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SUFU in SHH signalling mediated myogenesis

Suleyman Abdullah, *Western University*

Supervisor: Kelly, Gregory, *The University of Western Ontario*

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Abstract

Myogenesis is defined as the formation of skeletal muscle tissue during embryonic development and involves a multitude of cellular signalling pathways. Among these include the Sonic hedgehog (Shh) signalling pathway which must be deactivated for differentiation into muscle cells to occur. However, less is known regarding the pathways operation during cell differentiation and whether Suppressor of Fused (SUFU), the protein inhibitor of Shh signalling, plays a role. To address this, mouse C2C12 myoblast cells were utilized as a model and differentiated into muscle cells to identify the presence of SUFU during this time. Experiments in qRT-PCR show a decrease in Shh responsive gene transcripts after induced differentiation thus confirming the deactivation of the pathway during this time. Alongside this, immunoblotting results show the absence of SUFU during proliferation of the cells and its presence throughout differentiation. These results postulate a role for SUFU as an inhibitor during Shh mediated myogenesis.

Keywords

Skeletal muscle, Myogenesis, Myoblast, Myocyte, Myotube, Differentiation, Pluripotency, Embryonic development, Sonic hedgehog signaling, Suppressor of Fused, C2C12 cells

Summary for Lay Audience

During embryonic development, the structures and organs of the eventual adult body are built through differentiating cells. Cell differentiation is defined as the process whereby cells transform into that of a more specialized type with unique properties such as that of a muscle cell. For this process to occur, cells must receive a message in the form of a molecule or protein which binds to their surface. Upon successful delivery of this message, a chain reaction occurs within the cell whereby proteins pass along the newly delivered message to each other. The final protein in this chain then activates cell type specific genes needed for differentiation to occur such as genes involved in building the architecture of a muscle cell with the ability to contract. This successive passing of information is known as a cellular signalling pathway which exists in many different forms, each of which can signal cells to perform different actions.

The process in which undifferentiated cells transform into muscle cells is known as myogenesis. Many different signalling pathways participate in this process including the Sonic hedgehog (Shh) signalling pathway which must be deactivated for differentiation to occur. However, little is known regarding how this specific pathway is regulated during this time. Of particular interest to this thesis is the presence of the protein Suppressor of Fused (SUFU) during myogenesis which acts as an inhibitor to Shh signalling.

To identify the presence of SUFU during myogenesis, the C2C12 cell line was used as a cellular model for the experiments of this study to replicate differentiation as it occurs during development. These cells were originally isolated from mice and consist of myoblasts, the precursor to muscle cells. In this study, once C2C12 myoblast cell differentiation was induced, samples of the cells were collected at each day of differentiation and used in experimentation to determine the activation/deactivation of Shh signalling and presence of SUFU. Shh signalling was found to decrease throughout differentiation while SUFU was found to be present during this time. These results postulate that SUFU may play a role in the inhibition of Shh signalling during myogenesis.

Co-Authorship Statement

The studies and experiments in this thesis were all performed by Suleyman Abdullah under the supervision of Dr. Gregory M. Kelly who helped aid in project conceptualization, experimental design, data analysis, and financial assistance.

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List of Abbreviations

°C - Degrees Celsius

α-actin - Alpha actin

ADAM10 - A disintegrin and metalloproteinase domain-containing protein 10

ADP - Adenosine diphosphate

ALDH1A2 - Aldehyde dehydrogenase 1 family member A2

APC - Adenomatous polyposis coli

ATP - Adenosine triphosphate

BMPR - Bone morphogenetic protein receptor

BMP - Bone morphogenetic protein

Ca^{2+} - Calcium

CBF1 - C promoter binding factor 1

CK1 - Casein kinase 1

CDC42 - Cell division control protein 42

CYP26 - Cytochrome P450 26

DACH1 - Dachshund family transcription factor 1

DAMP - Damage-associated molecular pattern

DHH - Desert hedghog

Dll1 - Delta like-1

DHPR - Dihydropiridine receptors

Dvl - Disheveled

EB - Embryoid bodies

EPH - Epinephrine

ESC - Embryonic stem cell

EVC - Ellis-van Creveld protein

FGF - Fibroblast Growth Factor

FOXC - Forkhead Box C

FZD - Frizzled

GDP - Guanosine diphosphate

GRB2 - Guanine nucleotide exchange factor 2

GSK3β - Glycogen synthase kinase 3 beta

GTP - Guanosine triphosphate

HES7 - Hairy and enhancer of split families protein

HGF - Hepatocyte growth factor

HH - Hedgehog

HSMM - Human skeletal muscle myoblast

IGF - Insulin growth factor

IFT - Intraflagellar transport protein

IHH - Indian hedgehog

JAK - Janus kinase

KIF7 - Kinesin family member 7

LEF - Leukemia inhibitory factor

LRP - Low density lipoprotein-related protein

MAPK - Mitogen-activated protein kinase (also known as ERK)

MAPKK - Mitogen activated protein kinase kinase (also known as MEK)

MEF2C - Myocyte-specific enhancer factor 2C

MEOX - Mesenchyme Homeobox

MESP2 - Mesoderm posterior protein 2

MHC - Myosin heavy chain

MRF - Myogenic regulatory factor

MSC - Multipotent stem cell

MSGN1 - Mesogenin 1

mTORC1 - Mammalian target of rapamycin complex 1

MYF5 - Myogenic factor 5

MYOD - Myoblast determination protein 1

MYOG - Myogenin

NICD - Intracellular domain of Notch

NKX3.2 - NK3 homeobox 2

P - Cyclin-dependent kinase inhibitor

PTCH - Patched

PAX - Paired Box

PI3k/AKT - Phosphatidylinositol-3 kinase

PITX2 - Paired-like homeodomain transcription factor 2

PKA - Protein kinase A

PSC - Pluripotent stem cell

qRT-PCR - Quantitative real time – polymerase chain reaction

RAC1 - Ras-related C3 botulinum toxin substrate 1

RA - Retinoic acid

RAF - Rapidly accelerated fibrosarcoma

RTK - Receptor tyrosine kinase

RIPPLY - Ripply transcriptional repressor

RyR - Ryanodine receptors

SAG – Smoothened agonist

SAP18 - Sin3A associated protein 18

SERCA - Sarcoplasmic reticulum Ca^{2+}-ATPase

SHH - Sonic Hedgehog

SMO - Smoothened

SOS - Son of sevenless

STAT - Signal transducer and activator of transcription proteins

SUFU - Suppressor of Fused

TBX6 - T-box 6

TCF15 - Transcription factor 15

TCF - T cell factor

TGF-β - Transforming growth factor-beta

TSC - Tuberous sclerosis protein

T-Tubule - Transverse tubular system

T - T-box transcription factor T

Chapter 1

1 Introduction

Cellular differentiation is defined as cells altering their functional and phenotypical characteristics into that of a more specialized type. The process occurs whereby undifferentiated progenitor cells, or partially differentiated progenitor cells with the ability to indefinitely proliferate undergo various changes induced by regulatory proteins to give rise to various types of cells[1]. Stem cells are found both during embryonic development and within specific microenvironments in adult organisms[2]. As these cells differentiate, their level of cellular potency lowers, meaning that the potential pool of cell lineages that can be differentiated begins to decrease[3]. Cells with the ability to differentiate into all cell types of the adult organism as well as the placenta are known as totipotent cells. In mammals this includes the zygote and blastomeres during early embryonic development[4]. Conversely, cells that can only give rise to all adult cell types are known as pluripotent and consist of embryonic stem cells[4]. Finally, cells that can differentiate into a select few but highly related cell types are known as multipotent. These multipotent or progenitor cells can only replicate a finite number of times and are usually specific to a cell lineage relating to a single organ[4].

The process of cellular differentiation is responsible for the formation of complex systems of tissues and organs during embryonic development. One form of differentiation is myogenesis in which muscle cells are generated. Muscle is created as stem cells pass through multiple stages of differentiation resulting in mononucleated mesodermal progenitor cells fusing to form elongated multinucleated myofibers[5]. This differentiation is regulated through the activation of multiple transcriptional factors known as myogenic regulatory factors (MRFs), which upregulate muscle specific genes[6]. Furthermore, these MRFs are controlled through the activation/deactivation of many integral signalling pathways, one involving the ligand Sonic Hedgehog (Shh)[7]. Much of how this Shh signalling pathway operates during muscle differentiation has been explored with previous research showing its deactivation as a requirement for the proper formation of muscle cells.

However, the function of many of its protein players, including the inhibitor Suppressor of Fused (SUFU)[8], remains largely unknown. Understanding how this protein inhibitor may affect the pathway will help to further elucidate how Shh mediated myogenesis operates during embryonic muscle development.

In this chapter, I will provide a brief background on skeletal muscle anatomy and physiology. Then, I will detail the process of skeletal muscle development and muscle cell differentiation both *in vivo* and *in vitro*. Furthermore, I will discuss the different cellular pathways involved in myogenesis and highlight how Shh signalling pathway is involved. Finally, I will discuss my hypothesis and underlying objectives, with an emphasis placed on inhibiting the Shh pathway via SUFU.

1.1 Skeletal muscle

Muscle is a soft tissue consisting of multiple fibers of cells bundled together in a protective tissue with the unique ability to contract using specialized proteins[9]. There are three types of muscles in vertebrates, each of which is specific regarding their function and location in the body. This list includes skeletal, smooth, cardiac muscle, the latter which contracts involuntarily through the autonomous nervous system[10]. Cardiac muscle is also striated, meaning its muscle fibers show visibly repeating bands like skeletal muscle. These bands indicate the presence of sarcomeres, the smallest functional unit of muscle tissue which consists of highly organized bundles of cells that can contract in unison[11]. Cardiac muscle constitutes the walls of the heart and via contraction, circulates blood throughout the body[12]. Smooth muscle also contracts involuntarily but is considered non-striated due to individual muscle cells failing to perfectly align themselves to form sarcomeres[13]. Smooth muscle cells are found in the walls of many internal organs including the stomach, intestines, and bladder. As such, contractions in these organs are responsible for many bodily functions including moving food and waste[14]. Of particular interest for my research is skeletal muscle, which is both striated and uniquely voluntary in its contraction abilities due to the somatic nervous system. Skeletal muscle is attached to bones by tendons and is responsible for the movement of the entire body[9].

1.1.1 Skeletal muscle structure

Skeletal muscle is surrounded by dense irregular connective tissue or epimysium, which helps to protect cells from friction forces against other muscle and bones. Within the outer layer of epimysia exist groups of myofibers arranged in bundles called fascicles. Each fascicle is surrounded by another layer of collagenous connective tissue known as the perimysium, and the cells' membrane, or sarcolemma, envelopes each individual myofiber that makes up the myofibrils[15]. Desmin, a filamentous contractile protein is connected to the myofibrils and to the sarcolemma and where each myofibril is made up of myofilaments[16]. These myofilaments mainly consist of actin filaments and myosin protein[17] and the muscle regulatory proteins, troponin and tropomyosin[18] and repeat along the myofibrils forming contractile units called sarcomeres. Proteins contributing to the structural integrity of the sarcomere also include titin which binds to myosin to align the thick filament[19] as well as nebulin, which binds to actin to regulate the length of the thin filament[20]. Furthermore, α-actinin helps attach the actin myofilament to the Z disk, the lateral borders of a single sarcomere unit[21] **(Figure 1)**.

Within the sarcoplasm, the cytoplasm of muscle cells[22], other cellular organelles exist to help aid in the contraction of myofibers. An invagination within the sarcolemma exists known as the transverse tubular (T-tubule) system consisting of a network of tubules that connect the exterior of the cell to the interior[23]. This network is responsible for proper conduction of nerve action potentials towards the inside of the muscle cell[23], which it accomplishes alongside dysferlin, a protein that regulates calcium ion homeostasis[24]. The sarcoplasmic reticulum, another organelle like the smooth endoplasmic reticulum, is responsible for the storage and release of Ca^{2+} during contraction[25]. Within the sarcoplasmic reticulum the terminal cisternae can be found where calcium ions are stored and maintained by the protein known as the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA)[26]. Calsequestrin is also needed to bind calcium ions in the sarcoplasmic reticulum[27]. The energy (ATP) needed for muscle contraction is produced through a complex network of mitochondria, and in the presence of oxygen, within the muscle. These mitochondria are located close to the sarcolemma and are near oxygen delivering capillaries[28] **(Figure 2)**.

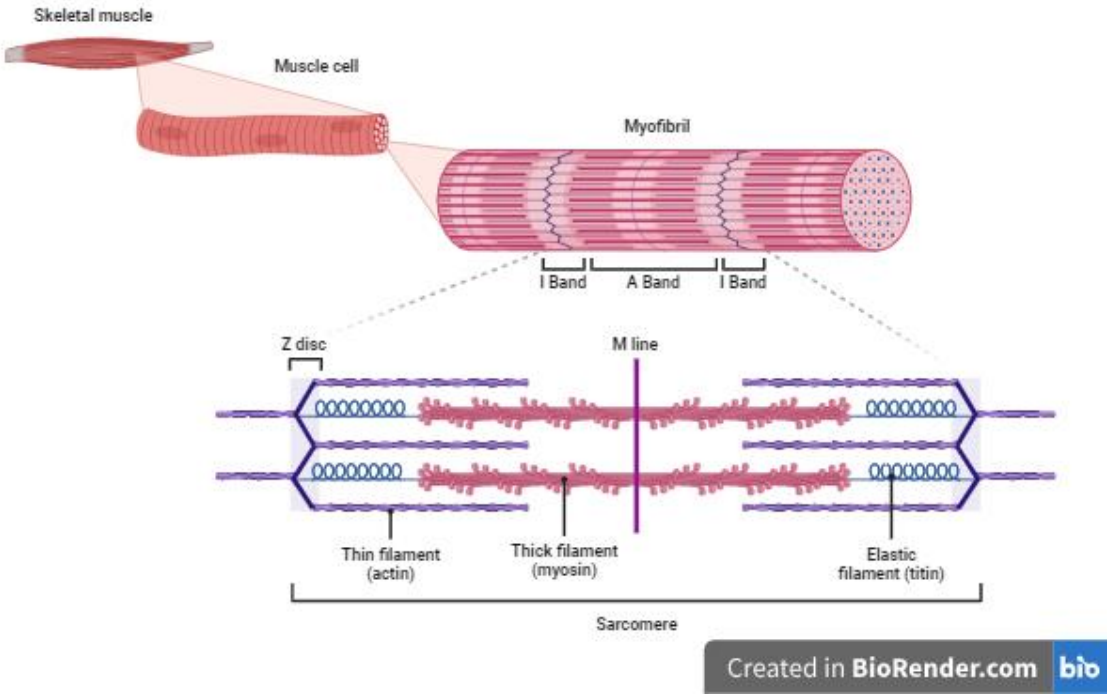


Figure 1: The structure of the skeletal muscle. Skeletal muscle consists of muscle cells or myofibers arranged in bundles. Each myofiber contains rod-like organelles known as the myofibril which consists of repeating contractile units known as sarcomeres. These sarcomeres are formed through filamentous actin and myosin proteins found parallel to one another. Reprinted from "Myofibril Structure" by BioRender.com.

1.1.2 Skeletal muscle function

The force needed to generate movement in skeletal muscle occurs through excitation-contraction coupling. During the initial excitation stage, an electrical action potential arrives at the sarcolemma from nearby motor nerves. The T-tubule system conducts the action potential into the interior of the muscle cell towards a structure known as the triad[29]. Triads consists of two terminal cisternae of the sarcoplasmic reticulum and the T-tubule[26]. Dihydropiridine receptors (DHPRs) on the T-tubule are sensitive to voltage and when depolarized conformationally change and allow in an influx of calcium ions[30] that triggers the opening of ryanodine receptors (RyRs) on the sarcoplasmic reticulum[31]. As a result, Ca^{2+} is released into the sarcoplasm and binds to troponin C, a protein bound to actin in the thin filaments[32] **(Figure 2)**. The head of myosin, which also binds ATP detaches from actin, allowing myosin to bind to another actin. ATP is hydrolyzed by the myosin head and the energy of this hydrolysis primes myosin to bind another actin[9,33]. Ca^{2+} bound to troponin C causes tropomyosin to move away from the actin binding site[9,32], allowing myosin molecules to firmly attach to this site and the inorganic phosphate from the ATP hydrolysis initiates a power stroke that causes a contraction in the sarcomeres. ADP is released from the myosin and the binding of ATP allows the myosin head to release from actin. This sliding of actin and myosin filaments opposite to one another generates the force needed for contraction[9,33]. Following contraction, the tropomyosin-troponin complex covers the actin binding sites and the muscle relaxes[9,32].

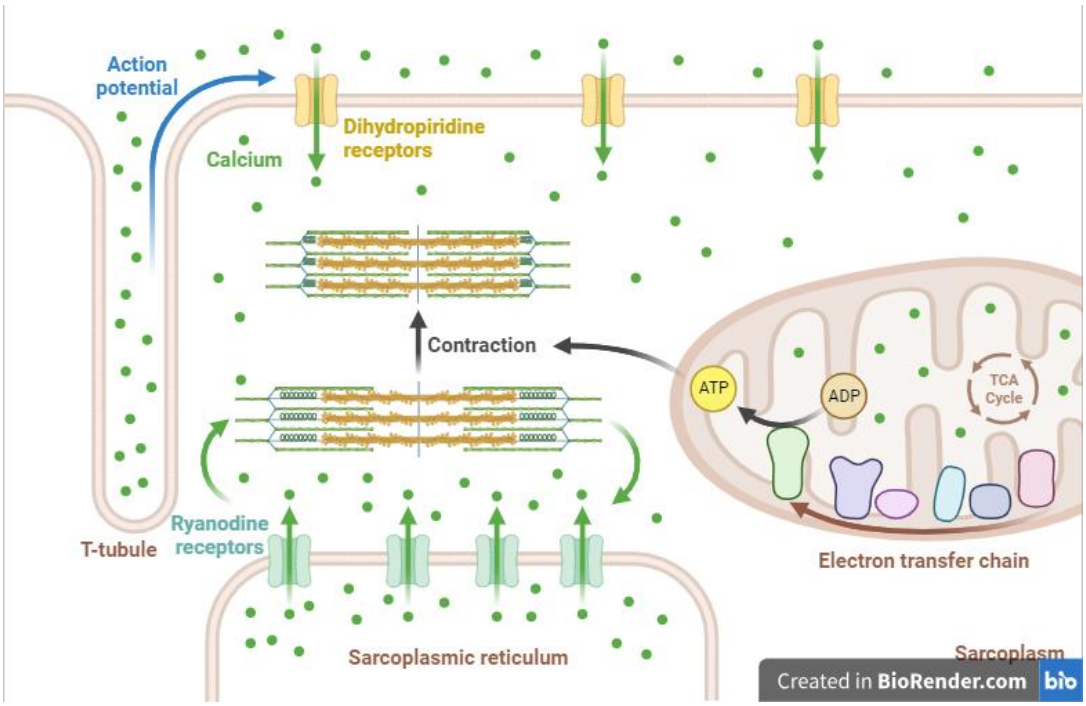


Figure 2: Skeletal muscle contraction. Skeletal muscle contraction occurs upon conduction of an action potential towards the interior of a muscle cell through the T-tubule. This results in the stimulation and opening of dihydropyridine receptors thus allowing in an influx of calcium which in turn opens the ryanodine receptors of the sarcoplasmic reticulum. This further releases calcium into the cell which alongside ATP produced by the mitochondria help cause contractions in the sarcomeres. Reprinted from "Cardiomyocyte Energetics" by BioRender.com.

1.2 Skeletal muscle development

1.2.1 Myogenesis *in vivo*

Skeletal muscle development in mouse begins during the gastrulation stage of embryonic development after the appearance of the primitive streak reorganizes a single-layered cell structure known as the blastula. This restructuring creates the gastrula, a multilayered cell structure containing all three germ layers which includes the ectoderm, mesoderm, and endoderm[34]. Within the primitive streak, a tissue known as the paraxial mesoderm develops at the posterior end of the embryo which bilaterally flanks both the notochord and neural tube. The paraxial mesoderm contains the presomitic mesoderm, a tissue that can be further divided into two parts including a posterior and anterior region[35]. The posterior presomitic mesoderm is unsegmented, whereas the anterior end gives rise to the somites. Somites serve as the origin point for myogenesis, where premyogenic progenitor cells proliferate and differentiate to eventually form multinucleated myofibers[35,36].

The formation of the primitive streak during gastrulation is dependent on the signalling factors Nodal and BMP4[35,37]. Within this region multiple types of progenitor cells exist with varying abilities regarding what tissues they can specify. One type of progenitor cell can give rise solely to the paraxial mesoderm[38], whereas another neuromesodermal progenitor can give rise to both the paraxial mesoderm and neural tube[39]. A third type of progenitor can differentiate into both the paraxial mesoderm and notochord[40], while a fourth type can differentiate into the paraxial mesoderm and lateral plate derivatives[41].

For specification of the paraxial mesoderm to occur, progenitor cells must receive signals from both Wnt[42] and Fibroblast Growth Factor (FGF)[43] ligands, signalling in respective pathways for cells to enter the posterior presomitic mesoderm. During differentiation, Wnt and FGF signalling increase the activity of transcription factors T[44], Tbx6[45], and Msgn1[46], all of which are needed in the patterning of the paraxial mesoderm. Furthermore, Wnt/FGF signalling is integral in regulating axis elongation in the developing embryo, as downregulation in signalling for both pathways occurs in the tail bud thus arresting further development of the paraxial mesoderm[47]. A regulatory loop exists between both Wnt and

FGF signalling in which both pathways must activate each other to properly develop the paraxial mesoderm[48,49]. Wnt signalling is activated by Fgf4 and Fgf8 in the presomitic mesoderm[48], while Fgf8 expression requires Wnt3a signalling[49]. Whereas both Wnt and FGF signalling are important to the anterior-posterior patterning of the mesoderm in the primitive streak, BMP signalling is needed for patterning along the mediolateral axis[50]. The posterior primitive streak secretes BMP4[51], while the axial structures of the embryo produce noggin, an antagonist of BMP signalling[52]. This antagonism creates a gradient in BMP signalling across the mediolateral axis where varying levels of BMP result in the formation of different mesodermal tissue including the notochord and lateral plate mesoderm[53]. The development of the paraxial mesoderm requires the suppression of BMP signalling and this is located where the gradient is at its weakest[54].

Development of the somites occurs through the segmentation clock, a process involving the oscillation of a multitude of different signalling pathways that help to periodically form individual segments of somites[55]. Segmentation occurs in the paraxial mesoderm, which can be divided into four regions. From the posterior to anterior end of the embryo, these regions consist of the tail bud, the posterior presomitic mesoderm, the anterior presomitic mesoderm, and the eventual developing somite[56]. A gradient of Wnt and FGF signalling occurs across these regions with the strongest signal found at the posterior end of the embryo and a steady decrease occurring in the anterior direction[49,57]. At the somitic region, retinoic acid is produced and acts as an antagonist to Wnt/FGF signalling[58]. The gradient that develops acts as a threshold in which high amounts of Wnt/FGF signalling help to maintain the paraxial mesoderm, whereas suppression of the pathways allows progenitor cells to form somites. These progenitors are initially found in the tail bud region where Wnt/FGF signalling is at its highest[49,57] and the enzyme Cyp26 helps to degrade and counteract retinoic acid[58]. Upon reaching the posterior presomitic mesoderm, progenitors can enter the segmentation clock where the genes, including *Mesp2*[59], *Pax3*[60], *Foxc1/2*[61], and *Meox1/2*[62], responsible for segmentation are expressed. Finally, in the anterior presomitic region, development of the somites begin because the suppression of Wnt/FGF signalling is at its greatest due to the high levels of retinoic acid[58].

Originally mesenchymal and mobile in nature[63], the progenitor cells become epithelial upon reaching the anterior presomitic mesoderm[64]. Here, these cells form a dorsal and ventral epithelial layer surrounding a mesenchymal core[65]. This process is caused by the activation of TcF15[66], a transcription factor induced by the receptor Frizzed 7 and the ligand Wnt6 in the dorsal ectoderm[67]. At the most anterior point of the presomitic mesoderm, a boundary soon develops between *Mesp2* expressing and non-expressing cells resulting in the formation of a block of mesodermal tissue[68]. This newly formed block is further remodelled by Eph-ephrins[69], cadherins[70], Cdc42[71], and Rac1[71] with Notch signalling needed to pattern both the anterior and posterior ends of the newly formed somite[68]. These somites are later sectioned into two parts, with one forming the dorsal epithelial dermomyotome that is responsible for giving rise to skeletal muscle, brown fat, and the dermis of the back[72]. The second region is the ventral mesenchymal sclerotome, which is responsible for the axial skeleton and tendons[72] **(Figure 3)**. This lineage specification is dependent on the signalling received from surrounding tissue where for the dermomyotome, inhibition of BMP signalling and increased Wnt signalling from the neural tube and dorsal ectoderm[73] leads to the upregulation of *Pax7*[74]*, Pax3*[74]*,* and *Myf5*[75]. Conversely, the sclerotome specification requires Shh signalling from the notochord and floor plate[76].

The dorsal epithelial dermomyotome is further segmented into multiple regions including a central domain, dorsomedial lip, anterior lip, posterior lip, and a ventrolateral lip[77]. Myogenesis begins within the dorsomedial lip, when progenitor cells begin to express *Myf5*[78] while the protein downregulates *Pax3*[79]. This signalling leads to the formation of the primary myotome, a cell layer found between the dermomyotome and sclerotome[80] **(Figure 3)**. Progenitors in the myotome differentiate into mononucleated myocytes, the initial skeletal muscle cells of the embryo[81] but later, Wnt11 signalling cause these myocytes to elongate across the somite along the anterior-posterior axis[82]. As more progenitors and eventually differentiated myocytes are added to the myotome, myofibers form through the fusion of multiple myocytes[83]. This fusion creates multinucleated myotubes that express the cytoskeleton proteins needed for contraction such as slow type 1 myosin heavy chains (MyHC)[83], α-actin[84], and desmin[81]. The process repeats with every sequential somite added throughout the development and elongation of the embryo[85]. Limb

muscles are formed later by cells in the lateral dermomyotome[86] and through the activation of transcription factors Pax3[87], Myf5[88], MyoD[88], MRF4[89], and Myogenin[89]. Activation of these factors help reorganize the myocytes into myofibers after they migrate into the developing limb buds. Myogenin is especially important for the differentiation of myoblasts into myocytes[90].

Signalling for myogenesis is induced by the tissues surrounding the somites such as the neural tube and lateral plate mesoderm[91]. In the case of the neural tube, neural crest cells migrate to the somite to help express Myf5 within progenitors in the dermomyotome[92]. These neural crest cells express delta like-1 (Dll1) and activate Notch signalling which inhibits GSK3ß thus allowing for the stabilization of Snail. This leads to the delamination and migration of the progenitors into the primary myotome[93]. The lateral plate mesoderm also supports myogenesis by secreting the signalling molecule hepatocyte growth factor (HGF) needed for the proper migration of myoblasts[94].

Thus, myogenesis initially begins early in embryonic development, serving as building block which adult muscle will later be built upon during the secondary stage of myogenesis[95]. This secondary stage takes place during fetal development and involves *Pax3* expressing progenitors upregulating *Pax7* while downregulating *Pax3*[96]. This signalling allows cells to fuse and form secondary myofibers defined by their expression of fast type 2 MyHC isoforms[97]. The addition of more myonuclei to the newly formed secondary myotome is what sustains this secondary stage of myogenesis[98].

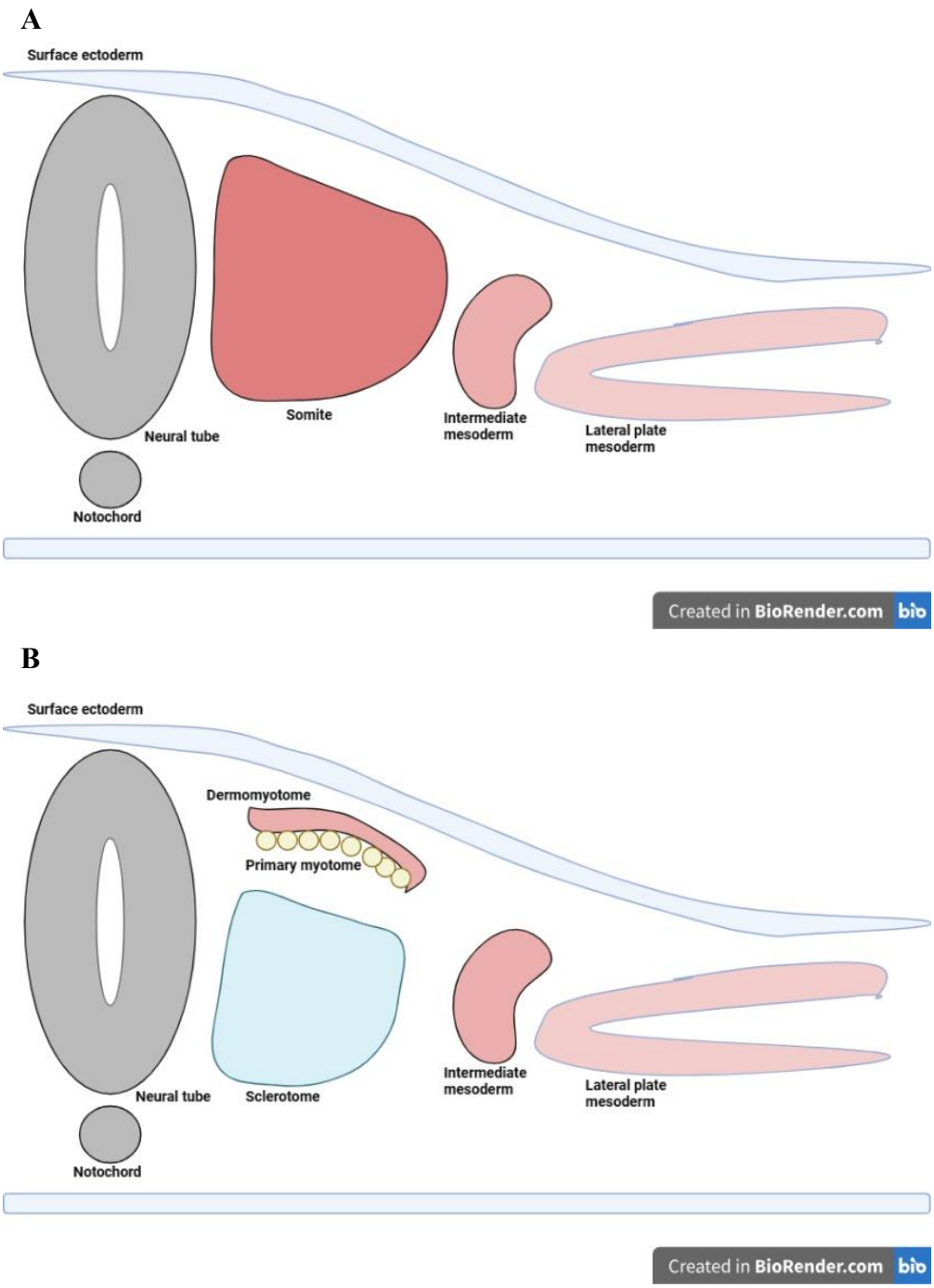


Figure 3: Formation of the somite and primary myotome during embryonic development. (A) Spatial relationship of the newly formed somite against the other structures of the embryo. **(B)** The somite eventually develops into separate regions including a dorsal dermomyotome and ventral sclerotome with a primary myotome later forming in between where myogenesis takes place. Created with BioRender.com

1.2.2 Skeletal muscle regeneration

Adult skeletal muscles can repair damaged tissue via satellite cells found sequestered in their own microenvironment between the sarcolemma and basal lamina of myofibers[99]. These adult stem cells are derived from the *Pax3*/*Pax7* expressing embryonic progenitor cells needed during postnatal muscle development[100]. To prevent migration during the absence of muscle injury, the cytoskeleton of satellite cells is bound to the basal lamina[101] through actin-integrin-a7 and actin-integrin-b1[102]. Satellite cells are considered to be quiescent or in the hibernating state where they lie dormant until activated[101,102]. Quiescence in satellite cells require Wnt[103] and Notch[104] signalling, which result in the expression of *Pax7* and suppression of M*yoD1* and *Myog*[103]. Furthermore, cell cycle inhibitors such as p21, p27[105], and DACH1[106] are expressed in quiescent satellite cells whereas myogenic regulatory factors including MYOG are absent[103].

During muscle injury, quiescent satellite cells are activated and migrate, proliferate, and differentiate into myofibers in order to replace damaged tissue. Upon activation, these cells upregulate PAX7 as well as myogenic regulatory factors such as MYOD1, MYOG, and MYF5, which help to initiate proliferation[107]. Differentiation into myocytes later occurs after downregulation of MYF5 and MYOD1[108], alongside consistent expression of MYOG, MEF2C, and MRF4[107]. Migration towards the site of injury is driven by chemoattractants secreted from inflammatory cells[109], including growth factors[110], damaged-associated molecular pattern molecules[111], and cytokines[112]. The latter has been linked to the activation of the JAK-STAT pathway, which promotes both satellite cell proliferation and differentiation[113]. During regeneration, a portion of activated satellite cells must return to the quiescent stage to maintain a pool of stem cells for future use[114].

1.3 Skeletal muscle development on a cellular level

1.3.1 Myogenesis *in vitro*

Many different methodologies have been implemented that recapitulate *in vivo* myogenesis within *in vitro* studies. One such method utilized mouse embryonic stem cells (mESCs),

which were differentiated into embryoid bodies (EBs)[115]. EBs are three-dimensional cell aggregates with the ability to give rise to cell types of all three germ layers[116]. Upon formation, EBs were tested for MRF expression along with the presence of myocytes[115]. However, EBs are heterogenous in regard to their cell lineage differentiation, with no simple way of controlling specification. To circumvent this issue new methods have been developed that focus on pluripotent stem cells (PSCs), which form homogenous cell monolayers that differentiate through multiple cell stages using specific signalling molecules[117].

The initial stage of muscle cell differentiation that occurs during *in vivo* development is that of the paraxial mesoderm precursor cells which help form the primitive streak. These cells are defined by their expression of the early mesodermal marker *Brachyury* and their ability to give rise to multiple different mesodermal subpopulations[118]. *In vitro* studies have shown that when treated with activin, PSCs differentiate into anterior primitive streak derivatives which serve as precursors to endodermal tissue[119]. Conversely, treatment with BMP leads to posterior primitive streak derivatives that are precursors to the lateral plate and extraembryonic mesoderm[120]. The mesodermal cells in these tissues serve as hematopoietic[121] and cardiogenic[122] progenitors. However, generation of the mesodermal progenitors in the primitive streak responsible for the paraxial mesoderm requires sequential activation of BMP[123] and Wnt[124] signalling **(Figure 4)**.

Production of paraxial mesoderm progenitor cells from PSCs requires activation of both Wnt[125] and FGF[126] signalling. These cells are defined by their expression of mesodermal markers *Msgn1* and *Tbx6*[125]. To generate cells with a posterior presomitic mesoderm fate, PSCs must initially be treated with BMP4 for differentiation into epiblast-like cells[127]. This is followed by activation of Wnt/FGF signalling, as well as inhibition of BMP signalling, to prevent the specification of lateral plate mesodermal cells **(Figure 4)**. The resulting cells express genes relating to the segmentation of somites such as *Hes7*[56]. During *in vivo* development of somites, *Msgn1* is downregulated at the determination front while segmentation genes *Mesp2*, *Ripply1*, and *Ripply2* are expressed along with the activation of Pax3 protein. These genetic and protein markers can be seen *in vitro* when cultured cells

maintain Wnt/FGF activation and BMP inhibition, and is indicative anterior of presomitic mesodermal cells[56].

The final stage of muscle cell differentiation *in vivo* occurs when myoblasts fuse to form myocytes; an obligatory predecessor of myofibers[81]. Myoblasts are derived from *Pax3*-expressing precursor cells in the dermomyotome, and to induce these cells *in vitro*, mESCs must again be maintained in conditions promoting Wnt signalling while inhibiting BMP[56]. The cells themselves produce their own retinoic acid (RA) through upregulation of the enzyme *Aldh1a2* and this promotes presomitic mesoderm differentiation[128]. Further treatment with HGH, IGF, and FGF leads to differentiation of cells into elongated mononucleated myocytes expressing myogenin[129] **(Figure 4)**. The increase in myocytes correlates with the replacement of *Pax3* expressing myogenic progenitors with those expressing *Pax7*[87,130].

Along with directed differentiation, other methods for *in vitro* cell differentiation include directed reprogramming, which involves the overexpression of certain transcription factors that cause cells to enter the myogenic lineage[117]. For instance, fibroblasts treated with 5-azacytidine, a demethylating agent that targets *MyoD*, leads to the reprogramming of these cells into myoblasts[131]. Furthermore, overexpressing *MyoD* in PSCs forces differentiation into a muscle specific lineage, skipping over early developmental stages, and results in the formation of myotubes after 10 days[132]. Myogenic differentiation can also be induced in PSCs through overexpression of *Pax3* and *Pax7*[133] **(Figure 4)**. In this instance, Pax3 upregulates the *Tcf15* and *Meox1* transcription factors and prevents differentiating cells from undergoing a cardiac specific lineage[134]. Direct reprogramming of PSCs can also be induced through MRFs, IGF2, HGF, and Mesp1. Mesp1, specifically permits the formation of progenitors with the ability to form either cardiac or cranial mesodermal cells[135].

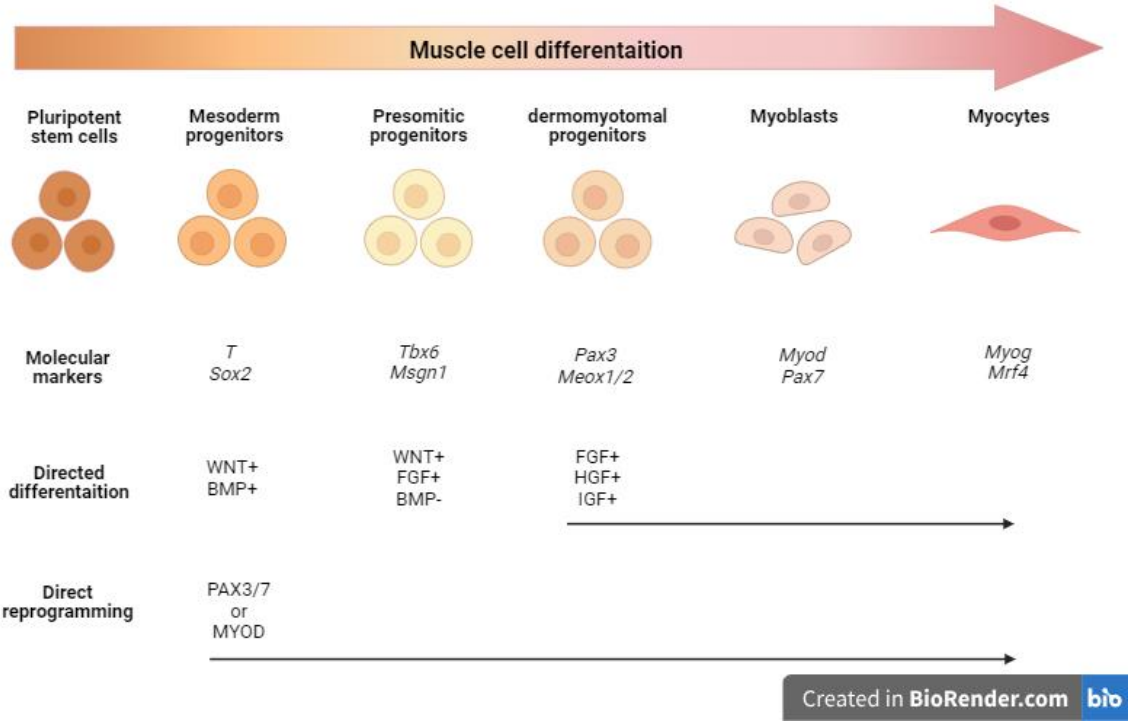


Figure 4: Muscle cell differentiation *in vitro*. Pluripotent stem cells (PSCs) can be differentiated into myocytes through a number of different techniques. These include directed differentiation requiring the sequential activation of numerous signalling pathways as well as direct reprogramming requiring the simple exogenous addition of certain myogenic factors. Each cell stage can be identified through numerous genetic markers. Created with BioRender.com.

1.3.2 C2C12 cells as a model for myogenesis

Embryonic Stem Cells (ESCs) and Pluripotent Stem Cells (PSCs) are integral in studying skeletal muscle development *in vitro* beginning at gastrulation stages, but multipotent stem cells (MSCs) offer a more efficient and direct way of studying differentiation at late stages[4]. MSCs allow for better targeted differentiation as they are already committed to a specific lineage with limited differentiation potential. Furthermore, MSCs having a more defined and linear differentiation pathway require less complex culture conditions[4]. Most commonly, myoblasts are the MSC of choice and multiple myoblastic cell lines exist including those from human (HSMM cells)[136], rat (L6 cells)[137] and mouse (C2C12 cells)[137].

Of interest to this research is C2C12 cells, an immortalized myoblast line developed by David Yaffe and Ora Saxel at the Weizmann Institute of Science in 1977. C2C12 cells were obtained through serial passage that stemmed from the thigh muscle of female C3H mice[138]. These cells are used extensively as a model of myogenesis in part due to their high division rate and high fusion rate into myotubules. During proliferation, C2C12 cells are mononucleated and have a radial branching morphology consisting of long fibers that extend in many directions. However, once differentiation begins, myoblasts elongate and form myocytes, which fuse to form multinucleated myotubule cells[139]. Differentiation occurs within 7 days and simply requires the exchange of high serum media (10%) to low serum media (1%), which starves the cells and allows them to exit the cell cycle and initiate differentiation[139]. This ease of handling and the relative timeline for muscle differentiation is why C2C12 MSCs have been selected for the purposes of this research related to cell signalling.

1.4 Cellular signalling pathways in muscle development

Proper muscle development requires the activation and deactivation of numerous different cellular signalling pathways. As of date 5 pathways in particular have been studied in depth regarding their roles in myogenesis. These include the Wnt and Fgf pathways of which signalling is required for the differentiation of muscle cell progenitors. This is in contrast to Bmp and Notch signalling which largely must be deactivated for

successful differentiation to occur. The following section will detail the roles each of these pathways play in myogenesis and end with a in depth look at the Shh pathway.

1.4.1 Wnts

The canonical Wnt signalling pathway plays an important role in the formation of numerous tissues during embryonic development as well as in adult tissue homeostasis. It is mainly known for its importance in defining the dorsoventral and anteroposterior body axes as well as its part in the formation of the nervous system[140]. In the absence of the Wnt ligand, a multi-protein complex consisting of CK1[141], GSK3B[141], Axin[142], and APC[143] hyperphosphorylates β-catenin thus causing its degradation by the proteasome and preventing gene transcription[144]. Conversely, the Wnt ligand when present binds to the co-receptors LRP5/6 and Frizzled (Fzd)[145], causing Disheveled to translocate to the plasma membrane which leads to the recruitment of Axin[146]. As a result, this multi-protein destruction complex does not phosphorylate β-catenin, and the latter enters the nucleus where it interacts with the TCF/LEF transcription factor to upregulate Wnt responsive transcription targets[147] **(Figure 5)**.

In the paraxial mesoderm several Wnt family members are required for the expression of many MRFs. *Wnt1* is expressed in the epaxial domain of the somite[148] and induces activation of *Myf5*[149], while *Wnt7a/Wnt6* are expressed in the dorsal ectoderm[150] and assist in the expression of *MyoD*[149]. Fzd7 is on the plasma membrane of the hypaxial region of the somite, where it is believed to interact with Wnt7a. Other receptors including Fzd1 and Fzd6 are expressed in the epaxial somite, suggesting other possible roles in aiding Myf5 expression in the region[151]. Wnt1, Wnt3a, Wnt4, and Wnt6 signalling from the surface ectoderm and neural tube maintain the levels of Pax3 and Pax7 in premyogenic cells within the somite[152], while the number of these cells is determined by transcription factors Lef1 and Pitx2[153]. Whereas in the developing dermomyotome, the absence of Wnt1 and Wnt3a leads to loss of the medial domain due to reduced expression of Myf5[154]. Wnt6 from the dorsal ectoderm is also required for the dermomyotome including the epithelial organization of cells in the somite[67]. Interestingly, β-catenin is required in the somite for the proper development of the dermomyotome and myotome and is necessary for the

resulting number of myofibers in the limb[155]. The elongation of such myofibers in the myotome requires Wnt1 and Wnt3a[154] signalling in the dorsal neural tube to induce Wnt11 activation within the epaxial dermomyotome[82].

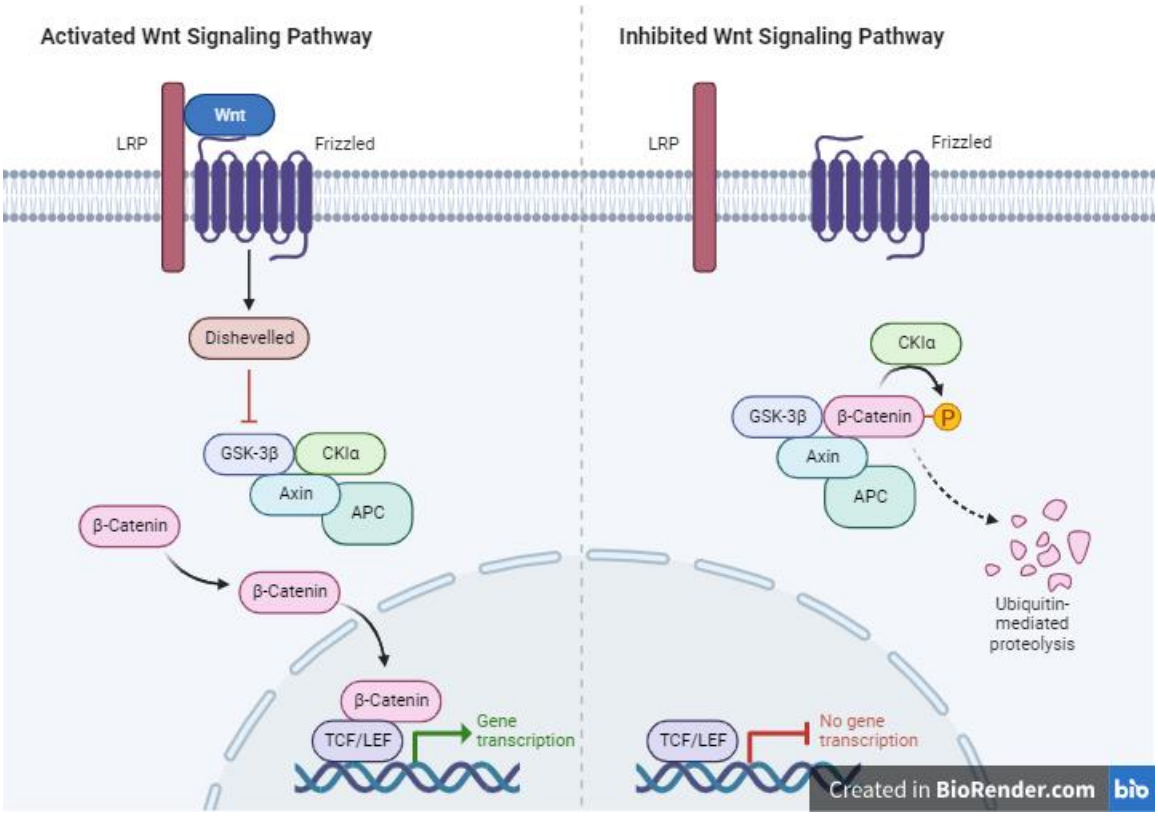


Figure 5: The Wnt signalling pathway. In the absence of the Wnt ligand, β-catenin is hyperphosphorylated by a multi protein complex consisting of CK1, GSK3B, Axin, and APC leading to its degradation by the proteasome. In the presence of the Wnt ligand which binds to co-receptors LRP5/6 and Fzd, Dishevelled recruits Axin leading to the destruction of the multi-protein complex. This allows β-catenin to translocate into the nucleus and bind TCF/LEF leading to the upregulation of Wnt responsive genes. Reprinted from "Wnt Signalling Pathway Activation and Inhibition" by BioRender.com.

1.4.2 Bone morphogenetic proteins

Bone morphogenetic proteins (BMP) are key regulators in the development of numerous tissues originally discovered by their ability to induce the formation of bone and cartilage[156]. BMP ligands belong to the Transforming growth Factor-beta (TGF-B) superfamily[157] and initiate signalling by binding to their respective serine/threonine kinase receptors including BMPR-1 and BMPR-2[158]. Upon ligand binding, BMPR-2 is activated and phosphorylates BMPR-1, which in turn phosphorylates the intracellular SMAD proteins SMAD1[159], SMAD5[160], and SMAD8[161]. These SMADs form a complex along with SMAD4 and translocate into the nucleus to upregulate BMP-responsive genes[162] **(Figure 6)**. Inhibitors of the pathway include Smad6 which blocks binding between Smad4 and Smad1[163] as well as noggin by binding Bmp4 and preventing it from binding its receptors[164].

Evidence from myoblast cell lines have shown BMPs act in an inhibitory role regarding differentiation. For instance, exogenous BMP ligand, when added to C2C12 cells, blocks myotube formation because genes related to myogenic differentiation such as *Myog* are not expressed. Instead, these cells experience trans-differentiation and are destined to form an osteogenic (bone) fate[165,166]. Conversely, myotube differentiation was successful in the presence of BMP inhibitors[167]. Parallels are seen during embryonic development with BMP signalling playing an inhibitory role regarding myogenic differentiation where the addition of the exogenous BMP ligand[168] or expression of Smad1[169] in mesodermal tissue in multiple organisms affected muscle determination. Conversely, when a bead containing noggin is grafted into the posterior primitive streak, cells that would otherwise give rise to the lateral plate were instead converted into a paraxial mesoderm fate and developed into somite's[164]. Such cases were seen in *Xenopus*[170] and chick embryos[171] where the addition of BMP inhibitors caused the increase of myogenic genes including *Myod* and *muscle actin*.

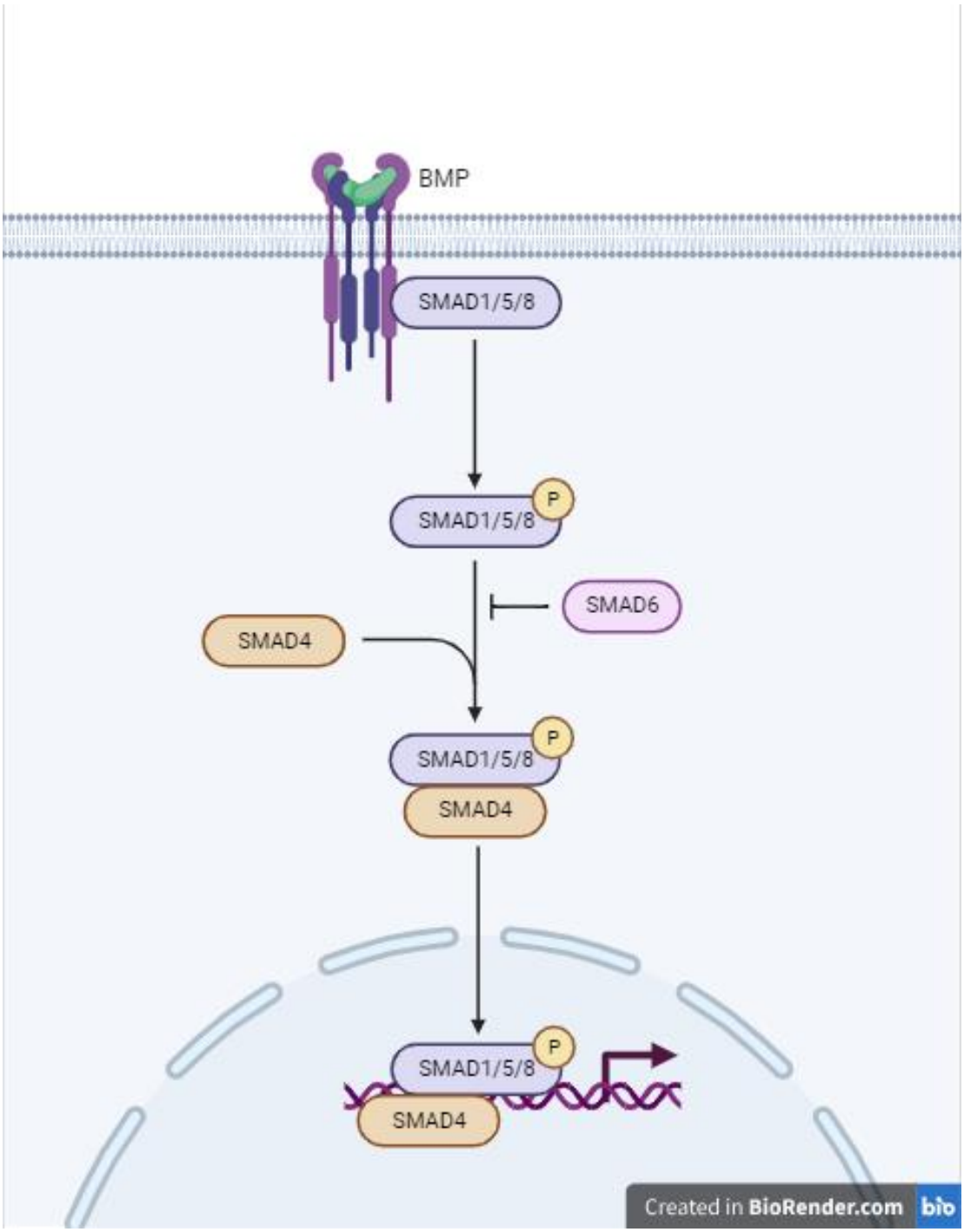


Figure 6: The Bone morphogenetic signalling pathway. Upon binding of the BMP ligand to the BMPR1/2 receptors, BMPR2 phosphorylates BMPR1 which then phosphorylates SMAD1/5/8 which go on to form a complex with SMAD4. This complex then translocates into the nucleus and upregulates BMP responsive genes. Reprinted from "BMP Signalling Pathway" by BioRender.com.

1.4.3 Fibroblast growth factors

Fibroblast growth factors (FGFs) govern many fundamental cellular processes including proliferation, migration, cell survival, and differentiation. Specifically in regards to development, FGFs are known to regulate anterior-posterior patterning as well as limb and neural development[172]. Signalling begins when the FGF ligand binds to a receptor tyrosine kinase (RTK) leading to its autophosphorylation[173] and activation of either the MAPK or AKT pathways[174]. In the MAPK pathway, an inactive GDP-bound Ras is converted into an active GTP-bound Ras by GRB2 and son of sevenless (SOS), leading to the activation of Raf[175]. This active Raf phosphorylates MEK and the cascade continues with the phosphorylation and activation of ERK1/2, which can phosphorylate a multitude of substrates. These substates include transcription factors, which upregulate other genes linked to proliferation[174,175]. Binding and activation of an RTK also sets in motion of the PI3K-AKT-mTOR pathway, leading to the pro-survival of cells receiving the signal[174]. In this pathway, PI3K converts phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5- triphosphate and results in the recruitment and phosphorylation of AKT[174,175]. Active AKT inhibits TSC proteins, leading to the activation of the mTORC1 complex which inhibits expression of genes related to autophagy, glucose metabolism, and the regulation of cell cycle proteins[176] **(Figure 7)**. Though these are only snippets of the actual activation leading to these results, there are many other proteins involved, as is the crosstalk of the signalling pathways leading to their activation.

FGF and Wnt signalling share concomitant roles during muscle development particularly regarding the formation of the paraxial mesoderm. As previously mentioned in section 1.2.1, Wnt/FGF are needed for the expression of transcription factors *T*[44], *Tbx6*[45], and *Msgn1*[46], all of which are needed for proper patterning of the paraxial mesoderm. Mice with mutations in *Msgn1* fail to develop the posterior presomitic mesoderm and as a result develop a large undifferentiated tail bud[177]. Furthermore, in the absence of either FGF[48] or Wnt[44] ligands, ectopic neural tissue forms and leads to a truncated mouse embryo. Thus, Wnt/FGF are needed for the specification of neuromesodermal progenitor cells towards a mesodermal fate as opposed to a neural one[178]. Late in embryonic development and during myogenesis, however, both pathways serve a more antagonistic role. Wnt functions to

promote myoblast fusion and differentiation[179] whereas FGF promotes myoblast proliferation and inhibits differentiation[180]. Although, during adult muscle regeneration, satellite cell activation requires signalling from both Wnt and FGF alongside IGF and HGF[179,181]. Interestingly, FGF shares another antagonistic role with BMP during the patterning of the paraxial mesoderm. This is evident in mouse embryos where the loss of BMP signalling showcased an expanded paraxial mesoderm, which is rescued and returned to normal by inhibiting FGF signalling[180].

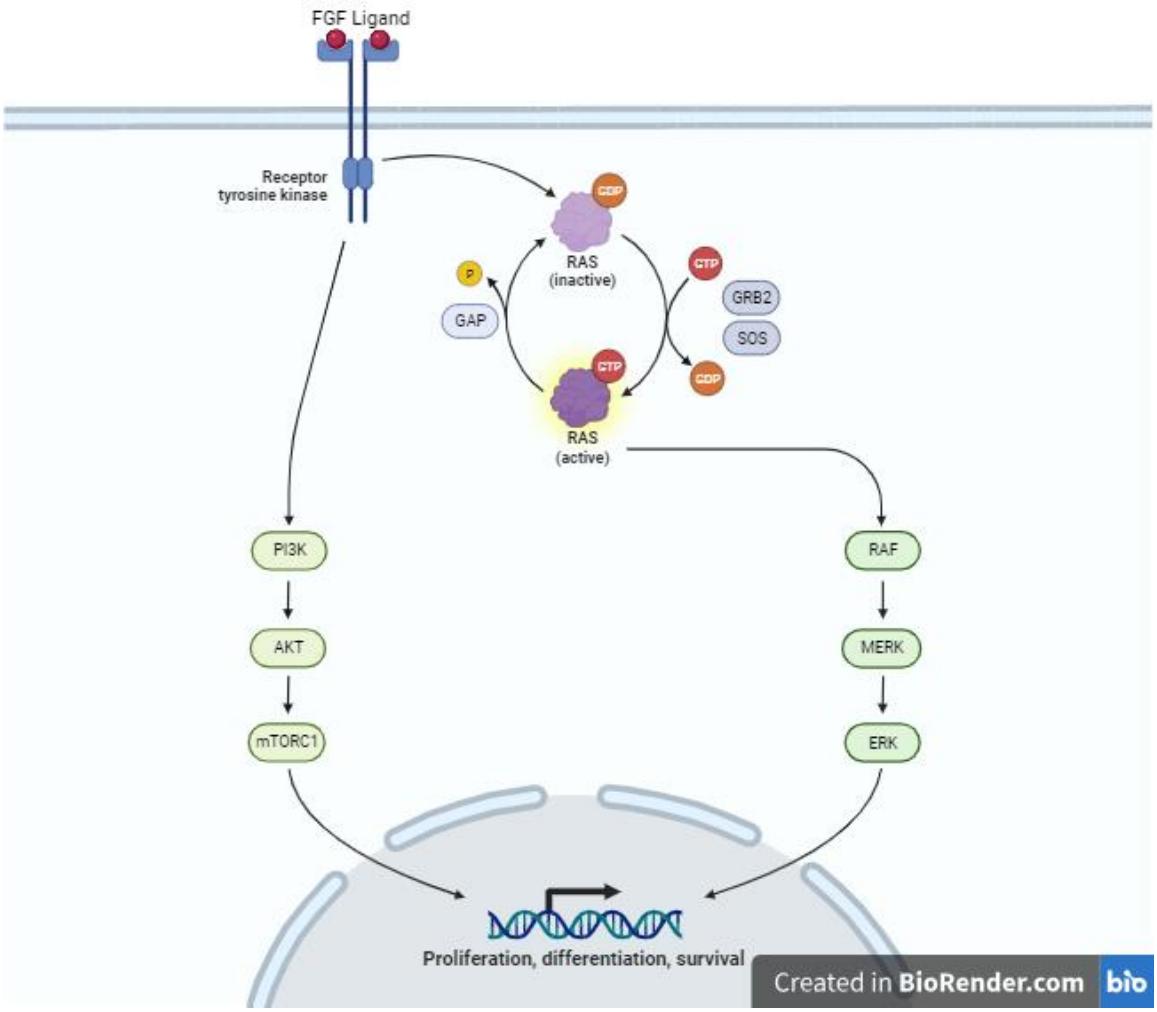


Figure 7: The Fibroblast growth factor signalling pathway. The FGF ligand binds to the RTK receptor resulting in its autophosphorylation and conversion of inactive GDP bound Ras into active GTP bound Ras by GRB2 and SOS. This results in the activation of either the MAPK or AKT pathways. In the MAPK pathway, Ras activates Raf which phosphorylates MEK. This results in the activation of ERK which helps to upregulate genes involved in proliferation. In the AKT pathway PI3K recruits and phosphorylates AKT which in turn activates the mTORC1 complex. This leads to the inhibition of genes related to autophagy. Adapted from "KRAS Signalling Pathways" by BioRender.com.

1.4.4 Notch

The Notch signalling pathway plays an important role in the formation, growth, and development of the embryo. Specifically, it is known to help regulate such processes such as neurogenesis and angiogenesis[182]. Initiation of the pathway occurs through a juxtracrine mechanism whereby the Notch receptor found on the membrane of one cell binds to the Delta ligand found on the membrane of an adjacent cell. This causes the activation of ADAM10 and γ-secretase proteases which leads to the cleaving and release of intracellular domain of Notch (NICD)[183]. NICD then translocates into the nucleus where it interacts with transcriptional factors and co-transcriptional regulators such as CBF1, recombination signal binding protein Jκ, and Mastermind. This all results in the upregulation of Notch responsive target genes[183,184] **(Figure 8).**

The Notch signalling pathway plays a critical role during skeletal muscle development as an inhibitor to muscle cell differentiation[185]. This can be seen *in vitro* through studies utilizing myoblast cell cultures in which forced activation of Notch signalling prevented differentiation into myotubes[186]. The same is true *in vivo* as determined by experiments dealing with muscle explants where asymmetric expression of Numb, an inhibitor of Notch, in proliferating myogenic progenitors was found[187]. Daughter cells expressing Numb were also found to express genes such as *Myf5* and *Desmin* which are needed for further progression in myogenic differentiation. No expression was found for markers such as *Pax3* which signify earlier pre-myoblast cell stages. Conversely, daughter cells without expression for Numb showed the opposite genetic profile[187]. Interestingly, Notch signalling is found to be activated after injury in post-natal muscle as seen by an increase in cleaved Notch1 induced by Dll1 activation[188]. Further studies with myoblast cell cultures showed that artificially activating Notch signalling led to an increase in proliferation while inhibition of Notch signalling led to a decrease in proliferation. These findings suggest that upon muscle injury Notch signalling is required for satellite cell proliferation while inhibition of the pathway is needed for eventual differentiation to take place[189]. Notch signalling also plays a role in distinguishing muscle stem cells from committed progenitors. Muscle stem cells are defined by their expression of *Pax7* and a lack of *Myf5*, whereas committed progenitors express both. Notch3 was found to be expressed highly in stem cells

while Dll1 was primarily expressed in progenitors suggesting that Notch signalling is needed to maintain proliferation in stem cells while its inhibition is required for further differentiation down the muscle cell lineage[190]. As previously mentioned in section 1.2.1, Notch signalling is also needed during somitogenesis where it specifies the borders of somites by imposing an anterior-posterior polarity[68]. This was first identified in mice embryos harbouring a mutated Dll1 that showcased dramatic muscle hypertrophy as caused by premature muscle differentiation and a limited Pax3/Pax7 expressing progenitor pool. As such, it is argued that Notch signalling prevents premature differentiation and helps to maintain a steady pool of progenitors[191].

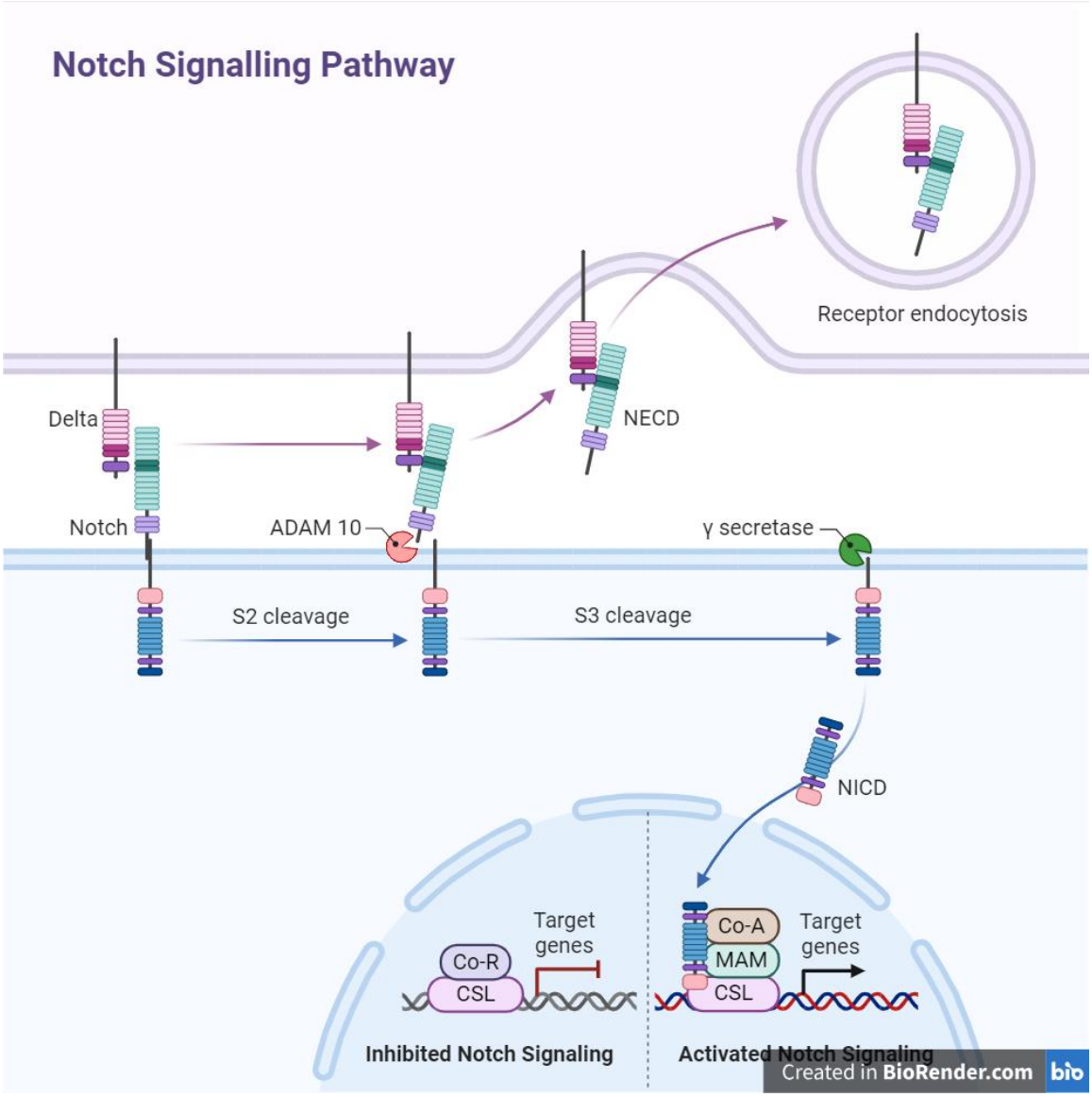


Figure 8: The Notch signalling pathway. Initiation of the pathway begins when the Notch receptor of one cell binds to the Delta ligand of an adjacent cell. This causes the activation of ADAM 10 and γ-secretase which cleave and release NICD. NICD translocates into the nucleus where it interacts with transcriptional factors and co-transcriptional factors to upregulate Notch responsive genes. Reprinted from "Notch Signalling Pathway" by BioRender.com.

1.4.5 Sonic hedgehog

The hedgehog (Hh) signalling pathway is known for transmitting information required for proper differentiation to embryonic cells during development[192]. Three different ligands exist in the pathway consisting of Desert Hedgehog (DHH), Indian Hedgehog (IHH), and Sonic Hedgehog (SHH). DHH is mainly known for its role in male germline development[193] whereas IHH regulates both bone development[194] and hematopoiesis[195]. Of interest to this research is the SHH ligand which has been heavily studied in neural development[196,197] and as of late myogenesis[7]. In the absence of the SHH ligand, the transmembrane receptor Patched (PTCH) inhibits the translocation of Smoothened (SMO) into the primary cilium[198,199] resulting in Suppressor of Fused (SUFU) isolating GLI and promoting its phosphorylation by Protein Kinase A (PKA), Glycogen Synthase Kinase 3β (GSK3β), and Casein Kinase 1 (CK1)[8]. GLI is then further ubiquitinated and as such is partially degraded by the proteasome into a form that lacks a transactivation domain while still retaining a repressor domain. As a result, the partially degraded GLI is able to translocate into the nucleus and act as a transcriptional repressor of Shh target genes[200]. Conversely, when the SHH ligand is present, it binds and inhibits PTCH thus allowing SMO to translocate into the primary cilium[201] leading to the dissociation of SUFU from GLI[202]. This allows the full length GLI to translocate into the nucleus and upregulate Shh responsive genes such as paralogues *Ptch1* and *Ptch2* which act as negative feedback for the pathway[203] **(Figure 9)**. Three GLI proteins exist including GLI1, GLI2, and GLI3. While both GLI2 and GLI3 can act as a transcriptional activator or repressor, the former is primarily an activator while the latter is a repressor[204]. Unlike GLI2 and GLI3, GLI1 does not contain a N-terminal repressor domain and thus can only act as a transcriptional activator[205].

Suppressor of Fused (SUFU), the protein of interest regarding this research, is the main inhibitor of the Shh signalling pathway. It operates by either sequestering and preventing the nuclear translocation of GLI proteins or promoting their proteasomal degradation into a transcriptional repressor of target genes[8]. In the presence of the Shh ligand, SUFU is disassociated from GLI after translocation from the primary cilium[202]. This action is caused by a complex consisting of SMO and EVC/EVC2 proteins which recruit KIF7, a

microtubule protein, into the primary cilium[206]. Intraflagellar transport proteins (IFTs) have also been identified as being involved in the dissociation of SUFU from GLI, specifically that of GLI3[202]. Furthermore, SUFU is highly regulated by multiple kinases such as with PKA and GSK3β which phosphorylate SUFU to stabilize the protein[207]. Conversely, SUFU is dephosphorylated by protein phosphatase in response to Hh signalling activation[208]. Post translational modifications have also been noted to help regulate the function of SUFU. Ubiquitination of SUFU on Lys63 leads to the binding and conversion of GLI3 into a transcriptional repressor[209]. SUFU has also been found to further repress gene expression by interacting with SAP18 and binding to DNA-bound Gli3[210].

During early embryonic development, somitogenesis is induced by Shh signalling from the notochord and Wnt signalling stemming from the dorsal neural tube through Wnt1, Wnt3a, and Wnt4[211]. Shh signalling from the notochord and floor plate is also needed to specify the sclerotome through downregulation of *Pax3* and upregulation of both *Pax1* and *Nkx3.2*[76]. Although recent studies have found that the pathway can also stimulate the formation of myotomal cells[212]. Experiments utilizing mice containing a knockout in either the *Shh*[213] or *Smo*[214] gene show as such with disrupted formation of the sclerotome and decreased expression of *Myf5* in the myotome. Furthermore, inhibition of Shh signalling in zebrafish resulted in an increase of *Pax3*/*Pax7* expressing cells in the somite but with a lack of further differentiation[215]. Conversely, ectopic expression of *Shh* in chicken embryos increased the expression of *Pax1*, a sclerotomal marker, while decreasing expression of *Pax3* in cells of the dermomyotome[216]. These studies suggest a role for the pathway in forming the *Myf5* expressing cells of the dermomyotome that have since downregulated their expression of *Pax3*/*Pax7*[217]. Further evidence of this is existence of a GLI binding site in the *Myf5* gene suggesting a possible way for activation by Shh signalling[148]. Interestingly, both Shh and Wnt seemingly have an antagonistic relationship with BMP. BMP4 signalling in the lateral plate mesoderm is needed to maintain a population of undifferentiated muscle progenitors through expression of *Pax3* and inhibition of *Myf5* and *MyoD* upregulation[218]. BMP signalling is however decreased by Wnt and Shh signalling in the dermomyotome through increase levels of Noggin[171]. This leads to the upregulation of *Myod* and myotome formation[52 220].

The Shh signalling pathway has also been implicated in myogenesis, the late and final stage of muscle development. A study utilizing C2C12 cells found that treatment with exogenous SHH ligand led to a decrease in differentiation and the generation of myotubes. Conversely, when treated with cyclopamine, an inhibitor of the pathway, differentiation was rescued and myotube formation increased[7]. A proliferation assay was also conducted with the cells under the same treatments. C2C12 cells treated with SHH showed increase levels of cell growth while those treated with cyclopamine showed decreased growth[7]. This indicates that Shh signalling is needed for myoblast proliferation while its inhibition is required for differentiation into muscle cells to take place. Further evidence of this can be seen in developmental muscle diseases involving the pathway. A study utilizing *mdx* mice, murine model organisms showcasing muscle dystrophy, found that Shh signalling was inhibited in these mice. Forced overexpression of Shh signalling lead to both an increase in myogenic cells and later the development of myofibers[219]. Less still is understood about SUFU and its role in muscle development with very limited research existing on the topic. One study posits a link between the protein and rhabdomyosarcoma, a cancer in skeletal muscle caused by constant proliferation of non-differentiating progenitor cells. Analysis of rhabdomyomas, benign tumors of muscle tissue, show high expression of Shh responsive genes such as *Gli1* and *Ptch1* whereas levels of SUFU are markedly decreased[220]. This suggests a possible role for SUFU as a regulator of Shh mediated myogenesis.

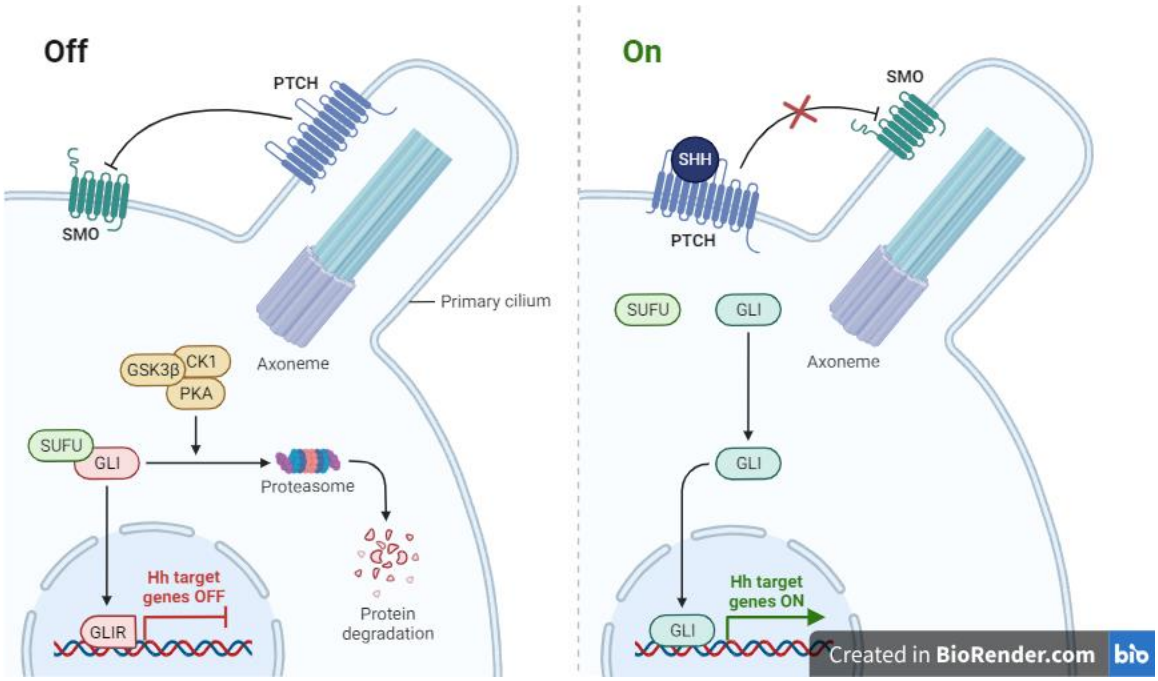


Figure 9: The Sonic hedgehog signalling pathway. In the absence of the SHH ligand, PTCH inhibits SMO. This leads to SUFU promoting the phosphorylation and degradation into a transcriptional repressor of GLI by CK1, PKA, and GSK3β. In the presence of SHH which binds PTCH, SMO is released from its inhibition which allows GLI to disassociate from SUFU in the primary cilium. GLI then translocates into the nucleus where it acts as a transcriptional activator of Shh responsive genes. Adapted from "Hedgehog Signalling Pathway" by BioRender.com.

1.5 Investigating the role of SUFU in myogenesis using cellular and molecular tools

Much research has been done on myogenesis and further uncovering the biochemical pathways involved. Still however, less is known about the Shh pathway and the intricacies of its relationship with muscle cell differentiation. Currently, it is known that the pathway must be deactivated for the successful formation of myotubules to occur. As such, the protein SUFU, an inhibitor of the pathway, is of much interest regarding its potential functional role during differentiation. With this research, I aimed to uncover the possible presence of SUFU during myogenesis and explore the effect its absence has on the process.

Given their ease of use and quick differentiation into myotubes, C2C12 myoblast cells are a convenient cellular model for use in this study pertaining to myogenesis. Alongside this, the CRISPR/Cas9 genome editing system is a molecular tool that can aid in studying the functional role of *Sufu* by knocking out the gene completely and ensuring that mutant cells used in experimentation have no traces of the protein. Various other techniques are also needed to produce proteomic and genetic data for analysis Western blotting can showcase the levels of SUFU present in differentiating cells as well as display protein markers for successful differentiation. Quantitative RT-PCR can provide the expression levels of key Shh responsive genes helping to identify the activation/deactivation of the pathway during myogenesis and whether this correlates with the presence of SUFU. Thus, this research will serve to uncover how exactly the Shh pathway is deactivated during muscle cell differentiation by further expanding on the knowledge surrounding the proteins involved in the pathway.

1.6 Rationale, hypothesis, objectives

Rationale of this study:

Myogenesis is defined as the formation of skeletal muscular tissue during embryogenesis. The differentiation of stem cells into muscle cells requires the activation/deactivation of numerous different cellular signalling pathways. Of particular interest is the one involving Sonic Hedgehog (Shh), known for its importance in embryonic development. This pathway plays a key role in muscle development with its activation promoting the proliferation of myoblasts and its subsequent inactivation promoting the differentiation of myoblasts into myotubes. Despite its role in myogenesis, there is still a lack of information regarding the specifics on how the Shh pathway is regulated during differentiation. While its deactivation is needed, the specific time, the cell stage of differentiation, and the key players in this regulation have not been detailed. As such, the aim of my research is to uncover when deactivation of the Shh pathway occurs, allowing myogenesis to continue, and how SUFU, serving as an inhibitory protein in the pathway, is involved in this deactivation. In the end, I hope that this new information will help to expand our knowledge of Shh signalling, and in particular how this pathway is regulated by SUFU during muscle differentiation.

Hypothesis of this study:

If the inhibition of Shh signalling is required to complete myogenesis, then the loss of the protein SUFU, a negative regulator of the pathway, should maintain the proliferative state of cells while preventing differentiation.

Objectives of this study:

1. Catalogue the presence of Shh pathway players during myogenesis.

2. Specifically examine SUFU levels before and after induced differentiation.

3. Determine if maintaining Shh signalling affects myogenesis.

4. Induce a loss of function in SUFU, then examine the effect on the Shh pathway during myogenesis.

Chapter 2

2 Materials and Methods

2.1 Cell culture

C2C12 cells were grown on adherent tissue culture plates (Sarstedt) within Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher Scientific) containing 10% fetal bovine serum (ThermoFisher Scientific) and 1% penicillin/streptomycin (ThermoFisher Scientific). Cells were maintained at a temperature of 37°C in 5% CO_2. Media was exchanged every 2 days with cells passaged before reaching a confluency of 70%. Differentiation was induced by replacing the growth media for differentiation media (DMEM containing 2% horse serum) (ThermoFisher Scientific) and 1% penicillin/streptomycin) when the cells reached a confluency of approximately 80-90%. Differentiation over the course of 7 days was examined and media exchanged every 2 days. For experiments involving Smoothened agonist (SAG; EMD Millipore) treated cells, 200nM SAG was added into the differentiation media.

2.2 Cell microscopy

Differentiating cells were grown on coverslips coated in 0.1% gelatin. At days 0, 2, 4, and 6 of differentiation, the cells were fixed in 4% paraformaldehyde for 10 min. Coverslips containing cells were then mounted onto microscopic slides using Slowfade Gold antifade reagent and imaged at the Integrated Microscopy Facility (Biotron, Western University, London, ON) using an Inverted Nikon T12E Deconvolution Microscope at 20X magnification. NIS-Elements Software was used to capture images of the cells.

2.3 RT-qPCR

Total RNA was collected from cells at days 0, 2, 4, and 6 of differentiation using a QiaShredder kit (Qiagen) and RNeasy kit (Qiagen). The quality and quantity of RNA obtained was assessed using a NanoDrop 200c Spectrophotometer (ThermoFisher

Scientific). RNA was reverse-transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). The presence of amplicons was first evaluated through endpoint PCR conducted using a reaction mixture containing 500 nM of forward and reverse primers (**Table 1**), 25 µL of DreamTaq Green PCR Master Mix (ThermoFisher Scientific), and 1 µL of cDNA template. Samples were run using a C1000 Touch Thermal Cycler (Bio-Rad Laboratories) with the resulting products visualized on a 2% agarose gel (1X TAE) containing RedSafe (iNtRON Biotechnology Inc) and imaged on a ChemiDoc Touch Imaging System (Bio-Rad Laboratories). *Gapdh* was used as reference gene to normalize the amplification of target genes. The relative expression levels of Shh target genes (*Gli1* and *Ptch1*) were determined by quantitative RT-PCR using a reaction mixture containing 500 nM of forward and reverse primers (**Table 1**), 10 µL of SensiFast SYBR No-ROX Mix (Bioline), and 1 µL of cDNA template. Samples were run in a CFX Connect Real-Time PCR Detection System (Bio-Rad) and the results were analyzed using the comparative Ct method (2-ΔΔCt). *Gapdh* was used as a loading control.

Table 1. Endpoint RT-PCR and qRT-PCR primer sequences for target genes

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Tm
Gli1	GGAAGTCCTATTCACGCCTTGA	CAACCTTCTTGCTCACACATGTAAG	55°C
Ptch1	AAAGAACTGCGGCAAGTTTTTG	CTTCTCCTATCTTCTGACGGGT	54°C
Gapdh	ATGTTTGTGATGGGTGTGAA	ATGCCAAAGTTGTCATGGAT	50°C

2.4 Immunoblotting

Protein was collected from cells at days 0-7 of differentiation by lysing them in 500 µL of 2% sodium dodecyl sulfate buffer containing 10% glycerol, 5% 2-mercaptoethanol, and 1:200 of 1X Halt Protease Inhibitor Cocktail (ThermoFisher Scientific). The protein samples were then sonicated and quantified using a DC Protein Assay (Bio-Rad). Approximately 10 µg of protein were loaded and separated on 6-10% polyacrylamide gels for 120 V for 1.5 h. Following this, the proteins were transferred to PVDF membrane (Bio-Rad) at 20 V in 4°C overnight. Membranes were then washed with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and then placed in TBS-T containing 5% skim milk

for 1 hour. After extensive washing, the membranes were incubated with primary antibodies in TBS-T containing 5% skim milk at 4℃ overnight. The primary antibodies used were SUFU (1:1000; Santa Cruz Biotechnology), MYOD (ThermoFisher Scientific), MYOG (1:1000; ThermoFisher Scientific), MHC (1:1000; ThermoFisher Scientific), and ß-ACTIN (1:1000; Santa Cruz Biotechnology). Following incubation, membranes were washed and then incubated with host-specific HRP-conjugated secondary antibody (1:10,000; Sigma) in TBS-T containing 5% skim milk for 2 hours. Membranes were washed once more and then incubated in Immobilon Classico Western HRP Substrate (Millipore). A Chemi Doc Touch System (Bio-Rad) was used to image membranes and ImageLab Software (Bio-Rad) was used to perform densitometric quantification on the bands of interest.

2.5 Generation of Sufu deficient cell lines

A pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene plasmid) with sgRNA for *Sufu* cloned into the vector (**Table 2**) was provided by Dr. D. Spice of the Kelly Laboratory. Approximately 2 µg of the plasmid and 10 µL of Lipofectamine 2000 (Invitrogen) were incubated in OptiMEM (ThermoFischer Scientific) separately for 5 minutes. The OptiMEM/DNA and OptiMEM/Lipofectamine volumes were then combined and incubated for another 20 minutes before being added dropwise into a 2 cm adherent tissue culture plate containing approximately 200,000 suspended C2C12 cells. After 6 hours, the media was exchanged, and the now adherent cells were left to grow over night. On reaching a confluency of 70%, the cells were passaged into a 96 well plate after serial diluting to a 1 cell/50 µL concentration. Once the cells had adhered to the plate, the growth media was exchanged for selection media containing 5 µg/ml of puromycin (BioShop). After 14 days, the media surrounding the remaining cells was exchanged with regular growth media and cells were left to proliferate. Single clonal cell colonies were chosen and collected after reaching a confluency of 70%. Mutant genotypes were identified by collecting RNA from the cells, and reverse-transcribing it into cDNA. The resulting samples were then amplified using end-point PCR with primers specific for *Sufu* including the sgRNA sequence (**Table 2**) and purified using a QIAquick PCR Purification Kit (Qiagen). The amplified DNA was sequenced at the London Regional Genomics Centre and the results were analyzed using

Geneious 2021 and Synthego Performance Analysis. Further tests were conducted by immunoblotting protein lysates to identify the presence of the SUFU protein.

Table 2. Primers used for CRISPR/Cas9

***Sufu* gRNA**	GGCTGATAACTGACATGCGG		
***Sufu* PCR**	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Tm
	CTCCATCCCACCTGTAGAGTTC	AGCAAGGTTTTCCTCACTCAAG	54°C

2.6 Statistical analysis

One-way ANOVA and Dunnett post hoc analysis was used to compare the levels of protein and expression of genes across different days of differentiation. Specifically, each day of differentiation was compared to the control day (day 0). A Shapiro-Wilk's test was used to determine the normality of data while a Levene's test was used to determine if the data contained equal variance. A transformation (square root) was applied to data considered to be violating parametric assumptions. A p-value of < 0.05 was considered significant and the error bars reported on all graphs represent the standard error of the mean. All data collected and analyzed is representative of 3 biological replicates. Statistical analysis was performed using Prism Software (Prism version 9.3.1).

Chapter 3

3 Results

3.1 Shh signalling decreases during myogenesis.

C2C12 cells were differentiated over the course of 6 days. In the undifferentiated state, the cells resembled circular myoblasts (**Figure 10A**). However, by day 2 of differentiation (**Figure 10B**) these cells elongated to form myocytes. By day 4 of differentiation (**Figure 10C**) cells had fused to form myotubular structures (myotubes), that later become more prominent by day 6 (arrows, **Figure 10D**). These gross morphological changes help to confirm the differentiation process.

To test for the activation of Shh signalling during this time, samples were collected from cells at multiple days. These samples were converted to cDNA for use in RT-qPCR experiments to determine the expression of Shh responsive genes *Gli1* and *Ptch1*. In the undifferentiated state, cells showed high levels of *Gli1* expression, but this decreased by day 6 of differentiation and indicated a decrease in Shh signalling (**Figure 11A**).

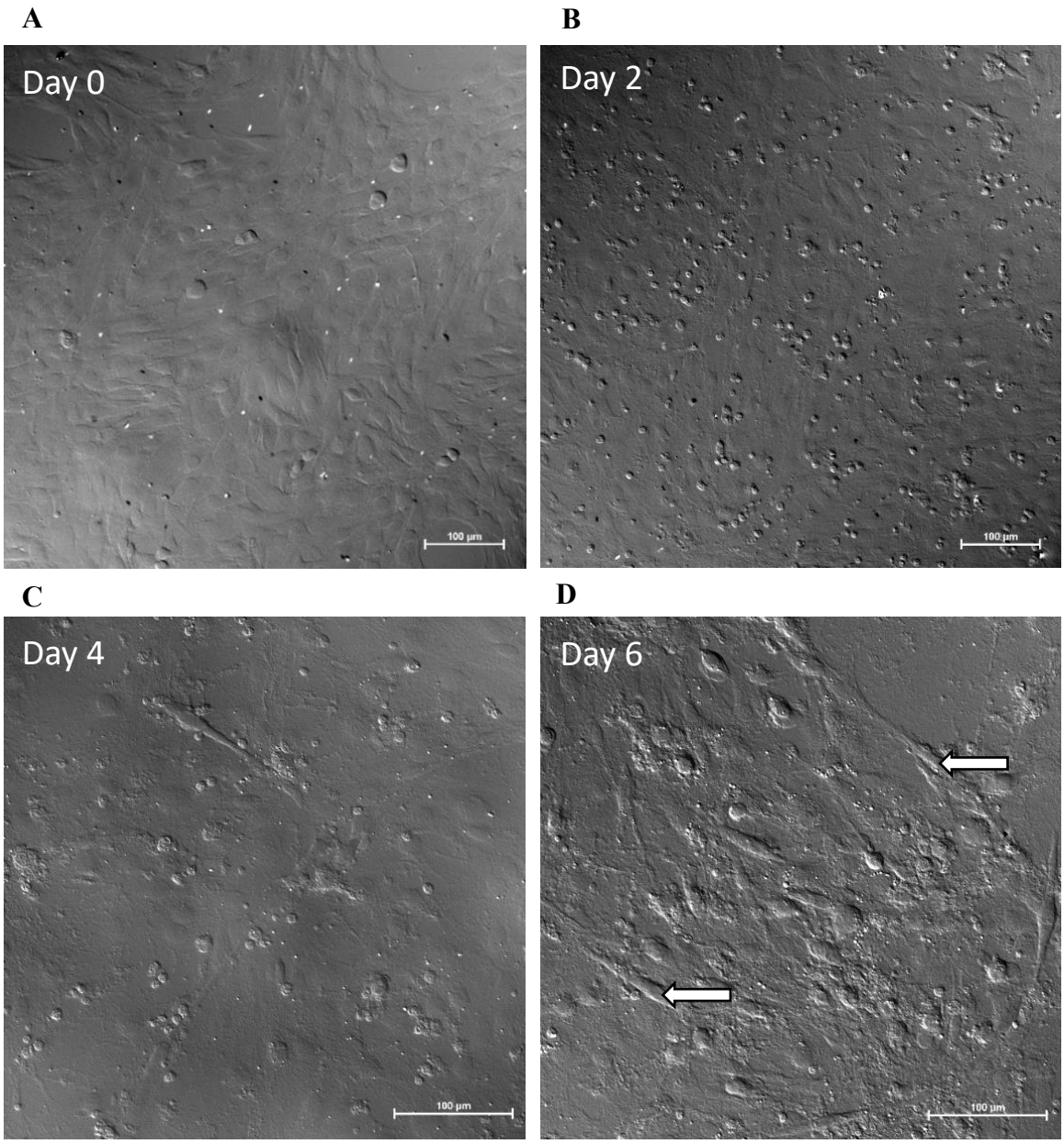


Figure 10: The differentiation of C2C12 myoblast cells into myotubes. Bright-field microscopic images showing the differentiation of C2C12 myoblast cells into myotubes at days **(A)** 0, **(B)** 2**, (C)** 4, and **(D)** 6 of differentiation. Arrows indicate myotubes. Scale bar = 100 µm.

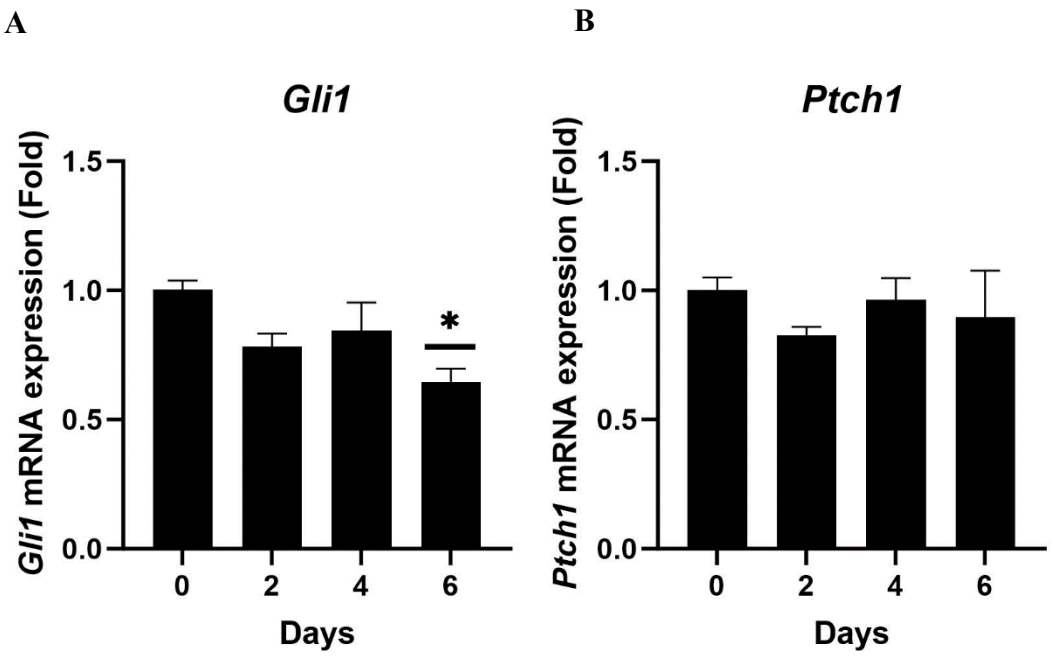
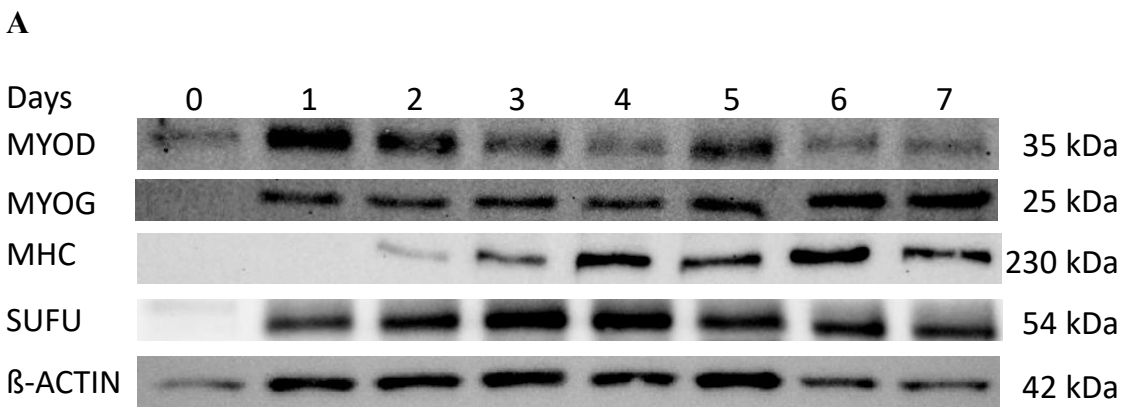


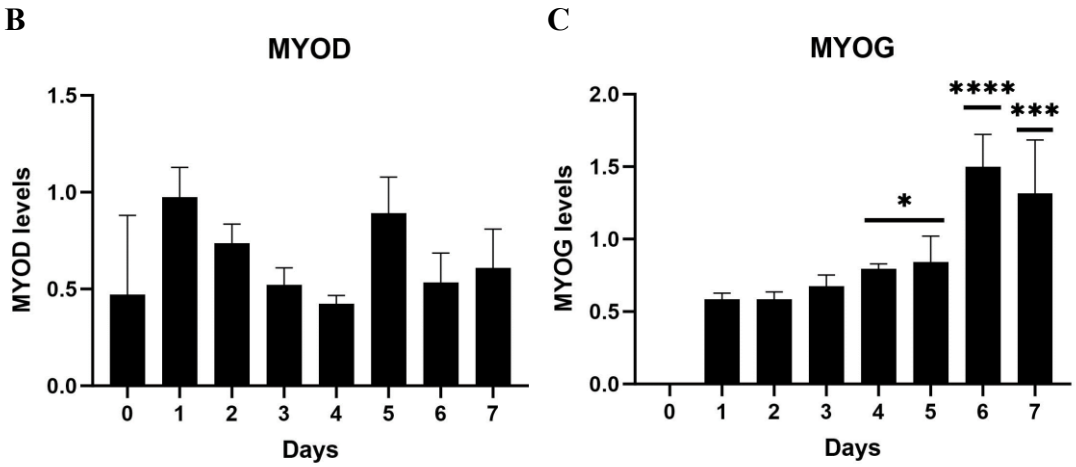
Figure 11: mRNA levels of Shh responsive genes in differentiating C2C12 myoblast cells. RT-qPCR results showing the expression of **(A)** *Gli1* and **(B)** *Ptch1* in differentiating C2C12 myoblast cells over the course of 6 days. *Gapdh* was used as a reference gene. N=3. Data was analyzed using a One-way ANOVA and Dunnett's post-hoc analysis with each day compared to the control day (day 0). Bars represent mean $\pm$ SEM. *P-value $\leq$ 0.05.

3.2 SUFU is present during myogenesis

Having established one of the markers (*Gli1*) declined during differentiation and suggested Shh signalling was reduced or absent during this time, SUFU was examined as it is known to negatively regulate Shh[8]. C2C12 protein lysates were collected at multiple days during differentiation and used in immunoblotting experiments with antibodies to detect markers of myogenesis. Since gross morphological analysis (**Figure 10**) suggested the cells had differentiated, I was expecting these myogenic markers changed accordingly and could be quantified by densitometric analysis. Myogenic differentiation 1 (MYOD), a marker of myoblast cells, was present before and after differentiation **(Figure 12A)**, and although the levels on blots appeared to change with the different days sampled, no significant differences were observed following the densiometric analysis **(Figure 12B)**. Myogenin (MYOG), a marker for myocytes, was not present in the undifferentiated state; however, it appeared starting at day 1 of differentiation and remained present at day 7 **(Figure 12A)**. Densitometric analysis showed that there was a significant increase in its levels at days 4-7 of differentiation in comparison to the undifferentiated state **(Figure 12C)**. Similarly, Myosin heavy chain (MHC), used as a marker for the myotubule cell stage, was absent in the undifferentiated state **(Figure 12A)**. However, and like MYOG, it appeared during differentiation with a significant increase noted in its levels at days 3-7 when compared to the undifferentiated state **(Figure 12D)**. These results corroborate the gross morphological changes I noted in **Figure 1**.

To test whether SUFU levels changed during differentiation, protein samples were probed with antibodies specific for SUFU and immunoblots were processed as above to detect changes in its levels. Results showed that SUFU was absent in cells that had not undergone differentiation but later was present like the myogenic markers MYOG and MHC **(Figure 12A)**. SUFU levels significantly increased when compared to the undifferentiated state at days 4 and 7 of differentiation **(Figure 12E)**.


A
Days 0 1 2 3 4 5 6 7
MYOD
MYOG
MHC
SUFU
ß-ACTIN
35 kDa
25 kDa
230 kDa
54 kDa
42 kDa


B
MYOD
MYOD levels
Days
C
MYOG
MYOG levels
Days
****
***
*

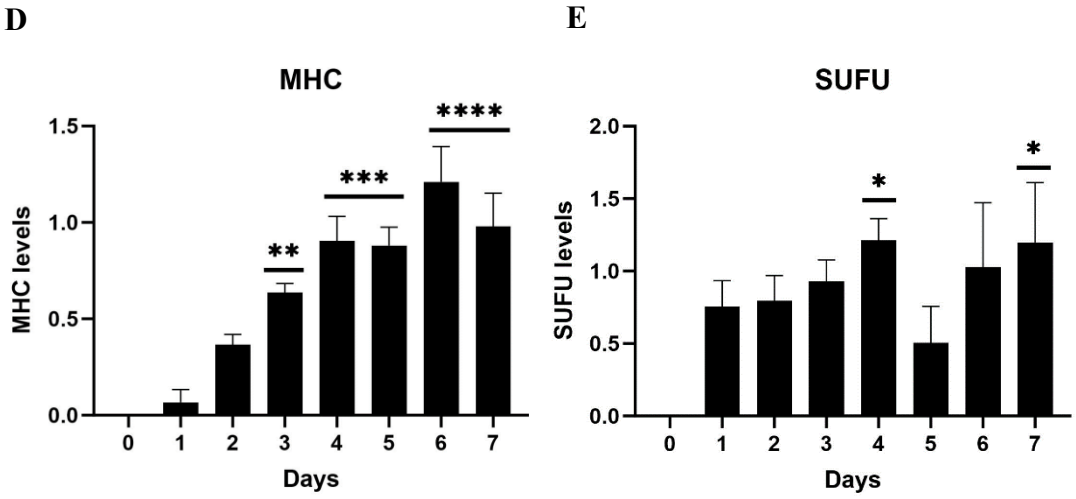

D
MHC
MHC levels
Days
**
***
****
E
SUFU
SUFU levels
Days
*
*

Figure 12: Protein levels of myogenic markers and SUFU in differentiating C2C12 myoblast cells. (A) Immunoblots showing the presence of MYOD, MYOG, MHC and SUFU in differentiating C2C12 myoblast cells over the course of 7 days. Densitometric quantification of immunoblotting results is shown for **(B)** MYOD, (**C)** MYOG, **(D)** MHC, and **(E)** SUFU. ß-Actin was used as a reference protein. N=3. Data was analyzed using a One-way ANOVA and Dunnett's post-hoc analysis with each day compared to the control day (day 0). Bars represent mean $\pm$ SEM. *P-value $\leq$ 0.05, **P-value $\leq$ 0.01, ***P-value $\leq$ 0.001, ****P-value $\leq$ 0.0001.

3.3 Constitutive activation of Shh signalling disrupts myogenesis

With confirmation of a decrease in Shh signalling occurring during myogenesis, the effect of constitutively activating the pathway during differentiation was tested. To test for this effect smoothened agonist (SAG), a chemical agonist of Shh signalling, was added to the media of differentiating C2C12 cells. Once again, samples were collected from differentiating cells at multiple days and converted into cDNA for use in RT-qPCR experiments in an effort to determine the expression of Shh responsive genes *Gli1* and *Ptch1*. It was observed that both *Gli1* and *Ptch1* significantly increased in expression by day 6 of differentiation when compared to the undifferentiated state **(Figure 13A&B)**. This indicates an increase in Shh signalling and further confirms the success of the SAG treatment in activating the pathway.

C2C12 cells treated with SAG were successful in differentiating throughout the course of the 6 days. Once again while in the undifferentiated state, the cells resembled circular myoblasts (**Figure 14A**) and later elongated to form myocytes after 2 days of differentiation (**Figure 14B**). At day 4 of differentiation **(Figure 14C)** myotubes formed which by day 6 (arrows, **Figure 14D**) became more prominent. However, the amount of myotubes generated by day 6 were observed to be greatly less than that of non-SAG treated cells **(Figure 10D)**. This gross morphology indicates that constitutive activation of Shh signalling disrupts myogenesis.

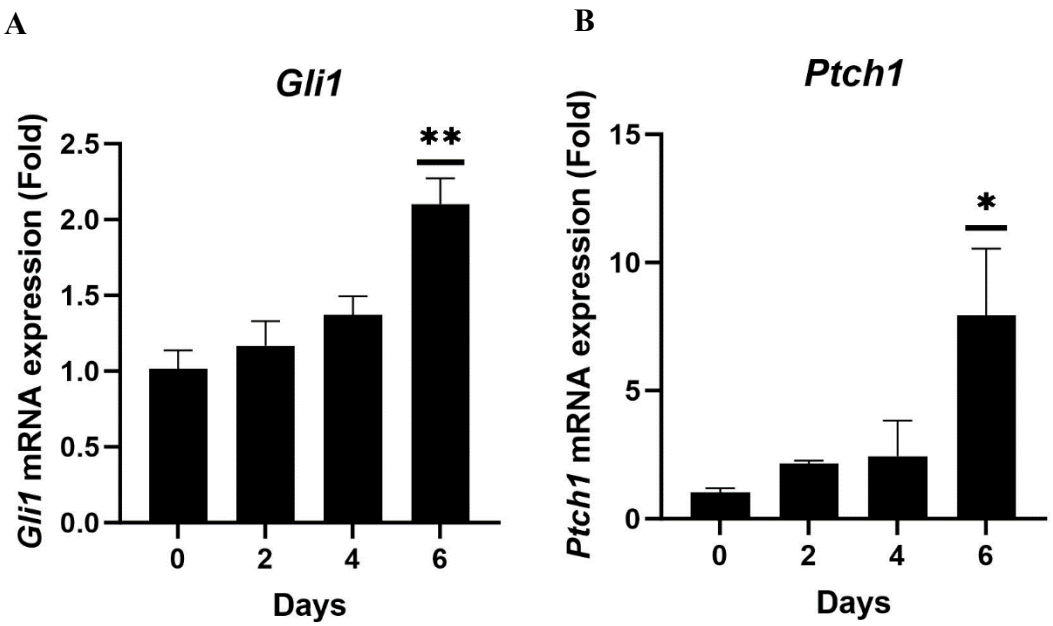


Figure 13: mRNA levels of Shh responsive genes in differentiating C2C12 myoblast cells treated with SAG. RT-qPCR results showing the expression of **(A)** *Gli1* and **(B)** *Ptch1* in differentiating C2C12 myoblast cells treated with SAG over the course of 6 days. *Gapdh* was used as a reference gene. N=3. Data was analyzed using a One-way ANOVA and Dunnett's post-hoc analysis with each day compared to the control day (day 0). Bars represent mean $\pm$ SEM. *P-value $\leq$ 0.05, **P-value $\leq$ 0.01.

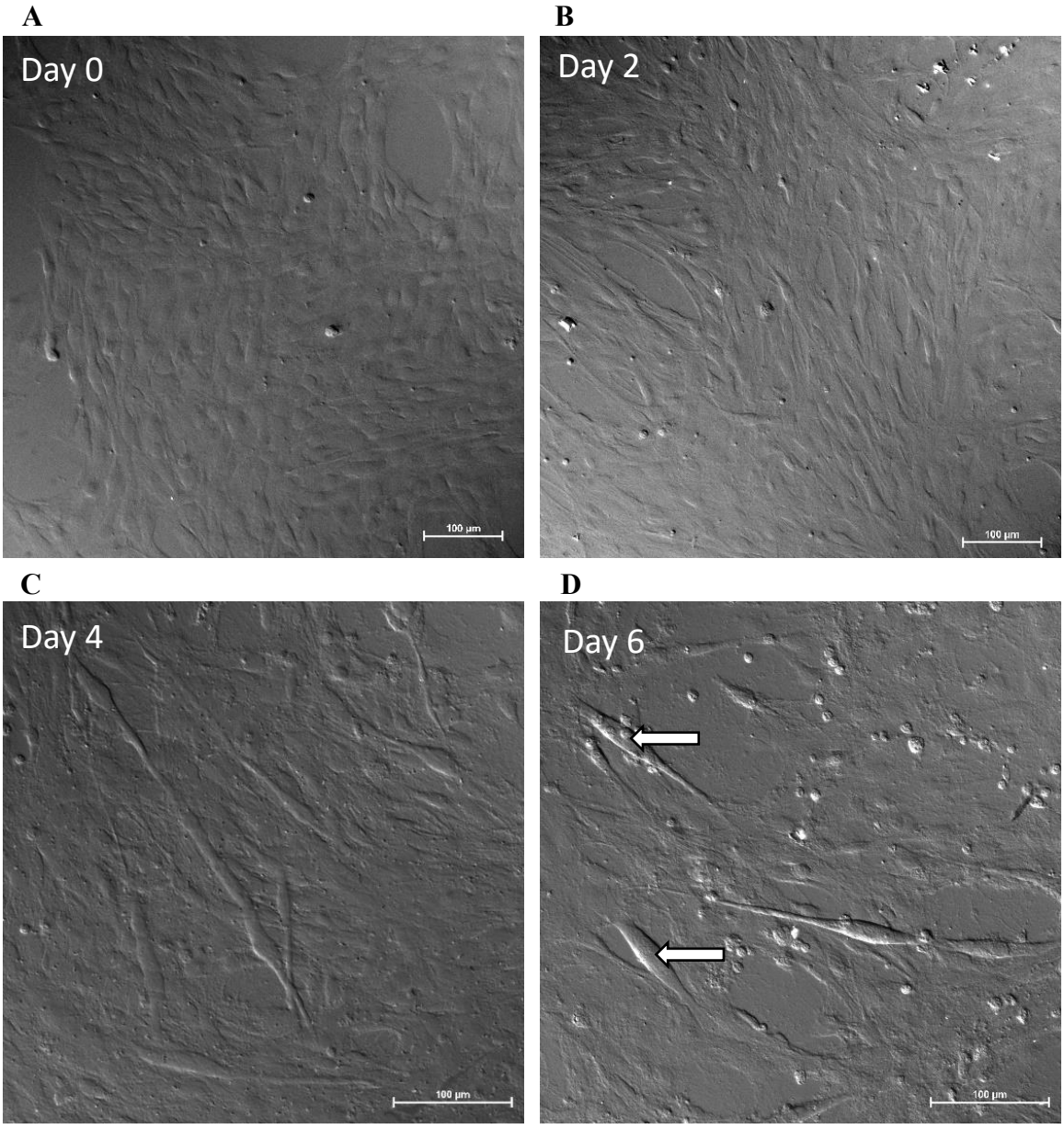


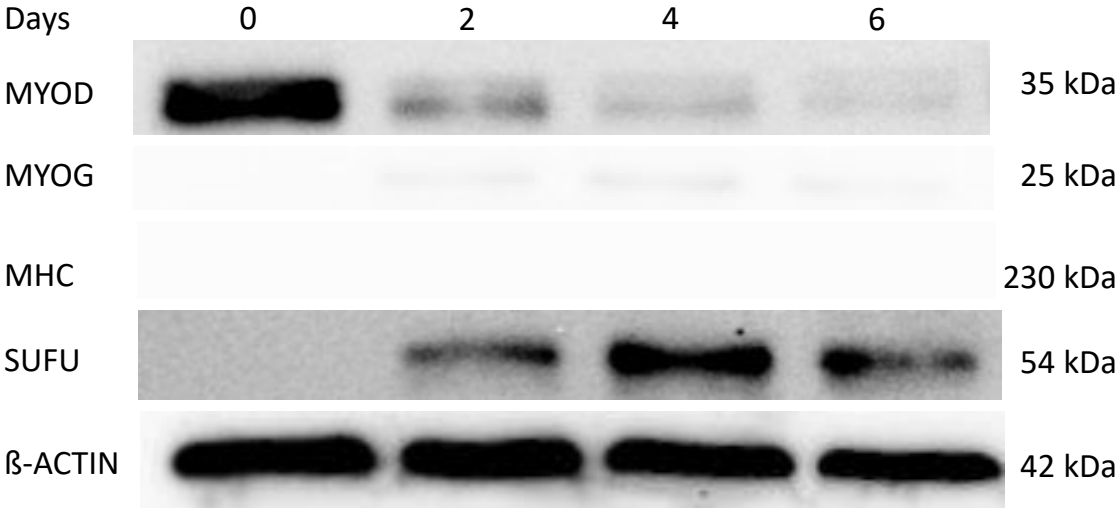
Figure 14: The differentiation of SAG treated C2C12 myoblast cells into myotubes. Bright-field microscopic images showing the differentiation of SAG treated C2C12 myoblast cells into myotubes at days **(A)** 0, **(B)** 2**, (C)** 4, and **(D)** 6 of differentiation. Arrows indicate myotubes. Scale bar = 100 µm.

3.4 Constitutive activation of Shh signalling has no effect on SUFU during differentiation

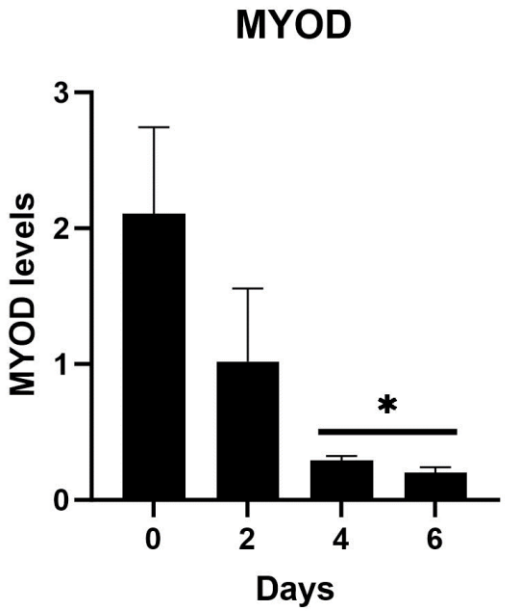
Having established that constitutive activation of Shh signalling in C2C12 cells leads to the disruption of myogenesis, the presence of SUFU was examined in these cells to determine whether the protein was also affected. Once again cell lysates of SAG treated C2C12 cells were collected at multiple days of differentiation for use in immunoblotting experiments to detect for the presence of molecular markers of myogenesis using antibodies. MYOD was found to be present in the undifferentiated state but began to decrease in levels during differentiation **(Figure 15A)**. A significant decrease was observed at days 4 and 6 of differentiation in comparison to the undifferentiated state **(Figure 15B)**. This contrasts with what was found in non-SAG treated cells which showed no difference in levels among the undifferentiated and differentiated days (**Figure 12B**). MYOG was not present in the undifferentiated state but appeared very faintly during all days of differentiation **(Figure 15A)**. Densitometric analysis showed a significant increase in levels at day 4 and 6 of differentiation when compared to the undifferentiated state **(Figure 15C)** matching what was seen in non-SAG treated cells **(Figure 12C)**. MHC was not present both before and after differentiation **(Figure 15A)** as opposed to its increase in levels during differentiation as seen in non-SAG treated cells (**Figure 12D**) thus indicating a disruption in myogenesis due to the increased Shh signalling. These results corroborate with the gross morphological changes noted in Figure 5 where SAG treated cells showed a marked decrease in myotube formation.

Interestingly, SUFU was absent in the undifferentiated state while present during all days of differentiation **(Figure 15A)**. A significant increase in levels is seen at days 2, 4, and 6 of differentiation **(Figure 15D)**. This matches closely with what was observed in non-SAG treated cells (**Figure 12A**).

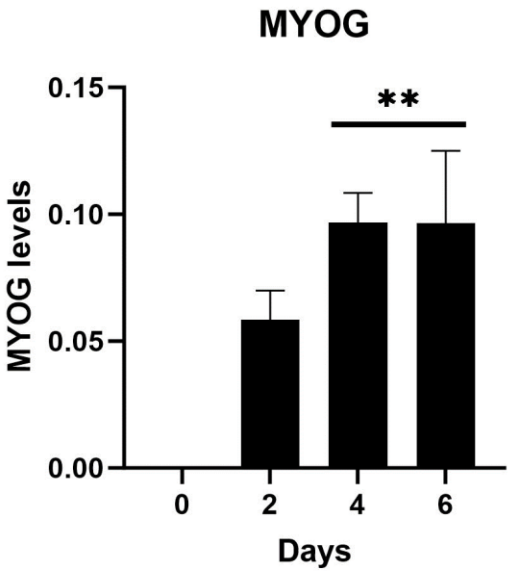
A


Days
0
2
4
6
MYOD
35 kDa
MYOG
25 kDa
MHC
230 kDa
SUFU
54 kDa
ß-ACTIN
42 kDa

B


MYOD
MYOD levels
3
2
1
0
*
0
2
4
6
Days

C


MYOG
MYOG levels
0.15
0.10
0.05
0.00
**
0
2
4
6
Days

D

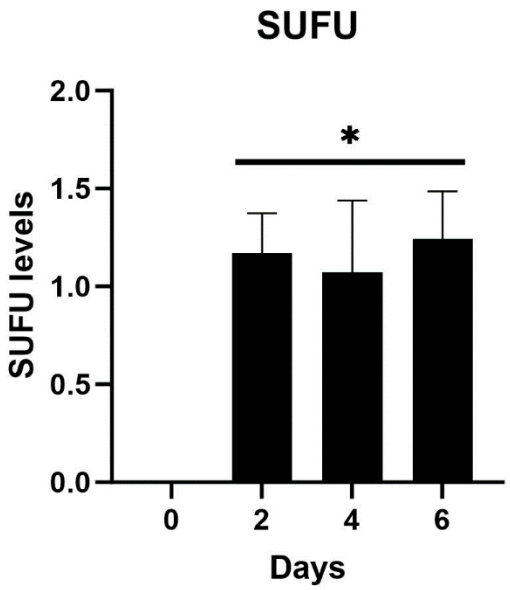
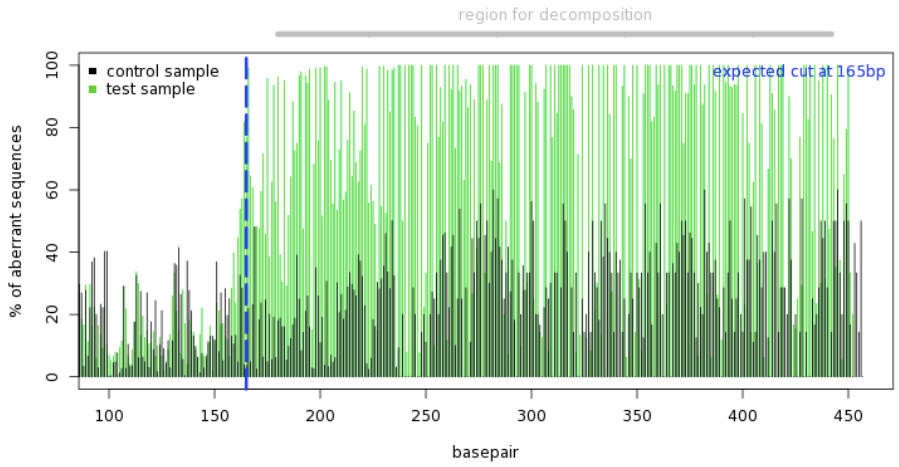


Figure 15: Protein levels for myogenic markers and SUFU in differentiating C2C12 myoblast cells treated with SAG. (A) Immunoblots showing the presence of MYOD, MYOG, MHC and SUFU in differentiating C2C12 myoblast cells treated with SAG over the course of 6 days. Densitometric quantification of the immunoblotting results shown for **(B)** MYOD, **(C)** MYOG, and (**D)** SUFU. ß-Actin was used as a reference protein. N=3. Data was analyzed using a One-way ANOVA and Dunnett's post-hoc analysis with each day compared to the control day (day 0). Bars represent mean $\pm$ SEM. *P-value $\leq$ 0.05, **P-value $\leq$ 0.01.

3.5 Utilizing the CRISPR/Cas9 genome editing system to generate *Sufu-/-* C2C12 cells

With the presence of SUFU during Shh mediated myogenesis confirmed, an attempt was made to test for the effect that a lack of the protein would have on myogenesis. The CRIPR/Cas9 genome editing system was used to generate *Sufu-/-* C2C12 cells for use in experimentation. After transfecting gRNA specific for *Sufu* into the cells and selecting for a single clonal cell line through selective antibiotic treatment, the resulting cells were analyzed to determine whether a successful knockout was created. Aberrant sequence analysis showcased an increase in the percentage of aberrant sequences after the expected cut site in the clonal cells indicating that the gRNA was successful in targeting the correct sequence **(Figure 16A)**. However, indel spectrum analysis detected 3 different possible mutant sequences within the clonal cell population, thus denoting that a single clonal cell was not successfully isolated **(Figure 16B)**. Furthermore, immunoblotting showed that the clonal cells still continued to express SUFU while differentiating **(Figure 17)**. As a result, it can be stated that *Sufu-/-* C2C12 cells were not generated.

A



B

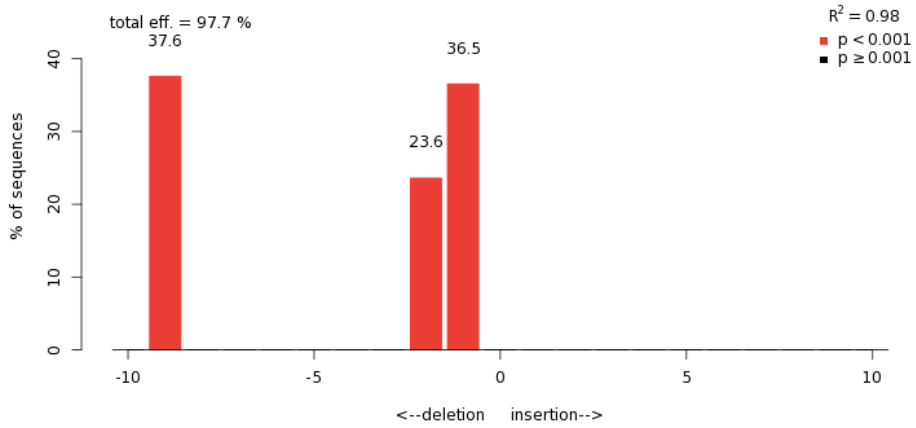


Figure 16: Genetic analysis determining the successful generation of a *Sufu-/-* C2C12 clone within a CRISPR-Cas9 generated mutant clonal pool. **(A)** Aberrant sequence analysis after sequence alignment between wild type and CRISPR-Cas9 generated mutant C2C12 cells. Percentage of aberrant sequences in the mutant is shown across a section of the *Sufu* gene including the Cas9 nuclease cut site. Black bars represent wild-type sequences while green bars represent mutant sequences. Dashed line indicates the cut sequence. **(B)** Indel spectrum analysis showing possible deletion or insertion mutations present in the clonal population after sequence alignment between wild type and CRISPR-Cas9 generated mutant C2C12 cells. Red bars indicate a significant presence of a mutant type in the population ($p<0.001$).

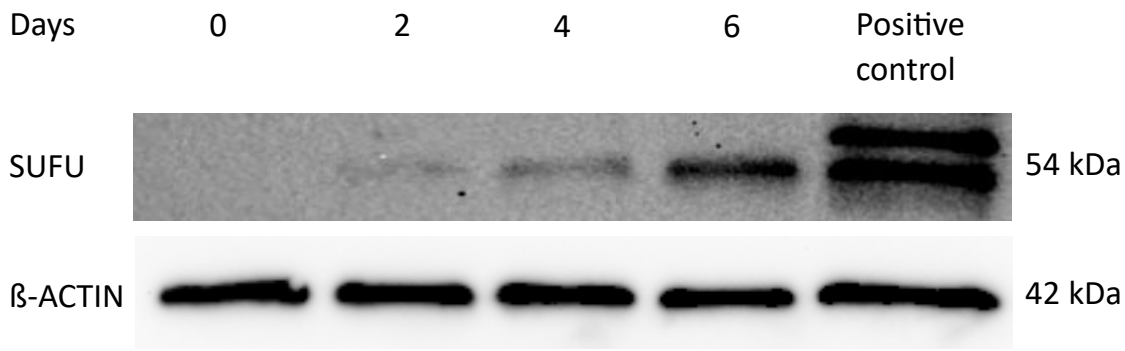


Figure 17: The presence of SUFU in CRISPR-Cas9 generated C2C12 mutant cells. Immunoblot showcasing the presence of SUFU in differentiating C2C12 myoblast cells containing a CRISPR-Cas9 generated mutant over the course of 6 days. Wild-type C2C12 cells at day 6 of differentiation were used as a positive control. ß-Actin used as a loading control.

Chapter 4

4 Discussion

4.1 Summary of findings

4.1.1 Modelling myogenesis in C2C12 cells

Myogenesis is defined as the formation of skeletal muscle tissue during embryonic development through the differentiation of stem cells into muscle specific cells[5]. The process is often studied starting at the myoblast stage, with undifferentiated progenitor cells having the ability to give rise to muscle cells. Once differentiation is initiated, myoblasts elongate to form myocytes, the initial stage in muscle cell generation[81]. Myocytes then fuse to form multinucleated cells known as myotubes, which are muscle fibers capable of contraction[83].

In vitro studies utilizing cell culture involving myogenesis often use signs of morphological change, as viewed through microscopy, to confirm the success of differentiation[221-223]. However, such methods do not allow for proper quantification of the rate of differentiation. Instead, the use of molecular markers offers a better alternative for assessing an increase or decrease in the generation of muscle cells during experiments. The most popular of these markers is myogenin (MYOG), a transcriptional activator required for myoblasts to begin differentiating[90] and myosin heavy chain (MHC), a major contractile protein of myotubes[83]. These two markers are often used interchangeably as successful indicators of myogenesis, despite signifying different stages of differentiation. Furthermore, these markers are preferably quantified by researchers through methods such as RT-qPCR[224-225]. While this approach is simpler and more efficient, it does not act as a proper confirmation of differentiation due to transcripts having no immediate direct cell function, instead simply serving as the intermediate step between genes and proteins. Essentially, expression of the gene does not indicate the presence of the protein.

To better model the process of myogenesis *in vitro*, I built a timeline to identify the different cell stages during differentiation using C2C12 myoblast cells as a model. As opposed to the quantification of gene expression, protein expression better served as molecular markers due to their role as the functional molecules of the cell responsible for its biological processes, including differentiation. To address this, immunoblotting was used to identify the presence of specific protein markers consisting of MYOG and MHC, along with myoblast determination protein 1 (MYOD), a myogenic regulatory factor highly expressed in the initial myoblast stage[88]. The resulting blots and densitometric quantification data showed a sequential pattern in the appearance of these myogenic markers. First off, MYOD was the only marker present in the undifferentiated state **(Figure 12A&B)** and served as a positive control for myoblasts that had yet to switch from proliferation to differentiation **(Figure 10A)**. MYOG appeared during the first day of differentiation **(Figure 12A&C),** which closely matches when myoblasts were observed elongating to form myocytes **(Figure 10B)**. MHC appeared weak starting at day 2 of differentiation and levels were higher later in the profile **(Figure 12A&D).** This increase corresponds to when myotube structures form in higher numbers **(Figure 10D)**. These results show that MYOG and MHC in my study cannot be used interchangeably as they appear to signify different timepoints during myogenesis. Together, MHC is better suited as a marker indicating the completion of myogenesis, whereas MYOG pinpoints when differentiation had begun.

4.1.2 Sonic hedgehog signalling in C2C12 cells

The sonic hedgehog (Shh) signalling pathway is an evolutionary conserved pathway that plays an important role in embryonic development and tissue patterning[192]. While mainly known for its importance in the development of the central nervous system[196,197], the pathway is also as equally important in the development of skeletal muscle[7]. In either case, the off state is characterized by the absence of SHH ligand and the presence of the transmembrane protein patched (PTCH) which inhibits the transmembrane co-receptor SMO and in turn prevents signalling from occurring[198,199]. For activation to occur, the sonic hedgehog (SHH) must bind to PTCH, thus inhibiting the function of the receptor and releasing SMO from its inhibition leading to further signalling[201]. One of the direct results

of this activation involves the signalling of GLI transcription factors to upregulate hedgehog responsive genes such as *Gli1* and *Ptch1*[203,204].

Previous studies have noted that Shh signalling plays an important role in multiple stages of skeletal muscle development. As one example, the development of the somite requires Shh signalling from the notochord in tandem with Wnt signalling from the dorsal neural tube[211]. Inhibition of the pathway after the formation of the somites can even prevent further differentiation of progenitors down the myogenic cell lineage as seen in zebrafish embryos[215]. The sclerotome is also formed through Shh signalling in the notochord and floor plate[76] with inhibition of the pathway leading to disrupted development in mice embryos[213,214]. Furthermore, activation of the pathway is needed for downregulation of *Pax3* in the dermomyotome[216] and the later upregulation of *Myf5* in the myotome[217] allowing progenitors to continue down the muscle cell lineage. During this time, both Shh and Wnt signalling work in tandem once again in order to antagonize BMP signalling[171] which works to maintain an undifferentiated population of cells and prevent myotome formation[218].

The pathway has also been linked to the later stages of myogenesis and eventual formation of myofibers. A *in vivo* study utilizing *mdx* mice, a model of Duchene muscular dystrophy found that Shh signalling was actively being inhibited, but over activating the pathway caused the increased proliferation of myogenic cells and generation of myofibers, essentially leading to the regeneration of the muscle[219]. The effect of Shh signalling on skeletal muscle development at the organ level helps confirm its importance in the process but does not specify how the pathway operates during this time. To explore this further, researchers identified how Shh signalling affects muscle cell differentiation utilizing C2C12 cells. When treated with exogenous SHH ligand to constitutively activate the pathway, differentiating C2C12 cells showed a decrease in the generation of myotubes. However, after the addition of cyclopamine, a chemical inhibitor of Shh signalling, differentiation is rescued and the successful generation of myotubes increased[7]. Furthermore, when they examined proliferation, treatment with exogenous SHH increased cell growth whereas the addition of cyclopamine caused a decrease[7]. Essentially, Shh

signalling promotes the proliferation of progenitor cells, whereas its deactivation is required for differentiation into muscle cells to occur.

The results of my study not only support the concept that the inhibition of Shh signalling is needed to switch precursor muscle cells from proliferation to differentiation, but also provides tangible evidence of the pathways deactivation during myogenesis occurs because of the loss or appearance of genetic markers such as *Gli1* and *Ptch1*. To demonstrate this, C2C12 cells were differentiated over one week and nucleic acid lysates were collected at multiple days for use in RT-qPCR and immunoblotting experiments. The expression of Shh-responsive genes *Gli1* and *Ptch1* were analyzed to determine the activation or deactivation of the pathway during differentiation. As opposed to the protein markers used to determine successful differentiation, mRNA/gene expression levels were used to identify Shh signalling as the upregulation of Shh responsive genes alone would indicate that the pathway is activated. The results showed a decrease in *Gli1* over the course of differentiation with a significant drop in levels by day 6 **(Figure 11A)**. Unexpectedly, *Ptch1* showed no changes in expression throughout differentiation **(Figure 11B)**. Nevertheless, previous studies utilizing *Ptch1* as a marker for Shh signalling deactivation have shown similar results with some cases, even reporting an increase in expression[226,227]. This increase may be due to the upregulation of *Ptch1* being needed for negative feedback to inhibit the pathway. Conversely, when differentiating cells are treated with Smoothened agonist (SAG), which should keep Shh signalling on, both *Gli1* and *Ptch1* increase in expression throughout differentiation, with a significant rise in levels occurring at day 6 **(Figure 13A&B)**. My results in SAG treated cells also correlate with a disruption in myogenesis as evident by the gross morphology of the cells and immunoblotting results throughout differentiation. When compared to controls **(Figure 10D)**, SAG treated cells showed fewer myotubes **(Figure 14D)**. Furthermore, immunoblotting results revealed that SAG treated cells had no MHC signals, a marker for successful myotube differentiation **(Figure 15A)**. Thus, SAG treatment shows that Shh signalling must be inhibited in order for myogenesis to occur and as such proposes a possible role for the protein inhibitor of the pathway, SUFU, to play during differentiation.

4.1.3 The presence of SUFU in differentiating C2C12 cells

One of the natural inhibitors of the Shh signalling pathway is the protein Suppressor of Fused (SUFU). In the inactivated state of the pathway, SUFU promotes the phosphorylation of GLI transcription factors through protein kinase A (PKA), glycogen synthase kinase (GSK3), and casein kinase 1 (CK1)[8]. The phosphorylated GLI is ubiquitinated and partially degraded by the proteasome into a transcriptional repressor that blocks Hedgehog-responsive gene transcription[200]. In the activated state SMO is free to translocate to the primary cilium[201] where it causes SUFU to disassociate from GLI, thus allowing the latter to act as a transcriptional activator of Hedgehog-responsive genes[202].

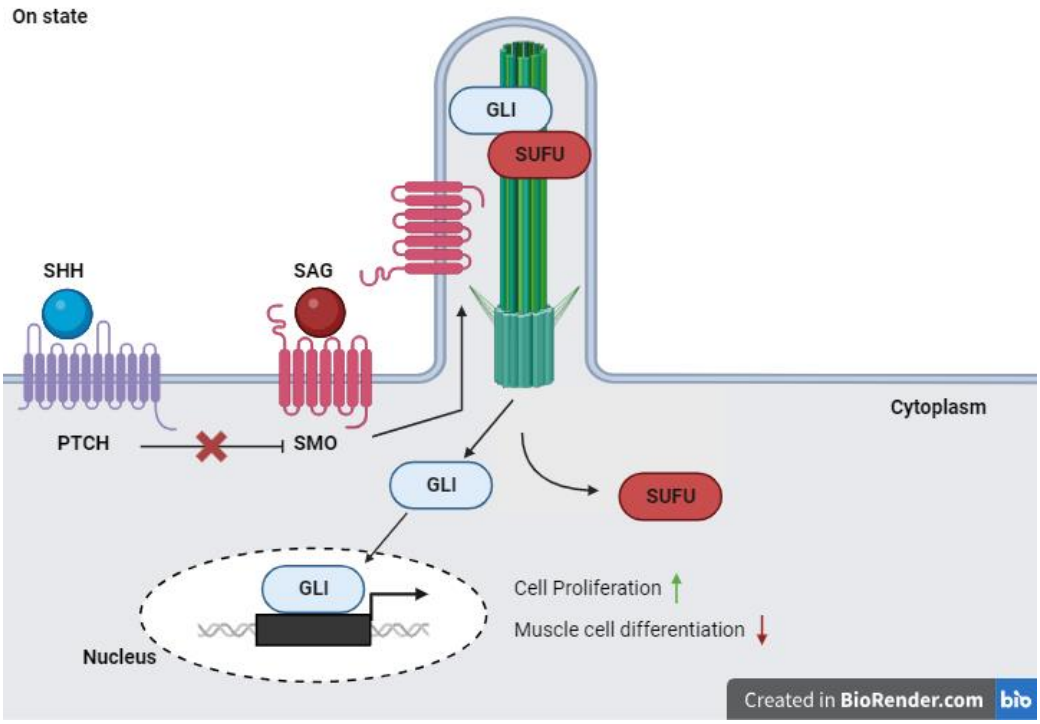
The role of SUFU as an inhibitor in Shh signalling has been shown to be conserved across many different tissues during development, primarily that of neural tissue. Experiments utilizing mouse embryos with a deletion in *Sufu* show neural tube closure defects[228] as well as a failure to develop the most ventral neurons of the neural tube[229]. The protein has been shown to play a role in the proper differentiation of retinal neurons[230], cerebellar neurons[231], and oligodendrocytes in the forebrain[232] while reducing astrocyte differentiation[233]. Beyond neurogenesis, SUFU has been implicated in both osteogenesis and angiogenesis. Inhibition of the protein improves osteogenic and angiogenic potentials in both bone marrow stromal cells (BMSCs) and human umbilical vein endothelial cells (HUVECs) through activation of Shh signalling[234]. Furthermore, overexpression of SUFU in mice was also found to inhibit skin wound healing by decreasing Shh signalling thus indicating a role in epidermal differentiation as well[235].

Though very little research exists revolving around the topic, SUFU has also been shown to play an important role in muscle development. One study speculates that *Sufu* mutations could be a cause of rhabdomyosarcoma, caused by the failure of proliferating mesenchymal cells to differentiate into muscle cells. Rhabdomyomas, benign tumors of straited muscle, caused by the disease, highly express Shh responsive genes such as *Gli1* and *Ptch1,* indicating activation of the pathway[220]. Toward that end, these researchers used immunostaining and reported SUFU was in tumors compared to the surrounding muscle tissue. Furthermore, tumors containing more differentiated muscle cells also showed a comparatively stronger signal for SUFU, and supports the notion that activation of the Shh

pathway is required for the proliferation of progenitor cells, whereas deactivation is needed for differentiation into muscle cells[220].

To better understand the connection between SUFU and Shh signalling that occurs during myogenesis, I attempted to identify the presence of SUFU throughout differentiation. C2C12 cells were differentiated and protein samples collected and used in immunoblotting experiments to detect the levels of SUFU. Results showed that SUFU was not present in proliferating cells. However once differentiation was initiated, SUFU was present at all days **(Figure 12A&E)**. Its initial appearance on day 1 coincided with the appearance of MYOG, the marker for myocyte differentiation **(Figure 12A&C)** and later MHC, the marker for myotubes **(Figure 12A&D)**. The presence of SUFU throughout differentiation is linked to the decrease in the expression of the Hedgehog-responsive gene *Gli1* **(Figure 11A)**, and would indicate that SUFU is required to deactivate the Shh signalling pathway during myogenesis. Interestingly, this SUFU pattern is similar to what was seen in non-SAG treated cells despite the increase in Shh signalling. These results indicate that the SAG treatment had no effect on the levels of SUFU protein during differentiation. Nevertheless, SUFU signals are absent in the proliferative state and present throughout the differentiative state **(Figure 15A&D)**. This pattern is seen and probably occurs after Shh signalling upregulates *Gli1* and *Ptch1* in the SAG treated cells **(Figure 13A&B)**, and the reduction in myogenesis as seen with corresponding low levels of MYOG and absence of MHC **(Figure 15A&C)**. Interestingly, studies examining the response to an overexpression in Shh signalling also report high levels of SUFU[236,237]. In this way, SUFU may be needed to act as a negative regulator during myogenesis and turn off the constitutive activation of the Hedgehog pathway.

A


On state
GLI
SUFU
SHH
SAG
PTCH
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Cytoplasm
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Cell Proliferation
Muscle cell differentiation
Nucleus
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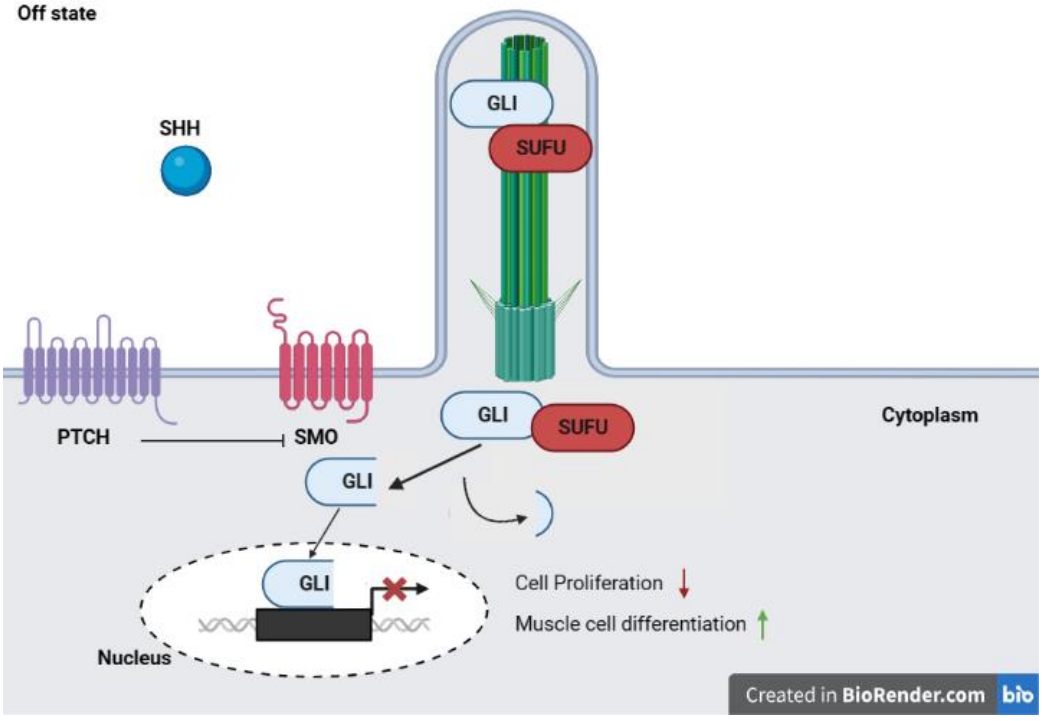
B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Cell Proliferation
Muscle cell differentiation
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Figure 9: Model for the role of SUFU during Shh signalling mediated myogenesis. **(A)** In the presence of the SHH ligand, PTCH is inhibited, and SMO is translocated into the primary cilium. This leads to the dissociation of GLI from SUFU allowing GLI to enter the nucleus and act as a transcriptional activator of Shh responsive genes. The pathway can also be activated by SAG through direct binding with SMO. **(B)** In the absence of the Shh ligand, PTCH inhibits SMO thus allowing SUFU to promote the phosphorylation and degradation of GLI into that of a transcriptional repressor of Shh responsive genes. Created with BioRender.com.

4.2 Limitations and future directions

Many different signalling pathways have been linked to the differentiation of progenitor cells down the muscle cell lineage such as with Wnt and FGF[178] signalling. Few studies, however, have been devoted to the Shh signalling pathway plays and its negative regulation that occurs in part by SUFU. This study attempted to uncover the potential role that SUFU plays in myogenesis, while also further modelling the activation/deactivation state of Shh signalling throughout muscle differentiation. Unfortunately, while SUFU was found during differentiation, its actual role could not be determined due to experimental issues with CRISPR-Cas9. Furthermore, other limitations also exist in this study and my explanations are presented below.

I believe one such limitation pertains to sample size. During this study, immunoblotting and RT-qPCR experiments were replicated a total of 3 times. In this case, N=1 represents a single biological replicate where both protein and nucleic acid samples were collected from a single petri dish of lysed cells per day of differentiation. While this is the same standard used by other studies, a small sample size can result in large variations[238]. This issue is seen in the densitometric quantification of immunoblotting results that show the levels of SUFU in differentiating C2C12 cells. Specifically, despite the trend of SUFU being present throughout all days of differentiation, there was no statistical significance in protein levels at days 1-3 and 5-6 in non-SAG treated cells **(Figure 12A&E)**. Thus, a larger sample size may have resolved this issue by reducing variation and increasing statistical power.

Another limitation pertains to the RT-qPCR results and relates to the molecular markers used for the activation/deactivation of Shh signalling. Both *Gli1* and *Ptch1* were used as markers, but only *Gli1* showed a significant decrease in expression throughout muscle cell differentiation and indicated Shh signalling was being inactivated **(Figure 11A)**. As discussed previously, *Ptch1* showed no changes in its expression **(Figure 11B)**, possibly due to its role as a negative regulator of the pathway whereby its upregulation is needed during muscle differentiation[203]. As such, it may be ideal to test for the inactivation of the Shh signalling pathway using other Hedgehog-responsive genes such as *Gli2* and *Smo,*

both of which are known to positively regulate the pathway. GLI2 being a transcriptional activator that upregulates Hedgehog-responsive genes[204] while SMO acts as the initial protein in the pathway that initiates the signalling cascade[201]. Previous studies have utilized these genes as markers and show results where their decrease in expression occurs concomitantly with the inactivation of Shh signalling[239,240].

Beyond limitations, there are also avenues to expand upon the study conducted in this thesis, particularly in regard to the initial presence of SUFU during myogenesis. The presence of SUFU during myogenesis and its absence during the proliferative stage prior to differentiation was encouraging **(Figure 12A&E)**. More specifically, SUFU levels appeared following the first day of differentiation along with MYOG, a marker for myocyte differentiation **(Figure 12A&C)**. This appearance was also before MHC, a marker for the presence of myotubes **(Figure 12A&D)**. Although not really a limitation of this study, future immunoblotting experiments should be conducted at earlier timepoints, between the initiation of and the end of the first day of differentiation. The results of such studies would therefore clarify whether SUFU appears before or after MYOG, and would indicate whether the protein is required for myocyte generation or only for myotube formation.

Overall, the results in this study only confirm the presence of SUFU during myogenesis and not its role in the process. To determine its role, I employed the CRISPR/Cas9 genome editing system to test how a loss of function in *Sufu* could affect muscle cell differentiation. Unfortunately, the objective of the study, to create a *Sufu*$^{-/-}$ C2C12 cell line, was not completed as there was a failure to generate a proper knockout cell line. I expect that while the gRNA used was successful in targeting and editing the correct sequence **(Figure 16A)**, a single clonal cell was not properly isolated because of my imprecision related to the serial dilution of cells **(Figure 16B)**. As a result, the cell line consisted of a population of multiple mutants, some of which continued to express *Sufu* and the encoded protein **(Figure 17)**, while others had the desired indels in the gene. Regardless, uncovering the role that SUFU plays during myogenesis through either knockdown or knockout studies remains an integral goal for future research.

Lastly, to further expand on the results obtained in this study it would be beneficial to repeat the experiments using different cell models. Replicating the findings in different cell lines, from a multitude of species, would ensure the robustness and validity that a common phenomenon exists for SUFU during muscle cell differentiation. Other myoblast lines that could be tested include rat cell lines such as L6 cells[137], RD cells[241], and H9c2 cells[242]. Human cell lines also exist such as HSMM cells[136] and LHCN-M2 cells[243]. Furthermore, it may be of interest to study how the Shh signalling pathway along with SUFU affects differentiation at an earlier cell stage prior to the myoblast stage. In this case, human embryonic stem cells would be of use with myogenic differentiation having been successfully induced in both H1[244] and H9[245] cell lines. That said, other stem cell lines including those representing mesenchymal stem cells should be tested as they can be reprogrammed to form skeletal muscle[246].

4.3 Conclusion

This thesis aimed to characterize the role of SUFU, a protein inhibitor of the Shh signalling pathway, during myogenesis. Throughout muscle cell differentiation, Shh signalling decreases over time as its inactivation is needed in order to switch precursor muscle cells from proliferation to differentiation. This is further supported morphologically as the cells showcase signs of disrupted differentiation when the pathway is constitutively activated. During the proliferative stage when Sonic Hedgehog is required, SUFU protein levels are below detectable levels. However, this changes with differentiation, and SUFU levels are significantly higher starting at the first day of differentiation and onwards, presumably to counteract and negatively regulate Hedgehog signalling. While results from this study have confirmed the presence of SUFU during myogenesis, further research is needed to determine its actual role in the events. Future experiments involving the creation of *Sufu*$^{-/-}$ cells are needed and is one essential feature that would address the role of SUFU in skeletal muscle formation. Understanding the role of SUFU as a potential inhibitor of Shh signalling will help to further elucidate how the pathway is regulated during muscle development.

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Curriculum Vitae

Name:	Suleyman Abdullah
Post-secondary Education and Degrees:	The University of Calgary Calgary, Alberta, Canada 2015-2020 B.Sc. The University of Western Ontario London, Ontario, Canada 2020-2023 M.Sc.
Honours and Awards:	**Mitacs Research Training Award**, 2020, $9000 **Alexander Rutherford Scholarship**, 2015, $2500 **Jason Lang Scholarship**, 2020, $1000
Related Work Experience:	**Teaching Assistant** Department of Biology The University of Western Ontario 2020-2023