

Full Length Research Paper

## Restriction enzyme mediated integration and FIV lentiviral transgenesis applied to amphibians

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Restriction enzyme mediated integration (REMI) transgenesis and lentiviral transgenesis are effective methods of introducing transgenes into the genome of frogs. One aquatic amphibian species, *Xenopus laevis*, and one land dwelling species, *Litoria caerulea*, were chosen as subjects for transgenesis. REMI produced *X. laevis* that expressed the fluorescent protein DsRed. REMI was unsuccessful in producing transgenic *Litoria*. Therefore, lentiviral transgenesis was attempted. Hatchling *Litoria* tadpoles were exposed to replication defective lentiviral particles containing the coding sequence for DsRed. Histological evaluation revealed the presence of DsRed in brain, heart, liver, kidney, and muscle tissues. Therefore, lentiviral transgenesis appears to be a viable technique for producing transgenic land-dwelling frogs.

**Key words:** DsRed, *Xenopus laevis*, *Litoria caerulea*, restriction enzyme mediated integration (REMI), Lentivirus.

### INTRODUCTION

In recent years, the African Clawed Frog *Xenopus laevis* has become a leading model organism for transgenesis. Restriction enzyme mediated integration (REMI) of DNA into sperm nuclei, and transplantation into unfertilized eggs, is a method that has been used previously to produce GFP transgenic lines of *X. laevis* (Sakamaki et al., 2004). Although positive results have been produced using the *Xenopus* cardiac actin promoter, low expression in tissues has been a problem (Sakamaki et al., 2004). The CAG promoter is a combination of the cytomegalovirus early enhancer element and the chicken beta-actin promoter, and it drives higher expression than the *Xenopus* cardiac actin promoter (Sakamaki et al., 2004). The CAG promoter has driven transgenic expression in most *Xenopus* tissues, including germline cells (Marsh-Armstrong et al., 1999). Transgenic *Xenopus* progeny show germline transmission without the CAG promoter, but expression is mosaic (Marsh-Armstrong et

al., 1999). DsRed is a 28-kDa red homologue of the fluorescent protein GFP, isolated from the *Discosoma* species of coral (Matz et al., 1999). The development of DsRed transgenic *Xenopus* would support the use of *Xenopus* as a model organism because DsRed emits in the visible light spectrum.

Although there have been several lines of transgenic *Xenopus* produced, no lines of transgenic land dwelling frogs exist. The main objective was to develop methods to generate transgenic land dwelling frogs. *Litoria caerulea* (White tree frogs) is a common land-dwelling tree frog found in Australia. Frequently found inhabiting human dwellings, *Litoria* are considered docile and have been bred in the pet trade. *Litoria* have been used to study parotoid gland secretion, natural insect repellents, lungworm infections, wet adhesion, skin water loss properties, and hind limb locomotion studies (Christian and Parry, 1997; Crockett and Peters, 2007; Pizzatto and

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Shine, 2006; Scholz et al., 2008; Smith et al., 2003; Williams et al., 2006).

## MATERIALS AND METHODS

### Frog husbandry methods

#### *Adult X. laevis* husbandry

*Xenopus* were maintained in sexed trios in two to five gallons of chlorine and chloramine free water at a room temperature of 21°C. Cage contents included PVC pipe and plastic plants for enrichment. Water was treated with 0.1 g/L Seachem equilibrium and 0.8 g/L Seachem Cichlid lake salts to bring the hardness to 215 ppm (Seachem Laboratories Madison, GA). *Xenopus* were placed on a 12/12 fluorescent lighting schedule. *Xenopus* were fed daily with *Xenopus* Express frog food (Brooksville, FL). Water changing took place once a week.

#### *Adult Litoria caerulea* husbandry

*Litoria* were maintained in sexed trios in 20-gallon aquariums at a room temperature of 27°C. Cage contents included a water bowl, drift wood branches, plastic foliage, with paper towel substrate. A 1 L water bowl was filled with 0.5 L sterile water. Frogs were on a 12/12 fluorescent lighting schedule. Feeding took place two days a week and consisted of four to six crickets per frog dusted with Rep-Cal Herptivite and Rep-Cal calcium with D3 (Rep-Cal, Los Gatos, CA). Water bowl and water were changed once a week when cage washing and autoclaved. Before sterilization, bowls and cages were sprayed with a 5% bleach solution. All procedures involving animals were approved by the North Carolina State University Institutional Animal Care and Use Committee.

#### *Tadpole X. laevis* husbandry

Tadpoles were first raised in an incubator with 0.1x MMR (0.01 M NaCl, 0.2 mM KCl, 0.1 mM MgSO<sub>4</sub>, 0.2 mM CaCl<sub>2</sub>, 0.5 mM HEPES (pH 7.8), 0.01 mM EDTA and 50 µg/mL Gentamicin) as a buffer. When tadpoles reached stage 35 of growth, they were transferred to a larger container (Nieuwkoop and Faber, 1994). Tadpoles were kept in 350 mL H<sub>2</sub>O and fed 2-4 drops of tadpole food daily (*Xenopus* Express Brooksville, FL). Water pH was between 7.0 - 8.5 and water hardness was brought to 200 ppm. Water hardness was achieved by adding Cichlid Lake Salts at 6.4 g/L, and Equilibrium Salts at 0.8 g/L with sterile water. A Tetra Whisper aquarium bubbler was used to aerate tadpole containers (Blacksburg, VA). Room temperature was 21-24°C.

#### *Tadpole Litoria* husbandry

*Litoria* tadpoles were kept in groups up to seven tadpoles per liter of water. Water was treated with 0.1 g/L Seachem Equilibrium and 0.8 g/L Seachem Cichlid Lake Salts to bring the hardness to 215 ppm (Seachem Laboratories Madison, GA). Water was heated with Flex Watt Heat tape to 27°C. A Tetra Whisper aquarium bubbler was used to aerate tadpole containers (Blacksburg, VA). Tadpoles were placed on a 12/12 fluorescent lighting schedule. Tadpoles were fed daily with *Xenopus* Express Premium Tadpole Diet (Brooksville, FL). Full container cleaning took place every other day, with half of the water changed every day to remove old food. Cleaning involved rinsing the containers with water and spraying and rinsing 2 times with 70% ethanol. Bowls were sterilized by baking in a laboratory

oven at 180°C overnight.

### Hormone injections

*X. laevis* were primed with a primary intraperitoneal (IP) injection of 200 International Units of Pregnant Mare's Serum Gonadotropin (PMSG) five to seven days before induction of egg laying. To induce laying, 500 IU of human Chorionic Gonadotropin (hCG) were administered 10-12 h before procedures were scheduled to begin. As soon as frogs were injected with hCG, they were transported to the lab for observation and egg collection.

Initially the *X. laevis* hormone doses of PMSG and hCG were applied to *Litoria* but *Xenopus* doses did not induce *Litoria* egg laying. *L. caerulea* were primed with a primary IP injection of 200-300 International Units of PMSG five to seven days before induction of egg laying. To induce egg laying, *Litoria* were then given two IP injections of 250 IU of human Chorionic Gonadotropin (hCG) with 2 µg Alarelin (synthetic luteinizing hormone releasing hormone) 12 h apart. The first hCG injection occurred 12 h before the procedure was scheduled to begin.

### Restriction enzyme mediated integration

#### Oocyte extract preparation

Oocyte extract was prepared following a published protocol detailed (Kroll and Amaya, 1996). *X. laevis* and *L. caerulea* were killed via double pithing after immersion in a 0.1% tricaine solution for 20 min until unresponsive to needle poke. Ovaries were collected and macerated in a salt solution of 87 mM NaCa, 2.5 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM Hepes, and 1% Penn-Strep pH 7.8 (OR2) with collagenase A to digest at 28°C. After washing in the salt solution, eggs were sorted in 15 mM Hepes and 50 µg/mL gentamycin (OR3), washed with OR2, and placed at 15°C overnight in OR3. Oocytes were then washed 4 times in 1X extract buffer salts (XB) and rinsed again 2 times in cyostatic factor extract buffer salts (CSF-XB) with protease inhibitors at a 1:1000 dilution. XB solution consisted of 1X XB salts, 50 mM sucrose, and 10 mM HEPES. CSF-XB solution consisted of 1X XB salts, 1 mM MgCl<sub>2</sub>, 10 mM HEPES (pH 7.7), 50 mM sucrose, and 5 mM EGTA. Protease inhibitors were 10 mg/mL Leupeptin, 10 mg/mL Chymostatin, and 10 mg/mL Pepstatin A. Samples were centrifuged for 1 min at 1000 rpm, excess buffer was removed, and solution was re-spun at 2000 rpm for 1 min or until 3 layers formed; the cytosolic layer was removed and centrifuged. The center most portion was harvested and frozen in liquid N<sub>2</sub>.

#### Sperm nuclei preparation

Males were injected with 250 IU of hCG for *Xenopus* and *Litoria* an hour before killing to increase sperm production. To isolate sperm nuclei, testes were harvested and placed into a Petri dish and rinsed with 1X Marc's modified ringers solution (1X MMR) and 1X nuclear preparation buffer (1X NPB). 1X MMR consisted of 0.1 M NaCl, 2 mM KCl, 1 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 5 mM HEPES (pH 7.8), and 0.1 mM EDTA. 1X NPB consisted of 250 mM sucrose, 15 mM HEPES, 0.5 mM spermidine trihydrochloride, 0.2 mM spermine tetrahydrochloride, 1 mM Dithiothreitol, and 2 mM of Ethylenediaminetetraacetic acid. Testes were then minced in 1X NPB and filtered through cheesecloth. Filtrate was centrifuged in an SS-34 rotor at 6,500 rpm (2,000 g) for 15 min at 4°C. *Xenopus* sperm pellets were warmed to room temperature and 50 nL of 10 mM/mL lysolecithin was added. For *Litoria*, lysolecithin alone did not subsequently de-tail the sperm; therefore an additional step of homogenizing with 100 strokes in a dounce homogenizer (Wheaton

Science Products, Millville NJ, Part # 357544) was needed before the sperm nuclei pellet was resuspended in 1X NPB/protease inhibitors/0.3% BSA. Protease inhibitors used were 10 mg/mL Leupeptin, 10 mg/mL Chymostatin, and 10 mg/mL Pepstatin A. Samples were centrifuged, and suspended in cold sperm storage buffer and frozen in liquid nitrogen in 20  $\mu$ L aliquots. Sperm nuclei storage buffer consisted of glycerol, 0.3% BSA, in 1X NPB. Sperm samples were stored at  $-80^{\circ}\text{C}$ . Nuclei quality was evaluated by mixing sperm nuclei with oocyte extract. Only nuclear preparation that exhibited visible sperm nuclei swelling were used for injection.

#### **De-jelly and post de-jelly solutions for transgenesis**

For *Xenopus*, a 15% sodium borate (Sb) buffer (10 mM NaOH pH to 8.5 with  $\text{H}_3\text{BO}_3$ ), 2.5% cysteine free base pH 8.5 solution was used to de-jelly the eggs (Kroll and Amaya, 1996). To remove de-jelly solution, eggs were rinsed 3 times in a solution of 0.5% bovine serum albumin (BSA) in 1x MMR.

*Xenopus* de-jelly solution was originally applied to *Litoria*, but the *Litoria* eggs are more delicate than the *Xenopus* eggs; they exhibited visual dehydration, and embryos did not develop. A final solution of 5% Cysteine free base solution in 0.1X MMR, pH 8.5 was determined as an appropriate de-jelly solution. To remove de-jelly solution; eggs were rinsed three times with a solution of 1% BSA in 0.4X MMR.

Attempts at injecting non-dejellied eggs involved laying a 1000  $\mu\text{m}$  mesh over the groups of eggs and injecting through the mesh using the same needle sizes and injection medias as with the de-jellied eggs.

#### **Injection Media and Post- Injection medias**

*Xenopus* eggs were injected in a solution of 6% Ficoll 400 and 0.5% BSA in 0.4X MMR. Following injection, *Xenopus* eggs were placed in a solution of 6% Ficoll 400 and gentamycin (50  $\mu\text{g}/\text{mL}$ ) in 0.1X MMR.

Due to the *Litoria* eggs failure to activate when the *Xenopus* injection solutions were used, the solutions were adjusted. *Litoria* eggs were injected in a solution of 20X MMR, Ficoll 400, and 10% BSA. Following injection *Litoria* eggs were placed in a solution of 0.4X MMR, 2% BSA medium with 50  $\mu\text{g}/\text{mL}$  gentamycin.

#### **REMI egg injection procedure**

The pCAG DsRed vector was chosen for transgenesis (Cambridge MA). Restriction enzyme *SpeI* and *ApaI* digests were performed on the pCAG vector. *SpeI* cut the plasmid once at 18 bp; *ApaI* cut the plasmid twice, at 4,010 and at 5,255 bp. The linearized plasmid contained an intact promoter and DsRed gene after restriction enzyme digest. Restriction enzymes were inactivated by placement in  $4^{\circ}\text{C}$  overnight. Sperm nuclei solutions used in nuclear micro injection to produce DsRed transgenic *Xenopus* and *Litoria* consisted of: sperm nuclei (50 sperm nuclei/nL), *ApaI* or *SpeI* digested p-CAG DsRed plasmid, 0.5 IU of *ApaI* or *SpeI*, oocyte extract, sperm dilution buffer,  $\text{CaCl}_2$  and  $\text{MgCl}_2$ . After de-jellying, eggs were rinsed 3 times with post de-jelly rinse and loaded into a Petri dish of injection media. Oocyte extract and sperm mix were combined and loaded into a 100  $\mu\text{m}$  beveled needle. Eggs were injected with 4-5 nL of sperm nuclei solution at a rate of 50  $\mu\text{L}/\text{hr}$ . Sperm solutions were used up to 30 minutes after thawing.

An unpaired student t-test was applied to the population data.

#### **Visual screening**

DsRed positive tadpoles were determined by observation using

epifluorescence illumination through a Texas Red filter set at 20x magnification with a LEICA DM IRB microscope (Richmond, Illinois). Pictures were taken with an RT slider Spot camera (Sterling Heights, Michigan).

#### **Lentiviral transgenesis**

A second protocol was developed to incubate hatched *Litoria* tadpoles in a solution containing lentivirus overnight to generate transgenic tadpoles. Hormone injections and timing were identical as used to stimulate sperm production and egg laying in the REMI protocol. To induce breeding, a rain chamber was constructed from a 20-gallon aquarium, which was filled halfway with water containing 0.1 g/L Seachem Equilibrium and 0.8 g/L Seachem Cichlid Lake Salts. The water had hardness (GH or general hardness) of 215 ppm heated to  $27^{\circ}\text{C}$ , and pumped into a container above the aquarium. Water was allowed to drip onto driftwood, a platform, and plastic plants to mimic the wet breeding season. Following PMSG injection, one to two female *Litoria* were placed into a rain chamber with three to six males. *Litoria* remained in the rain chamber for seven days or until eggs were laid. If eggs were laid, the adult *Litoria* were removed and eggs were allowed to hatch in the rain chamber.

#### **Lentivirus production**

The lentiviral vector system chosen for transgenesis was pCDF1 (System Biosciences, Mountain View, CA) a derivative of Feline Immunodeficiency Virus (FIV) requiring packaging plasmids for viral production (System Biosciences, Mountain View, CA). The gene isolated for transgenesis was DsRed from the plasmid pCAG, driven by the chicken beta actin promoter. A replication defective lentivirus was generated using the PEG-it kit (System Biosciences, Mountain View, CA). The DsRed construct was ligated into pCDF1. Subsequently, the plasmids were transfected into HEK 293 cells, which secreted the replication defective lentiviral particles into the cell culture media. Viral pseudoparticles were collected, frozen, and titered using NIH 3T3 cells. Cultures exhibited titers averaging  $1.6 \times 10^6$  TU/mL.

#### **Lentiviral infection media**

Three basal medias were chosen to select for lentiviral media infectivity: Dulbecco's Modified Eagle Medium (DMEM), NCTC 109, and Leibovitz 15 (L15) medium. NIH 3T3 cells were plated at  $1 \times 10^6$  cells/mL in three wells of three 6-well plates in 3 mL of media with 1  $\mu\text{L}/\text{mL}$  media of [100  $\mu\text{g}/\text{mL}$ ] polybrene. A day later cells were infected with replication defective lentiviral particles and observed for fluorescence three days later. Pictures were taken of the wells, and infected versus non-infected cell populations were recorded. Transformation units were determined between infected and non-infected cell population data (Table 4). Standard error was calculated and propagated for each well accordingly to give a total standard error for the media. A one-way analysis of variance (ANOVA) evaluation was performed on the infection populations to determine whether the medias caused the difference in infection rates. Means were separated using least significant difference (LSD) between each media.

#### **Lentivirus incubated tadpole method**

Immediately after tadpoles hatched, they were removed from the rain chamber and transported to the laboratory. Procedures were performed under a class II Biosafety Cabinet. Tadpoles were

**Table 1.** REMI production of transgenic *Xenopus laevis* categorized by restriction enzymes SpeI and ApaLI used on pCAG-DsRed.

Parameter	Restriction Enzyme SpeI	Restriction Enzyme ApaLI
Fluorescent tadpoles	88	49
Total tadpoles	392	433
Well formed tadpoles	129	217
Percent fluorescent	22%	11%

placed into 250 mL beakers with 20 tadpoles per beaker. As a media for the virus and the tadpoles, NCTC 109 media was diluted in half with sterilized water to a volume of 40 mL. To create frog water, 0.1 g/L Seachem Equilibrium and 0.8 g/L Seachem Cichlid Lake Salts was mixed in sterile water. The water had a hardness (GH or general hardness) of 215 ppm. To assist in transduction of viral particles into tadpole cells, 64  $\mu$ L polybrene (100  $\mu$ g/mL) was added to the solution. Lastly, 40 mL of virus was added to the solution, making the final ratios half virus, half frog water/NCTC 109 media/polybrene (50  $\mu$ g/mL). Tadpoles were incubated in virus for up to 12 h. Solutions with tadpoles were directly aerated. Tadpoles were observed every 15 min.

After incubating in the virus for 12 h, the tadpoles were moved to containers of the same size with water. When 20 min had passed, the tadpoles were moved to their final destination of 5 large 1.5 liter glass Pyrex bowls. Half of the tadpole water was changed every other weekday with a full change once a week. Tadpoles were cultivated until fully metamorphosed (typically after one to two months); samples of water were incubated with NIH 3T3 cells to ensure tadpoles were not shedding any retrovirus. No DsRed positive cells were observed.

#### *Litoria* histological evaluation

Histological evaluations were performed on 6 incubated *L. caerulea* tadpole tissues. *Litoria* samples were placed in an 80% PBS, and sucrose solution. Samples were stored over night at 4°C. A 2:1 solution of 20% sucrose OCT media was prepared as an embedding medium (Tissue Tek Sakura, Alphen aan den Rijn, Netherlands). *Litoria* were placed into dry embedding molds, which were filled with embedding medium. The mold was then taken and placed in a small container of methyl butane and floated over liquid nitrogen until the embedding media solidified. Ten micron thick sections were placed on glass slides and mounted in a glycerol based media containing 1 mg/mL p-phenylenediamine (Swartz et al., 1990). Cover slips were sealed with clear nail polish and samples were observed under a LEICA DM IRB microscope (Buffalo Grove, IL).

## RESULTS

### *X. laevis* REMI restriction enzyme results

The hatchability of REMI transgenic *Xenopus* tadpoles are listed in Table 1. Out of the 825 total tadpoles that hatched, 346 hatched well formed, and 137 expressed the DsRed protein. The total percent of tadpoles that visually expressed the DsRed protein were 22% of the tadpoles produced using SpeI, and 11% using ApaLI. Statistical analysis of an unpaired student t-test was

performed on the complete hatchability data, but no significant difference was found.

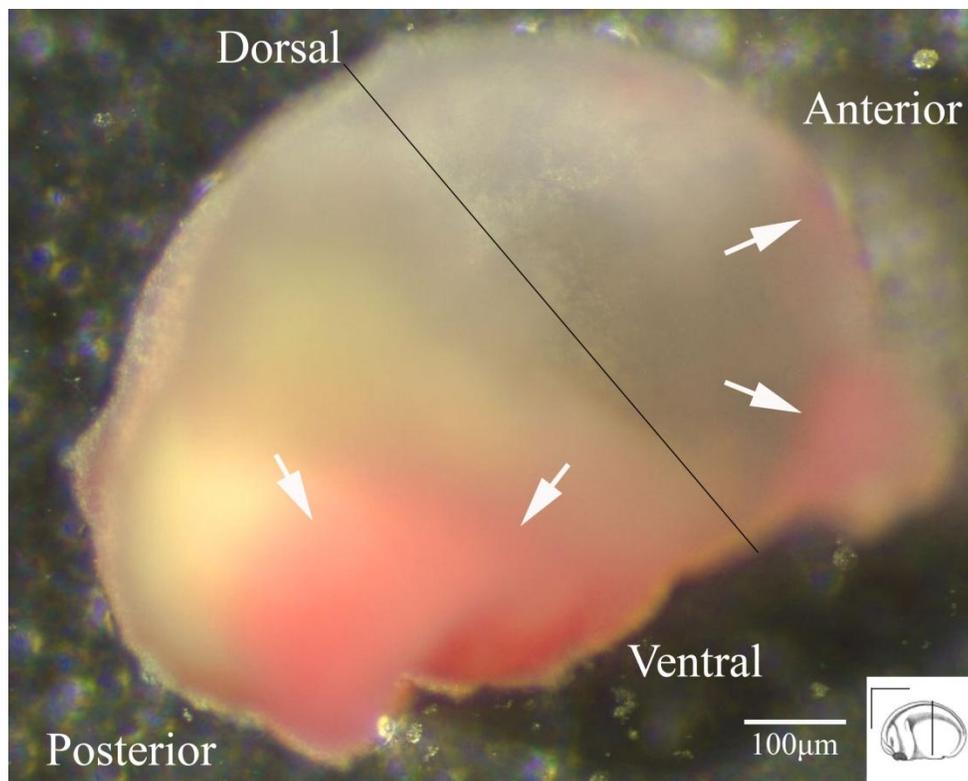
Pictures of DsRed-positive developing embryos and hatchlings were taken under incandescent illumination. Under incandescent illumination developing embryos and hatchlings display pink areas. (Figures 1 and 2).

### REMI De-jelly results

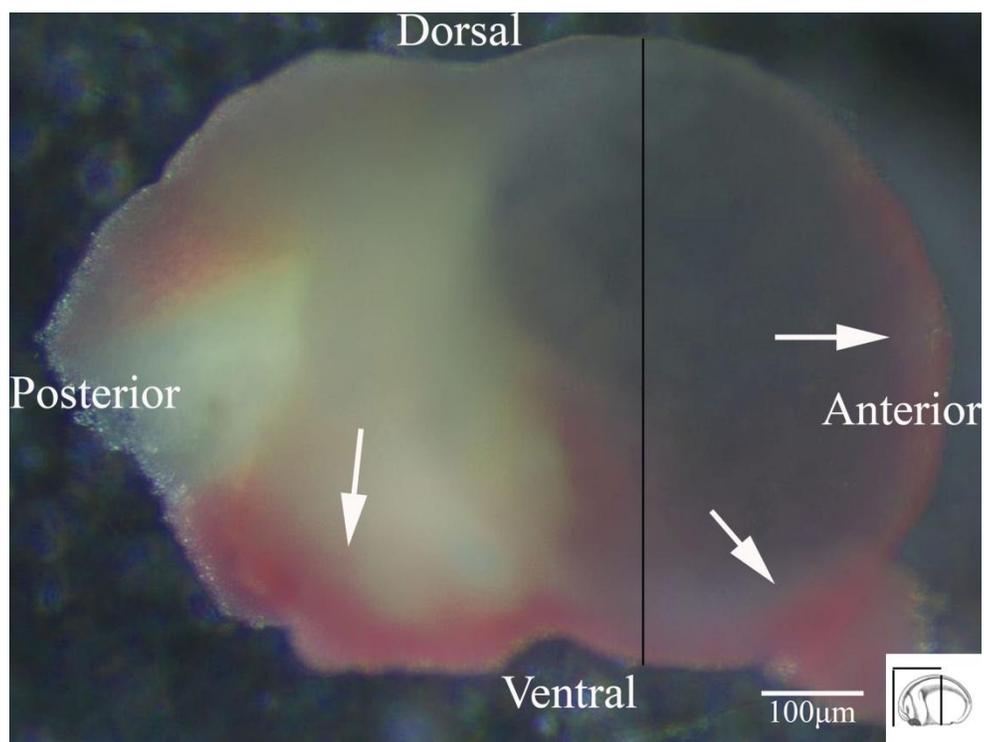
Several de-jelly solutions were applied to *Litoria* eggs to adjust for dehydration (Table 2). A de-jelly solution of 0.5% BSA and 3%-6% Ficoll in 0.4% MMR was found to qualitatively dehydrate *Litoria* eggs the least. After an adequate de-jelly solution was found, injection solutions were also varied using the optimal de-jelly solutions (Table 3). A solution of 6% Ficoll and 0.5% BSA in 0.4X MMR was found to assist in activating *Litoria* eggs. As a control injection through the jelly-coat was also attempted on *Xenopus* and *Litoria*. Mesh was used to hold the eggs in place and create a single layer of eggs for injection. Injection through the mesh and jelly coat proved difficult; the mesh obscured the location of the poles of the eggs for correct injection site and the eggs would move easily and tear when pierced with the needle. A second problem encountered with this method was the injection media used typically failed at activating the eggs. As a result few tadpoles were produced and it was determined de-jellying was an easier and more efficient method. The REMI method failed to produce embryo development past neural tube formation in the *Litoria* species, which may be related to tadpole management.

### Lentiviral infection media

Cells were plated at  $1 \times 10^6$  cells/mL per well. Total cell numbers for ten picture sample of the wells were as follows: 31,118 total cells for DMEM, 4,786 total cells for NCTC 109, and 3,937 for L15 media. For three media wells, the average transformation units were as follows; For DMEM media  $8.29 \times 10^2$  TU/mL,  $1.16 \times 10^5$  TU/mL for NCTC 109 media, and  $4.39 \times 10^4$  TU/mL for L15 media were counted (Table 4). A one way Analysis of Variance ANOVA was performed to determine whether there was a significant difference of infection rate among medias:



**Figure 1.** Stage 20 DsRed positive *Xenopus* embryo. Positive areas (pink) observed were of the embryos ventral hemisphere (denoted by arrows).



**Figure 2.** Stage 20 DsRed positive *Xenopus* embryo. Positive areas (pink) observed were of the embryos ventral hemisphere (denoted by arrows).

**Table 2.** REMI de-jelly solutions for *Litoria caerulea* and *Xenopus laevis* varied to decrease *Litoria caerulea* egg dehydration.

Species	De-jelly Solutions	De-jelly Results
<i>Litoria caerulea</i>	None- Injection through jelly coat	-Eggs were difficult to inject, none developed
	1 mg/mL Hyaluronidase in 1X MMR	-Eggs dehydrated and not de-jellied
	10% Cysteine free base in sterile water	-Eggs dehydrated and partially de-jellied
	2.5% Cysteine free base in 15% Sb	-Eggs dehydrated and de-jellied
	5% Cysteine free base in 1X MMR	-Eggs dehydrated the least and de-jellied
<i>Xenopus laevis</i>	2.5% Cysteine free base in 15% sodium borate (Sb) buffer (10mM NaOH pH to 8.5 with H <sub>3</sub> BO <sub>3</sub> )	-Well established method -Eggs dehydrated the least -Majority of transgenic tadpoles produced -Eggs did not activate well/method was difficult
	None- Injection through the jelly coat	-2 tadpoles/100 intact vs. 6 tadpoles /100 de-jellied

**Table 3.** REMI injection solutions for *Litoria caerulea* varied to increase egg activation.

Specie	Injection Media	Injection Results
<i>Litoria caerulea</i>	6% Ficoll in 0.1X MMR	Eggs dehydrated, No egg activation
	6% Ficoll in 0.4X MMR	Eggs dehydrated, No egg activation
	0.5% BSA and 6% Ficoll in 0.4X MMR	Eggs dehydrated, Egg activation

**Table 4.** Lentiviral titration of 3T3 cells in three medias to determine viral media preference.

Media	TU/mL	Standard Error
DMEM	8.29x10 <sup>2c</sup>	4x10 <sup>2</sup>
NCTC 109	1.16x10 <sup>5ab</sup>	4x10 <sup>4</sup>
L15	4.39x10 <sup>4b</sup>	1x10 <sup>4</sup>

ANOVA: Df= 2; P-Value= 0.0069193. Values with different superscript are significantly different (P<0.05).

there was significant difference found between DMEM and the other medias, but there is no significant difference between the means of NCTC 109 and L15 (Statistical Methods Table 4). To confirm the possibility of *Xenopus/amphibian* cell infectivity, embryo cells were cultured and transfected with DsRed (Figure 3). Positive cells exhibited fluorescence.

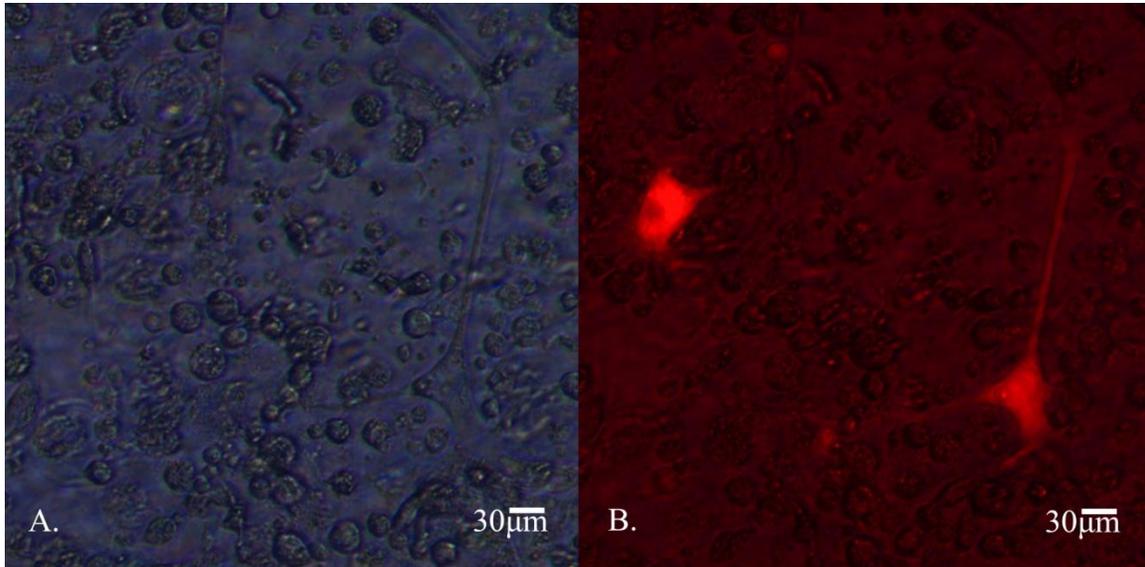
#### Lentiviral *L. Caerulea* microscopy

Transgenic *Litoria* tadpoles were successfully generated, cultivated, killed and observed. Samples were collected from transgenic and control froglets one to two months old. Tissue sections from control and treated tadpoles were examined for DsRed fluorescence under a epifluo-

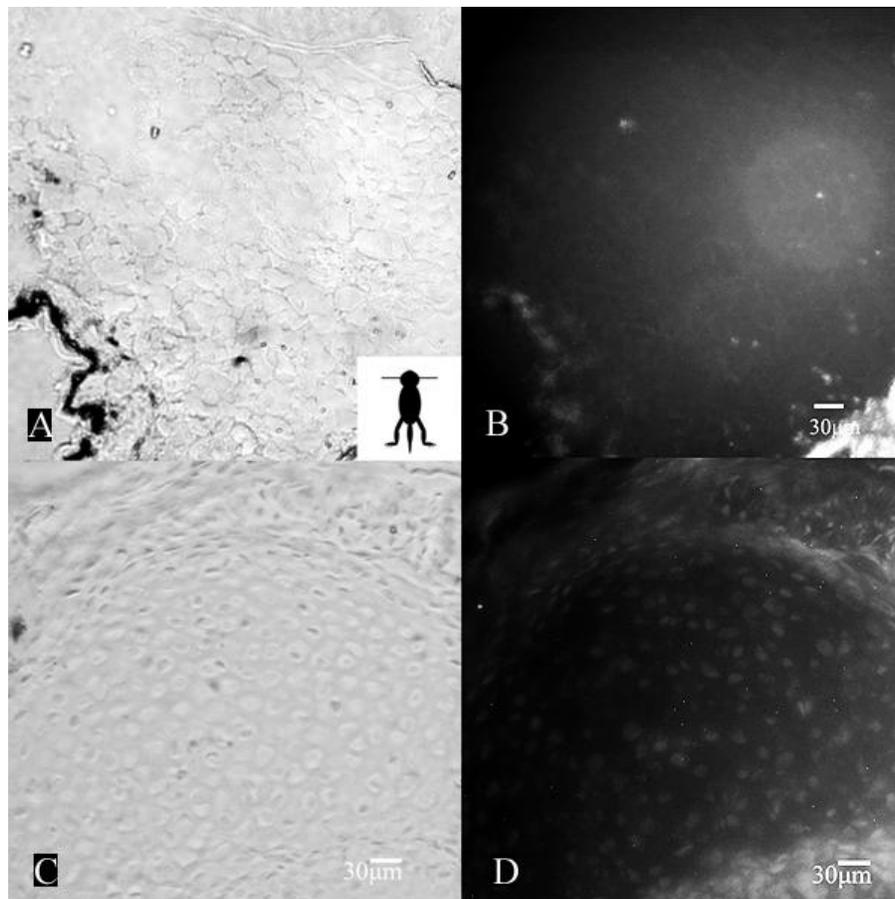
rescence illumination (Figures 4, 5, 6, and 7). Tissues observed to be positive for DsRed expression were brain, heart, muscle, and kidney.

#### DISCUSSION

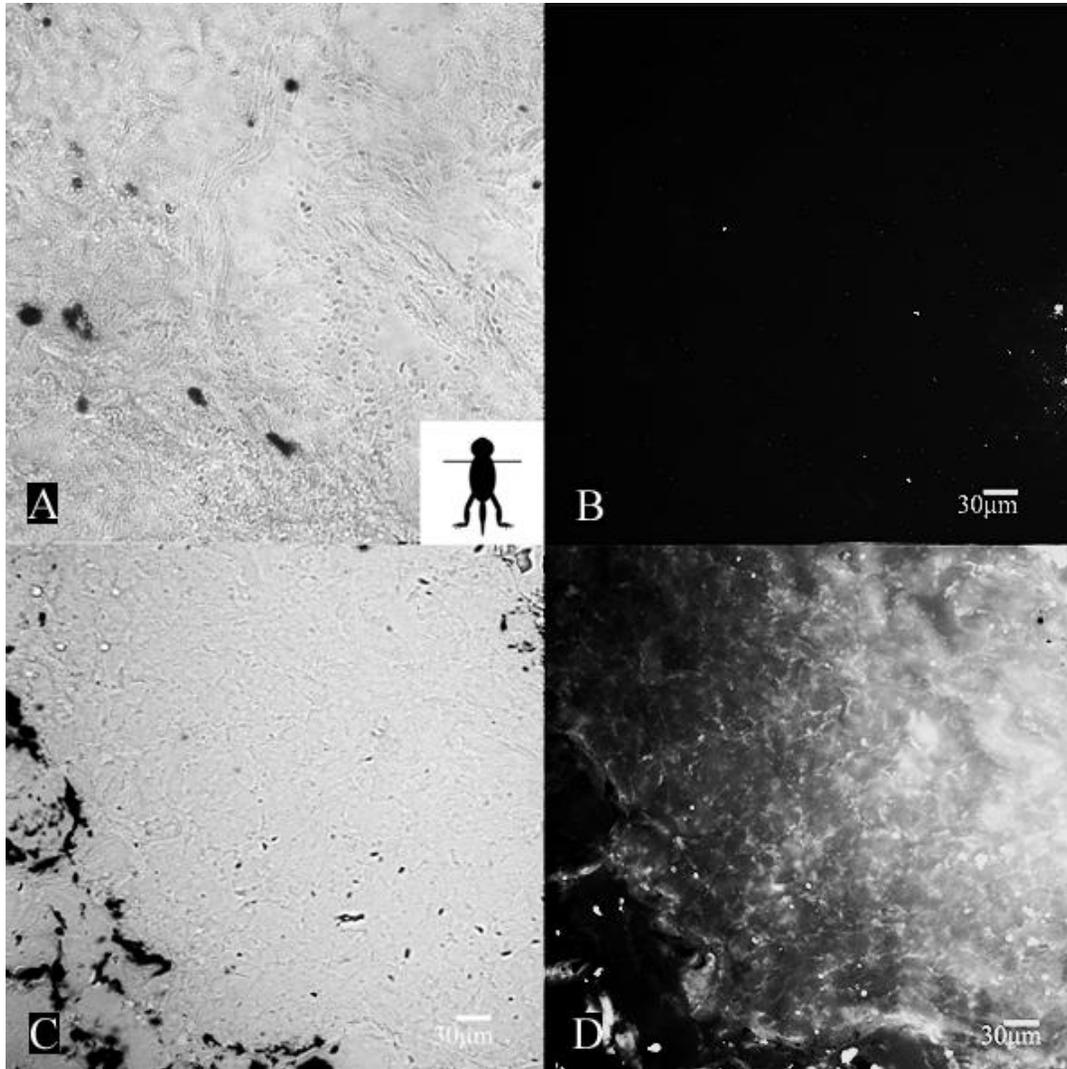
Restriction enzyme mediated integration (REMI) successfully produced transgenic *X. laevis*. After statistical analysis using an unpaired student t-test, it was found that SpeI and ApaI are both valid restriction enzymes to use in REMI. Because of the success in the *Xenopus*, the REMI protocol was attempted with *Litoria*. The *Xenopus* hormone injection failed to produce egg laying in the *Litoria*. PMSG was used as hormonal "priming" because it has been shown to increase the total number of early developing oocytes in the ovaries of *Rana cyanophlyctis* without increasing the number of mature oocytes (Pancharatna and Saidapur, 1984). Induction was first attempted in *L. caerulea* using a single dose of 1 mg/kg LHRH, which previously induced spermatozoa production in the tree frog *Hyla regilla*, with no success (Licht, 1974). It is likely that a single injection of LHRH did not induce egg laying in the *Litoria* because its effects are similar to PMSG; increasing the number of early stage oocytes without increasing maturation or ovulation. A second attempt using PMSG as a hormonal primer and a combination of



**Figure 3.** Brightfield (A) and epi-fluorescent (B) illumination of cultured DsRed *Xenopus laevis* tadpole cells that were transfected with DsRed. B. Fluorescence (Red) indicates positive results. Scale bar is 30  $\mu\text{m}$ .



**Figure 4.** Tissue sections from untreated *Litoria* tadpoles (A, B) and *Litoria* tadpoles treated with cell culture supernatant containing lentiviral particles encoding Ds Red (C, D). Schematic inset of frog shown within panel A illustrates the plane of tissue section from the whole animal. Images illustrate neural tissue. Sections are shown under bright field (A, C) and epifluorescence illumination (B, D). Scale bar is 30 microns.



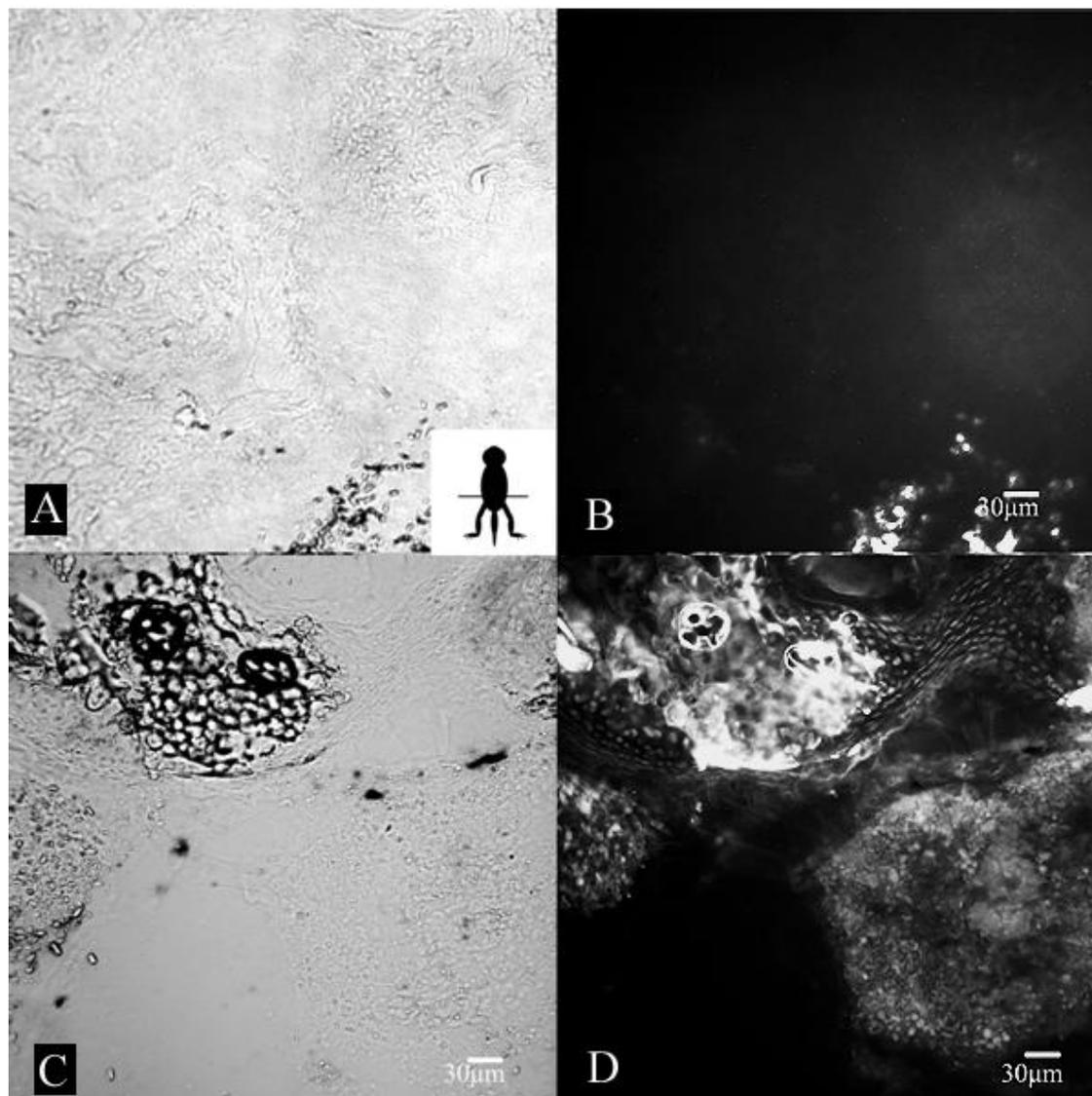
**Figure 5.** Tissue sections from untreated *Litoria* tadpoles (A, B) and *Litoria* tadpoles treated with cell culture supernatant containing lentiviral particles encoding Ds Red (C, D). Schematic inset of frog shown within panel A illustrates the plane of tissue section from the whole animal. Images illustrate heart tissue. Sections are shown under brightfield (A, C) and epifluorescence illumination (B, D). Scale bar is 30 microns.

hCG and Alarelin peptide hormone (synthetic LHRH) as an inducer, according to a protocol used to induce *Bufo baxteri*, was successful inducing consistent egg laying (Browne et al., 2000). *Litoria* were given hCG as an inducer because it has been shown to shorten oocyte maturation and stimulates ovulation in *X. laevis* (Browne et al., 2000).

REMI was attempted with the jelly coat intact, but the injection proved difficult; lower egg activation was observed and proper placement of the injection site was obscured because of the need for mesh to create a single layer of cells. Due to these problems, the de-jelly method was viewed as a more efficient method of production. Several de-jelly and injection medias were attempted to decrease the dehydration of *Litoria* eggs. Solutions

containing cysteine free-base appeared to be the most successful in de-jellying the eggs. A higher concentration of cysteine was required to de-jelly *Litoria* eggs than *Xenopus*. These differences contributed to why REMI failed to produce transgenic *Litoria*. Failure was likely due to a difference between the *Xenopus*, and *Litoria*'s tolerance to de-jellying the eggs. The jelly coat's function is to allow attachment to vegetation, provide protection, assist in sperm binding, prevent polyspermy, and act as a barrier to prevent embryo anoxia (Seymour and Bradford, 1995). With removal of the jelly coat, the embryo is more vulnerable to environmental influences.

It has been shown that egg quality and survivability depend on several factors including husbandry conditions of the adult females, water salinity, water contaminants (chloro-

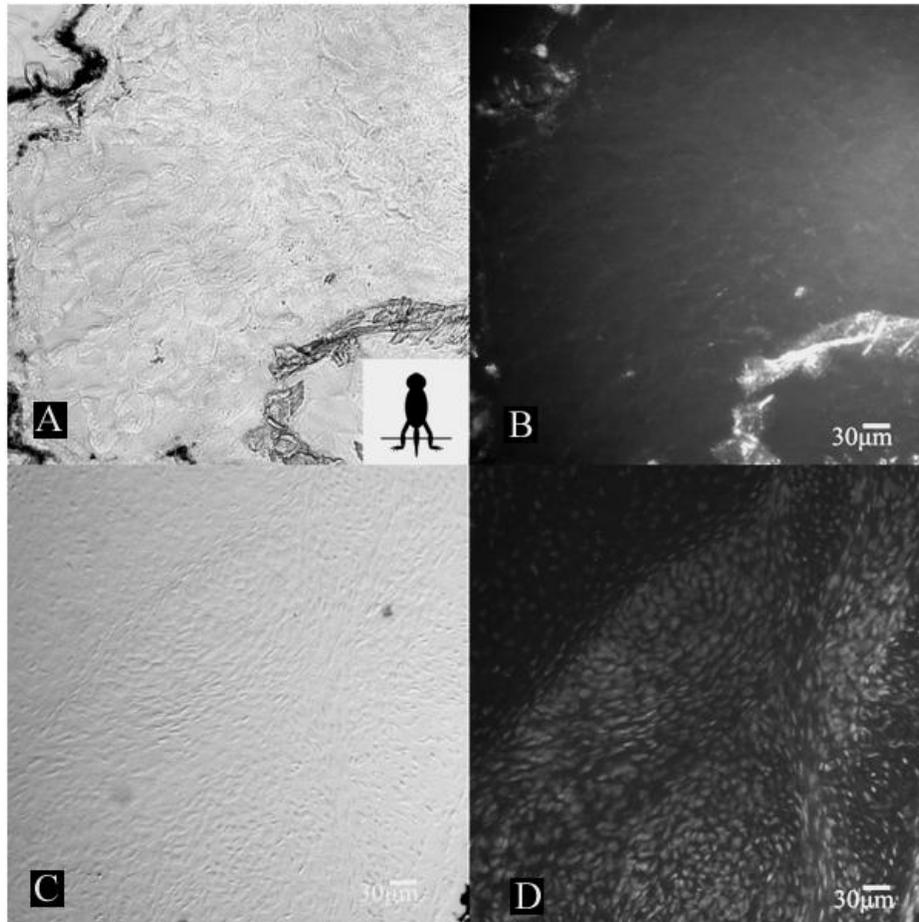


**Figure 6.** Tissue sections from untreated *Litoria* tadpoles (A, B) and *Litoria* tadpoles treated with cell culture supernatant containing lentiviral particles encoding Ds Red (C, D). Schematic inset of frog shown within panel A illustrates the plane of tissue section from the whole animal. Images illustrate kidney tissue. Sections are shown under brightfield (A, C) and epifluorescence illumination (B, D). Scale bar is 30 microns.

rine, ammonia, nitrate, and nitrite), and general hardness (GH) of the water; with hardness of water and presence of calcium and magnesium ions being more important than alkalinity or GH (Godfrey and Sanders, 2004). When *Xenopus* eggs were of poor quality, an increase in general hardness increased overall firmness of the vitelline membrane and normal development and survivability of embryos increased (Godfrey and Sanders, 2004). The protocol for maintaining transgenic embryos was modified from the *Xenopus* husbandry protocol to adjust for embryo fragility after de-jellying with an increase in GH. The de-jellying process may affect vitelline membrane elasticity. The vitelline membrane plays a significant role in osmoregulation of the developing embryo (Mild et al.,

1973). During the de-jellying process, it is possible the vitelline membrane of the *Litoria* eggs was damaged and the general hardness for the de-jellied egg medias needed to be increased further. It is possible that negative results were produced because the husbandry for the *Litoria* tadpoles needed to be adjusted further to account for different salinity, GH, and aeration.

It is also possible during the injection procedure more than one sperm nuclei were injected in some eggs producing abnormal embryo growth. In previous studies, it has been shown that aquatic frogs produce smaller ovum size than the terrestrial species allowing for more oxygenation to the embryo, which assists in faster development (Seymour, 1999). Removal of the jelly coat would influence



**Figure 7.** Tissue sections from untreated *Litoria* tadpoles (A, B) and *Litoria* tadpoles treated with cell culture supernatant containing lentiviral particles encoding Ds Red (C, D). Schematic inset of frog shown within panel A illustrates the plane of tissue section from the whole animal. Images illustrate muscle tissue. Sections are shown under brightfield (A, C) and epifluorescence illumination (B, D). Scale bar is 30 microns.

the embryos ability to respire and could account for slower development/embryo death due to inadequate oxygen/carbon dioxide regulation in the terrestrial species. Although unlikely, polyspermy causes abnormal development in monospermy species.

Transposon transgenesis is a common method used in biotechnology to create transgenic *Drosophila*, *C. elegans*, and plants (Grabher and Wittbrodt, 2009). The process is similar to REMI involving injection of transposon DNA with synthetic transposase mRNA to cause random insertion into the host genome. Transposon technology does not require a viral backbone like lentivirus transgenesis; the transposon itself is a naturally occurring mobile DNA element (Yergeau et al., 2010). Recently, this technology has been applied to vertebrate species, most notably *X. laevis* (Grabher and Wittbrodt, 2009). A major disadvantage to using transposon transgenesis is that its efficiency is inversely related to the inserted transposon's size, making it an inefficient candidate for large gene insertion (Grabher and Wittbrodt, 2009). Transposon

transgenesis also requires species-specific cofactors limiting its application to thoroughly studied species (Grabher and Wittbrodt, 2009). Mosaic expression is common due to the process requiring coinjection of transposase mRNA to catalyze the insertion reaction; the transposase mRNA may not be transcribed before the embryo completes its early stages, meaning the transposase enzyme will not be available to catalyze the reaction in those cells (Yergeau et al., 2010). For these reasons, transposon transgenesis was viewed as an inadequate method for this study.

Lentiviral transgenesis produced DsRed positive *L. caerulea* tadpoles. Lentiviral transgenesis only results in stable gene insertion into the genome (Cockrell and Kafry, 2007). The lentivirus vector pCDF1 was driven by the CMV/chicken beta-actin promoter, which drives protein production in the skin, liver, brain, heart, kidney, spleen and lung tissues (Fahim et al., 2009; Lois et al., 2002). Expression was visually confirmed in transgenic tadpole and frog tissues of the brain, heart, kidney, liver,

and muscle cells. The distribution of DsRed expression is similar to studies of GFP lentiviral transgenic mice, where the expression of transgene proteins was found in all tissues and organs analyzed including, skin, bone, muscle, lung, liver, stomach, intestine, kidney, brain, retina and gonads (Lois et al., 2002; Wiznerowicz and Trono, 2005).

Two transgenic species of amphibians were created using the methods of REMI and lentiviral transgenesis. Individuals in the *Xenopus* and *Litoria* species reach maturity at 1-1.5 years of age. Although germline transmission to the F1 generation was beyond the scope of the current study because of the long interval between generations, it is likely that successful germline transmission will occur, as evidenced by the previous success found in chickens, mice, and GFP transgenic *Xenopus laevis* (Lois et al., 2002; Marsh-Armstrong et al., 1999; Mozdziak et al., 2005).

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