relatively the same amount of protein. Among the samples taken 48 hours after injections the water and pCAX+571M samples seem to have relatively the same amount of protein. However, they both have significantly less protein than the pCAX+571 protein. Unfortunately protein concentrations were unable to be obtained for these specific samples, and thus we have to rely on the reprobed nitrocellulose as a means of relative concentrations. However, prior to performing the next Western blot, and to decrease confusion, a protein assay will be used to determine protein concentrations in each protein sample. Based on this information, it still appears that 500 pg of the pCAX+571 was not enough to knock down the expression of ephrin-A5.

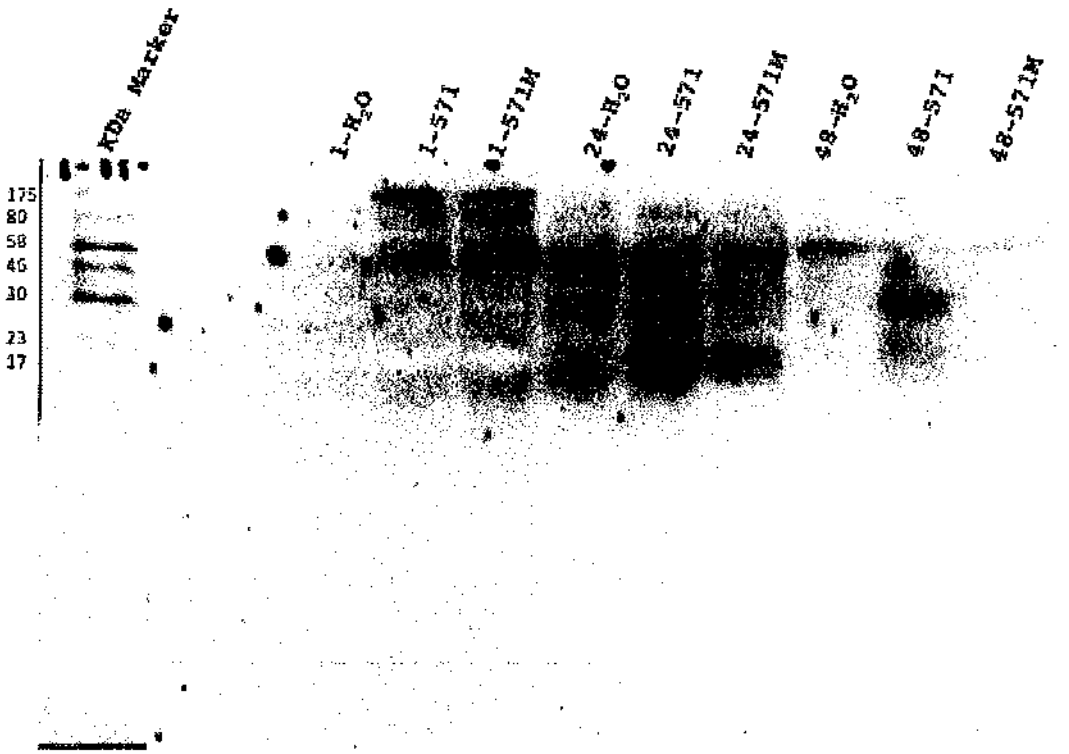

Figure 3. *Western blot reprobed with p190 antibodies from embryos injected with 500 pg of either pCAX+571 or pCAX+571M. The lanes, from left to right, are samples taken 1 hour, 24 hours, and 48 hours after injections from the water, pCAX+571, and pCAX+571M samples. The protein marker was added separately as a picture taken of the nitrocellulose with white light because the ladder disappeared during the washing process. Antibody-bound protein bands were visualized using a BioRad ChemiDoc XRS+ gel documentation system running Image Lab Software, using the chemiluminescence protocol. The nitrocellulose confirms the protein concentrations were not equal.*

To determine if a higher dose of pCAX+571 was capable of knocking down the expression of ephrin-A5, a new set of embryos was injected with 1 ng of either pCAX+571 or pCAX+571M. A protein assay was performed to determine the protein concentration in each of the protein samples taken from the embryos, which was used for determining how much sample to load into each well in the Western blot (See Table 1).

Table 1. *Protein assay of embryonic samples injected with 1 ng of pCAX+571, pCAX+571M, or water.*

H_2O	0.298	0.250	0.241	0.031	0.263
571	0.294	0.291	0.277	0.009	0.287
571M	0.270	0.249	0.305	0.028	0.275
H_2O	0.360	0.481	0.190	0.146	0.344
571	0.279	0.248	0.289	0.021	0.272
571M	0.246	0.244	0.344	0.057	0.278
H_2O	0.193	0.388	0.416	0.121	0.332
571	0.375	0.285	0.301	0.048	0.320
571M	0.222	0.217	0.333	0.066	0.257

The volume of each protein sample loaded into the SDS-PAGE for the Western blot of embryonic samples injected with 1 ng of plasmid can be seen in Table 2.

Table 2. *Volume of protein sample loaded into each of the wells in the Western blot of embryonic samples injected with 1 ng of plasmid.*

H_2O	19.5
571	17.9
571M	18.7
H_2O	14.9
571	18.9
571M	18.5
H_2O	15.5
571	16.1
571M	20.0

A second Western blot was performed to determine if the 1 ng dose of plasmid would knock down the expression of ephrin-A5. As seen in Figure 4, the band representing ephrin-A5 (21kDA) in each of the pCAX+571 wells does not decrease in intensity over time when

compared to the controls. This suggests that pCAX+571 is not capable of knocking down ephrin-A5 in *Xenopus laevis*.

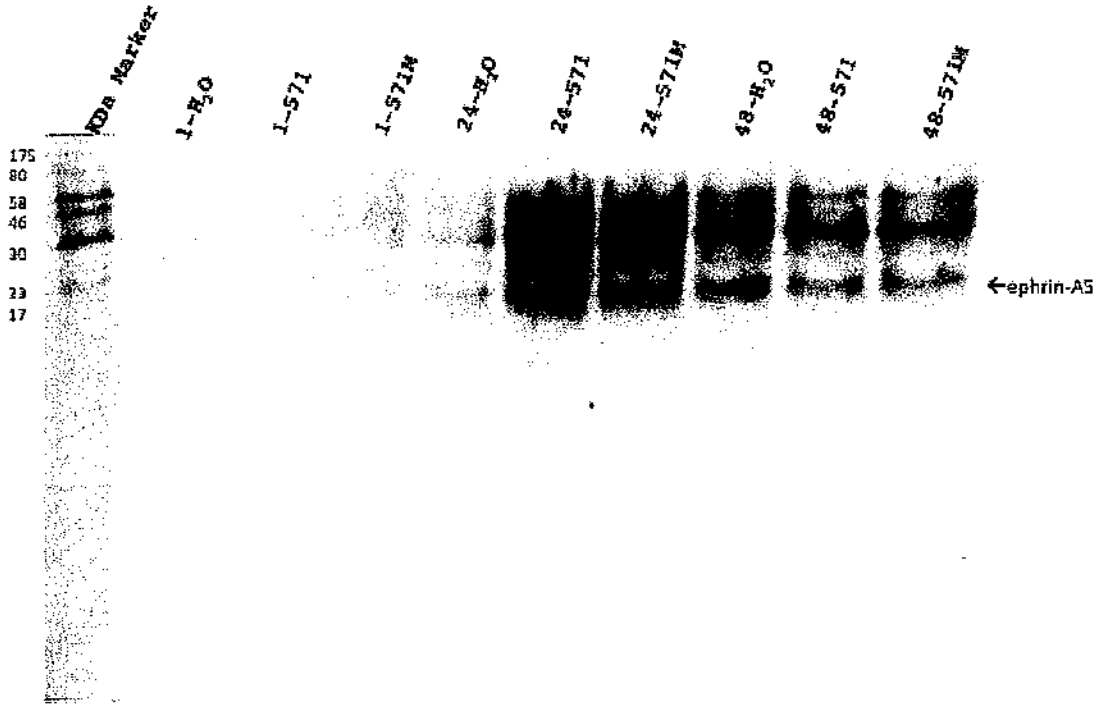

Figure 4. *Western blot of proteins extracted from embryos injected with 1 ng of either pCAX+571 or pCAX+571M. The lanes, from left to right, are samples taken 1 hour, 24 hours, and 48 hours after injections from the water, pCAX+571, and pCAX+571M samples. The protein marker was added separately as a picture taken of the nitrocellulose with white light because the ladder disappeared during the washing process. Antibody-bound protein bands were visualized using a BioRad ChemiDoc XRS+ gel documentation system utilizing Image Lab Software and the chemiluminescence protocol. The bands larger than ephrin-A5 can be attributed to proteins with similar epitopes to ephrin-A5 binding to the ephrin-A5 antibody and proteins that may interact solely with the secondary antibody. The persistence of 21 KDa band suggests that 1 ng of pCAX+571 was unable to knockdown the expression of ephrin-A5.*

The lane containing water injected embryonic protein samples taken 24 hours after injections looks significantly less concentrated compared with the other 24 hour samples. Based on this observation, the nitrocellulose was stripped and reprobed with anti-actin antibody, viewed at 43kDa, to verify even protein loading in each lane (Figure 5). After

viewing the newly probed nitrocellulose, it appears that the concentrations of proteins within each time set are relatively equal, however there is slightly less protein in the water injected 24 hour sample compared to the pCAX+571 and pCAX-571M 24 hour samples. It can still be assumed that any changes observed in the 1 ng Western blot were not due to a difference in protein concentrations. This does not change the results of the 1 ng Western blot. The wells containing proteins expressed in the cells bearing pCAX+571 did not show that the band at about 21 kDA, representing ephrin-A5, diminished in intensity at any of the time samples.

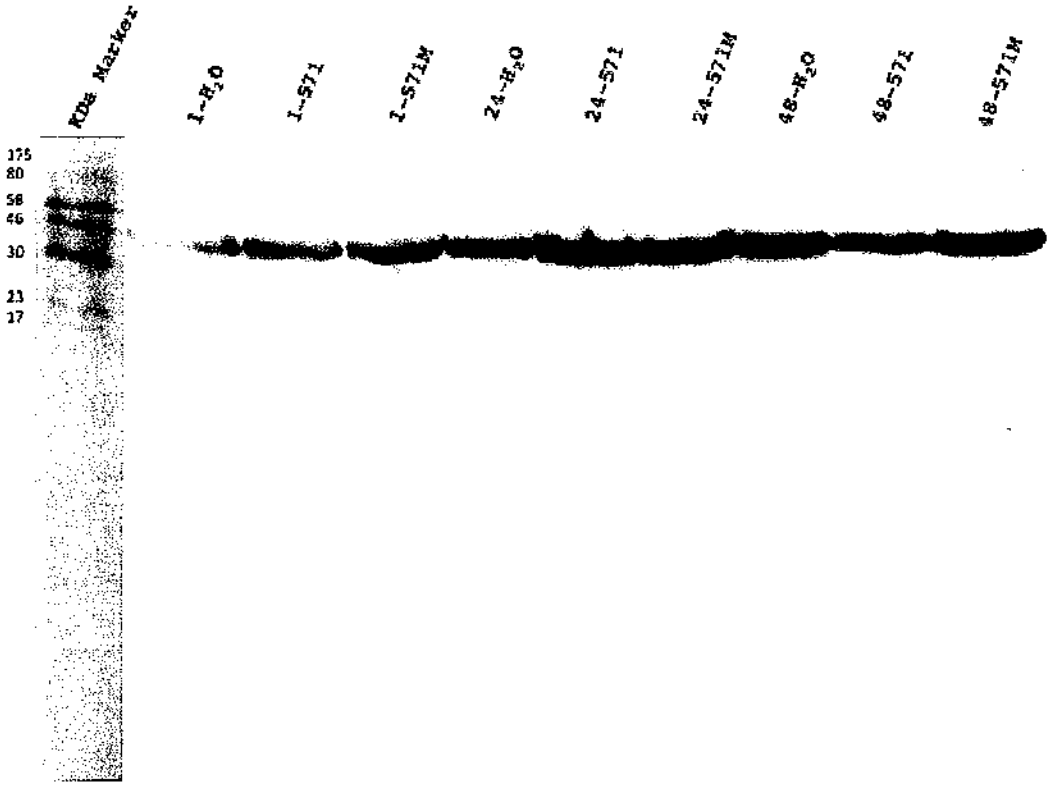

Figure 5. *Western blot of proteins extracted from embryos injected with 1 ng of either pCAX+571 or pCAX+571M reprobed with anti-actin antibodies,. The lanes, from left to right, are samples taken 1 hour, 24 hours, and 48 hours after injections from the water, pCAX+571, or pCAX+571M samples. The protein marker was added separately as a picture taken of the nitrocellulose with white light because the ladder disappeared during the washing process. Antibody-bound protein bands were visualized using a BioRad ChemiDoc XRS+ gel documentation system utilizing Image Lab Software and the chemiluminescence protocol. The image suggests that the total protein concentrations in each well were nearly equal relative to each sample set.*

To ensure that the band being considered ephrin-A5 on the Western Blots was in-fact the correct band with a size of 21kDa, a standard curve was created for the protein marker used in the SDS-PAGE and subsequently the Western Blot. Based on this curve and the length the assumed band traveled, it can be concluded that the band does sit at 21kDa and can be considered ephrin-A5. The standard curve and the weight of the significant bands on the Western blot can be seen in Figure 6.

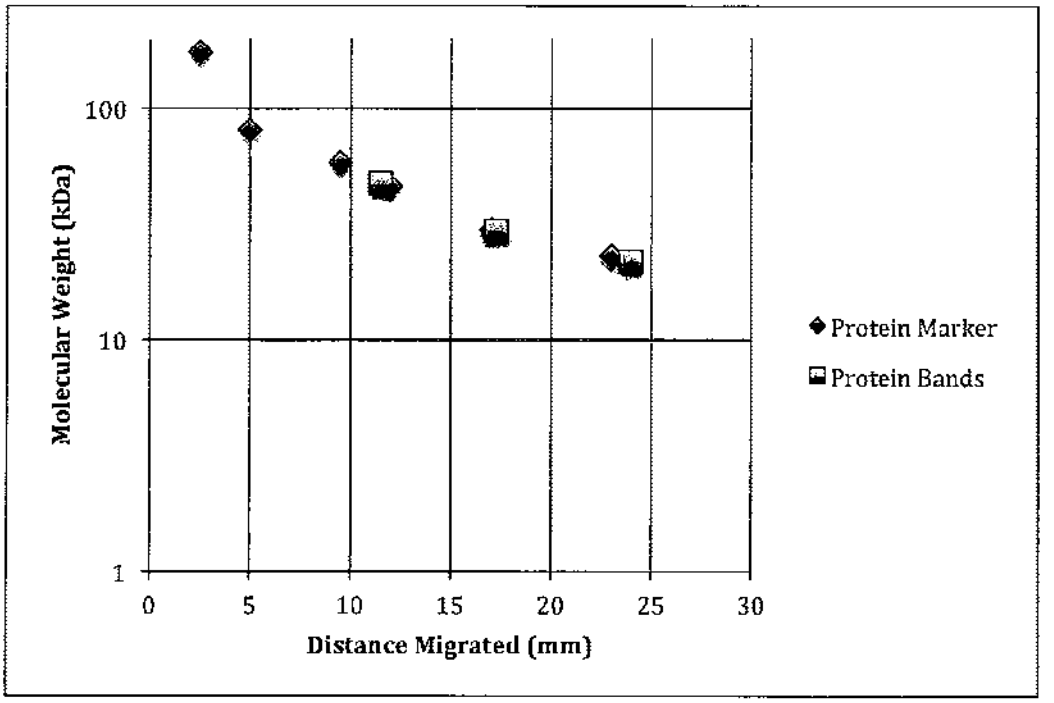

Figure 6. *The relationship between the molecular weight and the distance the protein migrated in the SDS-PAGE. The three prominent protein bands on the Western blot were compared to the standard curve of the protein marker used and their sizes were confirmed. This graph confirms that the smallest major band is 21kDa, and can be assumed to be ephrin-A5.*

Discussion

Ephrin-A5's presence at the zygote stage suggests that it plays a significant role in embryonic development. Using *Xenopus laevis* as a model organism, shRNA was used in an attempt to knock down the expression of ephrin-A5 in developing embryos. This method was used in a previous study that successfully knocked down the expression of ephrin-A5 in chicken embryos by 40% using a different plasmid, pCAX+236 (Winning and Krull, 2011). I attempted to use pCAX+571 to knockdown ephrin-A5 expression because the 571 sequence contains greater sequence identity to the *Xenopus* sequence than the 236 sequence (which was designed against the chicken gene). However, the results show that pCAX+571 is

ineffective in knocking down ephrin-A5 expression in *Xenopus laevis* embryos. This is similar with the findings in a previous study, where the pCAX+571 was not effective in knocking down ephrin-A5 expression in the hind limbs of chicks (Winning and Krull, 2011). Though one cannot assume the same outcome in chickens and frogs, the similarities between *Xenopus laevis* and chickens render similar outcomes possible. The results made it impossible to determine ephrin-A5's function in embryonic development. Nevertheless, it did confirm that using pCAX+571 is not the best choice to knock down ephrin-A5 expression. The experimental design, however, has been shown to be sound. Neither the microinjected water nor pCAX+571M hindered the expression of ephrin-A5 or the development of the *Xenopus laevis* embryos.

The Western blot showed interesting bands that were larger than the expected band representing ephrin-A5. These bands were unidentifiable proteins but may be attributed to various *in vivo* proteins cross-reacting with either the primary or secondary antibody. There may be proteins with similar epitopes to ephrin-A5 that can cross-react with the primary antibody (anti-ephrin-A5) or proteins that could have cross-reacted with the secondary antibody. To determine if any of the proteins interacted with the secondary antibody solely and the expected size of the proteins, another Western blot would need to be performed with only the secondary antibody used as a probe. Another possible reason is the fact that the anti-ephrin-A5 antibody used was tested against humans, mice, and rats, not frogs. This could have an impact because the difference in epitopes between mammals and amphibians may be great enough to alter the main protein being probed; thus causing different bands to be more prominent than the bands representing ephrin-A5. According to Novus Biologicals, the anti-

ephrin-A5 probe has not been used in *Xenopus laevis* previously, resulting in no comparable data available for review. According to Novus Biologicals, there is a possibility, however, that the band present at 29.5 kDa may be a glycosylated form of ephrin-A5, which would expand the interpretation of results to include this band. Including this new data does not change the results of pCAX-571, however. The plasmid still seems to be ineffective in knocking down ephrin-A5 expression. In regards to the 47.5 kDa band, Novus Biologicals attributed it to being either an aggregate or multimer of the ephrin-A5 protein. This further suggests that the full amount of ephrin-A5 cannot be seen at the expected band and may suggest that the full amount of the protein did not migrate into the separating gel, but that some may have remained in the stacking gel. If this were the case, then all three prominent bands would need to be considered to be ephrin-A5 and reinterpreted. However, even when including all three bands it still cannot be concluded that pCAX-571 was effective against ephrin-A5 synthesis.

In regards to why pCAX+571 did not knockdown ephrin-A5 expression, there may be many undetermined reasons. However, it has been shown that when testing shRNA plasmids, on average one in four will give ≥70% knockdown, one in four will give about a 50% knockdown, and half will be ineffective at knocking down the protein expression of interest (Life Technologies, 2006). The pCAX+571 was chosen as one of the possible plasmids to knock down ephrin-A5 expression in *Xenopus laevis*. However, the sequence of the shRNA (starting at position 571) was based on the sequenced *Xenopus tropicalis* genome, specifically its ephrin-A5 gene. This is due to the fact that *Xenopus laevis's* ephrin-A5 gene has not been completely sequenced.

Future research is needed to determine ephrin-A5's function in developing *Xenopus laevis* embryos and to test other plasmids' effectiveness to knock down ephrin-A5. Though pCAX+571 was not based on *Xenopus laevis's* sequenced ephrin-A5 gene, the genes should be nearly the same. This is based on the fact that *Xenopus laevis* and *Xenopus tropicalis* genomes are nearly identical (NCBI). The ephrin-A5 sequence starting at position 571 is identical between *tropicalis* and chickens, thus *tropicalis* and *laevis* are most likely identical as well. For example, based on a random protein expression search in UniGene small nuclear RNA auxiliary factor 2 in *Xenopus tropicalis* is 97.4% identical in function to *Xenopus laevis* and has a 99% positive base sequence match. Though pCAX+571 was ineffective in knocking down ephrin-A5 expression, it is unlikely that, if utilizing different plasmids based on *Xenopus tropicalis*, a knockdown will not occur. This, however, may not be necessary. Recently the first copy of *Xenopus laevis*'s genome has been released to the public (James-Zorn et. al 2012). Thus, shRNA plasmids may now be able to be based on *Xenopus laevis's* genome. With or without this new information, new shRNA plasmids need to be tested. Three plasmids encoding ephrin-A5 shRNA that should be tested start at positions 236, 248, and 373, which are three plasmids tested in a previous study and shown to be successful when transfected into developing chicks (Winning and Krull, 2011). Once a plasmid is found to knock down ephrin-A5, research can begin to determine ephrin-A5's function in developing embryos and provide increasing knowledge of the processes in which Eph-ephrin signaling is involved.

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