Fibroblast Growth Factor Receptor Function in Muscle Stem Cells

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ABSTRACT

Skeletal muscle has a remarkable ability to regenerate itself following injury and this is reliant upon a group of stem cells known as muscle stem cells Muscle stem cell dysfunction contributes to variety of muscle wasting diseases and insights into mechanisms that regulate MuSC function could lead to new therapies and further our understanding of stem cell biology in general. Fibroblast Growth Factor 2 (FGF2) is a protein that regulates muscle stem cell behavior through stimulation of cell surface tyrosine receptor kinases known as Fibroblast Growth Factor Receptors (Jones, 2001). Activation of the receptors by FGF will induce intracellular signaling pathways that control cell behaviors including activation, replication, differentiation and self-renewal. There are four Fibroblast Growth Factor Receptors (FGFR1, FGFR2, FGFR3 and FGFR4) and the role each of these individual receptors has in regulating muscle stem cell behavior is currently unclear. Receptor 1 and receptor 4 are highly expressed in muscle stem cells, while receptor 3 expression is weak and receptor 2 expression cannot be detected. Ablation of any single receptor has only a slight impact as compared to pharmacological inhibition targeting all four receptors, which significantly alters muscle stem cell behavior, thus suggesting that the receptors act in a complementary manner. My main goal in this thesis work is to genetically delete the Fibroblast Growth Factor Receptors individually and in combination to investigate their role in regulating satellite cell behavior. To knockout receptor expression I used both a CRISPR-Cas9 methodology and a Cre-Floxed approach. In both cases, I was able to achieve low-level knock down of receptors 1, 2, and 3, verified by RT-PCR and immunofluorescence. FGF receptor 1 and 3 double mutant muscle stem cells were slightly impaired compared to wild type controls. Overall however, my inability to entirely delete these receptors using well-established methods was unexpected. The deletion of FGF receptors and additional receptor complex proteins in turn proves to be much more problematic compared to other proteins possibly suggesting that these genes required for FGF signaling are vital to cell function and thus protected from modification.

Chapter 1:
INTRODUCTION

Skeletal muscle is an organized tissue composed of long cylindrical fibers, containing multiple nuclei per myofiber. Myonuclei within muscle fibers are post-mitotic and therefore the regeneration of muscle is largely mediated by a subset of stem cells located on the periphery of the myofiber. Muscle stem cells (MuSCs) are critical regulators of both non-injury tissue homeostasis and injury repair (Mauro 1969 and Murphy 2011). In un-injured muscle, MuSCs are in an inactive, quiescent state and upon injury the satellite cells will activate, rapidly expand, and differentiate into new muscle to rebuild myofibers and reconstitute a functional contractile apparatus (Relaix, 2012 and Rudnicki, 2011). Furthermore, a sub-population of MuSCs activated by injury will avoid terminal differentiation and return to quiescence to maintain the MuSC population (Dumont 2015) (Figure 1). The fate of MuSCs is coordinated on a molecular and cellular level and dysfunction can contribute to a variety of muscle wasting diseases including muscular dystrophy, age-related muscle wasting (sarcopenia) and cancer-induced muscle wasting, (He, 2013).

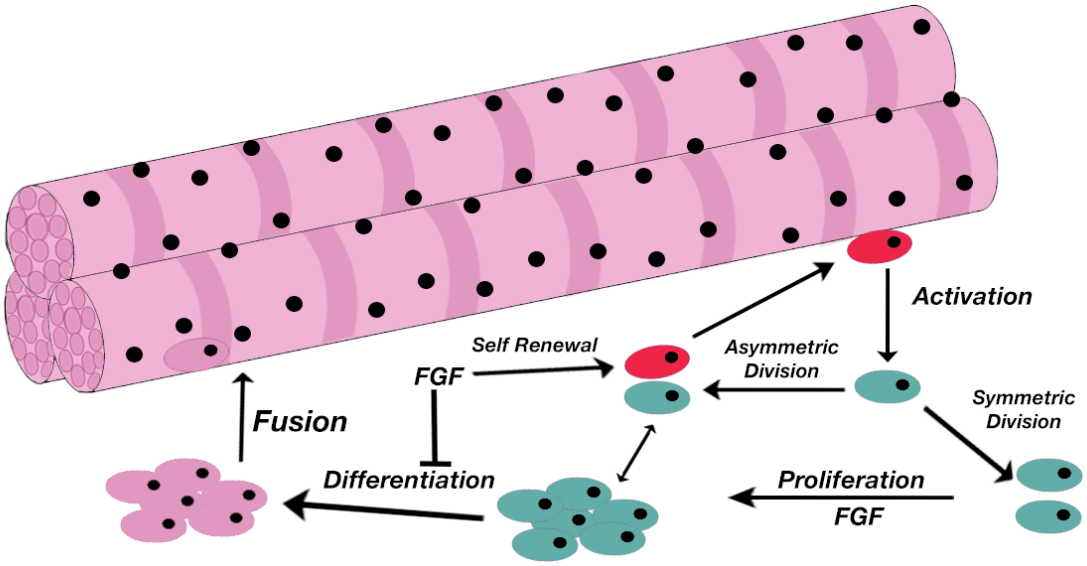

Figure 1- Muscle stem cells enable muscle regeneration. Upon injury, MuSc will activate and rapidly divide to either expand as myoblasts, differentiate to re-generate myofibers or self-renew. Self-renewal can occur through a process of asymmetric division. FGF represses terminal differentiation thereby potentiating self renewal. Adapted from Pawlikowski et al, 2017.

While many of the mechanisms controlling muscle stem cell behavior are poorly understood, extrinsic factors can regulate muscle stem cell fates, including members of the Fibroblast Growth Factor (FGF) family (Pawlikowski et al, 2017). The FGF family is composed of 22 proteins with high sequence homology, evolutionarily conserved across almost all tissue types (Ornitz and Itoh, 2001). FGF's are implicated in a wide variety of biological functions including embryonic development, metabolism, and injury repair (Ornitz and Itoh, 2015). Eighteen proteins of the FGF family are secreted factors, which bind to one of four membrane bound receptors (FGFR) (Fantl et al, 1993). FGF receptors are tyrosine kinases, a class of proteins with the ability to transfer a phosphate group to another protein and function as an on/off switch for signals within the cell. FGFRs are composed of an extracellular binding domain specific for FGF and an intracellular domain that contains the sites for subsequent recruitment of downstream signaling molecules (Lee, 1989).

FGF accomplishes the transmission of cellular signals by binding to and activating its receptor, which initiates a cascade of signaling events. In order for FGF to bind and fully activate receptor induced signaling, additional molecules such as heparan sulfate proteoglycans are required (Rapraeger et al, 1991)(Rozo et al, 2016). Heparan sulfates are a sulfated glycosaminoglycan with repeating disaccharides present on the surface of several membrane proteins residing in the cell surface. Variations in the sulfation of heparan proteoglycans dictate the affinity of FGF and in turn can regulate, positively or negatively, the levels of growth factor binding by acting as a reservoir for storage or as a co-receptor (Rapraeger, 1991). In addition, heparan sulfates associated with the receptor help bind and sequester FGFs to the receptor itself to promote stability. Syndecan-4, a muscle stem cell heparan sulfate proteoglycan, is required for FGF signaling, and regulates surface expression levels of FGFRs in muscle stem cells as it does in fibroblasts (Elfenbein et al, 2012).

In addition to Syndecan-4, integrins and fibronectin can regulate FGF mediated cellular signaling and can attenuate FGF insensitivity in muscle stem cells from aged mice (Rozo 2016). Losses of any of these molecules (syndecan 4, integrin, fibronectin) ablate or impair the capacity of FGF to induce downstream signaling. The regulation of FGF signaling is thus a complex process reliant upon availability of FGF, cofactors, and the addition of several other surface proteins and molecules.

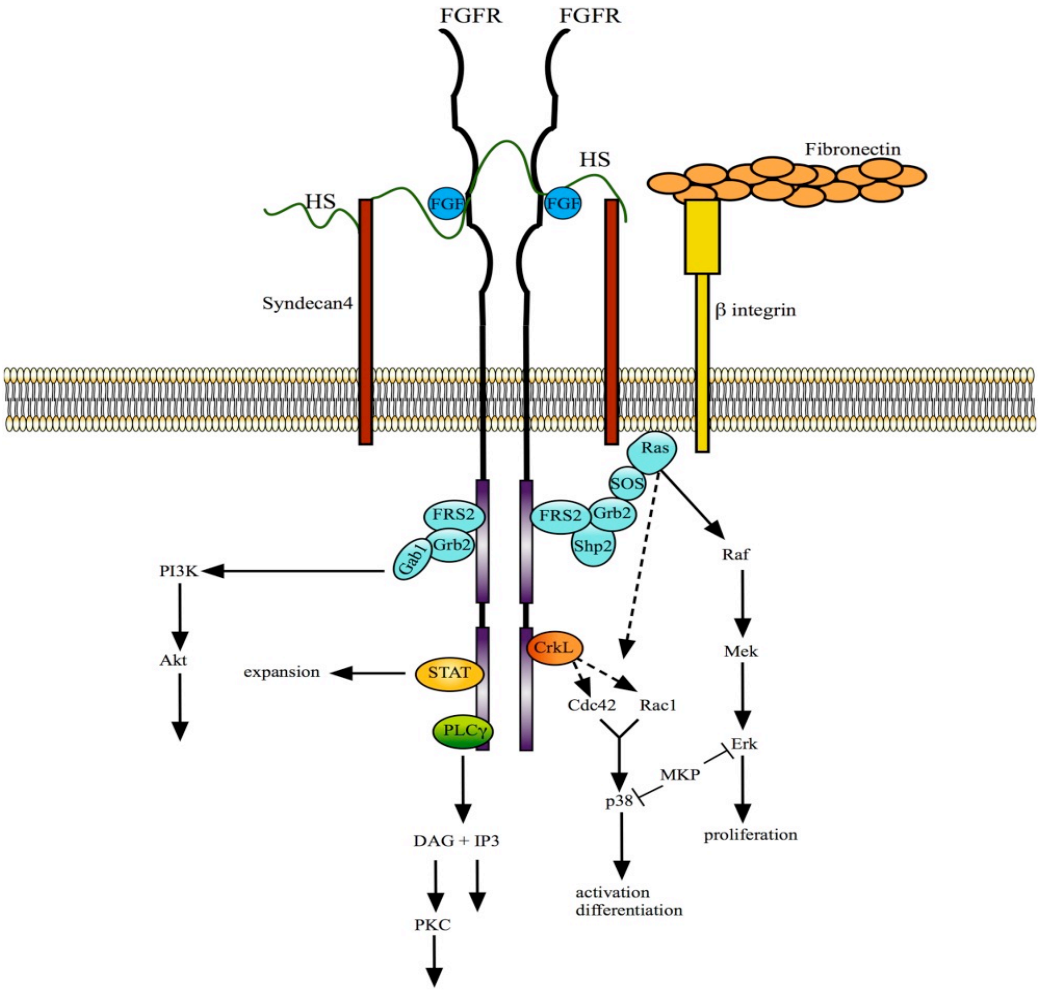

Figure 2- FGF signaling in satellite cells. Some FGF signaling is transduced via adaptor pro-teins (FRS2 and CRKL) that are phosphorylated upon activation of FGFRs, the mechanisms involved in p38a/b MAPK activation are not yet under-stood (dotted lines), whereas ERK MAPK activation is better characterized. FGF-induced activation of ERK MAPK is potentiated by the presence of B1-integrin and fibronectin, demonstrating that FGF signaling in satellite cells is complex, requiring multiple extracellular and transmembrane proteins, as deletion of either Syndecan-4, integrins, or fibronectin alters FGF-induced signaling and affects satellite cell behavior (Pawlikowski et al, 2017).

Once an FGF has become bound to an FGFR, the receptor is actived and triggers trans-autophosphorylation of tyrosine residues within the intracellular domain, initiating the downstream cascade of several signaling pathways including p38α/β MAPK, ERK MAPK, PI3 kinase, and Akt; activation of STAT; and stimulation of phospholipase C gamma/protein kinase C signaling (Ornitz and Itoh, 2015; Brewer et al, 2016) (Figure 2). These pathways regulate multiple cell behaviors such as proliferation, activation, and repression of terminal differentiation (Jones et al, 2005).

The general importance of FGF signaling is underscored by ablation experiments where FGF signaling is knocked out completely. For example, mice in which FGF has been genetically removed die embryonically at E 7.5-9.5 due to failed vascular formation (Su et al, 2014) and FGF signaling is required in the developing limb bud to establish myogenic precursors (Pownall et al 2010). In the MM14 muscle stem cell line, the inhibition of FGF signaling via the removal or inhibition of FGFR1 has a significant impact, resulting in rapid differentiation and the loss of pluripotency (Hannon, 1996; Kudla 1998). Additionally, treatment of isolated muscle stem cells in vitro with pharmacological inhibitors that block all FGF signaling result in impaired proliferation and increased differentiation (Bernet et al, 2016). Deletion of FGFR1 or FGFR4 individually in muscle stem cells generates no significant phenotypes. In vivo, the deletion of FGFR1 from MuSCs has only a minor phenotype and the downstream processes of FGF mediated signaling remained mostly intact (Yablonka-Reuveni et al, 2015;Weinstein et al, 1998; Zhao et al, 2006). Since, knockout of individual FGFRs in muscle stem cell do not generate significant phenotypes, while pharmacologic inhibition that block all FGFR signaling or expression of a dominant negative receptor that target all receptors has a strong phenotype, it is likely that the receptors act in a redundant mechanism to regulate behavior (Yablonka-Reuveni et al, 2015;Weinstein et al, 1998; Zhao et al, 2006).

Dysregulation of muscle stem cell behavior is associated with a variety of muscle wasting diseases and is of particular relevance to FGF signaling in muscle in age-induced muscle wasting called sarcopenia. The muscle stem cell population decreases with age and in turn this decline is correlated to deterioration in skeletal muscle function and muscle mass, highlighting the vital role of MuSCs in muscle homeostasis (Garber, 2016). Geriatric muscle stem cells become insensitive to FGF stimulation and this correlates with a decrease in muscle stem cells numbers, reduced self-renewal, and increase in differentiation (Bernet et al, 2014). FGF2 production is increased in muscle fibers from aged mice, perhaps acting to compensate for FGF insensitivity (Li et al, 2015). Additionally, mislocalization of FGFR cofactors such B1-integrin as well as Syndecan-4 are associated with aged muscle stem cells, indicating that alterations to the FGFR complex and subsequent impairment of FGF signaling can contribute to age induced muscle wasting (Lukjanenko et al, 2016; Pisconti et al, 2016). Thus, elucidating the mechanisms through which FGF signaling controls muscle stem cell behavior will provide valuable insight into the underlying causes of age associated muscle diseases.

The main goal of my work in this thesis is to understand how FGF signaling controls MuSC behavior. I hypothesize that multiple FGF receptors can mediate FGF signaling in MuSCs to control cell behavior, such that deletion of any single FGF receptor will be compensated by the other FGFRs and will thereby be insufficient to induce significant phenotypes. To test this hypothesis, two deletion approaches were utilized to delete FGF receptors individually and in combination. Chapter 2 describes the knockdown of FGFR1 and Sdc4 expression in cultured satellite cells using a lentiviral CRISPR-Cas9 based approach. Chapter 3 describes work with a Cre-flox conditional knockout mouse used to delete FR1, FR2 and FR3. Collectively, this thesis highlights multiple techniques that can be used to knockdown expression of FGFR and receptor complex components and furthermore establish methods for future studies to investigate the role of FGF signaling in regulating muscle stem cell behavior.

Chapter 2:
RESULTS

Lentiviral CRISPR-Cas9 Approach

CRISPR-Cas9 is a gene-editing tool that can be used to delete gene expression with high specificity (Mali, 2013), CRISPR-Cas9 is an enzyme complex composed of a guide RNA (gRNA) and the Cas-9 protein, which is a nuclease that cleaves double stranded DNA (Jinek, 2012). The gRNA, by binding to complementary DNA sequences within a target gene, directs Cas-9 enzyme to cut at specific DNA sequences within genes of interest (Wright et al, 2016). DNA cleavage by Cas-9 induces double stranded breaks within the genome, which is recognized and corrected by cells in one of two ways: either through Non Homologous End Joining (NHEJ) or Homologous Repair (HR) (Bothmer, 2017). NHEJ is the method most commonly used by the cells and this repair process fills in the double stranded gap with random DNA bases, disrupting the coding sequences of the gene that has been cut (Takata, 1998). Thus by expressing the Cas9 nuclease with a gRNA in the cells of interest, one can efficiently delete gene expression by inducing double stranded break at specific targets that are then incorrectly replaced by cellular DNA repair machinery.

To delete FGFR1 gene expression from MuSCs we used a replication incompetent lentirvirus to express Cas9 and an FGFR1 specific guide RNA in C2C12 cells. C2C12 cells are an immortalized myogenic mouse cell line that serves as a model to study MuSCs *in vitro*

(Yaffe, 1977).

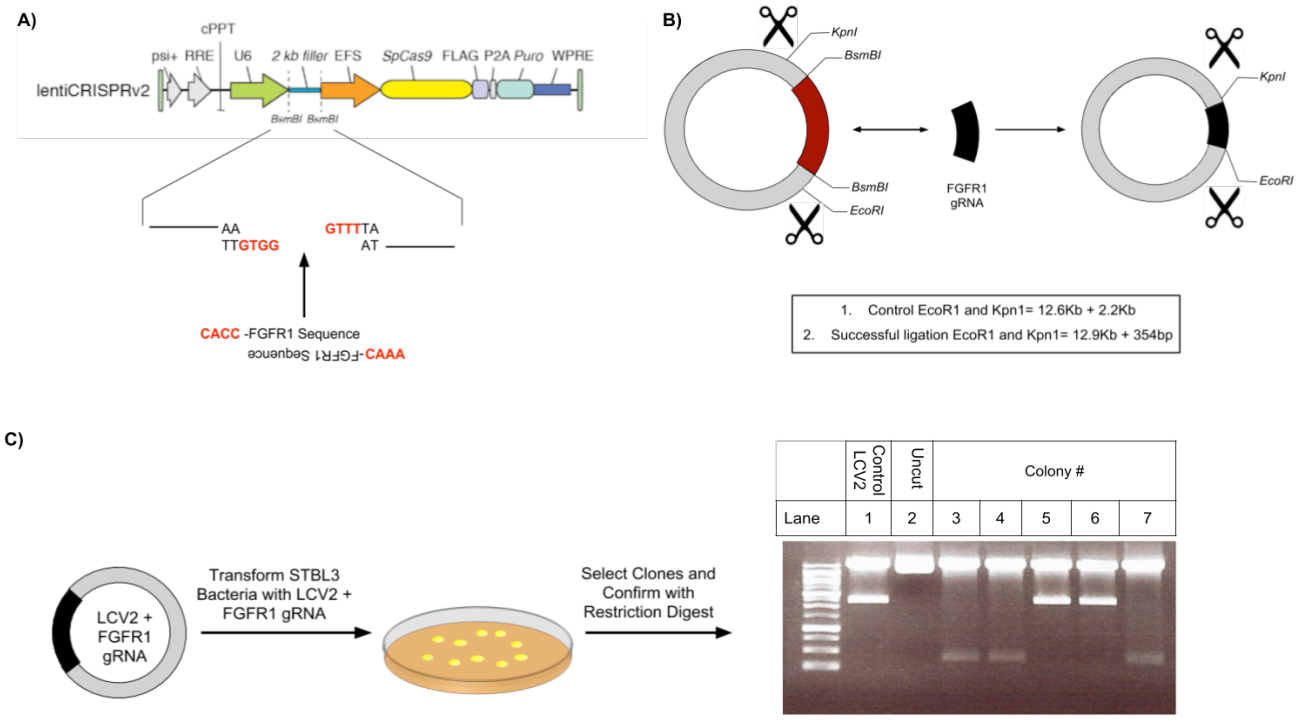

Figure 3- Schematic of the workflow to create LCV2 constructs containing a specific gRNA. **A)** A linear diagram of the LCV2 vector expression cassettes. *BsmbI* was used to digest and ligate FGFR1 gRNA with *BsmbI* sticky ends. **B)** Upon gRNA ligation into the vector, the *BsmbI* were removed. *KpnI* and *EcoRI* sites flanking the inserted region are used to confirm ligation. **C)** STBL3 bacteria was transformed with the ligated plasmid and plasmid DNA isolated, and screened via restriction digest to identify clones with correctly ligated inserts.

Replication deficient lentivirus containing sequences to make both the Cas9 enzyme and FGFR1 gRNA was generated as outlined in Figure 4 and detailed in the methods. Lentivirus was added to the media of C2C12 and left on overnight to infect cells. I infected C2C12 cells with a lentivirus containing a green fluorescent protein (GFP) as an assay to confirm our virus could efficiently infect C2C12 cells. The GFP coding lentivirus was added to media of C2C12 cells overnight and 3 days later cells were fixed and imaged to measure GFP expression. After infection, the majority of the C2C12 cells scored as GFP positive, indicating high levels of infection efficiency (Figure 4B).

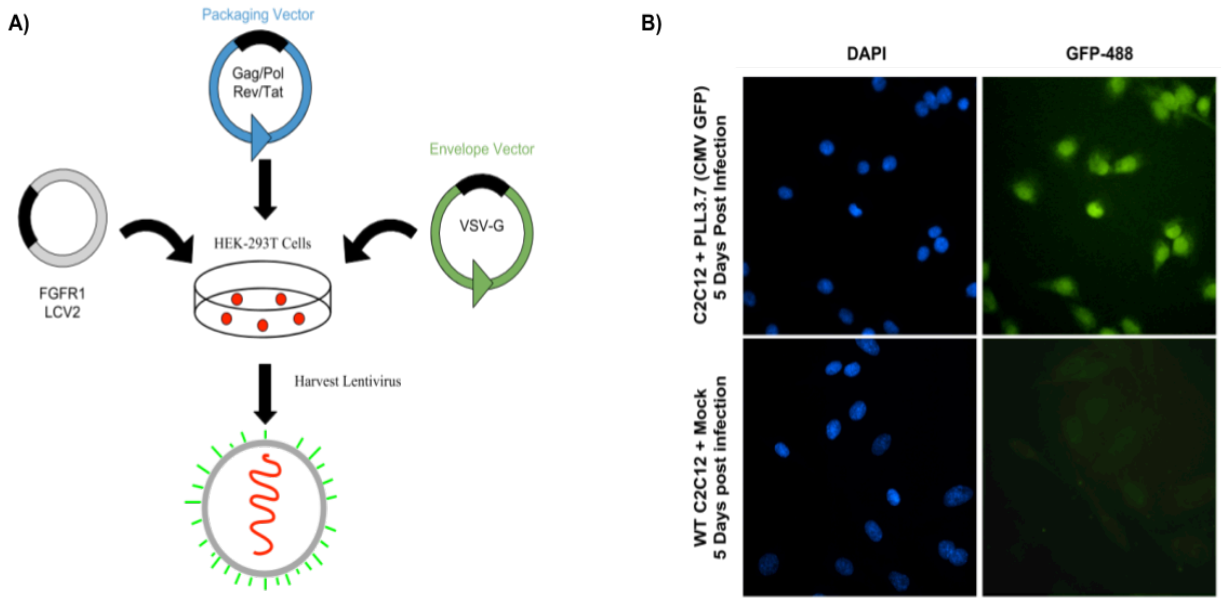

Figure 4- Lentivrus produced from HEK-293T cells is capable of infecting C2C12 cells. **A)** A cartoon schematic representing viral production and collection. FGFR1 LCV2 plasmid isolated from STBL3 bacteria was transfected into HEK-293T cells along with a packaging vector and envelope vector, required to produce lentivirus. **B)** Lentivirus with a CMV-GFP reporter harvested from HEK-293T cells is sufficient to infect C2C12 cells as compared to a mock control infection, measured via GFP fluoresence.

I next infected C2C12 cells with lentivirus coding for Cas9 and FGFR1 gRNA expression. Two different viruses (named FR1-LCV2 #2 and FR1-LCV2 #3) were made, each with a different guide RNA to FGFR1 to increase the chance of generating FGFR1 mutant cells (see Figure 3 (but make it Figure 4) for cloning details). The lentivirus used for infection also contains a puromycin resistance gene, which renders an infected cell the ability to grow in puromycin containing media (un-infected cells will die in the presence of puromycin). Following infection, cells were grown in the presence of puromycin for five days. To determine if FGFR1 gene expression had been deleted, mRNA was isolated from infected and control un-infected cells and used for RT-PCR analysis. The RT-PCR analysis showed a decrease in FGFR1 mRNA in cells infected with FGFR1 Cas9 lentivirus compared to uninfected C2C12 cells (Figure 5A). FGFR1 mRNA was not detectable in cells infected with FR1-LCV2 #2, while FR1-LCV2 #3 infected C2C12 cells display a decrease in FGFR1 expression. GAPDH is a metabolic gene independent of FGFR1 and therefore that should not be altered in FGFR1 mutant cells. Thus

using GAPDH as a comparison control allows the relative evaluation of expression levels between samples (Figure 5A). In a follow up RT-PCR analysis of the FGFR1 mutant cells, the FGFR1 transcript was detected and did not appear different than WT control cells (Figure 5B). These cells that appeared to have lost knockdown of FGFR1 were cultured and expanded for several passages prior to collection, possibly indicating that these knockout cells were selected against and were lost due to growth defects.

I also made additional CRISPR-Cas9 lentivirus that targeted FGFR1 cofactors Syndecan 4

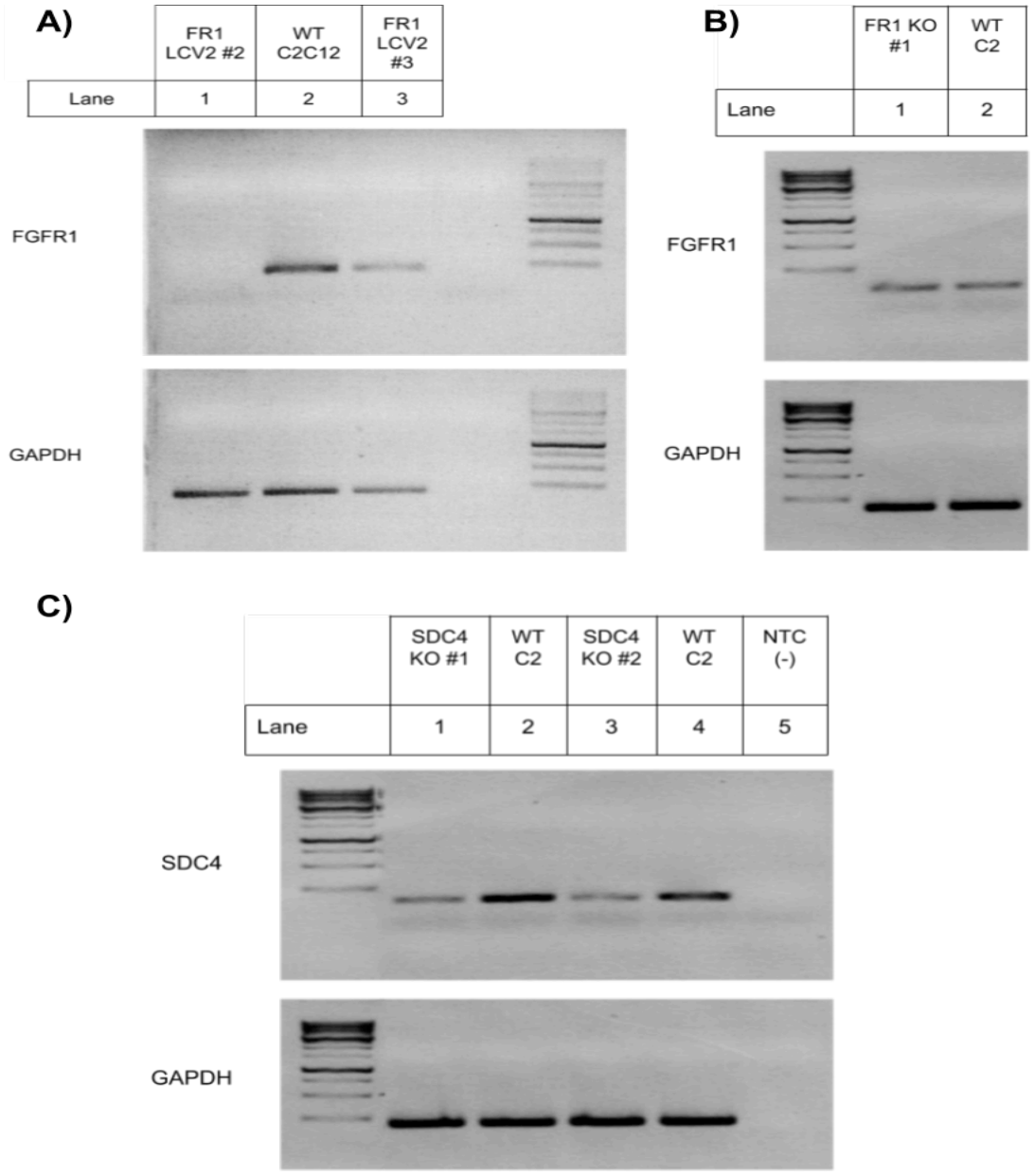

Figure 5- LCV2 is sufficient to knock down expression of targeted genes in C2C12's. **A)** RT-PCR of FGFR1 demonstrates decreased expression in C2C12's infected with FR1-LCV2. **B)** Repeating the experiment in panel A however demonstrated no significant decrease in FGFR1 expression. **C)** Infection with LCV2 lentivirus containing gRNA sequences against SDC4 was sufficient to decrease expression of SDC4 in C2C12's.

(Sdc4) and B1-Integrin. I made 2 CRISPR-Cas9 lentivirus with Sdc4 guide RNA (SDC4-LCV2 #1 and #2) and infected C2C12s with these viruses. Following infection, RT-PCR analysis showed knock down in the expression levels of Sdc4 as compared to the uninfected control (Figure 5C).

This process was also repeated with CRISPR-Cas9 lentivirus targeting B1-Integrin and this showed similar levels of knock down (data not shown), but no complete knock out was observed, most likely due to a heterologous population of infected cells. Cell culture heterogeneity possibly arose from a growth disadvantage in the mutant cells, thus causing a selective bias for wild type cells. Collectively, this set of experiments shows that lentiviral CRISPR-Cas9 is a viable option to knockdown expression of FGFRs and its associated proteins in C2C12 cells.

Chapter 3:
RESULTS

Cre-Flox Conditional Knockout Approach

A Cre-Flox gene knockout approach deletes genes by inducing DNA recombination at specific sites within a gene (Sauer, 1988). Cre recombinase is a bacterial enzyme that cuts and

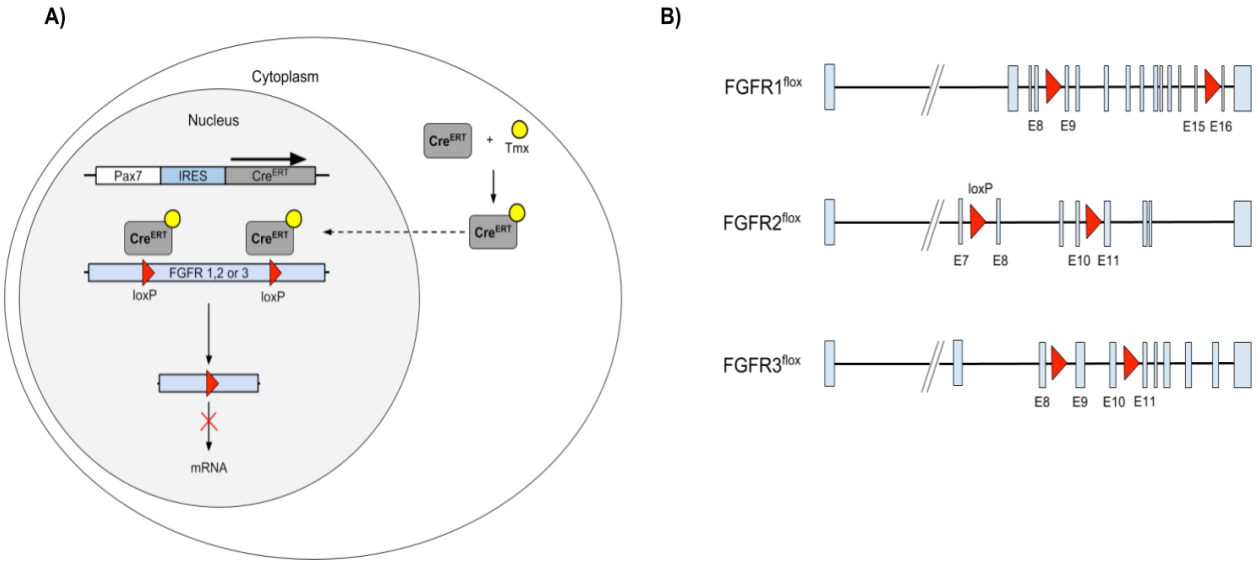

Figure 6- Schematic of the inducible Cre-Flox mediated recombination used to spatially and temporally knock down FGFR1-3 expression in muscle stem cells. **A)** Upon addition of Tamoxifen (Tmx), Cre-ERT is localized to the nucleus to induce recombination and subsequent knock down. **B)** Cartoon diagram of each floxed allele (FGFR1-3), indicating specific locations of each loxP site.

excises regions of DNA known as loxP sites, and recombines the flanking regions together. LoxP sites (also called floxed sites or alleles) are 34-base-pair DNA sequences that can be inserted into a gene of interest. When Cre recombinase is present, the DNA in-between the two loxP sites will be excised, essentially cutting out entire sections of DNA and preventing expression of that gene (Figure 6). To specifically delete FGFRs from satellite cells, we bred mice with LoxP sites inserted in the genes of FGFR1, FGFR2 and FGFR3.

We also obtained mice that make Cre recombinase only in MuSCs by having Cre expression driven by the Pax7 promoter, a gene highly expressed by MuSCs (Sean, 2000). Thus

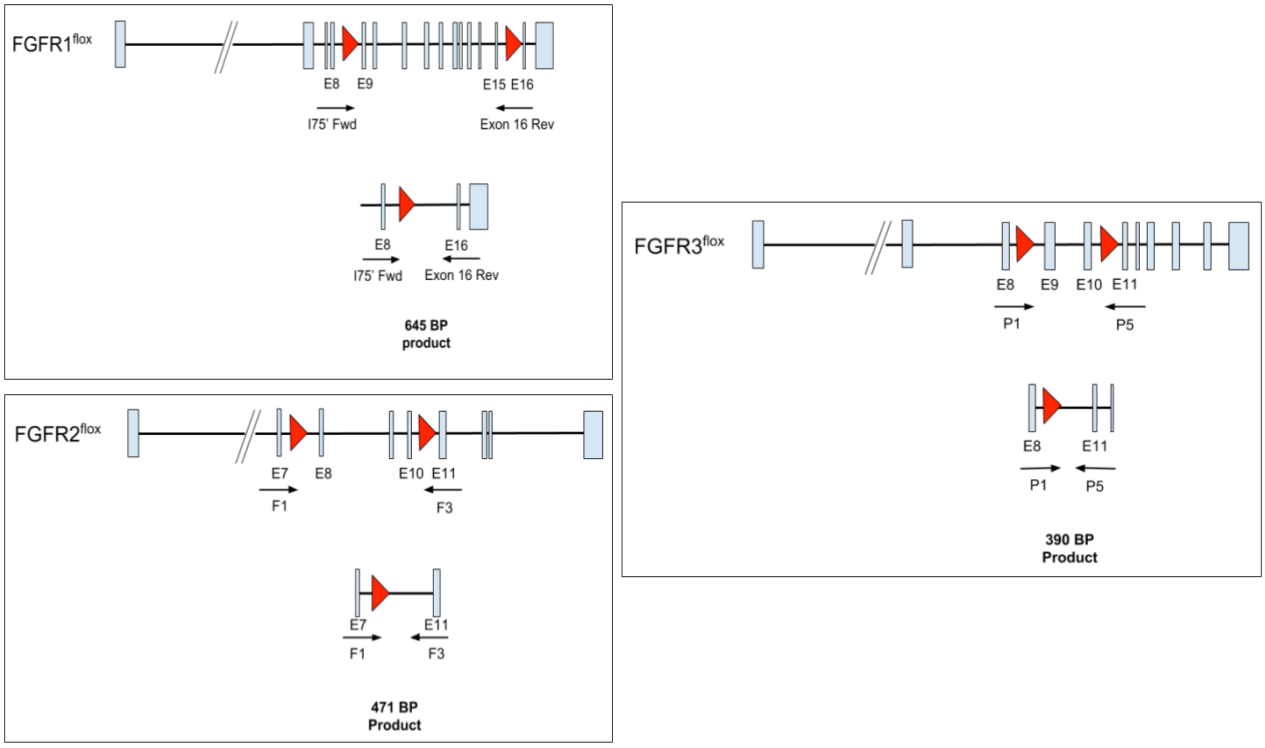

Figure 7- Representation of each floxed allele, diagramming the specific products created by recombination. After Cre/lox mediated recombination, primers flanking the loxp sites were used to produce the indicated PCR products. A 645bp band is produced following recombination of the floxed allele in FGFR1, using I75 Fwd and Exon 16 Rev. A 471bp band is produced after recombination using F1 and F3 in FGFR2. A 390bp band is produced after recombination using P1 and P5 primers in FGFR3.

only cells that make Pax7 (limited to MuSCs) will make Cre. In order to control temporal specificity of Cre recombination, the Cre protein has fused to it a mutated human estrogen receptor (ERT) domain that upon the addition of tamoxifen, an estrogen analog causes the protein to penetrate the nucleus and induce targeted recombination. Without tamoxifen, the Cre-

ERT remains cytoplasmic and will not induce recombination. By breeding mice that express the Pax7Cre-ERT allele with mice that have the FGFR1, 2 and 3 floxed alleles we can specifically delete FGFR expression only in satellite cells by injecting mice with Tamoxifen (Figure 6 and 7).

The final goal for these FGFR deletion experiments is to analyze the function of satellite cells from a mouse expressing the Pax7Cre-ERT allele and are homozygous for each floxed allele FGFR1$^{flox/flox}$, FGFR2$^{flox/flox}$, FGFR3$^{flox/flox}$ after treatment with tamoxifen to induce gene knockout. However, during my time in the lab we had only obtained mice that were Pax7Cre-ERT expressing and homozygous for one or two of the FGFR floxed alleles and no triple floxed mutant mice were available to study.

I began my analysis in mice expressing single FGFR floxed mutant alleles by carrying out a PCR assay on genomic DNA to detect if recombination of the FGFR floxed alleles had occurred. MuSCs were isolated and cultured from mice that were Pax7Cre ERT+ and homozygous for the FGFR1, FGFR2, or FGFR3 floxed allele. Tamoxifen in vivo is metabolized to produce 4'-Hydroxy Tamoxifen (4OHT), the pharmacologically active state of the drug, therefore 4OHT is added in place of standard tamoxifen in culture as these cells are unable to

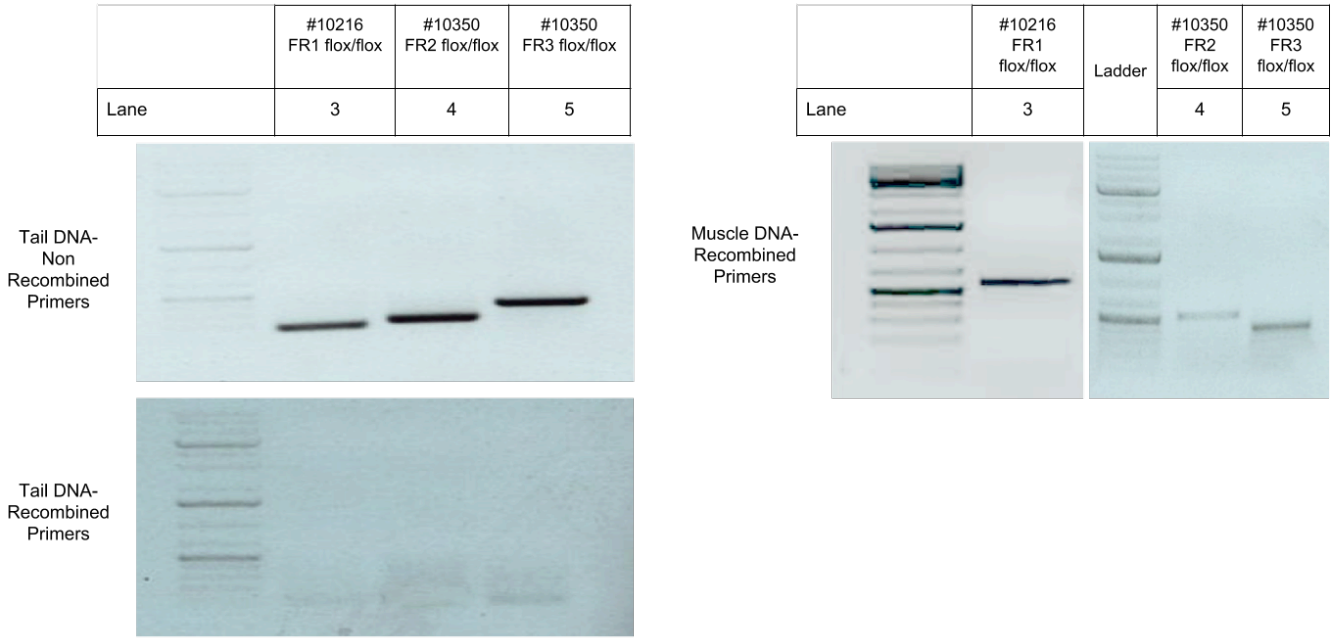

Figure 8-Cre Flox recombination is specific to muscle stem cells and induces knock down in FGFR1 and FGFR3 expression. PCR indicates the presence of floxed alleles in mice #10216 and #10350. Cre flox mediated recombination yields products that are only detectable in DNA isolated from muscle stem cells, as opposed to tail DNA.

process Tamoxifen into its active form. 4'-Hydroxy Tamoxifen was added to the cells at the start of culture and cells were grown for 3 days (72hr) in the presence of 4OHT. Cells were then harvested and genomic DNA was isolated. Primers specific to each FGFR floxed allele were used, primers that can distinguish between the non-recombined floxed allele and the recombined floxed allele (Figure 7, Table 1). Genomic DNA isolated from tail tissue was used as source for non-recombined DNA. The PCR analysis showed that muscle specific recombination occurred for each FGFR floxed allele, as expected in DNA isolated from MuSCs treated with tamoxifen but no recombination occurred in DNA isolated from the tail (Figure 8).

Genomic DNA Primers 5' - 3'		RT-PCR Primers 5' - 3'	
FRI		GAPDH-F	AGGTCGGTGTGAACGGATTTG
Exon16 Rev	GTTGGAGGTCAAGGCCACAA	GAPDH-R	GGGGTCGTTGATGGCAACA
i75' For	AGGTTCCCTCCTCTTGGATGA	FR1-F	GTGTGCCTGTGGAGGAACTT
i73' Rev	CTGGGTCAGTGTGGACAGTGT	FR1-R	GTACTGGTCCAGCGGTATGG
LoxP1 For	AATAGGTCCCTCGACGGTATC	FR3-F	CCGGCTGACACTTGGTAAG
		FR3-R	CTTGTCGATGCCAATAGCTTCT
FR2		Syn4-F	GCAGATACTTCTCTGGAGCCC
FR2-F1 For	ATAGGAGCAACAGGCGG	Syn4-R	GCACCAAGGGCTCAATCACTT
FR2-F2 Rev	TGCAAGAGGCGACCAGTCAG		
FR2-F3 Rev	CATAGCACAGGCCAGGTTG		
FR3			
FR3 P5 Rev	TGTAAAAGGGGTGGGGTGGTAG		
FR3 P1 For	GATGCCTCAACAATACTGGTAGCCC		
FR3 Common For	AGGACAAATTGGTACCATACAACGTGGG		
FR3 WT Reverse	GCTCCCTGCCTCGTGTTCTCCTAGAG		

Table 1- List of primers used for each allele to distinguish between floxed and non-recombined products based on size.

Having shown each FGFR allele had been successfully excised I next sought to determine if FGFR gene expression was knocked down using RT-PCR analysis. To measure FGFR gene expression, mRNA was extracted from MuSCs cultured for 72hr in the presence of tamoxifen and RT-PCR performed. As compared to wild type expression, FGFR mRNA expression from both FGFR1 and FGFR3 mutant mice showed a slight knock down (Figure 9). FGFR2

expression was not detectable in the WT or FGFR2 mutant samples. (data not shown). To further analyze the potential knockdown of FGFR1 protein expression in Pax7Cre-ERT; FGFR1 floxed mice, I carried out immunostaining analysis using a FGFR1 antibody. (Reliable antibodies to FGFR2 and FGFR3 for immunostaining analysis were not available). Individual muscle fibers were isolated from Pax7Cre; FGFR1 $^{flox\ flox}$ or control mice and attached MuSCs cultured for 60 hr in the presence of tamoxifen. Mice had been given 5 tamoxifen injections prior to isolation of the myofiber cultures. The myofiber MuSC cultures were then fixed and immunostained using an anti-FGFR1 antibody. In control cultures nearly 100% of satellite cells showed strong staining with the FGFR1 antibody (Figure 10). In myofiber cultures isolated from Pax7Cre; FGFR1 $^{flox\ flox}$ 79% of satellite cells showed strong FGFR1 staining. Thus, 21% percent of satellite cells from Pax7Cre; FGFR1 $^{flox\ flox}$ mice showed a distinct loss in FGFR1 expression.

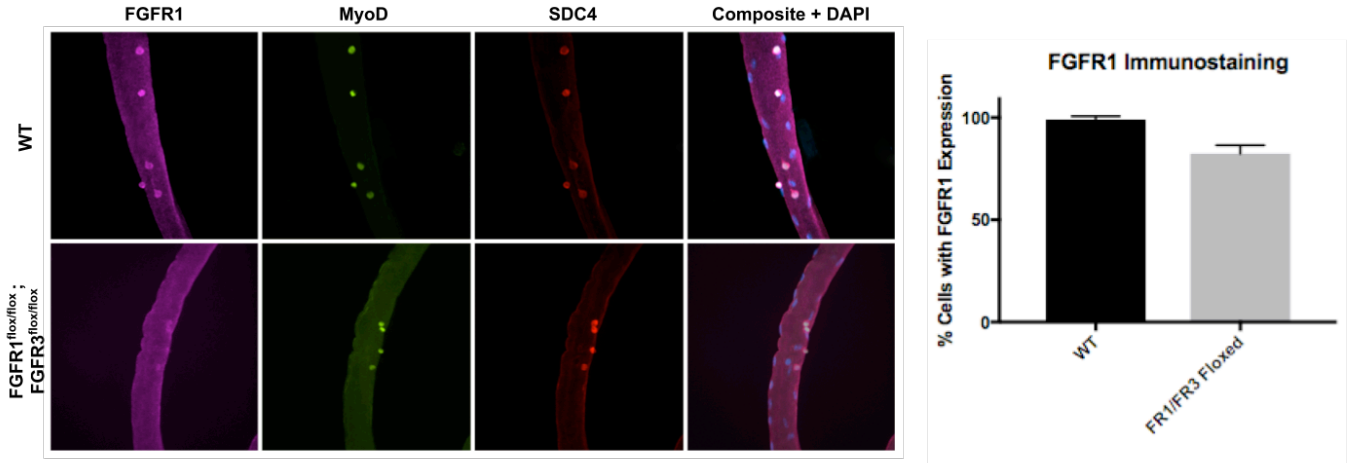

Figure 10- Immunostaining of muscle fibers from FR1 flox/flox mice displays a partial knock down of FGFR1 expression. Muscle fibers were isolated from a FR1 flox/flox mouse stained with FGFR1, MyoD, and SDC4 antibodies compared to wild type fibers show slight knockdown of FGFR1 expression in muscle stem cells after treatment with tamoxifen. The knock out cells were then quantified as a percentage over the entire muscle stem cell population.

I next carried out FGFR1 immunostaining analysis on MuSCs grown in standard primary cultures on coverslips (not attached myofibers). MuSCs were isolated from Pax7Cre; FGFR1 $^{flox\ flox}$ and control mice and plated onto gelatin coated cover slips and cultured for 72 hours with tamoxifen. The percent of FGFR1 positive cells was 99% for cells isolated from both control and FGFR1 mutant mice. I also co-stained these cultures with antibodies to Syndecan 4 , a muscle stem cell marker and FGFR co-receptor, and antibodies to MyoD, a marker of

proliferating satellite cells. There were also no observed differences in the percent of Syndecan4 or MyoD+ cells between control and mutant (Figure 11).

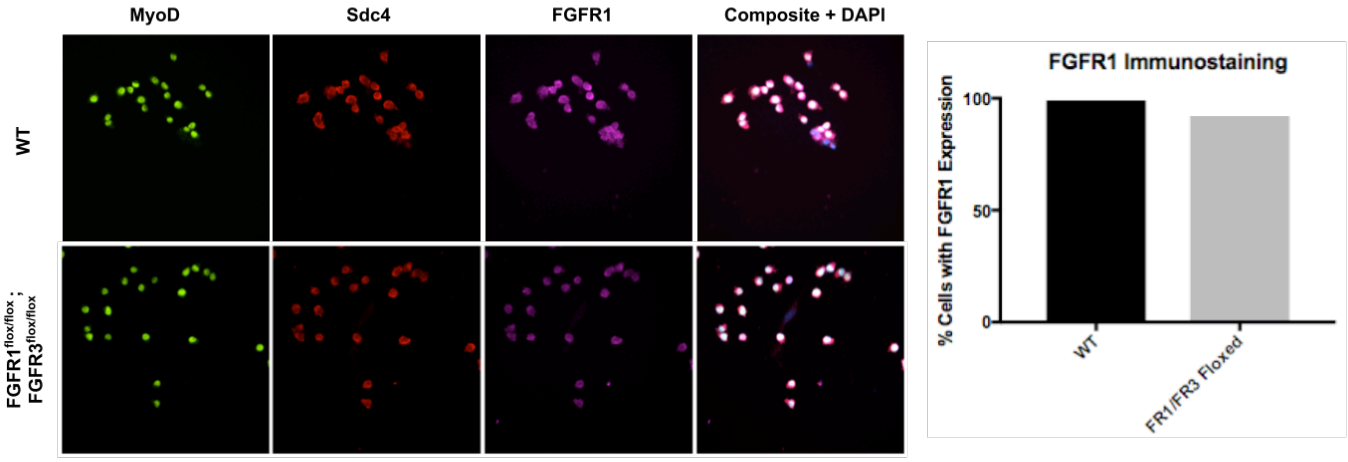

Figure 11- Muscle stem cells isolated from FGFR1,3 flox/flox mice cultured for 72 hours and treated with tamoxifen stained with FGFR1, MyoD, and SDC4.

To compare the phenotypic differences between deletions of a single receptor and multiple receptors, MuSCs were isolated from a double mutant FGFR1$^{flox/flox}$ FGFR3$^{flox/flox}$ mouse and grown in culture in the presence of tamoxifen, as was done in the previous experiments. Cells were grown on coverslips and stained for Syndecan4, MyoD, and FGFR1. A subset of cells were observed in culture that had weak FGFR1 expression and were also MyoD negative, indicating that these cells were no longer activated or had differentiated, possibly suggesting that ablation of FGFR1 and FGFR3 resulted in an impairment of the activation state of the muscle stem cell (Figure 12).

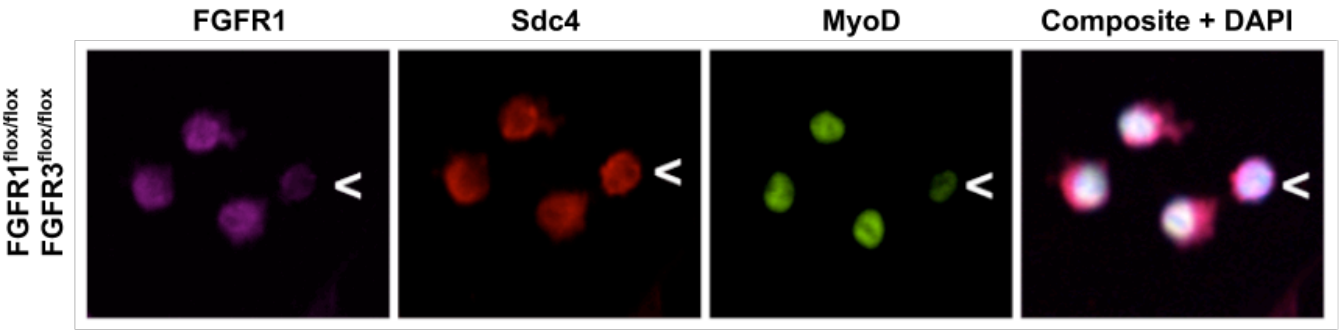

Figure 12- Immunostaining of muscle stem cells cultured for 72 hours treated with 4OHT stained with FGFR1, MyoD, and SDC4 isolated from FR1$^{flox/flox}$ FGFR3$^{flox/flox}$ mice that received IP tamoxifen 5 days prior to collection displays a partial knock down of FGFR1 expression. **B)** Quantification of Myod (-) FGFR1 (-) negative cells as compared to total SDC4 (+) cells.

Collectively, the data from my experiments with the floxed allele mice show that recombination of the floxed allele by Pax7CreERT occurs specifically within MuSCs upon the addition of tamoxifen ultimately resulting in knockdown of expression in a percentage of MuSCs.

Chapter 4:
DISCUSSION

The main goal of my thesis work was to test the hypothesis that FGF regulation of MuSC behavior is mediated by multiple FGF receptors. I sought to compare different approaches to gene deletion that would allow deletion of either individual or multiple FGFRs from MuSC to investigate FGFR function. My first approach utilized a lentiviral CRISPR-Cas9 method which I used to knockdown FGFR1 expression in the muscle cell line C2C12s. The second approach used a Cre Lox conditional knockout methodology to delete FGFR expression from MuSC in vivo.

Crispr-Cas9 gene manipulation has recently been established as a tool to delete or manipulate gene expression. To investigate the utility of Crispr-Cas9 to delete gene expression in MuSCs and develop a model to delete FGFR expression I carried out experiments using a lentiviral CRISPR-Cas9 to knockout FGFR1 and its associated protein Syndecan-4 from an immortalized cell line. I was able to show that both FGFR1 and Syndecan-4 mRNA expression were knocked down following lentiviral infection as measured by RT-PCR. I expected that following Cas9 mediated gene deletion I would see a total loss of mRNA and protein expression, however contrary to this both FGFR1 and Syndecan 4 displayed only a partial knockdown.

One reasonable explanation for seeing only a partial knockdown is that the infected cells existed as a heterogeneous population with some cells having undergone Cas9 mediated gene disruption while other cells within the population that did not. It is interesting to note however that these cells were able to survive puromycin selection, indicating that these cells most likely became infected with the LCV2 construct containing the puromycin resistance but did not undergo NHEJ and therefore did not have a knock out. To address this population heterogeneity, I attempted to isolate individual clones where the FGFR1 gene had been knocked down in order to establish a cell line with homogeneity. To isolate induvial clones, I plated infected cells at a very low “clonal density” in single wells of a 48 well plate, so that each well would contain only a single cell. After expanding these clonal homogenous cell populations, I collected and carried

out RT-PCR experiments to see if any of the clones had FGFR1 knockdown. Unfortunately, the RT-PCR analysis did not show any FGFR1 knockdown across dozens of selected clones, possibly due to a selection bias introduced by selective pressure.

It is possible that FGFR1 mutants having undergone NHEJ on only a single allele compensated for this loss by significantly increasing FGFR1 expression. Furthermore, cells deficient in FGFR1 may also exhibit slowed growth, therefore the cells with FGFR1 would have a competitive growth advantage and thus more FGFR1 would be detected.

One possible explanation for the finding that our infection with the Crispr-Cas9 virus generated puromycin resistant cells but did not induce gene delete is that the expression of the puromycin resistant gene was high enough to grant resistant but either the Cas9 or guide RNA expression driven by our lentiviral construct was insufficient. One way to improve this might be in increase our infection rate (more viral particles per cell to increase the number of copies of the Cas9 and gRNA genes per cell). To increase the infection rate per cell (MOI) in the future I would like to concentrate the lentivirus harvested from multiple HEK-293T cell dishes as opposed to using only un-concentrated viral media that I used in the experiments here. Several other groups that similarly have used the LCV2 system have reported excellent knock out after concentrating the lentivirus before infection (Sanjana, 2014).

The next approach utilized to measure the contributions of each individual FGFR was the conditional Cre-Flox approach. I obtained mice that were genetically engineered to express Cre only in MuSC (Pax7-CreERT) and had loxP sites inserted into the FGFR1, 2, and 3 genes. Following treatment with tamoxifen, I was able to detect that Cre-mediated recombination of the flox sites had occurred within each of the FGFR genes and specifically within MuSC as expected. However, my assays to measure both mRNA and protein expression following FGFR gene recombination showed that essentially normal levels of gene expression still persisted. One possible explanation for this is that the FGFR gene recombination was occurring at a very low frequency or only in a small percentage of cells. To address my potential low recombination efficiency, I tested whether increasing the exposure to tamoxifen might increase recombination rates by giving tamoxifen both in vivo (5 daily injections prior to collection) and in vitro (in the cell culture media). This increased exposure to tamoxifen caused only a slight increase in recombination as indicated by an increase in FGFR1 (-) cells seen in isolated muscle fibers as opposed to fibers only grown in the presence of tamoxifen in culture media.

Another possibility to explain the persistence of protein levels after recombination is that the excised product exists as a stable and functioning episome capable of synthesizing functional gene product. Other groups have demonstrated similarly that Cre mediated episomal products can exist within cells with low turnover rates, such as MuSCs. In order to verify this, PCR could be used to identify the presence of an episomal product within the nucleus of muscle stem cells (Turlo, 2010). To address this, the alleles would have to be redesigned so as to have a smaller distance separating the loxP sites. The mice used for this study have loxP sites in each receptor gene that span multiple exons. This was implemented to address the fact that each receptor has a high number of splice variants, and thus placing loxP sites across the length of the gene would cause recombination in all splice variant products. However, inserting loxP sites that span the gene in turn allow the recombined DNA to circularize and thus the episomal product to stably exist as a large segment of functional DNA. Therefore, placing the loxP sites in closer proximity could hopefully increase knock down levels by preventing episomal formation. It is also possible that the cells deficient in FGFR1 would be selected against and perhaps these cells died prior to collection of RNA.

The mice used for the conditional knock out were designed to have floxed alleles for FGFR1, 2 and 3 which is a useful tool to study these receptors, however I was unable to detect FGFR2 in muscle stem cells, and FGFR3 expression was evident but very weak in wild type cells. Having the ability to knockout FGFR2 and FGFR3 has potential importace if the MuSC were to compensate with the loss of FGFR1 by increasing FGFR2 or FGFR3. FGFR4 expression was also readily detected in MuSCs and therefore a possible future experiment would be to establish mice with FGFR1 and FGFR4 floxed alleles. Being that these are the most highly expressed receptors, it follows that alterations to these two receptors in combination would have the most significant impact on behavior as compared to the removal of receptors that have low expression.

An alternative approach to try for a genetic knockout of each individual receptor would be to create mice expressing an inducible dominant negative FGF receptor that would be able to competitively bind and inhibit all FGF receptors. This would be a very useful tool to observe if FGF can transduce a signal independent of the receptor, and to assay the extent to which FGF regulates MuSCs in vivo. In contrast to this, a constitutively active version of FGFR1 could be expressed in cells deficient in extracellular molecules such as Syndecan 4 or B1-Integrin to

observe if removal of these molecules is sufficient to prevent signal transduction in an active version of FGFR. Additionally, further studies could assay the formation of the receptor complex and determine what receptor complex molecules are interacting via either immunoprecipitation or through techniques such as proximity ligation assays.

If I had been successful in knocking down FGFR expression I would have carried out several phenotypic assays. I would have used both proliferation assays and biochemical assays to measure the levels of downstream signaling events such as activation of p38/MAPK or ERK1/2. If the receptors were acting in a compensatory manner, then deletion of all receptors would mirror the addition of a pharmacologic inhibitor, and downstream signaling transduction would be severely ablated, as compared to the removal of a single receptor. Additionally, the expression of the receptors could be assayed to observe if the deletion of a single receptor causes an increase in another receptor to compensate for this loss. Or in contrast, a specific receptor might increase expression in order to decrease the levels of FGF signaling by acting to sequester FGF and act in a dominant negative manner.

The complexity of FGF signaling in MuSCs highlights the capacity of FGF to control several cellular behaviors such as repression of terminal differentiation, self-renewal, and expansion in a context dependent manner. Illuminating the underlying intricate mechanisms through which FGF regulates MuSC behavior will require more additional work in vivo to accurately follow these behaviors. Since the discovery of FGF's role in myogenesis and muscle stem cell behavior, complex and incompletely understood mechanisms have been discovered, however much remains unknown. The observations linking age assosciated muscle wasting to FGF dysregulation provide significant clinical relevance in the hopes that continued discoveries might lead to new therapeutics. Overall, I was unexpectedly only able to partially knockdown receptor expression in muscle stem cells. These methods are highly established and work in other genes, suggesting that the genes I attempted to knock out are vital and the cell compensated in unknown ways to prevent alteration. Optimization of these techniques will likely prove useful in forwarding our understanding of how FGF regulates MUSC function.

Chapter 5:
METHODS

Lentiviral CRISPR Cas9

To make lentivirus capable of expressing Cas9 and specific guide RNAs we obtained the lentiCRISPR v2 (LCV2) vector as a gift from Feng Zhang (Addgene plasmid # 52961). The LCV2 plasmid contains three expression cassettes, hSpCas9 and the chimeric guide RNA, along with a puromycin and ampicillin resistance gene. The specific guide RNA sequences used to knockdown FGFR1, Syndecan 4 and B1 integrin were identified from the GeCKOV2 library and the gRNA oligos were cloned into the LCV2 plasmid, *BsmBI* restriction enzyme sites were used. After cloning the gRNA oligos into the LCV2 plasmid standard transformation of the annealed construct into STBL3 bacteria was performed. To screen the annealed plasmids for successful gRNA insert, digestion with Kpn1 and EcoRI, was used where plasmids containing the desired sgRNA sequence yield 12.9kB and 354 base pair products. Successful clones were expanded in 25ml LB + Amp cultures for 24 hour and extracted using MidiPrep plasmid kits (QIAGEN).

To produce lentivirus, the LCV2 plasmids were co-transfected with packaging plasmids pMD2.G and psPAX2 into HEK-293T cells using Lipofectamine 2000 (Life Sciences). The transfected cells are then grown in DMEM with 10% fetal bovine serum for 72 hours, after which the viral containing media is removed and frozen for storage.

To infect C2C12 cells with lentivirus the lentiviral containing media is supplemented with additional fetal bovine serum (to give a 20% final concentration) and added to C2C12's in a 6 well culture dish. The cells are centrifuged (1500 rpms) for 30 minutes at 35°C to increase infection efficiency. At 48 hours post-infection, puromycin was added at a concentration of 2 ug/ml puromycin for all wells including the uninfected controls without the addition of lentivirus. At 3 days post-infection, cells in all wells were split 1:5 to prevent any well from reaching confluence. New DMEM with 20% FBS media was supplemented with 2 ug/ul puromycin. After puromycin selection, the cells were harvested for RNA collection to measure expression changes using RT-PCR.

Mice

Mice were housed in a pathogen-free facility and the Institutional Animal Care and Use Committee at the University of Colorado approved all procedures and protocols. Mice were aged between 3 to 8 months before collection. Mice carrying the *Pax7 ires Cre* allele were a gift from

Dr. Kardon (Murphy, 2011). Mice with the FGFR1-3 floxed alleles mice were a gift from Dr. Ornitz. Where indicated, mice received 100ul of 2mg/ml tamoxifen injections 5 days prior to collection.

Satellite cell culture

Satellite cell cultures are established by isolating satellite cells from the leg muscle of mice. Hind leg muscles are dissected from mice, minced with scissors, and digested with collagenase for 1 hour. The digested tissue is then sequentially filtered through 100μM, 70 μM, and 40 μM filters to remove large chunks of tissue and cellular debris. The filtered cells are centrifuged and the supernatant removed. The pellet is re-suspended in F12-C growth media with 15% horse serum with 2ng FGF and plated for 2 hours to remove adherent fibroblasts. The cells are then transferred to gelatin coated plates and coverslips. The isolated muscle stem cells will attach to the cell culture dish and start dividing 24-36 hours after plating. The cells are then collected for RNA after 72 hours. Muscle Stem cells isolated from FGFRx3 KO mice are treated with 10mg/mL 4'OHT to induce Cre mediated recombination. Other cultures were additionally given AdenoCre virus at 200 MOI for 72 hours to induce Cre mediated recombination.
C2C12 cell lines were maintained in DMEM with 20% FBS and were serially passaged upon reaching confluence ($1x10^5$ cells/cm^2). Cells were passaged by rinsing with phosphate buffered saline (PBS) and then adding .25% Trypsin EDTA. Once removed, the cells were pelleted down by centrifugation at 1,500 RPM for 5 minutes and then re-suspended in 10mL of media.
To isolate live myofibers in culture, the extensor digitorum longus muscle of the mouse hindleg was dissected out and digested for 1.5 hours with collagenase. The muscle stem cell assosciated myofibers are then placed in F-12C growth media containing 15% horse serum and grown for 48 hours prior to fixation.

Immunostaining

For immunofluorescence analysis, cells grown on coverslips and myofibers were fixed in 4% paraformaldehyde for 10 minutes. They were subsequently permeablizied and blocked using .25% Triton in 3% BSA in PBS for 1 hour. Samples were then incubated with primary antibody for 1 hour at room temperature or 4°C overnight. Following this, samples were then incubated in secondary antibody for 1 hour at room temperatures and subsequently incubated in DAPI

(1:1000) prior to mounting. After each incubation period, samples were washed 5x for 5 minutes each in PBS. Fibers and cells were then mounted to slides using Mowiol and imaged using the spinning disk confocal microscope (black widow).

Primary antibody dilutions: mouse monoclonal FGFR1 at 1:125, rabbit polyclonal MyoD at 1:1000, chicken monoclonal Sdc4 at 1:500. Alexa Fluor 488, 555, and 647 conjugated secondary antibodies were used at a 1:750 dilution.

Works Cited:

Bernet, Jennifer D., et al. "p38 MAPK signaling underlies a cell-autonomous loss of stem cell self-renewal in skeletal muscle of aged mice." *Nature medicine* 20.3 (2014): 265.

Brewer, J. Richard, et al. "Fgfr1 regulates development through the combinatorial use of signaling proteins." *Genes & development* 29.17 (2015): 1863-1874.

Jones, Nathan C., et al. "ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion." *Journal of cellular physiology* 186.1 (2001): 104-115.

Ornitz, David M., and Nobuyuki Itoh. "The fibroblast growth factor signaling pathway." *Wiley Interdisciplinary Reviews: Developmental Biology* 4.3 (2015): 215-266.

Elfenbein, Arye, et al. "Syndecan 4 regulates FGFR1 signaling in endothelial cells by directing macropinocytosis." *Sci. Signal*.5.223 (2012): ra36-ra36.

Lin, Xinhua. "Functions of heparan sulfate proteoglycans in cell signaling during development." *Development* 131.24 (2004): 6009-6021.

Wright, Addison V., James K. Nuñez, and Jennifer A. Doudna. "Biology and applications of CRISPR systems: harnessing nature's toolbox for genome engineering." *Cell* 164.1-2 (2016): 29-44.

Sanjana, Neville E., Ophir Shalem, and Feng Zhang. "Improved vectors and genome-wide libraries for CRISPR screening." *Nature methods* 11.8 (2014): 783.

Su, Nan, et al. "Generation of Fgfr3 conditional knockout mice." *International journal of biological sciences* 6.4 (2010): 327.

Yin, H., F. Price, and M.A. Rudnicki, Satellite cells and the muscle stem cell niche. Physiol Rev, 2013. 93 (1):

Relaix, Frederic, and Peter S. Zammit. "Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage."*Development* 139.16 (2012): 2845-2856.

Jones, Nathan C., et al. "ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion." *Journal of cellular physiology* 186.1 (2001): 104-115.

He, Wei A., et al. "NF-κB–mediated Pax7 dysregulation in the muscle microenvironment promotes cancer cachexia." The Journal of clinical investigation 123.11 (2013): 4821-4835.

Mauro, Alexander. "Satellite cell of skeletal muscle fibers." *The Journal of biophysical and biochemical cytology* 9.2 (1961): 493-495.

Templeton, Thomas J., and Stephen D. Hauschka. "FGF-mediated aspects of skeletal muscle growth and differentiation are controlled by a high affinity receptor, FGFR1." *Developmental biology* 154.1 (1992): 169-181.

Rozo, Michelle, Liangji Li, and Chen-Ming Fan. "Targeting β1-integrin signaling enhances regeneration in aged and dystrophic muscle in mice." Nature medicine 22.8 (2016): 889.

Cornelison DD[1], Wilcox-Adelman SA, Goetinck PF, Rauvala H, Rapraeger AC, Olwin BB. "Essential and separable roles for Syndecan-3 and Syndecan-4 in skeletal muscle development and regeneration". Genes Dev. 2004 Sep 15;18(18):2231-6.

Turlo, Kirsten A., et al. "When Cre-mediated recombination in mice does not result in protein loss." *Genetics* 186.3 (2010): 959-967.

Takata, Minoru, et al. "Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells." *The EMBO journal*17.18 (1998): 5497-5508.

Yablonka-Reuveni, Zipora, et al. "Myogenic-specific ablation of Fgfr1 impairs FGF2-mediated proliferation of satellite cells at the myofiber niche but does not abolish the capacity for muscle regeneration." *Frontiers in aging neuroscience* 7 (2015): 85.

Weinstein, Michael, et al. "FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung."*Development* 125.18 (1998): 3615-3623.

Zhao, P. O., et al. "Fgfr4 is required for effective muscle regeneration in vivo delineation of a MyoD-Tead2-Fgfr4 transcriptional pathway." *Journal of Biological Chemistry* 281.1 (2006): 429-438.

Pawlikowski, Bradley, et al. "Pervasive satellite cell contribution to uninjured adult muscle fibers." *Skeletal muscle*5.1 (2015): 42.

Pawlikowski, Bradley, et al. "Regulation of skeletal muscle stem cells by fibroblast growth factors." *Developmental Dynamics* (2017).

Lukjanenko, Laura, et al. "Loss of fibronectin from the aged stem cell niche affects the regenerative capacity of skeletal muscle in mice." *Nature medicine* 22.8 (2016): 897.

Pisconti, Addolorata, et al. "Loss of niche-satellite cell interactions in syndecan-3 null mice alters muscle progenitor cell homeostasis improving muscle regeneration." *Skeletal muscle* 6.1 (2016): 34.

Fantl, Wendy J., Daniel E. Johnson, and Lewis T. Williams. "Signalling by receptor tyrosine kinases." *Annual review of biochemistry* 62.1 (1993): 453-481.

Ornitz, David M., and Nobuyuki Itoh. "Fibroblast growth factors." *Genome biology* 2.3 (2001): reviews3005-1.

Lee, Pauline L., et al. "Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor."*Science* 245.4913 (1989): 57-60.

Su, Nan, Min Jin, and Lin Chen. "Role of FGF/FGFR signaling in skeletal development and homeostasis: learning from mouse models." *Bone research* 2 (2014): 14003.

Hannon, Kevin, et al. "Differentially expressed fibroblast growth factors regulate skeletal muscle development through autocrine and paracrine mechanisms." *The Journal of cell biology* 132.6 (1996): 1151-1159.

Kudla, Arthur J., et al. "The FGF Receptor–1 Tyrosine Kinase Domain Regulates Myogenesis but Is Not Sufficient to Stimulate Proliferation." *The Journal of cell biology* 142.1 (1998): 241-250.

Pownall, Mary Elizabeth, and Harry V. Isaacs. "Fgf signalling in vertebrate development." *Colloquium Series on Developmental Biology*. Vol. 1. No. 1. Morgan & Claypool Life Sciences, 2010.

Murphy, Malea M., et al. "Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration." *Development* 138.17 (2011): 3625-3637.

Rapraeger, Alan C., Alison Krufka, and Bradley B. Olwin. "Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation." *Science* 252.5013 (1991): 1705-1708.

Bothmer, Anne, et al. "Characterization of the interplay between DNA repair and CRISPR/Cas9-induced DNA lesions at an endogenous locus." *Nature communications* 8 (2017): 13905.

Mali, Prashant, et al. "RNA-guided human genome engineering via Cas9." *Science* 339.6121 (2013): 823-826.

Jinek, Martin, et al. "A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity." *science* (2012): 1225829.

Sauer, Brian, and Nancy Henderson. "Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1." *Proceedings of the National Academy of Sciences* 85.14 (1988): 5166-5170.

Jones, Nathan C., et al. "The p38α/β MAPK functions as a molecular switch to activate the quiescent satellite cell." *J Cell Biol* 169.1 (2005): 105-116.

Seale, Patrick, et al. "Pax7 is required for the specification of myogenic satellite cells." *Cell* 102.6 (2000): 777-786.

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