Fibro/adipogenic Progenitor-Derived Follistatin-like 1 Expression is Reduced by Irradiation to Regulate Fibro/adipogenic Progenitor Fate Decisions

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Abstract

Introduction: Five-year cancer survivorship is increasing; however, the long-term effects of treatments are detrimental to skeletal muscle. We have found that fibro-adipogenic progenitors (FAPs) contribute to irradiation-induced muscle atrophy and fibrosis, in part, through alterations to their secretome that impairs muscle stem (satellite) cell (MuSC) fate. It is unknown; however, which specific FAP secreted factors are responsible for the irradiation-induced defect in MuSC differentiation and fusion. Preliminary data from our lab and others suggests that Follistatin-like 1 (FSTL1) may be a key, irradiation-sensitive secreted factor. We aimed to understand how irradiation impacts FSTL1 expression in FAPs and elucidate how FSTL1 expression impacts the MuSC niche.

Methods: *Fstl1 in vitro* gene and protein expression was analyzed on irradiated C3H10T1/2 (C3H) fibroblasts (a FAP cell line) and primary FAPs. *In vitro* FAP differentiation was analyzed on FSTL1 knockdown C3H cells. *Fstl1 in vivo* gene expression was analyzed on mice that had one hindlimb irradiated. The effect of FSTL1 repletion was analyzed *in vivo* on mice that had both hindlimbs irradiated and rFSTL1 injected intravenously.

Results: In C3H cells, *Fstl1* was significantly lower at 24-hours ($p<0.05$) and FSTL1 was significantly higher at 48-hours post-irradiation ($p<0.01$). In primary FAPs, FSTL1 was significantly lower at 12- and 48-hours post-irradiation ($p<0.01$). FSTL1 knockdown significantly lowered the area ($p<0.01$) and mean fluorescence intensity per cell ($p<0.0001$) of fibrogenically differentiated C3H cells. FSTL1 knockdown ($p=0.0701$) and irradiation ($p<0.01$) lowered the percentage of total C3H cells that were adipogenically differentiated. *In vivo* fractionated irradiation did not induce alterations in muscle-resident mononuclear cell populations, muscle fibrosis, and muscle cross-sectional area (CSA) at 3-days. Intravenous repletion of rFSTL1 raised

FSTL1 in circulation ($p<0.0910$) but not in skeletal muscle. **Significance:** The present study demonstrates that *Fstl1* expression is decreased by *in vitro* irradiation, that it upregulates FAP fibrogenic differentiation, and downregulates FAP adipogenic differentiation. We identified FSTL1 as a compelling candidate for mitigating cancer treatment-induced skeletal muscle atrophy and fibrosis. Future work using a novel intramuscular FSTL1 repletion protocol has the potential to unveil benefits in *in vivo* myogenesis following irradiation.

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List of Abbreviations

Ad libitum	As necessary
ANOVA	Analysis of variance
αSMA	Alpha smooth muscle actin
bFGF	Basic fibroblast growth factor
Bradford	Protein quantification assay
BSA	Bovine serum albumin
C2C12	Mouse myoblast cell line
C3H	C3H10T1/2 FAP cell line
C57BL/6	Wild-type mouse strain
$CaCl_2$	Calcium chloride
CD	Cluster of differentiation
CSA	Cross-sectional area
Cxcl14	C-X-C motif chemokine ligand 14
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
Dpp4	Dipeptidyl peptidase 4
ECM	Extra-cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EV	Empty vector condition
FACS	Fluorescence activated cell sorting
FAP	Fibro/adipogenic progenitors
FBS	Fetal bovine serum
FSTL1	Follistatin-like 1
$FSTL1^{kd}$	FSTL1 knockdown
G/S	Goat serum
gRNA	Guide RNA
HRP	Horseradish peroxidase
IL	Interleukin
IR	Irradiated condition
ITGA7	Alpha 7 integrin
Jax	Jackson Laboratories
MFI	Mean fluorescent intensity
MRF	Myogenic regulatory factor
MRF4	Myogenic regulatory factor 4
mRNA	Messenger ribonucleic acid
MuSC	Muscle satellite (stem) cells
Myf5	Myogenic factor 5
MyHC	Myosin heavy chain
MyoD	Myoblast determination protein 1
Myogenesis	Muscle regeneration
OCT	Tissue-embedding medium
Pax7	Paired box protein
PBS	Phosphate buffered saline
PDGFRα	Platelet derived growth factor receptor alpha

P/S	Penicillin/streptomycin
PVDF	Polyvinylidene fluoride membranes
qPCR	Quantitative real-time polymerase chain reaction
rFSTL1	Recombinant Follistatin-like 1
ROS	Reactive oxygen species
scRNA-seq	Single cell RNA-sequencing
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SMAD3	Mothers against decapentaplegic homolog 3
SPARC	Secreted acid and rich in cysteine family
TBST	Tris-buffered saline with tween-20
TGF-β1	Transforming growth factor beta 1
TGF-βR1	Transforming growth factor beta receptor 1
TNF-α	Tumour necrosis factor alpha
T_{reg}	Regulatory T cells
WISP1	Wnt family member 1 inducible signaling pathway protein 1

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1. Literature review

1.1. Introduction

With improved diagnoses and the aging baby boom generation, the rates of cancer incidence are increasing drastically[1]. It has been projected that by 2030, there will be approximately 26 million new cancer cases every year[1]. However, modern innovations in cancer therapies have improved survivorship such that approximately 84% of children and 64% of adults survive five or more years following diagnosis[2]. Increasing survivorship makes research into the long-term complications of cancer treatment imperative. 50% of cancer patients receive irradiation therapy over the course of their treatment[3–5]. Ionizing radiation (irradiation) contributes to approximately 40% of curative cancer treatments and is often used in conjunction with chemotherapy[3]. High energy irradiation is used to induce single and double stranded breaks in target cell DNA which blocks their ability to divide and proliferate further[3,4,6,7]. Double stranded breaks are particularly deleterious as the endogenous non-homologous end joining repair mechanism is inefficient and error prone[8]. Death occurs as cells attempt to divide. This is particularly devastating when it impacts stem cells as they are programmed to divide many times to maintain and replenish tissues[9]. Irradiation-induced secondary malignancies may occur that impair tissue growth, maintenance, and regeneration[4,6,7].

Irradiation exposure at a young age significantly raises the incidence of secondary malignancies compared to the older population[4,10]. Clinically, long-term follow-ups on pediatric cancer survivors have revealed patterns of tissue decline similar to accelerated aging[11–13]. Further, following equivalent doses of irradiation, pediatric survivors are 10-fold more sensitive to secondary malignancies than adult survivors[4,14]. Children are more susceptible due to their longer lifespan post-treatment and the proliferative nature of juvenile tissues[4]. In young, proliferative

tissues, the consequences of irradiation are severe[9] as these actively dividing cells are essential for tissue growth and their dysfunction cause life-long defects in development[5]. The accentuated consequences of irradiation treatment in pediatric survivors have created an emphasis on research into pediatric irradiation treatment-induced pathology.

1.2. Late effects of cancer irradiation treatment

Irradiation treatment causes dysregulation within tissue microenvironments that produce life-long effects[7]. Irradiation initiates damage at the molecular level through alterations in molecular signaling that disrupt homeostasis and overload the endogenous stress response[7]. Irradiation compromises tissues' innate healing ability and leads to significant atrophy long-term[15,16]. Irradiation exposure induces cell loss followed by an inflammatory phase mediated by cytokine and chemokine secretion from inflammatory monocytes[7,17]. The inflammatory phase is followed by a proliferative phase involving local cells, cells migrating from the periphery, or recruited mesenchymal cells[6,7]. The healing process is normally self-regulating with the final phase terminating with scar formation[6,7]; however, following irradiation the affected cells are damaged/transformed[4,7,18]. This damage to resident cell populations plays a significant role in downstream pathogenesis through direct autocrine signaling, or indirect paracrine signaling. Subsequently, a pro-inflammatory M1 macrophage-mediated destructive microenvironment is formed, characterized by a perpetual inflammatory cytokine cascade[7,17,19]. The impacted tissue microenvironment produces persistent mitochondrial damage through downstream generation of reactive oxygen species (ROS) that lead to chronic oxidative stress[7,20].

Skeletal muscle is a striated muscle tissue that is highly stable under normal conditions and exhibits an exceptional ability to regenerate after injury or stress[21]. It makes up approximately 40% of human body weight and is composed of contractile myofibres[21]. Skeletal muscle was originally

thought to be resistant to cancer therapy-induced damage due to its post-mitotic nature. However, mounting evidence indicates that irradiation-induced pathogenesis within skeletal muscle in the form of significant muscle atrophy and/or fibrosis is experienced by approximately 80% of cancer survivors[5,22–26]. Muscle does not have a high turnover rate at rest, so defects can accumulate slowly over time[9]. Clinically, researchers have observed reduced muscle strength, range of motion and motor performance in children following cancer irradiation treatment[27,28]. In mice, hindlimb irradiation has been shown to reduce muscle fibre cross-sectional area (CSA), the average proportion of small muscle fibres, and strength[5,25,28,29]. Age of exposure plays a role as hindlimb irradiation in young mice leads to significantly more skeletal muscle atrophy than in aged mice[25,29,30]. Specifically in juvenile mice, Bachman and colleagues found that young pups irradiated with the same dose as aged mice displayed uniquely lowered muscle weight and CSA[5]. Further, irradiation-induced impairment in muscle regeneration was associated with deficits in muscle satellite (stem) cell (MuSC) content[5]. These findings confirmed that prepubertal irradiation exposure can have long-term effects on muscle regeneration evidenced by life-long defects in MuSC proliferation and differentiation[5].

Studies from our lab and others have shown that pediatric irradiation promotes a fibrotic phenotype in muscle tissue. These studies demonstrated higher fibrotic gene expression, extracellular matrix deposition (fibrosis), upregulated fibro/adipogenic progenitor (FAP) senescence, and more FAP fibrogenic differentiation[5,23–26,31]. Our lab successfully confirmed these observations by displaying that juvenile irradiation exposure contributes to long-term muscle atrophy and fibrosis through widespread alterations in the MuSC niche[23,24]. These alterations may be due to impaired signaling between the various muscle-resident mononuclear cell populations.

Understanding how irradiation treatment affects communication between different muscle-resident mononuclear cell populations may represent a promising therapeutic avenue.

1.3. Muscle resident progenitor cells in muscle regeneration

MuSCs are the progenitor cells of skeletal muscle and are solely responsible for adult muscle regeneration (myogenesis)[32]. MuSCs reside between the basal lamina and the sarcolemma. They are composed of heterogeneous populations of stem cells and committed myogenic progenitors[33–35]. MuSCs are normally mitotically quiescent; however, in response to a variety of stimuli, they are activated to enter the cell cycle, give rise to myogenic precursor cells, undergo terminal differentiation, and fusion into multinucleated muscle fibres[21,32–34,36]. MuSCs are replenished throughout the lifetime; however; their proliferative capacity declines with age[9,33,37,38].

MuSCs are characterized by paired box protein (Pax7) expression[21,33,39,40]. Uncommitted MuSCs are identified as $Pax7^{+}/MyoD^{-}$ and as they exit quiescence, they begin upregulate Myf5, and upregulate MyoD to commit to differentiation[33,34,41]. As MyoD expression increases, MuSCs lose contact with the basal lamina to fully commit to the myogenic lineage and they are no longer able to return to quiescence[34]. However, MyoD alone is insufficient to support terminal differentiation, and it requires stepwise upregulation of other myogenic regulatory factors (MRFs) myogenin and MRF4 to complete myogenesis[42,43]. Once MuSCs terminally differentiate, they no longer express MyoD, Myf5, or MRF4 and begin to express Myosin Heavy Chain (MyHC) which is associated with mature myotubes and myofibres[32,41]. MRF4 expression is reliant on signals present within the MuSC niche which can be altered by stressors such as irradiation[5,32].

FAPs are a type of skeletal muscle resident mesenchymal stem cell that reside in the interstitial space between muscle fibres[44]. Two independent studies identified this bipotent cell population present in skeletal muscle in 2010[45,46]. Joe and colleagues termed these cells FAPs, and

displayed that they are abundant in healthy muscle, that they rapidly enter the cell cycle in response to muscle injury, and implied a role in muscle regeneration[45]. Proliferating FAPs are associated with regenerating myofibres, and their numbers peak 2-3 days following perturbation[45]. Undifferentiated FAPs secrete trophic factors that promote muscle regeneration and growth[45,47,48]. High FAP content is associated with high content of the MuSC differentiation markers MyoD, myogenin, and MyHC which implies a dose dependent effect of FAPs on MuSC terminal differentiation[45]. Blocked FAP proliferation and expansion has been associated with poor muscle regeneration following damage[49,50]. However, FAPs do not undergo myogenesis or fuse into existing myoblasts which suggests a separate developmental source from MuSCs[45]. A multitude of studies have shown that FAPs can differentiate into both fibroblasts and adipocytes depending on environmental conditions[44–46,51]. Fibrogenic FAPs produce components of the extracellular matrix (ECM) such as collagen, laminin, and fibronectin[47,52]. Under normal conditions, fibrotic differentiation generates a transient pro-differentiation myogenic niche that is followed by prompt termination of ECM growth to avoid pathological fibrosis and tissue degeneration[45,48,53]. However, following detrimental stressors such as irradiation, FAPs can be a source of infiltrating fibroblasts[54,55]. Aberrant overaccumulation of ECM components then replaces muscle with fibrous tissue and compromises the tissue microenvironment[5,45,54,55].

1.4. Cross-talk in the muscle satellite cell niche

Functional cross-talk between MuSCs and cells such as motor neurons, immune cells, endothelial cells, and FAPs is essential for muscle repair and homeostasis[9,48]. In response to muscle trauma, immune cells migrate to the damaged site to secrete cytokines and growth factors that regulate the regenerative niche[9]. Interleukin (IL)-1β secretion is upregulated early in the immune response to recruit other immune cells and amplify MuSC proliferation[56]. Eosinophils secrete IL-

4 and IL-13 which activate the regenerative capacity of FAPs by promoting their proliferation and inhibiting their adipogenic differentiation[48,57,58]. IL-4/IL-13 knockout mice exhibit persistent cellular debris and an absence of regenerated muscle fibres[57]. These findings implied that FAPs can also phagocytize necrotic debris which is crucial for successful completion of muscle repair[48,57]. IL-15 is a myokine expressed by skeletal muscle fibres that prevents intramuscular fatty infiltration by stimulating FAP proliferation and inhibiting their adipogenic differentiation to facilitate myofibre regeneration[48,59]. M1 pro-inflammatory macrophages rapidly secrete TNF-α upon muscle damage which regulates ECM production and mediates FAP apoptotic clearance[48,50,54]. When TNF-α-producing macrophages are depleted FAPs accumulate and muscle fibrosis is enhanced[48,50,54]. M2 anti-inflammatory macrophages secrete TGF-β1 which is a crucial regulator of fibrogenesis[41,54]. Under stress, macrophages secrete high levels of TGF-β1 which antagonizes TNF-α-mediated apoptosis and induces FAP fibrogenic differentiation[48,54,60,61].

Undifferentiated FAPs also secrete several important cytokines that influence the activity of muscle-resident mononuclear cell populations[48]. FAP-secreted trophic factors are essential for FAP-MuSC communication and play an important role in myogenesis[48]. IL-6 is secreted by FAPs and has a paradoxical impact on myogenic differentiation. IL-6 promotes both muscle hypertrophy and atrophy under different conditions[62]. During skeletal muscle overload and in models of resistance exercise, IL-6 is upregulated suggesting a role in skeletal muscle hypertrophy[48,62–64]. However, under stress IL-6 promotes skeletal muscle atrophy[55,65]. IL-6 inhibition counteracts muscle atrophy following muscle denervation[55]. Transient production of IL-6 plays a role in upregulating MuSC proliferation and muscle regeneration; however, long term elevation of IL-6 leads to significant muscle atrophy[48,55,62,65–67]. It is a pleiotropic cytokine whose tight regulation is important to maintain muscle regeneration[62]. IL-33 is another myokine secreted by FAPs

approximately 6-12 hours following injury which induces T_{reg} cell proliferation that promotes muscle repair[48,68]. IL-10 is a FAP-secreted myokine that is upregulated by muscle damage[69] to induce a pro-regenerative shift in macrophages that strongly promotes effective MuSC regeneration[48,69,70]. Wnt family member 1 inducible signaling pathway protein 1 (WISP1) secretion from FAPs plays an important role in MuSC expansion and asymmetric commitment to myogenic differentiation[38,48]. Lukjanenko and colleagues in 2019 found that aging impaired FAP activity primarily through lowered WISP1 secretion[38]. Lowered WISP1 secretion was associated with lower Pax7, MyoD, and myogenin content and smaller CSA in aged muscle. When WISP1 secretion was induced in aged mice, it enhanced MuSC commitment which implied that WISP1 is an important component of FAP-MuSC communication[38]. Follistatin is a natural antagonist of myostatin (part of the TGF-β1 superfamily) that is secreted by FAPs 12 hours following muscle injury[48,71,72]. Follistatin knockdown reduced MuSC differentiation and the formation of myotubes *in vitro* which suggested a critical role as a mediator of the pro-regenerative activity of FAPs[48,72]. The proper balance of FAP-secreted regulatory factors in the regenerative niche is key for effective muscle repair. These observations provide the rationale for studies that further characterize FAPs secretion profile of pro-myogenic trophic factors in response to stress such as irradiation.

1.5. Iradiation exposure impairs MuSC proliferation and differentiation

Several studies have demonstrated impaired MuSC function following irradiation. Previous work from our lab demonstrated that targeted mouse hindlimb irradiation immediately resulted in MuSC DNA damage and cellular oxidative stress[23]. These observations coincided with reduced MuSC proliferation, lower muscle CSA, and a lower proportion of type 1 muscle fibres[23]. Further, prepubertal irradiation has been shown to induce impairment in MuSC myogenic commitment which resulted in long-term muscle atrophy[5,73]. Bachman and colleagues in 2020

found that following targeted irradiation to one hindlimb, there were significantly fewer MuSCs in the irradiated limb[5]. Further, MuSC apoptotic markers were upregulated, which confirmed that MuSCs were both proliferating less and clearing themselves more in response to irradiation[5]. Myogenic commitment was also impaired, evidenced by a lower proportion of MyoD and myogenin positive MuSCs in irradiated versus control muscle[5]. Bachman and colleagues concluded that MuSCs subjected to prepubertal irradiation exhibit impaired proliferation and regeneration that results in lifelong deficits in MuSC number, myofibre CSA, and myonuclear number. Following pediatric irradiation, muscle fibres undergo significant remodeling through the MuSC niche that leads to reductions in hypertrophic muscle growth[5,25,74,75]. Previous attempts to mitigate irradiation-induced defects in muscle regeneration have been aimed at replacing lost cells through MuSC or myoblast transplantation[33,34,76]. MuSC transplantation only leads to terminal differentiation of surviving cells and does not contribute to the resident MuSC niche.[34]. Direct myoblast replacement also does not effectively repopulate the MuSC niche following irradiation due to immune rejection and cell death[77]. Even when an irradiation-resistant MuSC subpopulation was transplanted into irradiated skeletal muscle, muscle regeneration remained significantly impaired[76]. Combined, these observations suggest that irradiation induces widespread alterations in the skeletal muscle niche in addition to direct MuSC depletion.

1.6. Irradiation exposure reduces FAP content and upregulates FAP fibrogenic differentiation

As a major component of the skeletal muscle niche, it stands to reason that FAPs would also be affected by irradiation. Previous work from our lab and others identified a multitude of irradiation-induced alterations in FAP differentiation and signaling[5,23–26,31]. In 2023, Collao and colleagues found that irradiation *in vivo* induced acute (3-days) SMAD3 activation (downstream

of TGF-β1 signaling)[60] implying short-term activation of fibrotic pathways[23]. Long-term muscle fibrosis was also demonstrated through elevated Masson's trichrome staining, and higher content of fibrotic markers (αSMA and fibronectin protein) at 56-days post-irradiation[23,78]. The content of the overall FAP population was depleted at 56-days post-irradiation; however, the $CD142^+$ fibrotic FAP subpopulation was significantly higher[23]. Further, conditioned media from irradiated FAPs lowered MuSC fusion acutely (3-days) and both fusion and differentiation long-term (56-days) demonstrating that irradiation induces alterations to the FAP secretome[23]. In another study, our lab again found that *in vivo* irradiation depleted FAP content, alongside upregulated genes related to muscle fibrosis and inflammation[24]. In both cases, these alterations in FAP regulation coincided with a reduced proportion of type 1 muscle fibres, smaller overall muscle CSA, and lower type 1 fibre specific CSA[23,24]. Together, these findings indicate that irradiation treatment aberrantly upregulates FAP fibrotic differentiation and results in fewer FAPs[23,24]. Alterations in FAP regulation lead to higher fibrous tissue deposition with associated reductions in undifferentiated, pro-myogenic FAP content[23,24]. These findings implicate FAPs as a potential therapeutic target for treating irradiation-induced muscle atrophy and fibrosis.

1.7. Follistatin-like 1 as a candidate myogenic secreted factor

Follistatin-like 1 (FSTL1) is a secreted glycoprotein that is a member of the secreted acid and rich in cysteine (SPARC) family[79]. FSTL1 has been identified in vascular endothelial cells, smooth muscle cells, cardiomyocytes, and mesenchymal stromal cells[79,80]. FSTL1 is primarily expressed by cells of mesenchymal origin, including skeletal muscle FAPs[81,82]. By the end of gestation, FSTL1 expression becomes restricted to the mesenchymal component of most tissues[79,83]. FSTL1 was initially studied in the context of cardiomyocytes and revascularization. Researchers have seen a drastic upregulation of vascular wall fibrosis in cardiac muscle following

FSTL1 depletion[84]. In a model of vascular inefficiency, FSTL1 upregulation in the ischemic tissue enhanced capillary density and blood flow recovery through anti-apoptotic activity[85]. Thus, FSTL1 is secreted by mesenchymal cells and plays a role in general tissue fibrosis suggesting that it may be a FAP-secreted myokine that regulates muscle regeneration.

FSTL1 secretion is modulated by exercise and inflammatory stimuli, and its activity regulates muscle regeneration. Myotubes treated with IL-1β *in vitro* exhibited higher FSTL1 secretion into culture media[81,86]. In models of exercise and muscle injury, upregulation of FSTL1 activity is associated with normal muscle regeneration. In response to intense physical exercise FSTL1 secretion is upregulated[86]. Further, FSTL1 secretion from FAPs peaks at 4-10 days post-injury when MuSC-mediated muscle regeneration is occurring[80]. Two independent studies showed that FSTL1 secretion from FAPs is upregulated during muscle regeneration and it is associated with pro-regenerative FAP subpopulations[87,88]. FSTL1 treatment has been demonstrated to positively regulate myogenesis pre-clinically. Upregulation of FSTL1 leads to higher MuSC differentiation and recombinant FSTL1 treatment improves myogenesis following stress[72,89]. FSTL1 overexpression in mouse skeletal muscle enhances myoblast fusion, promotes muscle hypertrophy, and an oxidative fibre-type switch which leads to higher whole-body strength and fatigue resistance[90]. Importantly, FSTL1 content and signaling are impacted by various muscle stressors. Pre-clinically, cancer cachexia (aberrant muscle and adipose tissue loss associated with cancer) lowered FSTL1 content, which was associated with higher tissue fibrosis[90]. *Fstl1* transcript expression was also lowered in the skeletal muscle of the cachectic rats[90]. De Castro and colleagues in 2021 confirmed these observations clinically by showing that muscle FSTL1 expression was downregulated in the muscle tissue of patients with cancer cachexia [81,91]. They implied that FSTL1 deficiency caused long-term muscle wasting[81]. Furthermore, FSTL1 deficiency has been

implicated in upregulated TGF-β/SMAD3 signaling which lead to muscle wasting and fibrosis[7,84]. Since FSTL1 expression is elevated during normal muscle regeneration, and its deficiency is associated with muscle wasting, it is likely that altered FSTL1 function plays a role in irradiation-induced muscle defects.

Other groups have identified *Fstl1* as enriched in pro-regenerative FAP subpopulations and upregulated during myogenic commitment[87,88]. Preliminary single cell RNA-seq (scRNA-seq) experiments done by Dr. Nicolás Collao in our lab showed that *Fstl1* expression was enriched in the promyogenic $Dpp4^{+}$ and $Cxcl14^{+}$ FAP subpopulations[92] (Figure 1A-B; unpublished). His scRNA-seq data showed that *Fstl1* was significantly reduced at both 24-hours and 56-days following *in vivo* irradiation (Figure C-D; unpublished). These data implied that FSTL1 is an irradiation-sensitive FAP-derived secreted factor; however, qPCR and western blot confirmation *in vitro* are required to identify a causal link between irradiation and *Fstl1* expression. Further, Dr. Collao used CRISPR-Cas9 to generate a C3H cell line with FSTL1 knocked down ($FSTL1^{kd}$; Figure 2B; unpublished). He then cultured these cells and applied their conditioned media to C2C12 myoblasts differentiating *in vitro* (Figure 2A; unpublished). He found that the secretome of $FSTL1^{kd}$ C3H cells significantly reduced C2C12 differentiation and fusion compared to control and empty vector cells (Figure 2 C-D; unpublished). In a similar experiment, Dr. Collao subjected cultured C3H cells to a single 1.0 Gy dose of *in vitro* irradiation and applied their conditioned media with recombinant FSTL1 (rFSTL1) added to C2C12 myoblasts differentiating *in vitro* (Figure 3A; unpublished). This FSTL1 repletion resulted in significantly improved C2C12 differentiation and fusion (Figure 3B-C; unpublished). These data implied that FSTL1 exhibits a paracrine effect on MuSCs that is vital for their ability to differentiate into myofibres. Thus,

targeting FSTL1 may have therapeutic applications for treating irradiation-induced muscle regeneration impairment.

1.8. Statement of the problem and objectives of the present study

To summarize the presented literature: (1) Irradiation therapy has severe long-term side effects, especially in pediatric patients, (2) irradiation disrupts the muscle regenerative environment through alterations in MuSC cross-talk with its supportive niche, (3) FAPs are critical regulators of muscle regeneration, and they represent a promising therapeutic target for mitigating irradiation-induced muscle defects. FAPs contribute to irradiation-induced muscle atrophy and fibrosis[5,23–26,31]; in part, through alterations to their secretome that hinders MuSC fate[23]. It is unknown; however, which specific FAP-secreted factors are responsible for the irradiation-induced defect in MuSC differentiation. Preliminary data from our lab and others suggest that FSTL1 may be a key, irradiation-sensitive secreted factor. Thus, the purpose of the current study is to understand the role of FAP-secreted FSTL1 in irradiation-induced skeletal muscle atrophy and fibrosis. To address this purpose, I will complete the following specific aims:

1. Characterize how irradiation impacts FSTL1 expression in FAPs.
    a. We hypothesize that both *in vitro* and *in vivo* irradiation would deplete *Fstl1* transcript and protein expression.
2. Elucidate the role of FSTL1 in FAP fate decisions and secretome.
    a. We hypothesize that FSTL1 knockdown will lead to higher fibrogenic differentiation.
3. Elucidate the role of FSTL1 in myogenesis.
    a. We hypothesize that FSTL1 repletion will improve *in vivo* myogenesis in a murine model of *in vivo* irradiation.

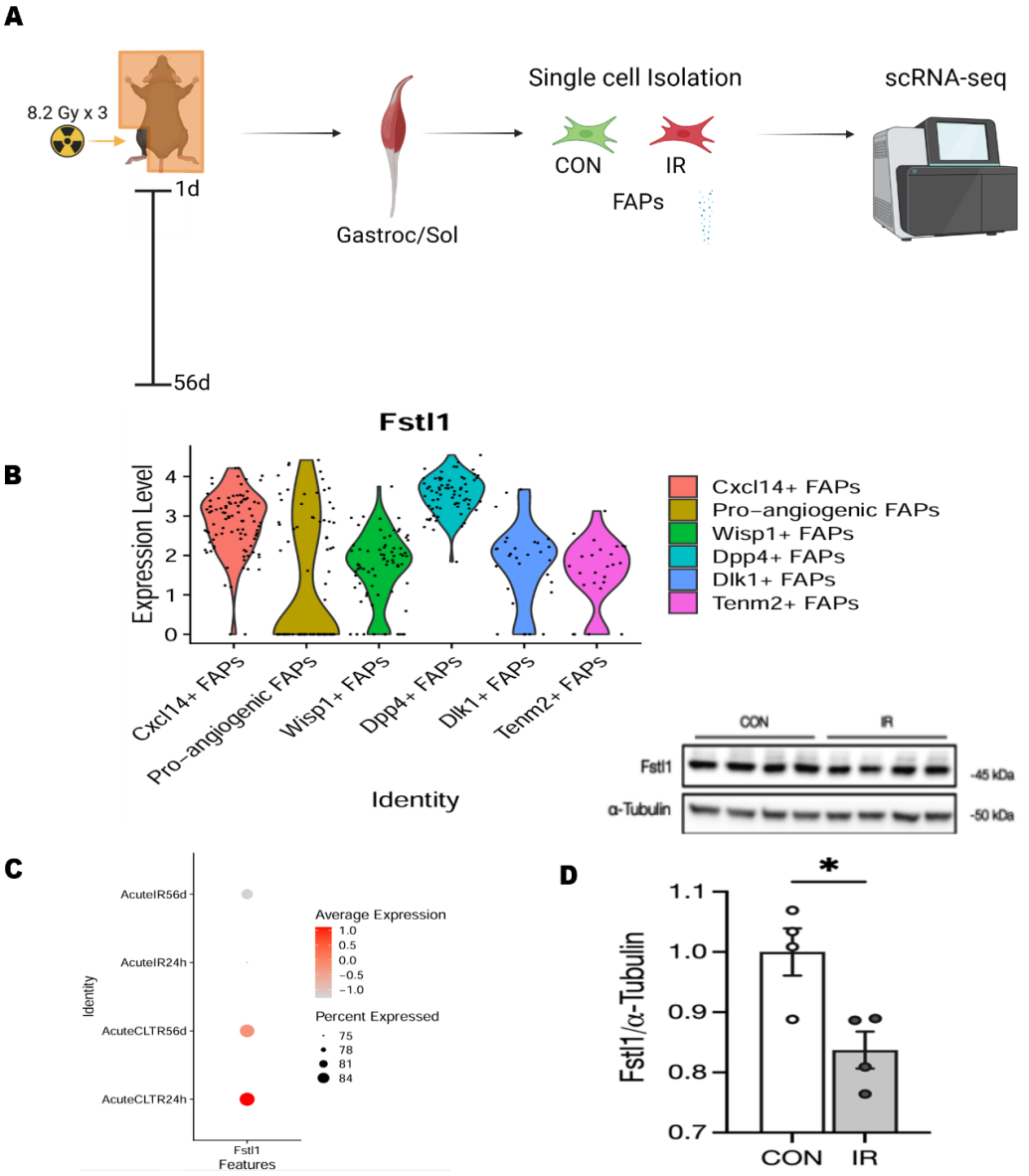

Figure 1: Follistatin like-1 (FSTL1) is enriched in promyogenic FAP populations and its expression is reduced by irradiation. (A) Mice received 3, 8.2 Gy doses of irradiation. FAPs were isolated from the irradiated (IR) and contralateral (CON) limbs at 1-, and 56-days post-irradiation for single-cell RNA-seq analysis. (B) Violin plot of *Fstl1* expression in 6 different FAP subclusters. $Dpp4^{+}$ and $Cxcl14^{+}$ are promyogenic subclusters. (C) Dot plot of *Fstl1* expression 24-hours and 56-days post-irradiation. (D) Bar graph of the impact of irradiation on FSTL1 protein content in whole skeletal muscle 56-days post-irradiation (*$p<0.05$; Student's t-test; n=4).

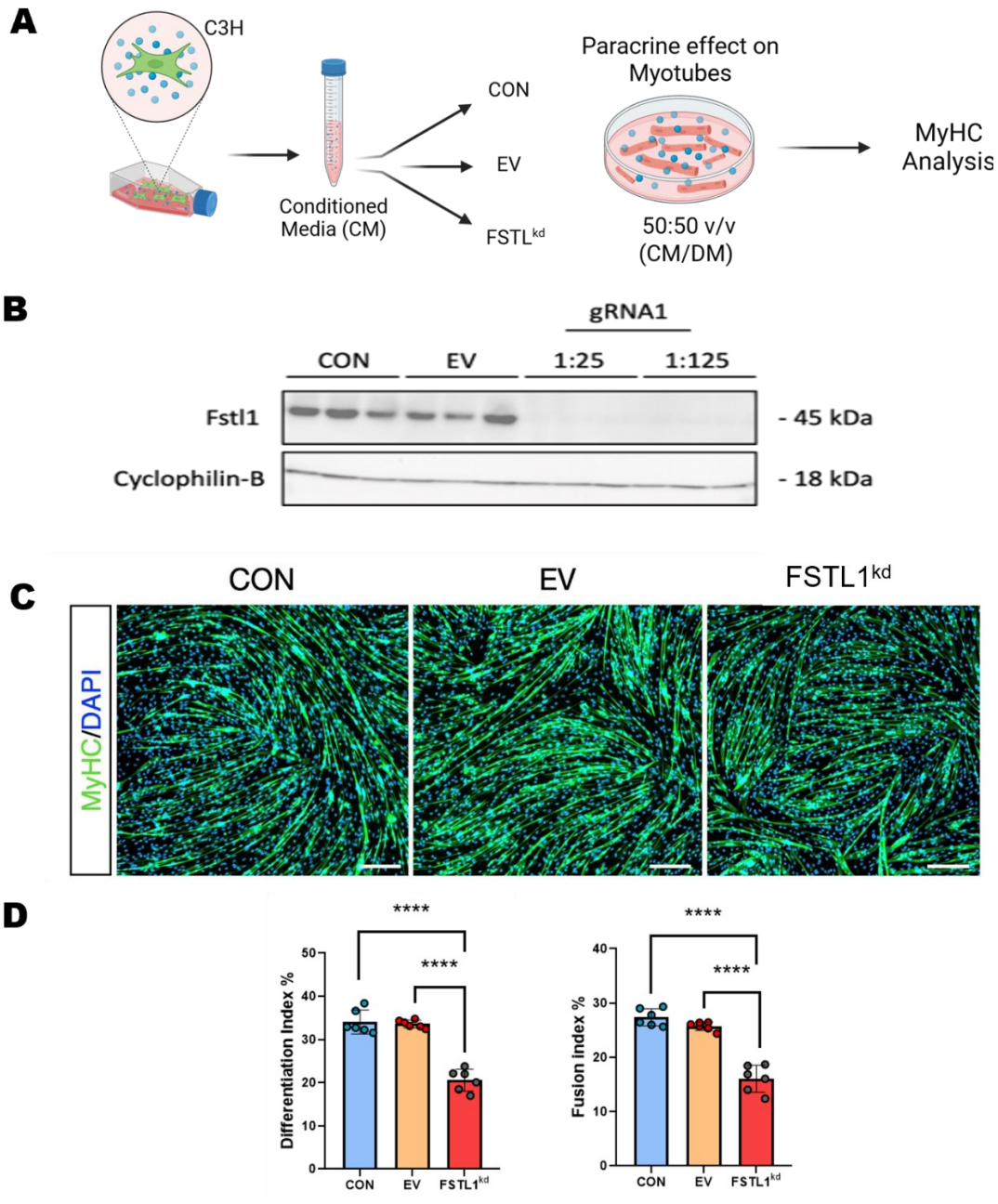

Figure 2: The secretome from FSTL1 knockdown FAPs inhibits C2C12 fusion and differentiation *in vitro.* (A) FSTL1 knockdown (FSTL1^{kd}), CON, and empty vector (EV) C3H fibroblasts were cultured and had their media applied to C2C12 myoblasts differentiating *in vitro.* (B) Representative western blot of CON, EV, and FSTL1^{kd} FAPs generated using 1:25 and 1:125 dilutions of *Fstl1* gRNA. (C) Representative images of differentiated C2C12 myoblasts grown in conditioned media. Cells were stained with Myosin Heavy Chain (green) and DAPI (blue). (D) Bar graphs of the impact of FSTL1 knockdown on C2C12 differentiation and fusion (****p<0.0001; One-way ANOVA with Tukey's post-hoc multiple comparisons test; n=6).

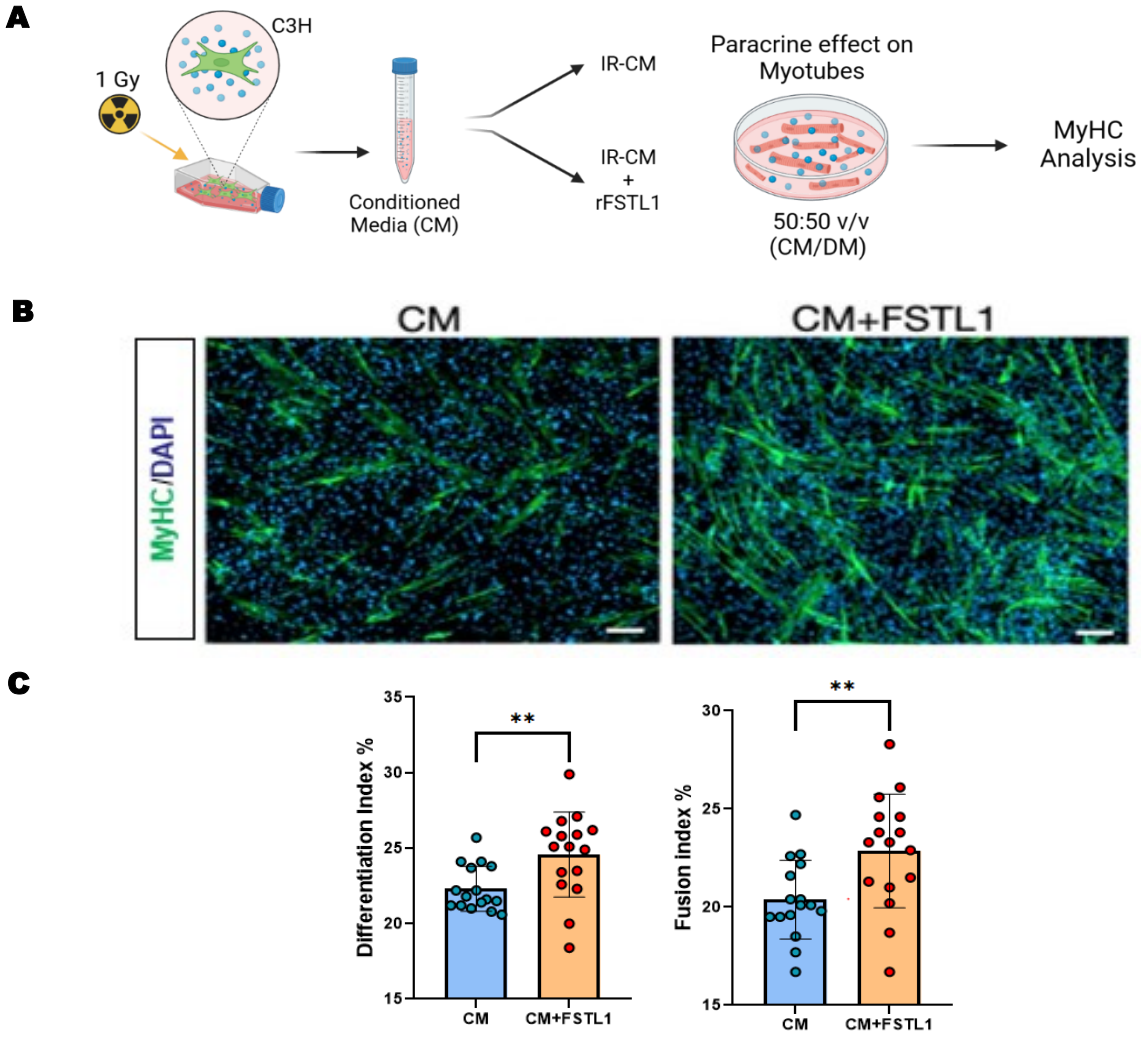

Figure 3: Restoring FSTL1 in irradiated FAPs media restores C2C12 fusion and differentiation *in vitro*. (A) Wild-type C3H fibroblasts were irradiated with a 1 Gy dose of irradiation and had their media applied to C2C12 myoblasts differentiating *in vitro* (CM). One group had 50 ng/ml rFSTL1 protein added to its media (CM+FSTL1). (B) Representative images of differentiated C2C12 myoblasts grown in conditioned media. Cells were stained with Myosin Heavy Chain (green) and DAPI (blue). (C) Bar graphs of the impact of FSTL1 repletion on C2C12 differentiation and fusion (**p<0.01; Student's t-test; n=16).

2. Methods

2.1. Ethics and mice

Experiments were conducted in accordance with ethical approval obtained from the University of Ottawa Animal Care and Veterinary Service Committee (ACVS), according to guidelines set by the Canadian Council on Animal Care. Mice were housed in a controlled pathogen-free environment (temperature: 22-25ºC, humidity: 30%, and a 12-hour light: dark cycle). Food and water were supplied *ad libitum*. Four-week-old male PDGFRα-CreER^{T2} (#032770) and female Ai14(RCL-tdT)-D (#007914) mice were ordered from The Jackson Laboratory (JAX; Bar Harbor, ME) and bred to produce a stable, tdTomato reporter colony (PDGFRα-tdT). Four-week-old male and female C57BL/6 (#000664) mice were also ordered from JAX. All mice were allowed to acclimatize to the animal facility for one week prior to any manipulation.

2.2. C3H10T1/2 and fibro/adipogenic progenitors cell culture

The C3H fibroblast cell line (ATCC, Clone 8 Cat# CCL-226^{TM}) was used as an *in vitro* FAP model[8,38,39]. C3H cells were cultured in either 100 mm (Cat# 130182; Thermo Fisher Scientific) or 60 mm (Cat# 130181; Thermo Fisher Scientific) cell culture plates in DMEM high glucose (DMEM++; Cat# 319-005-CL; Wisent Bioproducts), 10% fetal bovine serum (FBS; Cat# 12483-020; Thermo Fisher Scientific), and 100 U/mL penicillin/streptomycin (P/S; Cat# 15140-122; Thermo Fisher Scientific). Isolated primary FAPs were cultured in DMEM high glucose, 20% FBS, 100 U/mL P/S, and 5 ng/mL basic fibroblast growth factor (bFGF; Cat# F0291; MilliporeSigma).

2.3. *In vitro* irradiation model

For *in vitro Fstl1* mRNA quantification, C3H and primary FAPs were cultured in 6-well cell culture plates (Cat# 140675; Thermo Fisher Scientific) to 60-70% confluence. For protein quantification, C3H and primary FAPs were cultured in 100 mm cell culture plates to 60-70% confluence. Cells were then subjected to one or two 1.0 Gy doses of irradiation (XRAD 320; uOttawa Pre-clinical Imaging Core). Cells subjected to one dose had mRNA and protein extracted 12-, 24-, 48-hours post-irradiation. Cells subjected to two doses had 24-hours between their two irradiation doses to simulate clinical irradiation treatment, then mRNA and protein were extracted 24-hours following the second dose.

2.4. Tissue collection

Gastrocnemius/soleus complexes were carefully dissected following treatments and used for total weight, flow cytometry, gene expression, protein content, CSA, and FACS experiments. Muscles were either weighed for immediate digestion for flow cytometry or FACS; or cut in half with one half embedded in tissue-embedding medium (OCT; Cat# 4585, Thermo Fisher Scientific) for sectioning and one-half flash frozen for protein extraction. Frozen samples were preserved at -80^{o}C before analysis.

2.5. Flow cytometry and fluorescence activated cell sorting

Skeletal muscle preparation for flow cytometry and FACS was adapted from previously described protocols[9,42]. Gastrocnemius/soleus complexes were dissected, washed with cold-PBS, and connective tissue and fat were removed. Dissected muscles were transferred to Miltenyi GentleMACS C tubes (Cat# 130-093-237; Miltenyi Biotec) containing 1.5 U/mL Collagenase D (Cat# COLLD-RO; MilliporeSigma), 2 U/mL Dispase II (Cat# 42613-33-2; MilliporeSigma), and 10 µl/mL $CaCl_2$ in Ham's F10 media (Cat# 318-050-CL; Wisent Inc). Muscle tissue was minced

then dissociated by the gentleMACS Octo Dissociator (Cat# 130-096-427; Miltenyi Biotec). The muscle homogenate was diluted with 5 mL FBS first and then with 10 mL PBS before being passed through a 100 µm strainer (Cat# 22-363-549; Thermo Fisher Scientific) and subsequently through a 70 µm strainer (Cat# 08-771-2; Fisher Scientific). The filtered homogenate was then centrifuged at 400 x g for 10 minutes at 4ºC. The cell pellet was then resuspended in Red Blood Cell Lysing Buffer (Cat# R7767; MilliporeSigma), incubated at 4ºC for 3 minutes in the dark, and centrifuged at 400 x g for 5 minutes at 4ºC. For flow cytometry, the cell pellet was resuspended in 100 µl of an antibody cocktail containing 1:50 PDFRα (Brilliant Violet™ 605 anti-mouse CD140α (PDGFRα); Cat# 740380; BD Biosciences) in ice-cold FACS buffer (10% FBS, 3mM EDTA in 1xPBS) and incubated at 4ºC in the dark for 20 minutes. The cell suspension was quenched with 1 ml FACS buffer and then centrifuged at 400 x g for 5 minutes at 4ºC. The pellet was resuspended in 100 µl of an antibody cocktail consisting of 1:20 ITGA7 (PE anti-mouse Alpha 7 Integrin; Cat# 53-0010-05; AbLab Biologics), 1:50 CD45 (PE-Cy™7 anti-mouse CD45; Cat# 552848; BD Biosciences), and 1:50 CD31 (Brilliant Violet™ 421 anti-mouse CD31; Cat# 102424; BioLegend) in ice-cold FACS buffer and incubated at 4ºC in the dark for 30 minutes. The cell suspension was quenched with 1 ml FACS buffer and centrifuged at 400 x g for 5 minutes at 4ºC. The pellet was resuspended in 500 µl FACS buffer and filtered through a 50 µm strainer (Cat# MSPP-40042327; CellTrics). Flow cytometry was performed using the Attune NxT Flow Cytometer. For FACS, samples were prepared in the same way except PDFRα (PE anti-mouse CD140α (PDGFRα); Cat# 17-1401-81; Thermo Fisher Scientific), ITGA7 (647 anti-mouse Alpha 7 Integrin; Cat# 67-0010-05; AbLab Biologics), CD45 (FITC anti-mouse CD45; Cat# 103108; BioLegend), and SYTOX™ AADvanced™ (Cat# S10349; Thermo Fisher Scientific) were used in place of previously described antibodies. The AbC™ Total Antibody Compensation Bead Kit (Cat# A10497; Thermo

Fisher Scientific) was used for all antibody compensation. Fluorescence minus one and single-stained controls were used to establish gates for all cell populations. 0.3 µl SYTOX^{TM} Green (Cat# S34860; Thermo Fisher Scientific) stain was added right before analysis for classification of cell viability. Endothelial cells were identified as CD45^{-}/CD31^{+}. Hematopoietic cells were identified as CD45^{+}/CD31^{-}. MuSCs were identified as CD45^{-}/CD31^{-}/PDGFRα^{-}/ITGA7^{+}. FAPs were identified as CD45^{-}/CD31^{-}/PDGFRα^{+}/ITGA7^{-}.

2.6. *In vivo* irradiation model

At 5 weeks of age, C57BL/6 mice were subjected to 3 fractionated doses of 8.2 Gy irradiation localized to the right hindlimb delivered over alternating days (M/W/F)[5,93]. The rest of the body was covered with a lead murine abdomen shield and the contralateral non-irradiated limb was used as an internal control. 24-hours following the final dose, mice were sacrificed and both gastrocnemius/soleus complexes were prepared for FACS. Isolated FAPs were cultured on 6-well cell culture plates to 80-90% confluence and had mRNA extracted for qPCR analysis.

2.7. C3H10T1/2 *in vitro* differentiation and immunocytochemical analysis

C3H fibrogenic and adipogenic differentiation was initiated as previously described[23]. Briefly, C3H cells were cultured to 60-70% confluence in 24-well cell culture plates (Cat# 142475; Thermo Fisher Scientific). For fibrogenic differentiation, cells were incubated in DMEM++ with 2% horse serum (Cat# 16050122; Millipore Sigma), 1% P/S, and 5 ng/ml TGF-β1 (Cat# 100-21; PeproTech) for 3 days. Differentiated cells were stained for alpha-smooth muscle actin (αSMA; Cat# A5228; MilliporeSigma) and DAPI (Cat# D9542; Sigma-Aldrich) for analysis of αSMA area per cell and mean fluorescent intensity of αSMA per cell. For adipogenic differentiation, cells were incubated in the Mesencult^{TM} Adipogenic Differentiation Kit (Cat# 05507; Stemcell Technologies) for 3 days followed by adipocyte maintenance media consisting of DMEM++ with

20% FBS, 1% P/S, and 1 µg/ml insulin (Cat# I9278; MilliporeSigma) for 2 days. Differentiated cells were stained with perilipin (Cat# 9349S; CellSignaling) and DAPI for analysis of percent perilipin positive cells, area of perilipin per cell, and mean fluorescent intensity of perilipin per cell.

2.8. rFSTL1 intravenous repletion model

At 5 weeks of age, two groups of C57BL/6 mice were subjected to 3 fractionated doses of 8.2 Gy irradiation localized to both hindlimbs delivered over alternating days (M/W/F)[5,93]. The rest of the body was covered with a lead murine abdomen shield. A third group of age matched mice was used as a non-irradiated control group. 24-hours following the final dose, mice had 100 ng/g body mass of rFSTL1 (Cat# 1694-FN-050; R&D Systems) intravenously injected into their tail vein as previously described[94] (IR + FSTL1). The other irradiated group received the same volume/mass of 1xPBS (IR + SHAM) as did the control group (CON). Mice were exsanguinated, had one gastrocnemius/soleus muscle digested for flow cytometry, had half of one gastrocnemius/soleus muscle digested for western blot, and half of one gastrocnemius/soleus muscle mounted in OCT and sectioned for immunohistochemical analysis.

2.9. qPCR analyses

For mRNA extraction, cell culture plates were scraped (Cat# 08-771-1A; Fisher Scientific), and mRNA was purified using the Qiagen RNeasy Mini Kit (Cat# 74104; Qiagen Inc) according to manufacturer's instructions. Quantification of isolated RNA was performed using the NanoDrop™ 2000 Spectrophotometer (Cat# ND-2000; Thermo Fisher Scientific). RNA was reverse transcribed using the ProtoScript® II Reverse Transcriptase Kit (Cat# M0368S; New England Biolabs) according to manufacturer's instructions. Quantitative real-time PCR (qPCR) analysis was performed using Taqman gene expression assays (Cat# 4351368; Thermo Fisher

Scientific) for *Fstl1* (Assay ID: Mm00433371_m1) and *Ppia* (Assay ID: Mm02342430_g1). qPCR analysis was conducted with the CFX Opus 96 Real-Time PCR System (Cat# 12011319; Bio-Rad). Changes in gene expression of *Fstl1* was determined using the 2^{-ddCT} method[95] compared to the housekeeping gene *Ppia*.

2.10. Western blot analyses

Western blot from muscle samples and adherent cells were prepared as previously described[23]. For extraction from adherent cells, 150 µl RIPA buffer prepared with anti-protease (Cat# 04693116001; MilliporeSigma) and anti-phosphatase (Cat# 4906837001; MilliporeSigma) tablets were added to each 10 cm culture plate. The plates were then scraped and the collected volume was agitated for 35 minutes at 4ºC, centrifuged at 20,000 x g for 8 minutes at 4ºC, and the supernatant was stored at -80ºC, prior to analysis. For extraction from muscle tissue, flash-frozen muscle samples were homogenized in 10 µl/mg muscle mass RIPA buffer prepared with anti-protease and anti-phosphatase inhibitors using a bead homogenizer (Fisherbrand™ Bead Mill 24 Homogenizer; Cat: 15-340-163; Fisher Scientific) for two 1-min cycles at 6 m/s separated by 5 seconds. Samples were then centrifuged at 15,000 x g for 15 minutes at 4ºC and the supernatant was stored at -80ºC, for protein quantification by Bradford, prior to analysis. Samples were diluted with 4x Laemmli buffer and 35 µg of protein for FSTL1 (1:1000 in 5% BSA; Cat# NBP1-83425; R&D Systems), Cyclophilin B (1:1000 in 5% BSA; Cat# Ab16045; Abcam), TGF-β1 (1:1000 in 2% BSA; Cat# SAB4502954; MilliporeSigma), TGF-βR1 (1:1000 in 5% BSA; Cat# AP14647PU-N; OriGene), SMAD3 (1:500 in 2% BSA; Cat# MA5-14939; Thermo Fisher Scientific), pSMAD3 (1:500 in 2% BSA; Cat# 9520; CellSignaling), and Fibronectin (1:1000 in 5% BSA; Cat# F3648; MilliporeSigma) was resolved using SDS-PAGE. A standard sample was prepared using 15 µl from one sample from each condition to account for inter-gel variability. Resolved proteins were

then transferred to polyvinylidene fluoride membranes (PVDF; Cat# 162017; Bio-Rad) then stained with Ponceau S. Membranes containing FSTL1, Cyclophilin B, TGF-βR1, and Fibronectin were blocked in 5% milk-TBST for 1 hour, then stained for primary antibodies overnight at 4ºC. Membranes containing SMAD3, pSMAD3, and TGF-β were blocked in 5% BSA-TBST for 1 hour, then stained for primary antibodies overnight at 4ºC. After primary incubation, membranes containing FSTL1, Cyclophilin B, TGF-βR1, and Fibronectin were washed 3 times with TBST and incubated in secondary antibody (1:10,000 anti-rabbit HRP-linked antibody in 5% BSA-TBST; Cat# 7074S; CellSignaling). Membranes containing SMAD3, pSMAD3, and TGF-β1 were washed 3 times with TBST and incubated in secondary antibody (1:10,000 anti-rabbit HRP-linked antibody in 2% BSA-TBST; Cat# 7074S; CellSignaling). Membranes were then imaged on a ChemiDoc (Bio-Rad) imaging system and band intensities were measured with the Fiji software. For *in vitro* data in culture, protein of interest (FSTL1) data was normalized to the housekeeping protein (Cyclophilin-B). For *in vivo* data from muscle, proteins of interest (FSTL1, TGF-β1, TGF-βR1, SMAD3, and Fibronectin) data was normalized to Ponceau S.

2.11. Immunohistochemistry and microscopy

Sections of gastrocnemius/soleus muscle were cut to 10 µm thickness using an HM 525 NX Cryostat (Cat# 95-664-1EC; Fisher Scientific) and placed on frosted microscope slides (Cat# 12-544-2; Fisher Scientific). An average of 331 muscle fibres per mouse were analyzed for overall muscle fibre CSA and fibre-type specific CSA. Myosin heavy chain (MyHC) staining was performed as previously described[24,96]. Briefly, muscle sections were incubated in 1:200 anti-laminin primary antibody (Laminin β-2/γ-1 rat anti-laminin (IgG); Cat# MA1-06100; Thermo Fisher Scientific) in 5% goat serum (G/S; Cat# 16210064; Thermo Fisher Scientific) in 1xPBS overnight at 4ºC. Sections were then washed with 1xPBS and incubated in 1:300 Alexa Fluor 594

secondary antibody (Alexa Fluor Goat Anti-rat IgG (H+L); Cat# A-11007; Thermo Fisher Scientific) in 1% Bovine Serum Albumin (BSA; Cat# A9647; MilliporeSigma) in 1xPBS. Sections were incubated with a 1:25 dilution of the Mouse on Mouse Immunodetection Kit (M. o. M.; Cat# BMK-2202; Vector Labs) for 1 hour at room temperature. Sections were then washed and incubated in a cocktail of primary antibodies for MyHC-I (1:100; Mouse anti-myosin heavy chain type 1 (IgG-2b); Cat# BA-D5; DSHB), MyHC-IIa (1:100; Mouse anti-myosin heavy chain type IIa (IgG1); Cat# SC-71; DSHB), and MyHC-IIb (1:25; Mouse anti-myosin heavy chain type IIb (IgM); Cat# BF-F3; DSHB) in 1% BSA in 1xPBS for 45 minutes at 37ºC. Sections were then washed and incubated in a cocktail of secondary antibodies AF488-IgG1 (1:100; Alexa fluor 488 goat anti-mouse IgG, Fcγ subclass 1 specific; Cat# 115-545-205; Jackson Immunoresearch Lab), AF647-IgG-2b (1:100; Alexa fluor 647 goat anti-mouse IgG, Fcγ subclass 2b specific; Cat# 115-605-207; Jackson Immunoresearch Lab), and Cy^{TM}3 (1:50; Cy^{TM}3 goat anti-mouse IgM, μ chain specific; Cat# 115-165-020; Jackson Immunoresearch Lab) in 0.5% BSA in 1xPBS for 1 hour at room temperature to selectively bind the primaries. MyHC-IIx fibres were identified as fibres negative for any other fibre-type. A ZEISS CellDiscoverer7 microscope was used to capture fluorescent images.

2.12. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). A p value ≤ 0.05 was considered statistically significant and a p value ≤ 0.1 was considered a trend. The number of biological replicates (n) for each experiment is indicated in the figure legend. A Student's t-test was used to assess variance between two groups such as between the irradiated and control conditions in our *in vitro* and *in vivo* gene expression analyses, and in our differentiation analyses. A one-way ANOVA with Tukey's post-hoc multiple comparisons test was used to compare

variance between three or more groups such as between the control, irradiation + SHAM, and irradiation + FSTL1 groups in our flow cytometry, western blot, and muscle CSA analyses. All flow cytometry data were extracted using Attune NxT Software v4.0.0. All fluorescence intensity data was generated using the ImageJ Fiji software v2.9.0. All statistics and graphs were prepared using the GraphPad Prism software v.8.0.1. Mice were randomly allotted to their groups and all analyses were conducted by a blinded investigator.

3. Results

3.1. *Fstl1* in C3H cells is reduced 24-hours following *in vitro* irradiation

To determine the extent to which *in vitro* irradiation impairs *Fstl1* transcript and/or protein expression in C3H cells (aim 1), C3H cells were cultured to 60-70% confluence, subjected to a 1.0 Gy dose of *in vitro* irradiation, and had mRNA and protein extracted at 12-, 24-, and 48-hours post-irradiation. A group of cells was included that were subjected to a second 1.0 Gy dose of *in vitro* irradiation after 24-hours and had mRNA and protein extracted 48-hours after the initial dose to observe the effect of repeated irradiation on *Fstl1* as would be expected in a clinical setting (Figure 4A). *Fstl1* was significantly reduced at 24-hours following irradiation ($p<0.05$) but was not changed at any other timepoint (Figure 4B). FSTL1 was significantly higher at 48-hours post-irradiation ($p<0.01$) but not at any other timepoint (Figure C-D).

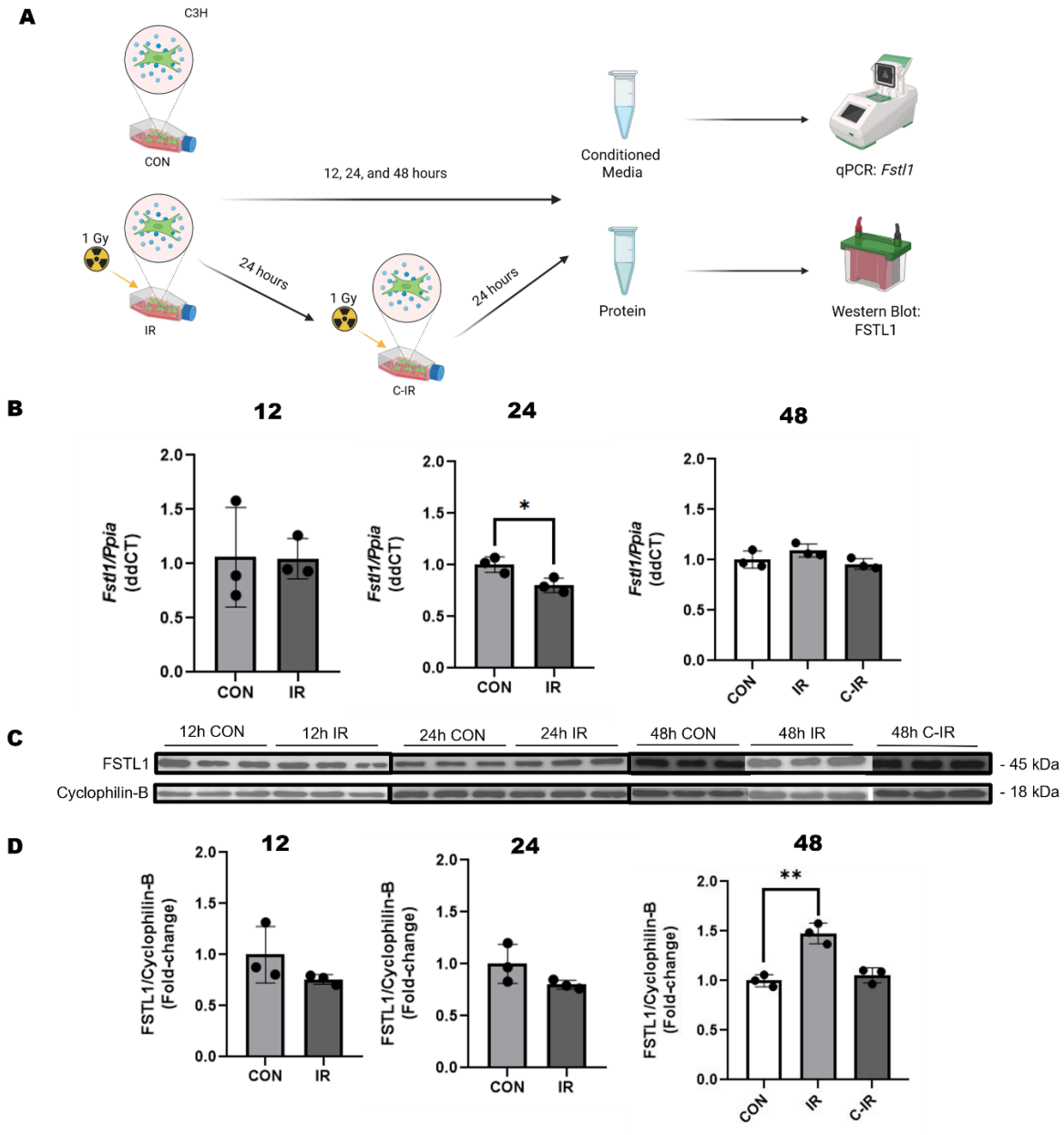

Figure 4: *In vitro* irradiation reduces *Fstl1* transcript abundance 24-hours following treatment in C3H10T1/2 fibroblasts. (A) Wild-type C3H cells were irradiated with 1 Gy irradiation and had mRNA and protein extracted 12-, 24-, and 48-hours following a single dose of irradiation, or 24-hours following two doses of irradiation (C-IR), for qPCR and western blot analysis. (B) Bar graphs of *Fstl1* transcript abundance (*$p<0.05$, Student's t-test). (C) Representative FSTL1/Cyclophilin-B western blots. (D) Bar graphs of FSTL1 protein abundance (**$p<0.01$; One-way ANOVA with Tukey's post-hoc multiple comparisons test; n=3).

3.2. FSTL1 in primary FAPs is reduced 12- and 48-hours following *in vitro* irradiation

Since *Fstl1* was reduced at 24-hours following *in vitro* irradiation in C3H cells; we asked if a similar trend could be observed in FAPs isolated from wild-type C57BL/6 mouse muscle (aim 1) using the same time course as for the C3H cells. Primary FAPs were cultured to 60-70% confluence, subjected to a 1.0 Gy dose of *in vitro* irradiation, and had protein and mRNA extracted 12-, 24-, and 48-hours post-irradiation. A group of cells was included that were subjected to a second 1.0 Gy dose of *in vitro* irradiation after 24-hours and had mRNA and protein extracted 48-hours after the initial dose to observe the effect of repeated irradiation on *Fstl1* as would be expected in a clinical setting (Figure 5A). *Fstl1* was not significantly altered at 12-, 24-, or 48-hours post nor following repeated irradiation (Figure 5B). FSTL1 was significantly reduced at 12- and 48-hours post-irradiation but not at 24-hours post nor following repeated irradiation (Figure 5C-D; $p<0.01$).

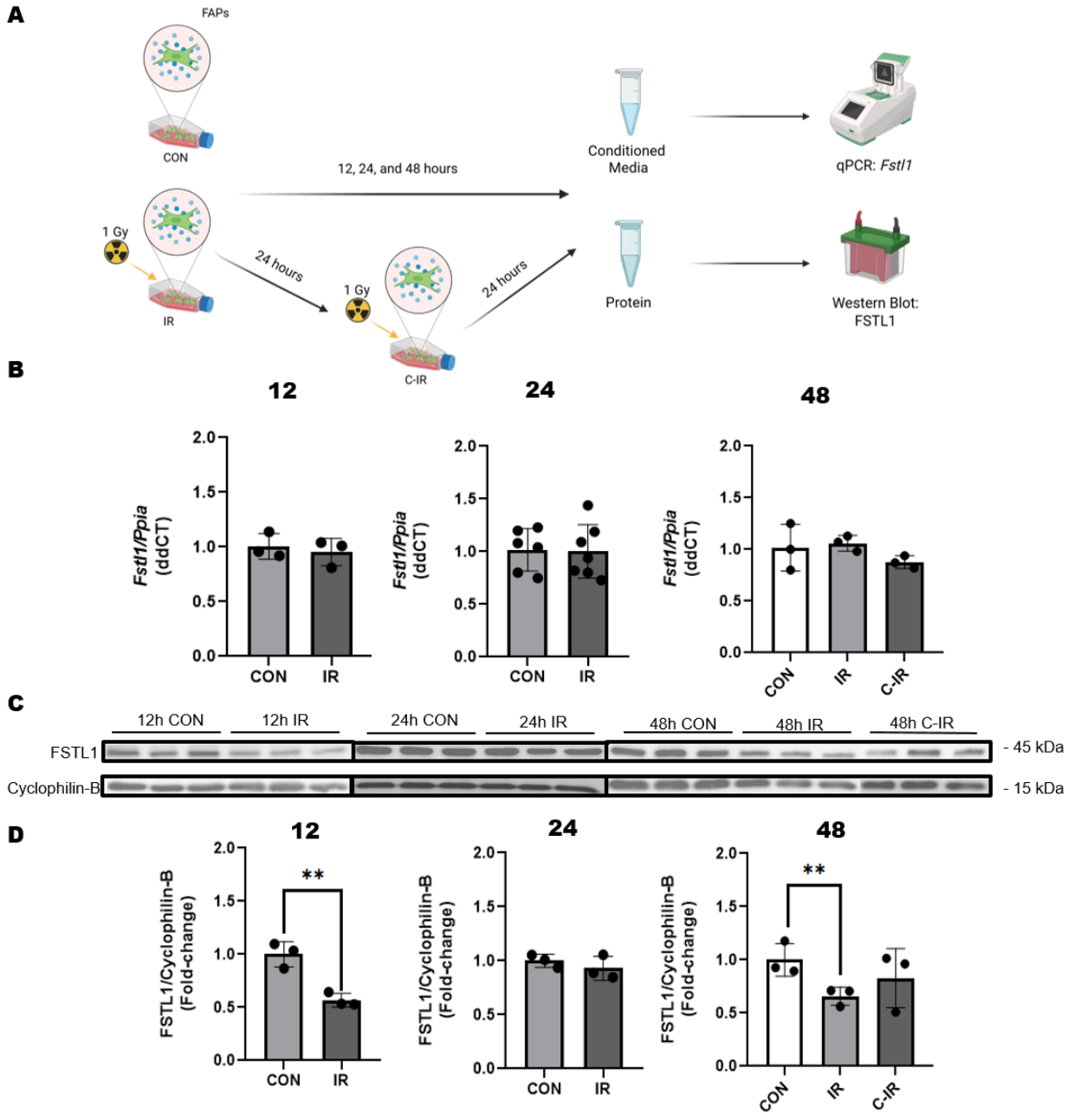

Figure 5: *In vitro* irradiation reduces FSTL1 protein abundance 12- and 48-hours following treatment in primary fibro/adipogenic progenitors. (A) Wild-type primary FAPs were radiated with 1 Gy irradiation and had mRNA and protein extracted 12-, 24-, and 48-hours following a single dose of irradiation, or 24-hours following two doses of irradiation (C-IR), for qPCR and western blot analysis. (B) Bar graphs of *Fstl1* transcript abundance. (C) Representative FSTL1/Cyclophilin-B western blots. (D) Bar graphs of FSTL1 protein abundance (**p<0.01; Student's t-test or One-way ANOVA with Tukey's post-hoc multiple comparisons test; n=3-6).

3.3. *Fstl1* is not reduced in FAPs isolated 24 hours following *in vivo* irradiation

To determine the extent to which *in vivo* irradiation reduces *Fstl1* (aim 1), the right hindlimbs of wild-type C57BL/6 mice were irradiated with three 8.2 Gy doses of fractionated irradiation delivered on alternating days (M/W/F)[5,93] (IR). The left hindlimbs were covered with a led shield to act as an internal contralateral control (CON). 24-hours following the last dose of irradiation, both gastrocnemius/soleus complexes were collected and their FAPs were isolated by FACS. Isolated FAPs were expanded in culture and had mRNA extracted for qPCR analysis (Figure 6A). *Fstl1* was not significantly altered 24-hours following *in vivo* irradiation (Figure 6B).

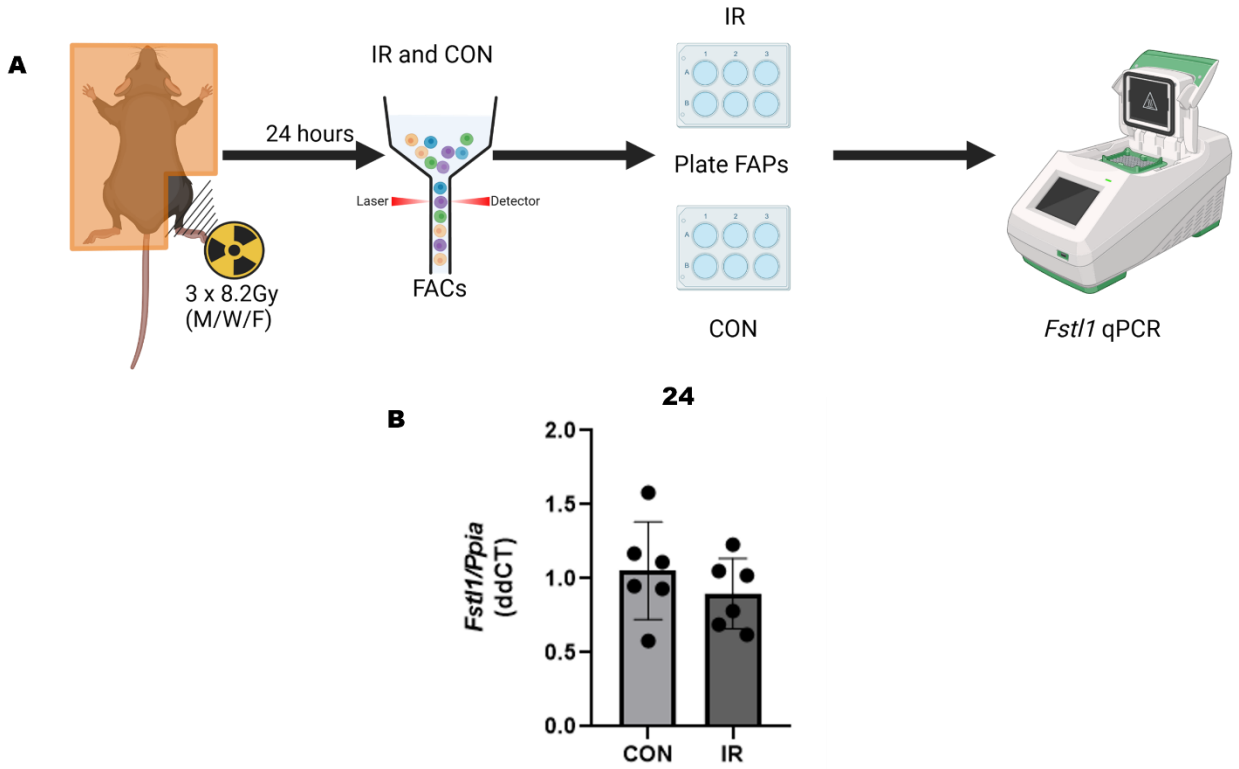

Figure 6: *Fstl1* transcript expression is not reduced in FAPs isolated 24 hours following *in vivo* irradiation. (A) Wild-type C57BL/6 mice had their right hindlimbs irradiated with three 8.2 Gy doses of *in vivo* irradiation delivered on alternating days (M/W/F). 24-hours following irradiation, FAPs were isolated from the irradiated (IR), and contralateral limbs (CON) and then cultured to 80-90% confluence for mRNA extraction. (B) Bar graphs of *Fstl1* transcript abundance (n=6).

3.4. FSTL1 knockdown upregulates FAP fibrogenic differentiation

To determine if FSTL1 regulates FAP fate (aim 2), fibrogenic differentiation analyses were conducted on FSTL1^{kd}, empty vector (EV), wild-type control (CON), and irradiated (IR) C3H cells. To examine fibrogenic differentiation, cells in each condition were cultured to 70-80% confluence and then had fibrogenic media applied (+TGF-β1)[23] for three days. Cells were stained with αSMA to measure the extent of differentiation (Figure 7A). FSTL1^{kd} cells displayed significantly higher mean fluorescent intensity (MFI) of αSMA expression compared to EV cells (Figure 7B-C; $p<0.01$). FSTL1^{kd} cells displayed significantly higher area per cell of αSMA positive cells compared to EV cells (Figure 7B-C; $p<0.001$). IR cells displayed no change in MFI of αSMA expression compared to CON cells (Figure 7D-E). IR cells displayed no change in area per cell of αSMA positive cells compared to CON (Figure 7D-E).

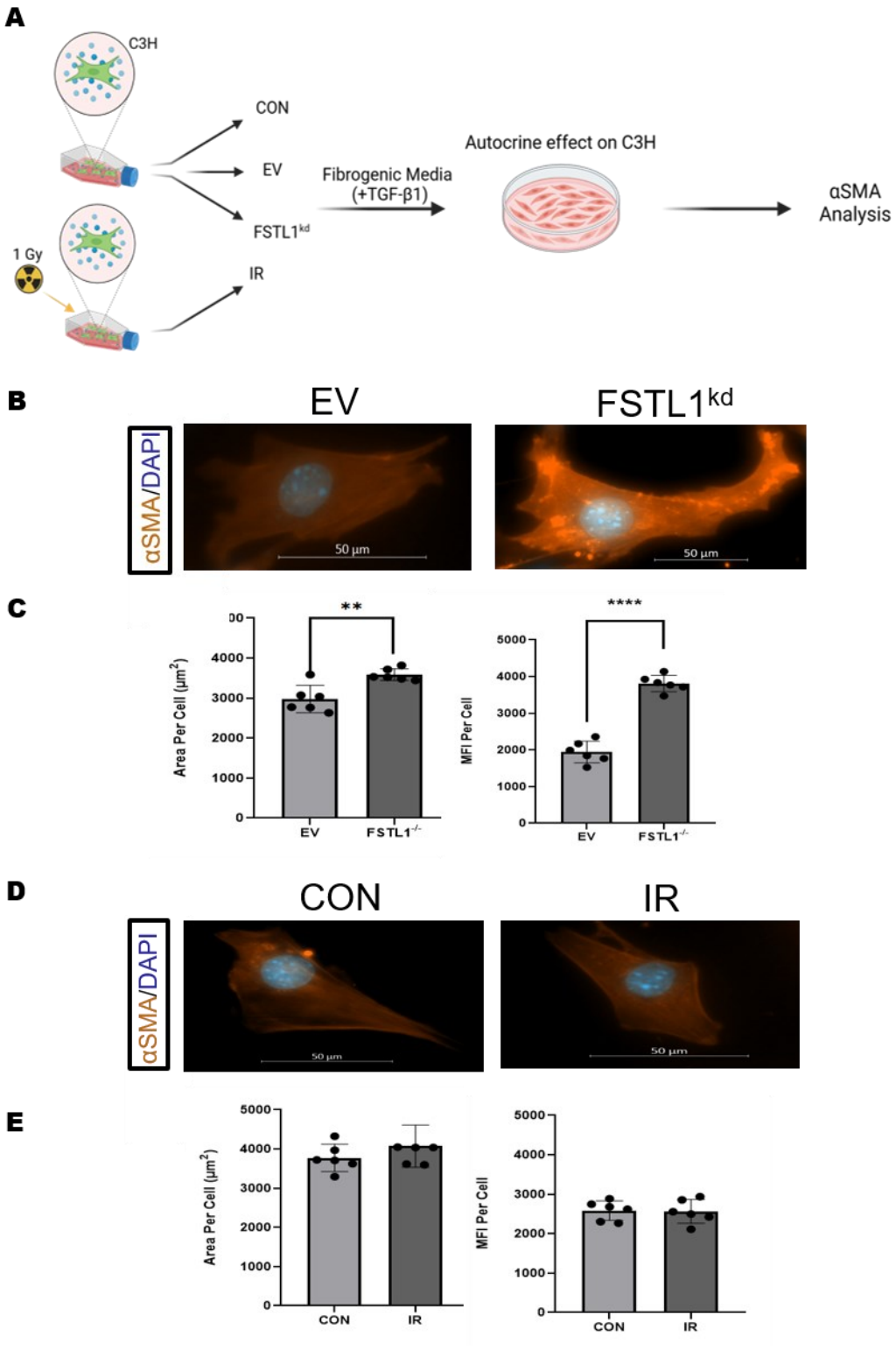
A
C3H
CON
EV
FSTL1kd
Fibrogenic Media
(+TGF-β1)
Autocrine effect on C3H
αSMA
Analysis
1 Gy
IR
B
EV
FSTL1kd
αSMA/DAPI
50 µm
50 µm
C
**
****
Area Per Cell (µm2)
MFI Per Cell
EV
FSTL1-/-
D
CON
IR
αSMA/DAPI
50 µm
50 µm
E
Area Per Cell (µm2)
MFI Per Cell
CON
IR

Figure 7: FSTL1 knockdown raised the density and area of FAP fibrogenic differentiation. (A) FSTL1 knockdown ($FSTL1^{kd}$), control (CON), empty vector (EV), and irradiated (IR) C3H fibroblasts were cultured to 70-80% confluence and then had fibrogenic media (+TGF-β1)[23] applied for three days. Cells were stained with αSMA which was analyzed to determine the density and size of fibrogenic cells. (B) Representative images of EV and CON C3H cells following fibrogenic differentiation. Cells were stained with αSMA (orange) and DAPI (blue). (C) Bar graphs of EV vs. $FSTL1^{kd}$ αSMA area per αSMA positive cell and mean fluorescent intensity of αSMA per αSMA positive cell (**p<0.01, ****p<0.0001; Student's t-test) (D) Representative images of CON and IR C3H cells following fibrogenic differentiation. Cells were stained with αSMA (orange) and DAPI (blue). (E) Bar graphs of CON vs. IR αSMA area per αSMA positive cell and mean fluorescent intensity of αSMA per αSMA positive cell (n=6).

3.5. *In vitro* irradiation lowers the number of adipogenic FAPs

To further examine how FSTL1 regulates FAP fate (aim 2), adipogenic differentiation analyses were conducted on $FSTL1^{kd}$, empty vector (EV), wild-type control (CON), and irradiated (IR) C3H cells. To examine adipogenic differentiation, cells in each condition were cultured to 70-80% confluence and then had adipogenic media (+insulin)[97] applied for five days. Cells were stained with perilipin to measure the extent of differentiation (Figure 8A). $FSTL1^{kd}$ cells displayed no significant difference in MFI of perilipin expression compared to EV cells (Figure 8B-C). $FSTL1^{kd}$ cells displayed no significant difference in area per cell of perilipin positive cells compared to EV cells (Figure 8B-C). The $FSTL1^{kd}$ condition displayed a trend of a decreased percentage of the total cells that were perilipin positive compared to the EV condition (p=0.0701; Figure 8D-E). IR cells displayed no significant difference in MFI of perilipin expression compared to CON cells (Figure 8F-G). IR cells displayed no significant difference in area per cell of perilipin positive cells compared to CON (Figure 8F-G). The IR condition displayed a significantly decreased percentage of the total cells that were perilipin positive compared to the CON condition (Figure 8H-I).

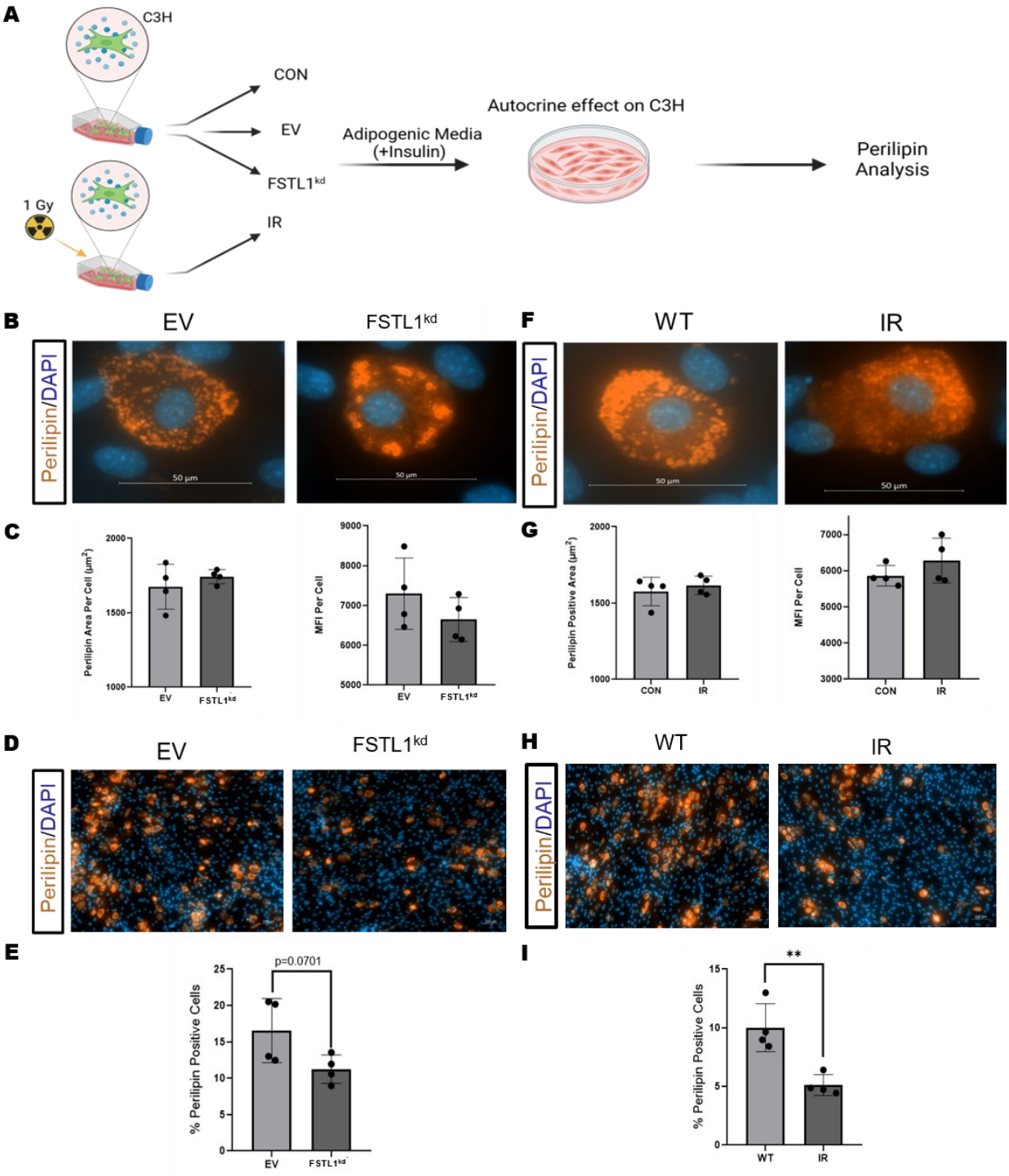
A
C3H
CON
EV
FSTL1kd
1 Gy
IR
Adipogenic Media
(+Insulin)
Autocrine effect on C3H
Perilipin
Analysis
B
EV
FSTL1kd
Perilipin/DAPI
50 µm
C
Perilipin Area Per Cell (µm2)
MFI Per Cell
EV
FSTL1kd
D
EV
FSTL1kd
Perilipin/DAPI
E
p=0.0701
% Perilipin Positive Cells
EV
FSTL1kd
F
WT
IR
Perilipin/DAPI
50 µm
G
Perilipin Positive Area (µm2)
MFI Per Cell
CON
IR
H
WT
IR
Perilipin/DAPI
I
**
% Perilipin Positive Cells
WT
IR

Figure 8: *In vitro* irradiation decreases the number of adipogenic FAPs. (A) FSTL1 knockdown ($FSTL1^{kd}$), control (CON), empty vector (EV), and irradiated (IR) C3H fibroblasts were cultured to 70-80% confluence and then had adipogenic media (+insulin)[37] applied for five days. Cells were stained with perilipin which was analyzed to determine the density and size of adipogenic cells. (B) Representative images of individual EV and $FSTL1^{kd}$ C3H cells following adipogenic differentiation. Cells were stained with perilipin (orange) and DAPI (blue). (C) Bar graphs of EV vs. $FSTL1^{kd}$ perilipin area per perilipin positive cell and mean fluorescent intensity of perilipin per perilipin positive cell (D) Representative images of plates of EV and $FSTL1^{kd}$ C3H cells following adipogenic differentiation. (E) Bar graph of EV vs. $FSTL1^{kd}$ percent perilipin positive cells ($p=0.0701$; Student's t-test). (F) Representative images of individual CON and IR C3H cells following adipogenic differentiation. Cells were stained with perilipin (orange) and DAPI (blue). (G) Bar graphs of CON vs. IR perilipin area per perilipin positive cell and mean fluorescent intensity of perilipin per perilipin positive cell (H) Representative images of plates of CON and IR C3H cells following adipogenic differentiation. (I) Bar graph of CON vs. IR percent perilipin positive cells. (**$p<0.01$; Student's t-test; $n=4$).

3.6. Acute irradiation depletes MuSCs in gastrocnemius muscle and intravenous rFSTL1 does not affect muscle resident mononuclear cell populations

To determine the extent to which repleting FSTL1 protein after irradiation can prevent irradiation-induced alterations in muscle-resident mononuclear cell populations[23] (aim 2), two groups of 5-week old C57BL/6 mice had both hindlimbs subjected to three 8.2 Gy doses of irradiation delivered on alternating days (F/Su/Tu or Su/Tu/Th)[5,93] with a third group acting as a non-irradiated control. 24-hours post-irradiation, one group was injected intravenously with 100 ng/g rFSTL1[94] and the other two groups were injected with the same volume/mass of PBS; resulting in the following three groups: (1) CON, (2) IR + SHAM, (3) IR + FSTL1. Three-days post-irradiation, cells were isolated from one gastrocnemius/soleus complex and flow cytometry analysis was performed to determine the impact of FSTL1 repletion on muscle-resident mononuclear cell populations (Figure 9A). Significantly fewer MuSCs were detected in both IR + SHAM and IR + FSTL1 compared to CON ($p<0.01$) with no change in IR + FSTL1 compared to IR + SHAM (Figure 9E-F). A trend for fewer hematopoietic cells was detected in IR + SHAM

compared to CON (p=0.053) with no change in IR + FSTL1 compared to CON, or in IR + FSTL1 compared to IR + SHAM (Figure 9B-C). No change was detected in FAPs in either IR + SHAM or IR + FSTL1 compared to CON or in IR + FSTL1 compared to IR + SHAM (Figure 9E&H). No change was detected in endothelial cells in either IR + SHAM or IR + FSTL1 compared to CON or in IR + FSTL1 compared to IR + SHAM (Figure 9B&D).

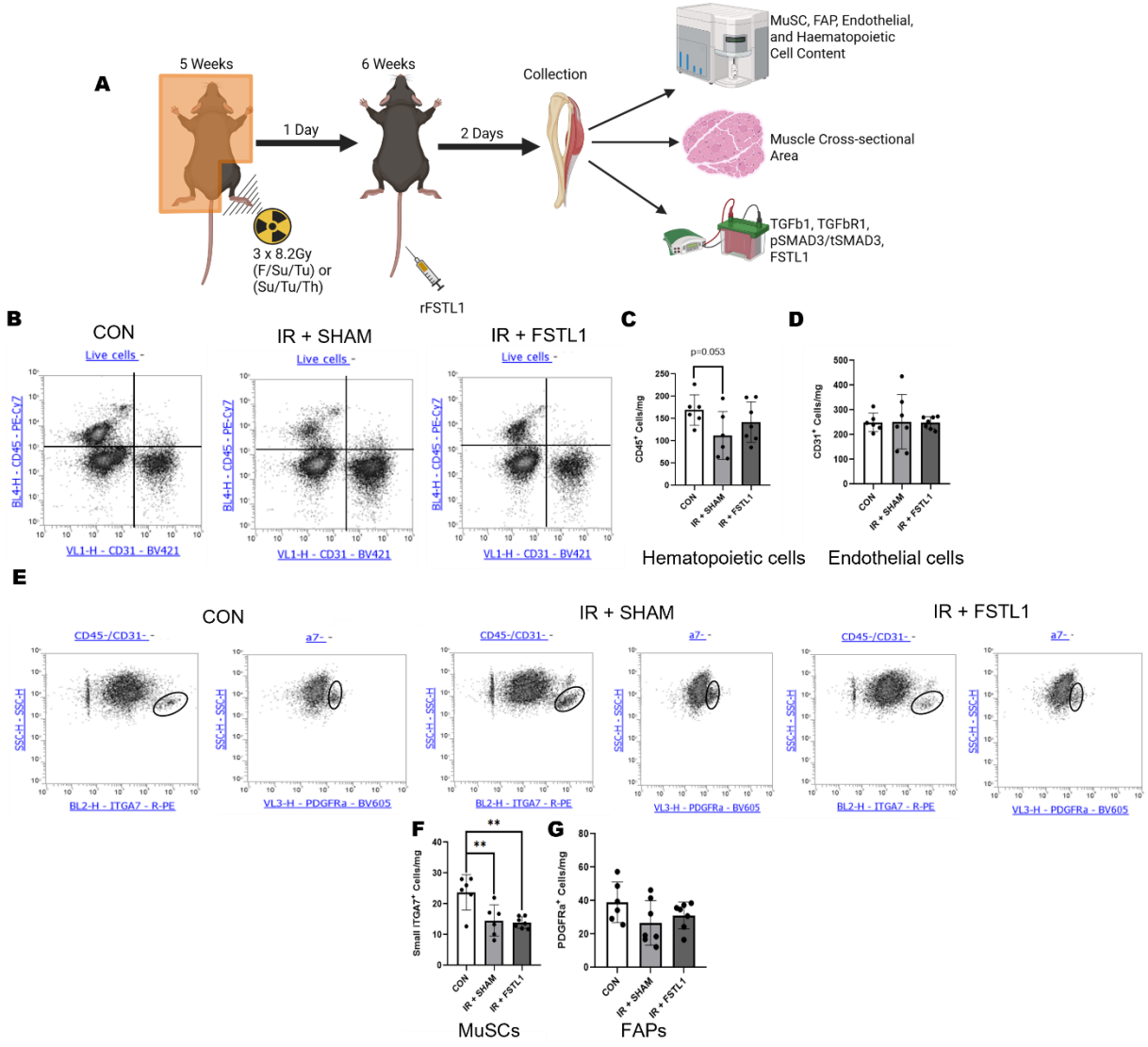

Figure 9: Intravenous rFSTL1 does not affect muscle resident mononuclear cell populations. (A) Two groups of 5-week old C57BL/6 mice had both hindlimbs subjected to three 8.2 Gy doses of irradiation delivered on alternating days (F/Su/Tu or Su/Tu/Th) with a third group acting as a non-radiated control (CON). 24-hours post-irradiation, one group was injected intravenously with 100 ng/g rFSTL1[94] (IR + FSTL1) and the other two groups were injected with the same volume/mass of PBS (IR + SHAM). (B) Representative flow cytometry plots of gating done to quantify hematopoietic cells ($CD45^+$) and endothelial cells ($CD31^+$). (C) Bar graph of quantified hematopoietic cells ($p=0.053$; One-way ANOVA with Tukey's post-hoc multiple comparisons test). (D) Bar graph of quantified endothelial cells. (E) Representative flow cytometry plots of gating done to quantify FAPs ($PDGFR\alpha^+$) and MuSCs ($ITGA7^+$). (F) Bar graph of quantified FAPs. (G) Bar graph of quantified MuSCs (**$p<0.01$; One-way ANOVA with Tukey's post-hoc multiple comparisons test; n=6-7).

3.7. Circulating FSTL1 is not incorporated into skeletal muscle and does not affect the expression of muscle resident fibrotic markers

To determine if repleting FSTL1 protein after irradiation can prevent irradiation-induced muscle fibrosis[23] (aim 2), three days post-irradiation, protein was extracted from half of one gastrocnemius/soleus complex and western blot analysis was conducted to determine the impact of FSTL1 repletion on common muscle fibrosis markers. Mice were also exsanguinated, and plasma was collected to assess levels of circulating FSTL1 following rFSTL1 injection. In plasma, a trend of higher FSTL1 was detected in IR + FSTL1 compared to CON ($p=0.0910$) but not in IR + SHAM compared to control or IR + FSTL1 compared to IR + SHAM (Figure 10A). In whole muscle, no change was detected in FSTL1, TGF-β1, TGF-βR1, tSMAD3, or pSMAD3/tSMAD3 in either IR + SHAM or IR + FSTL1 compared to CON or in IR + FSTL1 compared to IR + SHAM (Figure 10B-G).

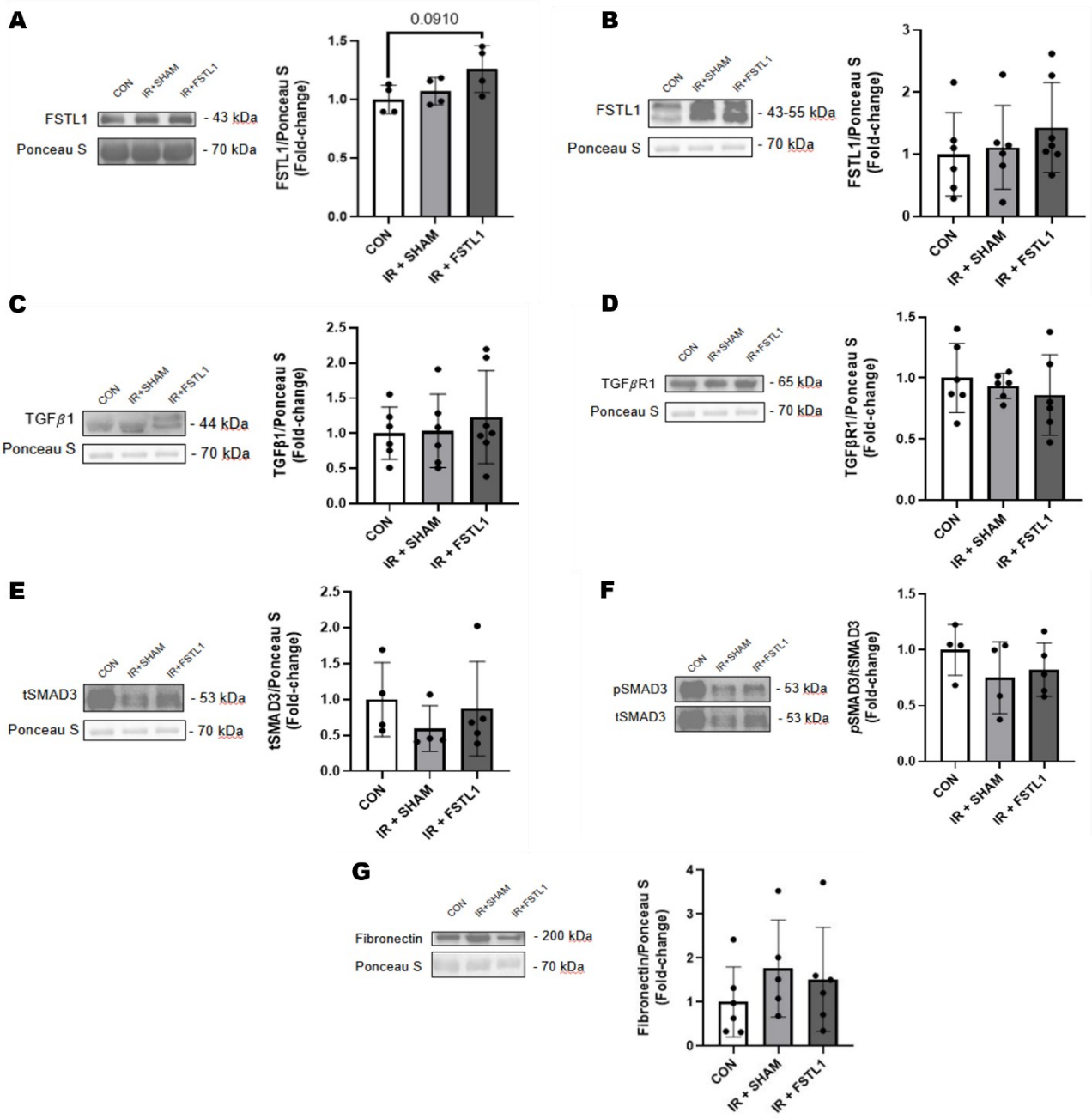
A
CON
IR+SHAM
IR+FSTL1
FSTL1
- 43 kDa
Ponceau S
- 70 kDa
0.0910
FSTL1/Ponceau S (Fold-change)
CON
IR + SHAM
IR + FSTL1
B
FSTL1
- 43-55 kDa
Ponceau S
- 70 kDa
FSTL1/Ponceau S (Fold-change)
C
TGFβ1
- 44 kDa
Ponceau S
- 70 kDa
TGFβ1/Ponceau S (Fold-change)
D
TGFβR1
- 65 kDa
Ponceau S
- 70 kDa
TGFβR1/Ponceau S (Fold-change)
E
tSMAD3
- 53 kDa
Ponceau S
- 70 kDa
tSMAD3/Ponceau S (Fold-change)
F
pSMAD3
- 53 kDa
tSMAD3
- 53 kDa
pSMAD3/tSMAD3 (Fold-change)
G
Fibronectin
- 200 kDa
Ponceau S
- 70 kDa
Fibronectin/Ponceau S (Fold-change)

Figure 10: Intravenous rFSTL1 injection does not affect the expression of fibrotic markers. (A) Representative western blot image and summary bar graph of quantified FSTL1 protein from plasma (p=0.0910; One-way ANOVA with Tukey's post-hoc multiple comparisons test). (B) Representative western blot image and summary bar graph of quantified FSTL1 protein from gastrocnemius/soleus muscle. (C) Representative western blot image and summary bar graph of quantified TGF-β1 protein from gastrocnemius/soleus muscle. (D) Representative western blot image and summary bar graph of quantified TGF-βR1 protein from gastrocnemius/soleus muscle. (E) Representative western blot image and summary bar graph of quantified SMAD3 protein from gastrocnemius/soleus muscle. (F) Representative western blot image and summary bar graph of activated (phosphorylated) SMAD3 protein from gastrocnemius/soleus muscle (G) Representative western blot image and summary bar graph of Fibronectin protein from gastrocnemius/soleus muscle (n=4-7).

3.8. Acute irradiation and intravenous rFSTL1 repletion does not affect muscle cross-sectional area

Lastly, to determine if repleting FSTL1 protein after irradiation can prevent irradiation-induced defects in muscle CSA[23] (aim 2), three days post-irradiation, half of one gastrocnemius/soleus complex was embedded in OCT for cryostat sectioning. Muscle sections were stained for Type 1, Type 2a, Type 2b fibres, and laminin. Type 2x was determined as fibres not stained for Type 1, Type 2a, or Type 2b (Figure 11A). Overall CSA was not impacted by irradiation or rFSTL1 repletion (Figure 11B). The percentage of small muscle fibres (<1200 μm^2) was not impacted by irradiation or rFSTL1 repletion (Figure 11C). CSA of Type 1, Type 2a, Type 2b, or Type 2x fibres were not impacted by irradiation or rFSTL1 repletion (Figure 11D-G).

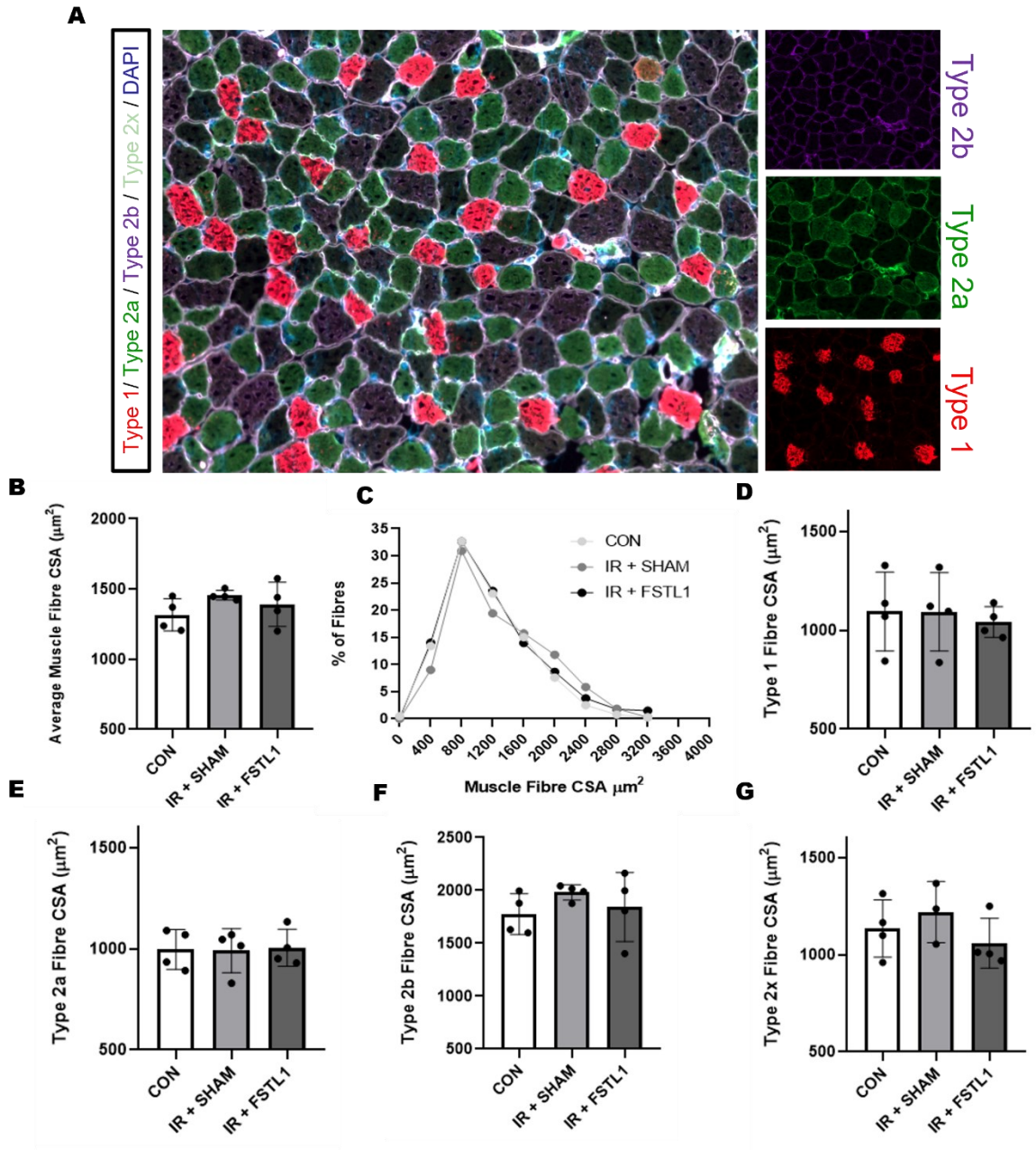

Figure 11: Acute irradiation and intravenous rFSTL1 repletion does not affect muscle cross-sectional area. (A) Representative image of myosin heavy chain immunofluorescence (Type 1: Red, Type 2a: green, Type 2b: purple, Type 2x: unstained, DAPI: blue). (B) Average overall fibre CSA (μm^2). (C) Average percentage of fibres within each CSA range (μm^2). (D) Average Type 1-specific fibre CSA (μm^2). (E) Average Type 2a-specific fibre CSA (μm^2). (F) Average Type 2b-specific fibre CSA (μm^2). (G) Average Type 2x-specific fibre CSA (μm^2) (n=4).

4. Discussion

FAPs contribute to irradiation-induced muscle atrophy and fibrosis[5,23–26,31], in part, through alterations to their secretome that hinders MuSC fate[23]. The overall objective of the present study was to investigate a previously described FAP-derived secreted factor, FSTL1, to understand its role in irradiation-induced defects in muscle regeneration. Our main findings were that irradiation affects *Fstl1* transcript and protein differently in the C3H line and isolated primary FAPs. These findings reject our first hypothesis that *in vitro* and *in vivo* irradiation would reduce *Fstl1* expression; and suggest that irradiation in an isolated model of FAPs, or their cell line, is insufficient to consistently deplete *Fstl1* expression. Furthermore, we determined that FSTL1 knockdown *in vitro* upregulated C3H fibrogenic differentiation while downregulating adipogenic differentiation. These findings confirm part of our second hypothesis that FSTL1 knockdown would upregulate fibrogenic differentiation. Acute irradiation (3-days) depleted MuSCs in gastrocnemius/soleus muscle tissue, but not other cell types. Lastly, intravenous rFSTL1 injection raised circulating FSTL1 protein, but not skeletal muscle levels. These findings reject part of our second hypothesis that FSTL1 repletion would improve *in vivo* myogenesis in a murine model; however, this result provides the basis for designing a novel intramuscular rFSTL1 injection protocol to address longer term irradiation exposure (56-days). The overall findings of this study provide further evidence that *Fstl1* expression is impacted by irradiation treatment and regulates FAP fate decisions.

Based on our preliminary scRNA-seq and western blot data (Figure 1C-D; unpublished), we expected to see both *Fstl1* transcript and protein expression depleted by irradiation. However, when we irradiated C3H cells *in vitro* we only observed depleted mRNA at 24-hours post-irradiation and higher protein levels at 48-hours post-irradiation (Figure 4). Further, when we

irradiated isolated primary FAPs *in vitro* we observed no change in the mRNA and depletion of protein expression at 12- and 48-hours post-irradiation (Figure 5). The differences in regulation between the C3H cell line and primary FAPs were interesting but not extraordinary. C3H cells are known to exhibit altered differentiation potential when compared to primary mesenchymal stromal cells (MSCs)[98]. MSCs are composed of heterogeneous cell populations that are capable of distinct spontaneous differentiation[98,99]. In contrast, C3H cells are an immortalized cell line with reduced adipogenic differentiation potential compared to primary MSCs[98]. The reduced adipogenic differentiation potential of C3H cells was confirmed by qPCR analysis which exemplifies that they display vastly different gene expression patterns than primary MSCs under the same conditions[98]. The same phenomenon has been observed in other cell types. When the Hepa1-6 cell line was compared to its primary counterpart; hepatocytes, proteomic analysis showed that expression of proteins related to cell cycle regulation, DNA synthesis and RNA polymerase were vastly different[100]. The immortalization process can alter transcription factor regulation which vastly change gene expression patterns and biological processes when compared to their tissues of origin[101]. As a secreted growth factor, it stands to reason that *Fstl1* expression patterns would be similarly altered when immortalized C3H cells are compared to their primary FAP counterparts.

Another interesting observation was that in both C3H cells and isolated primary FAPs, *Fstl1* transcript and protein expression was differentially regulated. In C3H cells, mRNA expression was lowered without correspondingly lowered protein content (Figure 4). In primary cells, protein content was lowered without corresponding lower mRNA expression (Figure 5). The Central Dogma of Molecular Biology states that genetic information is communicated from DNA to RNA through transcription and finally to protein through translation[102]. Theoretically, it implies that alterations in RNA content would be reflected in similar alterations in protein content.

However, in practice, there are many instances of post-transcriptional or post-translational modifications resulting in changes that are unique to transcript or protein. RNA-binding proteins (RBPs) are an example of a regulatory mechanism that cause changes in protein expression independently of changes in transcript expression. RBPs bind to mRNA strands and influence whether or not they progress to translation by altering their capacity to be transported out of the nucleus to the cytosol[103]. RBPs are modified under a wide range of stressors including DNA damage, oxidative stress, and irradiation[103,104]. Similarly, stress granules caused by oxidative stress (a by-product of irradiation treatment) induce 5^{1}-end RNA decay that causes translational initiation stress and detectable lowered protein content. These modifications lead to defects in translation not reflected in the associated transcript expression and may explain our observations in FSTL1 protein/gene expression in isolated primary FAPs. Alternatively, irradiation has been demonstrated to alter the transcript expression pattern of genes related to stress response without proportional changes observed in corresponding protein[105], similar to our observations in *Fstl1* expression in C3H cells. Our mRNA and protein data lines up with previous observations of inconsistent transcriptional and translation regulation[103–105] which opens the door to further investigation to understand mechanistically how each layer of FSTL1 expression and secretion is regulated.

When we examined *Fstl1* expression in primary FAPs isolated 24-hours following *in vivo* irradiation we did not detect any significant change compared to the control (Figure 6). These data did not align with our preliminary scRNA-seq data. The main difference in cellular preparation was that in the scRNA-seq experiment, genetic material was isolated immediately following cell isolation; while in our *in vivo* irradiation experiment, isolated cells were expanded in culture prior to mRNA extraction. We speculate that removing FAPs from signals present in the irradiated muscle microenvironment allowed them to recover FSTL1 expression to normal levels. Immune

cells within the MuSC niche secrete factors such as IL-1β, IL-4, IL-13, IL-15, TNF-α, and TGF-β1 that interact with FAPs to alter their differentiation dynamics and secretion profiles[9,44,48,54,57–59]. IL-1β specifically has been shown to regulate the secretion of FSTL1 into media following inflammatory stimuli[81,86]. Perhaps removing FAPs from the influence of these inflammatory factors returns FSTL1 expression to basal levels. This would also explain why we observed a similar trend in our *in vitro* irradiation experiments done on cultured FAPs. Future studies should evaluate gene expression immediately following FAP isolation to elucidate how the irradiated microenvironment impacts *Fstl1* expression. Similarly, *in vitro* co-culture experiments should be conducted where irradiation is delivered to a combination of FAPs and inflammatory cells to produce a more effective representation of the irradiated muscle microenvironment *in vitro*.

Another possible explanation for our findings is that FSTL1 secretion is being impacted by irradiation independently of alterations in gene expressions. In models of endoplasmic reticulum (ER) stress, it has been observed that paracrine factor secretion is reduced without corresponding changes in their gene expression[106]. The ER is responsible for processing proteins produced by a cell before secretion and its disruption leads to the buildup of misfolded proteins within the cell[106–108]. In astrocytes and microglia, induced ER stress results in decreased IL-6 and IL-8 secretion into culture media[106]. Similarly, in muscle, induced ER stress altered the secretion of inflammatory cytokines into cell media[109]. In both cases, no alterations in gene expression at the transcript or protein level were observed. The dysfunction was solely due to changes in protein processing[106,109]. Alterations in ER function represent an avenue of post-translational dysfunction caused by stress. In our experiments, it is possible that FSTL1 protein is being sequestered within FAPs such that its secretion is depleted without corresponding alterations in transcript or protein expression. Future conditioned media experiments should be conducted where FSTL1 protein levels in culture

media is compared between irradiated and control FAPs. It would be interesting to compare gene/protein expression data to secretion data to determine the differential impact of irradiation on *Fstl1* gene expression and secretion.

Previous work in our lab showed that following *in vitro* irradiation, C3H fibrogenic differentiation is upregulated compared to untreated cells[23]. In that experiment, fibrogenic differentiation media with TGF-β1 added was used as a positive control[23]. In the present study, irradiation did not add to the extent of fibrogenic differentiation compared to TGF-β1 cells. We speculate that the TGF-β1 added to the media maximizes fibrotic differentiation which did not allow us to detect higher levels of fibrotic differentiation following irradiation. In the future, we will repeat these experiments with a group of cells allowed to differentiation spontaneously without TGF-β1 as in our previous study[23]. However, FSTL1 knockdown did augment fibrogenic differentiation compared to an empty vector control in the same media (Figure 7). Alternatively, FSTL1 knockdown and irradiation both reduced adipogenic differentiation compared to their respective controls (Figure 8). This result implies an autocrine effect of FSTL1 signaling on FAP differentiation dynamics. Secreted factors from inflammatory cells are well described to differentially impact FAP differentiation[48]. IL-4, IL-13, and IL-15 have all been described to decrease FAP adipogenic differentiation and improve their proliferation capacity[57–59]. Even though these examples display opposite effects, they are documented examples of FAP adipogenic differentiation being impacted by interactions with secreted factors. However, TNF-α decreases FAP fibrotic differentiation during regeneration through mediation of apoptosis[48,50,54]; a process that is overcome by excessive TGF-β1 content[54,60,61]. Our findings that FSTL1 knockdown augmented fibrogenic differentiation in media containing TGF-β1 mirrored previous observations that FSTL1 deficiency upregulates TGF-β1/SMAD3 signaling[84]. We demonstrated that the

removal of FSTL1 accentuates the effect of TGF-β1. It is possible that FSTL1 is serving a similar purpose to TNF-α wherein its effective signaling is required to mitigate FAP apoptosis and regulate fibrotic deposition. Future experiments should be done using base culture media to determine the effect that FSTL1 knockdown has on fibrogenic differentiation without the addition of TGF-β1.

Based on previous literature, we expected to see smaller muscle CSA, lower MuSC and FAP content, and higher protein levels of markers related to muscle fibrosis following *in vivo* irradiation[5,23–25]. In the present study, the irradiation dosage schedule of 3 fractionated doses of 8.2 Gy delivered on alternating days did not impair muscle regeneration or fibrosis 3-days after the last dose (Figures 9-11). However, there are many examples in the literature that demonstrate that the impact of irradiation is dependent on the dose, age, dosage fractionation, time between fractions, and time after the last dose[5,23,25,30,74]. The magnitude of cell death following irradiation is strongly dependent on the dosage as it directly impacts the extent of DNA damage[6,7]. In one study, a single high dose of 16 Gy targeted irradiation decreased the CSA of Type 2a and Type 2b muscle fibres and led to a higher proportion of small diameter Type 2a and Type 2b fibres. However, the same dose fractionated into 4 x 4 Gy doses only lowered the CSA of Type 2b fibres[74]. This result aligns with previous work in our lab that demonstrated smaller muscle CSA and a higher proportion of small diameter muscle following a single high dose of 16 Gy[23]. In a similar experiment, mice subjected to 3 doses of 8.2 Gy irradiation delivered on alternating days exhibited lower MuSC proliferation, overall muscle CSA, and MuSC regenerative capacity 2-4 hours, 3 weeks, and 14 months following the last dose[5]. The fractionated dosage protocol was chosen to limit irradiation to the prepubertal mouse period[5]. This study provided the basis for our chosen irradiation protocol; however, we assessed muscle defects at 3-days following the last dose. The findings of our study add to previous literature that suggests that muscle defects following

irradiation are strongly influenced by the total dosage, dose fractionation, and time following the last dose. Future experiments will have to be done on longer term irradiation-induced defects at 56-days post-irradiation using the same dosage schedule.

Lastly, we found that repleting rFSTL1 intravenously through the tail vein is not an effective way to improve muscle architecture or fibrosis following irradiation exposure. In a previous study, rFSTL1 repleted in this manner was effective in preventing apoptosis and inflammatory responses in murine hearts[94]. They reported that following intravenous injection, FSTL1 levels were higher in plasma[94]. Our findings mirrored this by showing that 3-days following intravenous injection there was a trend for more circulating FSTL1 protein in murine plasma (Figure 10). However, this elevation in circulating FSTL1 was not detected in skeletal muscle (Figure 10). Repletion of FAP secreted factors represents a potential therapeutic avenue for treating muscle defects. Intraperitoneal injection of WISP1 has been demonstrated to rescue MuSC function and regeneration in aged mice[38]. Further, our preliminary conditioned media experiments displayed that repletion of rFSTL1 into the irradiated FAP secretome improved MuSC *in vitro* differentiation and fusion (Figure 3). These findings provide the basis for refining our FSTL1 repletion experiments by using an alternative injection method. Future experiments should be done using a novel intramuscular FSTL1 injection protocol to directly replete FSTL1 in muscle tissue.

Our findings combined with previous studies exemplify an opposing role of FSTL1 and TGF-β1 in the context of irradiation treatment. Although the present study didn't show consistent *Fstl1* gene expression alterations following irradiation, there were multiple examples of decreased expression (Figure 1C-D; Figure 4B; Figure 5D). Opposingly, irradiation treatment has been shown to upregulate TGF-β1 expression and signaling[5,23–26]. Irradiation upregulates FAP

fibrogenic differentiation; however, not to the same extent as adding TGF-β1 to the culture media meaning that it may transiently upregulate TGF-β1 signaling[23]. Since FSTL1 knockdown augmented the impact of TGF-β1 addition to FAP fibrogenic differentiation; and has previously been described to upregulate TGF-β1/SMAD3 signaling, it is possible that FSTL1 activity regulates TGF-β1 content. Therefore, a model can be proposed wherein irradiation treatment downregulates FSTL1 content that in turn upregulates TGF-β1 signaling to induce a fibrotic phenotype in FAPs. The promotion of a fibrogenic phenotype reduces the proliferative potential of FAPs and removes important pro-myogenic signaling factors from the MuSC niche[23,24]. The main limitations of the current study were that our *in vitro* assays were conducted in isolated model of FAPs that do not effectively mimic the muscle microenvironment. Similarly, culturing the cells prior to mRNA extraction in our *in vivo* expression assay created a confound of removal of inflammatory signaling. Lastly, in our rFSTL1 repletion model, we only reported on irradiation defects at 3-days post-treatment and used an intravenous injection model that has not been reliably shown to enhance FSTL1 content within skeletal muscle. Future steps need to be taken to address these limitations by conducting co-culture *in vitro* experiments, extract mRNA immediately following cell isolation, examine irradiation defects 56-days following the completion of 3 x 8.2 Gy doses of irradiation, and to assess the impact of intramuscular repletion of FSTL1 directly into skeletal muscle tissue. Intravenous FSTL1 injection was previously demonstrated to mitigate venous wall fibrosis[94] and our preliminary findings demonstrated the potential for FSTL1 repletion to improve myogenesis following irradiation *in vitro.* The present study built off these findings by attempting to understand how FSTL1 is impacted by irradiation and how its presence impacts FAP fate decisions. We used intravenous FSTL1 repletion to understand if FSTL1 can improve

myogenesis following irradiation *in vivo.* We identified FSTL1 as a compelling candidate for future work on mitigating the FAP fibrotic phenotype switch induced by irradiation treatment.

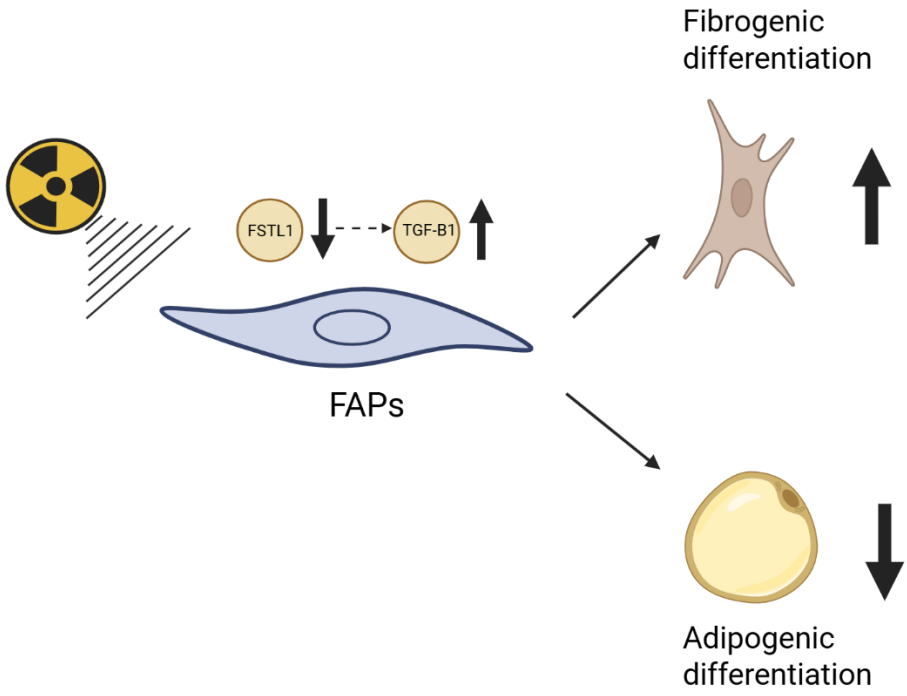

Figure 12: Working model. Irradiation treatment downregulates FSTL1 content that in turn upregulates TGF-β1 to induce a fibrogenic phenotype switch in FAPs evidenced by increased fibrogenic differentiation and decreased adipogenic differentiation.

5. Contributions

Dr. Michael De Lisio (MD), Dr. Nicolás Collao (NC), and Cooper Brabrook (CB) contributed to study design and direction. Abdelrhamen Fahmy (AF) and CB contributed to FAP differentiation image analysis. Alice Tate (AT) contributed to intravenous rFSTL1 injections. Lisa Ek Orloff (LEO) and CB contributed to running rFSTL1 repletion western blots and analyzing their data. Dr. Kayleigh Beaudry (KB), and CB contributed to rFSTL1 repletion cross-sectional area analysis. All other experimentation and analysis was completed by CB.

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Appendix A: Cell Culture Protocols

Fibro/adipogenic Progenitor Cell Culture

Growth Media (GM):

- FAP Primaries
  - DMEM high glucose media
  - 20% fetal bovine serum (FBS)
  - bFGF (5 ng/ml)
  - 1% penicillin streptomycin (P/S (100 U/ml))
- C3H Cell Line
  - DMEM high glucose media
  - 10% FBS
  - 1% P/S

Thawing and Plating Cells:

1. Warm growth media in the 37^{o} water bath
2. Remove cell tube from the liquid nitrogen and hold it half under the water in the water bath
   - Remove when most is thawed but make sure there is a small piece of ice remaining
3. Add 4 ml of GM to a 15 ml tube and then add the 1 ml of thawed cells to the 15 ml tube
   - Pipette up and down to mix
4. Centrifuge cells at 500 x G for 5 minutes at 4^{o}
5. While spinning, add 7.5 ml of GM to four 10 cm cell culture plates
6. After spin, remove the supernatant from the 15 ml tube and resuspend the cells in 1 ml GM
7. Add 150 µl of resuspended cells to each plate
   - Make an “S” shape when adding the cells
   - Make a figure 8 with the cell culture plate to evenly distribute the cells

Changing Media:

1. Warm GM, and 1x PBS in the 37^{o} water bath

2. Remove the GM from each cell culture plate
3. Wash each cell culture plate twice with 1x PBS
4. Add 8 ml of GM to each cell culture plate

Splitting Cells:

1. Warm GM, 0.25% Trypsin, and 1x PBS in the 37^{o} water bath
2. Remove the GM from each cell culture plate
3. Wash each cell culture plate twice with 3 ml 1x PBS
4. Add 1 ml of 0.25% Trypsin to each 10 cm plate
5. Incubate the cells in the 37^{o} incubator for 5 minutes
    a. Tap each plate before adding to the incubator
    b. Remove and tap each plate after 2.5 minutes to make sure the cells have detached from the plate
6. Add 3 ml of GM to each late and triturate the cells approximately 6 times
    a. Angle the plate and then take the GM and put it at the top of the plate to rinse the bottom and sides of the plate
    b. Take the GM and spread it around the plate to make sure you're taking as many cells out as possible
7. Remove cells from the plate and place them in a new 15 ml tube
8. Centrifuge the cells at 500 x G for 5 minutes at 19^{o}
9. While spinning, add 7.5 ml GM to each new plate
10. After spin, remove the supernatant and then resuspend the cells in 1 ml GM
11. Add 150 µl of cells to each plate
    a. Make an "S" shape when adding cells to the plate
    b. Make a figure 8 with each plate to evenly distribute the cells

Freezing Cells:

- Freeze Media is the normal GM + 10% DMSO
    - Typically do 300 µl DMSO in 2.7 ml of GM

1. Warm GM, 0.25% Trypsin, and 1x PBS in the 37^{o} water bath

2. Remove the GM from each cell culture plate
3. Wash each cell culture plate twice with 3 ml 1x PBS
4. Add 1 ml of 0.25% Trypsin to each 10 cm plate
5. Incubate the cells in the 37º incubator for 5 minutes
    a. Tap each plate before adding to the incubator
    b. Remove and tap each plate after 2.5 minutes to make sure the cells have detached from the plate
6. Add 3 ml of GM to each late and triturate the cells approximately 6 times
    a. Angle the plate and then take the GM and put it at the top of the plate to rinse the bottom and sides of the plate
    b. Take the GM and spread it around the plate to make sure you're taking as many cells out as possible
7. Remove cells from the plate and place them in a new 15 ml tube
8. Centrifuge the cells at 500 x G for 5 minutes at 19º
9. After spin, remove the supernatant and then resuspend the cells in 1 ml freeze media
10. Add the cell resuspension to a cryotube and put the cryotube in the Mr. Frosty and freeze in the -80º freezer
    a. After 24 hours add the cells to the liquid nitrogen

Adipogenic FAP Differentiation

Materials:

- Mesencult™ Adipogenic Differentiation Kit (Cat: 05507)
- L-Glutamine
- Insulin (Cat: I9278)
- FAP Growth Media

Protocols:

1. Adipogenic Media:
    a. Mix 5ml of 10x Supplemental Adipogenic Media with 45ml of Basal Medium
    b. Add 0.5 ml of L-Glutamine to create a final concentration of 2mM

2. Apply the adipogenic media to the cells for 3 days.
3. Adipocyte maintenance medium:
    a. Normal Growth Media
    b. 1ug/ml of Insulin
    c. The insulin stock concentration is 9.5 to 11.5 mg/ml, so use the 11.5 concentration.
4. After three days, remove the adipogenic media and then add adipocyte maintenance media for 2 days to mature the adipocytes

Fibrogenic FAP Differentiation

Materials:

- TGF-β1 (Cat: 100-21)
- DMEM glucose
- Horse serum
- Penicillin/streptomycin

Protocol:

1. Fibrogenic Media:
    a. DMEM high glucose
    b. 2% Horse serum
    c. 1% P/S
    d. 5 ng/ml TGF-β1
2. Incubate the cells in fibrogenic media for 3 days
    a. For TGF-β1 need to add 625 µl of 400 ng/ml stock for 50 ml media
    b. Store TGF-β1 stock (400ng/ml) at -20ºC
3. After 3 days, stain the cells with αSMA

Preparation of TGF-β1:

Use Prepotech Cat# 100-21 vial 10ug of recombinant human TGFB1 and reconstitute in 10mM Citric Acid pH 3.0.

RNA extraction from adherent cells

Materials:

- Qiagen RNA isolation minikit
- Cell scrapers
- 1xPBS
- P1000 pipette and tips
- 1.5ml eppendorf tubes

Protocol (Amounts indicated are for 6-well plates):

1. Remove cell media thoroughly with aspirator
2. Wash cells twice with 3 ml of cold 1xPBS
    a. Remove PBS thoroghly with the aspirator
3. Add 350 µl of Buffer RLT to all wells
4. Add 350 µl of 70% ethanol to all wells
    a. Diluted 70% in RNase free water
5. Scrape cells making sure to swish liquid around the plate to dissociate all cells
    a. SCRAPE FAST, **TIME SENSITIVE**
6. Remove 700 µl of the scraped solution and insert it into the Qiagen minikit spin column as close to the membrane as possible
7. Centrifuge at 8000 x G for 30 seconds at 4°
8. Go to Qiagen RNA extraction minikit instructions to extract RNA
9. End up with 50 µl of pure RNA in RNase free water
    a. Remember to remove 10 µl for nanodrop spectrophotometer quantification

Protein Extraction from adherent cells

Materials:

- Cell Scrapers
- Centrifuge tubes
- RIPA buffer (5ml aliquots)
- Anti-protease tablets (1/2 per 5ml buffer)

- Anti-phosphatase tablets (1/2 per 5ml buffer)
- P1000 pipette
- P1000 pipette tips
- Ice cold PBS
- PCR tubes

Protocol:

1. Prep RIPA buffer
    a. Thaw 5 ml aliquot from -20 freezer
    b. Add ½ anti-protease tablet and ½ anti-phosphatase tablet for 5 ml aliquot
    c. Vortex thoroughly until fully dissolved
2. Remove cell media from growth plates thoroughly with aspirator
3. Wash cells twice with ice cold PBS
4. Add RIPA buffer to the growth plate
    a. 150 uL for a 10 cm plate
    b. Dump it all over the cells
    c. After 3 hours throw out RIPA buffer
5. Open the cell scraper under the hood and begin scraping cells covered in RIPA buffer
    a. Start scraping down from one side of the plate to the bottom and then scrape all over making sure to scrape the entire surface of the plate
6. Keep the plate at an angle and transfer the 150 uL to a sterile centrifuge tube
    a. Put all of the volume in the tube and put it on ice immediately
7. Take tubes to the cold room and agitate them in the rotator at 17 degrees for 35 minutes
    a. While agitating, set the centrifuge to 4 degrees
8. After 35 minutes spin tubes at 20,000 x G for 8 minutes at 4^{o}
9. Separate the proteins into new tubes
    a. Prepare new sterile centrifuge tubes
    b. Remove supernatant (containing proteins) and transfer it to the new tubes

10. Transfer 10 uL to a sterile PCR tube for protein quantification

Store full samples and 10 uL aliquots in the -80 for western prep and Bradford

Appendix B: Immunochemistry Protocols

αSMA Staining

Materials:

- 4% Paraformaldehyde (PFA)
- 1xPBS
- FBS
- ddH2O
- 0.5% Triton X-100 (Cat: 9036-19-5) in 1xPBS
- 0.1% Triton X-100 in 1xPBS
- αSMA primary antibody (1:300; Cat: A5228)
- AF 488-Goat Anti-Mouse secondary antibody (1:300, IgG (H+L); Cat: A11001)
- DAPI (1:20,000; Cat: D9542)
- Block solution:
    - 1xPBS
    - 0.1% Triton X-100
    - 10% FBS

DAY 1:

1. Fix the cells with 4% PFA for 10 minutes at room temperature (RT)
2. Wash the cells two times with 1xPBS
    a. Amounts vary depending on size of well
    b. Approximately 500 µl per well on a 24-well plate
    c. Leave PBS on all wells until ready for the next step
3. Permeabilize the cells by adding 500 µl of 0.5% Triton X-100 in PBS for 5 minutes at RT
4. Wash the cells two times with 1xPBS
5. Block the cells by adding 500 µl of block solution to each well for 1 hour at RT
6. Dilute the primary αSMA antibody 1:300 in block solution and then add 500 µl of diluted solution to each well and incubate overnight at 4^{o}

a. Wrap plates in tinfoil and put each one in the fridge

DAY 2:

1. Wash the wells three times with 0.1% Triton X-100 for 5 minutes
   a. Add 500 µl to each well and leave it for 5 minutes each wash
2. Dilute AF-488-Goat Anti-Mouse secondary antibody 1:300 in block solution and then add 500 µl of the diluted solution to each well and incubate for 30 minutes at RT
3. Wash the wells three times with 0.1% Triton X-100 for 5 minutes each
4. Add 500 µl of 1:20,000 diluted DAPI to each well and incubate for 5 minutes at RT
5. Wash the cells once with 1xPBS and then once with ddH2O
6. Add 1 ml of ddH2O to each well for storage at 4^{o}
7. Can be stored in water for up to a week before imaging

Perilipin Staining

Materials:

- 4% Paraformaldehyde (PFA)
- 1xPBS
- FBS
- ddH2O
- 0.5% Triton X-100 (Cat: 9036-19-5) in 1xPBS
- 0.1% Triton X-100 in 1xPBS
- Perilipin primary antibody (1:300; Cat: 9349s)
- AF 488-Goat Anti-Rabbit secondary antibody (1:300, IgG (H+L); Cat: A11034)
- DAPI (1:20,000; Cat: D9542)
- Block solution:
  - 1xPBS
  - 0.1% Triton X-100

- 10% FBS

DAY 1:

1. Fix the cells with 4% PFA for 10 minutes at room temperature (RT)
2. Wash the cells two times with 1xPBS
    a. Amounts vary depending on size of well
    b. Approximately 500 µl per well on a 24-well plate
    c. Leave PBS on all wells until ready for the next step
3. Permeabilize the cells by adding 500 µl of 0.5% Triton X-100 in PBS for 5 minutes at RT
4. Wash the cells two times with 1xPBS
5. Block the cells by adding 500 µl of block solution to each well for 1 hour at RT
6. Dilute the primary perilipin antibody 1:300 in block solution and then add 500 µl of diluted solution to each well and incubate overnight at 4°
    a. Wrap plates in tinfoil and put each one in the fridge

DAY 2:

1. Wash the wells three times with 0.1% Triton X-100 for 5 minutes
    a. Add 500 µl to each well and leave it for 5 minutes each wash
2. Dilute AF-488-Goat Anti-Rabbit secondary antibody 1:300 in block solution and then add 500 µl of the diluted solution to each well and incubate for 30 minutes at RT
3. Wash the wells three times with 0.1% Triton X-100 for 5 minutes each
4. Add 500 µl of 1:20,000 diluted DAPI to each well and incubate for 5 minutes at RT
5. Wash the cells once with 1xPBS and then once with ddH2O
6. Add 1 ml of ddH2O to each well for storage at 4°

Can be stored in water for up to a week before imaging

Myosin Heavy Chain Staining (Adapted from OS' protocol)

Primary antibodies:

- BA-D5 (IgG-2b) Mouse anti-myosin Heavy Chain Type 1, DSHB
- SC-71 (IgG1) Mouse anti-myosin Heavy Chain Type IIa, DSHB
- BF-F3 (IgM) mouse anti-myosin Heavy Chain Type IIb DSHB
- Laminin β-2/ γ-1 Monoclonal (A5), rat anti-laminin (Cat: MA1-06100) (IgG)

Secondary antibodies:

- Alexa Fluor 488 AffiniPure Goat Anti-Mouse IgG, Fcγ subclass 1 specific (Cat: 115-545-205)
- Alexa Fluor 647 AffiniPure Goat Anti-Mouse IgG, Fcγ subclass 2b specific (Cat: 115-605-207)
- Cy™3 AffiniPure Fab Fragment Goat Anti-Mouse IgM, μ chain specific
- Alexa Fluor 594 Goat Anti-rat IgG (H+L) (Cat: A-11007)

Protocol:

DAY ONE:

1. Allow sections to thaw and air-dry (10-15min)
2. Circle muscle sections with immunopen
3. Line the bottom of a slide box with paper towel dampened with ddH2O, to prevent slide dehydration
4. Add 5% BSA in 1xPBS and incubate 1hour at room temperature (RT) to block
5. Dilute the laminin primary 1:200 in 5% goat serum (G/S) in 1xPBS to 1'AB sections
    a. Add 5% G/S only to the 2'AB sections.
6. Incubate overnight at 4°C

DAY TWO:

1. Wash slides three times with 1xPBS for 5 minutes each
2. Dilute AF594 anti-rat 1:300 in 1% BSA in 1xPBS
    a. Incubate for 1 hour at RT
    b. Add mixture to both sections
3. Wash slides three times with 1xPBS for 5 minutes each
4. Block with M.O.M. for 1 hour at RT diluted 1:25 (Drops) in 1xPBS
5. Wash slides twice with 1xPBS for 2 minutes each

6. Add 1:12.5 in 1xPBS M.o.M diluent for 5 min (1:12.5 in 1xPBS)
7. Make mixture of BA-D5 [1:100], SC-71 [1:100], and BF-F3 [1:25] in 1% BSA in 1xPBS and add it to the slides
8. Incubate in the heated incubator for 45min at 37°C
    a. Add 1% BSA in 1xPBS only to the 2'AB sections
9. Wash slides three times with 1xPBS for 5 minutes each
10. Make mixture of AF488-IgG1 [1:100], AF647-IgG-2b [1:100], and Cy3 [1:50] in 0.5% BSA, 5% G/S in 1xPBS and add it to the slides
11. Incubate for 1 hour at RT
    a. Add mixture to both sections.
12. Wash slides three times with 1xPBS for 5 minutes each
13. Add DAPI for 5 minutes
14. Wash slides three times with 1xPBS for 5 minutes each
15. Add fluoromount and the coverslip and let the slides dry for 10-15 minutes
    a. Do not press or move the coverslip after mounting
16. Let slides sit for at least 1 hour prior to imaging or store the slides long term at 4°

Appendix C: Murine Protocols

rFSTL1 reconstitution and injection

Materials:

- Sterile PBS
- rFSTL1 protein (biotechne catalog #: 1738-FN-050)
- Preferred needle size for I.V.

Notes:

- Protein comes as 50ug
- Can only inject up to 5ul\L/g per mouse
- Mice typically 15-20 grams at 6 weeks so 75-100ul max
- Reconstitution is 500ng/uL and must be diluted to 30ng/uL working solution
- Reconstituted stock is good for 3 months at -20

Protocol:

1. Open 50 ug rFSTL1 stock vial under the BSC and add 100 uL of sterile PBS
    a. Pipette up and down to mix
    b. Creates 500ng/uL stock solution that can be stored at –20
2. Dilute stock solution to 30ng/uL the week of your injections
    a. Add 60uL of the stock solution to 940uL of sterile PBS
    b. Pipette up and down and then vortex to mix thoroughly
3. For 15g mouse, need 1500ng of rFSTL1
    a. 1500/30 = 50uL of diluted solution

Fractionated Irradiation

In vivo:

1. The morning of each mouse radiation dosage ensure that you arrive early to warm up the X-RAD 320 (20 minutes)
2. Ensure that the correct filter is placed into the machine (animals or cells)
3. Bring the individual cages in carts to the machine

4. Sedate the mouse in the provided tank with isofluorane set to at 4.5

a. Make sure that both switches are flipped on so that isofluorane is going to the machine as well

5. Gently remove the sedated mouse from the tank and position it with its nose in the actively pumping nose cone inside the irradiator

a. Tape the back of its neck to the nose cone

b. Ensure that the beam of light signifying the irradiation beam is fully covering both limbs

6. Once all mice are in the X-RAD, lower the isofluorane dial to 2.5

7. Extend the irradiated legs of the mouse out and gently tape with skin-safe medical tape

8. Place the lead body shield over the mouse with the irradiated limbs sticking out and ensure that no other body parts of the mouse are exposed to the irradiation beam

9. Close the X-RAD door gently and ensure it doesn't slam

a. Set the dosage to 820 cGy

10. Hit start to begin the radiation process and step out of the room

11. After the radiation has finished, the light will go off and you can gently remove the mouse and put it back in its cage

12. Monitor the mouse after to make sure it has no issues waking up from the sedation

In vitro:

1. The morning of each mouse radiation dosage ensure that you arrive early to warm up the X-RAD 320 (20 minutes)

2. Ensure that the correct filter is placed into the machine (animals or cells)

3. Take a cafeteria tray and thoroughly spray it down with 70% ethanol

a. Place a piece of paper towel and place it on the tray and then spray down both with 70% ethanol

4. Remove cell culture plates from the incubator and thoroughly spray them down with 70% ethanol

5. Take the tray down to the X-RAD and spray it down thoroughly with 70% ethanol

6. Place the plates in the X-RAD making sure that the entire plate is inside of the beam of light

7. Set the dosage to 100 cGy and exit the room as it runs

8. Once all plates are done, thoroughly spray down the tray and plates again with 70% ethanol and return them to the incubator

Flash Freezing

Materials:

- Empty ice box
- Container of liquid nitrogen
- Can of isopentane
- Dissection tools
- Tin foil
- Time tape
- OCT
- Sample mounts

Protocol:

1. Prepare sample mounts
2. Pour liquid nitrogen in ice box and hang can of isopentane just outside of it
3. Right before dissection drop the can into the liquid nitrogen to cool it
4. Layer the sample mount with OCT
5. Dissect the mouse and lay the gas/sol complex flat on the OCT
6. Cover the muscle with one more layer of OCT
7. Take the can out of the liquid nitrogen and hang it off the side of the box
    a. Then take the sample mount with locking tweezers and hold the muscle fully submerged in the isopentane for 20-30 seconds
8. Remove the sample mount, quickly wrap it in labelled tinfoil and insert it right away into the –80
9. Use a box of dry ice to transport the samples to the basement -80

Muscle protein bead homogenization

Materials:

- RIPA buffer (5 ml aliquots)

- Anti-protease tablets (½ per 5 ml buffer)
- Anti-phosphatase tablets (½ per 5 ml buffer)
- Microcentrifuge tubes
- Homogenization beads
- Homogenizer tubes
- Weigh boats
- Scale

Protocol:

1. Fast temp the microcentrifuge to 4 degrees
2. Grab box of muscle samples and keep on dry ice until use
3. Prep thawed RIPA buffer with ½ phosphatase tablet and ½ protease inhibitor tablet
4. Weigh each muscle and place it into labelled homogenizer tube
    a. Add 10uL/mg prepped RIPA buffer to each tube
    b. Once the RIPA is in leave it on normal ice as much as possible
    c. Before RIPA leave the samples in dry ice or liquid nitrogen
5. Add 4 beads to each tube
6. Place samples in the bead homogenizer machine and run it twice for 60 seconds at max speeds
    a. Make sure to wait 5 seconds in between bouts
7. Transfer full sample to a new 1.5ml microcentrifuge tube using a 200uL pipette
8. Centrifuge the samples at 15000 x g for 15 minutes at 4 degrees
9. Transfer the supernatant to a new 1.5ml microcentrifuge tube
10. Aliquot 10uL into a PCR tube to use for Bradford assay
11. Put samples back on ice and place it into –80^{o} for storage

Appendix D: Single Cell Isolation and Staining

Muscle Progenitor Isolation (MACS)

Materials:

- Ham's F10 Media
- Collagenase B
- Dispase II
- FBS
- 1x PBS
- MACS tubes
- 25mM EDTA stock solution
- DMEM high Glucose
- P/S
- Tweezers and scissors
- 50 and 15ml tubes
- Flow tubes
- Eppendorf tubes
- 60 mm plates (1 for each sample)
- 70 um filters
- 100 um filters
- 5 ml syringes
- 0.2 um filters
- Red blood cell lysis buffer

FACS Media (50ml):

- 6ml of 25mM EDTA
- 5ml FBS
- 39ml of 1x PBS

Enzymatic Solution (50ml):

- 0.5 g of Collagenase D (final 1.5U/ml)
- 0.2 g of Dispase II (final 2U/ml)
- 500 uL $CaCl_2$
- 50ml of Ham ’s F10
- *Note: Prepare fresh from powder stock same day. Mix gently and filter in 0.2um. “warm up”

(This protocol is for two hindlimbs per MACS tubes)

1. Dissect hindlimbs making sure to get as much muscle as possible and clean as much fat and connective tissue off as possible
2. Place the muscle already clean (w/o Fat and connective tissue) into a 60mm dish containing 5ml of cold 1xPBS. (Place the dish on ice if you are dissecting more mice).
3. Add 5ml of enzymatic solution into the MACS tubes (respectively labeled) and add the pieces of muscle.
    a. Use 5ml for two hindlimbs, 3ml for one hindlimb and 400ul for
4. With scissors cut the muscle in small pieces into the MACS tube with the enzymatic solution in it.
5. Close the tube, invert it and place it on ice and go to the MACS gentle machine (4^{th} floor shared equipment.
6. Machine: user 1, Slice_FACS, program 27min.
7. After, add 5ml of FBS to each MACS tube and pipette up and down with the pipette gun (10ml).
    a. Pre prep your filters by running 1 ml of FBS through them
8. In a 50ml tube filter the solution with a 100um strainer (*coat the strainer with FBS)
9. Then, rinse the MACS tube with 10ml of 1xPBS, close the cap, shake it well and pass that through the filter as well.
10. Subsequently filter the flowthrough through an 70um filter
11. Split the 50ml tube containing your sample into two 15ml tubes and centrifuge at 400 x g for 10min at 4ºC (respectively labeled)
    a. Reduced speed from 600 x g to reduce debris flow through

12. Remove the supernatant with the pipette gun (10ml) and add 1 ml of Red blood lysis buffer to the first tube, resuspend the first pellet (mix well with the p1000), take all the solution and add it into the second 15ml and resuspend the other pellet in the original resuspension. Mix it well.
    a. Use 1 ml for two hindlimbs, and 400 ul for one hindlimb and one gastroc
    b. Leave on ice in the dark for 3 minutes
13. Add 10ml of cold FACS buffer, mix well
14. Centrifuge at 400 x g for 5min at 4^0C
    a. Reduced from 600 x g
15. Remove supernatant
16. Resuspend with 1ml FACS buffer
17. *From here, go the Flow cytometer and FACS sorting steps

For FACs sorting:

- Pre-make antibody cocktails
    - PDGFRa PE cocktail (amounts are per mouse)
        - 100 uL FACs buffer
        - 10 uL PDGFRa PE
    - Full stain for FACs (amounts are per mouse):
        - 100 uL FACs buffer
        - 2 uL CD31 BV421
        - 2 uL CD45 FITC
        - 2 uL ITGA7 647

1. After step 16 take the cell resuspension and centrifuge it again at 400 x g for 5 minutes at 4 degrees
    a. Reduced from 600 x g
2. Remove supernatant and resuspend the pellet in 100 uL of the PDGFRa PE cocktail
    a. Incubate the sample in ice in the dark for 20 mins
3. After incubation, add 1 ml of FACs buffer to quench the sample and then spin it again for 5 minutes at 400 x g at 4 degrees

a. Reduced from 600 x g

4. Resuspend the pellet in 100uL of the full stain cocktail
    a. Incubate on ice in the dark for 30 mins
    b. Tap the sample after 15 mins
5. After incubation, add 1 ml of FACs buffer to quench the sample and then spin it again for 5 minutes at 400 x g at 4 degrees
    a. Reduced from 600 x g
6. Remove the supernatant and resuspend the pellet in 1 ml of FACs buffer
7. Filter sample through a 50 um filter into a flow tube
8. Add 0.3 uL of Sytox 7AD to each sample tube, vortex, and keep in dark on the way to the FACs sorter in the OHRI

For Attune flow cytometer:

- Pre-make antibody cocktails
    - Full stain for flow (amounts are per sample):
        - 100 uL FACs buffer
        - 2 uL CD31 BV421
        - 2 uL CD45 PE-Cy7
        - 5 uL ITGA7 PE
    - PDGFRa (1:50 dilution)
        - 100 uL FACS buffer
        - 2 uL PDGFRa BV605
    - Zombie Yellow (1:300 dilution)
        - 300 uL PBS
        - 1 uL Zombie Yellow

1. After step 13 take the cell resuspension and centrifuge it again at 400 x G for 5 minutes at 4 degrees
    a. Reduced from 600 x g
2. Remove supernatant and resuspend the pellet in 100 uL of the zombie yellow cocktail

3. Incubate the sample at room temperature in the dark for 15 minutes
4. After incubation, add 500 uL of PBS to quench the sample and then spin it for 5 minutes at 500 x G at 4 degrees
5. Remove the supernatant and resuspend the pellet in 100 uL of the full stain cocktail
6. After incubation, add 1 ml of FACs buffer to quench the sample and then spin it again for 5 minutes at 400 x g at 4 degrees
    a. Reduced from 600 x g
7. Remove the supernatant and resuspend the pellet in 1 ml of FACs buffer
8. Filter sample through a 50 um filter into a centrifuge tube and take it to the flow cytometer on ice

Flow Compensation Beads

Materials:

- Microcentrifuge tubes
- Desired antibodies
- ABC beads (both positive and negative)
- 1xPBS

Protocol:

1. Label 1.5 ml tubes for each of the desired antibodies.
2. To prepare the ABC beads, vortex them in the bottle for 10 seconds.
3. Add one drop of the positive ABC beads to each tube.
4. Add 1 µl of the appropriate fluorochrome to each tube.
5. Vortex briefly to mix well.
6. Incubate tubes in the dark for 15 minutes at room temperature.
7. Add 1 ml of 1xPBS to each tube.
8. Centrifuge at 250 x G for 5 minutes at room temperature.
9. Remove the supernatant making sure to leave approximately 50 µl in the bottom as there isn't a visible pellet.
10. Resuspend in 500 µl of 1xPBS.
11. Prepare the negative ABC beads by vortexing them for 10 seconds.

12. Add one drop of negative beads to the resuspended beads and then pipette up and down to mix
13. Analyze with the Attune Flow Cytometer

Appendix E: Fiji Analyses

Counting DAPI stained nuclei in Fiji

1. Open image in Fiji – ImageJ
2. Go to image -> colour -> split channels
3. Remove all channels except the blue channel (written at top of image)
4. Go to image -> adjust -> threshold
    a. Adjust threshold such that individual cells can be made out but don't press apply
5. Go to process -> binary -> make binary
    a. Check "threshold pixels to foreground colour" and "remaining pixels to background colour" but not 'black foreground, white background"
    b. Click ok
6. Go to process -> binary -> fill holes
7. Go to process -> binary -> watershed
8. Go to analyze -> analyze particles
    a. Size: 30-infinity
    b. Check pixel units
    c. Circularity: 0.00-1.00
    d. Show: Outlines
    e. Only check Summarize and Include Holes
    f. Once all settings correct, click ok
9. Record count for your spreadsheet
10. Calculate mean of DAPI by just clicking measure on the blue channel image

Mean fluorescence intensity and area per cell of aSMA and Perilipin

1. Open image in Fiji – ImageJ
2. Go to image -> colour -> split channels
3. Remove all channels except the red channel (written at top of image)
4. Go to analyze -> measure
    a. Record area and mean in excel

5. Use counted number of cells to normalize to cell count in the image

% Perilipin Positive Cells

1. Open fiji (alongside Image --> Colour --> Channels Tool, and… Image --> Adjust --> Brightness/Contrast).
2. Open file in Fiji.
    - View stack with: Hyperstack
    - Color mode: Grayscale
    - Boxes: Check stitch tiles, auto scale, and split channels
        - Everything else unchecked
    - Open “Series 1”
3. Start with DAPI channel
    - Image --> Duplicate the DAPI channel. Use one as a reference, other will be converted to black/white binary.
4. Adjust the brightness/contrast on the reference image using the B&C tool to make strong signal and eliminate background.
    - Image --> adjust --> brightness and contrast
5. Image --> Type --> 8 bit on image to be analyzed.
6. Image --> Adjust --> Threshold (should look like reference image but in black and white, where cells are black and background is white). Click "Apply," then exit out of threshold.
    - Make sure to continuously check back to the reference image to make sure you aren’t excluding any cells with your set threshold
    - Manipulate the threshold until you can see well defined cells (no blobs of multiple cells)
7. Process --> Binary --> Make binary
8. Process --> Binary --> Dilate (makes cells bigger).
9. Process --> Binary --> Watershed (separates overlapping cells).
10. Analyze --> Analyze Particles.
    - Size (measure smallest - Infinity).
    - Circularity (0-1).

- Show: Bare Outlines.
- Boxes: Check Display results, Summarize, Include holes.
- Click OK.

11. Record Counts
12. Close DAPI image
13. Re-open image with same parameters
14. Close DAPI channel so that only adipocyte channel is open
15. Use the multi-point tool
    - Click on each adipocyte with the tool

Appendix F: Western Blot and qPCR Protocols

Bradford protein quantification

Materials needed:

- 96 well plate
- BSA stock (Millipore sigma: 0000355792)
- Bradford reagent (Bio-Rad Cat: #5000006)
- Multi-channel pipette
- Trough
- Multi-channel pipette tips
- 10uL pipette and tips

Protocol:

1. Prepare standard samples
    a. Serial dilution
    b. Make 2mg/ml stock by adding 100mg BSA powder to 50ml PBS
        i.20mg powder in 10ml PBS
    c. Set up tubes for 2mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml and 0mg/ml
    d. Take 400uL of stock BSA solution and add it to the 2mg/ml tube
    e. Add 200uL of PBS to every other tube
    f. Take 200uL from the 2mg/ml tube and add it to the 1mg/ml tube and vortex well
    g. Continue doing the same all the way down the series in the following pattern: 2mg/ml -> 1mg/ml -> 0.5mg/ml -> 0.25mg/ml -> 0.125mg/ml
    h. Vortex each tube well after mixing and then spin the solution down using the tabletop centrifuge
2. Dilute Protein samples
    a. Run a 10x and a 20x dilution
    b. Use the 10uL aliquot and add 5uL sample to 45uL PBS for a 10x dilution and add 2.5uL sample to 47.5uL PBS for a 20x dilution
3. Dilute Bradford reagent
    a. Use 1:4 dilution, 1 part Bradford, 4 parts PBS
    b. E.g. 15ml dilutant -> 12ml PBS, 3ml Bradford reagent

4. Add 5uL of standard curve, or sample, to each well
    a. Vortex sample, and then pipette up and down before pipetting
    1. Only go to first stop to avoid bubbles
5. Use multi-channel pipette to add 250ul Bradford dilutant to each well

Western Blot: Adapted from FB & LEO

Materials:

- 10x Running Buffer - to make 1x Running Buffer (with ddH2O)
    - 30.3g Tris Base (NOT Tris-HCl)
    - 144.0g Glycine
    - 10.0g Sodium Dodecyl Sulfate (SDS)
    - Top up to 1L with ddH2O
    - Adjust pH to 8.3 (Approximately 9.5 mL of 37% HCl)

- 10x Transfer Buffer - to make 1x Transfer Buffer (10% Transfer Buffer, 70% ddH2O, 20% Methanol)
    - 30.3g Tris Base (NOT Tris-HCl)
    - 144.0g Glycine
    - Top up to 1L with ddH2O

- 10x Tris-Buffered Saline (TBS) - to make 1x TBS (with ddH2O)
    - 204 24.2g Tris Base (NOT Tris-HCl)
    - 80g Sodium Chloride (NaCl)
    - Top up to 1L with ddH2O
    - Adjust pH to 7.6 (Approximately 12 mL of 37% HCl)

- 1.5 M Tris-HCl (for Running Gel) – 600 mL Preparation
    - 108.9g Tris Base (NOT Tris-HCl)
    - Top up to 600 mL ddH2O
    - Adjust pH to 8.8 (Approximately 18.5 mL of 37% HCl)

    - M Tris-HCl (for Stacking Gel) – 200 mL Preparation
        - 24.2g Tris Base (NOT Tris-HCl)
        - Top up to 200 mL ddH2O
        - Adjust pH to 6.8 (Approximately 15 mL of 37% HCl)

- 0.1% TBST (1 mL Tween 20 in 999 mL TBS) - Cut off end of 1 mL pipette tip with scissors. Tween is viscous and some will stay inside. Eject the empty pipette tip into the 1000 mL tube, cover with parafilm paper, and mix. Pour the fluid into a new capped glass jar.

- 10% Ammonium Persulfate (APS) (In -20°C freezer)
    - 1g Ammonium Persulfate
    - 10mL ddH2O
    - Aliquot in 1mL, store in -20°C freezer

- 4x Laemmli Buffer (In -20°C freezer)
    - 2.4mL 1M Tris-HCl (NOT Tris Base)
    - 0.8g SDS
    - 4mL Glycerol
    - 1mg Bromophenol Blue
    - 1mL β-mercaptoethanol
    - 2.8mL ddH2O
    - Aliquot in 1mL, store in -20°C freezer

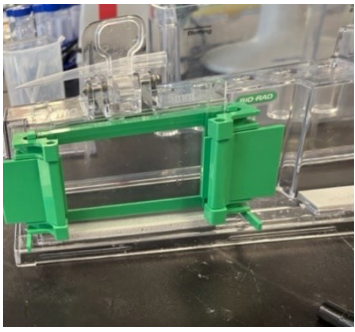
- 30% Acrylamide (In 4°C Fridge - Brown Bio-Rad bottle)
- TEMED (in flammables cabinet)
- APS (In -20°C freezer)
- 5% Milk-TBST (for 50 mL: 250 mg powder milk and top up to 50 mL with TBST)
- 5% BSA-TBST (for 50 mL: 250 mg powder BSA and top up to 50 mL with TBST)

- Prepare Resolving Gel (12%):
    - 8.2 mL ddH2O
    - 10 mL 30% Acrylamide
    - 6.3 mL Tris 1.5 M (pH 8.8)
    - 250 µL 10% SDS
    - 250 µL 10% APS - Only add this component when ready to form gel – it's an active component.
    - 25 µL TEMED - Only add this component when ready to form gel – it's an active component.

- Prepare Stacking Gel (5%):
    - 8.5 mL ddH2O
    - 2.1 mL 30% Acrylamide
    - 1.6 mL Tris 1.0 M (pH 6.8)
    - 125 µL 10% SDS
    - 125 µL 10% APS - Only add this component when ready to form gel – it's an active component.
    - 12.5 µL TEMED - Only add this component when ready to form gel – it's an active component.

- Prepare Running Buffer
    - 100 mL 10x running buffer (pre-made on shelf), 900 mL ddH2O

Protocol:

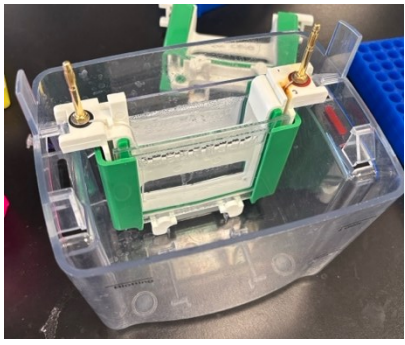


Day 1:

1. Clean plates with ddH2O and Kim wipes
2. Place plates in green holder (thick plate in back, thin plate in front) ->
    a. put 1000 µL pipette tip in clamp
    b. make sure that plates are flush to bottom

3. Prepare 12% resolving gel and 5% stacking gel
    a. 12% Resolving gel- fill to bottom of green holder
        i. 8.2 mL ddH2O
        ii. 10 mL 30% acrylamide (fridge)
        iii. 6.3 mL 1.5M tris buffer (pH 8.8) (shelf)
        iv. 250 µL 10% SDS (shelf)
        v. Add these components when ready to pour:
        vi. 250 µL 10% APS- keep in freezer until needed
        vii. 25 µL TEMED (flammable hood)
    b. Invert to mix
    c. Add 1000 µL of isopropyl alcohol (2-propyl) on top of resolving gel for leveling
    d. Let set for ~20-25 mins
    e. Discard isopropyl alcohol when set
    f. 5% Stacking gel- Fill to top of green holder
        i. 5.1 mL ddH2O
        ii. 1.25 mL 30% Acrylamide (fridge)
        iii. 960 µL Tris 1.5 M (pH 6.8) (shelf)
        iv. 75 µL 10% SDS (shelf)
        v. ~ Only add this component when ready to form gel
        vi. 75 µL 10% APS- keep in fridge until needed (freezer)
        vii. 8 µL TEMED (flammable hood)
    g. Invert to mix
    h. Add comb (flat side of the comb to the back, submerge fully into stacking gel, some will overflow)
4. Prepare running buffer

a. Add 900 mL of ddH2O to a 1000mL graduated cylinder
b. Then add 100mL of 10x Running buffer]

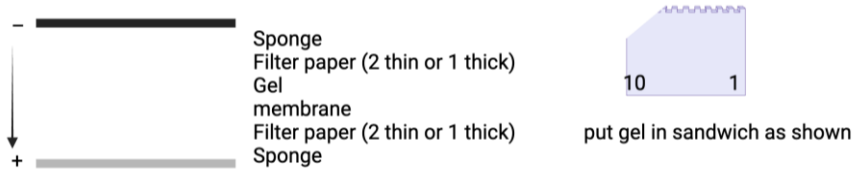


5. Boil samples at 95ºC for 5 mins before loading
6. Write down loading scheme of gel

Ex:

Lane	1	2	3	4	5
Sample	Ladder	25	50	70	Ladder
Volume (µL)	5	12.5	25	35	5

7. Once gel has set, remove gel from clamp setting and remove comb as well
    a. place plate in cassette (green and white holder) and place in container
    b. make sure to push up until plate is flush with green bumper
    c. if not doing even number of membranes, use a buffer dam
8. Partially fill cassette with running buffer to check for leaks, if there's no leaks: fill cassette until full then pour remainder into the container
9. Load ladder and samples
    a. Ladder in blue box in freezer with x on top
10. Put lid on holder and run at 90V for 30 mins (to get past stacking gel), then increase to 120V for ~ 60 mins or until bands get to the middle of the green bottom
    a. Keep checking periodically to make sure everything is going smoothly
11. Transfer proteins from gel to membrane
    a. Prep ddH2O, methanol (in chemical room), 1000 ml of transfer buffer
        i. Transfer buffer: 20% methanol, 10% transfer buffer, ddH2O to top
            1. 700 mL ddH2O
            2. 200 mL methanol
            3. 100 mL transfer buffer
    b. Prep sandwich
        i. Activate PVDF membrane in methanol for 10 seconds and activate filter paper and sponge in transfer buffer
        ii. Wet sponges and filter paper in transfer buffer
        iii. Go Methanol -> Transfer Buffer -> Water



iv. Add layers as shown above and use roller to roll out air bubbles evenly (press firm but don't tear gel)

c. Clamp and put into container

i. Add 2 ice packs and fill w/ transfer buffer as much as possible (transfers only as high as the buffer goes

ii. Run to red -> run at 120v for exactly 1hour in cold room

12. While waiting, prep 5% milk with TBST

a. 50mL TBST

b. 2.5g Milk powder

13. After transfer, add 10 ml of Ponceau to a dish and douse the membrane in it

a. Place on shaker for 5 minutes

14. Put in plastic sleeve with membrane #, name, date, protein and weight of proteins

a. Remember to label ladder before imaging

b. Smooth out and get rid of bubbles

15. Image membrane on the Biorad Chemidoc.

a. Ponceau- Auto optimal

16. Rinse quickly with ddH2O, then wash 3 x 5 minutes in TBST

17. Prep primary antibodies in 5% or 2% BSA-TBST

Primary Antibody	MW (kDa)	Dilution
FSTL1	42	1:1000 in 5% BSA-TBST
pSMAD3	53	1:500 in 2% BSA-TBST
SMAD3	53	1:500 in 2% BSA-TBST
TGF-β1	44	1:1000 in 2% BSA-TBST
TGF-βR1	56	1:1000 in 5% BSA-TBST
Cyclophilin-B	18	1:1000 in 5% BSA-TBST
Fibronectin	200	1:1000 in 5% BSA-TBST

18. Pour out TBST, add 1 º antibodies, seal with parafilm and place on shaker in cold room over night
    a. Keep antibody tube in freezer so it can be reused
    b. Mark off use

Day 2:

1. Take out membranes from cold rooms, put 1º antibody back into tube
2. Wash 3x 5 minutes in TBST
3. Prepare secondary antibody (Horseradish Peroxidase - HRP) in 5% BSA in TBST.
    a. Dilute HRP at 1:10,000 to make 10 mL total (1 µL HRP, 10 mL 5% BSA with TBST (0.5 g of BSA and 10 mL of TBST))
4. Add secondary HRP antibody to membranes and incubate for 1 hour at room temperature on the shaker
5. Wash membranes 3 x 5 minutes with TBST on shaker
    a. Once washes are done, put membrane not being imaged in ddH2O
6. Prepare chemiluminescence stain (ECL) (1:1 ratio) when ready to image - on shelf with other Western Blot reagents (ECL mixture).
    a. Prep 10 ml for all the membranes
    b. Take out glass plates
    c. Place 1 ml of ECL mixture on the glass plate and then place the membrane on top of it making sure to spread it around the membrane
    d. Add another 1 ml of the ECL mixture onto the membrane making sure to get it on the area of the proteins of interest
7. Prep plastic sleeve with membrane #, name, date, protein and weight of proteins
    a. Remember to label ladder before imaging
    b. Smooth out and get rid of bubbles
8. Image on the Biorad Chemidoc (manual for 5 mins of auto optimal and auto rapid don't work)
    a. Colorimetric – Auto (more than 5 mins, move on to auto rapid)
    b. Chemiluminescence – Auto (more than 7 mins, move on to auto rapid

        i. Make note of time
    c. Merge images together
    d. Transform to get best image (move H than G)
        i. Don't want bands to disappear but don't want blurry bands (smudged)
    e. Name image
9. Keep membrane in H2O while imaging next membrane (Repeat steps 8 to 7 for next step)
10. Once membranes are imaged, wash membrane 3x 5 minutes in TBST
    a. Keep membranes in ddH2O in fridge if not able to move on to next 1º antibody
        i. Can strip then keep in fridge or keep in fridge and strip when ready to move on)
11. Strip membrane using Restore Western Blot Stripping Buffer (brown bottle, not light sensitive)
    a. Add ~10 mL to dish
    b. Leave on shaker for 30 mins
12. Wash membrane 3x 5 minutes with TBST
13. Block in 5% milk for 1hr
14. Prep next 1º antibody
15. Wash membrane 3x 5 mins with TBST
16. Add next primary and leave it in the cold room overnight

Day 3:

1. Remove membrane from the cold room and return the primary antibody to its tube in the freezer
2. Add secondary HRP antibody to membranes and incubate for 1 hour at room temperature on the shaker
3. Wash membranes 3 x 5 minutes with TBST on shaker
    a. Once washes are done, put membrane not being imaged in ddH2O
4. Prepare chemiluminescence stain (ECL) (1:1 ratio) when ready to image - on shelf with other Western Blot reagents (ECL mixture).
    a. Prep 10 ml for all the membranes

b. Take out glass plates
c. Place 1 ml of ECL mixture on the glass plate and then place the membrane on top of it making sure to spread it around the membrane
d. Add another 1 ml of the ECL mixture onto the membrane making sure to get it on the area of the proteins of interest

5. Prep plastic sleeve with membrane #, name, date, protein and weight of proteins
    a. Remember to label ladder before imaging
    b. Smooth out and get rid of bubbles
6. Image on the Biorad Chemidoc (manual for 5 mins of auto optimal and auto rapid don't work)
    a. Colorimetric – Auto (more than 5 mins, move on to auto rapid)
    b. Chemiluminescence – Auto (more than 7 mins, move on to auto rapid
        i. Make note of time
    c. Merge images together
    d. Transform to get best image (move H than G)
        i. Don't want bands to disappear but don't want blurry bands (smudged)
    e. Name image
7. Keep membrane in H2O while imaging next membrane (Repeat steps 8 to 7 for next step)
8. Once membranes are imaged, wash membrane 3x 5 minutes in TBST
    a. Keep membranes in ddH2O in fridge if not able to move on to next 1º antibody
9. Save all images in the chemidoc according to membrane number and antibody designation
    a. Export them to the OneDrive and analyze in Fiji

Reverse Transcription

Materials:

- NEB First Strand cDNA Synthesis (Cat: M0368)
    - Protoscript II Reverse Transcriptase
    - Protoscript II Buffer
- dNTP Mixture (Cat: DD0056)
- Random Hexamers (Cat: 51-01-18-26)

- 0.1M DTT (Cat: B1034A)
- Microcentrifuge tubes (sterilized)
- PCR tubes (sterilized)
- DNase and RNase free water
- RNA (quantified by nanodrop)

Reverse Transcription Master Mix:

Reagent	Amount per sample (µl)	10 samples (e.g. x12)
10 mM dNTP	2	24
10x RT Random hexamers	2	24
5x ProtoScript BUFFER	8	96
0.1 M DTT	4	48
Nuclease-free water	8	96
ProtoScript Reverse Trascriptase	1	12
Total Volume	25	300

Protocol:

1. Make 25 µl RNA and nuclease-free water mix
    a. Use template on De Lisio Lab OneDrive to consistently dilute the RNA samples to 40 ng/µl
    b. Add the water first and then add the RNA
    c. Make sure to pipette up and down to thoroughly mix the samples
2. Make the RT Master Mix using the above table
3. Add 25 µl of the Master Mix to the tube containing your RNA and water mix
    a. You now have a total volume of 50 µl made up of your RNA + nuclease-free water + Master Mix
4. Pipette up and down and then briefly vortex to ensure thorough mixing
5. Spin the tubes using the table centrifuge
6. Place samples in the thermocycler and incubate them using the following specifications:

a. Incubate samples at 25ºC for 5 minutes
b. Incubate samples at 42ºC for 60 minutes
c. Incubate samples at 65ºC for 20 minutes to inactivate reverse transcriptase
d. Cool samples to 4ºC

7. Store the cDNA at -20ºC before dilution

Taqman qPCR Run

Materials:

- 20x TaqMan Gene Expression Assay (Cat: 4331182)
  - *Fstl1* (Mm00433371_m1)
  - *Ppia* (Mm02342430_g1)
- 2x TaqMan Fast Advanced Master Mix (Cat: 4444557)
- RNase-free water
- cDNA templates
- 96-well qPCR plate
- qPCR Microseal 'B' seal (Cat: MSB1001)

Taqman qPCR Master Mix

Component	Single Reaction (µl)	75 Reactions (e.g.)
20x Taqman Gene Expression Assay	1	75
2x Taqman Fast Advanced Master Mix	10	750
cDNA Template	2	
RNase-free water	7	525

Protocol:

1. Dilute the cDNA by adding 3 µl of cDNA to 97 µl of DNase free water
    a. Make sure to pipette up and down, vortex briefly, and spin down using the tabletop centrifuge
2. Make up the qPCR Master Mix using the above table with everything except the template cDNA
    a. Vortex thoroughly and mix with pipette
    b. Spin mix down using the tabletop centrifuge and then vortex right before plating
3. Set up a pipette box in the same formation as the plate you're using to keep track of which samples you have plated
4. Pipette 18 µl of the Master Mix into each well you're using making sure to minimize bubbles as much as possible
5. Add 2 µl of template cDNA to each well following your plating scheme
    a. Go to second stop extremely slowly to minimize bubbles
6. Gently tap the plate and spin it on the desk to mix wells as much as possible
7. Seal the plate with the qPCR Microseal 'B'
    a. Spin the plate again on the desk and tap it down to mix wells fully
8. Take the plate to the thermocycler and run the plate using the following specifications:
    a. Hold samples for 2 minutes at 50^{o}C
    b. Hold samples for 20 seconds at 95^{o}C to activate the polymerase
    c. Denature at 95^{o}C for 3 seconds followed by extension at 60^{o}C for 30 seconds
        i. Run this for 40 cycles with a read at this step

Appendix G: FAP Transplantation in a Model of Chemoradiation-induced Muscle Atrophy

This Appendix features the protocols for a project that I have been working on simultaneously with my proposed MSc project. My work on this project to date has been to establish a novel mouse colony and optimize *in vivo* experiments. Given that completion of the project described in Appendix G will exceed the timeline of an MSc, I decided to focus on the FSTL1 project as my main MSc research project.

Rhabdomyosarcoma Cancer Plus Treatment Model:

The cancer plus therapy model has been previously established by our group and others[24,25]. Briefly; at age four weeks, mice are injected with 1.0 x 10^5 RMS M3-9M into both lobes of the gastrocnemius muscle of both hindlimbs using an insulin syringe[24,25]. Four days later, mice ≥12 g (to minimize risk of death or severe illness due to vincristine toxicity) receive vincristine chemotherapy diluted in PBS (1 mg/kg of body weight)[24,25]. At age five weeks mice will receive five doses of 4.8 Gy irradiation delivered on five consecutive days[24,25]. Two days following the final irradiation treatment, mice will be injected with 1.0 x 10^6 FAPs isolated from PDGFRα/tdTomato reporter mice (Figure 13). FAPs will be injected into both lobes of the left gastrocnemius muscle with an insulin needle[51,110]. Saline will be injected into both lobes of the right gastrocnemius muscle with an insulin needle as an internal control. Both gastrocnemius/soleus muscle complexes will be collected from all mice at either two- or four-weeks post-FAP transplantation for downstream analyses of muscle architecture, muscle fibrosis, and mononuclear cell content (Figure 14).

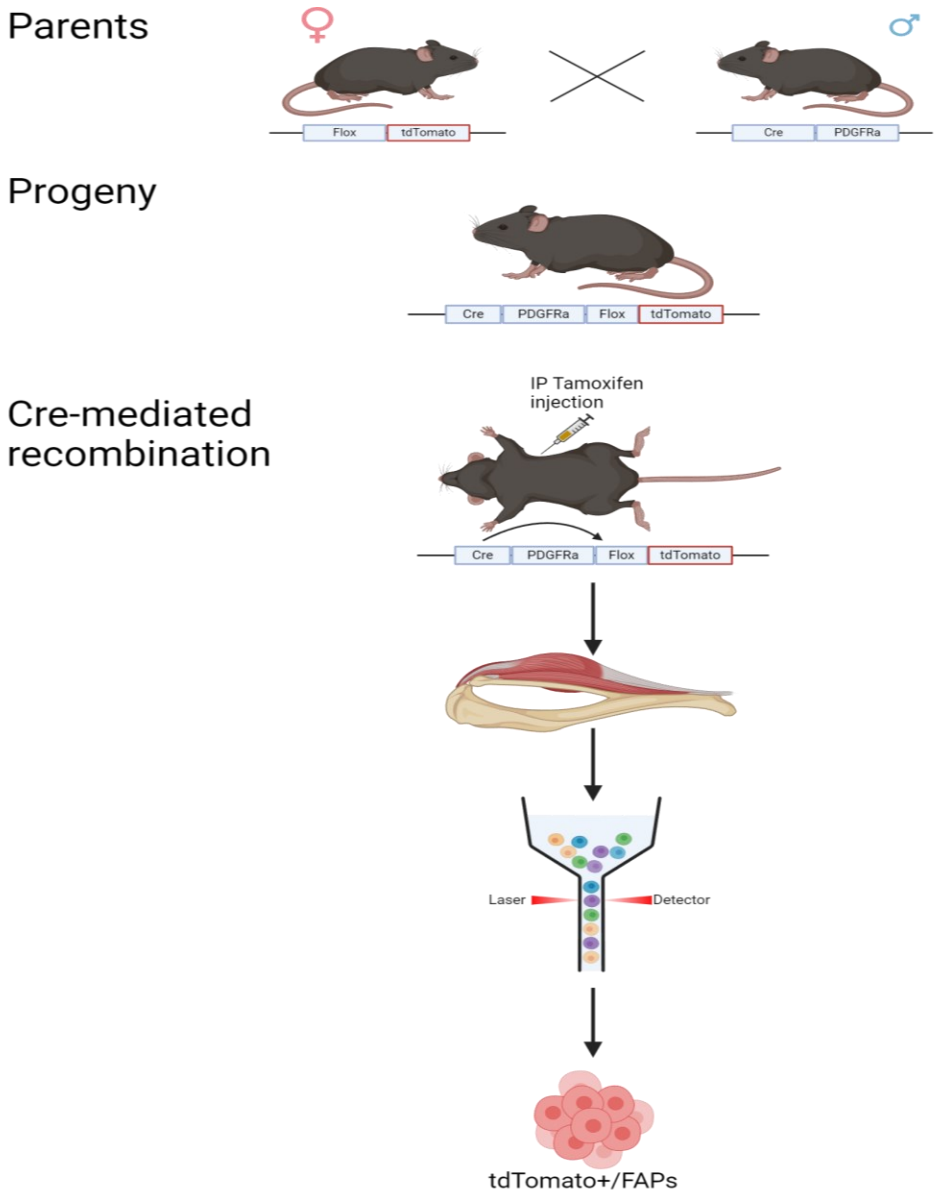


Figure 13: Cre-PDGFRα^{+}/WT; tdTomato^{+}/tdTomato^{+} reporter mouse breeding and tamoxifen induction.

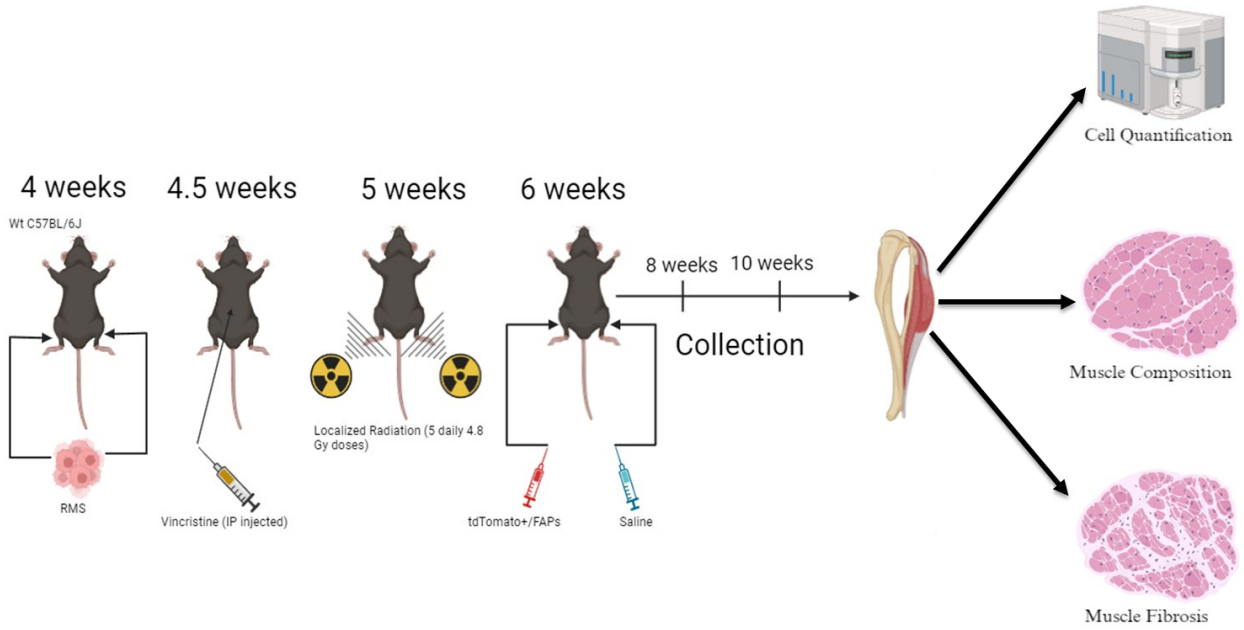


Figure 14: Rhabdomyosarcoma cancer induction plus chemotherapy, irradiation, and FAP injection treatment protocol.

DNA Extraction

1. Use earring apparatus to attach numbered tags to each mouse in each cage.
    a. Use odd numbers in left ears and even numbers in right ears.
2. Use fine scissors to clip small tissue samples from each mouse ear.
3. Store tissue samples in the -20 freezer prior to extraction.
4. Transfer all samples to individually labelled PCR tubes.
5. Add 50 uL of 50 mM NaOH to each tube.
6. Incubate all tubes at 98^0C for 60 minutes.
    a. Flick all tubes every 20 minutes.
7. Vortex tubes after incubation while still hot.
8. Add 50 uL of 300 mM NaOAc to each tube.
9. Add 150 uL of DNase free water.
10. Transfer full volume to individually labelled centrifuge tubes.

11. Centrifuge at 1500 x G for 3 minutes.
12. Transfer supernatant to different individually labelled PCR tubes and store in the -20 freezer.

10x TBE protocol

Materials needed for 1L:

- 108g Tris base
- 55g boric acid
- 40 ml 0.5M EDTA solution

Protocol:

1. Do entire protocol in a 1 litre beaker using a large stir stick spinning at 37^0.
2. Dissolve 108g Tris base in around 800ml DI water.
3. Add 55g boric acid.
4. Add 40 ml 0.5M EDTA solution.
5. Check that pH is 8.3 on a pH metre and adjust if needed.
6. Make up to 1 litre in a graduated cylinder with DI water.
7. Dilute to 1x by adding 100ml of 10x solution to 900ml DI water to use as working solution.

Agarose Gel Electrophoresis

1. Create 200 ml 2% agarose gel.
2. Weigh out 4 grams of agarose into 200 ml 1x TBE buffer in an Erlenmeyer flask.
3. Swirl solution while trying to keep as much powder off of the sides of the flask as possible.
4. Put solution in the microwave for 2 minutes and then take out and swirl.

a. Put it back in the microwave for 1 minute and then take out and swirl.

b. Put it back in the microwave for 30 seconds and then take out and swirl.

c. If not fully dissolved at this point repeatedly microwave for 15 seconds and swirl.

5. Set up large electrophoresis tank such that the comb is placed at the very top of the gel holder.
6. Leave dissolved agarose gel on the bench for 15 minutes to cool.
7. Once cooled, add 20 uL of Sybr Safe Dye to the gel (10uL per 100 ml gel).
8. Pour dyed gel into the holder and watch it until it has fully polymerized (solidified).
9. Once solidified, remove the comb and place the gel into the apparatus with the lanes at the black end.

    a. Make sure that the DNA is running from black to red.

10. Load 5uL of 100 bp DNA ladder into far left well and then 5uL of each PCR sample into the wells going from left to right.

    a. Make sure that you record in your lab book the order of PCR samples

11. Attach cables from the batterypack to the apparatus.

    a. Red to red, black to black.

12. Run the gel at 200 V for 40 minutes making sure to constantly check how well the dye front is travelling.
13. Once done, remove gel and take to the chemidoc to image and make sure to transfer results to USB stick for analysis.

Cre general PCR setup

Materials:

- Sterile PCR tubes

- P200 pipette and tips
- P10 pipette and tips
- Dnase free water
- 2x DreamTaq master mix (**Catalog number:** K1081)
- Cre Forward Primer
    - ATGTCCAATTTACTGACCG
- Cre Reverse Primer
    - CGCCGCATAACCAGTGAAAC

Protocol:

1. Dilute primer stock into a new tube with 30uL of primer into 270uL autoclaved ddH2O
2. Add components based on following chart:
    a. Add Dnase free water first, then master mix, and DNA template last.

Component	Volume
Dnase free water	22 uL
DreamTaq Master Mix	25 uL
Cre Forward Primer	0.5 uL
Cre Reverse Primer	0.5 uL
DNA Template	2 uL

3. Centrifuge all tubes using benchtop centrifuge to effectively mix components.
4. Setup PCR reaction on a thermocycler using the following parameters:

Temperature	Time	Cycles
95^0	2 minutes	1

95^0	30 seconds	40
58^0	30 seconds	40
72^0	1 minute	40
72^0	10 minutes	1
4^0	Hold	Hold

5. Remove samples from thermocycler and store at 4^0.
6. Run on 2% agarose gel.
    a. Cre^+ samples have bands of size 350 base pairs.

tdTomato PCR setup

Required materials:

- Sterile PCR tubes
- P200 pipette and tips
- P10 pipette and tips
- Dnase free water
- 2x DreamTaq master mix (**Catalog number:** K1081)
- tdTomato wild-type forward primer
    - AAG GGA GCT GCA GTG GAG TA
- tdTomato wild-type reverse primer
    - CCG AAA ATC TGT GGG AAG TC
- tdTomato mutant forward primer
    - CTG TTC CTG TAC GGC ATG G

- tdTomato mutant reverse primer
    - GGC ATT AAA GCA GCG TAT CC

Protocol:

1. Dilute primer stock into a new tube with 30uL of primer into 270uL autoclaved ddH2O
2. Add components based on the following chart:

a. Add Dnase free water first, then master mix, and DNA template last.

Component	Volume
Dnase free water	22uL
DreamTaq Master Mix	25uL
Wild-type Forward Primer	0.5uL
Wild-type Reverse Primer	0.5uL
Mutant Forward Primer	0.5uL
Mutant Reverse Primer	0.5uL
DNA Template	2uL

3. Centrifuge all tubes using benchtop centrifuge to effectively mix components.
4. Setup PCR reaction on a thermocycler using the following parameters:

Temperature	Time	Cycles
95^0	2 minutes	1
95^0	30 seconds	40
58^0	30 seconds	40
72^0	1 minute	40
72^0	10 minutes	1

4^0	Hold	Hold

5. Remove samples from thermocycler and store at 4^0.
6. Run on 2% agarose gel.
    a. Wild-type tdTomato band is at 297 base pairs.
    b. Mutant tdTomato band is at 196 base pairs.
    c. Homozygous mutant will only have the 196 base pairs band.
    d. Homozygous wild-type will only have the 297 base pairs band.
    e. Heterozygotes will have both bands.

Rhabdomyosarcoma (RMS) Plus Vincristine Protocol – Adapted from OS and NC

M3-9-M RMS cell line Media (500ml):

Glutamax (100x) Stock (Gibco, Cat# 35050-061)	**5 ml**
Non-essential amino acid (100x) Mem-Neaa (Gibco, Cat# 11140-050)	**5 ml**
HEPES Buffer (1M stock) (Gibco, Cat# 15630-080)	**12.5 ml**
15% Heat-Inactivated FBS (56C for 30min)	**75 ml**
RPMI 1640 (500ml, Multicell, Cat# 350-045-CL w/o HEPES, L-Glutamine and Phenol Red.	**397 ml**
P/S (100 IU/ml)/(100 ug/ml)	**5 ml**
50 uM of 2-Mercaptoethanol (55mM stock)	**454 uL**

RMS cell line injection:

1. To culture RMS M3-9-M cells, use 3-5 10cm plates and allow them to grow until they reach about 80% confluency. These cells grow quickly, so keep an eye on them.
2. Use specific RMS media made of RPMI 1640 supplemented with 1% L-glutamine (Cat# 07100; StemCell Technologies), 10% heat-inactivated fetal bovine serum (Cat# 12483-020; Gibco), HEPES (Cat# 15630-080, Gibco), 1% non-essential amino acids (Cat# 11140-050, Gibco), 1% sodium pyruvate (Cat# 11360-070, Gibco), 1% penicillin/streptomycin (P/S) (Cat# 15140-122, Thermo Fisher Scientific), and 50 mM 2-mercaptoethanol (Cat# 21985023, Gibco).
3. To prepare the cells for injection, lift them from the plate using trypsin, remove the cell debris in the supernatant, and re-suspend them in sterile PBS. Count the number of cells per ml of solution using a cell counter and adjust the volume of PBS accordingly to accurately inject 100,000 cells into each mouse. This should be done on the morning of the injections.
    a. Undergo the cell count protocol and dilute sample in order to inject 100,000 cells in 500 uL of solution or less.
4. Using isoflurane, sedate each mouse and remove the hair on the entire left hindlimb with clippers to ensure accurate location for the injection. Use a sterile insulin syringe to inject the M3-9-M cells into both lobes of the left GAS hindlimb of each mouse.

Chemotherapy Administration (Vincristine):

1. Start by dissolving solid vincristine sulfate (Sigma Aldrich, cat#. V8388) in sterile water and ensure it is combined homogenously to create a 20mg/ml concentration.
2. This solution can be stored in the -20c freezer for a few weeks. It is best to prepare this stock solution before it is needed and dilute it to the required concentration the day of the vincristine injections.
3. On the day of injection, freshly dilute the stock solution to a concentration of 0.2mg/ml (1:100) in PBS.

4. Weigh each mouse using an electronic scale on the day of the injections to ensure that each dosage is specific for each individual mouse.
    a. NOTE: It is important to weigh each mouse to ensure they weigh more than 12g during vincristine administration as a lower weight may be fatal.
5. Ensure each mouse receives 1mg/kg of body weight (5ul per gram).
6. Use a sterile standard insulin syringe to inject each mouse I.P.
7. Monitor the mice closely.

Tamoxifen Injection for Cre Recombination:

Materials:

- 5ml centrifuge tube
- Corn Oil
- Tamoxifen (Sigma-Aldrich), CAS # 10540-29-1
- Vortex
- 1ml syringe
- 26 gauge needle
- 5ml serological pipette tips
- 0.5ml syringe
- 18 gauge needle

PPE:

- Wear fitted N95 mask anytime tamoxifen powder or solution is open

Dosages:

- 5 daily doses of 100mg/kg mouse body weight

- Protocol creates 20mg/ml tamoxifen solution
- Conduct calculations to determine the amount of tamoxifen solution per dose based on mouse body weight
- Typically create 5 ml tamoxifen solution by dissolving 100mg tamoxifen in 5ml corn oil

Protocol:

1. Heat up corn oil to 50^0 in shaking incubator for 30 mins
2. While warming, remove tamoxifen powder from 4^0 fridge to allow it to equilibrate to room temperature
    a. Wrap tamoxifen stock in tinfoil as it is light sensitive
3. Transfer 5ml of 50^0 corn oil into a 5ml centrifuge tube
    a. Set shaking incubator to 37^0
4. Use Styrofoam holder to place corn oil on the electric scale and measure 100mg of tamoxifen into the tube
5. Place vortex in the fumehood, tape tamoxifen solution to it and vortex it for 10 minutes straight
6. Place tamoxifen solution in the shaking incubator at 37^0 and max RPM
    a. Remove from incubator and vortex 3 x 30 seconds every hour for 3 hours
7. Before leaving for the night, remove the tamoxifen solution from the incubator
    a. Open it in the fumehood in the dark and check for clumps of solute in the tube
    b. Break up any clumps using a 28 gauge needle on a 1ml syringe
8. Place the tamoxifen solution back in the shaking incubator and leave it to shake at 37^0 at max RPM overnight
9. Next morning, remove the tamoxifen solution from the incubator and vortex 3 x 30 seconds

10. Prepare 1ml syringes with 18 gauge needles in the fumehood to prepare for injections
11. Store all prepared syringes and tamoxifen stock solution at 4^0 for up to one month (label tube and syringes with date prepared)

tdTomato$^+$ FAPs FACS Sorting Protocol

- Pre-make antibody cocktails
    - Full stain for FACs (amounts are per mouse):
        - 100 uL FACs buffer
        - 2 uL CD31 BV421
        - 2 uL CD45 FITC
        - 10 uL ITGA7 647

2. After step 16 take the cell resuspension and centrifuge it again at 400 x g for 5 minutes at 4 degrees

    b. Reduced from 600 x g

3. Resuspend the pellet in 100uL of the full stain cocktail

    b. Incubate on ice in the dark for 30 mins

    c. Tap the sample after 15 mins

4. After incubation, add 1 ml of FACs buffer to quench the sample and then spin it again for 5 minutes at 400 x g at 4 degrees

    b. Reduced from 600 x g

5. Remove the supernatant and resuspend the pellet in 1 ml of FACs buffer
6. Filter sample through a 50 um filter into a flow tube
7. Add 0.3 uL of Sytox 7AD to each sample tube, vortex, and keep in dark on the way to the FACs sorter in the OHRI

Post-sort Culture:

1. Bring the cells down from the OHRI and keep them on ice while warming the FAP primary growth media
2. Centrifuge the cell tubes for 5 minutes at 400 x G at 4ºC
3. While spinning, prep cell culture plates (Size and amount depends on the amount of cells from the sort)
    1. Typically plate in 3.5 cm plates and then as they expand transfer to larger plates before freezing from 10cm plates
4. Remove the supernatant and resuspend the pellet in 1 ml of warm primary FAP media
5. Add the full volume of cells to the plate in an "S" shape
    1. Shake the plate in a lower case t shape to evenly distribute the cells

FAP Counting and Injection:

1. Need to expand these cells in culture to be able to inject 1.0 x 10^6 FAPs per mouse hindlimb
2. Full confluence of a 10 cm plate is approximately 2.0 x 10^6 FAPs
    1. Need one fully confluent plate for every two injections
3. When ready for injections, begin cell lifting protocol
4. Warm GM, 0.25% Trypsin, and 1x PBS in the 37º water bath
5. Remove the GM from each cell culture plate
6. Wash each cell culture plate twice with 3 ml 1x PBS
7. Add 1 ml of 0.25% Trypsin to each 10 cm plate
8. Incubate the cells in the 37º incubator for 5 minutes
    1. Tap each plate before adding to the incubator
    2. Remove and tap each plate after 2.5 minutes to make sure the cells have detached from the plate
9. Add 3 ml of GM to each plate and triturate the cells approximately 6 times

    1. Angle the plate and then take the GM and put it at the top of the plate to rinse the bottom and sides of the plate
    2. Take the GM and spread it around the plate to make sure you're taking as many cells out as possible

10. Remove cells from the plate and place them in a new 15 ml tube
11. Centrifuge the cells at 500 x G for 5 minutes at 19°
12. Remove the supernatant and resuspend the cells in 1 ml 1xPBS
13. Take 10 µl of the resuspension and add it to a PCR tube
14. Add 10 µl of Trypan Blue to the PCR tube and mix well with a pipette
15. Load 10 µl of Trypan-cells mixture and apply it to each side of the cell counting plate
16. Use the countess to count live cells in each side of the plate
    1. Take the count from either side and average it out to get the count
    2. E.g. A: 1.98 x 10^6 + B: 2.02 x 10^6 / 2 = AVG: 2.00 x 10^6/ml
17. Need to inject 250 µl of cells so centrifuge the cells at 500 x G for 5 minutes at 19°
18. Remove the supernatant and resuspend the cells in 500 µl of 1xPBS
19. Take the resuspended cells to the animal facility for intramuscular injection
20. Sedate the mice in the chamber with isoflurane set to 4.5
21. Once sedated, gently place the mouse in the nose cone on the bench and set the isoflurane dial to 2.5
22. Tape the mouse's head to the nose cone and gently shave the hair off of both hindlimbs with clippers
23. Use an insulin syringe to inject 250 ul of cells into one limb and 250 ul of 1xPBS into the other limb
    1. Line up the base of the needle with the tendon in the mouse's ankle and insert parallel to the tendon
    2. Go in approximately halfway up the needle and make sure you inject into both lobes of the gastrocnemius/soleus muscle
24. Return the mouse to its cage and monitor it as it regains consciousness