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Tracking the lineage of transplanted satellite cells

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Abstract

Satellite cells are the stem cells of the muscle and contribute to muscle homeostasis and regeneration by creating large amounts of cells which can fuse to create new muscle or repair existing muscle fibers. Muscle disorders such as Duchenne Muscle Dystrophy and age-related muscle wasting often demonstrate impaired satellite cell function. Providing patients of such disorders with a healthy population of satellite cells via transplantation may represent a viable strategy for improving muscle function and reducing frailty. My project sought to improve the ability to track post transplanted satellite cell behavior through use of a new lineage tracing tool, the Tet-on; H2B-GFP mouse. Once activated, the satellite cells from the H2B-GFP mouse are labeled with nuclear GFP that can be easily traced, whether the cells are mononuclear or a myonucleus in a multinucleated myofiber following cell fusion. I transplanted satellite cells from H2B-GFP mice into wild-type mice and measured the contribution of these cells to regenerating myofibers and the satellite cell pool. Our results show that most transplanted cells differentiate and contribute to regenerating myofibers but that some transplanted cells have the capability to self-renew and retain their satellite cell identity. The work presented here establishes a powerful tool for the optimization of stem cell transplantations by providing detailed tracking and quantification of post-transplant satellite cell behavior.

Introduction

Satellite cells are a specialized type of stem cell which occupy the periphery of muscle fibers and drive muscle generation and repair following injury. Similar to other adult stem cells, satellite cells are capable of self-renewal, in which they divide asymmetrically to form one cell which maintains its stem cell properties and one differentiated cell which goes on to perform specific cellular functions. Satellite cells reside in a niche on the outside of myofibers and are mostly quiescent in uninjured adult muscle [1]. Following muscle injury, satellite cells become activated and expand rapidly. These cells then fuse into already existing muscle cells in order to repair damaged myofibers, or fuse with each other to create new muscle fibers. While the majority of satellite cells fuse to rebuild myofibers, a small population of satellite cells return to the quiescent state within the niche [2]. In this way, there is a constant pool of satellite cells ready for future injury responses. Satellite cells are not only active during repair in the case of muscle damage, but are also critical for development and the functional homeostasis of all skeletal muscles [3-5]. The transcription factor Pax7 is uniquely expressed in satellite cells within skeletal muscles, providing a reliable way to identify satellite cells within dissected tissue.

In muscular dystrophies and sarcopenia (age-related muscle wasting), the ability of satellite cells to contribute to muscle repair is compromised. Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy in humans, affecting approximately 1 in every 3,500 live male births [6]. Patients of DMD experience debilitating muscle weakness which eventually necessitates the use of a wheelchair, and ultimately results in premature death at around age 30 due to failure of the diaphragm [7]. DMD and other forms of muscle pathology demonstrate impaired satellite cell function, such as reduced regenerative potential and

misregulated cell polarity [8]. Muscle loss is also a concern in aging, as humans lose an average of 1-2% of muscle mass per year after age 30, even in highly active individuals [9]. As sarcopenia progresses, elderly individuals may become too frail to live independently, and are at higher risk of sustaining injuries due to poor balance and coordination [10]. The satellite cell pool is diminished in elderly humans, and that satellite cell dysfunction may be one of the underlying causes in age-related muscle decline [11]. Developing strategies to replace or rescue dysfunctional satellite cells and better understand their behavior is a critical step in treating the many muscle disorders for which no cure yet exists.

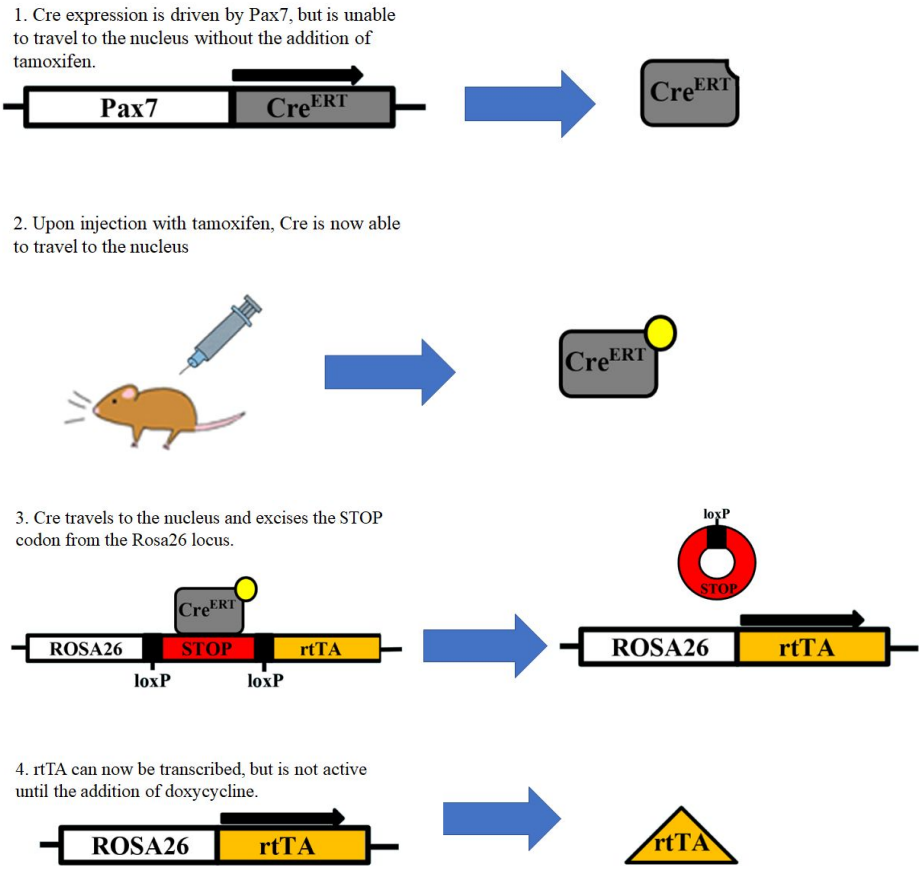
Transplantation of healthy satellite cells may thus be a potential strategy in treating muscular dystrophy and restoring healthy muscle regeneration in individuals suffering from sarcopenia. Using stem cell transplantations as a treatment for disease was first widely adopted with the use of hematopoietic stem cells [12]. With the transplantation of hematopoietic stem cells, patients are able to once more make healthy blood and immune cells to fight off diseases such as leukemia and lymphoma [13]. Given the success of hematopoietic stem cell transplantation, other types of stem cells could conceivably contribute treatment for a wide variety of diseases, including skeletal muscle pathologies.

The first experiments investigating the behavior of transplanted satellite cells began in the 1980s [14-16]. While these early experiments demonstrated poor retention of transplanted cells, more recent experiments have demonstrated the ability of transplanted satellite cells to contribute to improved muscle health [17-20]. Strategies have been developed to improve the efficacy of satellite cell transplants including transplantation of entire muscle fibers, selecting specific subpopulations of cells to transplant, and giving cells various pretreatments prior to

transplantation [19-23]. Both the quantification of transplanted satellite cell contribution to self-renewal and muscle regeneration, and the development techniques to increase the therapeutic potential of satellite cell transplants remain active areas of investigation.

For my project I sought to investigate satellite cell function and to test the viability of cell-based therapies by evaluating the efficacy of satellite cell transplantations in mice using a novel lineage tracing tool, the Tet-on H2B-GFP transgenic mouse (Figure 1). In this transgenic mouse, H2B-GFP expression is restricted to only satellite cells and occurs only when doxycycline is present [24,25]. This specificity is achieved by a Cre^{ERT2} allele expression being driven by Pax7, a satellite cells specific transcription factor. Following treatment with tamoxifen, the Cre^{ERT2} can translocate to the nucleus to drive transcription of rtTA, the reverse tetracycline-controlled transactivator. rtTA can combine with doxycycline (administered through injection or through treated chow) to promote the expression of H2B-GFP (Figure 1, Step 5). H2B is a histone protein involved in the packing of DNA within the nucleus of eukaryotic cells, and thus any H2B-GFP signal will be exclusively found in the nucleus. This is advantageous as the GFP signal will not diffuse through muscle fibers upon fusion of an H2B-GFP positive cell. This allows one to distinctly identify fused cells originating from transplants, whereas this had been difficult in earlier satellite cell transplantation experiments which relied upon fluorescent proteins localized in the cytoplasm to track transplanted cells. My main goal in this project was to track satellite cell behavior post-transplantation and measure the percent of satellite cells that self-renew versus the percent that fuse into myofibers. I performed experiments in mice quantifying the contribution of transplanted H2B-GFP satellite cells into wild-type mice by injected donor cells with a concurrent muscle injury and analysis at 4, 7, and 14 days

post-transplant. I quantified the transplanted cells using either immunostaining or flow cytometry analyses. H2B-GFP-expressing nuclei can be detected in both regenerated muscle fibers and among the self-renewed satellite cell populations. Overall, the data presented show a trend of decreasing quantities of transplanted cells retaining potential for self-renewal over a 14-day period and establish that the Tet-on H2B-GFP mice are a powerful tool to investigate and optimize stem cell transplantation methods.



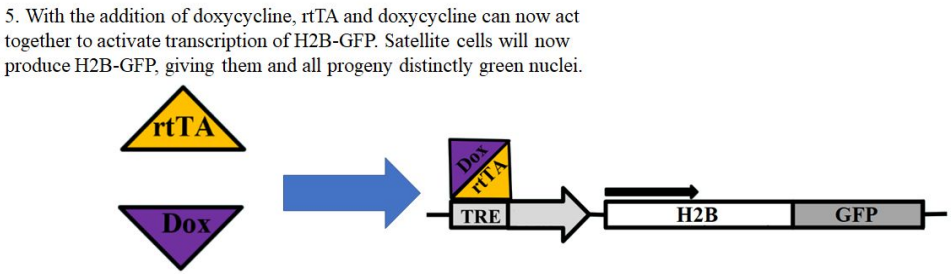


Figure 1: Demonstrates the mechanism of the H2B-GFP CreLox system. Figure adapted from illustrations made by Thomas Vogler.

Results

I first sought to verify that H2B-GFP-expressing satellite cells could be detected in Pax7Cre^{ERT2}; flox-stop-flox rtTA; Tet-on H2B-GFP mice (from here on called H2B-GFP mice). I gave H2B-GFP mice tamoxifen injections for three days to induce expression of the RTTA (Tet-on promoter), then gave the mice doxycycline-containing food starting one day prior to injury and kept the mice on doxycycline chow until muscle tissue was harvested. Injuries were induced to the left *Tibalis anterior* (TA) muscle by directly injecting 50 µL of $BaCl_2$. Seven days post-injury, I removed the injured tissue from the mice, and immunostained with antibodies to laminin, which marks the boundaries of muscle fibers, and Pax7, which marks satellite cells [26,27]. Analysis of results from cross sections of tissue immunostained with antibodies to Pax7 and laminin shows that many Pax7+ satellite cells are expressing nuclear GFP (Figure 2A). Quantification of cells within cross sections shows that 7 days post-injury, approximately 50% of satellite cells in the H2B-GFP mouse are positive for GFP (data not shown). Essentially zero GFP+ satellite cells could be seen in the contralateral un-injured TA muscle (Figure 2A). The lack of a GFP+ signal is expected in un-injured tissue as H2B protein has a very low turnover

rate in non-dividing cells and satellite cells are mostly quiescent. Strong GFP+ expression in satellite cells following injury in H2B-GFP mice was confirmed using flow cytometry, which showed a distinct GFP positive population of cells in injured TA muscle but not from an un-injured TA muscle (Figure 2B).

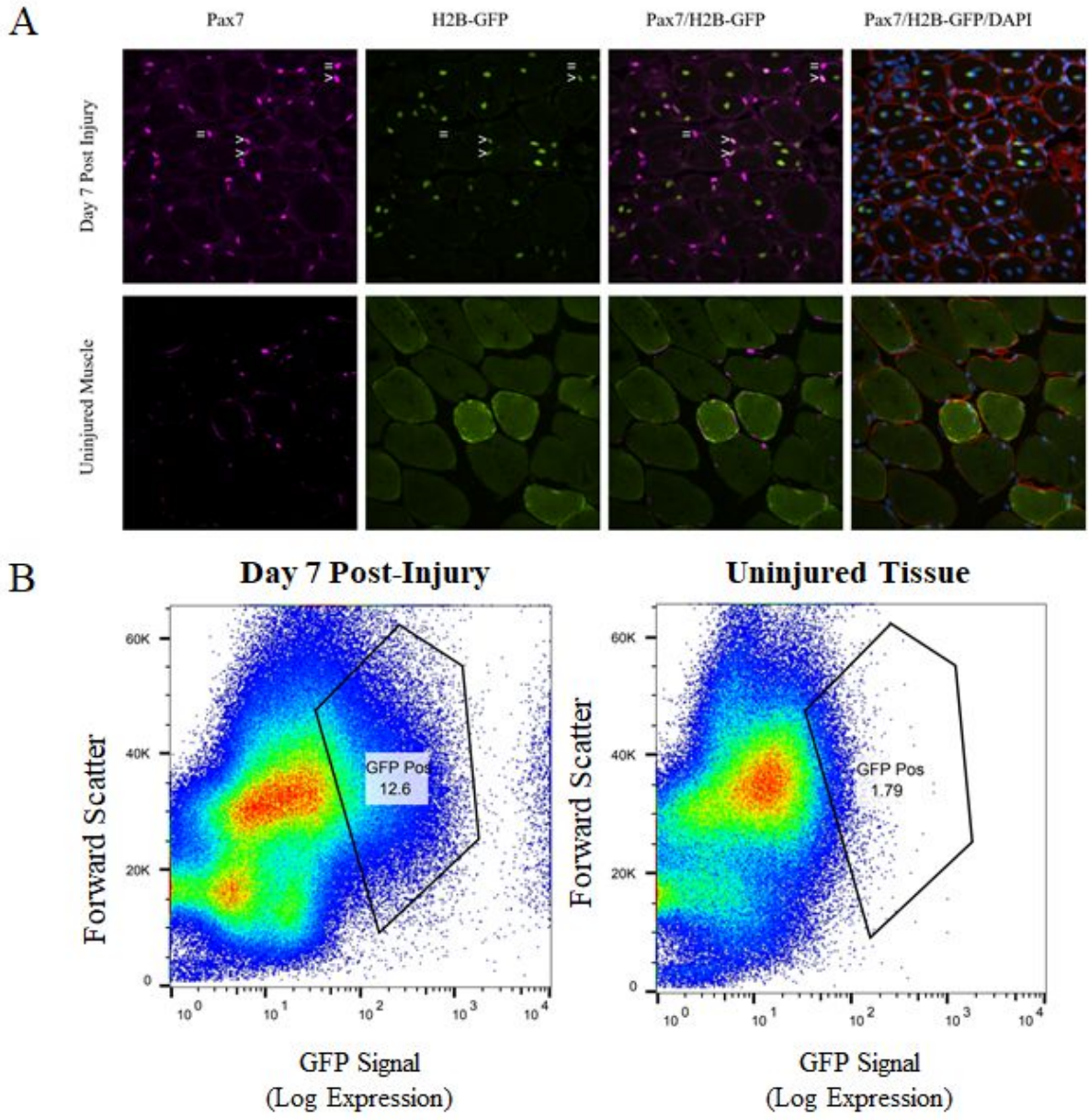


Figure 2: (A) Comparison of cross sections from injured H2B-GFP mouse tissue versus those from uninjured tissue. White arrows indicate some of the satellite cells which are also positive for GFP. The cells marked with the double bar indicate satellite cells negative for GFP. (B) Flow cytometry data from the TA of an H2B-GFP mouse 7 days post-injury. The cells within the black pentagon represent the population of GFP-positive cells.

We next performed a series of experiments transplanting H2B-GFP satellite cells into wild-type mice, concurrent with $BaCl_2$ injury, then extracting the injured tissue at time points of 4, 7, and 14 days post-transplant. In these experiments H2B-GFP mice were injected with tamoxifen four times and then satellite cells were isolated from hind leg muscles. Details of the treatments and procedures each mouse received are summarized in Table 1 below.

Table 1: Details of treatments mice received during experiments.

Transplant Donors	Pax7Cre H2B-GFP mice used. Treated with tamoxifen for three days prior to isolation of cells. No other treatment or injuries.
Transplant Recipients	Wild-type mice used. Treated with doxycycline for two days prior to transplantation and until tissue was collected. Injury given concurrent with cell transplant by injection of 50 uL of BaCl2. No tamoxifen administered.
Negative Controls	Wild-type mice used. Treated with doxycycline for same duration as transplant recipients. No tamoxifen or injuries received.
Positive Controls	Pax7Cre H2B-GFP mice used. Treated with tamoxifen for three days prior to injury, and doxycycline two days before injury until time tissue was collected. Given one BaCl2 injury at same time as transplant recipients. No transplantation received.

Approximately 100,000 satellite cells were isolated from each H2B-GFP mouse and then transplanted into 4 wild-type mice (~25,000 cells into each recipient). The recipient mice had been placed on doxycycline chow at least one day prior to transplant and were kept on doxycycline chow for the duration of the experiment. At each time point, the transplanted muscle tissue was removed from the mice, then sectioned for immunostaining or used in a flow cytometry assay. Frozen tissue sections were cut either in 10 μm cross sections or 30 μm longitudinal sections, then stained for with antibodies to laminin and Pax7. Figure 3 shows an image of 7-day post-transplant tissue that contains both H2B-GFP+ cells that are still Pax7+, and H2B-GFP+ cells that are Pax7 negative. Examples of GFP+/Pax7+ satellite cells are indicated by white arrow heads. Notice the straight line of GFP+ Pax7 negative cells indicated by arrows. A hallmark of regenerating muscle fibers are central located nuclei aligned in a straight line. Thus these GFP+Pax7- nuclei are transplanted cells that differentiated and contributed to the regeneration of the damaged myofibers. Transplanted H2B-GFP-labeled satellite cells can contribute to both the satellite cell population and the formation of newly formed myofibers at 7 days post-transplantation.

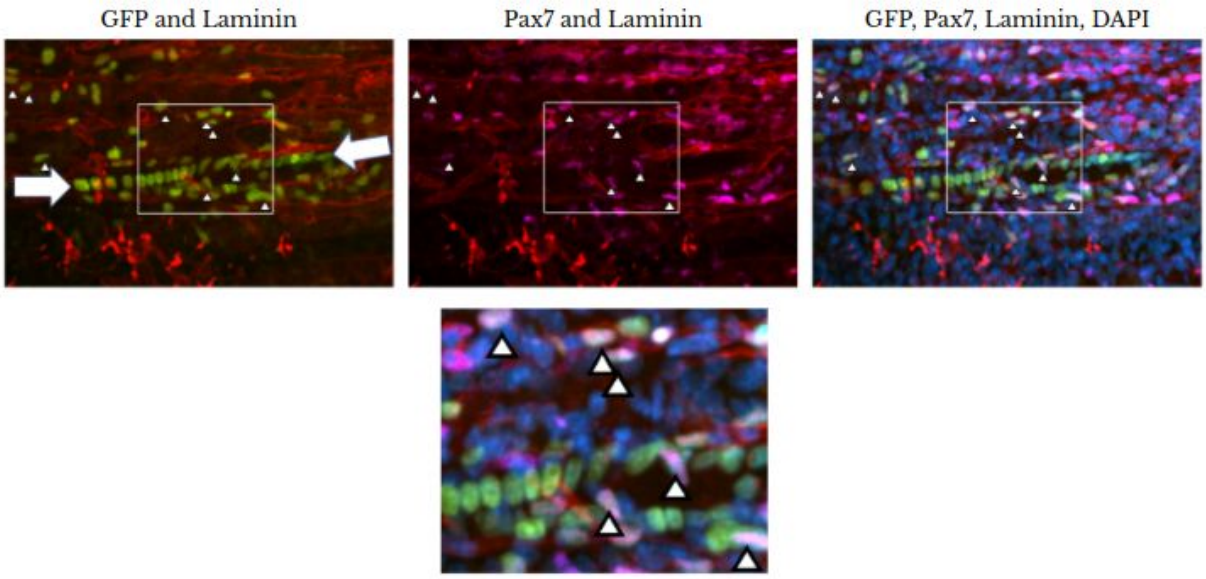


Figure 3: Longitudinal sections taken from mouse TA muscle 7 days after transplant. The first panel shows GFP positive cells, which represent transplanted cells. The second panel shows Pax7 staining, indicating satellite cells. The third panel shows the overlap of GFP, Pax7, and DAPI. Cells which are positive for all three markers are indicated with small white arrows. The panel below shows the area of the white box zoomed in. Not all cells positive for all three markers have been indicated. Laminin staining, which marks the edges of muscle fibers, has been shown in all panels.

I investigated if the percent of H2B-GFP+/Pax7+ transplanted cells would remain constant or change when compared at three time points post-transplant (4, 7 and 14 days). These time points were chosen because 4 days is the period in which there is the largest population of satellite cells which have divided to contribute to the muscle injury response, by 7 days cells have generally committed to fusion or self-renewal, and by 14 days the injury response is complete and satellite cells retraining their stem-cell-like properties should have returned to the niche. If the quantity of GFP+/Pax7+ cells remained constant from day 4 through day 14 this

would suggest that those GFP+Pax7+ cells present at day 4 have the potential to self-renew since most post injury self-renewal occurs after day 4 post injury (unpublished data). If the counts of GFP+/Pax7+ decline or are complete lost between day 4 and day 14 post-transplantation then this would suggest that the GFP+/Pax7+ present at day 4 post-injury will either differentiate or die (but not self-renew). I immunostained the tissue transplanted with H2B-GFP cells and collected at either day 4, day 7 or day 14 post-transplant (Figure 4). Unfortunately, sectioning and staining of the day 4 post-transplant tissue failed. However, quantification of GFP+/Pax+7 cells from the day 7 and day 14 post-injury tissue show that 27% of GFP cells are Pax7 positive at 7 days post-transplant and that 17% of GFP cells are Pax7 positive at 14 days post-transplant (Figure 5). These data show that the majority of H2B-GFP cells differentiate following transplantation but that some Pax7+/GFP+ satellite cells are still present at 14 days post-transplant. This suggests that, while some of GFP+/Pax7+ cells present at day 7 post-transplant will either die or differentiate, most of these cells have the potential to self-renew, as a significant percentage are still present at day 14.

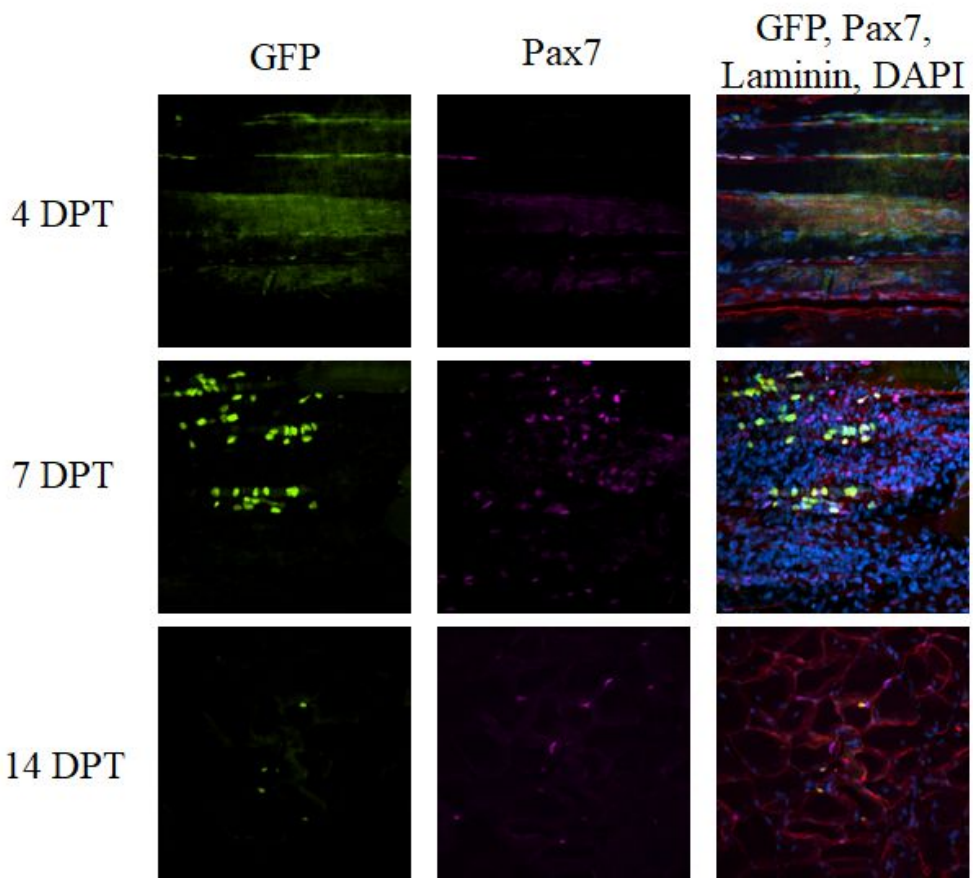


Figure 4: Comparison of stained tissue collected from all three time points. The 4-day and 7-day time points show 30 μm thick longitudinal sections, whereas the 14-day time point shows a cross section.

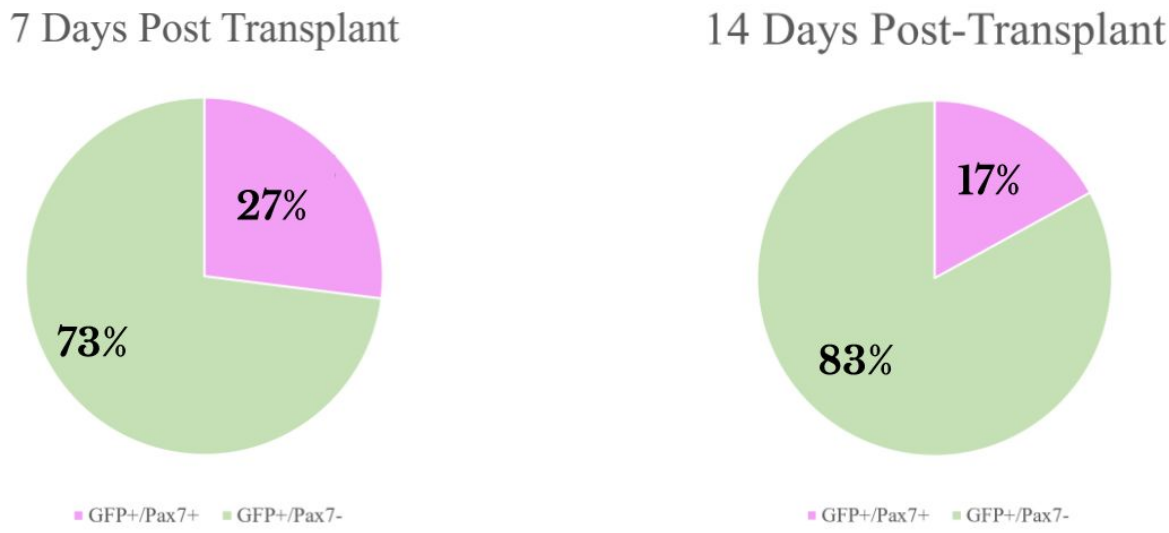


Figure 5: Comparison of total GFP positive cells found in sections versus the number of cells which were positive for both GFP and Pax7.

To complement my tissue staining analysis of transplanted H2B-GFP cells I next used flow cytometry to quantify the number of GFP cells at day 4, 7 or 14 days post-transplant. In the same manner as described above, H2B-GFP satellite cells were isolated and transplanted into wild-type mice on doxycycline-treated chow (which is used to continue driving the H2B-GFP signal). For the flow cytometry experiments, the transplanted tissue was removed, chopped, enzymatically digested, and filtered to remove large debris but retain individual cells. The isolated cells were then treated with DAPI, which is impermeable to living cells but will label myonuclei from that have been released from the muscle fibers. Thus, the H2B-GFP+ cells that are DAPI- represent transplanted cells that have not fused into a regenerating myofiber. Any GFP+ nuclei isolated from a myofiber will be identified by its DAPI+ signal. Total counts of GFP+/DAPI- cells for each transplant recipient group were compared to a negative control

(non-injured tissue from wild-type mice containing no GFP cells) and a positive control (H2B-GFP mice which had been injured and collected at the respective time interval). The flow cytometry profiles from the day 4 post injury tissue (Figure 6A and 6B) show a distinct H2B-GFP+ cell population with an average of 6084 GFP+ (n=2) cells at day 4 post-transplant (approximately 25,000 were transplanted). At day 7 post-transplant the flow cytometry analysis shows an average of 5689 GFP+ cells (n=2) (Figure 6C and 6D). Unfortunately, the positive and negative control profiles for the day 14 post-transplant tissue failed but profiles from the day 14 post-transplant tissue showed 3147 GFP+ (n=1) cells (Figure 6E). This data confirms that individual GFP+ cells can be detected up to 14 days post-transplant and provides further proof that transplanted cells have the capacity to self-renew because they are also positive for Pax7. The flow cytometry data also showed that the number of individual GFP+ cells decreases from day 4 post-transplant to day 14 post-transplant (Figure 7), matching the quantification obtained from our immunostaining analysis. This provides further support that some of the individual H2B-GFP+ cells present at day 4 post-transplant have the capability to retain their satellite cell identity and capacity to self-renew. Collectively, this data shows that flow cytometry can be used to count H2B-GFP cells post-transplant and can be an effective tool to analyze post-transplant cell behavior.

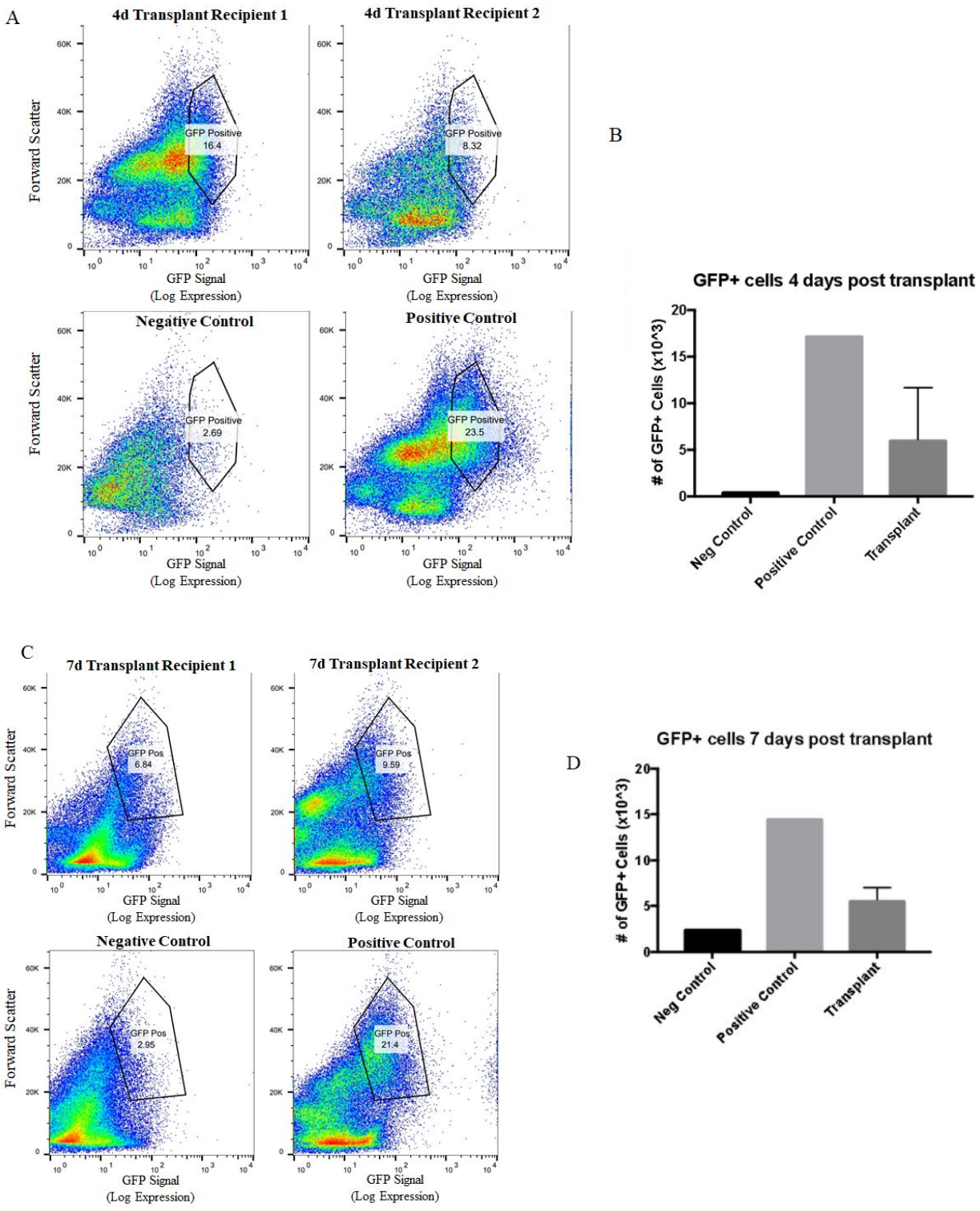


Figure 6: Comparison of flow cytometry data for 4 and 7 days post-transplant. The scatter plots on the left show GFP signal as a function of forward scatter (cell size). The graphs on the right

show total GFP cell counts in each group. For the transplant recipients group, the average was taken between the sum GFP cells in each sample.

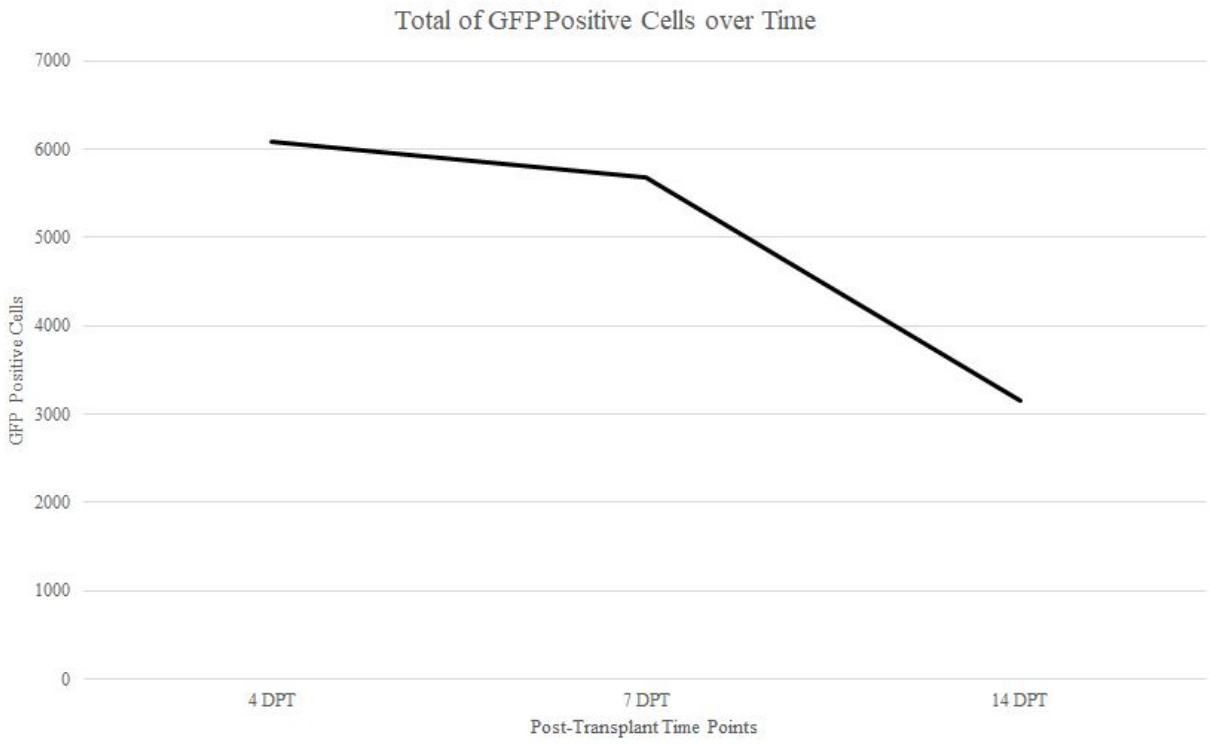


Figure 7: Comparison of GFP positive cells across the three time points collected. The 4- and 7-day time points represent the average amount of GFP cells found between two different transplant recipients. The 14-day time point represents GFP positive cells from a single transplant recipient.

Discussion

The main goal of my thesis work was to track satellite cell behavior following transplantation to advance transplantation techniques. I developed a novel lineage tracking method based on conditional expression of H2B-GFP that allowed for quantitative measurement of transplanted cell self-renewal and differentiation. Our data shows that most transplanted satellite cells fuse and contribute to regenerating myofibers, but some retain their satellite cell identify and have the potential for long term self-renewal. Overall, the work presented here shows that using H2B-GFP-labeled satellite cells is a powerful tool to examine and optimize stem cell transplantation methods.

My results show GFP positive cells that retained a satellite cell identity at each time point post-transplant, indicating that this methodology for transplanting satellite cells is generally successful and that the H2B-GFP mouse may be an effective tool in future self-renewal assays. The ability to effectively measure and optimize post-transplant self-renewal is clinically relevant because if healthy cells can be transplanted into a diseased tissue in manner that promotes self-renewal, healthy transplanted cells may be maintained indefinitely in patients after just a single transplant. Long term self-renewal of transplanted blood cells is common [28], but so far this has not been the case for muscle. A system such as this, which facilitates the effective study of post-transplant self-renewal in muscle, holds great potential for future discoveries. The immunostaining analysis identified GFP+/Pax7+, transplanted cells which have maintained a satellite cell identity, and may be able to contribute to long term self-renewal within the transplant recipient. Given that the doxycycline was only given to the transplant recipients and that H2B-GFP has little to no turnover in quiescent cells, this suggests that the GFP+/Pax7+ cells

identified may have already gone through self-renewal. Future experiments assaying for cell division can verify this result. Both the flow cytometry and immunostaining analysis data showed the amount of GFP positive satellite cells decreases over time. Future experiments will determine whether or not the population of transplanted satellite cells stays constant after 14 days, and to what extent these cells can participate in muscle repair and self-renewal. I would like to conduct future experiments measuring both the long-term retention of transplanted H2B-GFP cells, and how transplanted H2B-GFP cells respond to subsequent injuries. Overall, the identification of GFP+ cells that have retained their satellite cell identity at 14 days post-transplant is a promising result, and future studies will seek to verify these results and further investigate mechanism of self-renewal.

Another interesting and potentially clinically relevant finding from my studies was the high level of contribution that the transplanted cells made to regenerating myofibers. At each of the time points I looked at, I observed H2B-GFP cells which had fused into newly-formed myofibers. My quantifications from tissue sections suggest that the majority of transplanted H2B-GFP cells fuse into muscle fibers. The turnover of myonuclei inside myofibers is very low [29] and it may therefore be assumed that transplanted cells which have fused into myofibers will have long-term retention. Previous transplant experiments have equally shown high levels of fusion following transplant, but H2B-GFP-labeled cells have the advantage of allowing long term analysis of transplanted cells inside myofibers, something that has not been investigated before. Given that fused cells are likely to be retained inside myofibers for months or even years [30], transplanted satellite cells could be used as an effective vehicle for long-term delivery of healthy proteins in patients with muscle diseases. Overall, the future studies using H2B-GFP

mice to investigate and optimize both the fusion and self-renewal of transplanted cells hold high therapeutic relevance.

While the H2B-GFP mouse has proven to be a useful tool for investigating transplants, there are several technical issues which must be closely considered and reformed in future experiments. One issue with regard to the quantification of transplanted cells contributing to self-renewal is that not all satellite cells from the H2B-GFP mice were positive for GFP at the time of transplant. Earlier quantification demonstrated that after activation and treatment with tamoxifen and doxycycline, only about 50% of satellite cells within an H2B-GFP mouse contain detectable amounts of GFP. This means that it is possible that there is a subpopulation of transplanted cells which have contributed to self-renewal or muscle regeneration within the recipient muscle yet were not identifiable as exogenous cells due to their lack of a detectable GFP signal. This means that the quantity of transplanted cells within the recipient muscle may actually be significantly greater. This problem could be mitigated in future experiments by performing fluorescence-activated cell sorting prior to transplant to select out GFP positive cells alone. Quantification could then be performed with respect to the amount of GFP cells found in transplant recipient tissue compared to the amount of GFP positive cells transplanted.

Another limitation of the assays performed is that it is not possible to examine populations of transplanted cells over time within a single organism. To say that this data accurately depicts how the population of transplanted cells changes over time, one must make the assumption that the transplantation was equally successful in all cases. In actuality, it is unlikely that each transplant is equal due to the fact that one cannot see the exact location of the needle within the muscle when administering the transplant. For example, some mice may

receive “better injuries”, in that the injury affects more muscle fibers and stimulates a greater regenerative response. Due to the fact that this assay does not allow the number of transplanted cells to be monitored over time within a single mouse, it is thus difficult to say the extent to which the number of cells quantified is representative of typical behavior or rather a reflection of how the particular transplant was administered.

I chose to employ flow cytometry in this project with the goal of providing a more objective and complete quantification of GFP positive satellite cells within the muscle of the transplant recipient. Although using flow cytometry allowed us to quantify the entirety of GFP positive satellite cells within the injured muscle, several caveats come with the interpretation of the data. First of all, due to problems experienced with the flow cytometry machine, the data for one of the transplants and both of the controls was lost for the 14-day time point. This means that, although cells were counted for this time point and included in Figure 7, no real interpretation of the data is possible as adequate controls were not in place. Secondly, the GFP positive cells did not show up as a particularly distinct population within our flow cytometry experiments, which is a common problem in using flow cytometry on samples taken from muscle tissue. This means that it is possible that some cells were erroneously included or excluded from the total GFP positive cell counts. Finally, the negative controls used in these experiments were from uninjured tissue. While this discrepancy should not make any difference in our ability to properly distinguish GFP+ and GFP- cell populations, it is technically an inconsistency with the experimental design. These flow cytometry experiments should thus be conducted once more with the proper controls to verify the results, perhaps with the addition of a satellite cell-specific antibody which may facilitate determination of cell identities.

Quantification by analysis of tissue sections is inherently difficult in the case of satellite cells, due to the fact that satellite cells may be located anywhere along the muscle fiber. As such, any particular tissue section is not necessarily representative of the satellite cell population as a whole. To mitigate this difficulty, I made 30 μm thick longitudinal sections of the transplant recipient muscle, as opposed to the traditionally used 10 μm thick (transverse) cross sections. The advantage of using longitudinal sections is that I was able to visualize a much larger portion of the muscle than would typically be possible with cross sections. The disadvantages of this technique are that cells may not be as easily distinguished due to overlapping, and that it is sometimes unclear whether cells are inside or outside of muscle fibers. For reasons which remain unclear, the longitudinal sections in my project turned out very well for the 7-day time point, but were not usable for the other two time points. This means that the quantification of the 7-day and 14-day time points as shown in Figure 5 may not be a fair comparison, as the quantification of the 7-day time point was done in longitudinal sections whereas I used cross sections for the 14-day time point. Some optimization of tissue staining for longitudinal sectioning may be required, or these experiments may simply need to be repeated.

The original aim of this project was to find ways to improve transplantation efficiency. It was necessary to establish a baseline level quantification for the behavior of H2B-GFP transplanted cells, which was the purpose of the experiments shown here. Another experiment that had originally been planned was the transplantation of H2B-GFP cells into a strain of mice which have no endogenous satellite cells. While I was not able to perform this experiment due to technical limitations, such an experiment has the potential to teach more about the behavior of transplanted satellite cells and may give less ambiguous results from analysis via flow cytometry.

After the issues described above have been resolved, and the assays have been optimized to provide a reliable method of quantification, future directions include a variety of variations to the experimentation protocol which have the potential to increase the ability of transplanted cells to take up residence within the muscle stem cell niche. These include pretreating cells with pharmacological agents such as p38 inhibitors, transplanting specific subpopulations of satellite cells, and transplanting satellite cells which have been suspended within a hydrogel such that they are not released until after the initial injury response.

In conclusion, stem cell transplantation in skeletal muscle holds significant therapeutic potential and our data show that H2B-GFP mice may be a powerful tool to increase our understanding of this technique.

Materials and Methods

Mice

Mice were bred and housed according to National Institutes of Health (NIH) guidelines for the ethical treatment of animals in a pathogen-free facility at the University of Colorado at Boulder. University of Colorado Institutional Animal Care and Use Committee (IACUC) approved all animal protocols and procedures. H2B-GFP mice were purchased from The Jackson Laboratory (catalogue # 005104). The RTTA mouse was a gift from the laboratory of David Ornitz [31,32].

Isolation of Satellite Cells for Transplant

Mice were humanely sacrificed, then all muscle was dissected from both hindlimbs, and chopped with scissors for five minutes until a slurry of uniform consistency was achieved. Muscle was then added to 9 mL of F12C Media with 1 mL of 4,000 U/mL collagenase to release satellite cells from fibers. Collagenase digest would proceed for one hour at 37° C, while manually shaking the tubes every ten minutes. Collagenase reaction was quenched by 20 mL of F12 media with added horse serum. Cell mixture was filtered consecutively through 100 μm, 70 μm, and 40 μm cell filters. Cells would then be pelleted in centrifuge at 1500 RPM for five minutes. Supernatant was removed, and cells were resuspended in 1 mL of F12 + HS media, then divided equally (250 μL) each into 4 separate tubes. These cells would again be pelleted in a centrifuge at 1500 RPM for five minutes. After removing the supernatant from these tubes, cells would be put directly on ice and then be immediately used for transplant (sitting for no more than a maximum of 30 minutes). The final number of cells in each tube was approximately 25,000.

Mouse Injuries and Transplants

Prior to transplant of cells mice were anesthetized with 3% isoflurane such that the mice would be unconscious during the transplantation procedure. Isolated transplant donor cells were resuspended in 50 μL of 1.2% $BaCl_2$ in a 27 ½ gauge syringe and the left tibialis anterior was injected parallel to the muscle.

Tamoxifen injections

100 μL of 20 mg/mL of tamoxifen was administered through intraperitoneal injection into mice to activate CreER.

Tissue Sectioning

TA and EDL muscles were dissected, fixed for two hours in ice cold 4% paraformaldehyde, and then transferred to PBS with 30% sucrose at 4° C for 24 hours. Muscle was mounted in O.C.T. (Tissue-Tek®) and sectioning was performed at -20° C using a Leica cryostat to generate 10 μm cross sections or 30 μm longitudinal sections. Tissue sections were stored at −80° C until staining.

Immunofluorescence staining of tissue section

Tissue sections were post-fixed in 4% paraformaldehyde for ten minutes at room temperature (RT) and washed three times for five minutes in 1X PBS. Heat-induced epitope retrieval was performed in order to facilitate anti-Pax7 antibody staining, in which post-fixed slides were placed in citrate buffer (pH 6.0) and subjected to six min of high pressure-cooking in a Cuisinart

model CPC-600 pressure cooker set on high pressure. For immunostaining, tissue sections were permeabilized with 0.5% Triton-X100 (Sigma) in PBS containing 3% bovine serum albumin (Sigma) for 40 minutes at room temperature, incubated with primary antibody at room temperature for one hour, then with a secondary antibody at room temperature for one hour. Primary antibodies included mouse anti-Pax7 (DSHB) at 1:1000 and rabbit anti-laminin (Sigma-Aldrich) at 1:200. Alexa-555, and 647 secondary antibodies (Molecular Probes) were used at a 1:750 dilution. Tissue sections were incubated with 1 μg/mL DAPI for 10 min at room temperature then mounted in Mowiol supplemented with DABCO (Sigma-Aldrich) as an anti-fade agent.

Flow Cytometry

The left TA of each mouse was dissected then chopped with scissors for five minutes until a slurry of uniform consistency was achieved. Muscle was digested in 9 mL of F12C Media with 1 mL of 4,000 U/mL collagenase at 37° C for one hour, manually shaking every ten minutes. Collagenase reaction was quenched by 10 mL of F12 media with added horse serum. Cells were filtered through 100 μm and 40 μm cell filters, then pelleted by centrifuge at 1,500 RPM for five minutes. Cells were then resuspended in 250 μL of Hank's Balanced Salt Solution (Thermo Fisher) with added 3% bovine serum albumin. Cells were analyzed by flow cytometry (CyAN ADP Analyzer) and data analyzed in FlowJo 9.6. A gate was set to exclude small debris on the basis of previous analysis of freshly isolated SCs.

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