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# **Characterization of *MyoD* and *Myf5* Double-Knockout Muscle Stem Cells During Muscle Development**

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May 2017

Honors Thesis  
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University Scholar Project  
Advisory Committee: Dr. David Goldhamer, Dr.  
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## ***Abstract***

*MyoD* and *Myf5* are transcription factors that regulate myogenesis by promoting satellite cell transcription. The two genes are known to display functional redundancy. Both genes are considered myogenic determination genes and are expressed in satellite cells. When one gene is inactive, the other is able to compensate for it and carry out normal myogenic programming. *Myf5* is the first myogenic regulatory factor (MRF) to be expressed during myogenesis, followed by *MyoD*. The fate of myogenic precursors in the absence of both *MyoD* and *Myf5* remains largely unknown. We aimed to begin attaining this knowledge as part of this project. We utilized a CreLoxP system to control the expression of *MyoD* in mice lacking *Myf5*. *MyoD* was knocked out at embryonic day (E) 11.5 during myogenesis. Limbs were collected from experimental mice 2 days following tamoxifen injection. Additionally, recombined *MyoD*<sup>-/-</sup> *Myf5*<sup>-/-</sup> myogenic progenitor cells were plated and grown individually in culture for 13 days. Immunofluorescence staining was performed on whole tissue samples and cell culture samples to analyze potential fate changes. No signal that may indicate a fate change was detected in the limbs of experimental mice collected 2 days following the excision of *MyoD*. Cell culture data demonstrated the presence of signal that may indicate a potential fate change. However, making concrete conclusions from this data proved difficult due to the lack of positive controls available.

The second component of our project involved analyzing the function of *MyoD* in the regeneration of injured muscle. The CreLoxP system was utilized to excise *MyoD* from experimental mice containing a single allele of *Myf5*. Once again, *MyoD* was knocked out at E 11.5. Seven weeks following birth, mice limbs were injured and tissue

samples were collected. Immunohistochemistry was utilized to analyze potential fate changes. Our data did not appear to demonstrate increase in signal that marks a fate switch, but is difficult to interpret with certainty without further studies.

### ***Introduction***

Skeletal myogenesis, the development of skeletal muscle tissue, takes place in three stages and results in the formation of muscle fibers, as well as muscle stem cells, or satellite cells. At each stage, myogenesis accomplishes a different functional goal. In embryonic myogenesis, dermomyotomal cells begin to develop, the limb bud is formed, and myoblasts start to arise. Later on in embryonic development, these cells proliferate and then fuse to form myotubes and, ultimately, muscle fibers. This stage of development begins at E 9.5. At E 10.5, *MyoD* is first expressed. Shortly afterwards, at E 11.5, myogenic progenitors are identified. During fetal myogenesis, which occurs from E 14.5 until birth, the basic muscle scaffolding is established and its growth and development occurs<sup>8</sup>. Normal embryonic and fetal myogenesis leads to functional muscle, comprised of fibers and satellite cells.

Satellite cells function in postnatal myogenesis, particularly during the repair of damaged muscle<sup>8</sup>. These cells are first anatomically identified at E 16.5 and promote regeneration upon injury in adult myogenesis<sup>7</sup>. Found on the periphery of muscle fibers, satellite cells come into contact with the basal lamina, which is an extracellular matrix closely associated with the epithelium. A primary characteristic of adult stem cells is the ability to self-renew damaged tissue, which satellite cells have been shown to do. Upon injury, satellite cells demonstrate self-renewal and maintain homeostasis by repopulating muscle fibers as they proliferate. Because satellite cells divide asymmetrically, the result

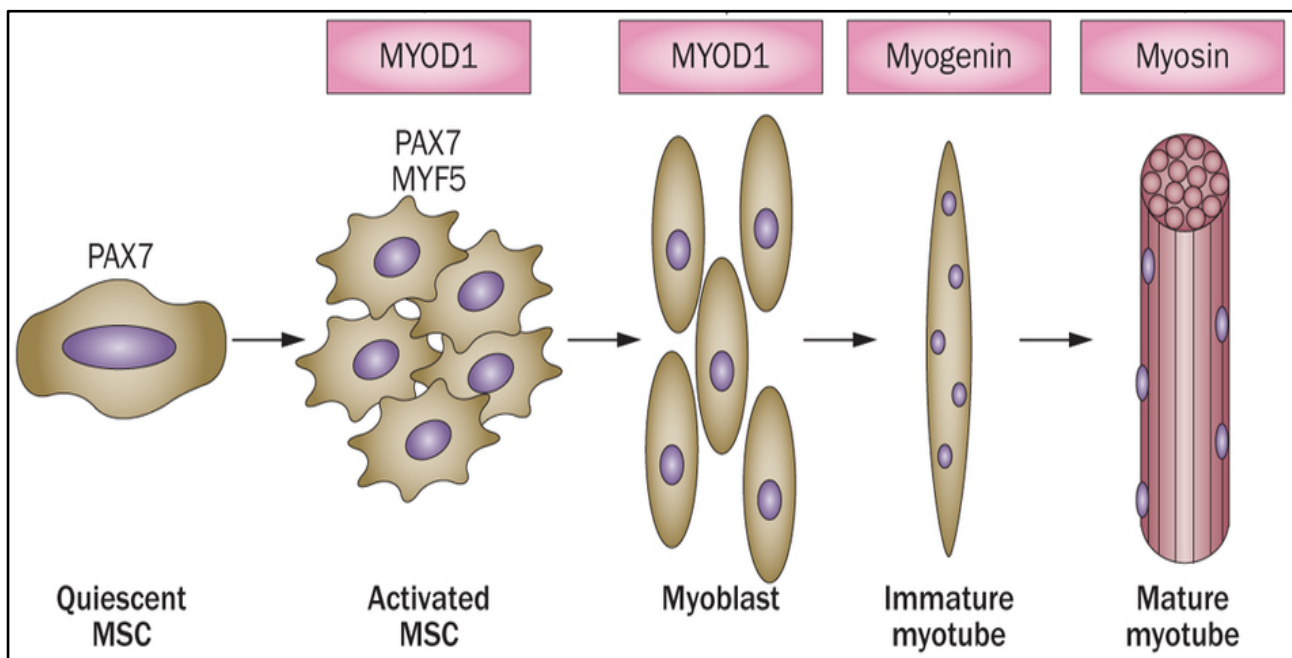
of this division is one cell that will be differentiated towards a myogenic fate and one new stem cell<sup>7</sup>.

Another important quality of satellite cells is that they express *Pax* genes, which are critical in myogenesis<sup>6</sup>. This set of genes, specifically *Pax3* and *Pax7*, regulate the entry of the satellite cells into the pathway of skeletal muscle differentiation<sup>2</sup>. Muscle fiber progenitors do not pass through the myogenic program without *Pax3* and *Pax7* and critical problems in the formation of skeletal muscle occur in animals that lack these genes<sup>2</sup>. Thus, *Pax3* and *Pax7* are vital for progression of adult satellite cells through the muscle development process.

When *Pax3* and *Pax7* promote the activity of adult satellite cells in myogenesis, these cells up-regulate the expression of myogenic regulatory factors (MRFs), *Myf5*, *MyoD*, *Mrf4*, and *Myogenin*<sup>1</sup>. The four MRFs are also active prior to adult myogenesis. These basic helix-loop-helix (bHLH) transcription factors are present and operate in different stages of development to contribute to muscle fiber formation.. The determination of cells towards the myogenic fate is governed largely by the activity of *Myf5* and *MyoD*, and in part by that of *Mrf4*. *Myogenin* is required for terminal skeletal muscle differentiation<sup>2</sup>. *Mrf4* is believed to act as a muscle determination gene in the embryonic stage of development. Although each MRF has a distinct role, there is functional redundancy among some of the genes in control of myogenic development<sup>10</sup>

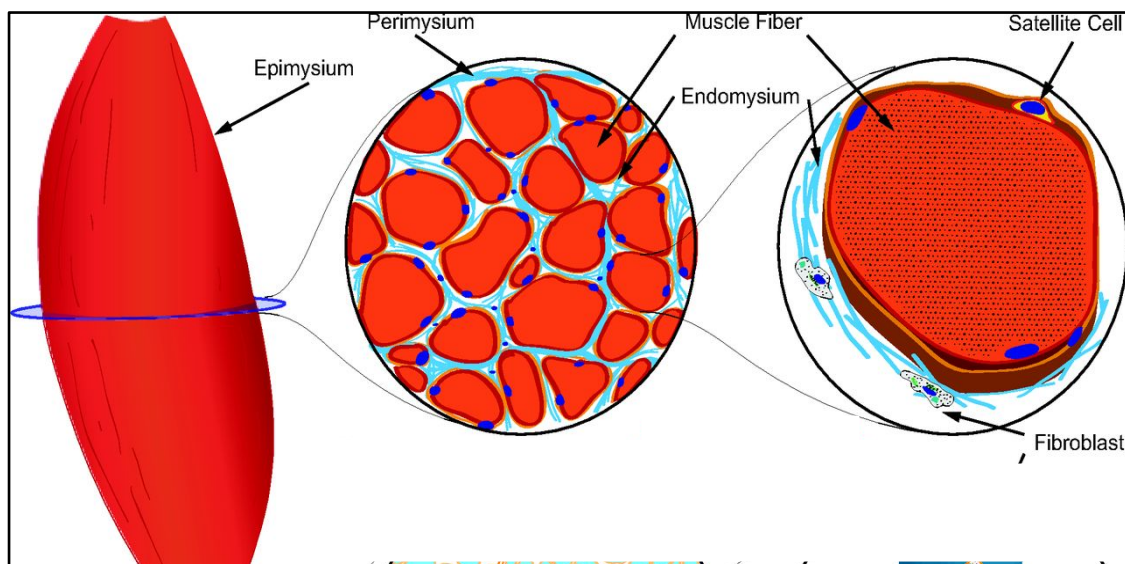
*Myf5* is the first MRF to be expressed during embryonic myogenesis, acting as a transcription factor for genes that regulate myogenesis<sup>1</sup>. *MyoD*, expressed shortly after *Myf5*, is also a transcription factor. The gene binds to enhancer sequences of muscle-specific genes to up-regulate their activity<sup>13</sup>. Although they are expressed at slightly

different time points, *MyoD* and *Myf5* are both considered myogenic determination genes, as each gene has been shown to initiate the myogenic program on its own. *MyoD* and *Myf5* are able to activate muscle genes in multiple differentiated cell lines, demonstrating their sufficiency for the downstream program of terminal muscle fiber differentiation<sup>13</sup>. Thus, although neither gene alone is necessary for myogenesis, they are both sufficient. Additionally, both genes are responsible for regulating satellite cell gene transcription. In the case of muscle tissue damage, *MyoD* is upregulated in order to promote the formation of new muscle fibers through satellite cell activation. As in myogenesis, *Myf5* has a similar function to that of *MyoD* in regeneration. Thus, *MyoD* and *Myf5* play significant, but overlapping, roles in determination and differentiation, as well as regeneration of myogenic cells.



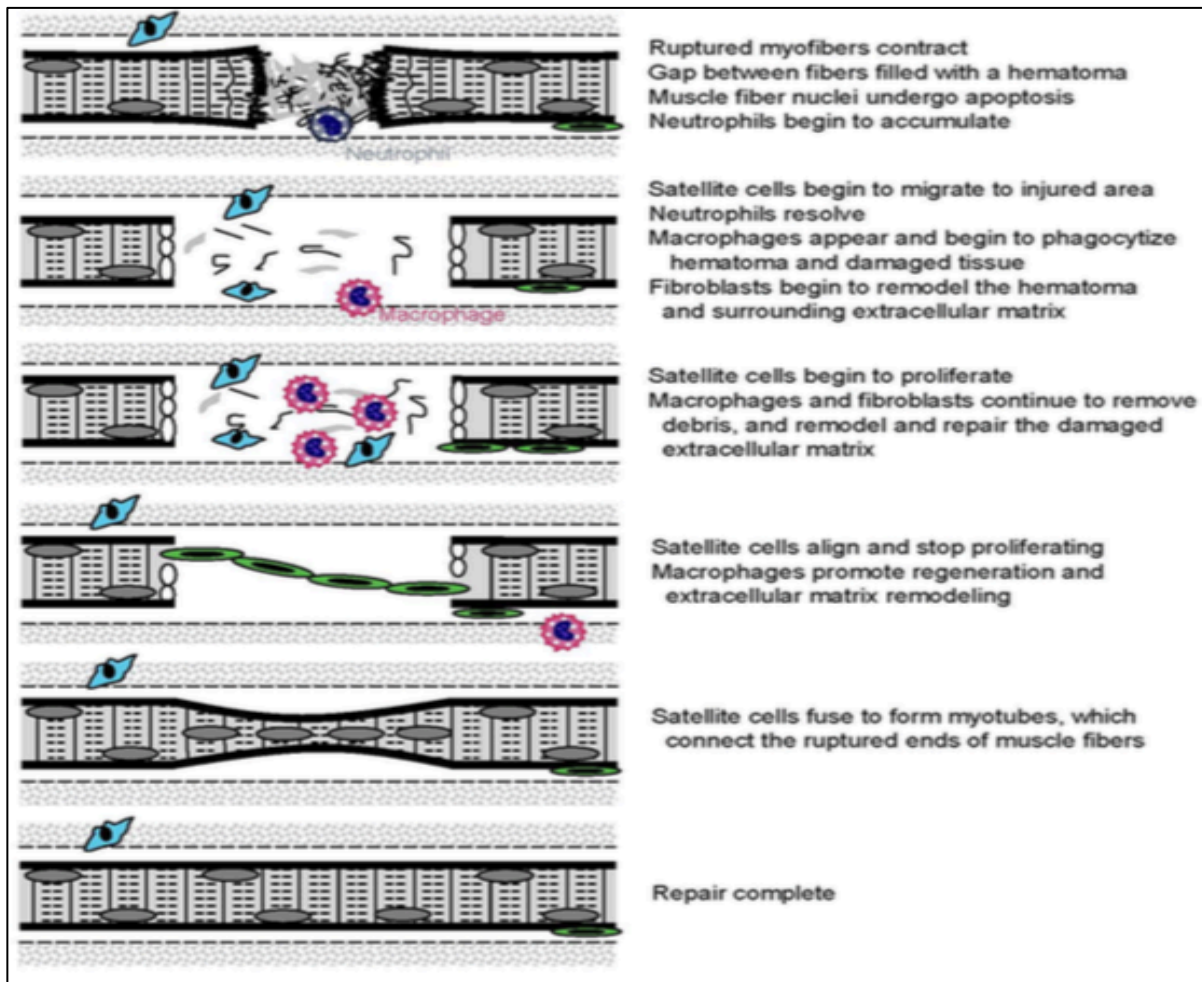
**Figure 1.** The roles of *MyoD* and *Myf5* during myogenesis. Adapted from Salvatore et al., 2014.

In 1992, Rudnicki et al. created a mouse model with a mutant knocked-out version of the *MyoD* gene. Surprisingly, the researchers found that the mice lacking *MyoD* were both viable and fertile. These mice showed normal levels of skeletal muscle-specific mRNAs and elevated levels of *Myf5* mRNA. Moreover, subsequent studies demonstrated that although limb myogenesis was somewhat delayed, these mutant adult mice appeared to have physiologically and morphologically normal muscle<sup>12</sup>. This data suggests that the activity of *Myf5* can compensate for the loss of *MyoD*<sup>9</sup>. The following year, researchers created a mouse model with both *Myf5* and *MyoD* knockouts. Because these mice completely lacked skeletal muscle and died perinatally, it was concluded that either *MyoD* or *Myf5* is essential for formation of muscle fibers<sup>10</sup>. The fate of satellite cells in the double knockout mice is still undiscovered, and exploring how satellite cell progenitors develop in mice lacking *Myf5* and *MyoD* was the goal of my research. Previous work in the Goldhamer lab has shown that when both *MyoD* and *Myf5* are absent, satellite cell progenitors acquire different fates and thus we anticipated observing fate changes in this population of cells.



**Figure 2.** Normal skeletal muscle anatomy. Adapted from Davis, et al. 2013.

When myogenesis is complete, functional skeletal muscle is formed, as presented in Figure 2. Multinucleated fibers are organized upon an extracellular matrix (ECM) that is comprised of three layers: the epimysium (outermost layer), the perimysium (intermediate layer) and the endomysium (innermost layer)<sup>7</sup>. When severe injuries occur, as during a disease or trauma state, muscle fibers atrophy and scar tissue accumulates throughout the ECM. An increase in scar tissue promotes a rupture in this organization of skeletal muscle, thereby perpetuating disease states.



**Figure 3.** Muscle regeneration following injury. Adapted from Dueweke, et al. 2016.



At the cellular level, injury causes nuclei at the damaged site to undergo apoptosis, as shown in Figure 3. To repair muscle, satellite cells become activated, migrate to the injured location of the muscle, and proliferate to repopulate lost cells<sup>6</sup>. At this stage, both *MyoD* and *Myf5* contribute to the influx of new myogenic progenitor cells. These satellite cells then differentiate into myoblasts, fuse to form myotubes, and ultimately become functional muscle fibers<sup>2</sup>. Following muscle regeneration, some satellite cells return to a quiescent state in order to respond to subsequent injuries. The second component of this project involved analyzing if and how muscle is able to regenerate in the absence of *MyoD*. Because *Myf5* and *MyoD* display functional redundancy, we hypothesized that regeneration would continue to occur.

To analyze the capacity of muscle to develop in the absence of *Myf5* and *MyoD*, a conditional knockout mouse model, which contains one conditional *MyoD* allele and is homozygous null for *Myf5*, was utilized. We utilized the CreLoxP system to knock out expression of *MyoD* at E 11.5, creating double-knockout experimental mice. The Cre protein enabled the recombination of the *MyoD* allele with the *R26<sup>NG</sup>* allele, which contains green fluorescent protein (GFP). As such, recombined alleles that lack *MyoD* were marked with GFP. However, recombination efficiency is not perfect and this can result in recombined cells that do contain the *MyoD* allele. Fusing the Cre protein to an estrogen receptor (ER) enabled us to control the function of the protein, and thus the expression of *MyoD*, with tamoxifen, a drug that mimics estrogen. In sum, when tamoxifen binds the Cre protein, the protein is able to enter the nucleus and subsequently excise *MyoD*. Therefore, the administration of tamoxifen done via intraperitoneal (IP) injection renders *MyoD* inactive in target cells.

In order to assess the roles of *MyoD* and *Myf5* in regeneration upon injury, we utilized the CreLoxP system coupled with tamoxifen injections to excise *MyoD* from mice that contained a functional allele of *Myf5*. In this way, we were able to knock out *MyoD* and examine if and how regeneration is able to occur solely through the effects of *Myf5*.

As part of my Honors Scholar Thesis, I have worked in Dr. David Goldhamer's lab to characterize the development of myogenic precursors that lack *MyoD* and *Myf5*. We first analyzed the development of these cells in the absence of *MyoD* and *Myf5* within the limbs. Subsequently, we plated cells of interest and grew them for 13 days, after which we analyzed potential fate changes through immunohistochemistry. Lastly, we explored the role of these genes in regeneration by knocking *MyoD* out of *Myf5*<sup>+</sup> experimental mice. Following injury, injured myogenic cells in mice were examined using immunohistochemistry. By being aware of the specific functions of genes involved in regulation of satellite cells, we can begin to understand the development of satellite cells within this gene regulatory network and in disease states involving muscle injury.

## ***Methods***

### I. Experimental Mouse Model

To begin our analysis, we utilized experimental mice lacking *Myf5*. Conditional *MyoD* alleles were knocked out using the CreLoxP system. To generate the mouse model utilized for these experiments, a Cre mouse was bred with a mouse that contained LoxP sites around the *MyoD* allele. The genotypes of the two mice crossed were as follows: *MyoD*<sup>iCreER/+</sup>*Myf5*<sup>-/-</sup> and *MyoD*<sup>cKO</sup>*Myf5*<sup>-/+</sup>*R26*<sup>NG</sup>. The Cre protein was fused with an estrogen receptor (ER) and its activity was controlled by tamoxifen, a drug that mimics

estrogen. After obtaining double-knockout animals with the genotype *MyoD<sup>iCreER/+</sup>Myf5<sup>-/-</sup>R26<sup>NG/+</sup>* we performed various experiments.

## II. Tissue Collection

First, tamoxifen was administered at E 11.5. Two milligrams of tamoxifen and 1 milligram of progesterone, included to minimize negative side effects of tamoxifen, were each diluted in corn oil and given to pregnant experimental mice through an IP injection. Following tamoxifen injections, the genotype of experimental mice was confirmed using polymerase chain reaction (PCR). Subsequently, tissue from experimental mice was collected. After dissection, muscle and whole embryo samples were fixed with paraformaldehyde, frozen in optimal cutting temperature (OCT) compound and cryosectioned at 10 micrometers. The first set of samples was imaged only with DAPI staining first. DAPI at a concentration of 0.1 µg/ml was added to slides for 10-15 minutes. Following imaging with an inverted fluorescence microscope, these slides were then stained with anti-GFP antibody to confirm the presence of GFP+ cells. The slides were placed in anti-GFP primary antibody overnight, washed 3 times for 5 minutes each in PBS, placed in anti-chicken secondary antibody, washed again with PBS as previously mentioned, cover-slipped and imaged. All other samples were stained as noted in Table 1.

## III. Immunohistochemistry Staining

Immunofluorescence staining was performed to identify PAX7+ cells, which were thought to be myogenic stem cells. The embryo and tissue samples were also stained for CD31, which is a marker of endothelial cells, Perilipin, which labels adipocytes, and Myosin Heavy Chain, which identifies a myogenic fate. Table 1 depicts the markers we utilized and their corresponding cell lineages. Data from the double-

knockout animals was compared to data from wild-type control mice that contained one functional allele of either *Myf5* or *MyoD*.

Samples that were to be stained for PAX7 were blocked in Mouse-on-Mouse (MOM) for two hours to reduce background signal. Similarly, samples that were to be stained for CD31 and Myosin Heavy Chain were placed in PBSMT, a blocking solution made of 1.5% Bovine Serum Albumin (BSA), 1.5% dry milk, and 0.1% TritonX in PBS for two hours. Samples that were to be stained for Osterix and Perilipin were blocked in a solution made up of 1.5% BSA, 10% goat serum, and 0.1% TritonX. Primary antibodies were diluted in their respective blocking solution, placed onto the samples, and left overnight. PAX7 primary antibody was diluted in PBSMT. The next day, samples were washed three times in PBS at five minutes per wash. Secondary antibodies were diluted in their respective blocking solutions and placed onto samples for 1 to 2 hours. PBS washes were repeated as previously stated. DAPI was placed onto samples for 10-15 minutes at a concentration of 0.1 µg/ml. Lastly, whole tissue samples on slides were mounted in Fluorogel, cover-slipped, and imaged using differential interference contrast (DIC) microscopy. Brightness of whole images was edited using Photoshop to allow better visualization.

<b>Lineage</b>	Satellite Cell	Endothelial	Adipogenic	Myogenic	Osteogenic
<b>Marker</b>	Pax7	CD31	Perilipin	Myosin Heavy Chain	Osterix
<b>Primary Antibody</b>	Mouse Polyclonal Pax7	Rat-Anti Mouse CD31	Sigma P1873	MF20	Rabbit Osterix
<b>Primary Antibody Dilution</b>	1:10	1:100	1:500	None	1:250
<b>Secondary Antibody</b>	Alexa Goat Anti-Mouse	Alexa Donkey Anti-Rat	Alexa Goat Anti-Rabbit	Alexa Goat Anti-Mouse	Alexa Fluor Anti-Rabbit
<b>Secondary Antibody Dilution</b>	1:250	1:500	1:500	1:500	1:500

***Table 1.** Possible fate changes as determined by immunohistochemistry markers, and respective primary and secondary antibodies utilized for the staining protocols.*

#### IV. FACS Sorting

The recombined myogenic progenitor cells, which were identified based on GFP signal in control and double-knockout animals, were analyzed through fluorescence-activated cell sorting (FACS) at E 13.5. FACS gating isolated live cells that were GFP+ and mononuclear, as indicated by DAPI signal (Figure 12).

#### V. Single Cell Plating

Single GFP+ mononuclear cells were plated into 96 well plates previously coated with collagen. Gibco Collagen I was diluted to 50 µg/ml in 20 mM acetic acid and 30 µL of the solution was added to each plate. The cells were then grown for 8 days. The growth media was made in Dulbecco's Modified Eagle Medium (DMEM) using 20% fetal bovine serum (FBS), 2.5 ng/mL fibroblast growth factor (FGF) and 0.1% Penicillin/Streptomycin. The media was changed after 48 hours of initial plating and then

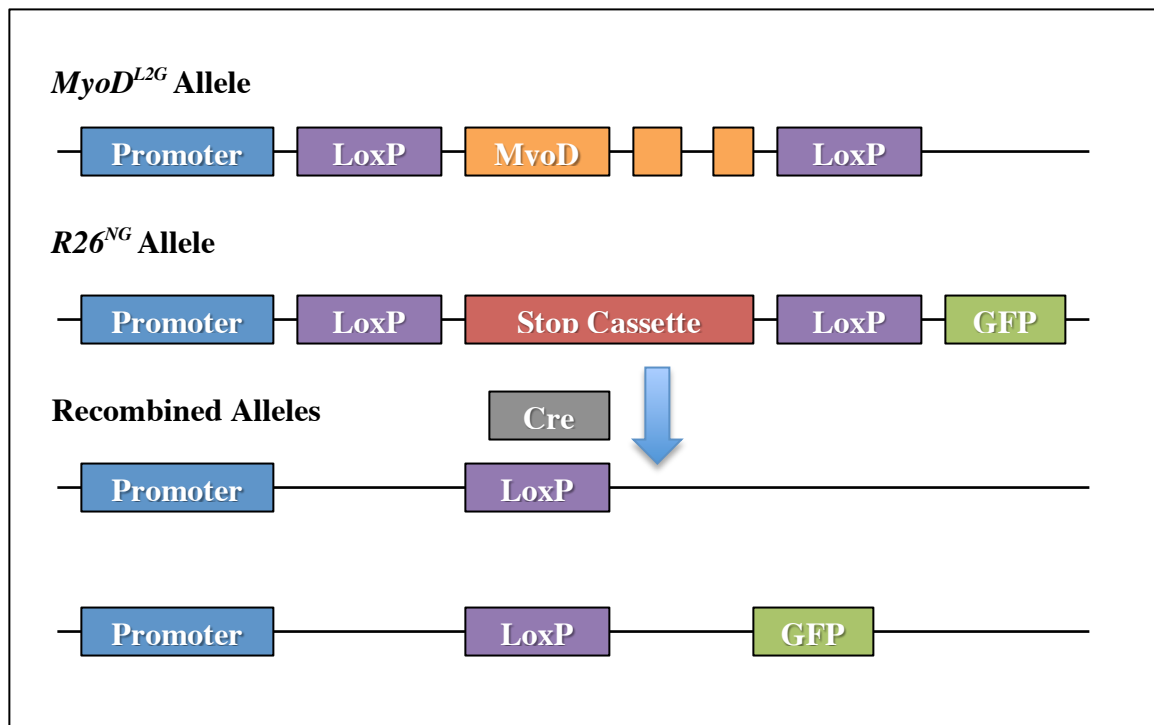
every 24 hours until day 8. After 8 days, cells were imaged using DIC microscopy. The cells were then placed in differentiation media, made in DMEM using 10% horse serum and 1% Penicillin-Streptomycin. After 5 days in differentiation media, the cells were subsequently washed, fixed, stained as shown in Table 1 and imaged. Cells from both wild type and double knockout mice were plated and treated as described above. We followed the same immunohistochemistry staining protocols for both limb tissue samples placed on slides and cell culture plates.

## VI. Injury Profile

To analyze the roles of *Myf5* in the absence of *MyoD* during regeneration, a CreER conditional *MyoD* knockout mouse model was utilized. Tamoxifen was again injected into pregnant mice at E 11.5. Seven weeks following the birth of the experimental conditional mice, hind limb muscles were injected with cardiotoxin, a snake venom that induces apoptosis. Tissue samples were collected 3 days following injury. Samples were subsequently dissected, fixed, frozen, and cryosectioned. Lastly, samples were analyzed using immunohistochemistry and imaged as described above.

## ***Results***

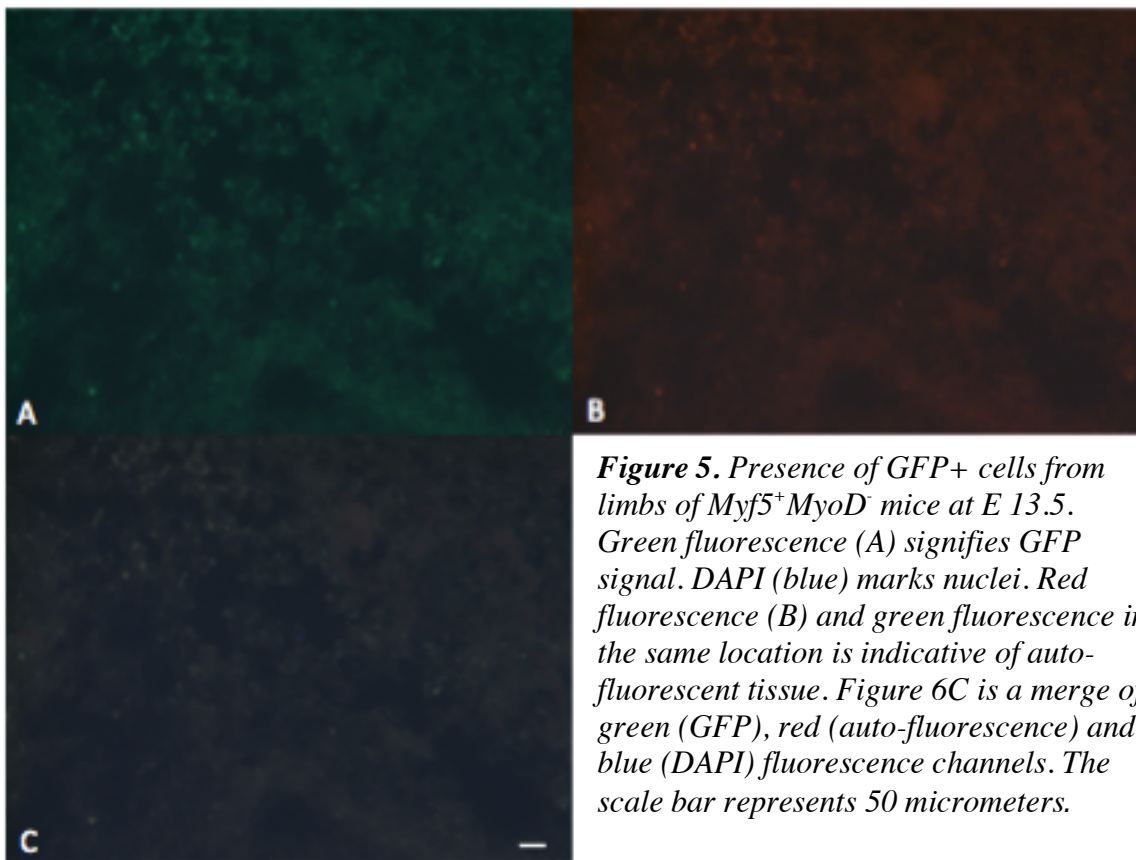
Using the CreLoxP system enabled us to create the necessary mice for our experiments, as shown in Figure 4. After crossing *MyoD*<sup>iCreER/+</sup>*Myf5*<sup>-/-</sup> mice with *MyoD*<sup>CKO</sup>*Myf5*<sup>-/+</sup>*R26*<sup>NG</sup> mice, we obtained *MyoD*<sup>iCreER/+</sup>*Myf5*<sup>-/-</sup>*R26*<sup>NG/+</sup> mice. Tamoxifen injections enabled the Cre protein to enter the nucleus and recombine the alleles of interest. As a result, GFP signal marked cells that lacked *MyoD*. However, this method does not guarantee perfect recombination frequency at the *MyoD* locus. Therefore, it is possible that some GFP+ cells contained *MyoD*.



**Figure 4.** *CreLoxP* recombination controlling the expression of *MyoD*. The *Cre* protein enters the nucleus upon tamoxifen injection and causes recombination, rendering *MyoD* inactive and *GFP* active. Thus, recombined cells that lack *MyoD* will be marked with *GFP*.

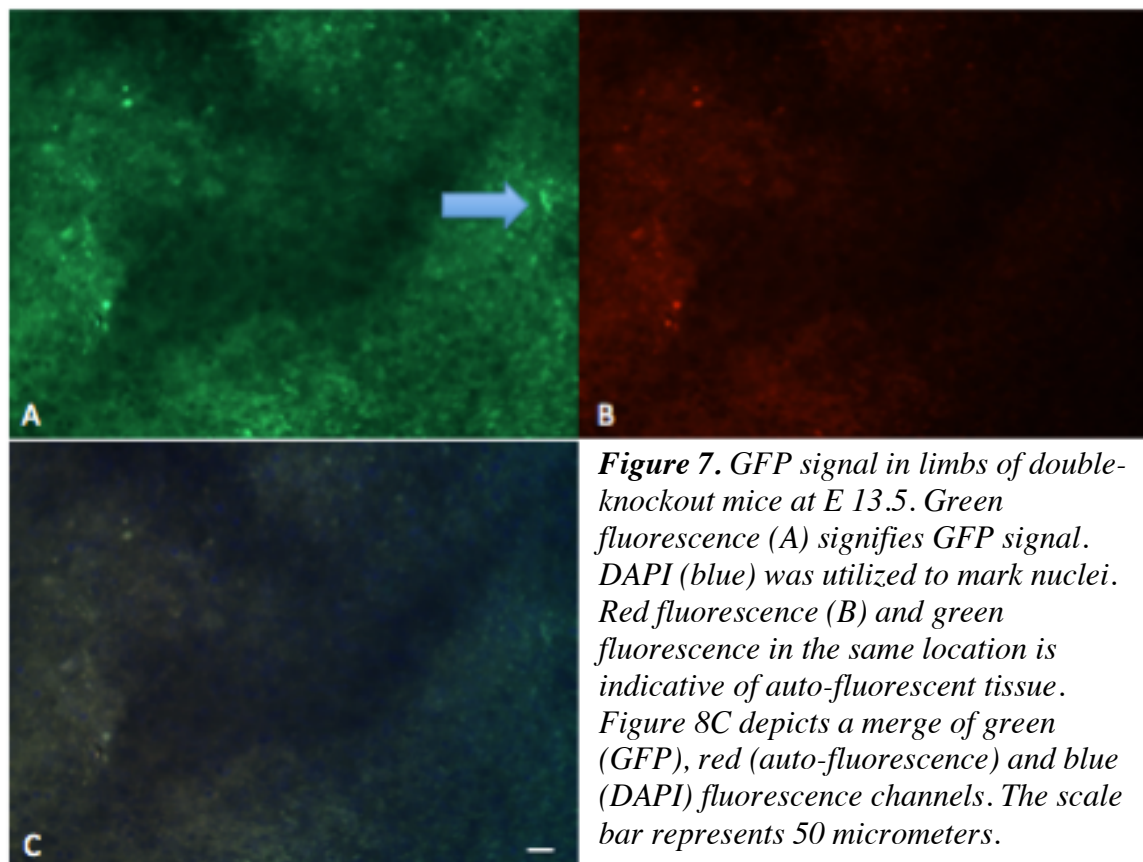
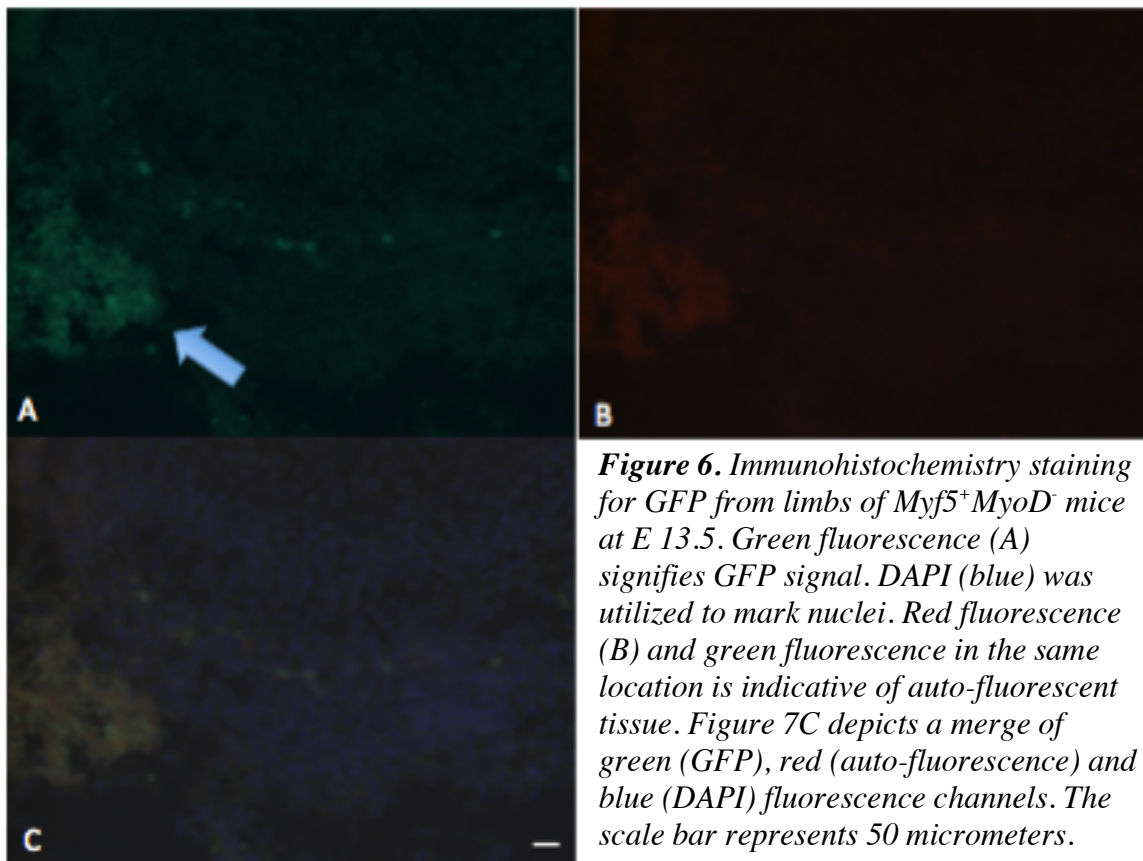
We began our experiments by sectioning and visualizing control animals containing one allele of *Myf5*. This data is shown in Figure 5. DAPI marks nuclei, which enabled us to detect the presence of cells apart from background or debris. The green channel depicts *GFP* signal, while cells that display signal in both the red and green channels are auto-fluorescent. Because the fluorescence signal appeared weak and we saw significant background interference, we stained the slides with anti-*GFP* antibody in order to amplify the signal of interest (Figure 6). We then compared these data to images of double-knockout animals at the same time point (Figure 7). Subsequently, we stained

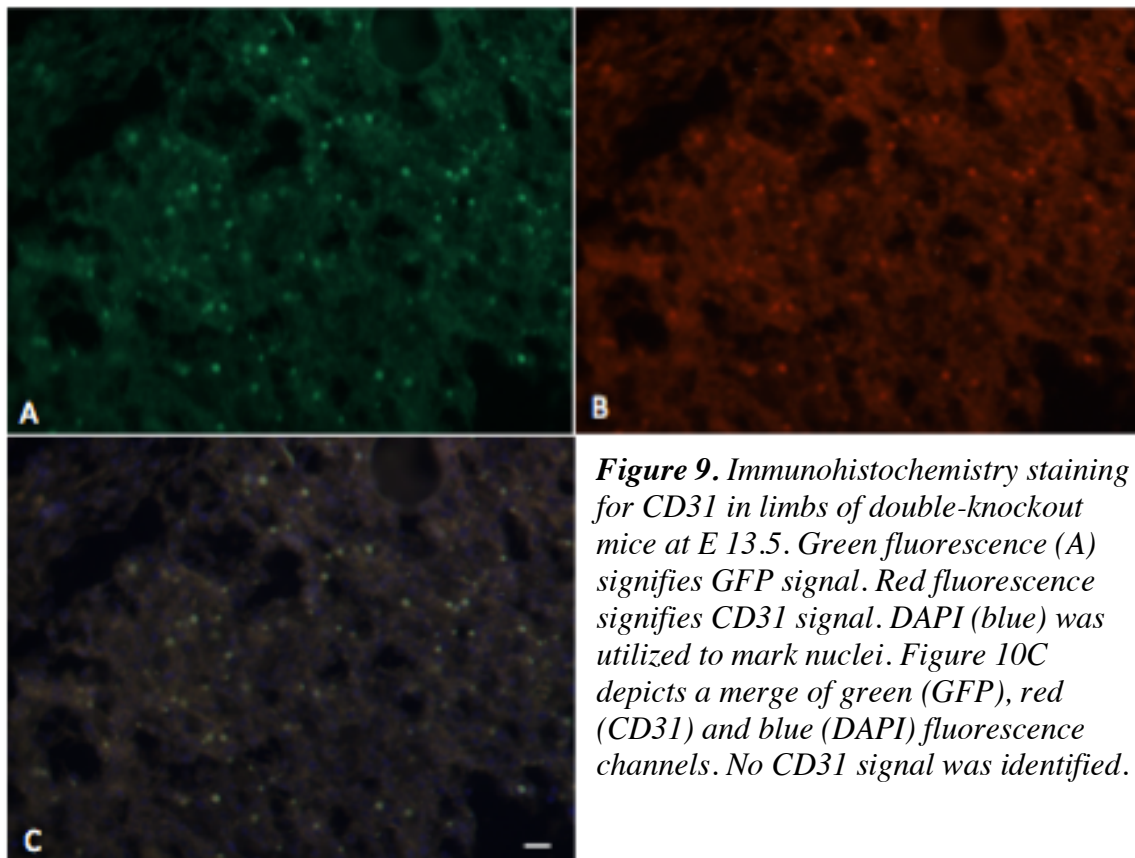
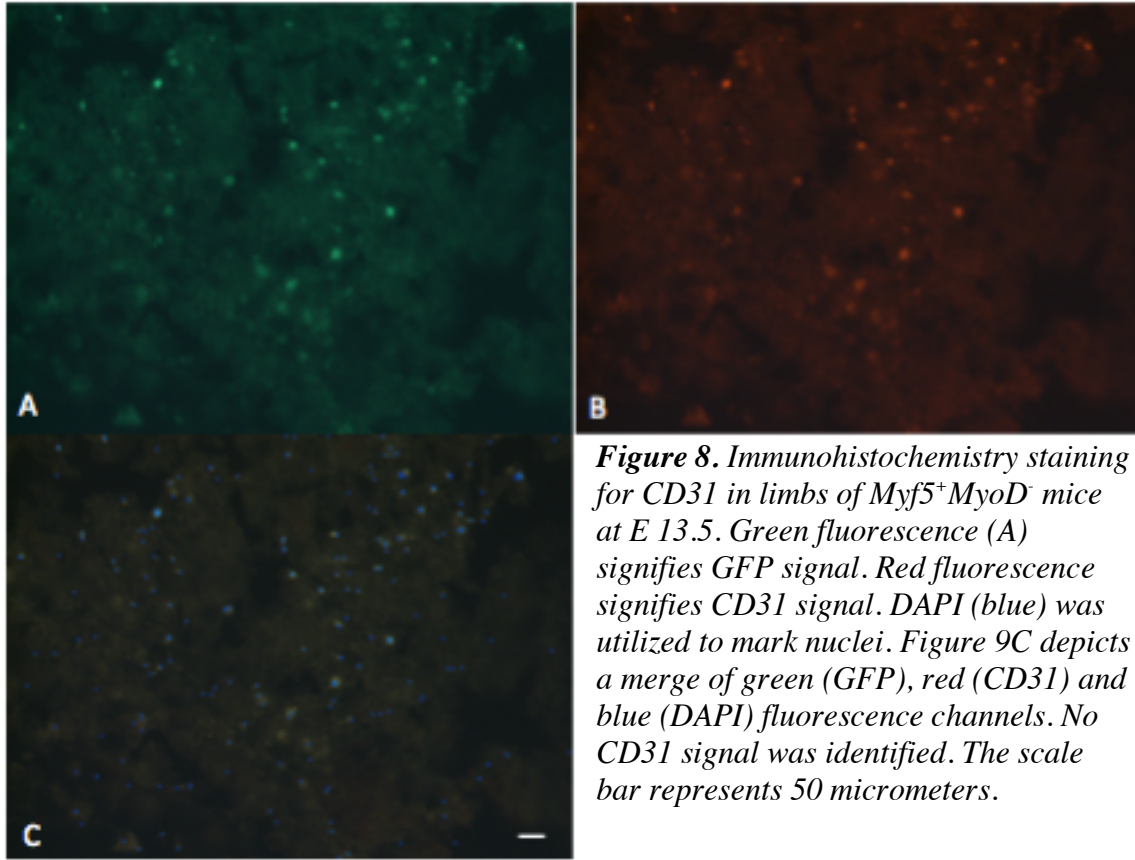
both control and double-knockout tissues collected at E 13.5. Figures 8, 9, 10, and 11 depict immunofluorescence staining for both CD31, an endothelial marker, and Perilipin, an adipogenic marker. We tested our tissue sample for these markers because we believed they were the most probable fates the double-knockout population would take. However, our data shows no signal for either markers at this time point. Even so, we are not able to draw unequivocal conclusions due to the lack of positive control for both Perilipin and CD31. Although it may be the case that satellite cell progenitors may have not yet made a change towards adipogenic or endothelial fates at E 13.5, it is also possible that staining failed to correctly mark adipocytes and endothelial cells or that these cells have not yet formed at this time point..

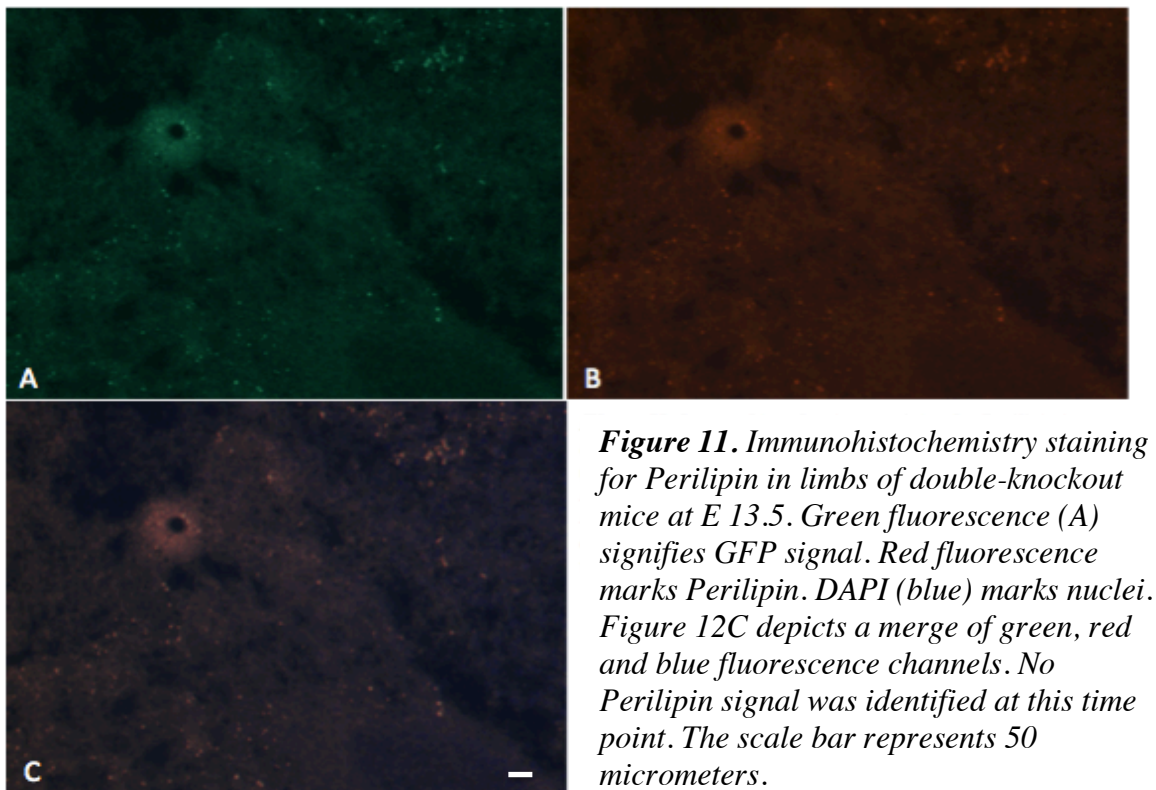
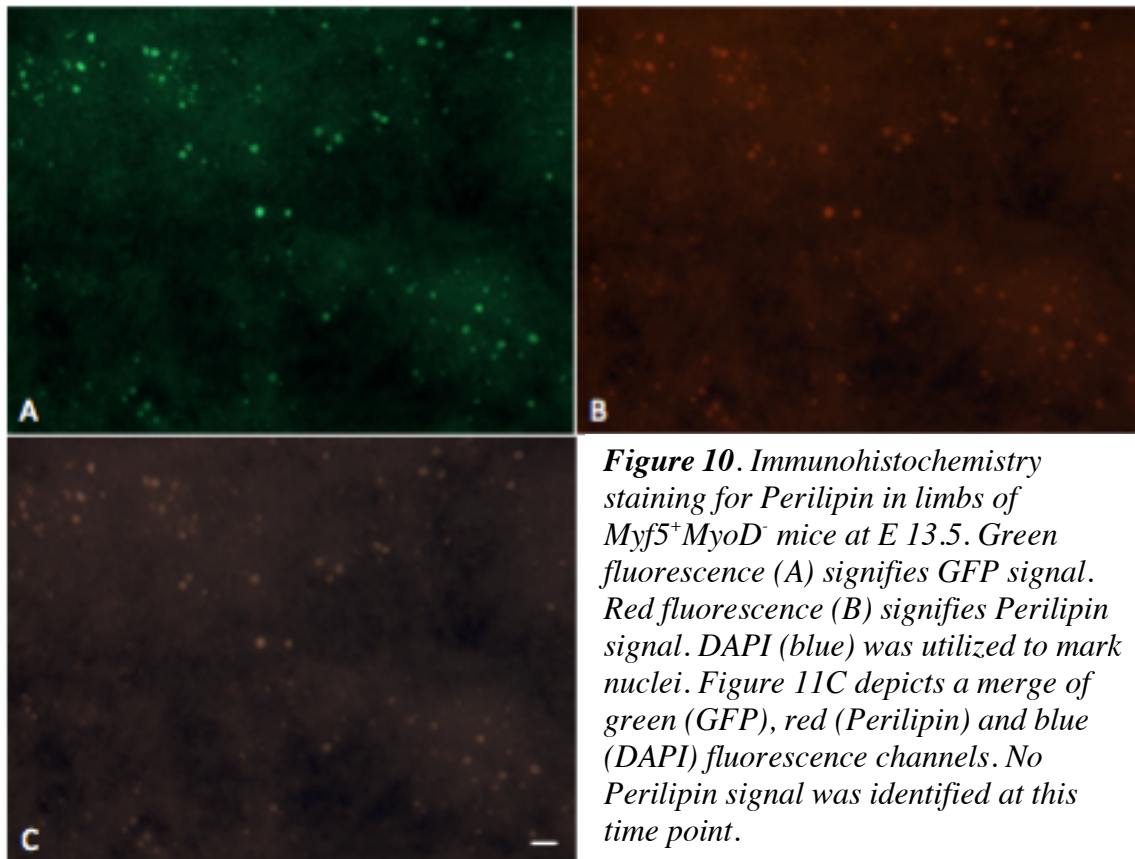


**Figure 5.** Presence of GFP<sup>+</sup> cells from limbs of Myf5<sup>+</sup>MyoD<sup>-</sup> mice at E 13.5. Green fluorescence (A) signifies GFP signal. DAPI (blue) marks nuclei. Red fluorescence (B) and green fluorescence in the same location is indicative of auto-fluorescent tissue. Figure 6C is a merge of green (GFP), red (auto-fluorescence) and blue (DAPI) fluorescence channels. The scale bar represents 50 micrometers.



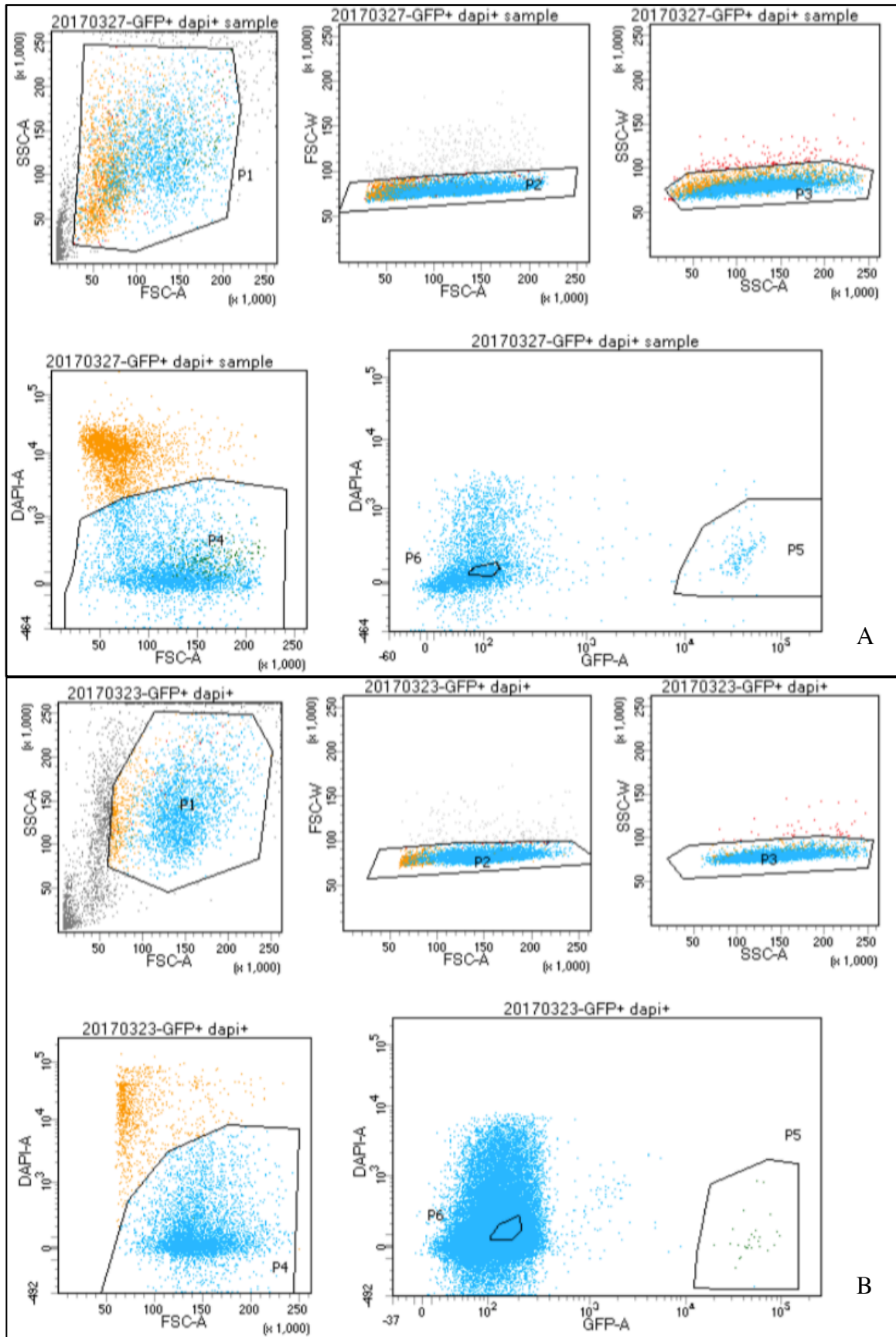






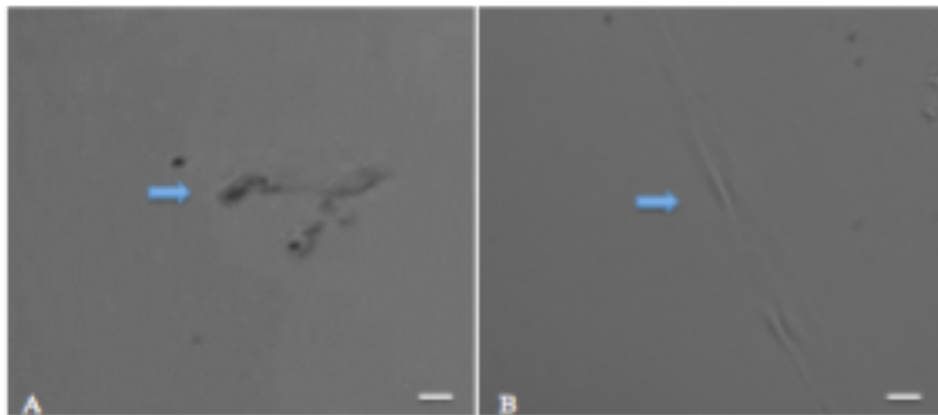
Immunofluorescence staining did not show any Perilipin and CD31 signal in double-knockout mice at E 13.5, but we were not able to conclude whether or not this staining was real. Additionally, we had issues with our sections sliding off of the slides. We tried using different time lengths and number of PBS washes during our staining protocol, but didn't seem to obtain significant improvement in the quality of our sections. Because we were not able to draw any concrete conclusions from immunofluorescence at E 13.5 in tissue, we decided to plate single GFP+ cells and trace their fate changes in order to determine if these cells have the capacity to acquire other fates at later time points on their own. Single cell plating was utilized to enable the analysis of the capacity of each cell to acquire a different fate. Additionally, single cell plating allows further RNA sequencing for the assessment of genes expressed. Although we were not able to complete this experiment in the interest of time, it is a logical next step. GFP signal was identified in the cells by FACS. Although GFP signal is indicative of recombination in the CreLoxP construct, recombination does not occur at the *MyoD* locus in every single cell. As such, we believe the majority, but not all, of our GFP+ cells lack *MyoD*.





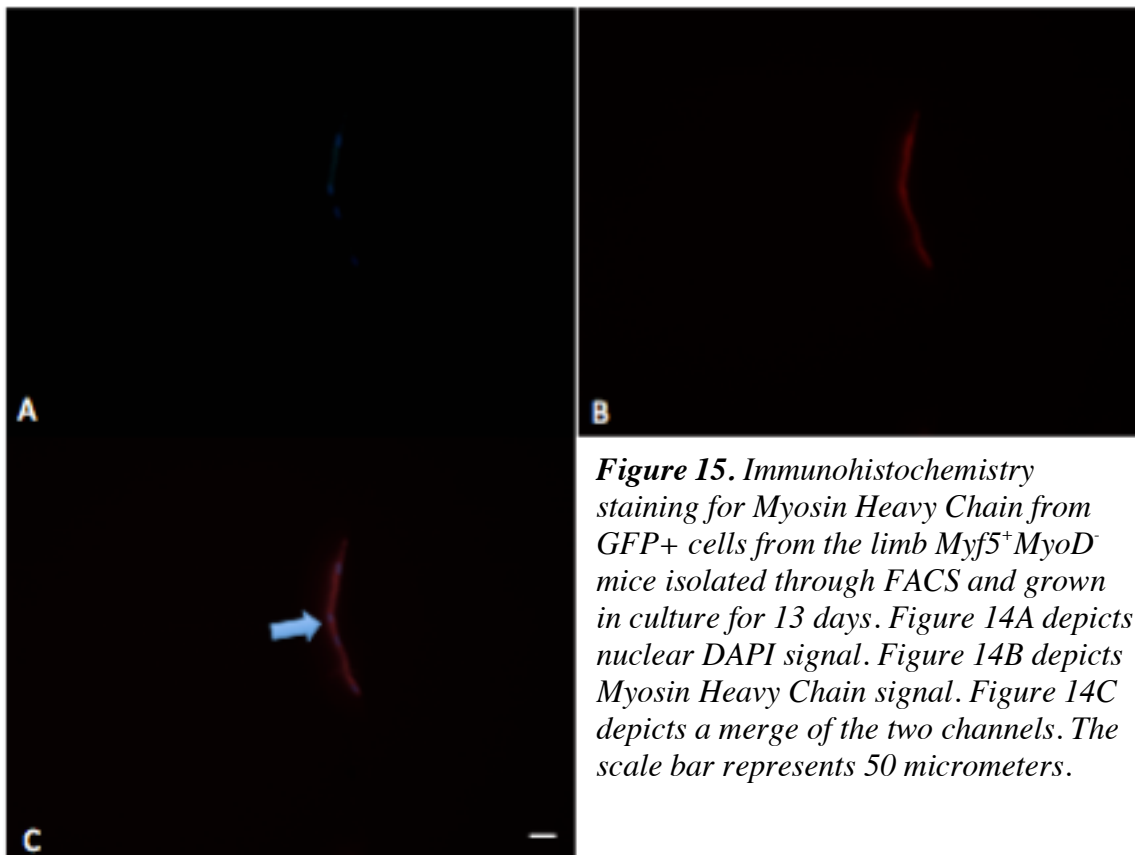
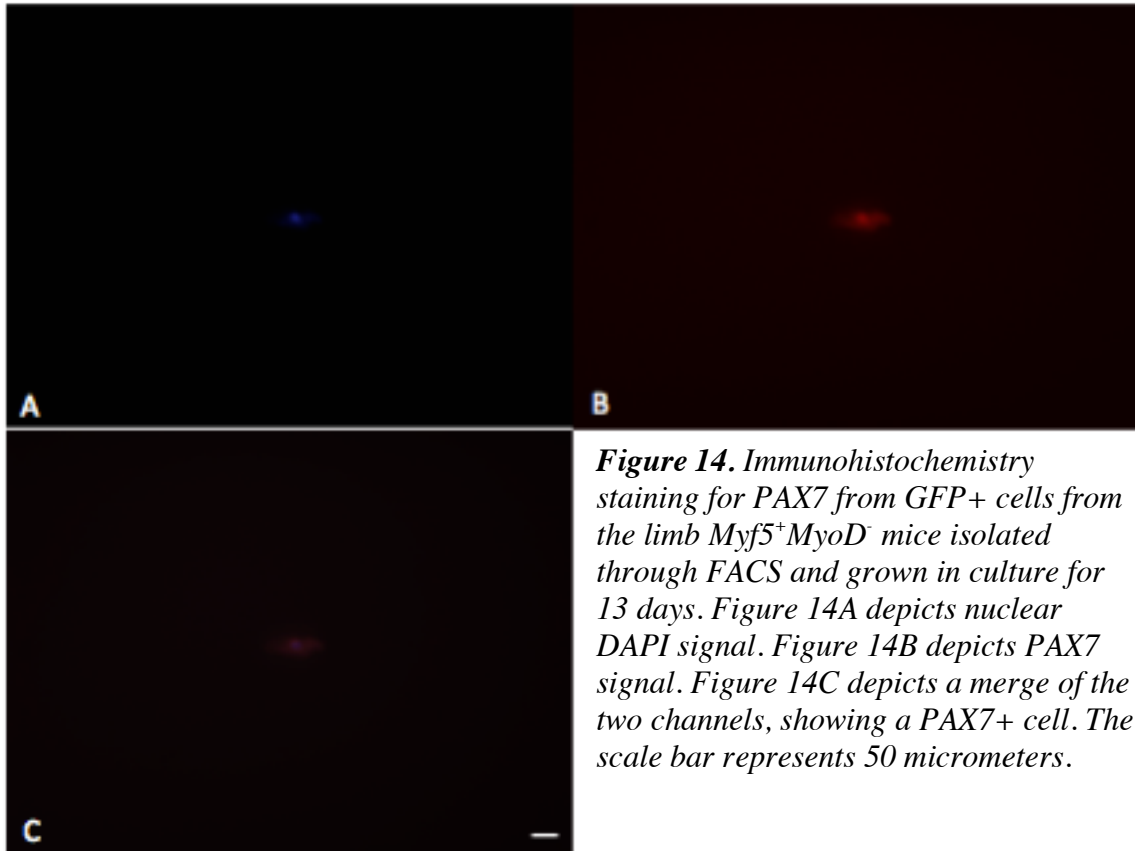
**Figure 12.** FACS gating parameters for index-sorted GFP<sup>+</sup> cells from the limbs of control (A) and double-knockout embryos (B) at E 13.5.

After we index-sorted our GFP<sup>+</sup> cells by FACS, we placed them in growth media for 8 days. Following plating, we checked the cells and were able to visualize GFP fluorescence right away. Although some plates lost GFP signal after a few days, we continued to track plates that had previously showed GFP signal under the assumption that live cells would remain adhered to the collagen on the plates. In the interest of time, we were not able to grow our cells to confluency. Because we did not stain our cells of interest until 13 days after plating, we were only able to analyze them through morphology and size up until that point. However, these analyses are far more subjective than immunofluorescence staining and do not enable us to draw any solid conclusions. Figure 13 depicts our findings after 8 days in growth media for wild type (Figure 13A) and control (Figure 13B) cells. There were numerous double-knockout plates that showed cells with the morphology depicted in Figure 13B. Thus, although we can't determine the fate of these cells based on morphology alone, the appearance of multiple cells with a similar morphology in Figure 13B only in the double-knockout plates could be significant.

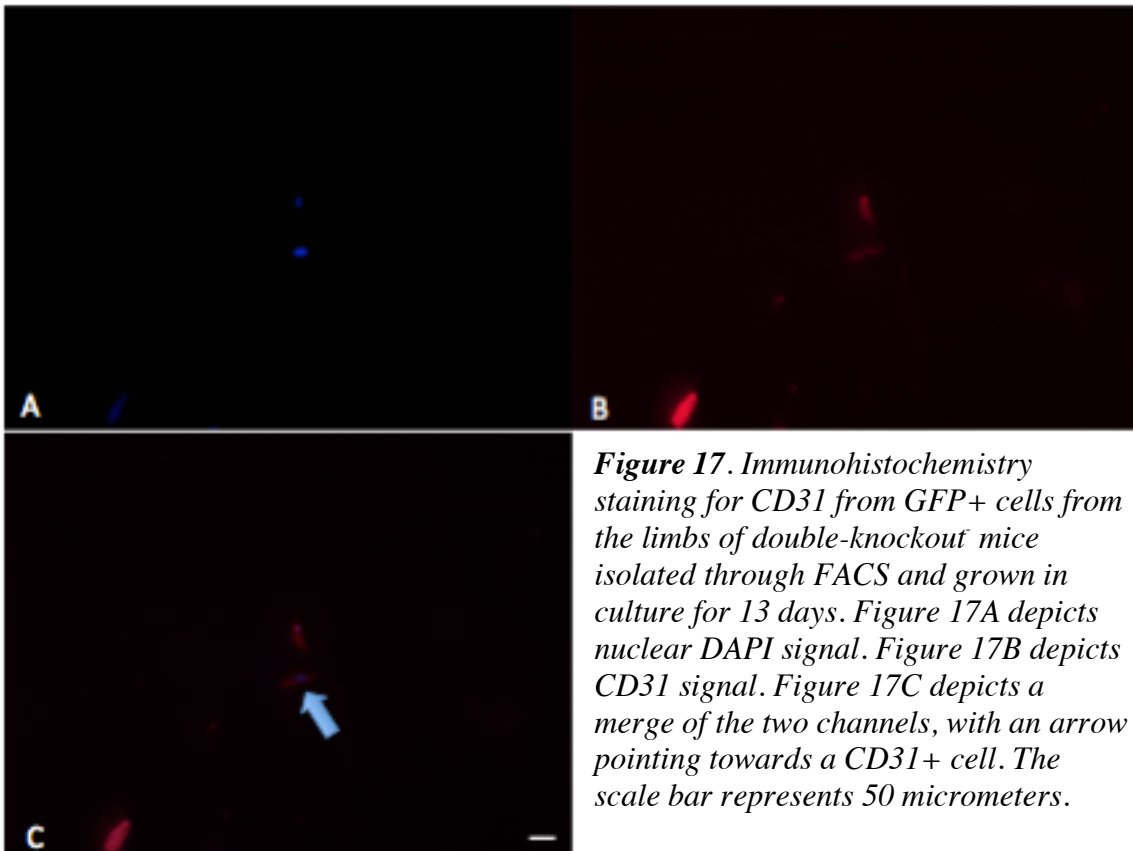
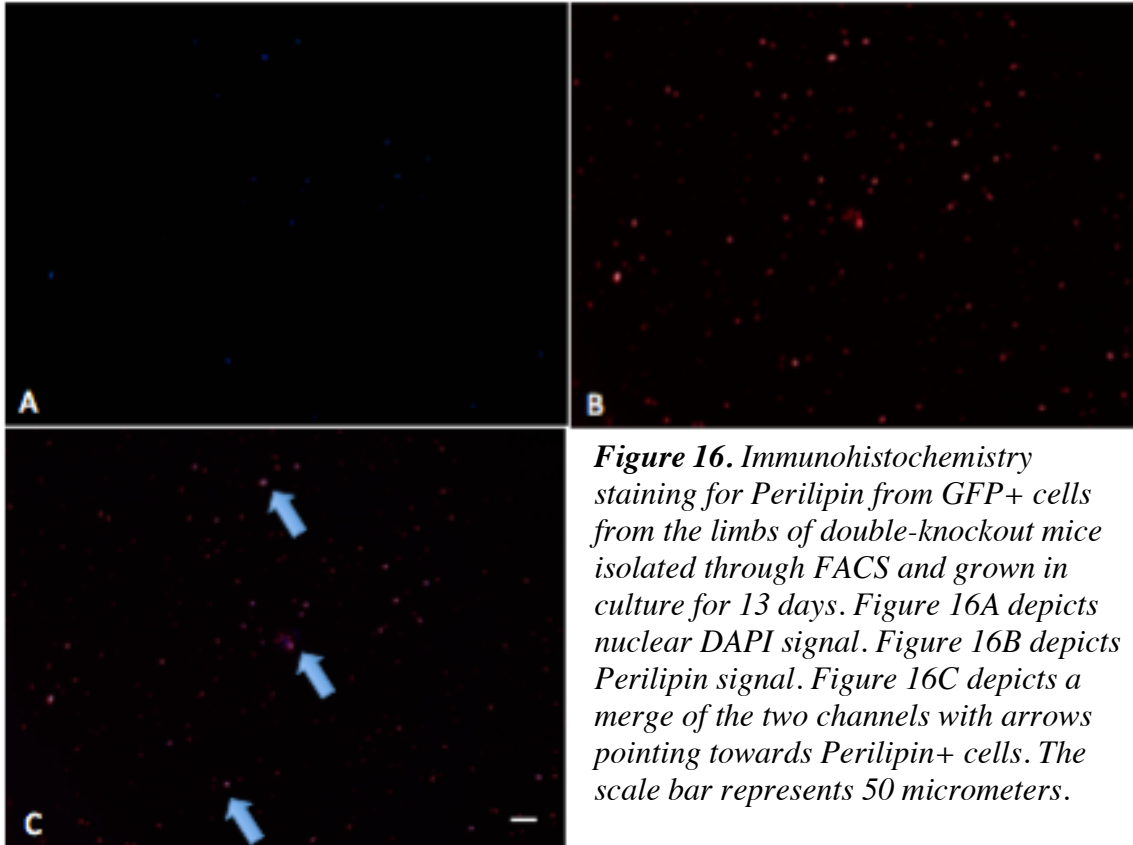


**Figure 13.** Index-sorted cells from the limbs of *MyoD*<sup>+</sup>*Myf5*<sup>+</sup> mice (A) and the limbs of double-knockout mice (B) at E 13.5 following eight days in growth media. The scale bar represents 20 micrometers.

Following 8 days in growth media, we placed our cells in differentiation media for 5 days and then stained them for CD31, Perlipin, and Osterix. Although the literature and previous lab experiments have shown that fate changes are largely adipocyte or endothelial directed, we wanted to ensure that we weren't missing another fate switch, perhaps towards bone. We also stained these cells for PAX7 and Myosin Heavy Chain. Although we did not necessarily anticipate a myogenic fate as marked by Myosin Heavy Chain, work in our lab has demonstrated that double-knockout satellite cells can express PAX7. We identified cells that were positive for CD31, and some that were positive for Perilipin (Figures 16 and 17) in our double knockout population. We did not detect any cells that were positive for Osterix, which marks differentiated bone, although it is certainly possible that satellite cell precursors could take a chondrogenic fate<sup>3</sup>. We also failed to identify any PAX7 and Myosin Heavy Chain signal in our double-knockout population (data not shown). Our data from the control population indicated the presence of signal that marks cells that are positive for Myosin Heavy Chain and PAX7 (Figures 14 and 15). However, further studies are needed to accurately determine whether these cells have truly acquired a fate change.

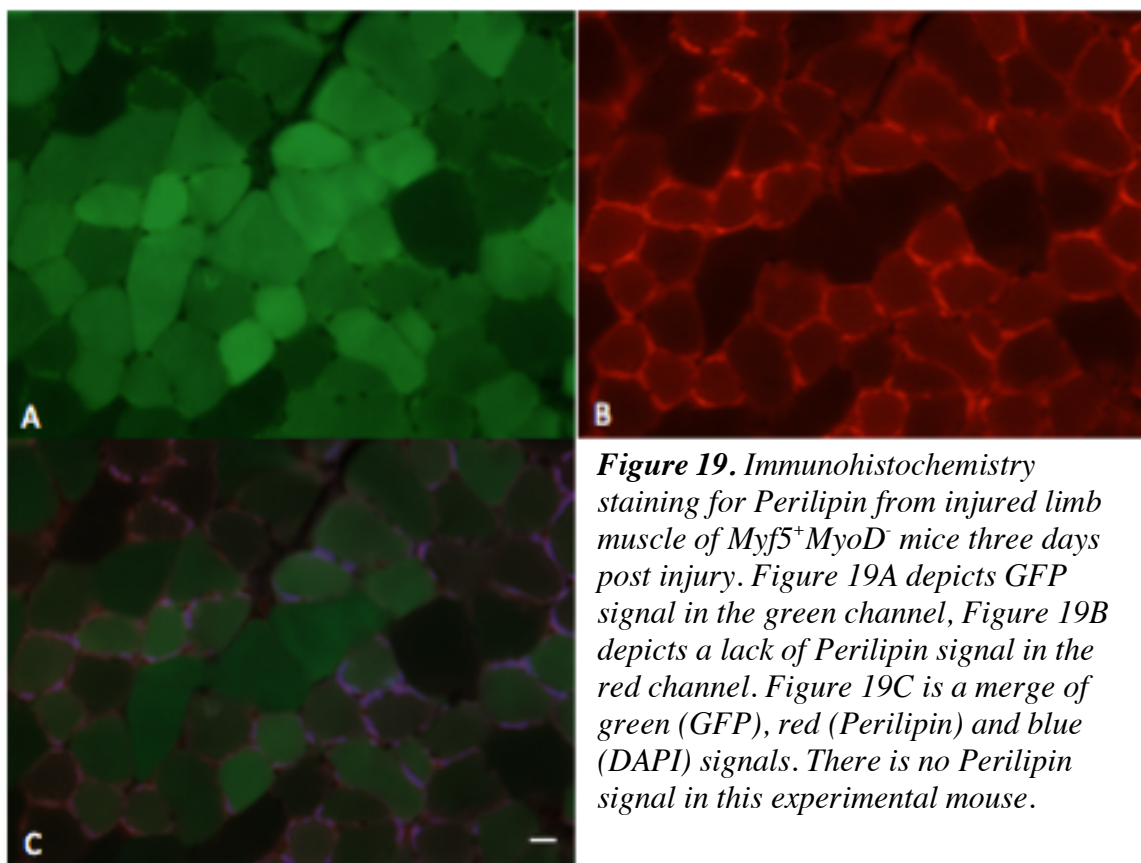
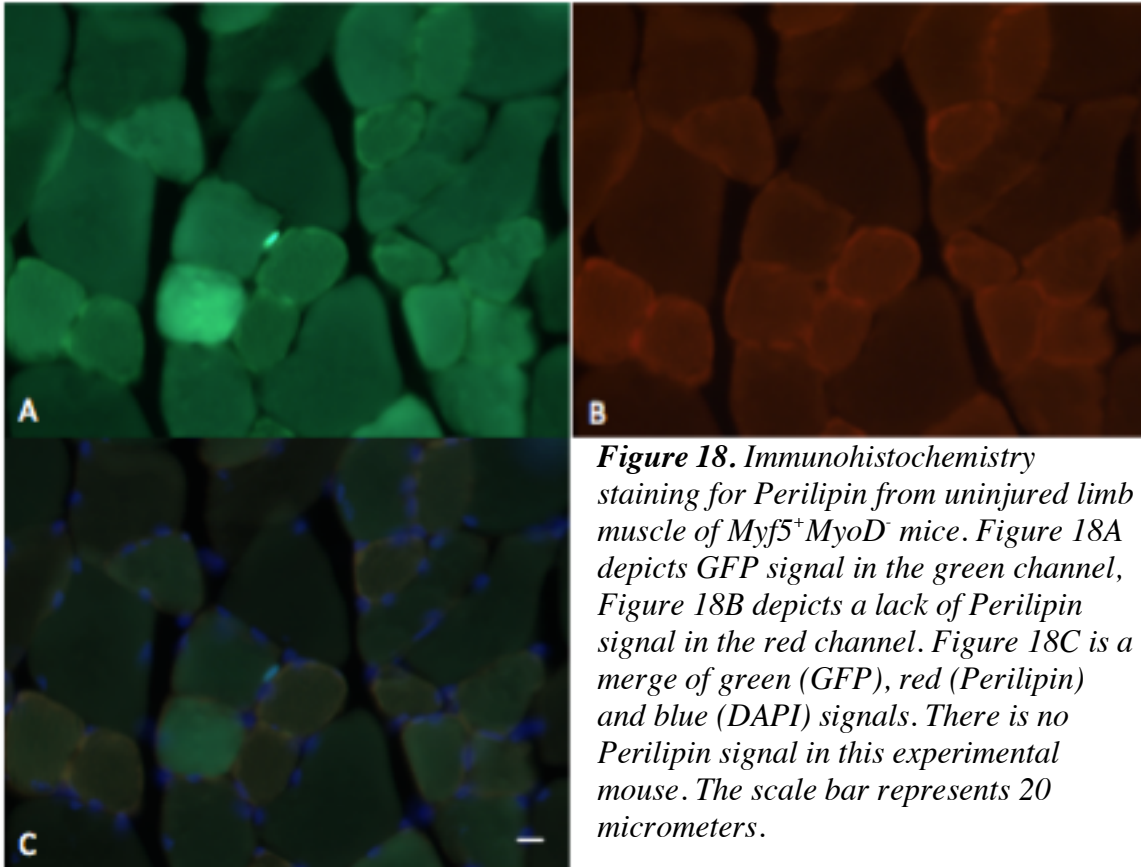


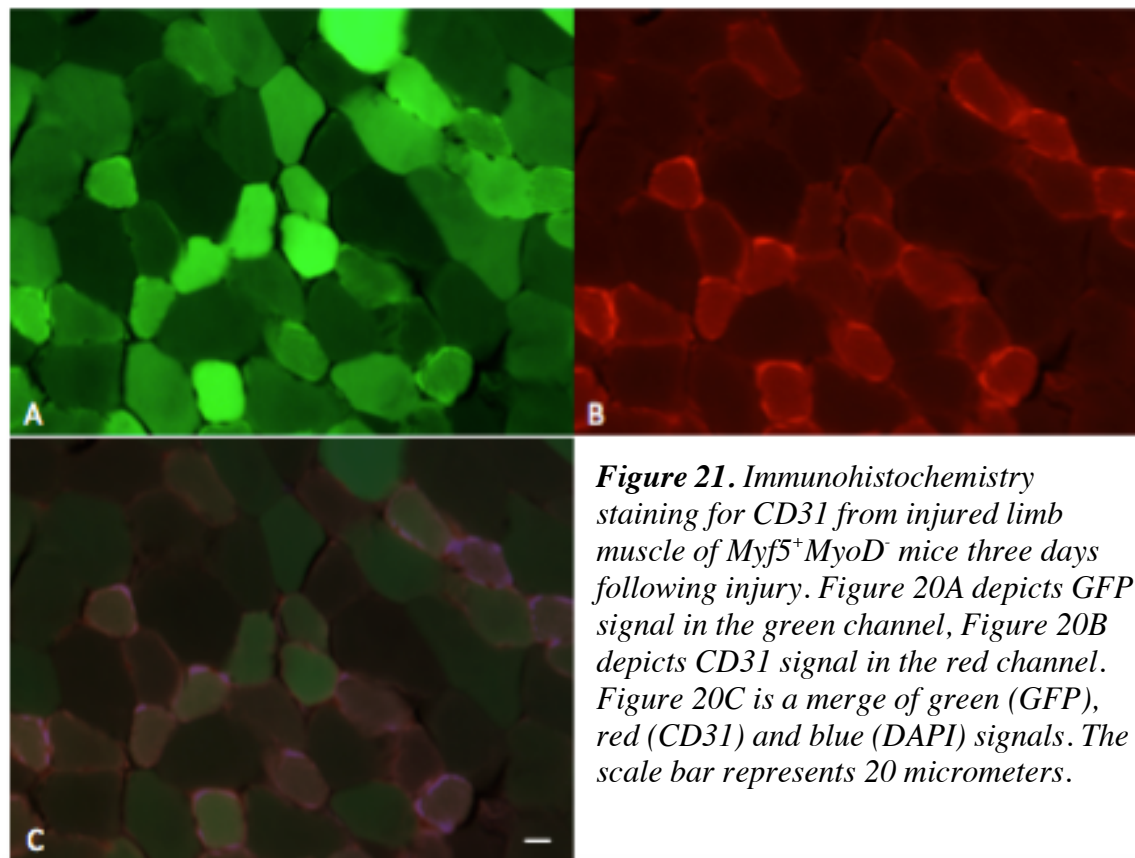
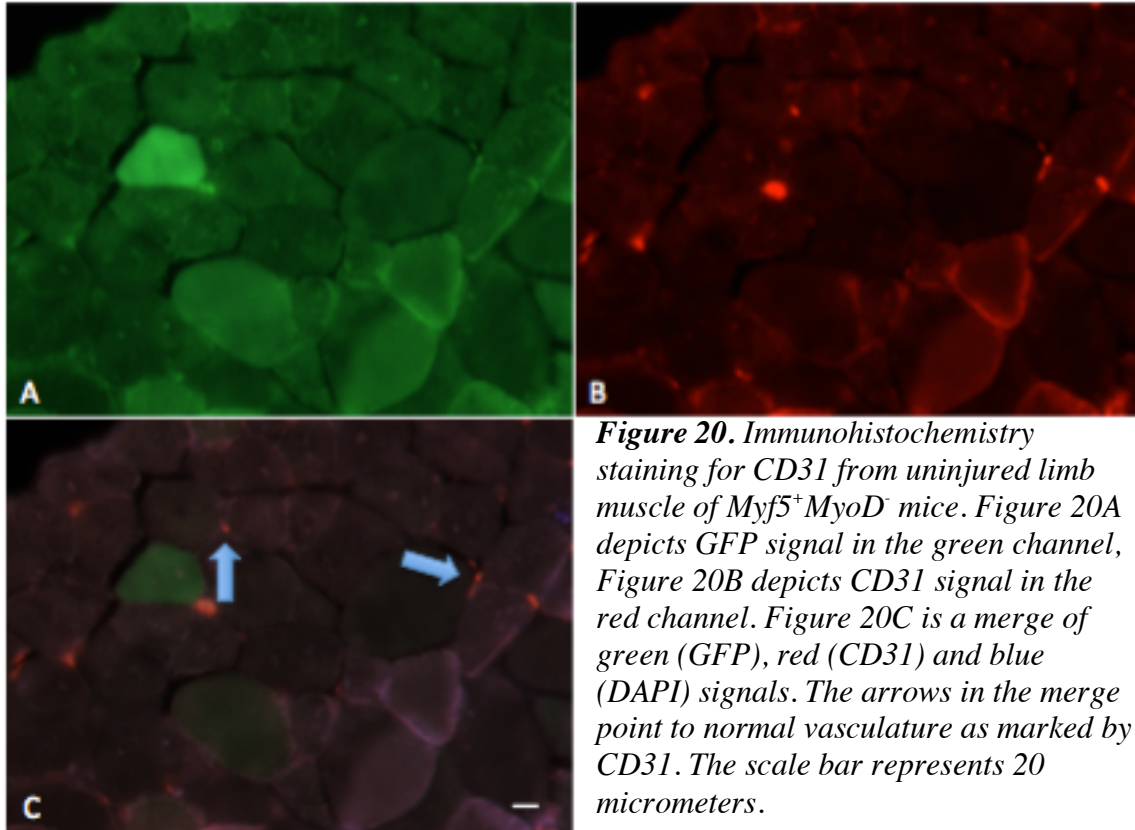




To analyze how regeneration occurs in the absence of *MyoD*, we stained our injured experimental animals with Perilipin and CD31, as we believed these would be the primary fate changes identified. Our data demonstrates no Perilipin and some CD31 signal present in muscle fibers three days following injury (Figures 18-21.. Although the CD31 signal may be an indication of a potential fate switch, the signal could also arise from autofluorescent tissue. Both outcomes are marked by an overlap of green and red signal, making it difficult to interpret the data.

Additionally, there are a significant number of GFP+ fibers in the regenerated muscle. As previously mentioned, GFP marks cells that have been recombined through the CreLoxP system and thus should lack *MyoD*. The presence of these fibers in regenerated muscle may indicate that regeneration occurred due to the downstream effects of *Myf5*, as *MyoD* was inactive. This data could show that *Myf5* was able to compensate for *MyoD* in regeneration in these experiments. This result is expected, as *Myf5* has been shown to compensate in the case of *MyoD* deficiency<sup>15</sup>. However, it is difficult to distinguish GFP+ vasculature in GFP+ fibers. Therefore, a fate switch that's unable to be identified could have also occurred, which would signify that *Myf5* alone is not able to regenerate muscle.





## ***Discussion***

In sum, our data showed that when *MyoD* is knocked out of *Myf5* negative experimental mice at E 11.5, satellite cell progenitors do not display any signal indicating a change towards adipogenic or endothelial lineages at E 13.5. This could mean that the time point is too early for a complete fate change to occur in the limbs of experimental mice. However, this data is also equivocal in the lack of positive controls for the immunofluorescence stains, as well as other staining showing the exact location of the GFP+ cells in the limbs. Coming to a significant conclusion is difficult without further experiments.

When isolated and grown in culture for 8 days, these cells appear to show signal that may indicate a fate change. The primary issue that we had with these cells was loss of GFP signal. Although after a few days in culture our cells no longer appeared GFP+, we believed that a significant quantity of the cells in the plates were *MyoD* knockouts. We first identified GFP signal in the embryo limbs upon dissection using fluorescence microscopy. Additionally, the FACS gating was specific for index-sorted live GFP+ mononuclear cells. Lastly, we assumed that these cells remained alive, in spite of a lack of GFP signal, because they did not lift up from the bottom of the plate. As such, we continued our analysis.

In our control population, we did not observe signal indicating fate changes towards an endothelial, chondrogenic, or adipogenic lineage. Rather, we saw primarily Myosin Heavy Chain+ cells following 8 days in growth media and 5 days in differentiation media, as in Figure 15. As single *Myf5*<sup>+</sup> animals have been shown to have the ability to carry out complete myogenic programming, we expected to observe a

myogenic fate lineage. We were able to observe Myosin Heavy Chain positive and some PAX7+ cells. The Myosin Heavy Chain is an indicator of differentiated muscle. PAX7 is expressed in quiescent satellite cells, before *Myf5* and *MyoD*, as shown in Figure 1. This indicates that the PAX7+ cells may be myogenic stem cells that have remained undifferentiated.

Although both samples contain GFP+ cells, there could be a fate change in the double-knockout embryos, while the control animals are shown to display signal that marks myogenic programming. After 8 days in growth media and 5 days in differentiation media, double-knockout satellite cell progenitors can be identified by a few different immunohistochemistry markers that indicate different cell lineages. The majority of these cells displayed signal that marks an endothelial or adipogenic fate. Because either *Myf5* or *MyoD* is required for proper differentiation to a myogenic fate,<sup>16</sup> we expected that *Myf5* and *MyoD* double knockout cells would not develop to form functional muscle or muscle progenitors. Instead, we expected to observe a fate switch. Because previous work in the Goldhamer lab has shown that this fate switch will be most likely towards adipose tissue, we expected to see an increase in Perilipin signal in the double-knockout mice when compared to wild-type mice. We believed the majority of satellite cell progenitors would show an adipogenic fate switch.

Although we stained our double-knockout population for osteogenic and myogenic markers, signal from markers indicating these fate changes couldn't be identified (data not shown). However, differentiation of cartilage and muscle is regulated by some common factors, such as Sox9 and Pax3<sup>3</sup>. Therefore, it is possible that chondrogenic cells were present in our population, but had not yet formed bone. In this

way, staining for Sox9 at this time point may have been more suitable. Additionally, we weren't able to detect the presence of PAX7+ cells either. This was true for both time points (8 days in growth media, 8 days in growth media followed by 5 days in differentiation media) in our single-cell plating culture experiments.

A fate change towards endothelial cells was also potentially expected. *Pax* family genes regulate both endothelial and myogenic cell lineages. During limb bud formation, this signal causes some myogenic progenitors to take an endothelial fate. Therefore, we anticipated seeing a potential increase in CD31 signal in double-knockout animals. However, we were surprised to observe a significant number of cells displaying markers for an endothelial fate, as opposed to an adipogenic fate. We expected to observe mostly adipose tissue signal, as adipose is usually the most prevalent lineage that satellite cell precursors take. However, it is possible that in the absence of *MyoD* and *Myf5*, *Pax* genes act to promote an endothelial cell lineage.

Lastly, we believed the possibility of myogenic progenitors or a myogenic fate to be unlikely, but possible. The other two myogenic regulatory factors, *Myogenin* and *MRF4*, should not have the ability to compensate for loss of *MyoD* and *Myf5* in myogenic differentiation. However, *MyoD* will have been active for only a short amount of time and it is possible that the gene may have contributed to the determination of a myogenic fate in this time. Another reason why we may have observed myogenic cells or myogenic precursors is due to recombination efficiency. Recombination in every cell at the *MyoD* locus is not guaranteed. As such, the occurrence of GFP+ cells that contain a *MyoD* allele is possible.

We did not necessarily anticipate observing a fate switch in our *Myf5* single-allele *MyoD*-knockout mice upon injury. Because *MyoD* is first expressed at E 11.5, knocking the gene out at that time point would allow it to be active during a short time in development, but not in regeneration due to injury. Additionally, as previously mentioned, *MyoD* and *Myf5* have redundant roles in myogenesis. Thus, we anticipated that injury and loss of muscle could lead to regeneration due to the downstream effects of *Myf5* up regulation.

Our injury profile data showed that that our *Myf5*<sup>+</sup>/*MyoD*<sup>-</sup> experimental mice do not depict signal indicating a fate change towards an adipogenic lineage (Figure 19). As in our double-knockout E 13.5 population, we observed signal indicating a potential fate switch towards an endothelial cell lineage, as marked by CD31 (Figure 21). Again, this could be due to the ability of *Pax* genes to regulate both myogenic and endothelial cells. However, endothelial and myogenic cells are closely associated, as previously mentioned. Thus, it could also be possible that the CD31 signal shown was due to normal vasculature. CD31 signal in GFP<sup>+</sup> cells may indicate the presence of an endothelial fate switch. Additionally, it is also possible that this is autofluorescent tissue. Consequently, without further experiments, whether these cells did take an endothelial fate remains unknown.

As previously stated, we saw a significant number of GFP<sup>+</sup> fibers in the regenerated muscle. GFP signal indicates that these fibers have been recombined and should lack *MyoD*. Due to imperfect recombination, it is not accurate to assume that each one of these recombined cells is *MyoD*<sup>-</sup>. However, because a significant number of the regenerated fibers are GFP<sup>+</sup>, we can assume that the majority of these fibers lack *MyoD*.



and regeneration occurred due to the effects of *Myf5*. Interestingly enough, this intense GFP signal appears to be depicted only in the regenerated fibers that were stained for CD31. This signal is not as fluorescent in the regenerated muscle that was stained for Perilipin. This occurrence could be explained by a lack of recombined fibers in the area imaged or due to an error in imaging.

In sum, we were unable to detect Perilipin signal in our injury profile data, as demonstrated in Figure 19. Although we identified CD31 signal, it is difficult to say with certainty that these cells are present due to a fate switch from myogenic precursors. *Myf5* and *MyoD* are both known to have the ability to carry out myogenic programming individually and they are both activated during satellite cell proliferation following injury, leading to regeneration of muscle fibers<sup>2</sup>

Although creating an injury profile for double-knockout animals would align better with the first component of my thesis, this experiment is unable to be completed due to embryonic fatality. Double-knockout animals have a low rate of survival for a full-term birth. This number decreases for mice that reach adulthood and would be exacerbated by cardiotoxin injection, rendering this experiment virtually impossible.

### ***Next Steps***

As previously mentioned, the single-cell plating in our experiment was initially done to further RNA sequencing analysis. Though we were not able to complete this step due to time constraints, this would be the next step in our research. In order to further support the possible conclusions that can be taken away from our data, we could also take bright field images showing the morphology of our cells of interest. For example, the detection of a lipid droplet in the cells that we believe may have taken a fate switch

towards an adipogenic lineage would confirm data from Perilipin staining. Additionally, the images in Figures 6-11 were taken from embryonic limbs. Imaging these sections using a light microscope or performing a hematoxylin stain would enable us to view the morphology and location of GFP+ cells within the limbs. Lastly, a positive control (i.e. staining adipocytes from our embryos for Perilipin) in the context of our experimental setting is necessary to confirm the accuracy of our results.

To further explore the roles of *Myf5* and *MyoD* in myogenesis, various experimental options could be performed. For example, our single-cell plating experiments could be repeated at different time points, such as E 15.5 and E 17.5. These are both time points in fetal myogenesis. At E 15.5, the basal lamina, located adjacent to satellite cells, is beginning to form. Similarly, the formation of satellite cells is still occurring at E 17.5. Completing these experiments would enable us to determine whether fate change occurs primarily during the embryonic stage or the fetal stage. Likewise, we could repeat FACS analysis. However, instead of single-cell plating, we could index sort GFP+ cells in populations to observe possible fate changes in this capacity. We initially plated single cells to evaluate the ability of each cell to acquire a fate change. Plating cells in populations may in turn allow us to see how neighboring cells influence fate changes and whether or not all cells in an adjacent location take the same fate.

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