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Altered Integrin Alpha 6 Expression As A Rescue For Muscle Fiber Detachment In Zebrafish (Danio Rerio)

Rose E. McGlauflin

ALTERED INTEGRIN ALPHA 6 EXPRESSION AS A RESCUE FOR MUSCLE FIBER DETACHMENT IN ZEBRAFISH (*DANIO RERIO*)

by

Rose E. McGlauflin

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Advisory Committee:

Clarissa A. Henry, Ph.D., Associate Professor of Biological Sciences, Advisor
Mary S. Tyler, Ph.D., Professor of Zoology
Mary Astumian, Henry Lab Manager
Michelle Smith, Ph.D., Assistant Professor of Biological Sciences
Mark Haggerty, Ph.D., Honors Preceptor for Civic Engagement

Abstract

Cells adhere to their extracellular matrix by way of integrins, transmembrane molecules that attach the cytoskeleton to the extracellular basement membrane (one kind of extracellular matrix). In some muscular dystrophies, specific integrins are disrupted and muscle fibers detach from the myotendenous junction and degenerate. This integrin disruption causes a constant cycle of regeneration and degeneration, which greatly harms the tissue over time. Congenital muscular dystrophy affects the integrin alpha 7 gene and prevents the muscle cell from producing the affiliated protein. In an attempt to rescue integrin alpha 7 dystrophies, this project over-expressed another integrin, integrin alpha 6, in zebrafish (*Danio rerio*). Preliminary data suggest it is possible to prevent fiber detachments in muscle, a characteristic of dystrophy, by over-expressing integrin alpha 6 in zebrafish lacking integrin alpha 7. Furthermore, a limited number of embryos, with both integrin alpha 7 and dystroglycan 1 deficiencies, show improved muscle structure when over-expressing the integrin alpha 6 protein. The integrin alpha 6 protein may hold promise for gene therapy applications because it already exists within the muscle fibers, reducing the risk of immunorejection. This protein may serve a useful therapeutic purpose if it can be both expressed uniformly and effectively in human skeletal muscle.

Acknowledgements

Taking on this thesis was incredibly challenging. I began this project with no idea where I was headed. Primarily, I would like to emphatically thank Clarissa Henry for giving me this opportunity as well as Jonathan Grant for convincing Clarissa to let me work in her lab. This project carried me through my senior year when I became bored with the tedium of classes. Clarissa's enthusiasm and ability to switch quickly between tasks and mindsets is a true inspiration. I would also like to express my thanks to Mary Astumian and Elizabeth Mason, who helped teach me all of the techniques required to complete these experiments. Mary, particularly, was a wonderful resource and possesses incredible patience. I could not have completed this without her. I also must thank Mark Nilan for extraordinary zebrafish care and maintenance. Finally, thank you to the graduate students in the Henry lab—Beth, Erin and Sarah—for their support and guidance throughout the entire thesis process. I loved doing this work and contributing to our collective knowledge; I am so grateful for this opportunity to do real science.

Table of Contents

List of Figures and Graphs

Introduction

Cells, within a tissue, do not exist in a vacuum. They are connected to an extracellular matrix (ECM) consisting of many types of proteins and fibrous collagen. Developmental biology is concerned with determining how cells become different from one another and interact with their extracellular microenvironment. A tissue is composed of cells that attach to a collagenous basement membrane. The basement membrane separates epithelial cells from the underlying mesenchymal tissue and consists of protein fibers. This basement membrane is a critical component of the ECM. Cells that make up the epithelium of a tissue rely on transmembrane protein complexes to maintain a connection with the basement membrane and the underlying supportive tissue. Pathologies exist that disrupt a cell's connection to its environment or, specifically, its basement membrane.

Of these pathologies are the muscular dystrophies, a group of diseases characterized by the progressive weakening and wasting of skeletal muscle. Clinical presentation of muscular dystrophy begins in early childhood and progresses through late adolescence, when patients often succumb to these diseases. The first recorded observations of muscular dystrophy were made in the mid-1800s and only in males, due to the X-linked recessive pattern of inheritance. This pattern requires a mutated gene on the X chromosome to be inherited. In females, both X chromosomes must carry the mutated gene in order for dystrophy to occur. In males, only the one mutated gene on the X chromosome (inherited from the mother) is sufficient to cause dystrophy. Because of this pattern, males are affected far more often than females though not all forms of dystrophy are X-linked recessive. When muscular dystrophy was first studied,

histological studies noted that the muscle cell membrane, also known as the sarcolemma, was disrupted or completely destroyed [1]. This is an observation researchers now know to be common to all forms of muscular dystrophy.

Types of muscular dystrophy include Duchenne, Congenital, Limb-Girdle, Emery-Dreifuss, Becker (a less severe form of Duchenne) and Distal muscular dystrophies. Genetic mutations associated with these dystrophies can include one gene, for example the protein dystrophin can be completely absent due to a genetic mutation. This change causes Duchenne muscular dystrophy; however, it is often much more complex than the loss of function of one gene. Frequently, dystrophies are the result of inadequate post-translational glycosylation of the dystroglycan protein, rather than a complete mutation in this protein [2]. Other dystrophies, such as congenital and Limb-girdle muscular dystrophy, have many associated genetic mutations and these mutations vary greatly between affected individuals. For example, congenital muscular dystrophy patients often have a mutation in laminin $\alpha 2$, but a rare mutation can exist in the integrin alpha 7 protein as well [3].

These mutations, resulting in absent or defective proteins, cause the sarcolemma (the cell membrane of muscle fibers, which is normally attached to the basement membrane) to detach. When the sarcolemma is damaged, a muscle enzyme called creatine kinase seeps into the serum. Normally, creatine kinase is found in cells that use large amounts of adenosine triphosphate (ATP) and acts as an important buffer in the regeneration of ATP. Because it seeps out of damaged muscle cells, creatine kinase is used as a biomarker to detect muscle degeneration. Muscle cell detachment from the basement membrane, known as fiber detachment, is the underlying cause of muscular

dystrophy [2]. When fibers detach, the body regenerates the muscle cell using muscular stem cells called satellite cells. The constant degradation and regeneration of muscle tissue depletes satellite cells and the muscle wastes away [4]. Gradually, muscle is replaced with fatty or fibrous scar tissues.

In order to understand muscular dystrophy, it is imperative to first understand how normal muscle develops. During development, short precursor cells elongate and protrude from a boundary called the myotendenous junction (MTJ). These developing skeletal muscle cells intercalate and adhere to their respective boundaries. Finally, myotubules form when the elongated cells develop a uniform shape. These cells reach their myotome boundaries and stop extending, this is known as boundary capture [5]. Muscle cell boundaries form the MTJ. In this region, contractile forces are transmitted from the muscle to the tendon. The MTJ is rich in fibronectin and laminin, key components of the ECM. Muscle cells can elongate outside of their designated myotome and cross the MTJ. This phenomenon is known as boundary crossing and is indicative of dystrophy. To summarize, muscle cells become dystrophic by extending outside of their natural boundaries and then by becoming detached from the basement membrane. Detached muscle cells cannot survive and the muscle is broken down and repaired continuously. A similar process happens in human muscle cell development.

When studying muscular dystrophy, the scientific community uses several model organisms. In recent years, zebrafish (*Danio rerio*) are a more commonly used model, though mice models are also frequently used. Zebrafish are ideal for studying developmental biological processes because they develop externally from the parent and are transparent. Zebrafish are perfect candidates for observing muscle development

because fast-twitch and slow-twitch fibers are segregated. Slow-twitch muscle fibers use aerobic metabolism to function while fast-twitch fibers can function without oxygen and can carry out cellular respiration via anaerobic means. Slow-twitch muscle cells are the first to differentiate around sixteen hours post-fertilization (hpf). Subsequently, these fibers migrate laterally and the myoseptum begins to form [6]. Furthermore, zebrafish are an excellent model organism for studying muscular dystrophies because the genes dystroglycan, dystrophin and sarcoglycans show conserved sequences between humans and zebrafish. Finally, zebrafish develop relatively quickly, compared to other model organisms like mice, and their muscle can be observed in the hours and days following fertilization.

Muscle cells are able to maintain contact with their basement membranes through specific transmembrane proteins called integrins. Integrins are crucial in signal transduction because they link the inside of the cell to the surrounding extracellular matrix (ECM). Signals regulated through integrins include both inside-out signals that relay information from the intracellular environment to the extracellular environment and outside-in signals that communicate information from the extracellular matrix into the cell. These integrins contain both an α and β subunit with large extracellular domains. Eighteen α dimers are known to exist as well as eight β dimers, creating a total twenty-four integrin heterodimers [7]. Within the cell, integrins attach to the actin microfilaments making up the cytoskeleton. Integrins span the membrane of the cell and attach to ECM components. One of these ECM proteins is laminin, a cross-shaped molecule that attaches to the collagen making up the basement membrane. This relationship between laminin and type IV collagen is required for the formation of the basement membrane [8]. When

adhesion between laminin and integrin or laminin and collagen is disrupted, dystrophy follows.

Muscular dystrophies occur because of mutations in a myriad of human genes. One of these mutations affects the integrin alpha seven (Itga7) gene. When cells adhere to the MTJ, fibronectin is first downregulated while laminin, Itga7, Itga6 and dystroglycan (Dag1) are upregulated. The Henry lab recently described the relationship among these genes and others in the Nrk2b pathway. Itga6 operates downstream of dag1 and Itga7 and is crucial for the formation of normal myogenic differentiation and myotubule formation [9]. Adding NAD^+ can improve the overall structure of Itga7 and Itga7/dag1 morphants when added during development [10]. A similar logic could be applied to integrins and instead of using NAD^+, Itga6 could potentially improve muscle structure. Because mutations in Itga7 and dag1 result in dystrophy in both zebrafish and humans, could over-expressing Itga6 rescue any embryo from dystrophy—or, rather, prevent embryos from developing dystrophy? This is the fundamental question of this project.

In order to determine if over-expressing Itga6 could rescue Itga7 or dag1 dystrophies, it is imperative to first understand these dystrophies. Itga7 morphant phenotypes have been extensively studied. In zebrafish, they are characterized by fiber detachments at around 22% of myotomes per embryo [10]. Of the integrin chains, only Itga6 and Itga7 are expressed during muscle development. Because Itga6 is one of the predominant integrins expressed during muscle development, it can potentially rescue Itga7 deficiencies. Itga6 is already found in these fish so there is less risk of rejection by the immune system.

Itga6 is especially prevalent in developing muscle tissue early on but expression then declines. In human embryonic muscle, Itga6 levels are relatively low [11]. However, the question is not as simple as rescuing Itga7 with Itga6. If Itga6 can effectively rescue Itga7 dystrophies, is it limited to rescuing *only* Itga7 dystrophies? The Henry lab, when examining NAD^+ and its ability to rescue muscle, examined Itga7 and dag1 double morphants. Dystroglycan (dag1) morphants experience progressive muscle atrophy [12]. Double morphants experience fiber detachments in approximately 76% of myotomes per embryo [10]. In early muscle development, NAD^+ supplementation rescued MTJ morphogenesis in double morphants by allowing appropriate adhesion to laminin [10]. Because Itga6 is expressed early, perhaps it can serve a similar function as NAD^+ in Itga7/dag1 double morphants. Or, perhaps the dystrophy in the double morphants will be too extensive to rescue but Itga6 might sufficiently rescue the Itga7 fish.

Rescue can be measured by counting the number of fiber detachments per embryo and calculating the percent found per embryo. Once this value is ascertained it can be compared to the previously known values. Measuring MTJ angles and comparing them to the known Itga7 MO angles and Itga7/dag1MO angles can also help determine if the Itga6 treatments effectively rescued dystrophy.

Materials and Methods

Zebrafish Husbandry

Embryos were collected from natural spawnings of zebrafish kept on a 16-hour light/8-hour dark cycle. These embryos were kept at 28ºC in ERM (embryo rearing medium).

Gateway and Morpholino (MO) Injections

Injected embryos consist of six groups (see Fig. 9). The Itga6 gateway with Itga7 MO and the Itga6 gateway with Itga7/dag MO were the two rescue groups. Injected alone, in order to confirm previous data, were the Itga6 gateway alone, Itga7 MO alone and Itga7/dag MO alone. AB fish were left uninjected as a control. One-cell embryos were injected at the animal pole with an Itga6 gateway β-actin GFP (green fluorescent protein) over-expressing construct. The injections consisted of 1.2μL of the construct, 1μL of transposase and 2.8μL of water for a total of 5μL. The construct was stored on ice until it was injected. Fish were screened over the next few days for muscle fluorescence, indicating the over-expressing construct is effective in the developing zebrafish muscle.

Morpholinos are antisense MO oligonucleotides engineered from Gene Tools, LLC. The Itga7 MO fish were injected with a 1-part Itga7 MO per 6-parts H_2O dilution. The morpholino was injected into the yolk of the embryo. Embryos were between the one and four cell stage when the morpholino was injected. The Itga7/dag1 MO was made from 1:6 dilutions. Combining 14.7μL of H_2O, 1.5μL of dag1 morpholino 1 (MO1) and 1.8μL of dag1 morpholino 2 (MO2) resulted in a dag1 M1 concentration of 1:12 and dag1 M2 at 1:10. These concentrations were equal to when dag morpholinos were

injected alone. By combining this mixture with equal volumes of Itga7 MO the resulting concentration was 1:12 for Itga7, 1:24 for dag1 MO1 and 1:20 for dag1 MO2.

AB fish were left uninjected. Fish were kept at 28ºC in an incubator for three days before fixing. These embryos were stored in ERM that is changed daily. Embryos were checked periodically and dead embryos were discarded. Embryos injected with Itga6 gateway were screened under a microscope around 24 hours post fertilization (hpf). Glowing fish expressed green florescent protein where Itga6 was over-expressed. Embryos that glow green within the developing muscle were kept, while others were discarded.

Antibody (Ab) Staining and Immunohistochemistry

At three days post fertilization (3 dpf), any embryos still within their chorion were dechorionated. This process allows the young fish to be fixed with their tails straight, instead of curled. Straight tails are important for clearly viewing and imaging the muscle structure. Embryos were fixed in 4% paraformaldehyde for four hours at room temperature, or overnight in the refrigerator, and then rinsed five times for five minutes each in Phosphate Buffered Saline-0.1% Tween20 (PBS-0.1% Tw) on a rocker. After this, they were permeabalized for 1.5 hours in PBS-2% Triton. Embryos were then incubated overnight in a 1:20 dilution of Alexa fluor 546 at 4ºC, a process called phalloidin staining. After phalloidin staining, embryos must be kept in the dark as much as possible. The tubes were covered in tin foil whenever they were not being put into another staining solution or rinse.

The next day, once rinsed, the fish were put in AB block (5% w/v Bovine Serum Albumin in PBS-Tw) for an hour at room temperature or up to four days in the

refrigerator. Fixed embryos were sorted by condition into tubes, with no more than ten embryos per tube. To continue staining, the fish were put in polyclonal primary antibody overnight at 4ºC; specifically, they were stained first with the laminin-111 antibody using a 1:50 dilution with AB block. This antibody is distributed evenly between tubes of fish. The next morning, embryos were rinsed every thirty minutes with AB block at room temperature. Blocking after the primary antibody and before the secondary antibody is meant to reduce nonspecific background.

After at least two hours of rinsing in AB block at room temperature, or up to a few days in the refrigerator, the secondary antibody was added. To add the secondary, 1µL of GAR (goat anti-rabbit secondary) of the desired color is mixed with 199µL AB block. These embryos were already stained with phalloidin (appearing red at 543nm). Itga6 fibers glow green due to the GFP (at 488nm), leaving laminin to stain blue, or far red (at 633nm). The embryos remained the secondary antibody overnight at 4ºC. The next day, the embryos were washed in PBS-Tw for at least two hours and are ready to be deyolked.

Deyolking and Imaging

Embryos were deyolked in PBS using forceps. During this time, the yolk was removed in order to allow the embryo to lie relatively flat when mounted on a microscope slide. The head of the embryo was also severed underneath the eyes. Muscle tissue is conserved as much as possible when deyolking. Fish were then mounted in 80%glycerol/20% PBS and ready for imaging. Images of embryos were obtained using a Zeiss Axio Imager Z1 microscope with a Zeiss ApoTome attachment. Embryos were viewed at the 10x or 20x objective. Averaging at 5 frames optimizes images.

Z-stack images were taken at a 1.5μm slice thickness. The red stain, representing the actin fibers stained with phalloidin, was crucial for showing any boundary crossings or fiber detachments within the myotomes. The GFP, seen as the background and within the myotomes, was the over-expressed green fluorescent Itga6 that was already screened for prior to fixing embryos. Finally, the blue stain represented laminin. Laminin was expected to stain particularly strongly at the MTJ because this is where the muscle fibers are attaching via integrins.

Results

To measure the levels of dystrophy in embryo muscle, fiber detachments were counted in each embryo. Operating under the assumption that all embryos have twenty-eight myotomes each at 3dpf, the percentage of fiber detachments per embryo was calculated. These values were compared in the following figures (Fig. 6, 7). The percentages of fiber detachment were not calculated for the AB control group or the Itga6 gateway only group. These fish, when examined, had normal tails and muscle. There is no reason to suspect over-expressing Itga6 would cause dystrophy. Healthy muscle structure in Itga6 over-expressing embryos was confirmed upon imaging.

The Itga7 MO Rescue

Most embryos within the Itga7 MO rescue group showed no fiber detachments (Fig. 1). The actin filaments, stained red with phalloidin in figure 1A and shown in grayscale in Figure 1B, were attached at the MTJ. This muscle looked very similar to the muscle of AB (control) embryos (Fig. 8). The rescued muscle showed far fewer fiber detachment events than when Itga7 MO is injected alone (Fig. 5). These initial observations suggest the rescue may have worked. The GFP in Fig. 1 was due to the over-expression of Itga6 due to the gateway injections. This group of embryos stained uniformly and, upon inspection under the microscope, showed a green background. There were also muscle fibers that stained more brightly than others—a normal phenomenon when looking for over-expression, but not ideal. This stain confirmed that Itga6 is indeed over-expressed within the muscle. The few zebrafish with curved tails showed many fiber detachments when examined under the microscope. Imaging these embryos was difficult because their curved tails keep them from lying flat beneath the coverslip. Despite this,

Figure 2 from the same group, showed many fiber detachments in nearly every myotome in the image.

Itga7 MO embryos experienced fiber detachment at approximately 22% of myotomes per embryo [10]. In the corresponding rescue group (Itga7 MO with Itga6 gateway), the average amount of fiber detachment decreased to 13%, (Fig. 6). Notably, the standard deviation for this population was very high at ±30%. Raw data show the percentage of myotomes varied from 0% in ten of the embryos, while a few embryos, with higher percentages, brought up the average substantially. Images were taken of both the large proportion of embryos with few fiber detachment events (Fig. 1) as well as the few embryos with very curved tails and many fiber detachments (Fig. 2). Because only twenty-six embryos were imaged (n=26), it is not yet possible to determine statistical significance; however, these preliminary data are promising. The five embryos with a large percentage of fiber detachments were all injected on the same day so potentially this is due to error in methodology. It is also possible the Itga6 gateway injections are not completely successful in overcoming Itga7 dystrophy. Either way, the experiment must be repeated.

The Itga7/dag1 MO Rescue

The Itga7/dag1 rescue was more difficult to measure. Though approximately equal numbers of embryos were injected in both rescue groups, the number of successful Itga7/dag1MO+Itga6 embryos dwindled either because the embryos died early on or they did not successfully express Itga6 in the muscle and were discarded after screening. The eight embryos that were imaged had relatively healthy muscle. This rescue group did not have a large discrepancy in the percentage of myotomes with fiber detachment like the

Itga7 rescue group. These embryos, at most, had fiber detachments in 7% of myotomes per embryo. For example, Fig. 3 showed the same embryo with two fiber detachments. Otherwise, the rest of the muscle fibers were attached at the MTJ. The colored image (Fig. 3A) showed Itga6 is over-expressed within the muscle. This image and Fig 3B, showing only phalloidin, when compared to the Itga7/dag1 MO alone (Fig. 4), were markedly healthier.

Itga7/Dag1 MO embryos have very unhealthy, dystrophic muscle (Fig. 4). Fiber detachment was around 76% of myotomes per embryo [10]. The few rescue embryos imaged showed an average fiber detachment of 2% with a standard deviation of ±3% (Fig. 7), an amount much smaller than when the morpholinos were injected alone. This is obviously very promising but more embryos need to be put through the experiment to determine significance.

Discussion

These data are very preliminary and only the beginning of answering the initial question: can Itga6 rescue Itga7 dystrophies? The n values for both rescue groups are too low to say statistically if dystrophy was rescued. There are ways the experiment could be tweaked, or more effectively executed, in order to better illuminate what is happening within the muscle. First, the Itga7 MO, when injected alone, showed some dystrophy but not as much as previously found in other publications [10]. There were potentially some consistency issues with the amount of morpholino injected. When embryos were injected, the amount injected into the yolk was only ascertained by looking at its size. This is not a perfect system and great care is needed to maintain a similar size morpholino injection among embryos. Even while examining the injected embryos for the appropriate size morpholino injection, this method is very subjective. If embryos are not injected with the same amount of morpholino, there will be discrepancies in the amount of dystrophy.

Embryos were fixed at 3dpf. The Igta7 MO embryos fixed at 3dpf looked fairly normal. There were a few fiber balls in most embryos but not enough to bring the percentage as high as 22%. Initially, this might suggest the rescue did not work because the fish never had dystrophy. Although this might be the case, a small number of Itga7 MO fish were fixed at 48hpf because they looked unhealthy and were likely to die. These embryos had extensive dystrophy and curved tails, typical of our previously known phenotype for Itga7 MO. Many embryos die during the three-day wait before fixing. If all of the embryos with significant dystrophy died prior to three days, they were pulled out of the data pool and discarded. In the future, it may be best to modify the experiment and fix embryos at 48 hpf before the dystrophic embryos die. This should put embryos well

enough into muscle development to see any potential dystrophies forming but hopefully allow for early fixing before the fish die within the incubator. Because the 48hpf did have dystrophy, it is probable that the morpholino was too effective, killing the embryos before they could be stained and imaged.

Obviously, there is huge dichotomy between the Itga7MO+Itga6 gateway rescue phenotypes. Most of the embryos had a small percentage of fiber detachments, if any, and one group of embryos, all injected on the same day, had curved tails and high levels of fiber detachment. This could be a dosage issue with the morpholino or a problem with the gateway injections. The Itga6 gateway injections are tricky because they must be injected at the one-cell stage into the animal pole. Timing is key and if embryos develop beyond one-cell, they cannot effectively express Itga6 uniformly when injected. Many of the Itga6 gateway injected embryos died. The doubly injected rescue groups (Itga7MO+Itga6 gateway, Itga7/dag1MO+Itga6 gateway) died even more frequently than the Itga6 gateway injected alone. These embryos were injected twice, meaning the chorion was pierced twice with a needle during very early development. This puts obvious strain on the embryo and high mortality rates are not surprising. This is another explanation for the low n value. There is also the possibility for bacterial contamination. Though the media in which embryos develop (ERM) contains methelene blue, a dye meant to keep bacteria at bay, there is always the potential for contamination.

The Itga7/dag1MO+Itga6 gateway rescue's n value could be low for any of the reasons already discussed, but it is also possible fewer embryos survived because muscle structure was so compromised with two morpholinos disrupting development on top of the strain of a second gateway injection. However, because there were eight embryos

with reduced fiber detachments compared to the Itga7/dag1MO alone (Fig. 4), there were embryos that can withstand these pressures and develop what appears to be healthy muscle. Repeating the experiment with many embryos (in anticipation of high mortality rates early on) will likely give more embryos for imaging and will also help determine if Itga6 can rescue both Itga7 deficiencies and dag1 deficiencies.

Once the embryos are about 24hpf, they are screened for glowing muscle, indicating the desired Itga6 over-expression. Many of these embryos do not uniformly express Itga6. Sometimes they do not express Itga6 at all or express it in the wrong place, for example within the gut of the embryo instead of in the muscle. Because these injections and expression are variable, injecting one hundred embryos may only yield twenty-five usable embryos. Again, the number of embryos injected per experiment should be very high to account for this. It also helps to have multiple injection apparatuses set up at once, so the Itga6 gateway can be injected immediately and then be followed by the morpholino before the cell develops beyond the one-cell stage.

Though these preliminary data are exciting, they only reflect the fiber detachment events within the rescue group embryos. This is not the only method of measuring dystrophy. Often, when dystrophy occurs, the somites go from V-shaped to C-shaped—the MTJ angles increase. Determining the average MTJ angle is another way to conclusively say if these embryos are rescued, not only in the muscle fibers, but also at the MTJ itself. Another possibility for defining dystrophy is a motility assay. The muscle structure may look pristine, but can these embryos effectively respond to a stimulus? Can they swim away from the stimulus in a reasonable amount of time when compared to the control embryos? Can they swim at all? The motility assay is useful for determining if the

Itga6 rescue is successful, not just at the cellular level, but at a mechanical level sufficient to allow locomotion.

If the experiment were repeated and the results were further fleshed out to show Itga6 rescuing Itga7 dystrophies, the Itga6 protein would be a particularly good candidate for gene therapy application. Currently, gene therapy is a scientific work in progress and cannot cure disease—nonetheless, there are limited examples of physicians and scientists treating illness by over-expressing specific genes in humans. Gene therapy effectively reduced muscle myopathy in mouse models by over-expressing basement membrane components in these animals [13]. Over-expression of integrins in order to overcome dystrophy is a completely novel approach in muscular dystrophy gene therapy and this experiment has never been carried out in any context, let alone in zebrafish; however, other genes and their affiliated proteins are currently being considered as candidates for treating muscular dystrophy.

In order to develop gene therapies, scientists must ascertain the best potential protein for treating an illness, but they must also find a mechanism for sneaking these proteins into cells without raising an immune response. For example, patients with Itga7 muscular dystrophies cannot receive a transfusion of Itga7 because the innate and acquired immune responses will cause systemic infections [14]. Muscular dystrophy already causes an immune response in the muscle. When muscle regenerates, the tissue is rich with major histocompatability complex (MHC) class I molecules [15]. These cell surface molecules bind to antigens and tag them for destruction. This puts dystrophic muscle, already saturated with MHC Class I, at a disadvantage for receiving the potential therapeutic benefits of gene therapy. Though the immune system would recognize and

attack the foreign protein despite the tissue type, dystrophic muscle is even more likely to trigger a systemic immune response due to the ongoing myofiber necrosis [14]. A perfect gene therapy would introduce the effective protein into a tissue while also minimizing toxic effects due to immunorejection.

Treating any disease with gene therapies is daunting because physicians are not expressing a gene in a one-cell embryo, as researchers might in a laboratory. Muscular dystrophy patients are full, complex organisms with trillions of cells. Forty percent of the body's mass is made up of skeletal muscle. Researchers are challenged to deliver a lacking protein to all the affected cells within the body. Presently, this feat is impossible. A fully differentiated human cannot receive Itga6 into every cell to rescue an Itga7 deficiency. Regardless, it may be possible to locally treat cells with a therapeutic protein in the attempt to alleviate symptoms of disease. Research suggests hundreds of intramuscular injections could be used to improve the quality of life for these patients. A protein could also be introduced into the vascular system and, with the help of vasodilators like histamine and papaverine, can make contact with the muscle fiber's surface [16]. Scientists are also exploring gene transfer via stem cells by taking hematopoietic muscle-derived stem cells out of a patient, modifying the cells with the appropriate gene, using a virus, and then transplanting these cells back into the patient [14]. Similar methods are already being used to alleviate suffering in patients with other diseases.

There is no denying the challenges of gene therapy. The Itga6 bypasses some of the immunorejection challenges of gene therapy by over-expressing a gene that embryos, and perhaps someday patients, already have and express within their cells. The Itga6

over-expression could capably deceive a patient's own immune system and at the same time greatly improve muscle structure and function. The initial data exploring Itga6's ability to rescue these dystrophies are encouraging. Much more data are needed to determine if Itga6 can stealthily prevent and preserve muscle from deterioration.

Figures and Graphs

A

B

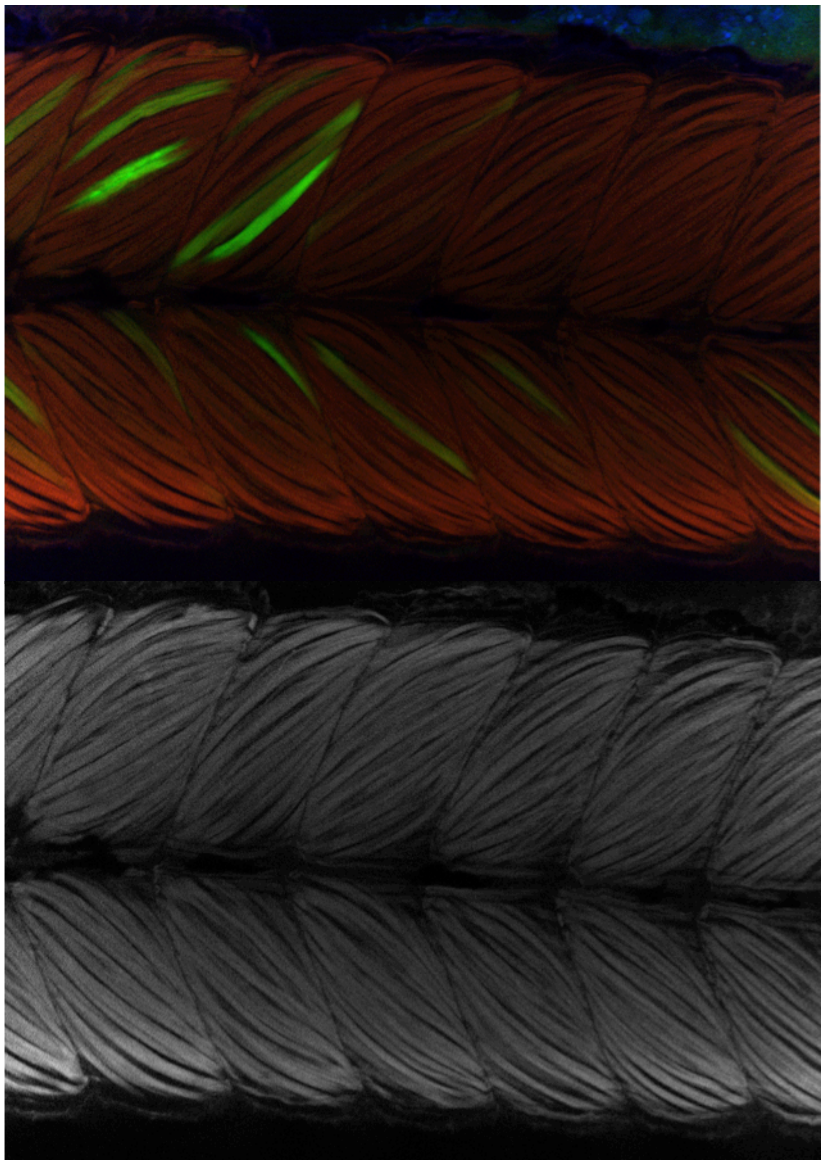


Figure 1. 3dpf Itga7 MO+Itga6 gateway, 200x.
A shows all three stains: laminin (stained blue and very faint), green fluorescent protein (GFP) staining over-expressed Itga6 and phalloidin staining actin filaments. B shows only the actin filaments stained with phalloidin. No fiber detachments are visible in this embryo.

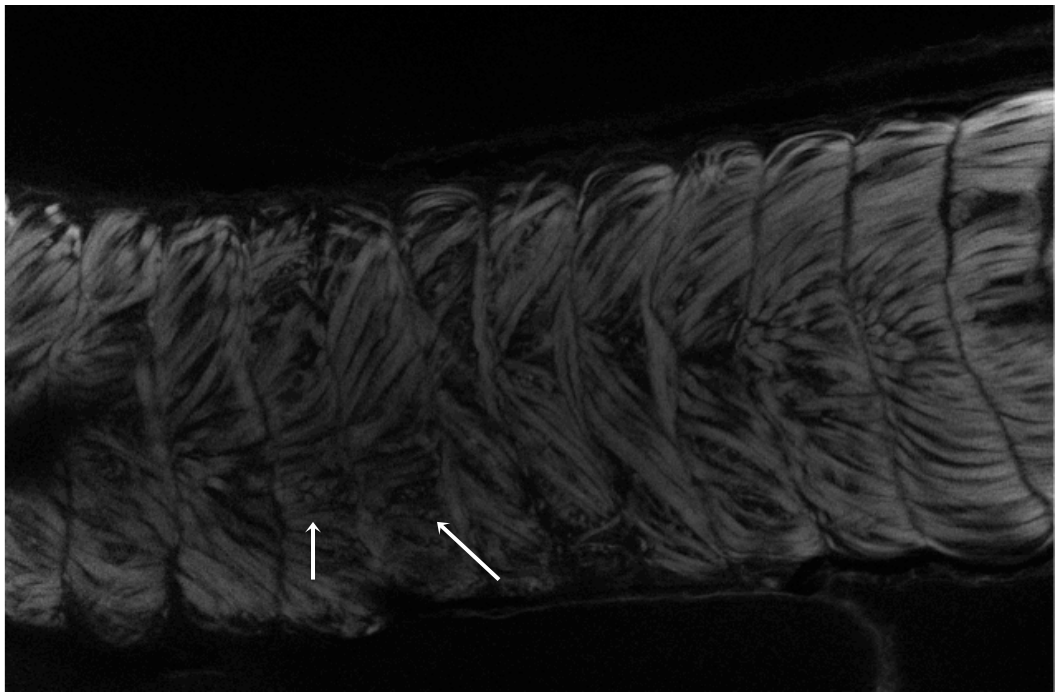
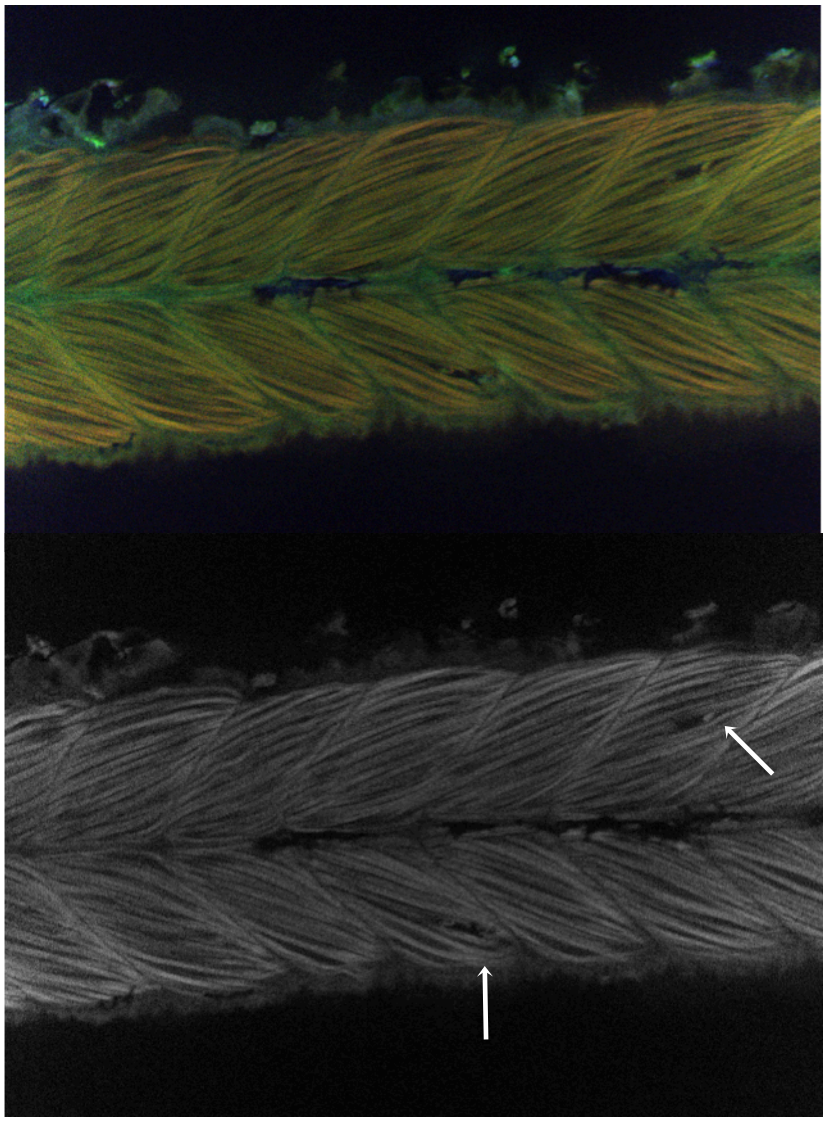


Figure 2. 3dpf Itga7 MO+Itga6 gateway, 200x.
Embryo is shown with phalloidin stain of actin filaments. There are numerous fiber detachments forming fiber balls, see arrows, and very wide MTJ angles.

A



B

Figure 3. 3dpf Itga7/dag1 MO+Itga6 gateway, 200x.
A is stained with all three stains in the series: phalloidin, GFP and laminin-111. B shows only phalloidin and white arrows refer to fiber detachments.

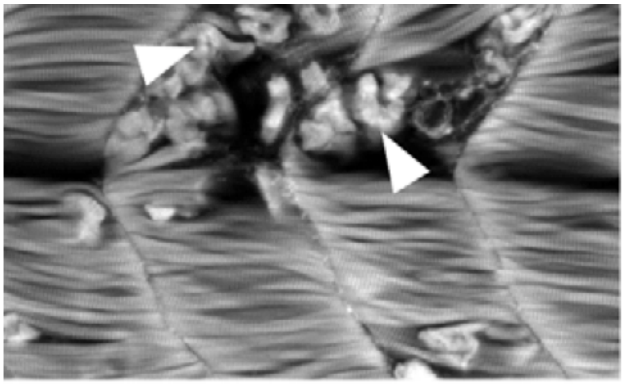


Figure 4. 3dpf Itga7/dag1 MO[10].
Itga7/dag1 MO with many fiber detachments. These embryos average fiber detachments in 76% of myotomes per embryo.

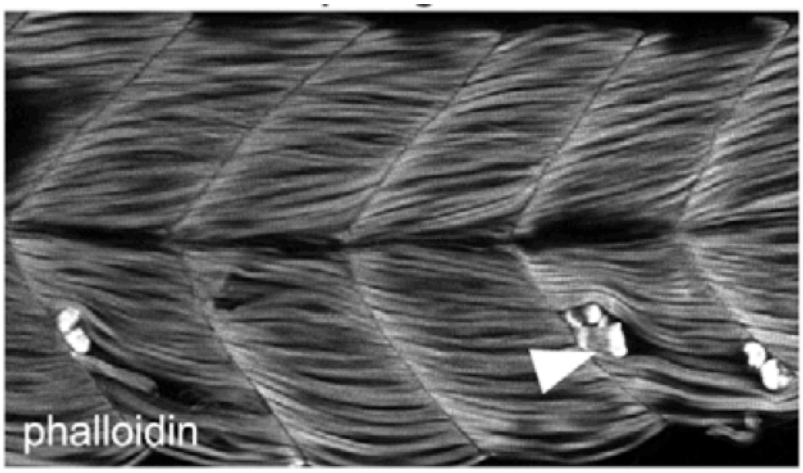


Figure 5. 4dpf Itga7 MO[10].
Itga7 MO image stained with phalloidin. These embryos average fiber detachments in 22% of myotomes per embryo.

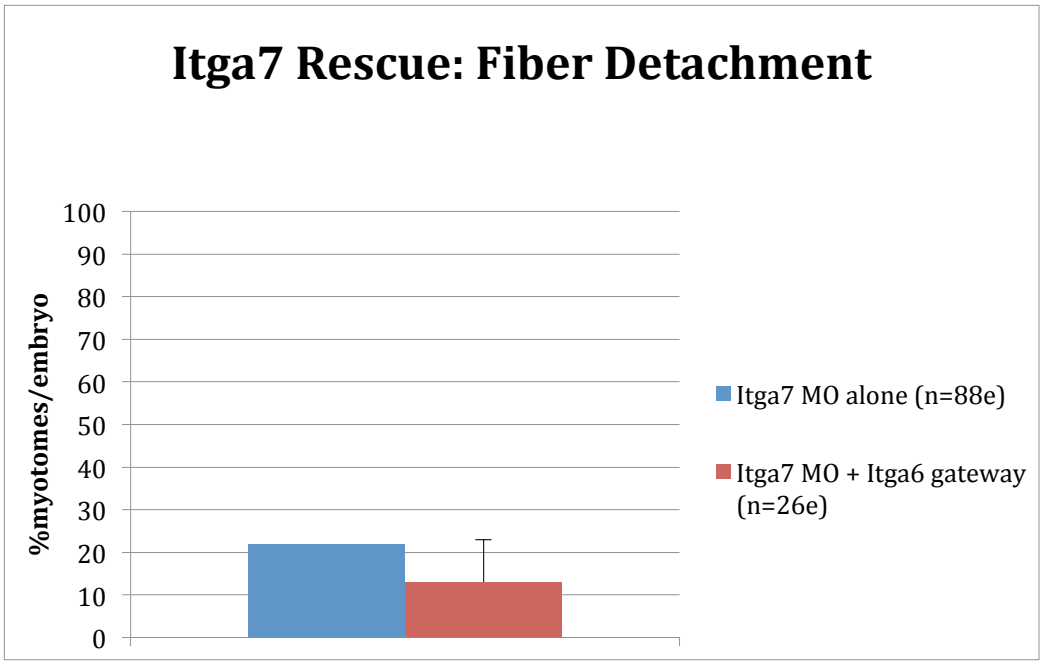


Figure 6. Graph of average fiber detachments in Itga7 MO and rescue.
On average, 22% of myotomes per embryo show fiber detachments[10]. Comparatively, only 13% of myotomes per embryo show fiber detachments in the Itga7 MO + Itga6 gateway rescue. The error bar represents standard deviation and is missing from the left bar because this data set was taken from Goody, 2012.

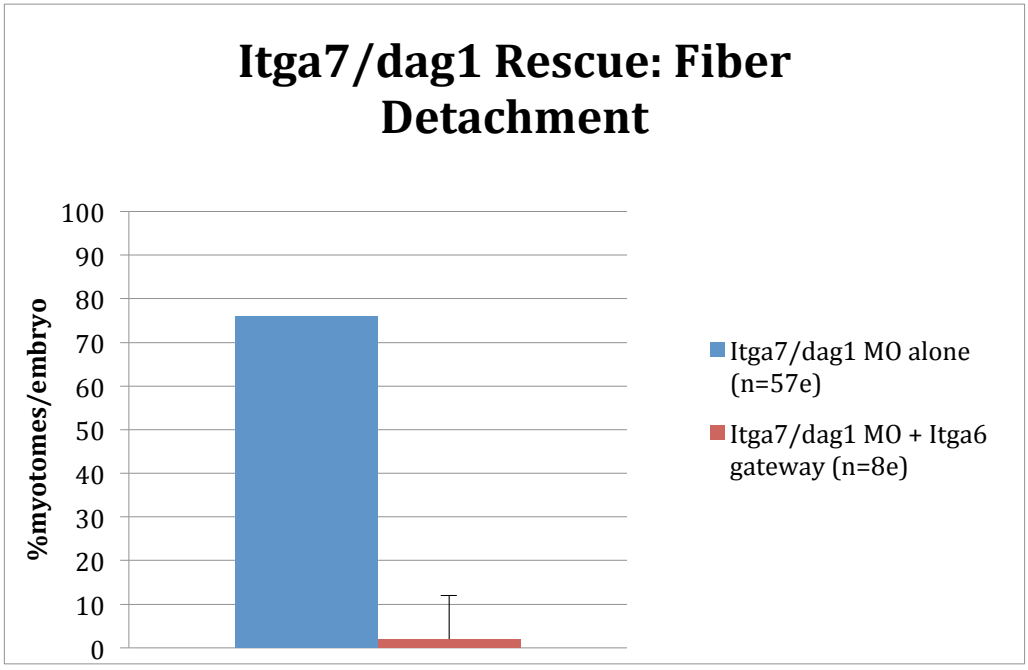


Figure 7. Graph of average fiber detachments in Itga7/dag1 MO and rescue.
On average, 76% of myotomes per embryo show fiber detachments[10] in Itga7/dag1 MO fish. Comparatively, only 2% of myotomes per embryo show fiber detachments in the Itga7/dag1 MO + Itga6 gateway rescue. The error bar represents standard deviation and is missing from the left bar because this data set was taken from Goody, 2012.

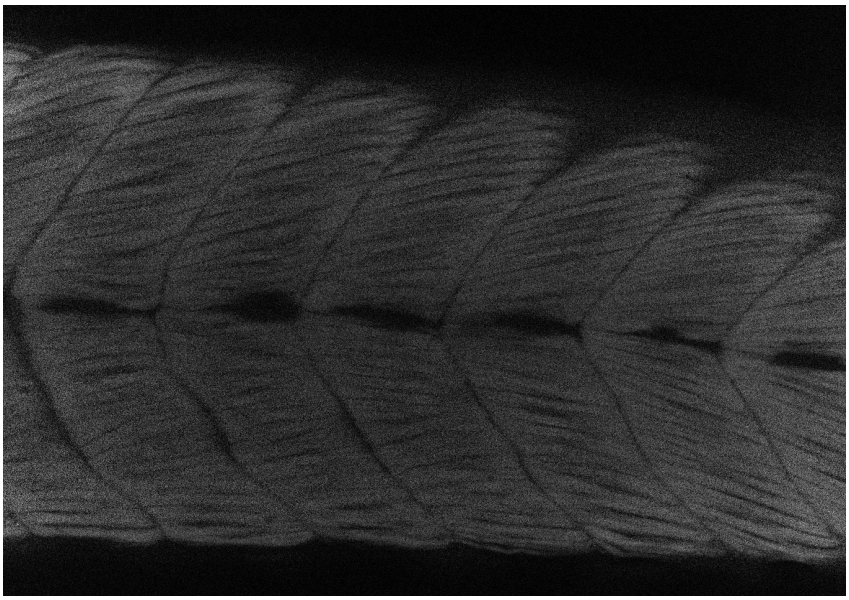


Figure 8. 3dpf AB control embryo, 200x.
Stained with phalloidin.

Group	Experimental Purpose
AB (control)	Observe and confirm embryos are developing normally
Itga7 MO alone	Look for fiber detachment levels similar to those found in previous experiments [10]
Itga7 MO+Itga6 gateway (rescue group)	Determine if fiber detachment levels decreased when compared to the Itga7 MO alone
Itga7/dag1 MO alone	Look for fiber detachment levels similar to those found in previous experiments [10]
Itga7/dag1 MO+Itga6 gateway (rescue group)	Determine if fiber detachment levels decreased when compared to the Itga7/dag1 MO alone
Itga6 gateway alone	Conclude the gateway injection is effective in over-expressing Itga6 and also to determine our expected mortality rate regardless of morpholino injections

Figure 9. Table of six injected groups of embryos and their importance in the experiment.

References

[1] Emery A (2002) The muscular dystrophies. The Lancet 359: 687-95

[2] Rahimov F, Kunkel LM (2013) Cellular and molecular mechanisms underlying muscular dystrophy. J. Cell Biol 201: 499-510

[3] Hayashi YK, Chou F, Engvall E, Ogawa M, Matsuda C, Hirabayashi S, Yokochi K, Ziober BL, Kramer RH, Kaufman SJ, Ozawa E, Goto Y, Nonaka I, Tsukahara T, Wang J, Hoffman EP, Arahata K (1998) Mutaions in the integrin α7 gene cause congenital myopathy. Nature Genetics 19: 94-97

[4] Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, Morgan JE (2005) Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122: 289-301

[5] Snow CJ, Goody M, Kelly MW, Oster EC, Jones R, Khalil A, Henry CA (2008) Time-lapse analysis and mathematical characterization elucidate novel mechanisms underlying muscle morphogenesis. PLOS Genetics 4: 1-18

[6] Bassett DI, Currie PD (2003) The zebrafish as a model for muscular dystrophy and congenital myopathy. Human Molecular Genetics 12: 265-270

[7] Takada Y, Ye X, Simon S (2007) The integrins. Genome Biology 8: 215

[8] Goody MF, Henry CA (2010) Dynamic interactions between cells and their extracellular matrix mediate embryonic development. Mol Reprod. Dev 77: 475-488

[9] Wilschut KJ, van Tol HTA, Arkesteijn GJA, Haagsman HP, Roelen BAJ (2011) Alpha 6 integrin is important for myogenic stem cell differentiation. Stem Cell Research 7: 112-123

[10] Goody MF, Kelly MW, Reynolds CJ, Khalil A, Crawford BD, Henry CA (2012) NAD+ biosynthesis ameliorates a zebrafish model of muscular dystrophy. PLOS Biology 10: 1-17

[11] Terpe HJ, Stark H, Ruiz P, Imhof BA (1994) Alpha 6 integrin distribution in human embryonic and adult tissues. Histochem Cell Biol 101: 41-49

[12] Parsons M, Campos I, Hirst E, Stemple D (2002) Removal of Dystroglycan causes severe muscular dystrophy in zebrafish embryos. Development 129: 3505-3512.

[13] Moll J, Barzaghi P, Lin S, Bezakova G, Lochmüller H, Engvall E, Müller U, Rueg MA (2001) An agrin minigene rescues dystrophic symptoms in a mouse model for congenital muscular dystrophy. Nature 413: 302-307

[14] Chamberlain JS (2002) Gene therapy of muscular dystrophy. Human Molecular Genetics. 11: 2355-2362

[15] Hartigan-O'Connor D, Kirk CJ, Crawford R, Mulé JJ, Chamberlain JS (2001) Immune evasion by muscle-specific gene expression in dystrophic muscle. Molecular Therapy 4: 525-533

[16] Greelish JP, Su LT, Lankfort EB, Burkman JM, Chen H, Konig SK, Mercier IM, Desjardins PR, Mitchell MA, Zheng XG, Leferovitch J, Gao GP, Balice-Gordon RJ, Wilson JM, Stedman HH (1999) Stable restoration of the sarcoglycan complex in dystrophic muscle perfused with histamine and a recombinant adeno-associated viral vector. Nat. Med. 5: 439-443

Appendix

All procedures involving zebrafish (*Danio rerio*) described here are reviewed and approved by the IACUC at the University of Maine.

Animal Care Certification:

Completed on: September 4, 2009

Certification number: 2009070

Author's Biography

Rose McGlauflin is a bright, young whippersnapper with a knack for solving mysteries. She is from Mount Vernon, Maine and earned a Bachelor of Science in biology from the University of Maine. After graduation, she is faced with the daunting task of becoming an actual adult, which will either mean attending medical school or moving to Barbados and adopting a pug while learning to fly single-engine airplanes—both options are equally compelling. Don't miss the next installment of Rose's majestic existence in *Rose McGlauflin Hangs Her Degree on the Wall and Stares At It* and *Rose McGlauflin's Caper Through the "Real World."*