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Quantifying apoptosis induced by the fusion of EWS-FLI1 by means of terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) using the zebrafish model

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Senior Honor's Thesis

August 2016

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Abstract

Ewing sarcoma is a pediatric cancer that is found most commonly in the bone but also in soft tissues. Though rare, it is the second most common bone cancer in children, affecting approximately 200 children annually. Patients are typically treated with multimodality chemotherapy, surgery, and radiation. Although these treatments can result in cure, roughly 30% of diagnosed patients do not survive[6]. Emerging studies have attempted to find links between EWS-FLI1; an oncogene which encodes for an aberrant transcription factor that helps drive tumorigenesis in Ewing sarcoma, and apoptosis / cell cycle regulation[13]. However, the mechanism behind this regulation is still poorly understood. We intended to create a quantitative apoptotic index using TUNEL assays in order to validate the link between EWS-FLI1 and apoptosis, but also prepare for further studies on potential genes that interact with EWS-FLI1 to bypass apoptosis and cause cell proliferation. An experimental setup was designed in which embryos were injected with CMV:EWS-FLI1-GFP and CMV:GFP as a control. After injections, embryos were fixed and stained using TUNEL. This study found a 4-fold increase in the average number of apoptotic cells per unit area for 48 hour old embryos while also indicating zero change in mean apoptotic cells per unit area for 24 hour old embryos. A second experiment was then designed in order to confirm the preliminary results obtained from the first experiment. The second study focused only on 48 hour old embryos and found that there is a 3-fold increase in mean apoptotic cells per unit area for 48 hour old embryos.

Introduction

Ewing sarcoma arises when a chromosomal translocation occurs between the amino-terminal domain of the EWS gene from chromosome 22 and a number of possible DNA-binding transcription factors that are part of the ETS family of transcription factors. Most commonly, this fusion takes place with the FLI1 gene as shown in figure 1[10]. It is shown that this fusion is required for tumorigenesis[12], however this fusion alone is not sufficient to drive tumorigenesis[11]. There are not many known secondary events that help to drive tumorigenesis. Recent studies suggest that a potential mechanism for Ewing sarcoma tumorigenesis lies within its ability to bypass cell cycle checkpoints and apoptosis[3].

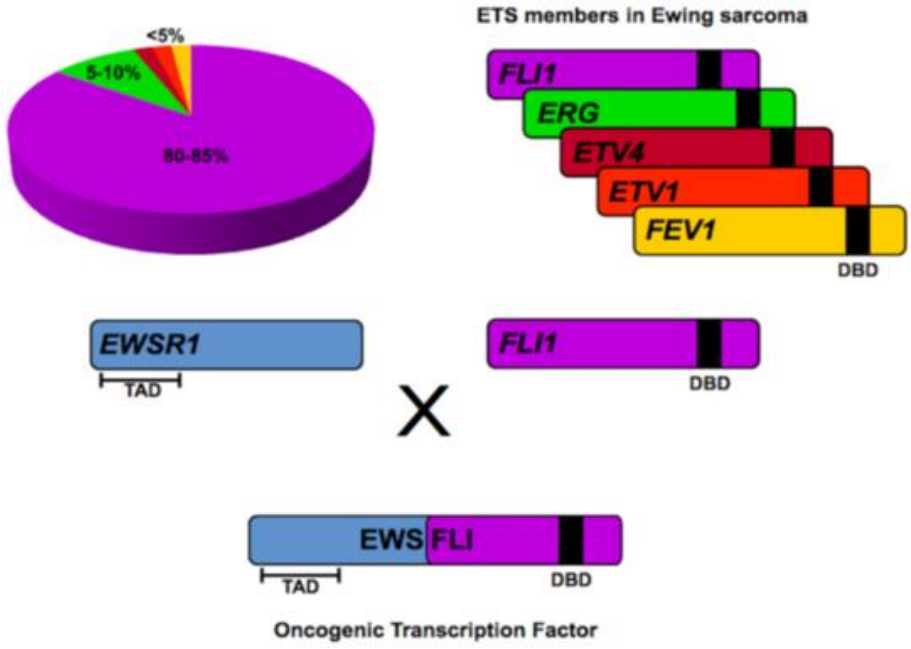


Figure 1. Percent chance of fusion between EWS and members of the ETS family of transcription factors (Monument et al 2012).

The cause of Ewing sarcoma is unclear. It has been shown that it is not a hereditable disease and no environmental exposure has been linked to the disease. New research in Ewing sarcoma aims to target the disease at the genetic and cellular level.

Zebrafish model for Ewing sarcoma

Zebrafish serve as an ideal model for studying the genetics of various cancers like Ewing sarcoma. Because zebrafish are vertebrates, they share a high degree of sequence and functional homology with humans. Moreover, any genetic mutation or human manipulations are easier to see due to their embryos being transparent. Also, zebrafish can produce progeny weekly and can lay 100-200 embryos at one given time. Zebrafish tumor susceptibility is comparable to that of humans. This makes Zebrafish an excellent candidate for studying Ewing sarcoma. Establishing an animal model of Ewing sarcoma is difficult due to the fact that many times cells will silence the gene and undergo apoptosis rather than constitutively expressing the EWS-FLI1 gene. Human Ewing sarcoma is characterized as a small round blue cell tumor. This histology is similar to that of zebrafish Ewing sarcoma[9].

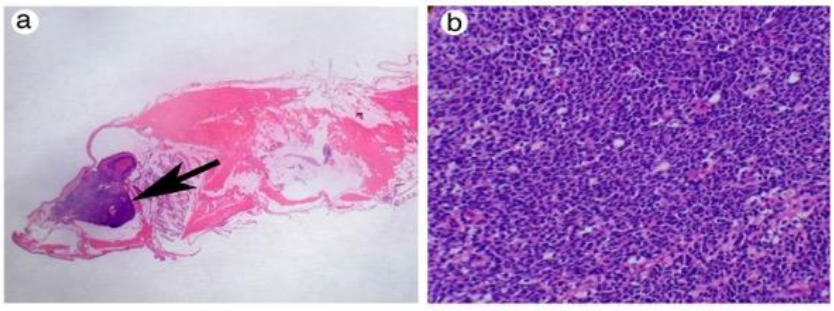


Figure 2. Gross (a) and microscopic (b) histology of tumor arising in mosaic EWS-FLI1 transgenic fish. From Leacock et al 2012

Generating transgenic fish using the Tol2 transposon system

Previous methods for creating transgenic zebrafish required the use of plasmid DNA and pseudo-typed retrovirus's[8]. The use of Plasmid DNA, which was injected into fertilized embryos, was effective in integrating green fluorescent protein (GFP) into certain tissues and organs; however, this method was mostly ineffective in producing transgenic fish that were able to produce transgenic offspring as only 5% of injected fish were able to produce transgenic

offspring[7]. On the other hand, injecting a pseudo-typed retrovirus into blastula stage embryos allows for the chromosomal integration of the virus's complementary DNA. This method can yield up to 100% transgenic germ line transmitting founder fish and therefore a larger number of transgenic offspring[4]. Although transgenic offspring yield is high, using a pseudo-typed retrovirus is very laborious and therefore, makes it difficult to produce a stable transgenic line[8]. In order to overcome the limitations of previous methods, the Tol2 transposon system was developed. This system allows for the integration of genetic constructs into genomic DNA in which transgenic fish can yield large amounts of transgenic offspring (50-70%) in larger frequencies as compared to previous methods[8]. The Tol2 transposon is roughly 4.7 kilobases (kb) in length with a gene encoding for a transposase gene, which consists of four exons. Figure 3 shows the structure of a Tol2 transposable element with the Tol2 vector[8]. The RNA transcribed by the Tol2 which encodes for the transposase protein is shown at the top of the illustration by solid lines (exons) and dotted lines (introns)[8]. The final mRNA construct which encodes for the transposase protein causes transposition through a cut-and-paste mechanism. This mechanism causes 8bp repeats at the target site; however, it does not cause any rearrangements or other modifications[8].

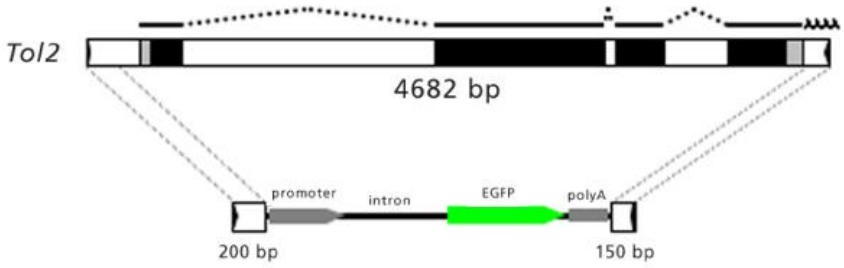


Figure 3. Structure of the Tol2 transposable element with the Tol2 vector. From Kawakami et al., 2007.

The Tol2 transposon system has been shown to be active in all vertebrate cells that have been tested thus far[7]. Generating transgenic zebrafish using the Tol2 system requires the use of

plasmid DNA. First, a transposon donor plasmid is co-injected with the mRNA encoding for the transposase. After the transposase protein is translated and folded, the transposase construct is excised and is allowed to fully integrate itself into the genome[7].

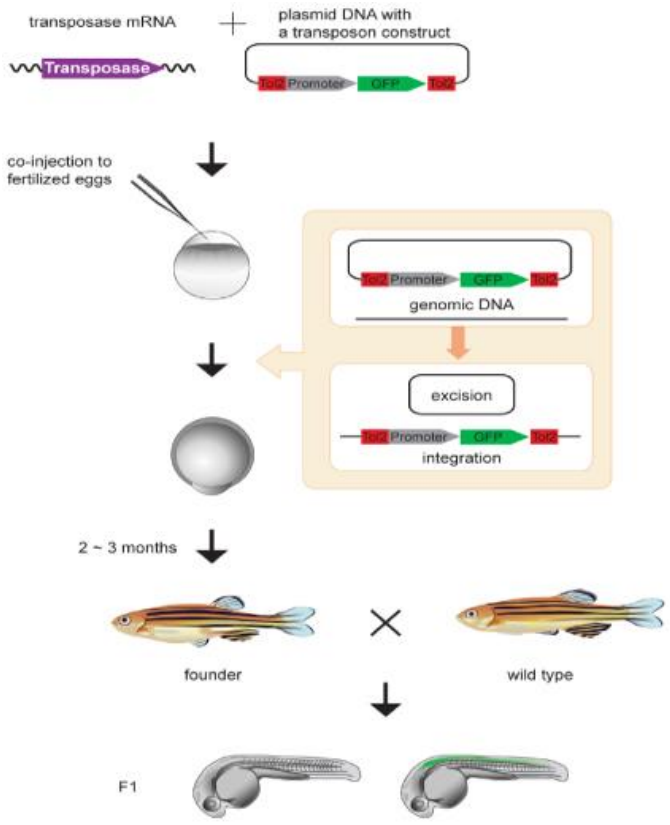


Figure 4. Tol2 transposon system as a way to integrate plasmid DNA into genomic DNA (Kawakami et al., 2004).

EWS-FLI1 expression in zebrafish embryos

The expression of EWS-FLI1 in zebrafish embryos relys on the promotor attached to it. In the experiments discussed in this paper, the cytomegalovirus (CMV) promoter was used due to its high expression in muscle cells. This expression can be visualized with the attachement of GFP to the construct of interest. Once EWS-FLI1 is integrated and expressed, the embryo begins to change morphologically depending on the level of expression. High levels of EWS-FLI1

expression results in a high degree of morphological deformity as shown in figure 5. This deformity may be due to the organism undergoing apoptosis at higher rates than normal.

Although it is clear that EWS-FLI1 induces a morphological change, It is difficult to establish a morphological apoptotic index because it is shown that an organism can undergo 25% tissue regression per day while only having 2-3% of its cells undergo apoptosis[1]. Because of this, it is important to establish a quantifiable method in which apoptotic cells can be better detected.

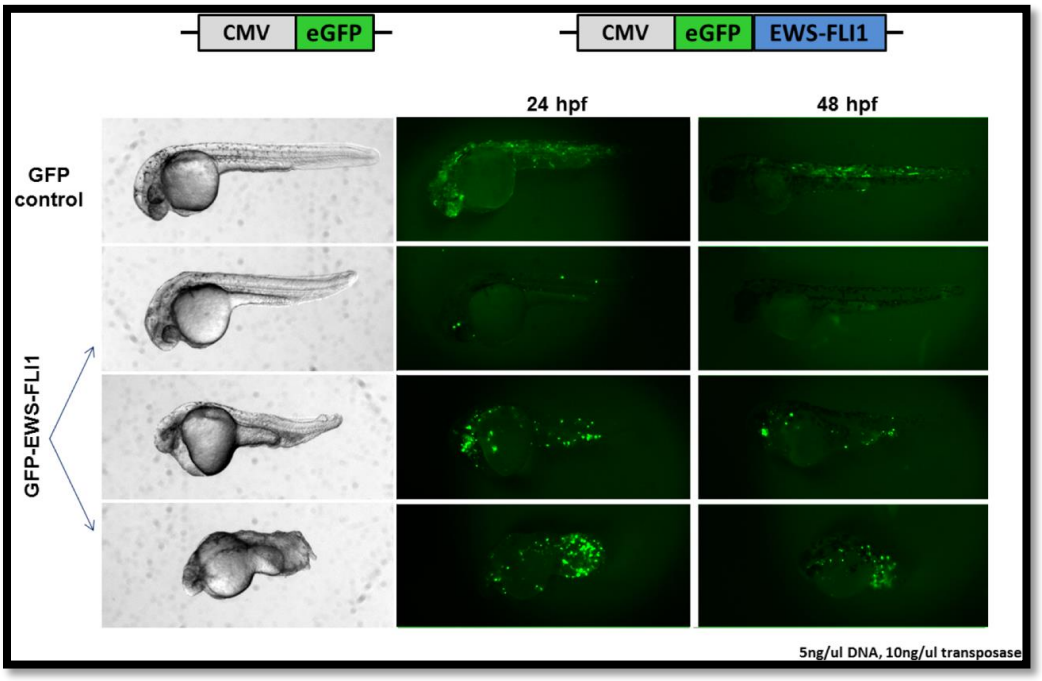


Figure 5. Various levels of EWS-FLI1 and GFP (control) expression at 24hpf & 48hpf at 5ng/ul DNA, 10ng/ul transposase

TUNEL Assay

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) is an assay for locating DNA fragments in situ after they have undergone apoptosis. This assay is template independent and relies on the identification of blunt ends of double stranded DNA breaks by the

enzyme terminal deoxynucleotidyl transferase (TdT)[5]. TdT catalyzes the addition of labeled dUTP's onto the 3'OH terminus of DNA strands[3]. These nucleotides form an oligomer composed of digoxigenin, which then allows for the addition of an anti-digoxigenin conjugate antibody[10]. This antibody contains a rhodamine fluorochrome which stains bright red.

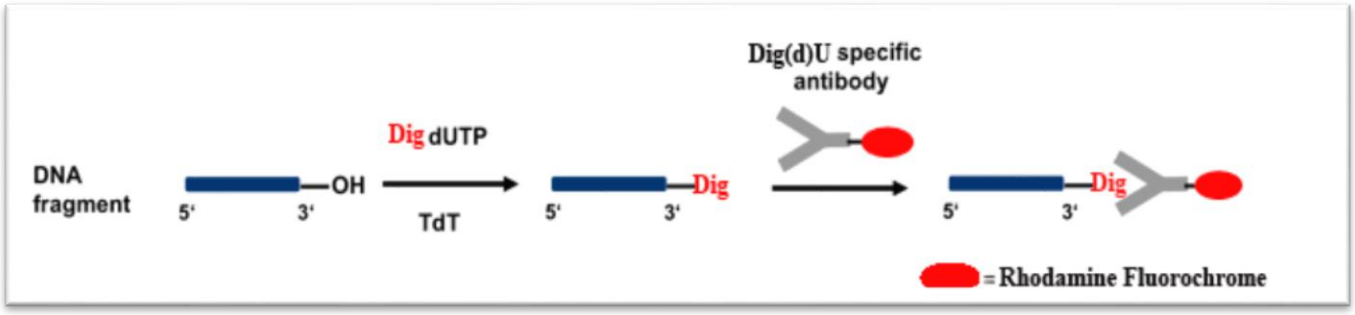


Figure 6. Visual representation of how a TUNEL assay works. TdT adds Dig-labeled dUTP onto 3'OH ends. Dig specific antibody with rhodamine fluorochrome attaches itself onto Dig.

Experimental Methods – A

Injections and TUNEL stain preparation

Two injection mixes were prepared at 10ng/µl for both CMV:EWS-FLI1-eGFP and CMV:eGFP. These two mixes were injected into fertilized zebrafish embryos within 45 minutes to an hour after being laid. Following injections, each petri dish was placed inside an incubator at 29-30 degrees centigrade. At 24 hours post injection, embryos were screened for GFP and then subsequently fixed overnight in 4% paraformaldehyde (PFA)/1X phosphate buffered saline (PBS). The next day, these embryos were prepared for TUNEL staining by further fixation in methanol at -20 degrees centigrade followed by rehydration with 3:1, 1:1, and 1:3 solutions of Methanol/PBS. After two washes in 1X phosphate buffered saline with Triton X-100 (PBST) for 5 minutes each wash, the embryos were digested by proteinase K in PBST (10µl/ml) for two minutes and then two subsequent washes with PBST were done. Further fixation in 4% PFA/1X PBS was performed for 20 minutes followed by two washes in PBST. Another fixation in 2:1 ethanol/acetic acid was then carried out for 10 minutes at -20 degree centigrade. After three rounds of washes in PBST, embryos were incubated in an equilibration buffer for one hour at room temperature. Lastly, a mixture of 70% reaction buffer - containing modified digoxigenin labeled dUTP's, with 30% terminal deoxynucleotidyl transferase (TdT) - was prepared and added to fixed and equilibrated embryos. These embryos were incubated in a water bath at 37 degrees centigrade overnight. The same steps were performed for the injected 48 hour-old embryos.

Preparing embryos for GFP staining

After embryos are fixed and prepared for TUNEL staining, any previous GFP that was visible using fluorescence microscopy become undetectable. To compensate for this loss, green fluorescent antibodies were used to localize GFP presence. Following the fixing and preparation for staining, a stop/wash buffer was added for 1 hour at room temperature. Next, a block was performed using 1% bovine serum albumin (BSA) for 30 minutes. Lastly, a 1:200 dilution of rabbit anti-GFP/1% BSA was prepared and added to the embryos. Embryos were then placed on top of a rocker in 4 degrees centigrade overnight.

TUNEL and GFP stain

After adding the primary antibody for GFP as well as the modified digoxigenin dUTP's for the TUNEL stain, a mixture containing 52% blocking solution, 47% Anti-digoxigenin conjugate, and 1% AlexaFluor 488 goat anti-rabbit IgG was prepared. Embryos were incubated in the solution for 4 hours at room temperature in the dark. Following incubation, embryos were washed three times in PBST for five minutes each time. Embryos were stored in PBST in a 4-degree centigrade refrigerator.

Quantifying the TUNEL stain

Attempting to obtain quantifiable results from whole-imaged embryos after TUNEL staining is difficult due to the large circular yolk sack located in the middle of the embryos body which causes the images to be blurred and unquantifiable. In order to succeed in dealing with this issue, each embryo's tail was cut so that the yolk sack was not involved in the data as shown in figure 7. Next, each tail was imaged using fluorescent microscopy. Following imaging, each tail was measured for its area in pixels using ImageJ software. In order to normalize the data, a

unit area in pixels was determined by using the smallest tail in each separate group (24 and 48 hours) to represent the unit area. The unit area for the 24 hour old embryos was 71763 pixels and 87861 pixels for the 48 hour old embryos. Next, each tail was individually counted three times, and an average for TUNEL positive cells was determined for each separate tail. All of the averages obtained for each tail were then added and a new average for each data set was determined. Finally, a two-tailed T test was done in order to determine statistical significance.

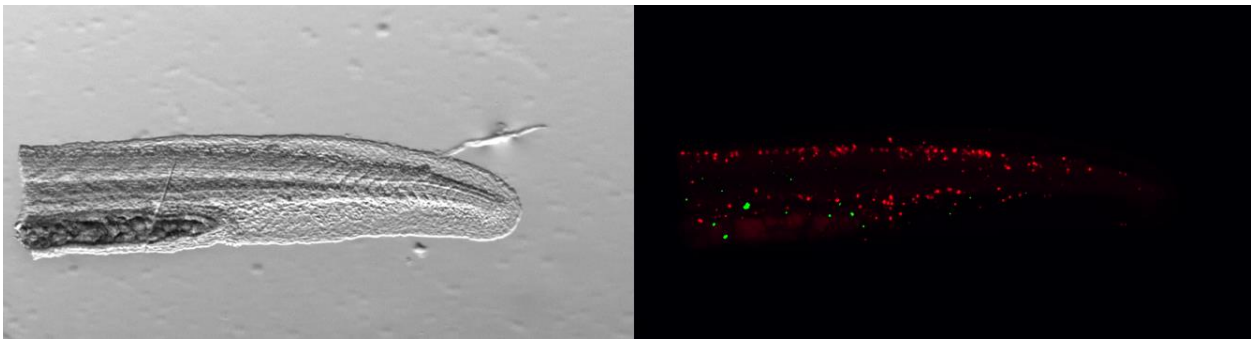


Figure 7. Zebrafish embryo tail post TUNEL assay. Bright-field (left) and TUNEL positive (red) / EWS-FLI1 positive (green) (right).

Results – A

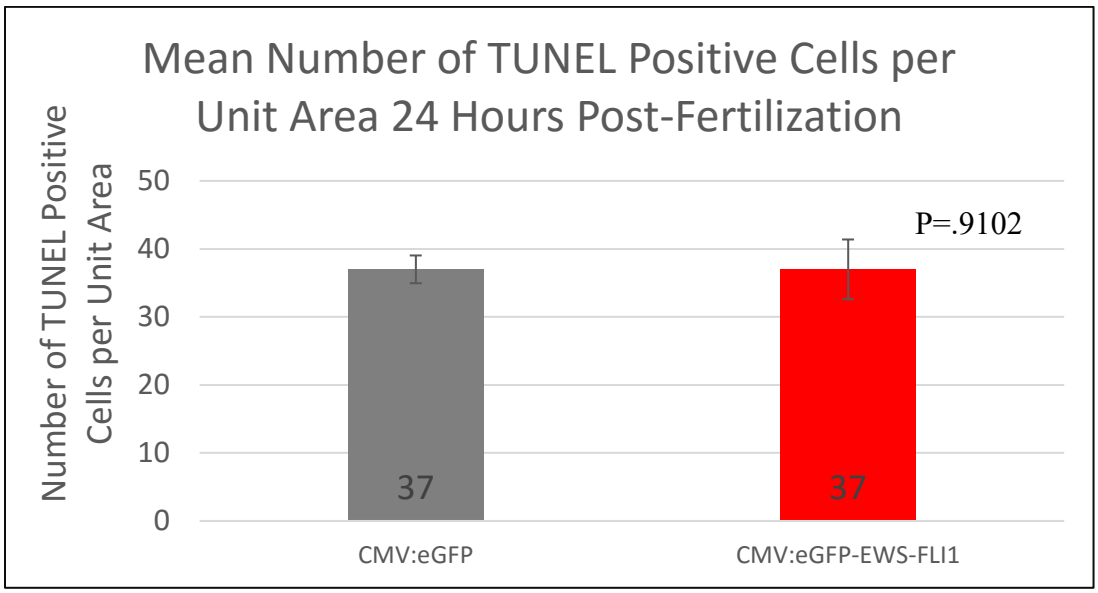


Figure 8. Average number of TUNEL positive cells 24 hours post fertilization did not increase when compared to the control

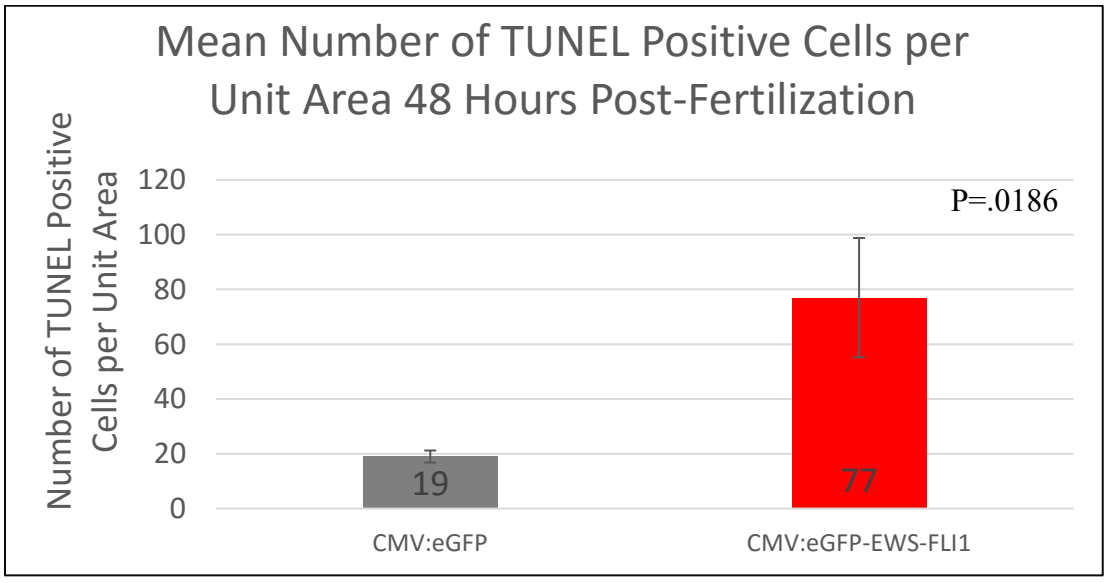


Figure 9. Average number of TUNEL positive cells 48 hours post fertilization drastically increased (4 fold) when compared to the control.

Discussion - A

Based off the results shown in figures 7 and 8, the average number of apoptotic cells does not change for 24 hour old embryos while significantly increasing (4 fold) for 48 hour old embryos. The reason there is no increase in apoptosis at 24 hours post fertilization could be due to the fact that approximately 70-80% of the 24 hour old embryos died before reaching 24 hours. This means that the TUNEL assay was performed on embryos that had less severe morphological deformity. Also, a high mortality rate made it difficult to obtain a large sample for each data set. Another possible reason for the lack of difference in apoptotic cells for 24 hour old embryos could be due to the timing mechanisms required for inducing apoptosis. Considering that embryonic transcription in zebrafish does not start until the 1000 cell stage, which is roughly 4-6 hours post fertilization[2], the amount of time it takes for EWS-FLI1-GFP to be transcribed, translated, folded, and finally induce apoptosis could take longer than 24 hours. Another limitation of this experiment was the GFP staining. Most embryos displayed small green dots scattered all throughout the tail in a manner that was inconsistent with previous GFP expression. This could be due to nonspecific binding of the primary antibody. The drastic increase in apoptosis for 48 hour old embryos indicates that EWS-FLI1 does induce more apoptosis than the GFP control. However, the 4-fold increase in apoptosis may have been skewed by one outlier that had 210 TUNEL positive cells. Due to the limitations presented by this experiment, another study must be conducted in order to validate and to be able to better quantify the results obtained from this experiment.

Experimental Methods – B

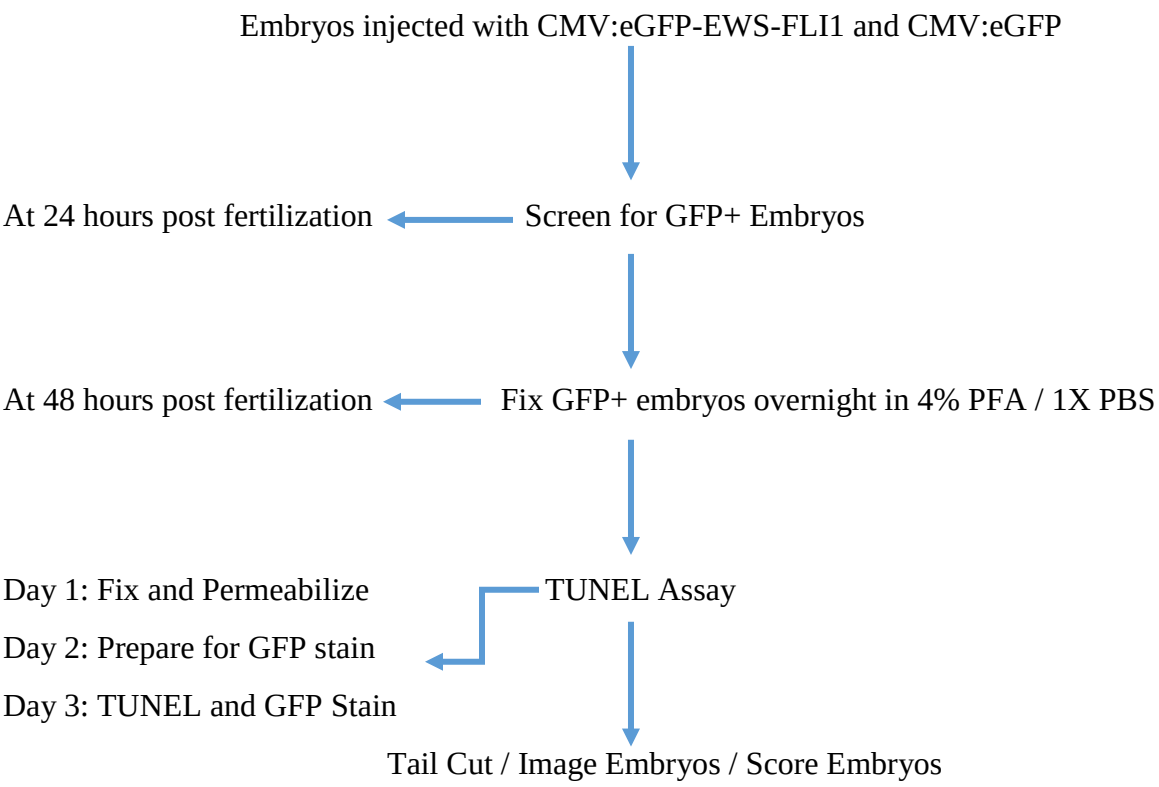
Changes made from Experimental Methods – A

In order to test the preliminary results obtained from figures 7 and 8, another experiment was designed with the following changes made from the first experiment:

- Only 48 hour old embryos were tested
- The concentration of the injection mix was lowered to 5ng/µl for both CMV:eGFP-EWS-FLI1 and CMV:eGFP
- Dilution of rabbit anti-GFP/1% BSA was increased from 1:200 to 1:500
- Unit area used for normalizing the data: 142714 pixels

All other aspects of this experiment remained constant with the previous study.

The method behind this experiment can be summarized by the flowchart shown below:



Results – B

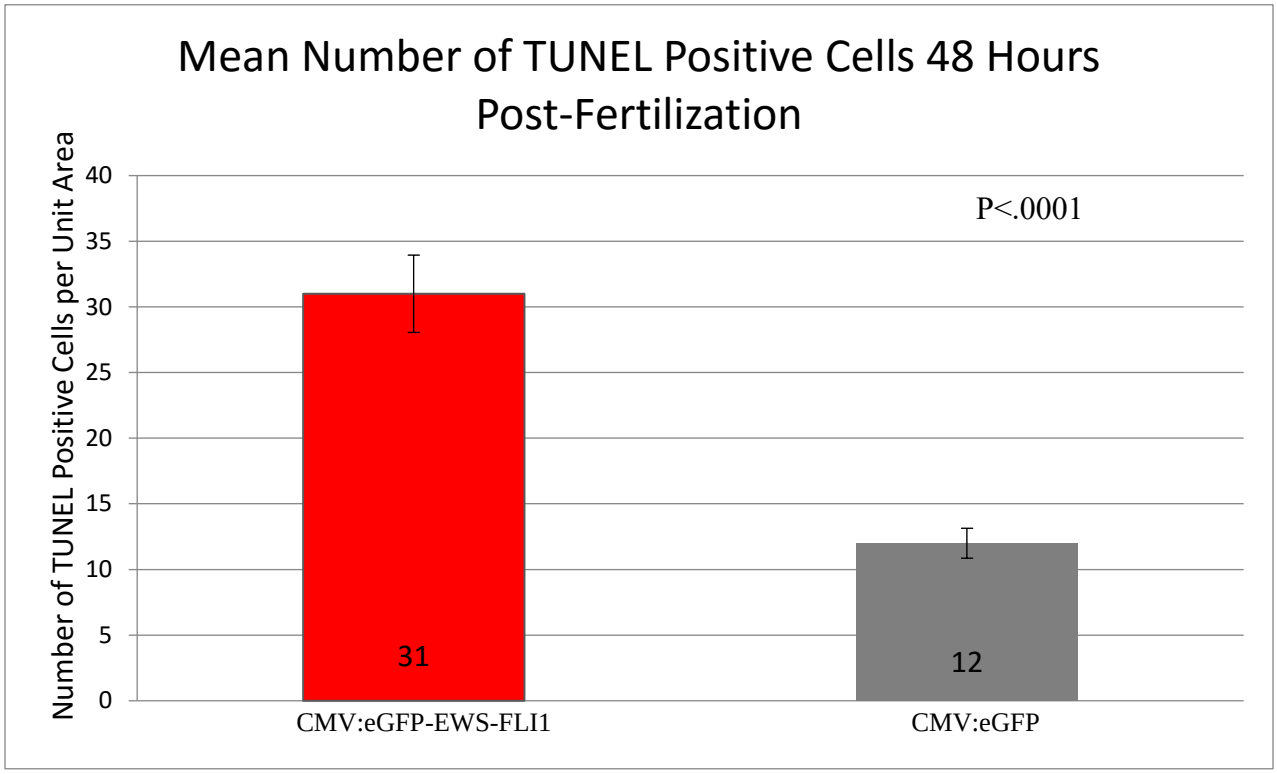


Figure 10. Average number of TUNEL positive cells 48 hours post fertilization increased (3 fold) when compared to the control.

Discussion - B

The results obtained from the follow up experiment confirmed the preliminary results obtained from figure 8. In this experiment, only 48 hour old embryos were used because the preliminary results suggested no change in 24 hour old embryos. Also, studying only one time point allowed for a larger sample size to be collected. Lowering the injection mix concentration from 10ng/µl to 5ng/µl lowered mortality rate from 70-80% to 40-50% while also decreasing the average number of EWS-FLI1 TUNEL positive cells from 77 to 31. This indicates a direct correlation between the amount of EWS-FLI1 injected and the number of TUNEL positive cells. Increasing the primary antibody dilution for GFP from 1:200 to 1:500 decreased the amount of nonspecific antibody binding; however, the GFP stain still did not accurately represent previous GFP expression. Further studies will need to be conducted in order to attempt to localize GFP expression.

Conclusion

The experiments conducted and discussed in this paper aimed to understand if EWS-FLI1 induces apoptosis more than the control. Based off the findings obtained from both experiments, it can be concluded that EWS-FLI1 induces 3-4 times the normal amount of apoptosis. The significance of these results suggest that one potential mechanism in which Ewing sarcoma is able to proliferate could be through bypassing cell cycle checkpoints which would lead to a decrease in the number of apoptotic cells. However, more research will need to be conducted in order to validate this claim. The results also establish a baseline for any future experiments in which the amount of apoptotic cells are compared between EWS-FLI1 and potential candidate genes that may aid or hinder EWS-FLI1 in cell proliferation / tumorigenesis.

Acknowledgements

I would first like to thank Deborah McGill, Anne Leazar, and Dr. Mihaela C. Stefan for inspiring me to apply to the Green Fellowship program. I also want to thank Dr. Nancy Street, Dr. Dean Sherry, and Vanessa Powell for allowing me the opportunity to participate in this program. Also, I want to thank Nikit Venishetty for his contribution to the project. Finally, I want to thank Dr. James F. Amatruda and Dr. Joanie C. Neumann for their guidance and mentorship throughout my time as a Green Fellow.

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