SATELLITE CELLS IN DIABETES MELLITUS

IMPACT OF DIABETES MELLITUS AND ASSOCIATED CHANGES ON SKELETAL MUSCLE AND ITS STEM CELL POPULATION

By

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ABSTRACT

Diabetes Mellitus is chronic lifelong condition that continues to be a global health concern. Despite the development of insulin therapy in 1921, many diabetics are likely to endure a number of co-morbidities that impact their quality of life. Today, the search for additional diabetic therapies incorporates the investigation of various organ systems for their potential in attenuating disease development. Skeletal muscle is a striated tissue that is integral to metabolism, movement, and overall wellbeing, yet its significance to Diabetes Mellitus remains understudied, as compared to other metabolic tissues. Previous work has identified that diabetes promotes adverse changes to skeletal muscle physiology, function, and morphology, contributing to a complication referred to as diabetic myopathy. The capacity to adapt to changing internal and external cues, as achieved through skeletal muscle plasticity, permits the maintenance of skeletal muscle health; a term encompassing its metabolism, function, and/or structure. This malleability is primarily regulated by the function of muscle progenitor stem cells, referred to as satellite cells. While past research has shown that satellite cells are hindered in various diabetic states, the precise mechanisms through which these observations occur remain to be elucidated. The data presented herein identify impaired satellite cell activation in two sub-types of diabetes (Pre-Diabetes and Type 1 Diabetes), and shows that such results are mediated by alterations to intrinsic signalling cascades. Additional insight into a potential unifying mechanism mediating this response led to the identification of Lipocalin-2 and its influence on satellite cell function and muscle plasticity. The results uncovered in

these studies have enhanced our understanding of the response of satellite cells in diabetes, and have identified a prospective therapeutic target for the attenuation of diabetic myopathy.

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Billy, I am so glad that we were able to complete this journey together, and am excited to see what the future has in store.

Finally, I am indebted to the mice sacrificed, and the human participants involved.

PREFACE

This thesis is a "sandwich" style thesis. Chapter 1 provides a review of the concepts and ideas relevant to this thesis in the form of a general introduction. Chapters 2, 3, and 4 have been published (Chapters 2 and 3) or will be submitted for publication in a peer-reviewed journal (Chapter 4). A preface that describes the significance of each work is found at the beginning of each chapter, along with the individual contributions of all authors included in the work.

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LIST OF ABBREVIATIONS AND SYMBOLS

ADAMs: A Disintegrin And Metalloprotease

AGE: Advanced Glycation End Product

ANOVA: Analysis of Variance

ATP: Adenosine tri-phosphate

AU: Arbitrary Unit

BSA: Bovine Serum Albumin

CAV1: Caveolin-1

CON: Control

CTX: Cardiotoxin

CME: Crushed Muscle Extract

DAPI: 4,6-diamidino-2-phenylindole

DAPT: *N*-[2*S*(3,5-Difluorophenyl)actyel]-L-alanyl-2-phenyl-1,1-dimethyletyl -ester glycine

DIO: Diet-Induced Obesity

DLL1: Delta Like Ligand 1

DMS: d-erythro-N, N-dimethylsphingosine

ECM: Extracellular Matrix

EDL: Extensor Digitorum Longus

ERK1/2: Extracellular Signal-Regulated Protein Kinase 1 and 2

GP: Gastrocnemius-Plantaris

GPS: Gastrocnemius-Plantaris-Soleus

H&E: Hemotoxylin and Eosin

HFF: High Fat Feeding

HFD: High Fat Diet

HGF: Hepatocyte Growth Factor

IMCL: Intramyocellular Lipid Content

***Ins2*$^{\text{Akita+/-}}$:** Heterozygotic C57BL6/J-Ins2Akita/J

LCN2/NGAL: Lipocalin-2/Neutrophil Gelatinase Associated Lipocalin

MMP: Matrix Metalloproteinase

MYH3: Embryonic Myosin Heavy Chain/Myosin3

MYF5: Myogenic Factor 5

MYOD: Myogenic Differentiation

ND: Normal Diet

NFκB: Nuclear Factor Kappa-Light-Chain-Enhanced of Activated B Cells

NICD: Notch Intracellular Domain

NIDDM: Non-insulin dependent diabetes mellitus

PAI-1: Plasminogen Activator Inhibitor-1

SC: Satellite Cell

STZ: Streptozotocin

TA: Tibialis Anterior

T1D/T1DM: Type 1 Diabetes Mellitus

T2D /T2DM: Type 2 Diabetes Mellitus

TNF-α: Tumour Necrosis Factor Alpha

uPA: Urokinase Plasminogen Activator

WT: Wild Type

CHAPTER 1: GENERAL INTRODUCTION AND OBJECTIVES

PREFACE

Over the past three decades, the number of adults diagnosed with Diabetes Mellitus has doubled, with these values continuing to rise worldwide (1). In the study of diabetes, skeletal muscle is considered an integral facet based on its function as the largest metabolic organ in the body (2), as well as its overall contribution to whole-body metabolic capacity (3). Skeletal muscle is subject to a number of unfavourable changes in diabetes progression; a complication referred to as 'diabetic myopathy'. A specific feature that may contribute to diabetic myopathy is the inability to appropriately adapt to physiological stressors/stimuli. A primary contributor to this plasticity of skeletal muscle is the resident muscle progenitor cell population, termed satellite cells (SC). Comprehensive studies on the effect of the diabetic environment on SC content and/or function remain few and far between.

This introductory chapter presents a summary of findings related to the major topics investigated in this thesis, and is divided into two main parts. First, we review the pathophysiology of diabetes and its relevance to diabetic myopathy, and then present literature on the regenerative capacity of skeletal muscle, especially within various diabetic conditions. In the second half of this chapter, literature is provided regarding SCs in diabetes, insight on various factors influencing SC function in diabetes, as well as an introduction to Lipocalin-2, a potential regulator of SCs and muscle repair.

1.1 Diabetes Mellitus

Diabetes mellitus is a multifaceted metabolic disorder originating from the inability to maintain glucose homeostasis. It is predicted to affect more than 422 million people worldwide, with these numbers increasing annually (1). Two major categories of diabetes currently exist, and are divided based on their dependence (Type 1 Diabetes Mellitus; T1D), or independence (Type 2 Diabetes Mellitus; T2D) on exogenous insulin.

Type 1 Diabetes is as an autoimmune disease leading to the destruction of the pancreatic β-cells and a resultant inability to produce insulin. Current estimates indicate that 20 million people worldwide are affected by T1D (4). It is surmised that T1D occurs in genetically susceptible individuals that become exposed to an environmental trigger that leads to the autoimmune destruction of pancreatic β-cells (5). Clinical symptoms are not observed until about 80-90% of the β-cells have been destroyed (5), with onset typically observed in children (4-5 years of age) or adolescents (6).

Type 2 Diabetes (T2D), also known as non-insulin dependent diabetes mellitus (NIDDM), is characterized by the inability to effectively respond to insulin, referred to as a state of insulin resistance (7,8) that is attributed to adverse changes in fatty acid metabolism (9). The etiology of T2D differs from T1D, since elevations in blood glucose levels occur as a result of reduced glucose uptake by peripheral tissues, including skeletal muscle. This, in turn, is a result of reduced insulin production in response to glucose stimulation due to a decrease in β-cell mass, as caused by augmented rates of β-cell apoptosis (10). Factors contributing to the development of T2D may be genetic, behavioral, or environmental in origin, and include a sedentary lifestyle, poor nutrition,

stress, aging, and obesity. In a majority of cases, T2D onset occurs as a result of the advancement of 'Pre-Diabetes' (11), an important predictor in the development of T2D. Pre-diabetes is a condition defined as a state of impaired fasting glucose, impaired glucose tolerance, and/or elevations in glycated hemoglobin (12). Though fasting blood glucose levels are typically elevated in Pre-diabetes (6.1-6.9mmol/L), they are not considered to be within the diabetic range (7mmol/L or higher). Pre-Diabetes presents with physiological features such as increased body mass index, central distribution of adiposity, enhanced serum lipid levels, elevated blood pressure (13), as well as a 40% decline in β-cell mass (10). Pre-Diabetes is a growing concern in our society (1), but can be eliminated with appropriate interventions in place (14-16). Consequently, the study of Pre-Diabetes and its associated complications serves as a logical place to understand and prevent T2D progression.

The loss of insulin secretion caused by the destruction of β-cells in all forms of diabetes discussed is associated with adverse metabolic changes that contribute to the development of macro- and micro-vascular complications following disease onset (17). In particular, the persistence of hyperglycemia is believed to be a contributing factor in the development of these complications (18). While there have been many diabetic complications identified and examined, the investigation of unfavourable changes to diabetic skeletal muscle remains limited, but is believed to be intricately connected to disease progression since skeletal muscle serves as a major contributor to whole-body metabolism (19).

1.2 Diabetic Myopathy

Diabetic myopathy refers to adverse changes to various aspects of skeletal muscle health; which encompasses metabolic, functional and/or structural properties of skeletal muscle. Myopathic features may negatively influence the maintenance of healthy metabolism; which is characterized by the ability to respond to energy demands, healthy muscle morphology; defined by normal muscle structure and the presence of normal muscle constituents (i.e. actin, myosin, etc.), or normal muscle function; as determined by the ability for muscles to contract when invoked by stimuli. Adverse changes to one or more of these features of skeletal muscle health likely deters the other aspects of muscle health, as all components are believed to be intricately linked (for review of healthy skeletal muscle, see 20).

In the following section, we compare and contrast work completed in rodent and human samples obtained from different models of diabetes to identify consistent features of diabetic myopathy. Furthermore, we highlight unique myopathic features observed in certain types of diabetes, and relate these features to disease progression.

1.2.1 Diabetic Myopathy in T1D

Skeletal muscle derived from T1D rodent samples demonstrate a number of deleterious structural and physiological changes that manifest into functional abnormalities. For instance, severe atrophy of fast-twitch fibers (21–24) coincides with a decline in muscle mass (25), and reflects what has been observed in human T1D samples (26–29). Altering muscle morphology influences muscle function, as results show a loss of force production both in T1D rodents (30–33) and humans (34–37). Interestingly, this

response is attributed to shifts in Ca^{2+} kinetics and Ca^{2+} ATPase activity (38), but can vary depending on the method of diabetes induction (22). Like morphology and function, metabolism of T1D skeletal muscle is also affected, however results remain inconclusive due to divergent findings obtained from different models of T1D. For instance, although the expression of lipid transporters was enhanced in T1D samples, when compared to healthy controls (22,39–41), it is unclear if this directly corresponded with changes to Intramyocellular Lipid Content (IMCL), as there are opposing findings (22,42). Additionally, some studies have noted a decline in T1D skeletal muscle lipid metabolism (22,43,44) and ATP availability (44,45), but this was not demonstrated in a genetic model of T1D (22). This ambiguity in findings makes it hard to relate rodent data to that of humans, and is further deterred by the fact that human studies assessing T1D skeletal muscle metabolism remain limited. Recently, a link between mitochondrial dysfunction and insulin resistance was identified in T1D youth (46), and coincides with previous findings (47,48). Clearly, additional studies are warranted to further characterize human T1D skeletal muscle metabolism.

Collectively, these studies demonstrate that T1D adversely influences skeletal muscle metabolism, structure, and function. Future work assessing T1D myopathy should be completed in rodent models that more closely mimic the human T1D condition to avoid confounding variables.

1.2.2 Diabetic Myopathy in Pre-Diabetes/T2D

Like T1D, reductions to glycolytic fiber size have also been identified in Pre-Diabetic and T2D rodents, as well as human skeletal muscle (49–53). However, there are

some unique myopathic characteristics that are restricted to Pre-Diabetic/T2D skeletal muscle, including an increased prevalence of hybrid fibers, (49,54), as well as the presence of ectopic lipids (55) that coincides with a decline in protein synthesis (56), and reduced intermyofibrillar mitochondrial density (57). The excess lipid content associated with T2D development facilitates the production of adipokines, factors predominantly secreted from adipose tissue, as observed in young (58) and aging (59) T2D populations, that detrimentally impact skeletal muscle metabolism (60,61). Such metabolic and structural adaptations to T2D skeletal muscle promote what is referred to as a 'metabolically inflexible' muscle that does not respond easily to changes in substrate availability (62).

In addition to metabolism and morphology, Pre-diabetic/T2D skeletal muscle function is also impacted. However, divergent findings regarding skeletal muscle function are apparent when examining rodent models of Pre-Diabetes/T2D (63–66), and may be connected to variations in muscle metabolism. Specifically, Ostler and colleagues hypothesize that an inverse correlation exists between enhanced inflammatory/glucocorticoid signalling and reduced muscle growth/function in T2D (63). In comparison, the literature regarding human T2D skeletal muscle function proves less variable, as studies have demonstrated a negative impact of T2D on skeletal muscle function. Specifically, studies have shown that the poor glycemic control associated with T2D contributes to the formation of severe neuropathy, increased protein catabolism, and an elevated inflammatory state (67,68).

Taken together, the findings of these studies on skeletal muscle morphology, metabolism, and function in diabetes confirm that diabetes hinders the maintenance of skeletal muscle health. While some myopathic characteristics, such as fiber atrophy and reduced function, were evident in all types of diabetes discussed, there were distinct metabolic characteristic (i.e. substrate usages, lipid availability) that were restricted to one form of diabetes and not the other. This suggests that though skeletal muscle metabolic capacities may differ between the types of diabetes investigated, changes in muscle structure are unified, and result in a decline in muscle function. The capacity for skeletal muscle to repair and regenerate is a distinguishing feature of this tissue, and enables it to adapt to external stimuli in an effort to sustain its function and structure. As such, evaluation of the regenerative potential of skeletal muscle diabetes is especially relevant to the study of diabetic myopathy.

1.3 Skeletal Muscle Regeneration

Skeletal muscle plasticity is a vital feature that enables this tissue to adjust to its environment either by altering the quantity and/or type of proteins expressed (69).The plasticity of skeletal muscle is exemplified in its capacity to repair and regenerate following injury. The regeneration of skeletal muscle is comprised of a degenerative and regenerative phase. In the degenerative stage, damaged myofibers rupture and undergo necrosis, while the surrounding extracellular matrix (ECM), a structure that is an integral part of the muscle remodelling process (70), is also degraded through the activity of inflammatory cells such as macrophages and neutrophils. These cells aid in the transition to the reparative phase of muscle regeneration as factors sequestered in the ECM are

degraded, released, and in turn, activated (71). Degradation is facilitated by the action of matrix metalloproteinases (MMPs), which are secreted by damaged myofibers and inflammatory cells (72,73). Skeletal muscle contains two main populations of MMPs, MMP-2 and MMP-9. These two proteases participate in the regenerative process following muscle injury, albeit through different means (74). In addition to activation factors, cytokines are also released, and attract additional inflammatory cells to enhance the turnover of injured muscle tissue. Furthermore, nerve, blood vessels, and muscle cells infiltrate the wound area to form a new infrastructure at the site of damage. Newly generated myofibers subsequently mature and attach to the surrounding ECM (75–77). Regeneration of skeletal muscle spans from several days to weeks, with the degenerative phase occurring immediately after injury, followed by infiltration of inflammatory and non-inflammatory cells that promote repair, and the subsequent rebuilding of skeletal muscle in the reparative phase that restores normal muscle function (78). Impairments at any step of this process will impede normal skeletal muscle repair. Consequently, it is important to decipher whether disease states such as diabetes impact a specific stage (or stages) of the regenerative process. The following sections provide literature regarding the regenerative response of skeletal muscle in diabetes.

1.3.1 Skeletal Muscle Regeneration in Diabetes Mellitus

1.3.1.1. T1D

Regeneration of T1D skeletal muscle has been investigated over the course of the past few decades. Initially, regeneration was examined through transplantation studies in diabetic or normal hosts. Investigators observed that regeneration was greatly hindered when normal healthy muscle was transplanted into a diabetic host, and attributed these

findings to changes in metabolic enzyme function, as well as altered skeletal muscle innervation and vascularity (79,80). Interestingly, similar results were obtained in investigations that used myotoxins as a means of inducing injury (25,32). Manipulation of ECM remodelling provides a clue to the potential origin of these impairments, as identified by our lab (25), and others (81). Dysregulation of T1D muscle repair appears to be muscle-specific, and is attributed to variations in ECM protease activity (82). Interestingly, elevated blood glucose facilitates the formation of Advanced Glycation End products (AGEs). These products have been found to associate with ECM components in sensory neurons, modifying their structure and function, and subsequently contributing to the development of T1D neuropathy (83). While evidence of this specific phenomenon in T1D skeletal muscle is currently unavailable, it is postulated that comparable changes in T1D skeletal muscle ECM composition is likely, leading to the observed changes in ECM remodelling following T1D skeletal muscle injury.

In contrast to data regarding muscle repair in T1D rodent models, a dearth of information is available on the human response. In fact, the only study to date is taken from the assessment of rotator cuff repair following rotator cuff tears in 30 T1D and non-diabetic patients. As predicted, T1D patients demonstrated poor repair and wound healing compared to healthy patients (84). Evidently, more information regarding the repair and regeneration of human T1D skeletal muscle is warranted to determine whether associations can be made between the muscle regenerative response in T1D rodents and humans.

1.3.1.2 Pre-Diabetes/T2D

The regeneration of skeletal muscle has been examined using various models of diet-induced obesity (DIO), a model of Pre-Diabetes that includes the provision of a high fat diet (60%) to mice for a long or short term period. Unfortunately, these results remain somewhat inconclusive due to the diversity of studies investigated. For instance, three weeks of high fat feeding in young mice led to a decline in the repair of the Tibialis Anterior (TA) following cold-injury (85). Similarly, mice subjected to 12 weeks of high fat feeding also demonstrated impaired TA muscle regeneration that was linked with increases in inflammatory markers, and decreases in myokines and the protein synthetic pathway (86). In contrast, Nguyen and colleagues observed no difference in regenerative areas of Extensor Digitorum Longus (EDL) muscles between normal chow and high fat chow groups, but were able to identify impairments in regeneration in more extreme models of T2D (87). This discrepancy between studies is attributed to fiber type specific responses following diet-induced obesity, as more injury was observed in the TA (85,86), than EDL (87), and is supported by work completed over a longer duration of diet-induced obesity (88). Together, these data provide proof of the negative influence of Pre-diabetes/T2D on rodent muscle regeneration, and highlight the significance of ECM composition and inflammation (85–88), as well as enhanced lipid uptake (89) as prime factors that impede normal repair.

The translation of these findings to the human condition becomes difficult given the limited amount of literature available regarding the response of Pre-Diabetics and Type 2 Diabetics to muscle injury. Thus far, studies have shown that T2D subjects are less likely

to recover following muscle-related surgical procedures (90,91), thus supporting findings from the rodent studies completed.

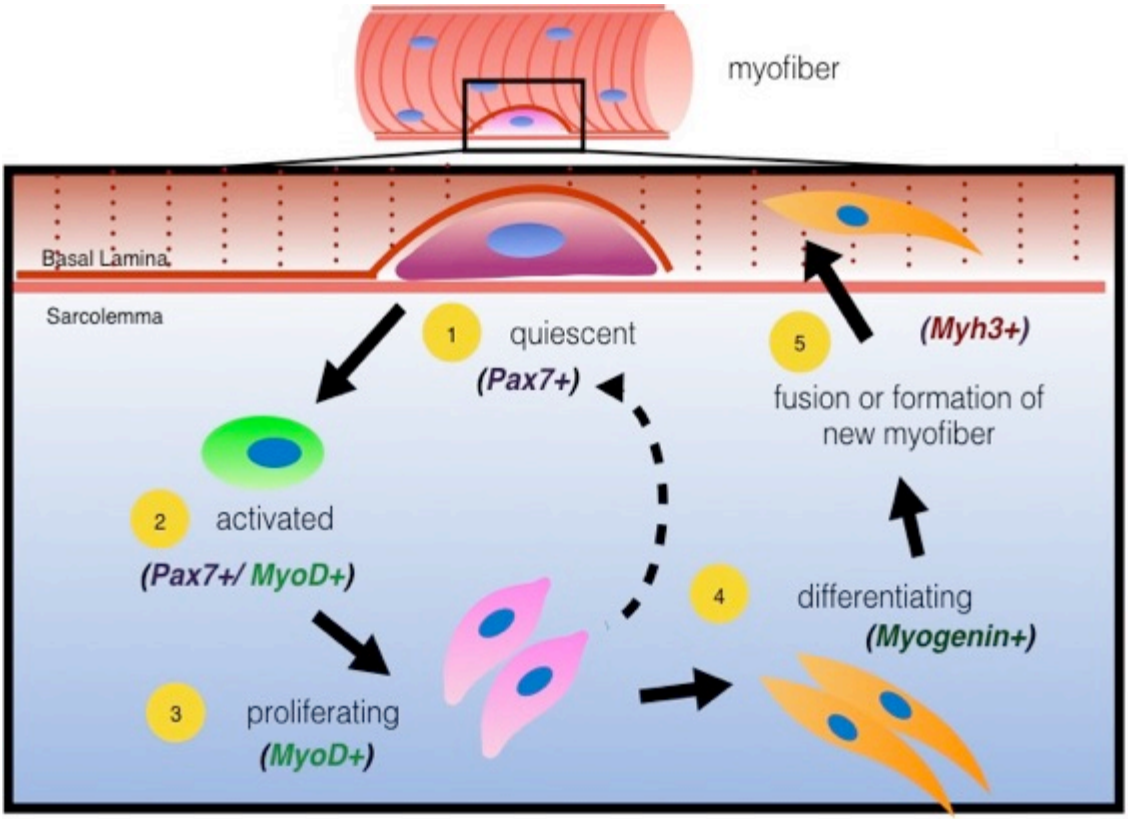
In addition to skeletal muscle metabolism, morphology and function, the literature presented here demonstrates that skeletal muscle plasticity is also negatively affected in diabetes. These studies suggest that impaired repair in diabetes may be attributed to changes in vascularity, ECM remodelling, inflammatory cell profile, and/or progenitor cell function (progenitor cell function to be discussed in sections 1.4.1 and 1.4.2). Given the relevance of SCs to skeletal repair (92), for the purpose of this thesis, we have narrowed our focus to specifically elucidate changes to SCs in diabetes. Before assessing the response of SCs in diabetes, it is necessary to discuss the fundamentals of SC function in normal skeletal muscle repair.

1.4 Role of the Satellite Cell in Muscle Regeneration

The satellite cell serves a central role in the preservation of skeletal muscle structure due its contribution to muscle repair. While other cell populations contribute to skeletal muscle repair (93–95), satellite cells are widely established as the primary players in muscle regeneration (96). This muscle stem cell population accounts for about 3-11% of myonuclei, with a dramatic decline in density observed following birth (97,98). A majority of SCs are quiescent, or inactive, but can become activated through the release of various growth factors (99,100), and/or through changes to their local environment (101). Disruption of the sarcolemma and basal lamina alters calcium availability and promotes the release of growth factors, prompting SCs to exit quiescence and enter the cell cycle (102–104). Following activation, SCs proliferate, and some will asymmetrically

divide to facilitate replenishment of the quiescent population. Those SCs committed to the myogenic lineage, termed myoblasts, differentiate into newly regenerating myofibers, or may fuse to existing damaged myofibers to promote muscle repair (76; Figure 1.1).

Figure 1.1 The Life Cycle of the Satellite Cell. SCs normally reside in a quiescent state (1), but may become activated by various stimuli, including exercise and/or injury (2). Activation promotes entry into the cell cycle, facilitating their proliferation (3). A fraction of these cells replenish the quiescent population, while the remainder differentiate into myotubes (4), and subsequently fuse with damaged myofibers, or form new myofibers (5). Various cellular markers may be used to identify the status of the SC in the myogenic process (shown in brackets).



The next section of this Introduction features literature regarding the study of SCs in T1D (1.4.1) and Pre-Diabetes/T2D (1.4.2), and is an excerpt taken from the following review article:

D'souza DM, Al-Sajee D, Hawke TJ. Diabetic myopathy: impact of diabetes mellitus on skeletal muscle progenitor cells. Front Physiol. 2013; 4:379.

1.4.1 Satellite Cells in T1D

Satellite cells from Streptozotocin (STZ)-treated diabetic mice fail to activate properly, resulting in failed regeneration following chemically induced muscle injury (105). This extreme catabolic state has previously been shown to promote the fusion of SCs to adjacent muscle fibers in T1D mice, as this is thought to promote the release of factors that function to sustain muscle integrity in this less than favorable metabolic condition (106). Furthermore, Aragno et al. (107) reported reduced myogenic regulatory factor expression and impaired differentiation in T1D-derived myoblasts. Attenuated muscle repair has also been observed in T1D mice (25,82). The impaired regeneration with diabetes was attributed to an elevation in plasma PAI-1 resulting from attenuated extracellular matrix (ECM) turnover. The delay in ECM turnover inhibited macrophage and SC migration into the damaged/necrotic regions of injured muscle. Interestingly, despite systemic increases in PAI-1, the impaired regeneration occurred in a muscle-specific pattern (82), indicating that muscles are intrinsically resistant to the T1D environment. It is becoming increasingly clear that alterations to muscle protein turnover cannot, by itself, account for diabetic myopathy. Although studies investigating SCs in T1D remains limited, evidence indicates that functionality is affected. Clinically, it is

important to appreciate that T1D-onset almost always occurs during childhood/adolescence, a period of extensive muscle growth. Thus, understanding alterations to the SC population in T1D is essential for the development of therapeutic strategies to maximize muscle health during this vulnerable time.

1.4.2 Satellite Cells in Pre-Diabetes/T2D

While studies directly assessing SC function in T2D remain limited, a number of recent investigations have evaluated SC behavior with hyperglycemia and/or lipotoxicity. For instance, 3 weeks of a high fat feeding (HFF) affected SC content and functionality, with the latter classified as the quantity of regenerating fibers present following injury (108). Hu et al. (88) demonstrated reduced muscle regeneration after 8-months HFF that was attributed to a delay in myofiber maturation, rather than SC activation or proliferation. In vitro studies have also shown that SCs incubated in high glucose medium have an increased propensity to differentiate into adipocytes (109), suggesting that SC myogenic capacity may be impacted by uncontrolled diabetes. This is further substantiated through the use of genetic models of obesity and diabetes. The Obese Zucker Rat (OZR), a model for the metabolic syndrome, displays reduced SC proliferative capacity though quiescent SC percentages remain unchanged (110); findings consistent with observed alterations to Akt signalling and myogenic regulatory factor expression (110). Similar results were obtained in transgenic (*ob/ob*, *db/db*) models of T2D. Specifically, impaired SC proliferation and activation were observed and were reflected in measurable impairments of muscle regeneration (87). A critical, but as of yet unanswered, question is the role of altered leptin signalling in mediating changes to SCs in these animal models.

Interestingly, these authors found no difference in SC function or regenerative capacity in HFF mice (87).

In addition to altered myogenic potential, SCs derived from T2D patients were found to retain a "diabetic phenotype" upon isolation and culturing. These T2D-derived SCs displayed reduced lipid oxidation (111), increased secretion of inflammatory markers leading to altered cell signalling (112), impaired glucose transport (113), and insulin-resistance (114). These modifications were based on T2D-induced epigenetic changes to muscle cell gene programming, modifying protein expression of factors essential to myogenesis, thereby permanently affecting muscle SCs (115). Taken together, these findings suggest that the degree of T2D disease severity (i.e., diet vs. genetic model) will differentially influence SC function. A more severe T2D phenotype, as is found in genetic models of T2D, results in impairments to the early stages of myogenesis (proliferation, activation), while the HFF models will alter the differentiation potential of the SCs. Finally, long-term exposure to T2D may promote detrimental epigenetic changes to SCs that will inevitably affect their functionality, and ultimately, overall skeletal muscle health.

[END OF EXCERPT]

These studies suggest that the diabetic environment negatively affects SC function, however certain gaps in the literature still exist. Firstly, some of the data collected are inferred from results obtained in the study of SC progeny (i.e. myoblasts), and not SCs directly. Additionally, it is unclear whether SCs respond similarly in both genetic and chemical models of T1D, while the specific effect of the Pre-Diabetic environment on SC

function also remains to be determined. Finally, results obtained from rodent samples should be compared and contrasted with diabetic human skeletal muscle SCs to discern whether a comparable response occurs. **To contribute to the literature, the first two studies of this thesis assess SC function in a Diet-Induced Obesity (DIO) model of Pre-Diabetes and a genetic model of T1D, with the latter study focused on addressing whether similar results are obtained when comparing SCs derived from T1D rodent and human samples.** Following these studies, it will be of interest to identify a unifying factor (or factors) that modulates SC function in the diabetic environment. In this regard, it becomes necessary to describe current pathways and proteins that regulate SC behaviour.

1.5 Distinct Variables Influencing SC Function

A wide variety of variables may be at play in the regulation of SC function in diabetes. In this dissertation, we have chosen to specifically examine three notable variables based on their control of certain aspects of myogenesis, and their relation to the types of diabetes examined.

1.5.1 Notch Signalling

Notch signalling has been implicated in a variety of processes related to the development of diverse organs and tissues (116,117). To activate the canonical Notch pathway, a Notch ligand (DSL family) will bind to one of its receptors (Notch 1-4), leading to the enzymatic cleavage of the Notch receptor, and the release of its active form, Notch Intracellular Domain (NICD) into the cytoplasm. NICD will subsequently

translocate to the nucleus where it associates with the transcriptional mediator, Rbpj (118), as well as promotes the activity of transcriptional co-activators. As a result, Notch target genes, such as Hes1, are up-regulated and influence cellular processes (for review of the Notch pathway, refer to 119).

In skeletal muscle, Notch signalling is evident during development (120–122), and continues to play a post-natal role in the regulation of SC behaviour. Targets genes, such as Hes and Hey family members that are induced by Notch activity, are particularly relevant to myogenesis as they have been shown to inactivate MyoD through the formation of heterodimers (123;Figure 1.2).

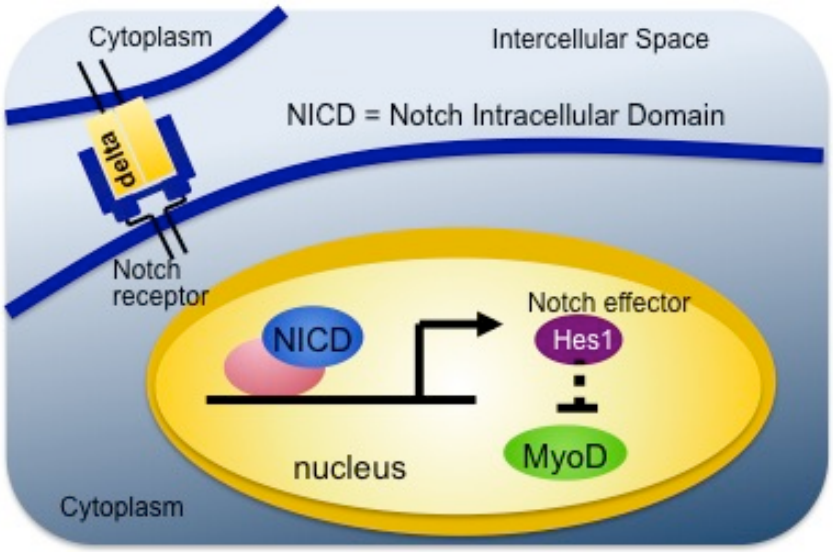


Figure 1.2. Role of Notch Activity in SC Activation. A Notch ligand binds to a Notch receptor via cell-to-cell communication to initiate the cleavage of the Notch Intracellular Domain (NICD). Once cleaved, NICD translocates to the nucleus where it promotes the transcription of Notch effectors that ultimately inhibit the myogenic factor, MyoD, to hinder myogenesis.

Based on the data, it is not surprising that Notch signalling promotes the maintenance of the quiescent SC population through regulation of SC turnover (124,125). A modification in Notch signalling is directly correlated with the availability of its ligands, as evident in studies comparing young and aged SCs (126,127). The decline in Notch activity observed in newly activated SCs is a result of the SCs physical disassociation from the myofiber, thus removing it from its ligand source (128). The ligands and receptors involved in the Notch pathway are trans-membrane proteins, and thus, cell-to-cell communication is required to facilitate Notch signalling (129,130). The correlation between SC cell cycle behaviour and the down-regulation of the Notch pathway is proof of the pivotal function of Notch in the tight regulation of myogenesis. Not only does Notch promote SC quiescence (131,132), it is also known to regulate SC self-renewal (133), based on its enhanced expression 4-5 days post-injury (128). In addition to its direct regulation of SC function, Notch signalling has also been implicated in the assembly of the basal lamina surrounding newly synthesized SCs (134). Consequently, Notch activity facilitates the replenishment and maintenance of the quiescent SC population in skeletal muscle through direct and indirect methods. While there have been studies that link Notch activity to prominent features of diabetes (135,136), additional insight is necessary to establish whether Notch activity is altered in diabetic SCs, and if this is associated with the development of myopathy.

1.5.2 Hepatocyte Growth Factor (HGF) Signalling

Unlike Notch activity, which is primarily associated with the maintenance of quiescent SCs, the activity of Hepatocyte Growth Factor (HGF) is well established in its influence on SC behaviour. HGF constitutes an 82 kDa protein that is sequestered in its latent form in the ECM of uninjured skeletal muscle fibers (137), and released in its active form following muscle injury (138). Its levels directly correspond with the degree of muscle injury incurred (72, 139, 140), as it increases in expression following myofiber isolation and culture (104). Activation of HGF signalling occurs through HGF binding to c-Met; a tyrosine kinase receptor, leading to phosphorylation of two tyrosine residues (Tyr1349 and Tyr1356; 141). An overview of the c-Met/HGF signalling pathway is shown in Figure 1.3.

HGF and its receptor, c-Met, are expressed in quiescent SCs (142), and are implicated in a variety of SC-related functions, including activation, proliferation, and differentiation (104, 143-146). This is mediated, in part, through its modulation of myogenic regulatory factors (144,146), the cell cycle (147), and SC migration and fusion following muscle injury (148).

In the context of HGF and diabetes, a recent study identified a concomitant increase in HGF and insulin resistance in DIO rats (149). However it is unknown whether the HGF/c-Met axis is dysregulated specifically in diabetic skeletal muscle, and if this subsequently impedes normal muscle regeneration. Therefore, the importance of HGF signalling to diabetic skeletal muscle regeneration remains to be elucidated.

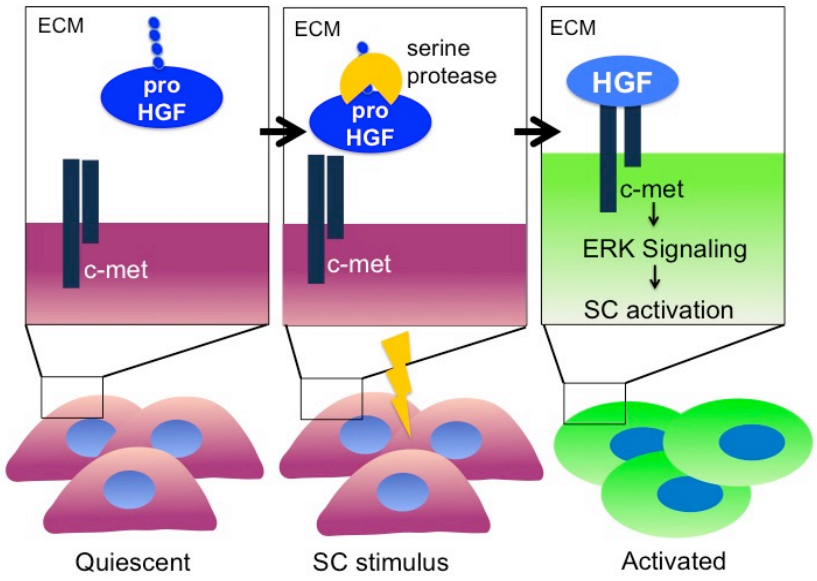


Figure 1.3. HGF Signalling and SC Activation. c-Met is located in both quiescent and activated SCs. Upon an injury stimulus, pro-Hepatocyte Growth Factor (pro-HGF) is cleaved by serine proteases found in the extracellular matrix (ECM) to promote formation of HGF. Binding of HGF to its receptor, c-Met, triggers ERK signalling to promote SC activation.

1.5.3 The Extracellular Matrix (ECM)

While Notch and HGF represent signalling pathways that may directly exert their influence on SC behaviour through ligand-receptor interactions, the extracellular matrix (ECM) presents a different type of stimulus, and is likely to influence both Notch and HGF activity in its potential to interfere with ligand availability. As previously mentioned in section 1.3 of this chapter, the ECM represents an important facet in skeletal muscle regeneration. The ECM is comprised of connective tissue made of collagens, fibronectin, glycosaminoglycans, laminins, and proteoglycans (150), and shares a complex relationship with SCs, as the ECM exerts its effects on these cells through several means. For instance, the ECM contributes to the structural integrity of the SC niche (151), as

demonstrated in studies that have assessed the response of SCs to various ECM coatings (152,153). The ECM also communicates with the SC through focal adhesions in order to dictate SC fate. A rigid ECM structure is more likely to facilitate the differentiation of SCs to a more myogenic lineage, as compared to a softer or extremely rigid structure (154). Finally, and most importantly, constituents of the ECM are directly involved in regulating the activation status of SCs, as it consists of factors bound to proteoglycans (such as HGF) that can activate quiescent SCs (155–157). Alterations in ECM composition has been observed in differing disease states (158–160), as well as ageing (161–163). Thus, it becomes necessary to determine whether specific factors that regulate ECM composition are altered in conditions such as diabetes, as it may contribute to dysregulated SC function, and as such, diabetic myopathy. *Preliminary work from our lab has identified an ECM-related protein, referred to as Lipocalin-2 (LCN2), that was also found to be up-regulated following muscle injury (Krause and Hawke, unpublished observations), and is predicted to participate in skeletal muscle repair.*

1.5.4 Lipocalin-2 (LCN2)

First discovered in human neutrophils more than two decades ago (164,165), Lipocalin-2 (LCN2), also known as neutrophil gelatinase-associated lipocalin (NGAL) or 24p3, is expressed in a variety of tissues ranging from immune cells (166) to adipose tissue (167). While not directly investigated in skeletal muscle, LCN2 is known to associate with the skeletal muscle proteinase, MMP-9, in order to enhance MMP-9 availability (168). In the context of this thesis, uncovering the role of LCN2 in skeletal muscle is of interest, considering the plethora of studies that have shown that its associated protein, MMP-9,

plays a primary role in skeletal muscle regeneration (74, 169, 170). Consequently, direct assessment of LCN2 in skeletal muscle regeneration is warranted, as it will provide a better understanding of LCN2 in skeletal muscle.

In addition to ECM remodelling, LCN2 is also linked to metabolic and inflammatory conditions (171), as it is up-regulated by pro-inflammatory stimuli (172). LCN2 has a diverse array of functions, such as its capacity to bind to hydrophobic molecules (173), as well as sequester iron (174). LCN2 participates in immunity and inflammation, and is associated with chronic kidney disease, obesity, and diabetes. LCN2 is involved in the acute innate immune response by binding to, and therefore depriving bacteria of, iron (174,175). This function of LCN2 is supported by the fact that mice deficient in LCN2 have an increased susceptibility to bacterial infection (174,176,177). LCN2 is highly expressed in adipose and liver tissue, and is secreted as a glycoprotein that modulates inflammation associated with metabolic syndrome (178). During inflammation, LCN2 serves as a chemo-attractant for neutrophils (179), and permits adhesion of neutrophils at the site of inflammation (180). Overall, these responses likely contribute to its role in chronic kidney disease (CKD), obesity, and diabetes. LCN2 is evident with increasing severity of CKD, as it associated with tubular cell damage and tubulointerstitial fibrosis (181). Transgenic mice with reduced and/or ablated LCN2 expression exhibit increased glucose tolerance (182), and improved insulin action (167, 183), and is indicative of its correlation with obesity and diabetes. In fact, its relevance to diabetes has become a topic of interest lately, however its precise function in this disease remains unknown.

1.5.4.1 Lipocalin-2 in T1D

The evaluation of LCN2 expression in T1D remains limited when considering data acquired from T1D rodent and human studies. While there have been a few publications to date examining its expression in T1D rodents, all investigations have been completed using STZ mice. Furthermore, most of these studies evaluated the potential for LCN2 in its relation to the development of diabetic nephropathy. A study published in 2012 identified that LCN2 was increased prior to classic markers of diabetic nephropathy, and is indicative of its potential as an earlier detector of this microvascular complication (184). Elevations in LCN2 expression is primarily observed in serum at 30 days following STZ injection, but is negated with the availability of insulin. Consequently, the rise in LCN2 associated with T1D development is attributed to changes in glucose homeostasis and its impact on kidney function (185), as has been recently confirmed (186).

Akin to the rodent data collected, a paucity of information is available regarding LCN2 expression in T1D humans, and is restricted to observations regarding its expression as a marker of diabetic kidney damage (187,188).

1.5.4.2 Lipocalin-2 in Pre-Diabetes/T2D

The association between LCN2 and T2D has been investigated more extensively as a number of studies have been completed in T2D rodent and human samples. LCN2 was first identified in its relation to insulin resistance based on its expression in adipocytes, and its promotion of insulin resistance in cultured hepatocytes and adipocytes (167). As previously mentioned, knockout models of LCN2 (LCN2-/-) have produced some inconsistent findings regarding its phenotype, however it is worth mentioning that a

majority of these studies report improvements in insulin sensitivity in LCN2-/- mice following a period of high fat feeding (182,183,189). The exact mechanism of action is not fully known, but appears to be associated with the expansion of distinct fat pads (189). Hence, it seems as though mice lacking LCN2 are protected against the development of insulin resistance.

Results from the investigation of LCN2 expression in human diabetic patients also indicate variability in the correlation between LCN2 and insulin resistance, as some studies report a decrease in serum levels of LCN2 (190), while others report an increase when compared to healthy controls (191,192). The diversity of these studies is such that it is hard to truly compare and contrast the results from one study to another, and may be attributed to variations in glycemic control and/or severity of insulin resistance in each group of samples investigated. In future work, it is suggested that serum concentrations of LCN2 be investigated over a time course in humans from a period of Pre-Diabetes to T2D, and correlated with the degree of insulin resistance and/or glycemic control to discern the relationship between LCN2 and diabetes pathogenesis.

In these studies, we identify that LCN2 is correlated with T1D diabetic nephropathy in the kidney, and contributes to insulin resistance in T2D via its expression in adipose tissue. However, if attempting to elucidate its significance to diabetes pathogenesis, its influence on other metabolic tissues, including skeletal muscle, must be considered. Skeletal muscle significantly contributes to whole body metabolism (3), and therefore, it is of interest to determine the effect of LCN2 on skeletal muscle. Minimal information is available regarding its expression and activity in healthy and diseased

skeletal muscle, offering an opportunity for further examination. To date, there has been only one publication, to our knowledge, that examined the association between LCN2 and skeletal muscle, but was completed within the context of obesity-related inflammation (193). Based on this, and its regulation of the skeletal muscle ECM proteinase, MMP-9 (194), future work should first aim to understand the role of LCN2 in skeletal muscle. Following this, a proper assessment of its role in diabetic myopathy may be completed. ***For the purpose of this thesis, we have concentrated on the significance of LCN2 in normal skeletal muscle remodelling via investigation of its role in SCs. In doing so, it is hoped that future studies may glean information on its role in skeletal muscle and consider this in the study of diabetic myopathy.***

1.6 Main Objective

Comprehensive studies on the effect of a diabetic environment on SC content and/or function remain scarce. Much of the research completed on the study of T1D SCs has been completed using STZ (105,107), a chemical means to induce T1D that is, in itself, a confounding factor in the study of myogenesis (195). It is therefore difficult to fully comprehend the response of SCs to the T1D state. In comparison, while there are data available regarding the impact of insulin-dependent diabetes on SCs, a majority of insulin-independent studies were completed under a T2D framework, with less data available on the sole influence of a Pre-Diabetic state. Our lab has characterized skeletal muscle structure and function following exposure to long and short term periods of DIO (66; 196), and thus a solid foundation is available for use of this DIO model in the study of the effect of Pre-Diabetes on the muscle stem cell. *As such, the purpose of this thesis is to examine the response of SCs in a genetic model of T1D (Akita) and a Pre-Diabetic model (DIO) to fully comprehend whether this progenitor muscle cell population vastly differs in function between sub-types of Diabetes Mellitus. Furthermore, identification of the mechanisms (or mechanism) involved in modulating SC behaviour is also of interest as it may permit the development of skeletal muscle-based therapies that attenuate diabetes progression.*

1.7 Specific Aims

1) Assess satellite cell content and function in a Pre-Diabetic murine model (Diet Induced Obesity; DIO);

2) Assess satellite cell content and function in a T1D genetic murine model (Ins/Akita mice, herein referred to as Akita);

3) Elucidate the significance of Lipocalin-2 (LCN2) in skeletal muscle regeneration, with focus on its impact on the satellite cell.

1.8 Major Hypothesis and Minor Hypotheses

Major Hypothesis:

*Satellite cells exposed to the diabetic environment will be adversely affected due to intrinsic changes in cellular pathways that modulate SC function, and extrinsic factors that affect the SC environment***.**

Minor Hypotheses:

1) Diet-induced Obesity (DIO) subjected to muscle injury will demonstrate impaired regeneration, due in part to modifications to the content and/or function of SCs.

2) In T1D, SC function will be negatively affected due to alterations to intrinsic cellular pathways that regulate quiescence and activation.

3) Lipocalin-2 (LCN2) contributes to skeletal muscle regeneration through its influence on SC activation, and in its absence, muscle repair is hindered.

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CHAPTER 2

Diet-Induced Obesity Impairs Muscle Satellite Cell Activation and Muscle Repair Through Alterations in Hepatocyte Growth Factor Signalling

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PREFACE

Significance to thesis

Previous research from our lab has comprehensively characterized skeletal muscle physiology following a period of Diet-Induced Obesity (DIO), a model of Pre-Diabetes. However it remains unclear of the regenerative response of skeletal muscle to DIO. The primary goal of this work was to examine the impact of DIO on skeletal muscle repair, with an emphasis placed on elucidating whether SC content and/or function would be affected. A secondary goal of this study was to identify a means through which SC activation was impacted in DIO. Pre-Diabetes is a rapidly growing health problem in society, and thus, assessing the response of SCs in DIO contributes to our investigation of SCs in Diabetes Mellitus. Furthermore, identifying a method for impaired SC function provides an avenue for the development of therapies to attenuate unfavourable changes to SCs in Pre-Diabetic skeletal muscle, and may subsequently contribute to the maintenance of skeletal muscle structure.

Author's Contributions:

Donna M. D'souza and Karin E. Trajcevski contributed equally to the preparation of this manuscript.

Donna D'souza contributed to Figures 3, 5(B, D, E, &F), 6(A&B), 7(C). She aided in the design of the study, researched data, completed microscope image analysis, performed statistical analysis, rewrote the manuscript, and worked on refining this draft and the revisions based on editorial review.

Karin E. Trajcevski contributed to Figures 1, 2, 4, 5 (A-E), 6(C), and 7 (A&B). She aided in the design of the study, researched data, completed microscope analysis, performed statistical analysis, and wrote an initial draft of the manuscript

Dhuha Al-Sajee assisted in experiments and microscope image analysis.

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Melissa M. Thomas assisted in experiments and animal care.

Judy E. Anderson contributed to the design of the study, assisted with CME experiments, and revised the manuscript.

Thomas J. Hawke contributed to the design of the study, assisted in experiments, and worked on refining drafts of the manuscript and the revisions based on editorial review.

ABSTRACT

A healthy skeletal muscle mass is essential in attenuating the complications of obesity. Importantly, healthy muscle function is maintained through adequate repair following overuse and injury. The purpose of this study was to investigate the impact of diet-induced obesity (DIO) on skeletal muscle repair and the functionality of the muscle satellite cell (SC) population. Male C57BL/6J mice were fed a standard chow or high-fat diet (60% kcal fat; DIO) for 8 weeks. Muscles from DIO mice subjected to Cardiotoxin (CTX) injury displayed attenuated muscle regeneration, as indicated by prolonged necrosis, delayed expression of MyoD and Myogenin, elevated collagen content, and persistent embryonic myosin heavy chain expression. While no significant differences in SC content were observed, SCs from DIO muscles did not activate normally nor did they respond to exogenous hepatocyte growth factor (HGF) despite similar receptor (c-Met) density. Furthermore, HGF release from crushed muscle was significantly less than that from muscles of chow fed mice. This study demonstrates that deficits in muscle repair are present in DIO, and the impairments in the functionality of the muscle SC population as a result of altered HGF/c-Met signalling are contributors to the delayed regeneration.

INTRODUCTION

In addition to our physical capacities, skeletal muscle also provides a major contribution to our whole-body metabolic control by regulating blood glucose and fatty acid (FA) levels through uptake, followed by utilization and/or storage. In fact, skeletal muscle comprises ~40% of our body weight (in non-obese subjects), accounts for approximately 1/3 of our resting oxygen uptake, and is the site for up to 90% of our exercising oxygen uptake (1). Given the extensive contribution of skeletal muscle to our whole-body metabolic capacity, one can appreciate that in individuals who have a reduced relative muscle mass, lead a sedentary lifestyle, and/or suffer from a chronic illness, there would be a negative impact on their basal metabolic rate and their capacity to adequately manage circulating lipids and glucose (2).

Our laboratory has previously demonstrated initial positive adaptations in skeletal muscle with high-fat feeding, followed by a decline in muscle health and the development of muscle insulin resistance (3-5). Additionally, previous literature using various models of obesity has already established that a number of unfavorable changes occur within skeletal muscle of obese rodents (for review, see 6). Elucidating the response of skeletal muscle to stimuli that promote damage and/or injury is relevant in the study of skeletal muscle health in obesity. Skeletal muscles from obese samples demonstrate an increased susceptibility to damage, as identified using both human (7) and animal (8) models of obesity. As a result of this, diet-induced obesity is often associated with an atrophic rather than hypertrophic response by skeletal muscle (9). Given the importance of skeletal muscle function and metabolism in the pathogenesis of obesity, it becomes clear that

understanding the capability of (and deficits to) skeletal muscle in those with diet-induced obesity (DIO) to undergo growth and repair is of paramount importance to identify potential avenues to circumvent the loss of skeletal muscle health in the obese condition.

Muscle regeneration following injury is a temporally sensitive and complex series of events with necrosis, phagocytosis, satellite cell (SC) activation/proliferation, de novo myofiber formation, and maturation all overlapping (10-12). A primary contributor to the repair process is the SC population, which is activated from its normally quiescent state in response to injury (13). Once activated, the SC begins to proliferate extensively and ultimately undergoes differentiation and fusion to generate new myofibers, or repair existing myofibers (14). To date, the few studies that have investigated the effect of DIO on muscle repair and SC functionality, though interesting and well executed, have offered conflicting results. These disparities are likely due to variances in diet composition, diet length, and injury type (15-17). Furthermore, such design differences between and within studies make it difficult to extrapolate findings as detailed analyses of many variables (e.g., derangements to metabolism, insulin sensitivity, and muscle fiber type/ composition) that impact the regenerative process in DIO mice remain undefined.

Thus, the purpose of this investigation was to provide a more detailed study of muscle repair and satellite cell functionality in a model system that has been consistently used and thoroughly characterized (3-5). We hypothesized that DIO mice subjected to muscle injury would display deficits in the transition from the degenerative to regenerative phase in the muscle repair process, when compared to standard chow-fed mice. Specifically, we hoped to demonstrate a failure for DIO muscle to properly repair itself following injury as

a result of alterations to the content and/or function of inflammatory and skeletal muscle cells that participate in muscle regeneration. Furthermore, we wanted to identify whether differences in hepatocyte growth factor (HGF) signalling contributed to any differences in SC function observed between experimental groups.

RESEARCH METHODOLOGY

Animal care. All experimental protocols were approved by the McMaster University Animal Care Committee (AUP #09-08-29) in accordance with the Canadian Council for Animal Care guidelines. Male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature- and humidity-controlled facility with a 12/12 h light/dark cycle and had ad libitum access to water and food. At approximately 10 weeks of age, following the diet period of 6 weeks, mice were divided into the following experiments: muscle injury using Cardiotoxin (CTX) for analysis of muscle mass, histology and protein expression by immunofluorescence of cross-sections (N=6 per group), single fiber isolation (N=11–27 fibers per CON group, N=18–31 fiber per DIO group), the content of crushed-muscle extract (N=4 animals per group), and proliferation of myofiber-derived myoblasts in culture (N=6–8).

Diet-induced obesity. Following a 1-week acclimatization period, animals were randomly assigned to either a high-fat diet (TestDiet, cat #58126: energy [kcal/g] from protein [18.3%], fat [60.9%], carbohydrate [20.1%]) or standard mouse chow (LabDiet 5015 Mouse Diet: energy [kcal/g] from protein [20%], fat [25%], carbohydrate [55%]). Mice were maintained on the high fat diet (DIO) or standard chow (CON) for 6 weeks before euthanasia.

Skeletal muscle injury. Intramuscular injections of CTX (Latoxan, Valence, France; 10 μmol/L) were performed as previously described (18) into the gastrocnemius-plantaris (GP), tibialis anterior (TA), and quadriceps muscles of the left leg of DIO and ND mice. Mice were sacrificed and tissue was collected at 5 or 10 days post-injury.

Tissue collection. Mice were euthanized by CO_2 inhalation followed by cervical dislocation. Injured and uninjured TA and GP muscle complexes were excised, weighed, and covered in optimum cutting temperature embedding compound, and frozen in isopentane cooled by liquid nitrogen. Quadriceps muscles from injured and uninjured legs were snap-frozen and stored in -80°C.

Histochemical and immunofluorescent analyses. Muscles were cut into 8 μm muscle cross-sections and mounted on glass slides to be stained as described below.

Picrosirius Red for collagen. Muscle sections were immersed in Picrosirius Red solution (0.1% w/v Direct Red 80 [Sigma 365548] mixed in a saturated aqueous solution of picric acid [Sigma p6744]) for 1 hr. Following this, sections were washed with 0.5% glacial acetic acid, dehydrated, cleared, and mounted. This stain enables appropriate quantification of collagen content using fluorescence microscopy, as the collagen retains the fluorescent-red stain, while myofibers appear yellow. The area of red pixels was expressed as percentage of the total injured area per muscle section.

Immunofluorescence. Sections were fixed with ice-cold 2% paraformaldehyde for 5 min at 4°C, blocked (PBS with 10% NGS and 1.5% BSA), and incubated with primary antibody overnight at 4°C (rabbit anti-dystrophin, Abcam, 1:200 dilution; rabbit anti-laminin, Abcam, 1:80 dilution; mouse anti-Myosin3, DSHB, used neat; mouse anti-Pax-7,

DSHB, neat; mouse anti-MyoD, DAKO, 1:500; chicken anti-laminin, Abcam, 1:250; mouse anti-caveolin 1, Abcam, 1:100; and rat anti- F4/80, AbD Serotec, 1:200). The appropriate Alexa secondary antibody (Invitrogen, Carlsbad, CA) was used for detection of each primary antibody, and nuclei were counterstained using 4,6-diamidino-2-phenylindole (DAPI, 1:10000).

Image analysis. Images were acquired with a Nikon 90-eclipse microscope (Nikon, Inc., Melville, NY) and analyzed using Nikon Elements software (Nikon, Inc., Melville, NY). Analysis included determination of necrotic regions, Myosin3-positive area, collagen-positive area in regenerating muscles, and BrdU incorporation on single myofibers. In sections, necrotic fibers were identified by the absence of a distinctive dystrophin ring circling a fiber photographed at 20x magnification. Necrotic fibers also exhibit a disruption and reduction in the expression of laminin surrounding them, as previously reported (19).

Single myofiber isolation and immunofluorescence. Single myofibers were obtained by collagenase digestion of EDL and peroneus muscles, as previously described (11). Briefly, following collagenase digestion, muscles were triturated with plastic Pasteur pipettes and moved to cell culture dishes with a glass Pasteur pipette. Floating cultures were achieved by coating dishes with 10% normal horse serum prior to addition of plating media (10% normal horse serum, 0.5% chick embryo extract [MP Biomedicals] in low-glucose [1 g/L] Dulbecco's modified Eagle's medium [DMEM; Invitrogen]). Single myofibers were incubated in one of three conditions: basal (plating media alone), Recombinant Mouse HGF (R&D, 10 ng/mL), or dimethylsphingosine (DMS, 10 μmol/L)

for 45 min, prior to the addition of BrdU to the medium. Satellite cell activation was determined by performing immunofluorescence on single myofibers following a 24-h incubation in 5-bromo-2-deoxyuridine (BrdU; Sigma, 10 μmol/ L), as previously described (18). The number of BrdU-positive SCs per myofiber were counted on at least 12 myofibers per treatment group as a measure of the functionality of SCs becoming activated by HGF.

Crushed muscle extract. Crushed muscle extract (CME) was harvested as previously described (20) from resting muscle of CON and DIO mice.

Western blot analysis. About 100 μg of protein derived from crushed muscle extracts and uninjured muscle were run out on a separate acrylamide gel, transferred to PVDF membrane (BioRad, Mississauga, Ontario), blocked with 5% skim milk for 1 h at RT, and then incubated overnight at 4°C with primary Active-HGF antibody (Abcam). A similar western blot protocol was completed for the analysis of c-Met (Sigma Aldrich) and myogenin (Novus Biologicals) protein content, using 30 μg of protein from lysates of uninjured and 5 days post-injury muscles, respectively. The appropriate horseradish peroxidase-conjugated secondary antibodies were incubated for 1 h at RT, and the blot was visualized using SuperSignal Chemiluminescent reagent (Thermo Scientific). Images were acquired using a Gel Logic 6000 Pro Imager (Carestream, Rochester, NY) and the area density of each band was analyzed using Adobe Photoshop.

Primary myoblasts to assay SC proliferative capacity. Primary myoblasts were derived from single myofiber cultures attached to a basement membrane matrix (Matrigel; 1:10 dilution) similar to that previously described (18), with the following exceptions:

Following 2 days in plating media, single fibers (1/well in a 24-well plate) were removed and proliferation media (10% FBS, high glucose DMEM) was added to SCs that had migrated off the myofiber. Myoblast number was counted at 0 and 24 h following the addition of proliferation media.

Statistical analysis. For all experiments, the appropriate t-test or two-way ANOVA with Tukey's post-hoc analysis was performed to analyze differences between DIO and CON groups. Two- way ANOVA was run on data sets with dependent variables measured over time, while one- or two-tailed t-tests were carried out on data with only a single comparison. Data are presented as standard error of the mean with $P \leq 0.05$ considered significant (denoted by asterisk).

RESULTS

Relative muscle mass following injury unaltered with DIO. Absolute muscle mass of the uninjured tibialis anterior (TA) and gastrocnemius/plantaris muscle groups (GP) was previously found to be unaltered after high-fat feeding (3). Similarly, we report no differences in muscle mass of injured (left) relative to uninjured (right) muscle 5 or 10 days post-injury between diet groups (Fig. 2.1 A, B).

Prolonged necrosis present in muscle following injury with DIO. In the damaged muscles of DIO mice, the area of necrosis was found to be significantly larger 5 days post-injury compared to that of CON mice, and suggests that these muscles are unable to appropriately transition from the degeneration to regeneration phase (Fig. 2.2A,B). This greater area of necrosis in muscle from DIO mice remained 10 days post-injury compared to CON (Fig. 2.2C).

Collagen content, but not macrophage density, is enhanced 5 days post-injury in DIO muscle. Consistent with a delay in transitioning to the regenerative phase, injured DIO muscles exhibited a significantly higher collagen content 5 days post-injury (Fig. 2. 3A) which was resolved by 10 days post-injury (Fig. 2.3B). Greater collagen content during muscle repair can impair cellular influx (21). However, there was no reduction in total (F4/80 positive) macrophage content at 5 or 10 days post-injury with DIO in either the necrotic or regenerating areas (Fig. 2.3C–F).

Myofiber repair is delayed in muscle with DIO. The persistence of necrotic tissue in regenerating DIO muscle indicated a delay in the muscle regenerative process. This led to the quantification of regenerating fiber sizes between diet groups to determine whether alterations in the muscle repair process would be evident. Newly regenerating myofibers express developmental isoforms of myosin heavy chain, including embryonic myosin heavy chain (Myosin3; Fig. 2.4A) prior to expressing more mature forms of myosin, thus rendering Myosin3 an appropriate marker for myofiber regeneration (10, 21, 22). We observed a significantly smaller area of Myosin3 staining in fibers in regenerating DIO muscle at 5 days post-injury compared to CON (Fig. 2.4B). By 10 days post-injury, much of the damaged CON muscle had transitioned to more mature myosin isoforms, while damaged DIO muscle expressed nearly twice as much Myosin3 as the controls (Fig. 2.4C).

To support the conclusion of delayed regeneration, we plotted the cross-sectional areas of Myosin3-positive fibers in CON and DIO muscles at 10 days post-injury. As shown in Figure 4D, there were significantly smaller Myosin3-positive fibers in DIO

muscles compared to CON (Fig. 2.4D), resulting in a leftward shift in newly regenerative fiber size distribution when compared to CON.

DIO does not alter satellite cell content. Considering the importance of satellite cells to regeneration, changes to SC functionality and/or content impact the repair of skeletal muscle (23). There was no difference in SC content of resting muscle between diet groups as determined by evaluating the percentage of Pax7-positive myonuclei (Fig. 2.5A,B). The basal activation state of SCs was investigated by determining the number of MyoD-positive myonuclei in a given area of resting muscle fibers. DIO mice, similar to CON, had very few positive MyoD nuclei (data not shown) demonstrating that there was no increase in basal SC activation with DIO.

DIO skeletal muscle does not respond to HGF and releases less of it following injury. We next ascertained the ability for SCs from DIO and CON muscles to appropriately respond to external activation stimuli. SC activation has been demonstrated to occur when HGF binds to its receptor, c-Met, on SCs (24). HGF activates sphingosine kinase 1 (SK1), the enzyme that converts sphingomyelin to sphingosine-1-phosphate (S1P), which itself has been shown to initiate proliferation of reserve cells (25,26). We therefore assessed the ability of HGF to enhance SC activation on freshly isolated myofibers from both CON and DIO mice, as has been previously demonstrated (27,28), as well as examined the involvement of S1P in this process. HGF administration (10 ng/mL) successfully activated SCs, promoting their entry into the cell cycle (as demonstrated by BrdU incorporation) on CON muscle fibers (Fig. 2.5C,D) but had no effect on isolated myofibers from DIO muscle (Fig. 2.5E). Co-incubation of myofibers with both HGF and

a sphingosine kinase inhibitor (d-erythro-N, N-dimethyl- sphingosine, DMS; 26) significantly reduced the number of BrdU-positive SCs on CON myofibers (Fig. 2.5D), but had no effect on the number of BrdU-positive SCs observed on DIO myofibers (Fig. 2.5E). In support of HGF signalling being down-regulated, increased Caveolin-1 protein content, normally down-regulated by HGF following injury (29), was found in DIO compared to CON muscle 24 h post-injury (Fig. 2.5F).

The increased collagen content evident in regenerating DIO muscle could affect SC activation by impeding the interaction between active HGF and its receptor, c-Met, as has been reported in aged skeletal muscle (30,31). No difference in the intramuscular protein content of the active form of HGF or its receptor, c-Met, was observed in uninjured muscle between DIO and CON groups (Fig. 2.6A,B). Furthermore, we determined that there was significantly less HGF released from crushed muscle extracts (CME) from resting DIO mice compared to CON mice (Fig. 2.6C).

Myoblast proliferative ability is impaired ex vivo and in muscle 5 days after injury with DIO. To assess the impact of lower HGF release from DIO muscles on the proliferative capacity of satellite cells from DIO and CON muscles, primary SC cultures were isolated and permitted to proliferate. Significantly fewer myoblasts were counted in cultures derived from DIO muscles than from cultures of CON muscles (Fig. 2.7A). To support these in vitro results, the number of MyoD-positive cells was quantified in immunofluorescently stained sections from DIO and CON muscles at 5 days post-injury. In healthy control muscles, a peak in myoblast (MyoD-positive) content has been reported at ~3 days following CTX injury (32,33) with a reduction in MyoD-positive cells

thereafter. We observed a strong trend (P = 0.07) toward more MyoD-positive cells in injured DIO muscles compared to CON at 5 days post- injury, supporting the findings of attenuated proliferative capacity in the SC population of DIO muscles (Fig. 2.7B). Western blot analysis of regenerating muscles at 5 days post-injury indicated significantly more Myogenin in DIO muscle compared to regenerating CON muscles (Fig. 2.7C), further corroborating the disruption in SC functionality. Taken together, these results suggest that SCs originating from DIO muscle fail to respond to activating stimuli in a timely fashion, an effect that ultimately results in an attenuated regenerative capacity of the skeletal muscle.

DISCUSSION

A healthy skeletal muscle mass is integral to the success of efforts to reduce obesity and insulin resistance and, ultimately, in attenuating the progression toward Type 2 Diabetes Mellitus and associated complications. A primary attribute of healthy skeletal muscle is the capacity for efficient maintenance and repair from overuse or injury. In the present study, we observed delayed muscle repair in DIO mice compared to standard-chow controls, as identified by the enhanced presence of necrotic areas and collagen content, as well as a reduction in regenerative fibers in DIO muscle at 5 days post-injury. While a deficit in muscle repair has been noted by some, but not all investigations involving DIO (15-17), this is the first study to report decrements specifically in muscle SC function and provide evidence for an underlying cause for this impairment. We demonstrate here that DIO induces changes within skeletal muscle that alters HGF

signalling, prompting intrinsic impairments in SC activation and proliferation, and a concomitant delay in muscle repair following injury compared to control mice.

Intramuscular injection of cardiotoxin results in significant muscle damage and is an established means to investigate changes to muscle regeneration pathways (14). Following injury, the muscle will undergo two distinct phases of muscle repair. The degenerative phase comprises removal of damaged cells, accompanied by SC activation and an increase in the collagen matrix. The degenerative phase is followed by the regeneration phase, wherein SCs proliferate and differentiate to repair the damaged muscle (14, 34). This regenerative phase also involves extensive remodeling of the extracellular matrix (ECM) as new and regenerated myofibers replace the collagen matrix that infiltrated the damaged muscle. Indicators of muscle repair, including morphometric and SC assessments, are often used to detect differences in the repair process (10,12,35,36). The degenerative phase involves influx of inflammatory cells and necrosis of damaged myofibers which subsides by ~5 days post-injury. The continued presence of necrotic areas is indicative of a persistent degenerative phase and/ or a slowed regenerative process (31). The regenerative phase is characterized by the appearance of numerous small, newly regenerated myofibers (11). As regeneration progresses, the new myofibers increase in size and the expression of myosin heavy chain isoforms transitions from developmental to mature isoforms (11,12,37). Previous work reported no difference in muscle fiber area and muscle repair with DIO, as quantified histologically by the number of regenerating fibers and the percent area of regenerating muscle after injury (16). While this method detected significant reductions in the measured variables for

other mouse models of diabetes (ob/ob and db/db), it only detected trends for deficits in mice fed a high-fat diet (60% kcal fat) for 12 weeks. Though their study (16) may have been underpowered for these specific measurement techniques in the subtler phenotype of the DIO mouse model, it should also be noted that their mice were only 4 weeks old at the time of high-fat diet commencement. It has previously been reported that young, growing mice fed a high-fat diet do not display many of the impairments exhibited when the diet is initiated in adulthood, likely the result of a much higher metabolic rate in youth (5). Furthermore, quantifying newly regenerating muscle fibers at 5 days post-injury by counting centrally nucleated myofibers in hemotoxylin–eosin-stained sections can be difficult given the very small size of the most recently generated myofibers, especially if there is delayed growth after treatment. In an effort to improve sensitivity to newly regenerating fibers in the present study, we used software-mediated quantification of newly regenerating myofibers through Myosin3 immunofluorescence (22). At 5 days post-injury, we observed that DIO muscles had an increased number of very small, newly regenerated myofibers and the relative size of the regenerating area (with respect to the whole area of injury) was smaller than that measured in CON muscles. The presence of persistent, larger necrotic areas within injured DIO muscle was hypothesized to be the result of higher levels of collagen relative to CON muscles, which has been reported to impair macrophage infiltration in T1DM mice (21). Our investigations into this hypothesis revealed no difference between diet groups in overall macrophage content within either necrotic or regenerating areas of the damaged muscles. However, while total macrophage content was not different, it is possible that the elevated fibrosis influenced

the timely transition of macrophages from a pro-inflammatory (M1) to an anti-inflammatory (M2) phenotype (12,38). It is possible that an increased abundance of M1, but not M2, macrophages in DIO muscle may contribute to the lack of HGF production observed in crush-injured DIO muscle, as prior work has identified M2 macrophages as a source of HGF production in injured muscle (36). While we do not directly quantify the two distinct macrophage populations in this study, others have shown that in obese samples, there is an increased presence of inflammatory macrophages (M1) that coincides with an overall increase in total macrophage population (39). Furthermore, past work has demonstrated that only 3 weeks of high-fat feeding can promote an excess of M1 macrophages in uninjured muscle (40). As such, it seems plausible that a contributing factor to the increased presence of necrosis in DIO muscle is a result of an overall enhanced inflammatory state, thereby attenuating the regenerative process.

Perhaps even more influential to the regenerative process, besides the presence of anti-inflammatory macrophages, is the skeletal muscle satellite cell (SC). Past research has suggested that the cause of impaired regeneration in DIO muscle is primarily associated with reductions in SC content. (17). We did not observe a difference in SC content between diet groups in the present study. Rather, we measured significant impairments in SC functionality in DIO muscle compared to control muscle. The attenuation of SC activation in DIO muscles led us to investigate the HGF signalling pathway for its well-characterized role in activating satellite cells from quiescence (41). Following injury, HGF is released by injured skeletal muscle and resides in the ECM where it binds to ECM components much like other growth factors (42). A significant

reduction in HGF release from crushed DIO muscles, compared to CON, was observed in the present study providing a possible mechanism for impaired SC activation. Interestingly, while HGF release from crushed muscle was lower in DIO mice, the total HGF content contained within the muscles did not differ between groups. This would suggest defective HGF release from sequestration in DIO muscles rather than a reduction in total HGF content. The active form of HGF has been found in skeletal muscle ECM (43), and while there is no direct evidence relating changes to ECM composition after a period of high-fat feeding with the release of HGF, it is predicted that DIO unfavorably alters ECM composition, deterring the release of HGF from the ECM following muscle injury. Human data examining men subjected to a 10% weight gain induced by overfeeding found evidence for skeletal muscle ECM remodelling, subsequently resulting in skeletal muscle fibrosis (44). The results from this human study raise the possibility that the findings of the current study could very well be linked to a failure in the release of HGF from the remodelled ECM in DIO skeletal muscle.

To further elucidate the importance of HGF in DIO SC activation, we examined whether SCs from DIO muscles would respond appropriately to exogenous HGF administration. Exogenous HGF led to a significant increase in CON SCs exiting quiescence and entering the cell cycle (BrdU-positive) as expected. However, this same protocol was completely ineffective at activating SCs on myofibers isolated from DIO muscles. Components of the ECM remain intact following single fiber isolation (45,46), and thus the lack of response observed in DIO single fibers treated with exogenous HGF might be attributed to the inability of HGF to properly communicate with SCs due to an

altered/thickened ECM. The absence of HGF-mediated in vitro activation of SCs on single DIO muscle fibers prompted further investigation of the HGF/c-Met signalling pathway. Caveolin-1 (Cav1) is a member of the caveolin family of scaffolding proteins that play a role in the formation of caveolae in the plasma membrane (47,48). Cav1 is expressed on SCs and aids in maintaining the quiescent state (29). Following muscle injury, Cav1 is down regulated by HGF, thus stimulating the progression of SCs through myogenesis (29). While down-regulation of Cav1 is evident in CON muscle at 24-h post-injury, this same response is not evident in injured DIO muscle, and supports the impairment of HGF- mediated SC activation and proliferation. These results coincide with the delay in Myosin3 expression, a marker of differentiation, in DIO muscle at 5 days post-injury compared to controls. Overall, the results of the present study indicate that the HGF pathway is not being activated appropriately, and is predicted to be a result of aberrant ECM remodelling in DIO muscle.

The present study demonstrates a novel mechanism for decrements in the repair of DIO skeletal muscle. It should also be noted that HGF administered to myotubes in culture enhanced glucose uptake and metabolism (49). Moreover, glucose uptake and metabolism are impaired in obese, insulin-resistant states despite concomitant elevations in adipose and serum HGF levels (50). Taken together with our present findings, we hypothesize that a common mechanism, defective HGF signalling, may be contributing to the impairments in SC functionality, muscle repair and skeletal muscle metabolism. Such findings are integral to the study of skeletal muscle health in DIO as it highlights the significance of the SC to skeletal muscle growth and repair in DIO. By hindering SC

function, as identified through increases in fibrosis and impairments in activation signalling, SCs are unable to function appropriately, and over time, could contribute to the decline in skeletal muscle health of obese individuals. This deficit in skeletal muscle health and function would negatively impact physical capacities, further perpetuating the obese condition and accelerating the onset of additional co-morbidities due to the pivotal role of skeletal muscle in overall health. This condition may worsen with increasing age as other factors also impair satellite cell function, ultimately leading to the state of sarcopenic obesity (51).

FIGURES

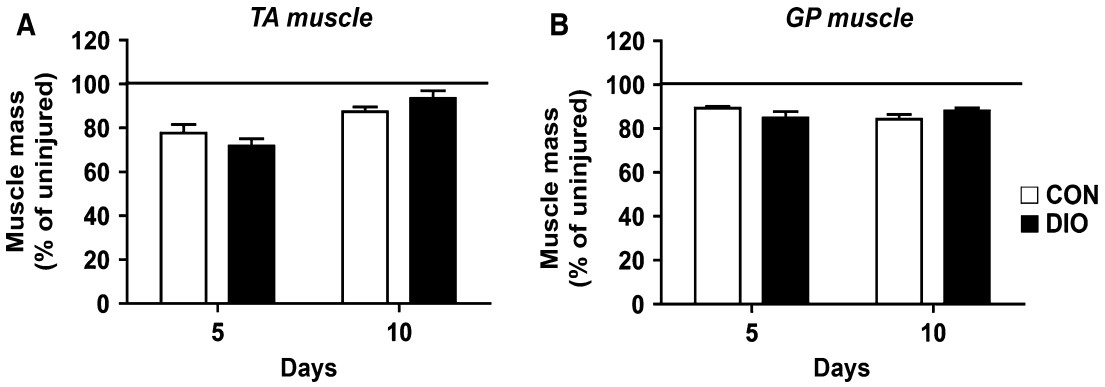

Figure 2.1. Diet-induced Obesity does not alter muscle mass 5 and 10 days following CTX injury. Injured muscle mass relative to uninjured contralateral leg 5 or 10 days after CTX injury in the tibialis anterior (TA) muscle and the gastrocnemius/plantaris (GP) muscle complex (n = 6). Significance was determined by two-way ANOVA performed for each muscle type with Tukey Multiple Comparison post-hoc tests, *P < 0.05. Control diet (CON, white bars), diet-induced obesity (DIO, black bars)

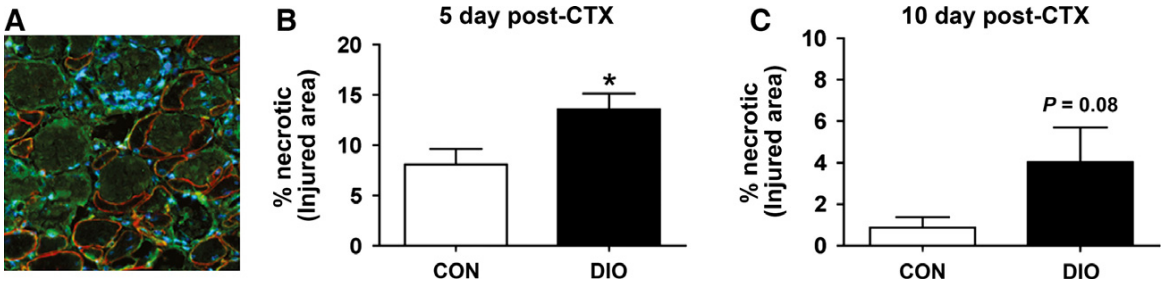

Figure 2.2. Enhanced necrotic area in CTX-injured skeletal muscle following diet-induced obesity. (A) Representative image of CON GP muscle 10 days post CTX stained with laminin (green) and dystrophin (red), which both outline healthy fibers (nuclei stained with DAPI, blue). Arrows indicate necrotic fibers identified by the absence of dystrophin. (B) Percentage area of necrosis in the injured area after 5 days (n = 5). (C) Percentage area of necrosis in the injured area after 10 days (n = 6 CON, 5 DIO). Control diet (CON), diet-induced obesity (DIO). Significance was determined by student's t-test, *$P < 0.05$.

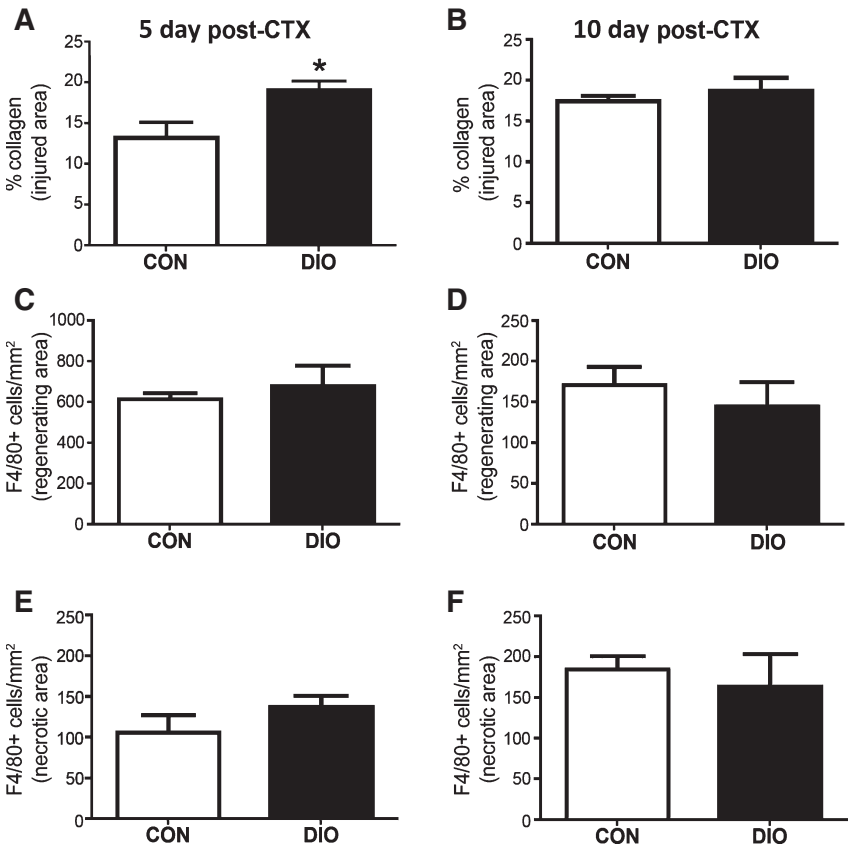

Figure 2.3. Diet-induced obesity increases collagen content during early muscle regeneration. (A and B) Percent collagen content in injured areas at 5 (n=5 both groups) and 10 days (n=4 both groups) following injury. (C–F) Macrophage-positive cells (determined by staining for F4/80) in necrotic and regenerating areas at 5 (n=5 both groups) and 10 days (n=4 both groups) following injury. Control diet (CON), diet-induced obesity (DIO). Significance was determined by student's t-test, *$P < 0.05$.

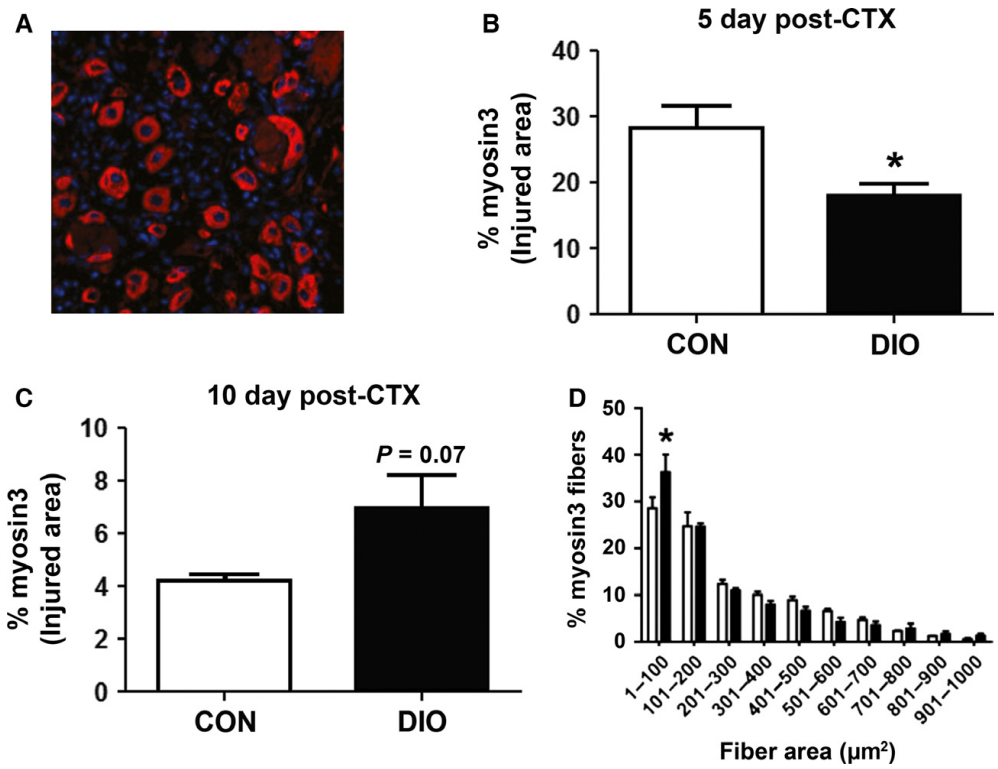

Figure 2.4. Diet-induced obesity delays muscle regeneration in muscle following CTX injury. (A) Representative image of CON GP muscle 5 days after injury stained with Myosin3 (Myh3, red; nuclei stained with DAPI, blue). (B) Percentage area of Myh3 content in the injured area after 5 days (n=5). (C) Percentage area of Myh3 content in the injured area after 10 days (n=4). (D) Distribution of Myh3-positive fiber area at 5 days following injury. Control diet (CON), diet-induced obesity (DIO). Significance was determined by student's t-test, *P < 0.05**.**

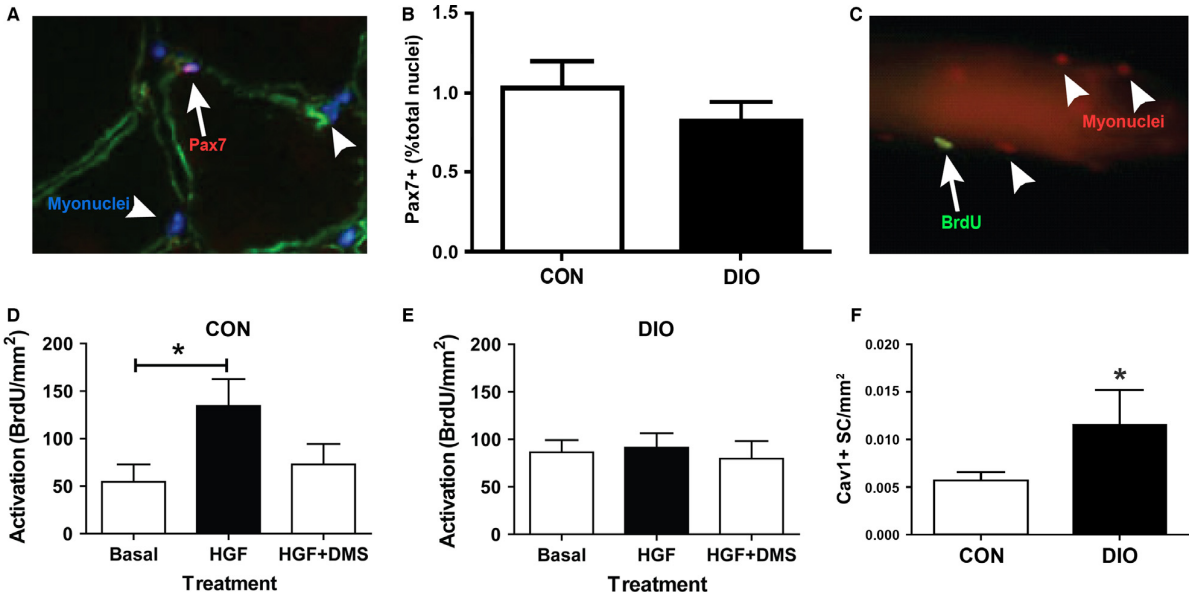

Figure 2.5. Satellite cell activation is impaired in diet-induced obesity. (A) Representative image of a Pax-7 positive SC (red; nuclei blue) located under the basal lamina (laminin, green). (B) Pax-7 content in uninjured TA muscle (n =3 CON, n=5 DIO). (C) Representative image of a single myofiber with an activated, Bromo-deoxy-uridine (BrdU) positive SC (green; nuclei red). (D and E) Activated SCs (BrdU positive) per fiber relative to fiber area (CON, n=11–27 fibers/treatment; DIO, n=18–31 fibers/treatment condition) in response to plating media (Basal), plating media with hepatocyte growth factor (HGF) or plating media with HGF and dimethyl sulphoxide (DMS) in floating cultures of (D) CON and (E) DIO myofibers. Control diet (CON), diet-induced obesity (DIO). (F) Caveolin-1 positive SCs 24 h following CTX injury in GP muscle. (B and F) Significance was determined by student's t-test, *$P < 0.05$. (D and E) Significance was determined by a one-way ANOVA with Tukey's Multiple Comparison post-hoc test, *$P < 0.05$.

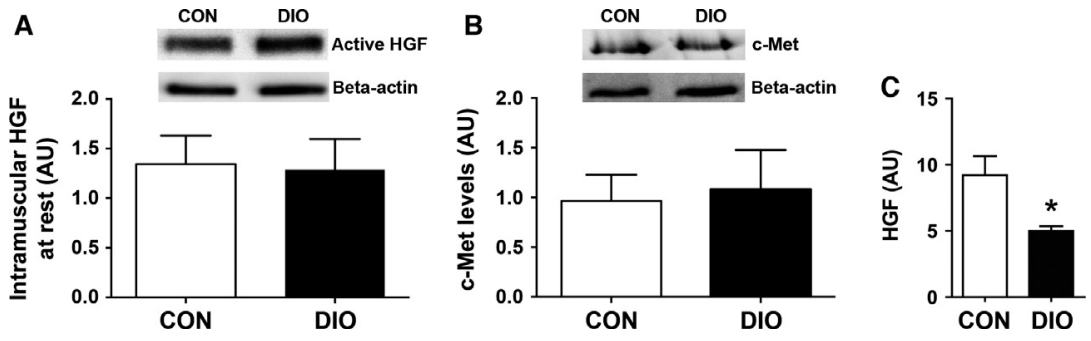

Figure 2.6. Diet-induced obesity impairs HGF release from injured muscle. (A) Representative blot and graph of intramuscular active HGF in CON and DIO uninjured muscle (n=4). (B) Representative blot and graph of c-Met expression in WT and DIO uninjured muscle (n=4). (C) Release of active HGF (both α&β chains) relative to the marker of injury myoglobin in crushed muscle extract (n=4). Control diet (CON), diet-induced obesity (DIO). Significance was determined by student's t-test, *$P < 0.05$.

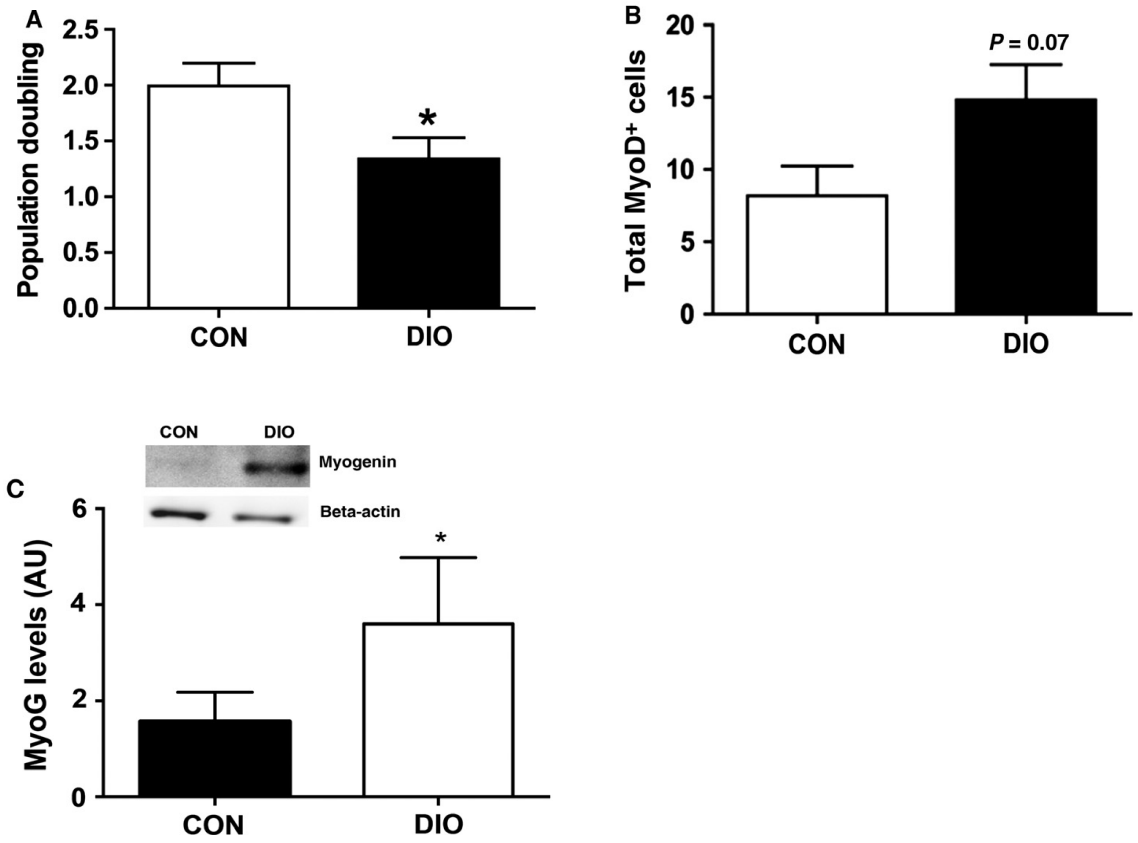

Figure 2.7. Diet-induced obesity impairs SC proliferation, and attenuates the expression of myogenic markers 5 days following CTX injury. (A) Myoblast proliferation assessed from day 2 to 3 post-harvest in cell culture (n = 6 CON, 8 DIO). (B) MyoD positive cells in TA muscle cross-sections at 5 days post-injury (n = 5). (C) Representative blot and graph of myogenin (MyoG) 5 days following injury (n = 3). Significance was determined by student's t-test, *P < 0.05.

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CHAPTER 3

Decreased Satellite Cell Number and Function in Humans and Mice With Type 1 Diabetes Mellitus is the Result of Altered Notch Signalling

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PREFACE

Significance to thesis

The goal of this research was to further our understanding of SCs in Diabetes Mellitus by investigating the response of SCs to T1D, another form of this disease. Other research has shown that SC function is hindered with exposure to T1D conditions, but a majority of this work was completed using a chemical model of T1D that is less than ideal to study SCs and skeletal muscle repair. While our lab has successfully identified impaired skeletal muscle regeneration using the genetic model of T1D implemented in this study (Akita mice), little information is available regarding the response of SCs in Akita skeletal muscle. The primary goal of this study was to establish whether SC content and/or function would be negatively impacted in Akita mice, as well as elucidate a possible mechanism of action. Evaluation of the response of SCs from this chapter (T1D) and the previous chapter (DIO) will allow us to determine whether SC response is comparable across various types of Diabetes Mellitus. A secondary goal of this study was to translate murine findings to human T1D samples. Assessment of human tissue is novel as it will be the first characterization of human T1D SCs, and will enable a better understanding of whether comparable changes in murine and human diabetic samples are evident.

Author's Contributions:

Donna M. D'souza contributed to the design of the study, researched data, completed microscope image analysis, performed statistical analysis, wrote the manuscript, and worked on refining this draft and the revisions based on editorial review. Sarah Zhou assisted in performing experiments and completed microscope analysis.

Irena A. Rebalka performed animal care and assisted in the collection of human samples.

Blair M. MacDonald recruited human participants and assisted in the collection of human samples.

Jasmin Moradi performed some experiments and completed some data analysis.

Matthew P. Krause assisted in some animal care and revised the manuscript.

Dhuha Al-Sajee assisted in quantifying satellite cell density in rodent skeletal muscle.

Zubin Punthakee recruited human participants, contributed to the design of the study, and revised the manuscript.

Mark A. Tarnopolsky collected human samples, contributed to the design of the study, and revised the manuscript.

Thomas J. Hawke contributed to the design of the study, assisted in experiments, and worked on refining drafts of the manuscript and the revisions based on editorial review.

ABSTRACT

Type 1 Diabetes (T1D) negatively influences skeletal muscle health, however, its impact on muscle satellite cells (SCs) remains largely unknown. SCs from T1D rodent (Akita) and human samples were examined to discern differences in SC density and functionality compared to their respective controls. Examination of the Notch pathway was undertaken to investigate its role in changes to SC functionality. Compared to controls, Akita mice demonstrated increased muscle damage following eccentric exercise, along with a decline in SC density and myogenic capacity. Quantification of components of the Notch signalling pathway revealed a persistent activation of Notch signalling in Akita SCs, which could be reversed with the Notch inhibitor DAPT. Similar to Akita samples, T1D human skeletal muscle displayed a significant reduction in SC content and the Notch ligand, DLL1, was significantly increased compared to controls- supporting the dysregulated Notch pathway observed in Akita muscles. These data indicate that persistent activation in Notch signalling impairs SC functionality in the T1D muscle, resulting in a decline in SC content. Given the vital role played by the SC in muscle growth and maintenance, these findings suggest that impairments in SC capacities play a primary role in the skeletal muscle myopathy that characterizes T1D.

INTRODUCTION

The prevalence of Type 1 Diabetes (T1D) continues to rise globally in youth populations (1). This autoimmune disorder is characterized by the destruction of pancreatic β-cells, leading to hypoinsulinemia and the loss of glucose homeostasis. While exogenous insulin therapy is currently available for these afflicted individuals, this treatment is not curative. Failure to properly maintain blood glucose through insulin therapy promotes periods of extreme glycemic levels and, over time, the development of diabetic complications.

Diabetic myopathy is an often-overlooked diabetic complication, but is believed to adversely impact the health and wellbeing of individuals with T1D. Although skeletal muscle is a largely resilient tissue that is capable of adapting to changing conditions, the skeletal muscle of T1D individuals exhibits a decline in physiological function and performance compared to healthy skeletal muscle, including significant impairments to its reparative capacities (2–7).

The skeletal muscle stem cell population, referred to as satellite cells (SCs), are a primary contributor to the maintenance and repair of skeletal muscle and thus play a central role in skeletal muscle plasticity (8). Though fundamentally involved in maintaining the health of skeletal muscle, few studies have investigated the impact of T1D on the muscle SCs and no study, to the best of our knowledge, has investigated SC populations in young T1D human populations to ascertain whether the changes occurring in rodent studies are translatable to the human condition.

The purpose of the current study was to examine SC content and function in the

Ins2^{Akita} mouse model (herein referred to as "Akita") and young adult T1D humans. Single fiber isolation experiments were completed to examine markers of SC quiescence and activation in Akita and wild-type (WT) mice, allowing for the identification of intrinsic differences in SC function between experimental conditions. Disparities in SC function within T1D human biopsies where also investigated to determine whether these changes in rodents were translatable. We hypothesized that SCs derived from muscle of diabetics would display impairments in SC function that detrimentally impact overall muscle health, and that this would be attributed to modifications to unique intracellular pathways that regulate SC quiescence and activation in T1D muscle. Specifically, we chose to assess the influence of the Notch signalling pathway and its effect on SC function in T1D skeletal muscle due to its well-established role in post-natal myogenesis (9), and its regulation of SC self-renewal during muscle regeneration (10).

RESEARCH METHODOLOGY

Animals. Male C57BL/6-Ins2$^{Akita/J}$ (hereafter referred to as Akita) mice and their wild-type littermates (WT) were housed in a temperature and humidity controlled facility with a 12/12h light/dark cycle and were given free access to food and water. Akita mice spontaneously develop Type 1 Diabetes at ~4 weeks of age due to a heterozygous mutation in the *Ins-2* gene. Akita mice were monitored for diabetes onset (blood glucose >15 mM) following weaning through the use of blood and urine analyses. All experimental protocols were carried out with approval of the McMaster University

Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines.

Endurance Exercise Test. To compare the functional capacity of Akita and WT skeletal muscle, mice with 16 weeks of diabetes (i.e. 20 weeks of age) from each experimental condition were subjected to an endurance exercise test (N=3 WT, N=4 Akita). The acclimation period lasted for 2 days, and consisted of placing mice on the treadmill (Columbus Instruments, Columbus, OH), with a gradual increase in treadmill speed and duration up to 10m/min for 5 minutes. The exercise test was performed with mice starting at a speed of 8m/min for 5 minutes, with a subsequent increase to 9m/min for 3 minutes. Following this, the speed was increased by 1m/min every 10 minutes until the mice reached exhaustion (11).

Eccentric Exercise Protocol. A fraction of WT and Akita animals (8 weeks of diabetes, N=3 per group) were randomly assigned to a 4-day Eccentric Exercise Training Protocol to assess changes to muscle repair following subjection to a physiologically relevant stimulus to induce muscle damage. These mice were placed on a treadmill with a 15 degree downhill incline to promote eccentric exercise, as previously described (modified from 12). Mice were tested at this specific age to compare and contrast data from a previous study completed by our lab that utilized a chemical means to induce muscle damage using this same mouse model (13).

Tissue Collection. Animals were euthanized by CO_2 inhalation followed by cervical dislocation. The Tibialis Anterior (TA) muscles were excised from WT and Akita mice, with the left muscle coated in tissue-mounting medium and frozen in liquid nitrogen-

cooled isopentane, while the right muscle was snap frozen in liquid nitrogen. Left and right extensor digitorum longus (EDL) and peroneus muscles were harvested to isolate single muscle fibers, while remaining hind limb muscles (gastrocnemius-plantaris-soleus complex and quadriceps) were snap frozen and used for protein analyses.

Patients and Ethics Statement. Skeletal muscle biopsy specimens were taken from the vastus lateralis using a 5-mm Bergstrom needle, as previously described (14). Samples were taken from healthy, non-diabetic (Control; N=5) and type 1 diabetic (T1D; N=6) males aged 18-24 years of age (Table 3.1). Subjects gave written consent after being informed of the procedure and associated risks involved with the study. This portion of the study was approved by the Hamilton Health Sciences Research Ethics Board (REB#14-649), and conformed to the Declaration of Helsinki regarding the use of human subjects as research participants.

Single Muscle Fiber Isolation. Single muscle fibers were obtained from Akita and WT mice at 12 weeks of age (8 weeks of diabetes) from the left and right EDL and peroneus muscles, as previously described (15). Fibers were either fixed immediately following isolation (referred to as Control fibers) or placed in culture dishes with plating media [10% normal horse serum, 0.5% chick embryo extract in Dulbecco's modified Eagle's medium (DMEM)] overnight (18 hours; referred to as Activated fibers). Note that for all single fiber experiments, the minimum and maximum number of fibers analyzed are provided in a range (i.e. 15-38) and is derived from at least 3 mice per experimental group.

To investigate the role of Notch signalling, isolated myofibers were treated with 10 μm *N*-[2*S*-(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl-1,1-dimethyletyl ester glycine (DAPT, Sigma Aldrich, St. Louis, MO) following myofiber isolation, and subsequently left in culture, as previously described (16). Specifically, isolated single fibers from WT and Akita skeletal muscle were placed in the presence or absence of DAPT for 24 hours to permit assessment of their capacity to become activated with (DAPT treatment; DAPT Tx) or without (Activated) Notch inhibition. Fibers were subsequently fixed and stained for Pax7+ nuclei to determine changes in the quantity of SCs between experimental groups. A direct comparison of the number of Pax7+ nuclei on DAPT Tx and activated fibers from each experimental group was completed, and represented as a fold-difference.

Satellite Cell Activation. Satellite cell activation was assessed in floating cultures by adding 10 μM 5-bromo-2-deoxyuridine (BrdU) to the plating media and incubating newly isolated single fibers for 24 hours. Fibers were fixed and stained for BrdU (Abcam, Cambridge, MA), as previously described (15). Satellite cells that became activated and entered the cell cycle incorporated BrdU. As myonuclei are post-mitotic, BrdU positive nuclei would represent SCs that became 'activated' and have entered the cell cycle SC activation was therefore analyzed by the number of BrdU-positive nuclei per muscle fiber.

*Western Blot Analyses***.** Approximately 100 μg of protein from mouse or human whole muscle lyates was run out on a separate acrylamide gel, transferred to PVDF membrane, blocked with 5% skim milk for 1 hour at room temperature (RT), and then incubated overnight at 4°C with primary DLL1 antibody (mouse, Abnova, Taipei, Taiwan; human,

Cell Signaling, Danvers, MA). The appropriate horseradish peroxidase-conjugated secondary antibodies were incubated for 1 hour at RT, and the blot was visualized using SuperSignal Chemiluminescent reagent (Thermo Scientific, Waltham, MA). Images were acquired using a Gel Logic 6000 Pro Imager (Carestream, Rochester, NY) and the area density of each band was analyzed using Adobe Photoshop.

Skeletal Muscle Histology. Hematoxylin and eosin (H&E) stains were used for the determination of muscle morphology, with greater than 75 muscle fibers analyzed per section. Muscle injury induced by the eccentric exercise protocol was determined by the presence of centrally-located nuclei, pale cytoplasm, and infiltrated muscle fibers, as has been previously established (12). Each incidence of muscle injury was annotated to obtain a value that was then corrected for by the total number of fibers analyzed. The amount of muscle injury in each experimental condition was then expressed relative to the degree of injury observed in the WT sedentary group (WT REST)

Immunofluorescent Staining. Tibialis Anterior muscle sections from WT and Akita mice were fixed with 4% PFA, while human Vastus Laterialis muscle sections were fixed using the same protocol. Single muscle fibers isolated from WT and Akita mice were either immediately fixed using 4% PFA (control fibers), or following an activation period (activated fibers). Muscle sections from mice and humans were stained for Pax7 (DSHB, Iowa City, Iowa), using TSA amplification, and Dystrophin (Abcam). Single fibers were stained for antibodies against Pax7 (DHSB), MyoD (Abcam), Myogenin (Novus Biologicals, Littleton, CO), Notch Intracellular Domain (NICD, Abcam), and Hes1 (Abcam). The appropriate secondary antibodies were applied: Alexa Fluor 594,

biotinylated secondary antibody, Alexa Fluor 488 (Thermo Scientific). Nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI).

Image Analyses. All stained fibers were viewed using the Nikon 90-eclipse microscope (Nikon, Inc., Melville, NY) and analyzed using Nikon Elements software. Analyses include examining of muscle morphology, quantification of protein expression on single fibers, and quantification of SC content (Pax7+/DAPI+) in muscle sections. All images were examined at 20x magnification.

Statistics. Measures were assessed using a Two-Way ANOVA with Bonferroni post-hoc test, or where appropriate, Student's t-test. Significance was set at a P value of less than 0.05. All statistical analysis was performed using GraphPad Prism 5 (La Jolla, CA) software. Data are presented as means ± standard error of the mean (SEM).

RESULTS

Diabetic Akita mice display greater evidence of muscle damage following eccentric exercise. Following 8 weeks of overt diabetes (~12 weeks of age), there were significant reductions in skeletal muscle masses (Figure 3.1A, $p<0.05$) relative to non-diabetic (WT) controls, along with a 17% decrease in body weight and a 60% decrease in epididymal fat mass (data not shown), as has been previously observed in T1D rodent models (7,17,18). The reduction in muscle mass led to the evaluation of muscle function, determined by an endurance exercise test. When compared to their age-matched WT counterparts, Akita mice were found to reach exhaustion faster (Figure 3.1B, $p<0.05$). We then investigated if T1D rodent skeletal muscle were more susceptible to muscle damage following eccentric exercise. WT and Akita mice underwent a 4-day eccentric exercise training protocol. The

increased presence of muscle injury in Akita muscle sections, as observed histologically (Figure 3.1C), and in a graphical representation (Figure 3.1D, $p<0.05$), confirms that T1D skeletal muscles are more susceptible (i.e. display a greater degree of damage) to a physiologically relevant muscle injury stimulus; a finding consistent with previous work using Evans Blue Dye incorporation into the muscles of downhill run diabetic and WT mice (19).

T1D SCs display impairments in activation and content. The importance of SCs to skeletal muscle repair and regeneration has been well established (20-21), and was therefore a primary focus for the current study. Based on observations of a decline in skeletal muscle health in Akita mice, particularly after eccentric exercise, we were interested in evaluating the response of SCs. An important characteristic of SC function is the capacity to exit quiescence in response to a stimulus, a process termed 'activation'. We had hypothesized that SC activation would be enhanced given the myopathy which characterizes the skeletal muscle of T1D subjects.

SC activation was examined using single fibers isolated from Akita and WT muscles that were fixed either immediately following isolation or after an in vitro activation period. Fibers were stained for nuclei and Pax7, a transcription factor used to demarcate the SC (Pax7; Figure 3.2A). Compared to the quiescent period, SCs present on Akita myofibers did not increase in content following an in vitro activation period, as evidenced by a 40% difference in Pax7+ nuclei on Akita-activated versus WT-activated myofibers (Figure 3.2B, $p<0.05$). Individual data points from this analysis are shown (Supplementary Figure 3.1A, $p<0.05$). Furthermore, BrdU incorporation at 24 hours post-isolation was found to

be lower in SCs on myofibers isolated from Akita muscle compared to WT (Figure 3.2C,D, $p<0.05$), further confirming that activation is lower in Akita diabetic SCs.

It is well established that a failure for SCs to properly activate and progress through myogenesis hinders their ability to replenish their own population, leading to an eventual decline in SC content (22). Given the impairments in SC activation we observed in T1D muscles, assessment of SC content was completed to determine whether a failure to properly activate SCs altered total SC density in T1D muscle. Quantification of SC density revealed a 31% reduction in Akita diabetic compared to WT skeletal muscle (Figure 3.2E, $p<0.05$).

Following activation, most SCs will progress down the myogenic lineage (termed myoblasts) including expansive proliferation and fusion with one another or with existing, damaged myofibers (8). Additional markers of myogenesis, MyoD and Myogenin, were examined to assess the progression of Akita SCs down the myogenic lineage. In activated Akita myofibers, MyoD-positive nuclei were found to be 2.7-fold lower in expression than the WT (Figure 3.2F, $p<0.05$), while 2-fold fewer Myogenin-positive nuclei were observed in Akita myofibers when compared to the WT (Figure 3.2G, $p<0.05$).

Hyper-activation of Notch Signalling in T1D SCs. A tight regulation of Notch signalling is imperative for normal myogenesis (23), as it is typically found to increase with activation in order to promote Pax7 expression and SC self-renewal (24), but must return to a negligible level to facilitate the progression of the SC through the remainder of myogenesis. Given this, it was hypothesized that Notch signalling would remain elevated in T1D muscle SCs, resulting in a reduced capacity for activation and progression down

the myogenic lineage. Assessment of the active form of Notch-1, referred to as the Notch Intracellular Domain (NICD), and its downstream effector, Hes1, was achieved through immunofluorescent staining of single myofibers (co-stained with Pax7; Figure 3.3A). No difference between groups was observed in the expression of NICD+/Pax7+ cells on quiescent fibers between WT and Akita myofibers, while a 1.9-fold increase in NICD+/Pax7+ nuclei was evident in activated Akita myofibers compared to activated WT myofibers (Figure 3.3B, $p<0.05$). Similar to the NICD data, it was determined that Hes-1+ SC number did not differ in expression between quiescent (control) Akita and WT SCs, but was down-regulated in activated WT SCs while remaining significantly elevated in activated Akita SCs (Figure 3.3C, $p<0.05$). Pharmacological repression of the Notch signalling pathway was completed through use of the Notch inhibitor, DAPT, in vitro. While no difference was observed in the expression of Pax7 on treated and untreated WT single fibers, a 1.6-fold increase in Pax7 expression was identified when comparing DAPT treated and untreated Akita single fibers (Figure 3.3D, $p<0.05$). Raw data from this experiment is demonstrated in Supplementary Figure 3.1B. Taken together, these data provide evidence that inhibiting Notch signalling facilitates an increase in Pax7 expression in Akita single fibers, thereby supporting a role for Notch in impairing SC activation in T1D.

To determine if the increase in Notch signalling in Akita SCs was the result of increased Notch ligand presence on the myofiber, we quantified Delta like 1 (DLL1) by Western blot in WT and Akita skeletal muscle. No significant difference between groups was noted in DLL1 expression (Figure 3.3E) suggesting that the Notch pathway is being

activated by means other than a direct up-regulation of DLL1.

Satellite cell content is decreased in young adult T1D humans. To determine if the observations made in T1D mouse SCs were comparable to T1D human SCs, we assessed SC content and the expression of the Notch ligand, DLL1, in the skeletal muscle of T1D and non-diabetic young adults (18-24 years old). A 39% reduction in Pax7 expression was observed in T1D skeletal muscle cross-sections in comparison to healthy age- and sex-matched Controls (Figure 3.4A,B, $p<0.05$). Since analyses of single muscle fibers from human skeletal muscle (including the aforementioned activation protocol) was not available using the Bergström biopsy procedure, we investigated changes to Notch signalling through quantification of DLL1 protein expression in whole muscle lysates from T1D and healthy human skeletal muscle. In contrast to our findings in mouse skeletal muscle, DLL1 protein expression in human T1D skeletal muscle was found to be significantly elevated compared to non-diabetic muscle (Figure 3.4C, $p<0.05$), and may identify a species-specific difference in the availability of different Notch ligands.

DISCUSSION

Skeletal muscle represents the largest insulin-sensitive organ within the body and is the site for approximately 80% of whole body glucose uptake (25). Given this level of contribution to glycemic control, one can appreciate that impairments to skeletal muscle health in T1D could be a primary factor in the progression of other diabetic complications. Satellite cells play an important role in the maintenance of healthy skeletal muscle mass due to their function in maintenance and repair (26), however little is known

about this cell population following T1D development. In the present study, we demonstrate for the first time, that exposure to the T1D environment adversely affected muscle satellite cell content- a finding consistent in both rodent and human skeletal muscles. Akita diabetic mice exhibited a significant reduction in SC content that was mirrored in young adult T1D humans. We also observed a significant impairment in SC activation in Akita mice that was consistent with our results. The mechanism for these defects appears to be impaired SC activation as a result of an over-activation of the Notch signalling pathway within this cell population. Indeed, inhibition of Notch activity in Akita myofibers through in vitro DAPT treatment led to an increase in the expression of the SC marker Pax7, and thus an increase in SC activation, verifying the role of Notch in the regulation of T1D SC activation.

The decreased exercise capacity of Akita mice observed in this study is supported by previous work in rodent models of T1D (27-28), as well as T1D humans (29–32). While the precise cause for this diminished capacity remains controversial, a number of factors are thought to contribute to this decline, as observed in healthy muscle (for review, see 33-34). A paucity of information is available regarding the response of T1D skeletal muscle to a more physiologically-relevant stimulus, such as exercise-induced damage (33-34). Literature from our lab has demonstrated that Akita skeletal muscle displays functional deficits (17), and supports work done by others in regenerating and uninjured Akita skeletal muscle (6). While we and others have established that rodents with T1D demonstrate a failure to repair following extreme damage, such as transplantation or toxin-induced injury (5,6,13,35), data presented here are the first to

show a decline in skeletal muscle function following exposure to a mild muscle-damaging stimulus, such as eccentric exercise and corroborates work from Howard et al. (7), who found that myocytes from diabetic mice failed to repair from laser- and contraction-induced plasma membrane injuries in vitro. We predict that the decline in Akita skeletal muscle function, as demonstrated by the rapid time to exhaustion, is a result of a slow rate of muscle repair following damage, as has been identified previously (35). Given our data, it is clear that diabetic skeletal muscle is more susceptible to muscle injury, and likely endures a downward spiral of repeated damage and delayed repair that ultimately hinders normal functionality.

The more pronounced damage in Akita mice compared to WT mice exposed to the same stimulus led us to investigate the effect of T1D on the SC population, a pivotal player in muscle growth and repair. We hypothesized that SCs from the diabetic group would be more activated, or would be more readily activated [a state referred to as G_{alert} (36)], as SCs are known to respond to stimuli such as muscle injury (37). Unexpectedly, we found a reduction in Pax7-positive cells in Akita muscles following an activation stimulus compared to WT. We verified this observation by investigating BrdU incorporation into activated/proliferating satellite cells on isolated single fibers, as well as the number of MyoD- and Myogenin-positive satellite cells on isolated fibers. In all of these analyses, a significant impairment in SC activation was noted; in agreement with past work (38). A previously published report in STZ-treated rats had also noted a decreased expression of myogenic factors by Western blotting (39). Though consistent with our present findings, that study was investigating the effect of oxidative stress

induced by chronic hyperglycemia on genes involved in protein muscle synthesis, thus, a specific analysis of the muscle satellite cell was not undertaken.

Given the observed decrements to SC activation, we next wanted to ascertain whether SC content would be negatively influenced as this relationship has previously been described (22). Here we examined SC density in both rodent and human T1D muscle samples. Despite our T1D mouse model being provided no exogenous insulin and our young adult human T1D cohort receiving exogenous insulin, a similar decrement in SC density was observed. To our knowledge, this is the first quantification of satellite cell density in young adult T1D patients, and while these patients receive exogenous insulin therapy, it is interesting to note that the decline in SC density is comparable to data derived from rodents with acute (8 weeks) uncontrolled T1D. As such, it appears that aberrant changes to the T1D SC population may be largely independent of insulin availability. Clearly, future studies using insulin pellets in rodents would shed further light on the temporal changes in SC density with exposure to T1D.

The impaired satellite cell activation observed on isolated single fibers suggested that the declines in SC function were either intrinsic to the SC, or were mediated through the myofiber-SC microenvironment, a niche which is maintained in the isolated fiber protocol. As the Notch pathway fit this theory, and has been implicated in the maintenance of the SC population and SC quiescence (40-41), it seemed the most appropriate pathway to interrogate. In the adult, Notch signalling plays an important role in satellite cell expansion (42) and constitutive Notch activity in muscle stem cells results in SC self-renewal, inhibition of MyoD and Myogenin expression (43), and impaired

muscle regeneration (24). Therefore, the elevated Notch signalling observed in Akita skeletal muscle would repress MyoD and Myogenin expression in response to an activation stimulus, and ultimately delay the exit of SCs from quiescence. Interestingly, a reduction in Notch activity has also been reported to delay regeneration in aged skeletal muscle (44). Thus, the influence of Notch activity on SC function appears to be situation-specific, and suggests that changes to the SC niche may alter the availability of those factors (such as Notch ligands) that modulate Notch signalling.

Although we expected to identify an increase in the Notch ligand, DLL1, as a primary mechanism through which Notch activity was enhanced in T1D rodent and human skeletal muscle, this was not observed in both species. Instead, a discrepancy exists in the expression of DLL1 between human and rodent T1D skeletal muscle. The lack of increase in DLL1 in T1D rodent muscle could be attributed to that fact that alternative Notch ligands regulate Notch signalling in rodent skeletal muscle. For instance, Jagged-1 is expressed in activated murine SCs, and has been used to determine its activation status (45). In another study, Jagged-2 was highly expressed in regenerating/damaged myofibers in both experimental cohorts examined, and was higher in abundance than DLL1 following the injury stimulus (16) suggesting that the availability of Notch ligands may only be quantified when the muscle has been subjected to a stimulus that disrupts its environment (such as exercise or injury). Future studies will aim to evaluate various Notch ligands in exercised and/or damaged Akita skeletal muscle to determine if differences in their quantity are observed when compared to the WT.

While the underlying cause for an increased DLL1 in human skeletal muscle was

not elucidated in this study, exposure of cells to high glucose has been found to alter Notch signalling pathway members (46-47). The hyperglycemia observed in diabetic mice (and consistent with poorly controlled young adult T1D humans; 48) coincides with the enhanced Notch signalling in T1D skeletal muscle. Additionally, extracellular matrix remodelling is important for SC function (13, 49), and it is clear that the capacity for extracellular matrix remodelling, through reduction in matrix metalloprotease activity, is negatively impacted in T1D skeletal muscle (13,31). As these proteases (MMPs, ADAMs, etc.) are known to cleave Notch prior to its translocation into the nucleus, a reduced capacity or abundance of these extracellular proteases, as seen in T1D, could account for the persistent Notch signalling. The influence of a high glucose environment and aberrant protease activity on Notch signalling in T1D SCs represents an interesting area for future investigation.

The data collected from our human subjects is the first to identify that such impairments in skeletal muscle health, via the SC, occur in young adults with T1D despite the availability of insulin therapy. The comparable changes to SC density observed in rodent and human T1D samples is promising as an avenue for future investigation in translation research as it suggests that, like what has been observed in rodent T1D SCs, human T1D SC function may be hindered in skeletal muscle as a result of dysregulated Notch activity.

In summary, our present findings highlight losses to the primary muscle stem cell population in T1D humans and rodents, a novel finding that we would propose is the result of hyper-activated Notch signalling impairing SC function. Given the vital role of

the satellite cell in the maintenance of skeletal muscle health, identification of intrinsic changes to the SC in T1D is integral to the development of therapeutic strategies to attenuate diabetic myopathy.

TABLES AND FIGURES

Table 3.1 Subject demographics for Control and T1D human participants.

Characteristic	Control	T1D
N=	5	6*
Age (yrs)	22 ±0.55	20 ±0.52
Weight (kg)	82.98 ±3.85	72.20 ± 3.50
Height (m)	1.83 ± 0.01	1.78 ± 0.04
BMI (kg/m^2)	24.90 ± 1.19	22.80 ± 0.33
Diabetes Duration (yrs)		7.80 ± 1.16
HbA1C		8.40% ± 0.27%

Data comparing subject demographics between T1D and Control human muscle indicates no significant difference for all parameters except age (*P<0.05). Statistical analysis was completed using Student's t-test to identify statistical significance between the two experimental groups. Differences exist in the number of samples used for each experiment based on the method of preparing the biopsy, and the specific sample size used for each analysis is defined within the figure legends.

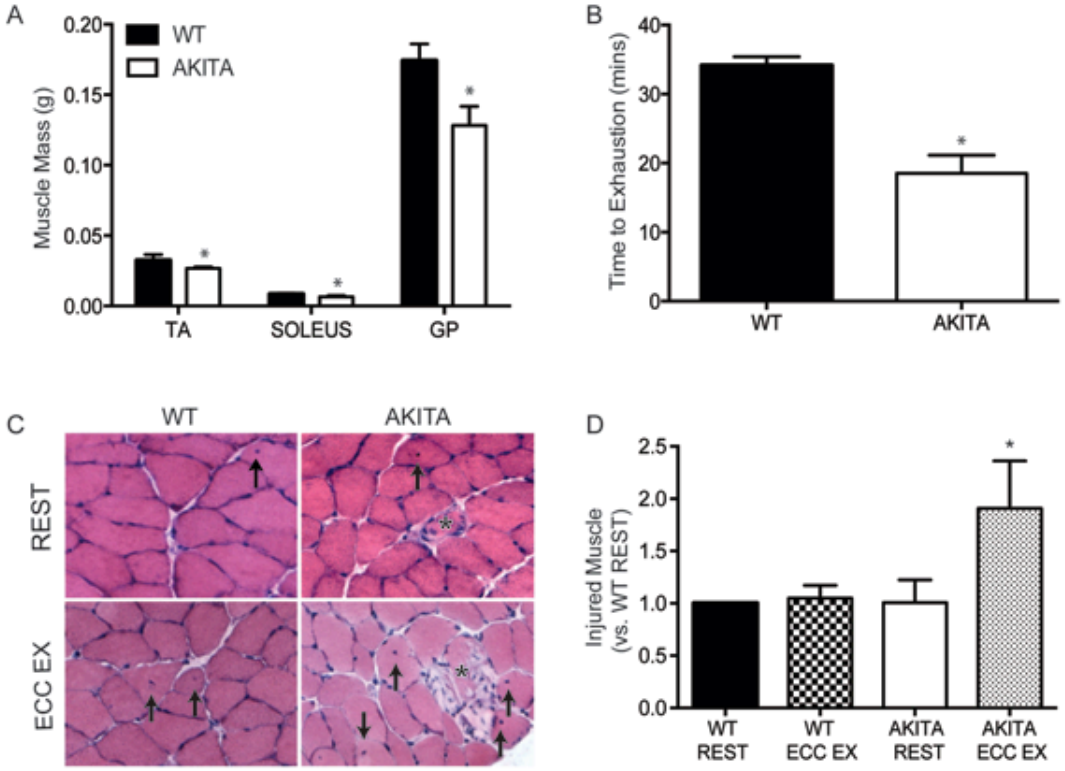


Figure 3.1. T1D skeletal muscle display hallmark characteristics of myopathy. (A) Muscle masses from Tibialis Anterior (TA), soleus, and Gastrocnemius-Plantaris (GP) muscles are decreased in 12 week Akita mice, n=3. (B) WT and Akita mice subjected to an endurance stress test demonstrate that Akita mice are quicker to exhaust than their WT counterparts, n=3 WT, n=4 Akita. (C) WT and Akita mice were eccentrically exercised to induce mild muscle damage, with exercised Akita mice displaying the greatest indices of muscle damage. Black arrows identify central located nuclei, while black asterisks identify necrotic tissue. (D) Quantification of muscle injury (see methods for criteria) indicate that Akita skeletal muscle is more damaged following eccentric exercise, n=3. *P<0.05 vs. WT.

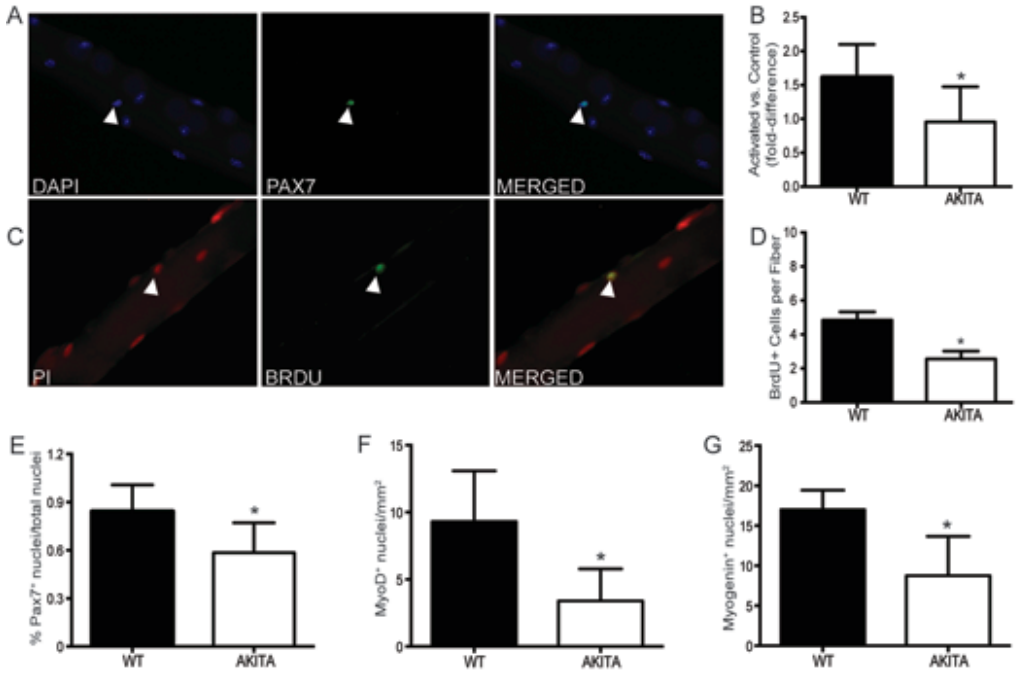


Figure 3.2. SC activation and content is decreased in T1D skeletal muscle. (A) Single myofibers were isolated from WT and Akita muscle, and stained for nuclei and Pax7. The white arrowheads note a positive signal for a satellite cell. (B) The difference in Pax7 content between activated and control myofibers was determined, and indicates that Akita SCs demonstrate a failure to become activated when compared to the WT, n=15-38 myofibers per experimental group. (C) Representative images of BrdU incorporation, a measure of SC activation, are shown in a WT myofiber. Single myofibers were stained with propidium iodide (PI) as a marker for nuclei and BrdU. The white arrowhead indicates a positive signal for BrdU incorporation. (D) SC activation was found to be lower in Akita mice at 24 hours following isolation, when compared to WT single myofibers, n=7-21 myofibers per experimental group. (E) SC content, determined by Pax7 expression in muscle sections, is lower in T1D skeletal muscle, n=5. (F&G)

Markers of myogenesis, MyoD and Myogenin, were stained for on activated WT and Akita single myofibers. Compared to WT, T1D SCs display reduced expression of MyoD (n=14-16 myofibers) and Myogenin (n=4-5 myofibers). *P<0.05 vs. WT.

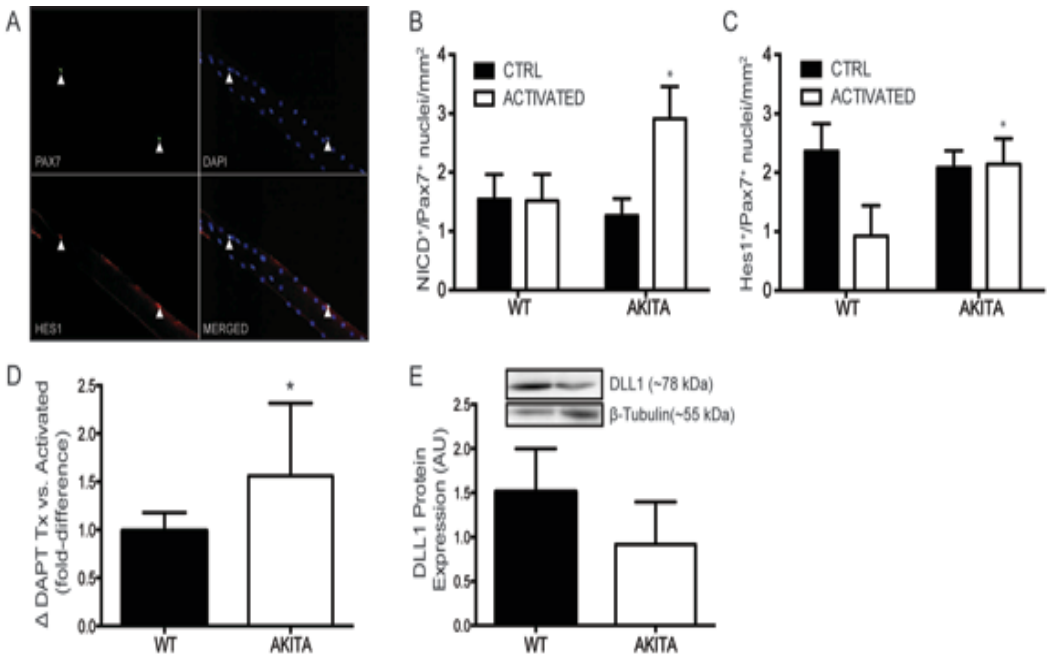


Figure 3.3. Hyper-activation of notch signalling alters SC behaviour in T1D muscle, but is restored with notch inhibition. (A) Representative images of the evaluation of the Notch target, Hes1, in single myofibers. White arrowheads identify a positive signal for Hes1+/Pax7+ SCs. (B) Hyper-activation of Notch activity is evident in activated Akita SCs when compared to WT SCs, n=7-9 myofibers per experimental group. (C) Hes1 is repressed in activated WT SCs but remains elevated in Akita SCs, confirming enhanced Notch activity in T1D SCs, n=6-13 myofibers per experimental group. (D) Activated WT and Akita single myofibers were treated with the Notch inhibitor DAPT (DAPT Tx), and compared to untreated activated single myofibers from each respective experimental condition (Activated). Notch inhibition with DAPT treatment led to a significant increase in Pax7 expression in activated Akita single myofibers, while no difference in Pax7 expression was determined in activated WT myofibers, n=6-12 myofibers per

experimental group. (E) The Notch ligand DLL1 shows a trend ($p=0.09$) towards a decrease in expression in whole muscle lysates from diabetic samples, n=3. *$P<0.05$ vs. WTActivated.

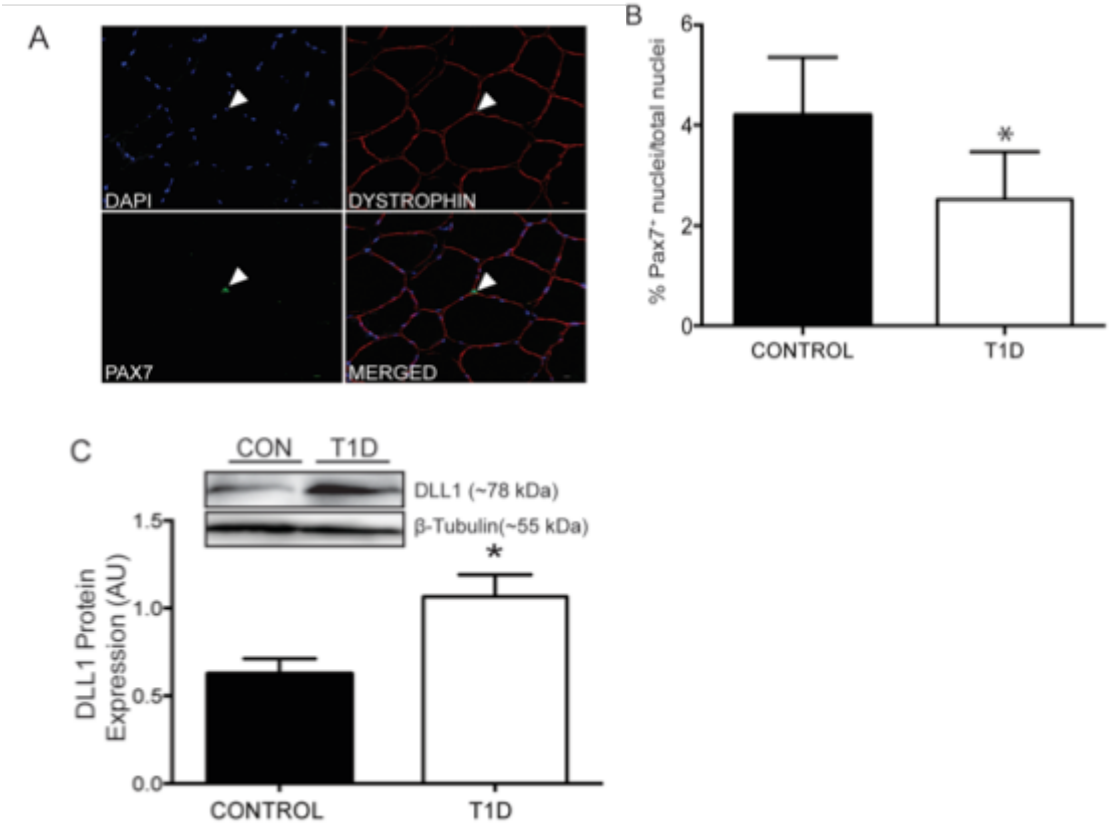
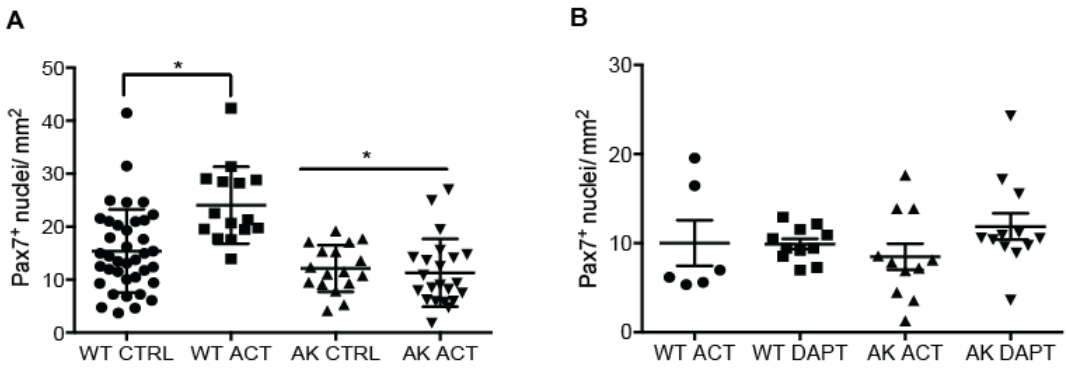


Figure 3.4. SC content is decreased in the skeletal muscle of humans with T1D. (A) Representative image of SC content in a T1D human muscle section. Sections were co-stained with DAPI, Dystrophin, and Pax7. White arrowheads indicate a positive signal for a SC. (B) The corresponding quantification of SC density is shown, n=5 Control, n=5 T1D. (C) To ascertain whether activation of the Notch pathway was evident, protein expression for the Notch ligand DLL1 was quantified, showing enhanced expression in T1D human muscle, n=3 Control, n=4 T1D. *P<0.05 vs. Control.



Supplementary Figure 3.1. SC Activation in WT and Akita mice with or without DAPT treatment (A) Raw data points demonstrating the number of Pax7+ nuclei per single myofiber area (mm^2) in WT and Akita muscle, n=15-38 myofibers per experimental group. (B) Raw data points demonstrating the number of Pax7+ nuclei per single myofiber area (mm^2) in activated myofiber (ACT) and activated myofibers treated with DAPT for 24 hours (DAPT), n=6-12 fibers per experimental group. *P<0.05 vs. WT ACT.

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CHAPTER 4

A Role for Lipocalin-2 in Skeletal Muscle Repair through its Influence on the Satellite Cell

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PREFACE

Significance to thesis

Data obtained from Chapters 2 and 3 demonstrated that SCs poorly activate in various forms of diabetes, and is likely attributed to adverse changes to intrinsic cellular pathways that regulate SC quiescence and activation. Previous work from our lab and others has identified the importance of the extracellular matrix (ECM) and its regulators to skeletal muscle repair and SC activity. In this study, we focus on the role of an ECM-associated factor referred to as Lipocalin-2 (LCN2) that is known to be dysregulated in diabetes, and has been shown to influence ECM composition. Since the primary focus of this thesis is on the SC, a goal of this work was to investigate the significance of LCN2 to the SC, as well as discern its relevance to skeletal muscle repair. Establishing whether LCN2 is required for normal SC activity, and thus, skeletal muscle regeneration, will help to determine its relevance to the maintenance of skeletal muscle structure, and as such, skeletal muscle health. This information will be considered in subsequent investigations regarding LCN2 in Diabetes Mellitus, and specifically, the role for LCN2 in diabetic SCs.

Author's Contributions:

Donna M. D'souza contributed to the design of the study, researched data, completed microscope image analysis, performed statistical analysis, wrote the manuscript, and worked on refining this draft.

Sarah Zhou assisted in performing experiments and completed microscope analysis.

Irena A. Rebalka performed animal care and assisted in sample collection.

Thorsten Berger contributed to the design of the study, provided LCN2 deficient mice and performed animal care.

Thomas J. Hawke contributed to the design of the study, assisted in experiments, and worked on refining drafts of the manuscript.

ABSTRACT

Lipocalin-2 (LCN2) has been previously established for its contribution to energy metabolism and innate immunity, and is also considered to be an extracellular matrix (ECM) regulator based on its association with the ECM protease, MMP-9. Preliminary work from our lab has shown that it is elevated in regenerating skeletal muscle, more so in healthy versus diabetic samples. To establish its function in healthy skeletal muscle, we examined its localization and expression in uninjured and 48-hour injured wild-type (WT) skeletal muscle. LCN2 was highly elevated in 48-hour injured skeletal muscle, and was found to be present in both macrophages and satellite cells (SCs). SC content was reduced and SC activation was attenuated in skeletal muscle from LCN2 whole body knockout mice (LCN2-/-) at 2 and 5 days post-injury. These results coincided with an overall increase in M1 macrophages in LCN2-/- muscle at 4 days post-injury, as well as a trend for reduced MMP-9 expression in LCN2-/- muscle at 2 days post-injury. Taken together, these data indicate that in the absence of LCN2, normal skeletal muscle regeneration is negatively affected, and occurs via defects in SC function and altered MMP-9 availability. This study is novel in that it is the first to identify a role for LCN2 in healthy skeletal muscle regeneration. Based on this work, and studies that have shown LCN2 to be linked to the presence of diabetic complications, future studies will be focused on examining its influence on diabetic SCs and skeletal muscle repair to better understand its contribution to the development of diabetic myopathy.

INTRODUCTION

Skeletal muscle is a malleable tissue capable of adapting to a variety of pathological and physiological stimuli. Its plasticity is attributed to its ability to regenerate, which encompasses a complex series of events that includes both the degeneration and regeneration of injured muscle fibers (1). Skeletal muscle regenerative capacity is an important component in the maintenance of skeletal muscle mass and function, as has been established (2). In various disease states, such as Diabetes Mellitus, skeletal muscle repair is impaired, leading to the advancement of diabetic myopathy, a condition characterized by detrimental changes to muscle morphology, metabolism and/or function (3). Several studies have demonstrated a failure for muscle to properly regenerate in both Type 1 Diabetes Mellitus (T1D; 4–7) and Type 2 Diabetes Mellitus (T2D; 8–11). Taken together, these studies imply that despite variances in the etiology of these types of diabetes, skeletal muscle derived from diabetic individuals demonstrates similar characteristics following exposure to injury stimuli, including enhanced fibrosis caused by abnormal ECM remodelling, changes in vascularity, altered inflammatory cell presence, and most importantly, adverse modifications to the satellite cell, a muscle progenitor cell that significantly contributes to muscle repair (12). Accordingly, it is surmised that Type 1 and 2 diabetic muscles may share a common feature (or features) that contribute to the development of myopathy, and as such, it becomes necessary to elucidate the potential factor(s) involved.

Preliminary work from our lab has identified a factor, referred to as Lipocalin-2 (LCN2), that was highly up-regulated following CTX injury in diabetic mouse muscle

(Krause and Hawke, unpublished observations), and that has an assortment of roles related to diabetes, including a link to insulin resistance (13, 14), participation in innate immunity (15) and an association with obesity-related inflammation and hyperglycemia (14). LCN2 was also shown to be a key predictor of diabetic kidney injury (16), as it is induced by a diverse array of inflammatory and metabolic factors (17). Perhaps most relevant to our interest in the study of skeletal muscle repair were studies that showed that LCN2 forms a complex with the ECM protease, MMP-9, in order to maintain its stability and prolong its lifespan (18,19). Considering this, along with data that demonstrate that MMP-9 is integral to muscle regeneration (20), and is produced by SCs (21), it was hypothesized that LCN2, through its association with MMP-9, modulates SC function, and thus contributes to skeletal muscle regenerative capacity. Accordingly, the purpose of the current study was to determine the impact of LCN2 deficiency on skeletal muscle regeneration, with a primary focus on the muscle SC population.

Using WT and LCN2-/- whole body knockout mice, we demonstrate that LCN2 expression is enhanced following muscle injury, and is localized in both inflammatory cells and SCs during the early phases of skeletal muscle repair. In the absence of LCN2, impairments in SC content and function were observed, resulting in delayed regeneration at 2 and 5 days following CTX injury. While a specific mechanism of action remains to be fully elucidated, it is speculated that the response of LCN2-/- skeletal muscle to injury is attributed to both intrinsic and extrinsic factors related to the SC, along with changes to the presence of inflammatory cells early in the repair process. This study is novel as it is the first to demonstrate the presence of LCN2 in SCs, and is related to the study of

diabetic myopathy as it establishes a purpose for LCN2 in healthy skeletal muscle subjected to injury. This information may be subsequently implemented in the investigation of diabetic myopathy.

RESEARCH METHODOLOGY

Animals. All experimental protocols were approved by the McMaster University Animal Care Committee (AUP#13-09-32) in accordance with the Canadian Council for Animal Care guidelines. Female C57BL6/J mice were obtained from Jackson Laboratories (wild-type; WT), while previously generated Lipocalin-2 knockout (LCN2-/-) females with a C57BL6/J background (22) were generously donated. Animals were housed in a temperature- and humidity-controlled facility with a 12/12h light/dark cycle and had *ad libitum* access to water and food. At approximately 5-7 months of age, mice were divided into the following experiments: single fiber isolation of uninjured mice and CTX injury for the analysis of muscle regeneration and protein expression through immunofluorescence and western blotting.

Skeletal muscle injury. Intramuscular injections of CTX (Latoxan, Valence, France; 10uM) were performed as described previously (23) into the left Tibialis Anterior (TA) muscle or Gastrocnemius-Plantaris-Soleus (GPS) complex of WT and LCN2-/- mice. Mice were sacrificed and harvested at 2 or 5 days post-injury.

Tissue Collection. Mice were euthanized by CO_2 inhalation followed by cervical dislocation. Uninjured (right) and injured (left) TA muscles were covered with optimum cutting temperature embedding compound, as was the left GPS complex. The right GPS complex and Quadriceps were snap-frozen. All tissues were stored at -80°C.

Single Myofiber Isolation and Immunofluorescence. Extensor Digitorum Longus (EDL) and peroneus muscles from uninjured WT and LCN2-/- mice were digested by Type I Collagenase (Invitrogen, Carlsbad, CA), as previously described (23). Single fibers were triturated from digested muscle bundles and then transferred to 10% horse serum-coated cell culture dishes with a glass Pasteur pipette. Floating cultures were either placed in plating media [10% normal horse serum, 0.5% chick embryo extract in low-glucose (1g/L) Dulbecco's Modified Eagle Medium (DMEM; Invitrogen)] for an overnight activation period, or fixed immediately with 2% paraformaldehyde (PFA) for 5 min at room temperature (referred to as 'Control Myofibers'). Following the activation period (~18 h, overnight), single fibers from each experimental group were also fixed with 2% PFA for 5 min at room temperature (referred to as 'Activated Myofibers'). Single fibers were stained for primary antibodies against Pax7 (1:10, DSHB, Iowa City, Iowa), MyoD (1:100, Abcam, Cambridge, UK), and/or LCN2 (1:500, Abcam), and subsequently stained for the appropriate secondary antibodies (Invitrogen). Nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI). Note that for all myofibers staining, at least 3 mice per experimental group were used to isolate myofibers. The specific number of myofibers isolated per experiment is noted in the figure legends.

Histochemical and Immunofluorescence Analyses of Muscle Sections. Skeletal muscle sections from TA or GPS muscles embedded in OCT were transversely cut into 8μm muscle cross-sections and mounted on glass slides.

Hematoxylin and Eosin stain. Hematoxylin and Eosin (H&E) stains were completed to assess muscle morphology between animal cohorts at rest, or after 2 and 5 days post-

injury. More than 75 fibers were examined per muscle section to provide information regarding fiber size, fiber morphology, and the appearance of myopathic markers (i.e. necrotic fibers, central located nuclei, pale cytoplasm, inflammatory cells).

Immunofluorescence. Sections were fixed with 2% PFA for 5 min at room temperature, blocked (PBS with 1.5% NGS and 1.5% NHS), and incubated with primary antibody overnight at 4°C. Muscle sections were stained for LCN2 (1:500, Abcam), F4/80 (1:250, Abcam), Pax7 (DSHB) with TSA amplification, MyoD (1:100, Abcam), Dystrophin (1:250, Abcam), Embryonic Myosin Heavy Chain (DSHB), or CD206 (1:250, Abcam). Nuclei were counterstained with DAPI, while the appropriate Alexa secondary antibody was used for each primary antibody.

Image Analysis. Images were acquired using a Nikon 90-eclipse microscope (Nikon Inc., Melville, NY) and analyzed using the Nikon Elements Software (Nikon Inc.). Analyses included identification of muscle morphology following CTX injury (as described above), determination of regenerating areas (Myh3+ area), localization of LCN2 in SCs (LCN2+/PAX7+), Macrophage polarization (F480+/CD206+ or F480+/CD206-) and Pax7+ and MyoD+ nuclei on single myofibers and/or muscle sections. Images were taken at 20x magnification.

Western Blot Analyses. Approximately 20 or 35ug of protein derived from injured or uninjured whole muscle lysates were loaded on a 10% acrylamide gel, transferred to PVDF membrane (BioRad, Missisauga, Ontario), blocked with 5% skim milk for 1 h at room temperature, and then incubated overnight at 4°C with primary antibody. Proteins assessed include LCN2 (1:500, Abcam), MyoD (1:250, Abcam), and MMP-9 (1:5000,

AbCam). The appropriate horseradish peroxidase-conjugated secondary antibodies were incubated for 1 h at room temperature, and the blot was then visualized using SuperSignal Chemiluminescent reagent (Thermo Scientific, Waltham, MA). Images were acquired using a Gel Logic 6000 Pro Imager (Carestream, Rochester, NY), and the density of each band was analyzed using Adobe Photoshop.

Statistical Analysis. Measures were assessed using Student's t-test, or where appropriate, a Two-Way ANOVA with Bonferroni post-hoc tests. Significance was set at a P value of less than or equal to 0.05 (denoted by asterisk). All statistical analysis was performed using GraphPad Prism 5 (La Jolla, CA) software. Data are presented as means ± standard error of the mean (SEM).

RESULTS

LCN2 is expressed immediately post-injury in skeletal muscle. Representative Western blots from various time points following CTX injection in WT Gastrocnemius-Plantaris-Soleus (GPS) muscle complexes demonstrate an enhanced expression of LCN2 at 24 and 48 hours following injury, while minimally detectable levels of LCN2 expression were observed at rest, 5, or 7 days post-injury (Figure 4.1A). Quantification of LCN2 in TA muscles 48 hours following injury demonstrates a 3-fold increase in its expression, as compared to uninjured muscle (Figure 4.1B, $p<0.05$).

LCN2 is localized to cell populations pertinent to skeletal muscle regeneration following injury. The enhanced expression of LCN2 following CTX injection in skeletal muscle led to the evaluation of LCN2 in injured WT muscle sections (48 hours post-CTX injection). Based on its role in innate immunity (15), it was predicted that LCN2 would be

primarily identified in inflammatory cell types, and was confirmed through immunofluorescent co-staining of LCN2 with the macrophage marker, F4/80 (Figure 4.2A). The identification of LCN2 in other cell types prompted assessment of LCN2 expression in SCs. LCN2 was minimally detected at rest, but was found in SCs at 48 hours post-injury (Figure 4.2B). To further confirm the co-localization of LCN2 in SCs, single myofibers were isolated from WT skeletal muscle and co-stained for LCN2 and Pax7 in activated single myofibers. As predicted, a direct overlap of LCN2 and Pax7 was observed (Figure 4.2C).

LCN2 influences SC density and activation, as demonstrated by its in vivo ablation.

Based on the newfound localization of LCN2 in SCs, we hypothesized that changes to LCN2 expression in skeletal muscle could influence SCs. As such, SC content was quantified in muscle sections obtained from age- and sex-matched LCN2-/- and WT mice. SC density was 35% lower in LCN2-/- mice, compared to the WT, as shown by the relative reduction of Pax7 in muscle sections (Figure 4.3A) and graphical quantification (Figure 4.3B, $p<0.05$). Following this, single myofiber experiments were completed using WT and LCN2-/- muscle to further ascertain the impact of LCN2 on SC activity. Differences in Pax7+ nuclei observed between Activated and Control myofibers reflects the SCs ability to enter the cell cycle and proliferate. While WT activated single fibers demonstrate an approximate 2-fold increase in Pax7 expression, activated single fibers from LCN2-/- displayed only a 1.5-fold change as compared to LCN2-/- control single fibers, indicating impaired activation (Figure 4.3C, $p<0.05$). Individual data points demonstrating differences in Pax7+ nuclei on control and activated single myofibers from

each experimental group are shown in a supplementary graph (Supplementary Figure 4.1A, $p<0.05$). This phenomenon is further supported by a 2.9-fold decrease in MyoD+ nuclei in activated LCN2-/- single myofibers, in contrast with WT (Figure 4.3D, $p<0.05$).

LCN2-/- mice display delayed muscle regeneration at 2 and 5 days post-injury. The observed change in SC density and/or function following LCN2 manipulation led to the assessment of muscle regeneration in WT and LCN2-/- muscle. Both cohorts of mice were subjected to CTX injury of TA or GPS complex muscles, with muscles harvested at 2 or 5 days post-injury. Relative to the WT, the weight of LCN2-/- TA muscles were increased at 2 days post-injury, and then decreased by 5 days post-injury (Figure 4.4A, $p<0.05$). Immunohistochemical H&E staining of resting and injured WT and LCN2-/- muscles shows morphological differences, as fewer and smaller regenerating fibers are identified in LCN2-/- muscle at 2 and 5 days, respectively (Figure 4.4B). Muscle repair was quantified at 5 days post-injury by staining for embryonic myosin heavy chain (Myh3+) fiber, a well-established marker for regenerating fibers (24; Figure 4.4C). Fewer and smaller newly regenerating fibers were identified in 5-day injured LCN2-/- muscle, as compared to injury-matched WT muscle sections (Figure 4.4D,E; $p<0.05$). These data, along with the morphological changes identified through H&E staining, provide evidence of delayed skeletal muscle repair in LCN2-/- mice.

Defects in SC activity and macrophage polarization in the early phases of regeneration contribute to abnormal muscle repair in LCN2-/- skeletal muscle. To further verify that SC function was hindered in the absence of LCN2, expression of the SC activation marker, MyoD, was determined in WT and LCN2-/- whole muscle lysates

48-hours following CTX injury. As expected, there was a 2.4-fold decrease in MyoD protein expression in injured LCN2-/- muscle (Figure 4.5A, $p<0.05$). Defective SC function, and thus, muscle repair, may be attributed to a variety of factors that modulate SC behaviour (25). Given the role of LCN2 in the regulation of MMP-9 (26), and the importance of MMP-9 in the early phase of muscle regeneration (27), we examined the expression of active MMP-9 at 48 hours post-injury between experimental groups. Our preliminary results indicate that MMP-9 expression is reduced in LCN2-/- injured whole muscle lysates, as depicted in Figure 4.5B and 4.5C.

The significance of macrophages to SC function, and thus, muscle regeneration has been established (28), therefore we examined different subsets of macrophage populations at 4 days post-injury to discern if differences in macrophage polarization existed between experimental groups. Note that this specific time point was selected as it was hypothesized, based on data obtained regarding delayed LCN2-/- muscle repair, that macrophage populations would vary between WT and LCN2-/- injured muscle. Macrophage polarization was determined by immunofluorescent co-staining for the macrophage markers F4/80 and CD206 (Figure 4.5D). LCN2-/- skeletal muscle displayed a 14-fold increase in the number of M1 macrophages (F480+/CD206-), and a 57% decline in the number of M2 macrophages (F480+/CD206+) in areas of regeneration, in contrast to regenerating WT muscle samples (Figure 4.5E,F, $p<0.05$).

DISCUSSION

In the present study, we investigated the influence of Lipocalin-2 (LCN2), a factor that appears to be dysregulated in Diabetes Mellitus, on SC function and skeletal muscle

regeneration. To our knowledge, this is the first study to demonstrate that LCN2 is found within SCs, and in its absence, delays normal skeletal muscle regeneration. The main findings of this study can be summarized as follows: [1] LCN2 is expressed immediately post-injury in skeletal muscle, and is localized in inflammatory cells and muscle SCs; [2] in the absence of LCN2, SC function is adversely affected and macrophage polarization is modified, interfering with the initial stages of muscle repair.

The immediate up-regulation of LCN2 at 24 and 48 hours following CTX injury is not surprising given its established role in innate immunity (15), and more importantly, its close association and stabilization of the ECM protease, MMP-9 (26). The inflammatory response of skeletal muscle to injury is beneficial as it promotes the degradation of materials, and is largely regulated by inflammatory and myogenic cells (29). The first cell population to be found at the site of injury are neutrophils, as they peak in concentrations at 24 hours-post injury, followed by their depletion (30, 31). Given the significant increase in LCN2 protein expression at 48 hours post-injury, it was expected that alternative cell types, rather than neutrophils, serve as the source for LCN2. Following immunofluorescent staining, LCN2 was found in macrophages, which are primarily present in the early stages of muscle repair (32–34). Expression of LCN2 in macrophages is likely related to its ability to sustain MMP-9 function, which is known to facilitate macrophage migration (35, 36). Completion of immunofluorescence analyses revealed that LCN2 was localized not only in macrophages (F4/80+), but other cell types (F4/80-). Since MMP-9 was discovered in SCs (21), we postulated that LCN2 would also be found in SCs. LCN2 was only evident in SCs found in muscle sections from 48-hour

injured muscle, and SCs found on 24-hour isolated myofibers. Accordingly, it seems that LCN2 is exclusively expressed in activated, rather than quiescent, SCs.

Defining the effect of LCN2 on SC function was achieved by evaluating LCN2-/- skeletal muscle. In the absence of LCN2, SC density and function were negatively affected. Furthermore, the decline in MyoD+ nuclei 48-hours post-injury confirms the negative regulation of SC behaviour following muscle injury in LCN2-/- muscle. Although the precise cause for this remains unclear, there are a number of proposed mechanisms believed to be involved. Firstly, though no study to date has identified LCN2 in SCs, the findings of this study parallel results obtained from investigations assessing MMP-9 in the SC. There have been a variety of studies that have shown that MMP-9 is linked to SC activation (20,37), as well as downstream processes of myogenesis (38). In a recent study, MMP-9 was found to be necessary for the proliferation, but not differentiation, of SCs based on its nuclear localization (39). In their study, Zimowaska and colleagues identify that localization of MMP-9 in the nucleus contributed to nuclear matrix proteolysis and turnover, facilitating SC proliferation (39). Though none of these studies evaluated LCN2 in their investigation of MMP-9 activity in SCs, the decrease in MMP-9 expression in 48 hour injured LCN2-/- muscle from the present study corroborates the idea that LCN2 and MMP-9 are closely intertwined. Therefore, in the absence of LCN2, it is postulated that MMP-9 availability declines in activated SCs, resulting in aberrant proliferation. Following this, replenishment of the quiescent SC pool in LCN2-/- skeletal muscle likely becomes difficult, prompting a reduction in SC density. LCN2 may not only exert its effect on the intrinsic machinery governing SC activity. It is

surmised that reductions in the number of Pax7+ and MyoD+ nuclei in LCN2-/- myofibers observed at 24 hours post-isolation is suggestive of an extrinsic mechanism through which LCN2 in modulates SC status. Physical dissociation of SCs from their niche has been found to contribute to activation, and is orchestrated by the function of MMPs and other proteases, as demonstrated in aged muscle (40,41). Although myofibers isolated from WT and LCN2-/- muscle were investigated in vitro, the isolation procedure permits that components of the ECM remain intact (42,43), hence it is likely that SCs derived from LCN2-/- myofibers differ in their niche components as compared to WT SCs, abnormally altering the sensitivity for SCs derived from LCN2-/- muscle to become activated. In addition to influencing intrinsic SC function and the extrinsic SC environment, the ablation of LCN2 is postulated to modify the interaction between inflammatory cells that participate in regeneration and the SC population. This concept has been observed in alternative animal models (44, 45), and was confirmed in the present study through data identifying changes in macrophage polarization, as has been previously shown (46). The persistence of pro-inflammatory macrophages in 4-day injured LCN2-/- muscle, versus that of the WT, likely facilitates attenuated muscle repair as has been identified (47). To better discern the specific influence of LCN2 on SCs, future studies should include analyses of isolated LCN2-/- SCs to determine if they are capable of differentiating into functional myoblasts in vitro, as well as muscle-specific deletion of LCN2 in vivo to examine individual responses of muscle and non-muscle cells following skeletal muscle injury.

Modifications to SC function has often resulted in unfavourable changes to muscle regeneration (48), and thus we expected defective LCN2-/- muscle repair at 2 and 5 days post-injury, as determined by differences in LCN2-/- muscle mass, morphology, and the expression of Myh3. The delay in muscle regeneration is attributed to abnormalities in SC behaviour, as has been observed by previous work from our lab that has examined muscle regeneration in specific murine models of diabetes (49, 50). Although these studies were not focused on elucidating the function of LCN2 in diabetic muscle regeneration, there are some interesting comparisons to be made between these studies. For instance, inhibition of plasminogen activator inhibitor-1 (PAI-1), an upstream inhibitor of MMP-9, augmented muscle repair in T1D mice (49, 51), and corroborates current data that shows the opposing effect caused by the ablation of LCN2. Furthermore, SC activation was also found to be impaired in murine models of Pre-diabetes and T1D (50, 52), and parallels the findings from this investigation. These results suggest that a common underlying factor evident in all studies likely contributes to the impairment of normal SC function. Based on the absence of LCN2 in the current study, and its regulation of MMP-9, it is surmised that alterations in ECM composition, as brought forth by the ablation of LCN2 in this study, or via the onset of T1D and Pre-diabetes in previous work, greatly influences the fate of the SC. Further assessment of ECM composition in various murine models of diabetes should be investigated and compared to that of LCN2-/- skeletal muscle to establish this theory. Additionally, the manipulation of LCN2 in divergent models of diabetes is necessary to elucidate its use as

a novel therapeutic target for the maintenance and/or improvement of skeletal muscle structure, which represents a key component of skeletal muscle health.

Collectively, the results from this study demonstrate a role for Lipocalin-2 in the regulation of skeletal repair, via its modulation of SC function, and thus, skeletal muscle regeneration. Uncovering a novel function for LCN2 in SCs is critical to our understanding of skeletal muscle plasticity, and contributes to knowledge regarding the development of treatments that work to maintain and/or enhance skeletal regeneration in various disease states, including diabetes.

FIGURES

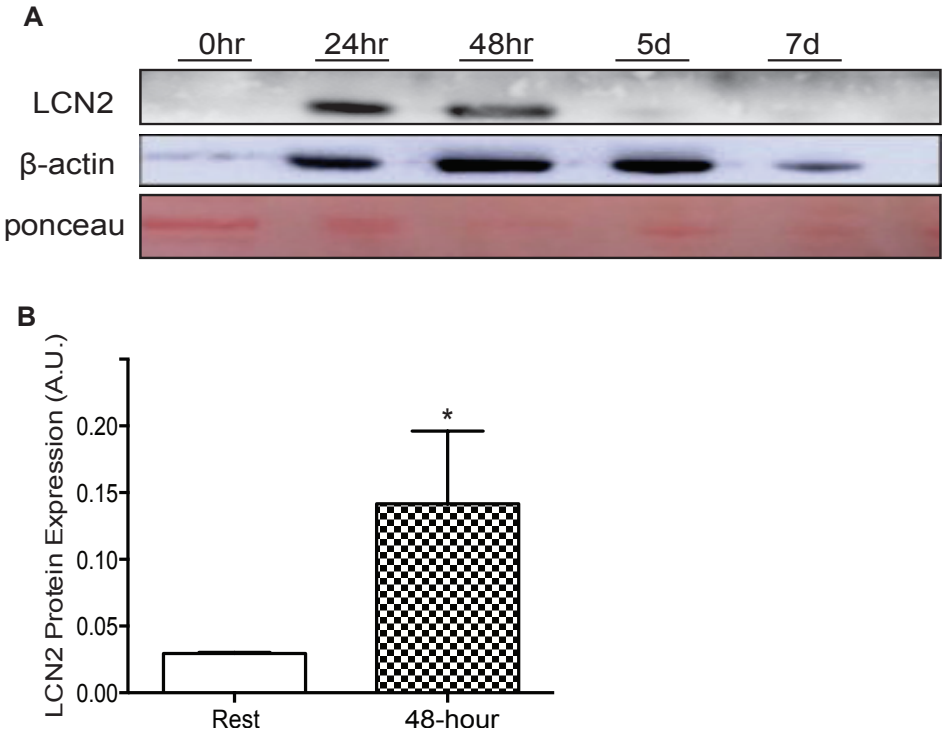


Figure 4.1. Lipocalin-2 is expressed immediately post-injury in skeletal muscle. (A) Representative western blot of LCN2 expression in WT GPS muscles at increasing time points post-injury. (B) Quantification of LCN2 protein expression in TA muscles at 48-hours post-CTX injection, n=3. *P<0.05 vs. Rest.

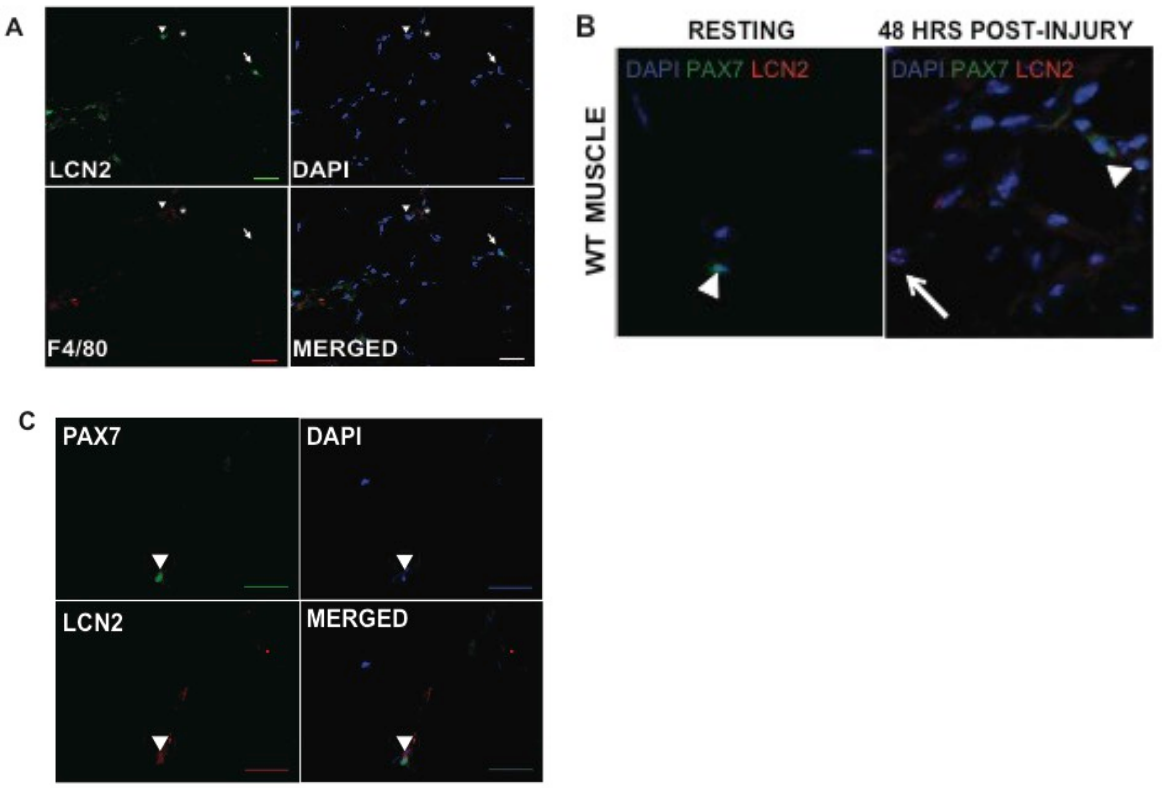


Figure 4.2. LCN2 is expressed in inflammatory cells and satellite cells following injury, and is not observed in uninjured muscle. (A) Muscle sections taken from 48-hour CTX-injured WT muscles were stained for LCN2, F4/80, and DAPI, and reveal localization of LCN2 in F4/80+ cells (white triangle) and F4/80- cells (white arrow). Note that LCN2 was not found in all F4/80+ cells (white asterisk). (B) Comparison of LCN2 localization in SCs (white triangle) between uninjured and 48-hour injured WT muscle sections. LCN2 was not found in SCS from uninjured muscle, but was evident in both SCs and non-SCs (white arrow) following injury. (C) LCN2 expression in activated SCs (white triangle) was confirmed by staining WT myofibers with LCN2 and Pax7. Scale bars=0.2cm.

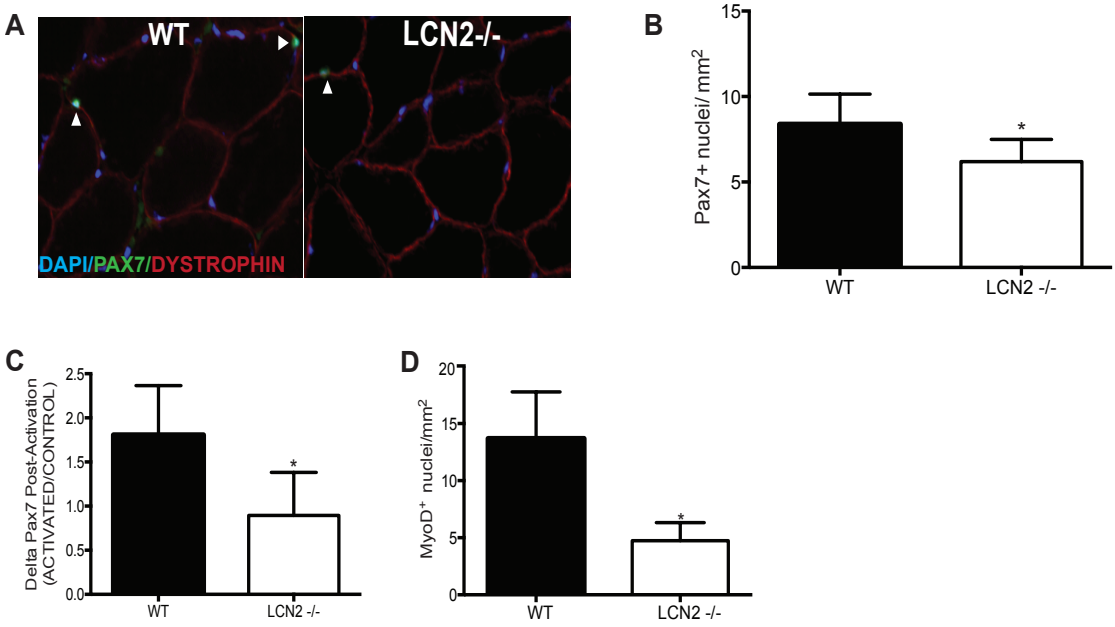


Figure 4.3. LCN2 modulates SC density and function. (A&B) SCs were quantified in WT and LCN2-/- muscle sections (white triangle), and were found to be lower in LCN2-/- skeletal muscle, as compared to WT, n=4. (C) SC activation was determined by the difference in Pax7+ nuclei between activated and control myofibers isolated from WT and LCN2-/- mice, n=7-16 fibers per experimental group. (D) The number of MyoD+ nuclei was determine on activated WT and LCN2-/- myofibers, n=4-6 fibers per experimental group. *P<0.05 vs. WT. Scale bars=0.02mm.

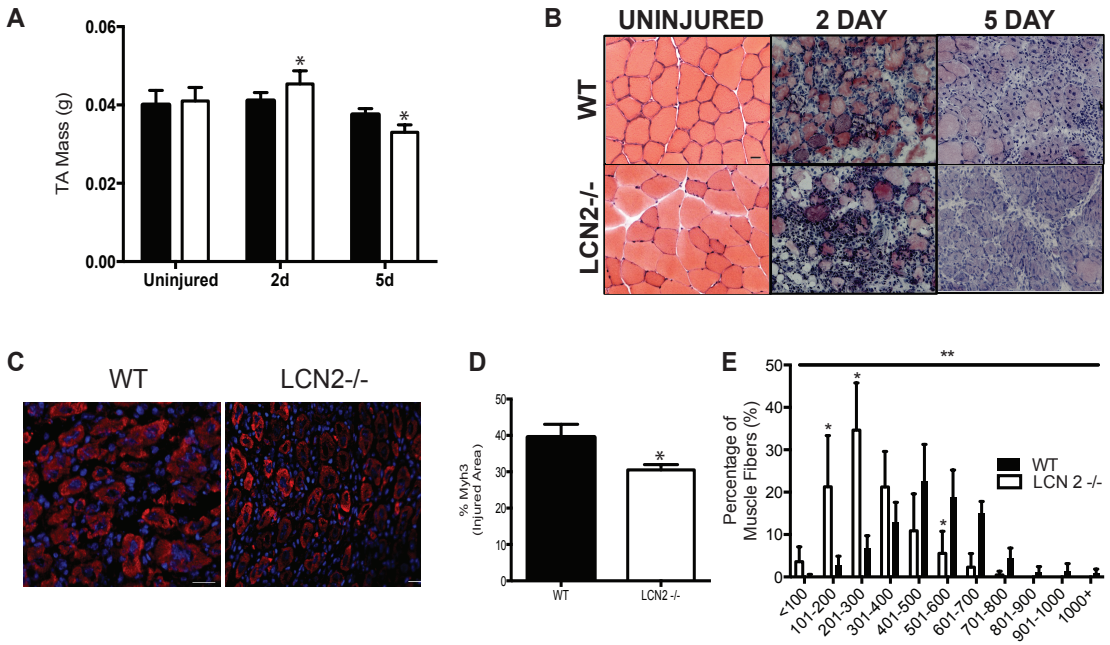


Figure 4.4. LCN2-/- skeletal muscle displays impaired skeletal muscle regeneration. (A) Mass of WT and LCN2-/- TA muscles was quantified from uninjured muscle, as well as from muscle taken at 2 and 5 days post-injury, n=5. (B) Morphology of TA muscle sections from each time point investigated were compared and contrasted between WT and LCN2-/- experimental groups. (C) A representative image of WT TA muscle section stained at 5 days post-injury for embryonic myosin heavy chain (Myh3+, red; nuclei stained for DAPI, blue) (D) Graphical representation of the percentage of Myh3+ in the injured areas of WT and LCN2-/- muscle, n=3. (E) Distribution of regenerating myofiber area between WT and LCN2-/- muscles, n=4 WT, 3 LCN2-/-, *P<0.05 vs. WT. **P<0.05 Interaction effect. Scale bars=0.02mm.

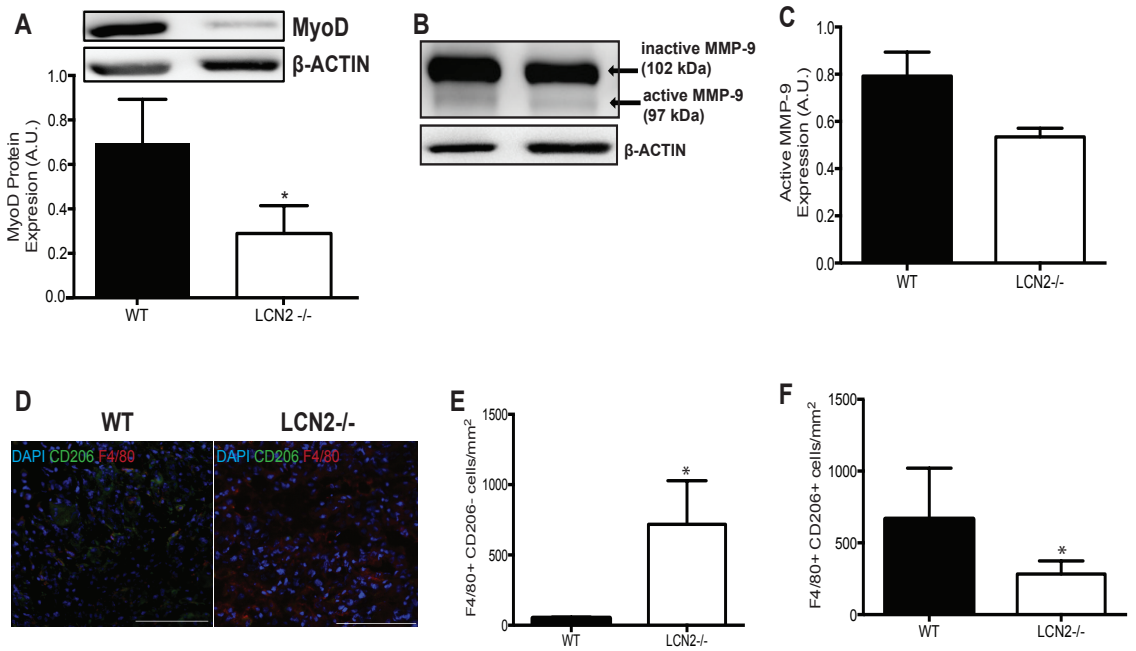
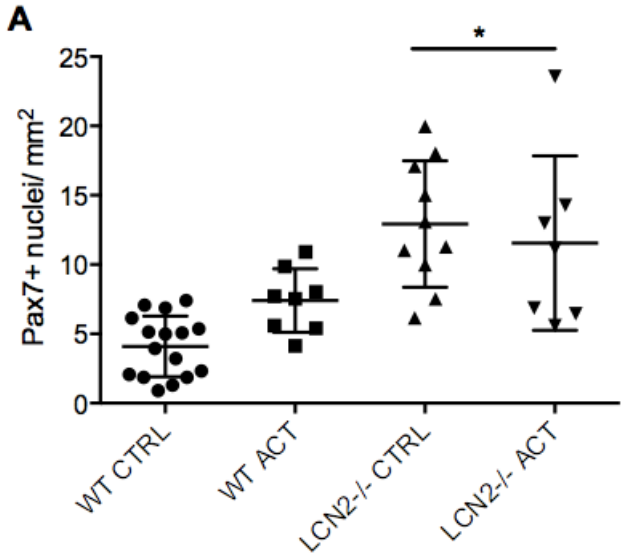


Figure 4.5. Defects in SC activation and macrophage polarization in LCN2-/- skeletal muscle contribute to impaired regeneration. (A) Representative blot and graphical presentation of MyoD protein expression in 48-hour injured WT and LCN2-/- TA muscle lysates, n=3. (B&C) Representative blot and graphical presentation of pro- and active forms of MMP-9 in 48-hour injured WT and LCN2-/- TA muscle lysates, n=2-3. (D) Representative GPS muscle sections from WT muscle shows the presence of M1 (F4/80+, red, CD206-, green) and M2 (F4/80+, CD206-) macrophages at 4 days post-injury. (E&F) Quantification of M1 and M2 macrophages in WT and LCN2-/- muscle sections at 4 days post-injury, n=4. *P<0.05 vs. WT. Scale bars=0.1mm.



Supplementary Figure 4.1. SC activation in WT and LCN2-/- muscle. (A) Raw data points demonstrating the number of Pax7+ nuclei per single fiber area (mm2), n=7-16 fibers per experimental group. *P<0.05 vs. WT CTRL. .

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CHAPTER 5:
General Discussion and Conclusions

5.0 Significance of Studies

Despite being the subject of intense scientific and clinical investigation for many years, Diabetes Mellitus continues to burden the Canadian healthcare system, costing Canadians approximately $12 billion in 2010, with a projected increase to approximately $17 billion by the year 2020 (1). Subtypes of diabetes include Type 1, Type 2 and Pre-Diabetes, with increasing incidences of each form evident not only in Canada (1), but also worldwide (2).

Skeletal muscle tissue is intricately linked with diabetes progression, based to its function as the largest site for glucose uptake (3), and the biggest (by mass) endocrine organ of the body (4). A key feature of skeletal muscle is its plasticity; a feature which allows the tissue to adapt and repair in response to changes in its environment (for review, see 5). Studies have shown that the diabetic state negatively impacts skeletal muscle (6), partly through altering its capacity to regenerate (7–10). The precise mechanism(s) underlying impaired skeletal muscle regeneration in diabetes remains poorly understood.

In this thesis, I have focused my studies on a distinct population of muscle stem cells, termed Satellite Cells (SCs), integral to muscle repair. By examining two different types of diabetes, I address the question of whether the mechanism(s) for attenuated regeneration in diabetes is the result of impaired SC function. SCs were selected as the primary topic due to their significant contribution to skeletal muscle development, repair, ageing, and muscle disease (11), as well as their contribution to the maintenance of healthy uninjured skeletal muscle (12). Though data has been collected regarding the function of SCs in different diabetic states, the studies completed herein represent the first

body of work that investigates SCs in two unique types of diabetes, identifies similarities and differences in SC function in these conditions, compares and contrasts changes to the SC in rodent and human diabetic samples, and attempts to establish a link between diabetes development and impaired SC function through investigation of LCN2.

5.1 Examining the SC in DIO, Akita and Human T1D samples

In study 1 of this thesis, we assessed changes to SCs of mice subjected to 8 weeks of high fat feeding leading to diet-induced obesity (DIO). Results from this first study show that SCs were poorly activated in DIO skeletal muscle following muscle injury, and is mediated by changes in HGF/c-Met signalling. In contrast to DIO mice that obtain their phenotype through high fat feeding, the evaluation of T1D SC function in study 2 was determined using a transgenic mouse model (Akita mice) that has been used to examine a diverse array of diabetic complications (13–15). SCs derived from T1D myofibers displayed impaired activation and reduced SC density, the latter of which paralleled findings observed in T1D human muscle. The animal models were chosen for their strengths in recapitulating the human condition, while minimizing confounding variables that are seen in other models of Pre-Diabetes/T2D and T1D. The DIO model of Pre-Diabetes was incorporated in our investigations as it has been found to represent many characteristics of the human condition (16), and is relevant due to increasing rates of Pre-Diabetes (1). Successful characterization of skeletal muscle physiology in young (17) and adult (18) mice subjected to DIO has been completed, and our first study compliments these findings by adding to the literature regarding skeletal muscle and its response to the DIO environment. Meanwhile, the use of a genetic model of T1D (Akita), rather than

chemical-induction of T1D (STZ or the like), avoids interference of the cell cycle in the study of SCs, as has been previously noted (19). Additionally, the inclusion of T1D human data in chapter 3 of this thesis represents the first study, to our knowledge, that simultaneously investigated SCs in T1D rodent and human samples. Despite differences in exogenous insulin availability (uncontrolled in Akita mice versus insulin therapy in humans), SC density was reduced in both diabetic groups. This finding is significant when considering human diabetic therapy, as it suggests that insulin therapy is insufficient, by itself, to maintain and/or restore deficits to T1D SC activation, and should be considered along with previously established data investigating the influence of insulin on SC function (20-21). Furthermore, these results validate the use of the Akita animal model in the study of T1D, including the investigation of uncontrolled diabetes.

Although SC activation is impaired in both types of diabetes examined, SC content was reduced only in T1D rodent and human T1D muscle. This discrepancy between studies is attributed to differences in the activity of the two SC-related pathways investigated. Notch is closely associated with SC quiescence and self-renewal (22), and therefore it is postulated that its hyper-activation in T1D attenuates the capacity for SCs to exit quiescence to enable proper proliferation and self-renewal. In contrast, SC activation, and not content, is targeted in DIO skeletal muscle and was linked to impaired HGF/c-Met signalling. Literature has shown that HGF is vital for SC activation (23), and is also known to improve glucose homeostasis (24). It not surprising, therefore, that DIO muscles that were unable to obtain proper binding of HGF to c-Met displayed impaired SC activation.

While Notch activity is augmented in T1D skeletal muscle, HGF/c-Met signalling appears hindered in DIO. These observations led us to try and elucidate the particular factor, or factors, involved in mediating such changes to these SC-related cellular pathways. The extracellular matrix (ECM) is an interesting avenue for such an investigation, as both signalling pathways may be affected by alterations to ECM composition and/or proteins that regulate ECM turnover.

5.2 Assessing the Influence of ECM on SCs: Investigation of LCN2

ECM composition, especially within the diabetic skeletal muscle setting, is relevant to the study of diabetic SC function, as both HGF/c-Met and Notch are likely to be influenced by the ECM. HGF resides in the ECM (25), in both its active (HGF) and latent (pro-HGF) forms (26). Pro-HGF becomes active through cleavage by urinary plasminogen activator (uPA; 27), another key member of the plasminogen activator system. Likewise, Notch signalling may be affected by altered expression of ECM proteases that influence ligand availability. Following binding of a ligand to its receptor, proteases such as A Disintegrin And Metallaoprotease (ADAMs) will cleave its receptor at the S2 site, subsequently leading to the release and translocation of an active form of Notch (28). ADAM-10 cleaves the Notch receptor to promote SC quiescence (29) and is associated with MMP-9 (30); an ECM protease closely connected to SC activation (31) that is inversely correlated with diabetic muscle repair (10). Considering these data, it is proposed that expression and/or activity of ECM regulators, such as MMPs or ADAMs, influence signalling pathways such as HGF/c-Met and Notch that regulate the life cycle of the SC. Indeed, work from our lab has found that inhibition of PAI-1 led to improved

muscle repair in Akita mice (10,13), and is comparable to work that has identified PAI-1 in its association with muscle atrophy (32). Of particular importance is the idea that the soleus muscles were largely unaffected by elevations in PAI-1 in T1D skeletal muscle and demonstrated comparable regeneration to the WT cohort. This phenomenon was attributed to enhanced presence of the protease MMP-9 (10). Given the positive results regarding muscle repair obtained through the inhibition of PAI-1 in Akita mice, as well as the importance of ECM composition in the activity of SC-related signalling pathways, our lab became interested in uncovering additional ECM-related factors that modulate skeletal muscle regeneration that are dysregulated in diabetes. Microarray analyses comparing gene expression in uninjured and injured WT and Akita mice indicate that while LCN2 is substantially higher in uninjured T1D muscle, as compared to the WT, its increase following muscle injury is subdued in diabetic muscle (1.4-fold), whereas it is dramatically elevated in the WT (4.3-fold; Krause and Hawke, unpublished observations). Based on this preliminary data, we speculated that its relative failure to increase post-injury in diabetic skeletal muscle, in comparison to WT, highlights a potential source for aberrant diabetic muscle repair. Review of literature on LCN2 revealed that it is a modulator of MMP-9 availability (33), and is dysregulated in various forms of diabetes (34–36). While it would have been ideal to examine an overexpression of LCN2 in our diabetic models, the dearth of information currently available regarding LCN2 function in skeletal muscle led to its evaluation in WT skeletal muscle regeneration and whole-body LCN2 knockout mice (LCN2-/-). We surmised that if LCN2 were dysregulated in diabetes as a result of its association with adipose tissue (34-35) or its relation to diabetic

nephropathy in T1D (36), then its absence in skeletal muscle would improve the regeneration process. Through our study, we show that SCs derived from LCN2-/- mice displayed impaired activation that leads to attenuated muscle repair. It is interesting to note that SC activation is hindered in all three studies completed in this thesis, despite our prediction that LCN2 absence in SCs would enhance regeneration via improved SC function. It is somewhat difficult to try and relate these findings to other work on LCN2 in skeletal muscle, as little is known. However, if we consider data on the function of MMP-9 in healthy and diseased muscle, it may enable a better understanding of the results of the third study. Elimination of MMP-9 in dystrophic skeletal muscle has been found to improve muscle regeneration (37-40), while its ablation in normal healthy muscle results in reduced fiber size and altered fiber type distribution (41), with the latter results paralleling the findings observed in our LCN2-/- muscle. It is interesting to note that like muscular dystrophy, diabetes presents as a disease condition with augmented skeletal muscle inflammation and fibrosis (42). However, unlike results from dystrophic rodents (37-40), MMP-9 was decreased in diabetic skeletal muscle (13, 56), and was inversely correlated with enhanced collagen deposition (13). As a result of these and previous findings, we predict that, like MMP-9, LCN2 will be reduced in diabetic skeletal muscle, with this decrease likely contributing to the poor skeletal muscle remodeling observed following an injury stimulus. Future investigations should aim to manipulate LCN2 in diabetic skeletal muscle to discern its roles in the advancement of myopathy. Furthermore, the precise effect of LCN2 in diabetic SCs will also be of interest to

elucidate whether the main effect of LCN2 on diabetic muscle repair occurs through its regulation of SC activity.

5.3 Overarching Hypotheses and Future Experiments

While the first two studies of this thesis demonstrate attenuated SC activation in diabetic skeletal muscle, as mediated by an increase or decrease in specific SC-related pathways, and the third study eludes to the importance of a specific ECM regulator, LCN2, in influencing SC fate, the specific sequences of events linking diabetes to changes in ECM composition, and thus, SC function, remains to be fully determined. In this section, we discuss potential overarching hypotheses that could contribute to the observations made.

5.3.1 HGF/c-Met and Notch are interconnected in diabetes

As previously mentioned, we chose to examine different SC-related cellular pathways in study 1 and 2 due to variations in SC density results. While SC content was unaffected in DIO, we found a decline in SC quantity in T1D, and therefore speculated that an alternative pathway, other than HGF, was responsible for these results. However, despite the study of SCs in two different types of diabetes and via two different SC-related pathways, SC activation was attenuated in both investigations. While we believe that ECM composition, as mediated by ECM regulators such as LCN2, influence the availability of ligands that are integral to the activation of each pathway, it is also likely that there may be some interaction between HGF/c-Met and Notch that could lead to their convergence on modulating diabetic SC activation. In damaged myocardium, it has been

shown that activation of c-Met up-regulates Notch expression, which in turn, down-regulates c-Met expression (43; Figure 5.1).

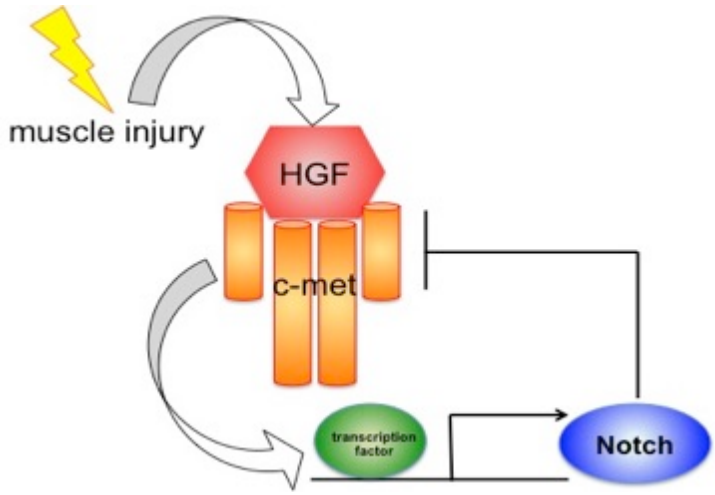

Figure 5.1. HGF/c-Met Interacts with Notch. Upon muscle injury, HGF is released from the ECM, binds to c-Met, and promotes the up-regulation of Notch. Following this, Notch hinders c-Met expression, leading to a negative feedback loop between c-Met/HGF and Notch (43).

There was no noted difference in c-Met protein content between ND and DIO skeletal muscle samples, however this was only analyzed in uninjured skeletal muscle, and it is possible that injured DIO skeletal muscle will demonstrate a decline in c-Met content. To further delineate the activity of this suggested negative feedback loop between HGF/c-Met and Notch, future studies will aim to examine each pathway in both models of diabetes investigated, as well as in T2D. We believe that hyper-activation of Notch will be shown in T2D skeletal muscle based on previous work displaying increased expression of Notch ligands in high glucose conditions (44) and increased Notch activity with high fat feeding (45).

5.3.2 Key attributes in diabetic skeletal muscle are associated with altered ECM composition

In addition to teasing out the interaction between HGF/c-Met and Notch in diabetic skeletal muscle, it is also of interest to determine what is occurring upstream of these changes in cell signalling. As previously mentioned, the ECM is thought to modulate the activity of HGF/c-Met and Notch, and as was shown in study 3, the absence of LCN2, a regulator of ECM proteases, led to a decline in SC function that coincided with findings from studies 1 and 2. It is of interest to determine the specific mechanism (or mechanisms) through which diabetes induces changes to the ECM. As diabetes progresses, several distinguishing features of diabetes are evident, including oxidative stress, inflammation, and the subsequent development of insulin resistance. Interestingly, oxidative stress is observed in T1D (46), T2D (47), and DIO (48), and is known to promote an inflammatory state in diabetic tissue (49,50) as a result of the formation of Advanced Glycation End-products (AGEs; 51–53). Past research has found that oxidative stress and inflammation promotes skeletal muscle insulin resistance (54), which has been linked with irregular ECM remodeling (55). Recent work has demonstrated that the presence of AGEs contributes to irregularities in the ECM (56). Collectively, it is hypothesized that oxidative stress, inflammation, and insulin resistance serve as the primary attributes of diabetes advancement, and likely converge to adversely modify ECM composition. As result of these changes to the ECM, SC function will be detrimentally affected, thereby promoting the development of diabetic myopathy (Figure 5.2).

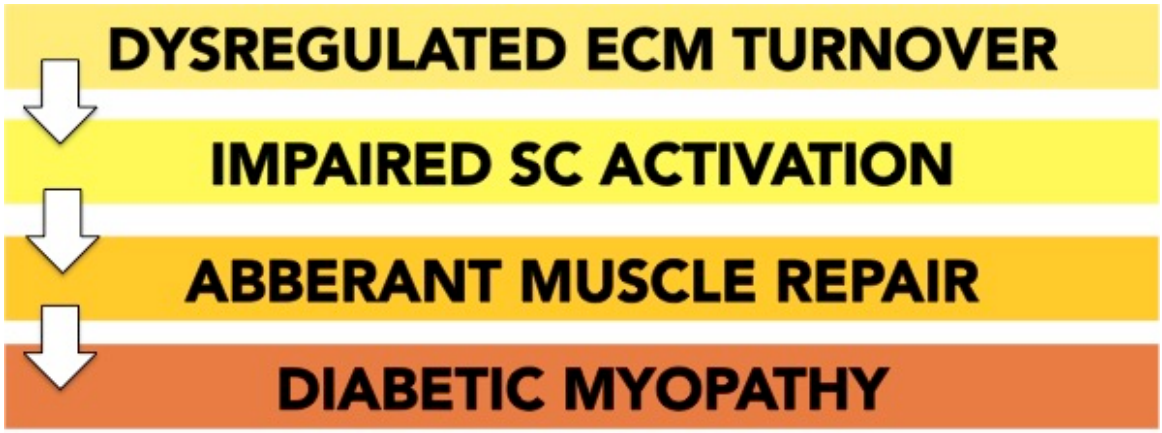

Figure 5.2. Proposed theory linking ECM composition to Diabetic Myopathy. Modifications to ECM composition of diabetic muscle has been previously observed (13, 55), and likely contributes to impaired SC activation observed in this thesis, subsequently resulting in attenuated muscle repair that may contribute to the formation of diabetic myopathy.

A number of future studies remain to be completed to provide a more thorough understanding of the diabetic SC. Firstly, it is imperative to investigate the activity of HGF/c-Met and Notch in both models of diabetes to confirm whether both signalling pathways are affected in the specific subtypes of diabetes investigated. Additionally, the elucidation of LCN2 in the diabetic state will also be relevant to confirming its regulation of the ECM, as well as whether its manipulation (through overexpression or ablation) will enhance or hinder SC function in diabetic muscle. It is also of interest to assess whether manipulation of LCN2 in one or more types of diabetes has any effect on HGF/c-Met signalling and/or Notch activity. It will then be beneficial to correlate data with results obtained from all types of diabetic human samples. This thesis was limited in its

presentation of reduced SC content in young adults with T1D. We have yet to examine SC activation in these samples, and did not assess additional Notch ligands. To fully comprehend the relevance of Notch to human diabetic SCs, we must determine species-specific Notch ligand(s) that promote Notch hyper-activation in T1D muscle, establish the distinct stage at which T1D SCs are affected, as well as relate these findings to sex and age matched Pre-diabetic and T2D human samples. .

5.4 Conclusions

Although myopathy remains a poorly investigated complication of diabetes, an emphasis should be placed on examining specifically how skeletal muscle is adversely affected in the diabetic condition. Skeletal muscle mass has been shown to be closely associated with glycemic control and insulin sensitivity (50). Thus, the examination of SCs, a key modulator of skeletal muscle repair and maintenance (57), is relevant in the assessment of diabetic myopathy. Collectively, the studies of this thesis demonstrate that SC function is hindered in the diabetic state, and may be attributed to alterations in its surrounding environment, as our preliminary work show that loss of LCN2 adversely influences the SC.

Akin to the research completed in other disease states such as muscular dystrophy or ageing, establishing and understanding the etiology of impaired SC function in diabetes serves as one step towards identifying potential therapies that would maintain and/or improve skeletal muscle structure, a integral facet when regarding overall skeletal muscle health. Though insulin is currently the gold standard of treatment in these diseases, it is not likely to prevent aberrant changes to SC function, as revealed in our

data comparing mouse (non-insulin treated) and human (insulin-treated) SC function in study 2. As a result, future research on the treatment of diabetic myopathy must focus on evaluating the use of a 'cocktail' of therapies, rather than the implementation of insulin alone, to assess their capacity to improve and/or maintain all or some key aspects of health muscle health (i.e. metabolism, structure, and function).

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