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Profiling changes in glycosylation during C2C12 myoblast differentiation

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Profiling changes in glycosylation during C2C12 myoblast differentiation

A thesis submitted in partial satisfaction of the requirement for the degree
Master of Science in Biomedical Engineering

by

Brian James McMorran

2012
Duchenne muscular dystrophy (DMD) affects 1 in 3500 boys. Systemic lack of the protein dystrophin disrupts muscle function by preventing the formation of the transmembrane dystrophin-glycoprotein complex (DGC). The DGC connects the actin cytoskeleton with the ECM, and disruption of this connection causes necrosis, degeneration and ultimately muscle wasting. Under physiologic conditions, a homologous complex, the utrophin glycoprotein complex (UGC) where utrophin substitutes for dystrophin, is restricted to the neuromuscular junction (NMJ). Alpha dystroglycan (αDG), the cell membrane associated glycoprotein that completes the last link between transmembrane βDG and laminin in the extracellular matrix, differs in glycosylation depending upon association with the DGC vs. the UGC. αDG at the DGC binds the lectin wheat germ agglutinin (WGA) while αDG associated with the UGC
preferentially binds the lectin *Wisteria floribunda* agglutinin (WFA). Some current therapies aim to ameliorate DMD by manipulating the complex association-dependent glycosylation of αDG, by increasing WFA reactive DG extrasynaptically and therefore redistributing the utrophin-glycoprotein complex in an effort to substitute for the missing dystrophin.

The current work utilized a panel of 12 lectins to perform comprehensive profiling of changes in glycosylation of C2C12 murine myoblasts following differentiation. Significant increases were observed in the binding of PNA and all five GalNAc binding specific lectins following 2 and 7 days of differentiation respectively. Three GalNAc binding lectins (WFA, VVA-B4 and HPA) showed increased binding following only 2 days of differentiation, illustrating differences in time dependency for changes in glycosylation. Lectin binding epitopes were mapped via the use of a pharmacological inhibitor to complex N-glycan creation. Importantly, not all GalNAc binding lectins were dependent upon complex N-glycan creation for reactivity.

It is important to note that there is no comprehensive knowledge surrounding changes in glycosylation of myoblasts as the cells differentiate into myotubes, or differences in glycosylation between healthy and DMD muscles. The current work profiled changes in C2C12 glycosylation during differentiation. Future work will 1) identify changes during human myoblast differentiation, 2) note differences in glycosylation between DMD and wildtype human myotubes, and 3) aim to manipulate human myofiber glycosylation, similarly to current mouse work, to ultimately increase utrophin retention at the cell membrane, improve laminin binding and enhance muscle function.
The thesis of Brian James McMorran is approved

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CHAPTER ONE

Introduction
Protein glycosylation is ubiquitous and critical for life

All cells are encased by a lipid bilayer membrane filled with transmembrane and cell surface proteins. These cell surface proteins participate in cellular interactions with the external environment, and transduce signals into the cell which allows the cell to react to its surroundings. Almost all transmembrane and cell surface proteins are glycosylated. Protein glycosylation provides a large degree of heterogeneity to the cell membrane and can impact the interactions of cells with the extracellular milieu [1]. Glycan modifications exist in various forms on different protein and lipid backbones (Figure 1-1). The focus of the current work is on glycan modification of glycoproteins. Glycoproteins can be modified with two types of glycans-N- or O-glycans. N-glycans are attached to the amine of asparagines residues within a protein while O-glycans are attached to the hydroxyl of either seronine or threonine residues.

Specific glycosylation of a protein backbone is the result of enzymatic processes occurring in the late endoplasmic reticulum and Golgi complex. Appropriate glycosylation of a protein is a dynamic process and depends upon many variables: enzyme activity and availability, donor substrate pool size and acceptor substrate availability. Specific glycan structures result from the appropriate sequential enzymatic processing of protein backbones. While the requisite enzymes and protein backbones are genetically encoded, specific glycan structures result from the many variables and dynamic nature of glycan processing. Therefore, for a functional glycan to be produced all steps in the enzymatic process must occur in the right order, at the right time. Due to this stringent processing sequence, for example, Mga1−/− mice lacking an enzyme responsible for N-glycan processing (N-acetylglucosaminytransferase-1; GlcNAcT1) are developmentally stunted, most notably neurologically, and die by day E10.5 [2]. Therefore, the
appropriate and sequential processing of glycans is critical for life as demonstrated by the embryonic lethality of $\text{Mgat}^{1/-}$ mice.

Glycan modifications of proteins play many roles. Protein glycosylation can change structure, increase molecular weight and/or relative charge and therefore ultimately impact protein function. Glycosylation of proteins acts as a control measure of protein synthesis and for glycoprotein targeting \[3\]. Furthermore, interactions between glycans and extracellular binding partners, known as carbohydrate binding proteins or lectins, can increase cell surface retention rates \[4\]. It is also important to note that glycosylation is known to differ across developmental stages, between tissues and even between cell types \[5-7\]. Due to the many impacts glycan modifications can have on many proteins, defects in glycosylation can have a profound effect on physiology and pathology.

*Glycosylation plays a critical role in muscle function*

Proper glycosylation of surface proteins embedded in the sarcolemma (or cell membrane) of muscle fibers (myofibers) plays an integral role in muscle integrity. It is known that alpha dystroglycan ($\alpha$DG) and various integrin isoforms, the two main myofiber ligands for extracellular matrix (ECM) receptors, are highly glycosylated \[8, 9\]. Interactions between receptors in the ECM and sarcolemmal glycoproteins anchor myofibers to the basal lamina. The anchorage at the sarcolemma provided by these interactions is what provides myofibers the necessary stability to withstand the force generated by contraction cycling \[10\].

The integral role that sarcolemmal protein glycosylation plays in muscle pathology can be seen by evaluating the pathologies of the congenital muscular dystrophies (CMDs). CMDs result from the loss of function in one of the glycosyltransferases requisite for the appropriate
glycosylation of αDG. Individuals suffering from a loss of function in these glycosyltransferases present with varying pathologies due to the impact that loss of specific glycan modifications on αDG has on muscle function. Walker-Warburg Syndrome, one of the most severe CMDs, results from the loss of the glycosyltransferases protein O-mannosyltransferases 1 and 2 (POMT1/T2) which prevents the creation of mannose O-glycans [11]. Muscle-eye-brain disease results from the loss of function of the glycosyltransferase protein O-linked-mannose beta-1,2-N-acetylgalcosaminyltransferase 1 (POMGnT1) [12]. POMGnT1 is involved in the addition of the next sugar to the mannose transferred by POMT1/T2 and its loss results in discontinuity between muscle and neurons [12]. Fukuyama Muscular Dystrophy results from the loss of function of the protein Fukutin [13] and to a lesser extent Fukutin-related protein (FKRP). While the precise activity of these proteins has not yet been elucidated, both Fukutin and FKRP play a role in the glycosylation of αDG [14].

It has also been reported that patients with a deficiency of Dol-P-Man Synthase Subunit DPM3, a congenital disorder of glycosylation (CDG) in which the precursors critical for complex N-glycan creation are absent, have presented with a dystrophic phenotype [15]. Interestingly, boys suffering from Duchenne Muscular dystrophy (DMD), while not lacking the function of a specific glycosyltransferase, do exhibit changes in muscular glycosylation due to a loss of stability of sarcolemmal protein complexes. Therefore, there is a clear role played by glycosylation in healthy muscle function.

*Duchenne Muscular Dystrophy (DMD) and the Dystrophin-Glycoprotein Complex (DGC)*

DMD is a severe muscular dystrophy. An X-linked recessive disorder resulting from the loss of the cytoskeletal linker protein dystrophin [16], DMD affects 1 in every 3500 boys
worldwide. As the lack of dystrophin is global, skeletal as well as cardiac function is ultimately impaired in boys suffering from the disease. Disease onset occurs by late infancy; most patients are wheelchair bound by puberty and rarely live to the third decade of life. Most boys succumb to either diaphragm failure related pulmonary complications or cardiac failure. While treatment regimens exist that prolong and improve quality of life, currently no cure for DMD is available.

The protein dystrophin acts as a cytoskeletal linker between the intracellular actin cytoskeleton and the transmembrane protein dystroglycan (Figure 1-2) [17]. Dystroglycan then completes the link to the ECM by binding laminin as well as other extracellular binding partners [8, 18, 19]. The complex which supports this transmembrane connection is known as the dystrophin-glycoprotein complex (DGC; Figure 2) [20]. Functionally, the DGC acts to support the transmission of forces between the actin cytoskeleton and the basal lamina or ECM. Acting functionally as an anchor [21], the DGC is expressed almost universally throughout the skeletal muscle cell membrane, or sarcolemma [22]. An important exception is made at the neuromuscular junction of myofibers where utrophin, a homologue of dystrophin, is substituted into the complex creating the homologous utrophin-glycoprotein complex (UGC, Figure 1-2) [23].

The DGC/UGC is composed of many component proteins. Intracellularly dystrophin (or utrophin) binds the transmembrane protein beta dystroglycan (βDG). The DGC is further anchored at the sarcolemma through the association of βDG with a complex of glycosylated proteins known as sarcoglycans. This association between the sarcoglycans and βDG in the membrane is further stabilized by the tetraspan-like protein sarcospan. Alpha dystrobrevin has also been shown to interact with βDG allowing for the further association of other proteins (syntrophins, neural Nitric Oxide synthase [nNOS], Grb2) with the DGC. Extracellularly, alpha
Dystroglycan (αDG) binds to βDG noncovalently, completing the connection between the DGC and the ECM via interactions of αDG with laminin. This connection is thought to stabilize myofibers in vivo during the muscle contraction cycle.

**Dystroglycan is highly glycosylated**

αDG is a dumbbell shaped, membrane associated glycoprotein which associates noncovalently with its transmembrane partner βDG (Figure 1-2) [24]. As a dumbbell, αDG is composed of three main domains: two globular domains, one at the N-terminus and at the C-terminus (which interacts with βDG), as well as an extended, highly glycosylated mucin domain which separates the two termini. Alpha and beta dystroglycan are both the translational product of the dag1 gene. Translated as one 895 residue precursor, dystroglycan is cleaved into alpha and beta subunits post-translationally. The cleavage site is conserved amongst vertebrates [8, 25] and has been shown to be integral in the appropriate glycosylation and localization of αDG to the membrane [26]; mutagenesis of one of the cleavage site residues (S654A) is known to cause dystrophy in a mouse model [27]. Via expression of a recombinant βDG fragment, it has also been shown that the extracellular binding epitope of βDG is capable of binding deglycosylated αDG [28], indicating that αDG and βDG interact via protein-protein interactions, and that the cytoplasmic domain of βDG is not required for this interaction.

Glycosylation increases the molecular weight of αDG significantly. While glycosylation varies temporally during development and across tissues, consistently almost half of the relative molecular weight of αDG results from glycosylation. The predicted molecular weight of αDG from the amino acid sequence is 74 kDa, however in brain and nerve αDG has a molecular weight of 120 kDa [29], while in cardiac muscle it is 140 kDa [6, 20, 30] and in skeletal muscle
it can be greater than 200 kDa [6]. It is known that in skeletal muscle, glycosylation of αDG is dependent upon both innervation and developmental progression of the myofiber [31].

Properly glycosylated αDG has been shown to be decorated by three types of glycan: N-type, mucin type O-glycans and mannose type O-glycans. αDG has three canonical sites of N-glycosylation which can be identified by the amino acid sequence Asn-X-Ser/Thr, where X is any residue except proline. Two of these sites exist on the C-terminal globular domain; an additional site is located on the N-terminus and has been show to be occupied [32]. Importantly, it has been shown that mutating the site of N-glycosylation closest to the α/β-DG cleavage site renders the DG precursor non-cleavable while mutating a site of N-glycosylation in the βDG peptide sequence reduced levels of α and β dystroglycan expression [26].

The mucin domain of αDG is known to be highly glycosylated by both mucin type and mannose type O-glycans. The glycosylation of this mucin domain is what provides αDG its length, as the high level of glycosylation extends the polypeptide which, without modification, could collapse on itself. Nilsson et. al. showed that there are 25 different sites of O-type glycosylation on the mucin domain of human skeletal αDG [33]. Similarly, Stalnaker et. al. detected 23 O-glycans on rabbit αDG, 9 of which were mannose type O-glycans while also noting that two specific peptide sites exhibited the possibility of having either form of O-glycosylation [34]. Recently, Tran et. al. demonstrated that modification of specific threonines and serines with O-mannosyl sugars can play a role in whether or not subsequent residues are modified with mucin type sugars [35]. This interdependence of glycan type specific modification further demonstrates the necessity for appropriate and sequential processing of O-glycans on αDG.
Significant research has been done on the O-mannosyl glycans of αDG allowing for significant inroads to be made regarding the function they perform. The complete O-mannosyl type glycans of αDG require the activity of the glycosyltransferase LARGE. The glycan product of this enzyme LARGE is required for the laminin binding activity of αDG [36]. It is also known that the O-mannosyl glycans must be phosphorylated in order for appropriate laminin binding activity [37]. Inamori et. al. demonstrated that in order to bind laminin αDG required the xylosyl- and glucuronyltransferase activity of LARGE [38]. This shows that not only is αDG modified with specific types of elongated glycan chains known as glycosylaminoglycans (GAGs) but also that laminin binding is dependent upon these modifications. In addition, regarding the structure and function of αDG, Hara et. al. revealed that the modification of two threonine residues most proximal to the globular N-domain of αDG by the enzyme LARGE provides the vast majority of laminin binding function [39]. The depth of research surrounding O-mannosyl glycans on αDG exhibits the contribution specific sites of glycosylation make to αDG function.

Importantly, it is known that the specific glycosylation of αDG correlates with cellular localization and protein association. αDG associated with the dystrophin containing DGC is known to preferentially react with wheat germ agglutinin (WGA) a lectin which binds N-acetylglucosamine (GlcNAc) [17]. αDG which associates with the utrophin containing UGC preferentially reacts with Wisteria floribunda agglutinin (WFA), a lectin that binds N-acetylgalactosamine (GalNAc) though reactivity with WGA is retained (Figure 1-2). [40]. This differential lectin binding reveals that αDG associated with the DGC is differentially glycosylated compared to αDG of the UGC. This distinction in reactivity is important and will be addressed further in later sections.
How can the glycosylation of sarcolemmal proteins be characterized?

Lectins are a class of proteins which bind sugar moieties present on protein backbones but have no enzymatic activity. First described extensively in plants and some invertebrates, lectins were not robustly isolated or studied until the 1970s even though the first lectin was described as early as 1888 [41, 42]. Prior to the era of monoclonal antibodies, lectins provided a straightforward means of determining the carbohydrate structures present on cells of differing blood types. Initially called hemagglutinins for their ability to preferentially agglutinate differing types of blood cells, lectins provide an easy means for detecting sugars present on varying cell types [42]. This cell type specificity, coupled with extensive research surrounding lectins since the 1970s, has allowed for the increased use of lectins as biochemical tools.

Why not use antibodies? For one, lectins are relatively cheap. More importantly however, most current antibodies against mammalian carbohydrate moieties are also protein specific. The monoclonal antibodies (mAbs) IIH6 and VIA-4 both recognize the LARGE modification on αDG and the mAb 1D4 is known to recognize O-glycans specifically on CD43. While the use of these antibodies would provide a researcher a high level of specificity, that specificity is what limits mAb use. Lectins allow for a broader profiling of changes in cell wide glycosylation as their recognition is protein independent.

Lectins have previously been used to characterize muscle glycosylation. In 1982 Sanes and Cheney demonstrated that the lectin *Dolichos biflorus* agglutinin (DBA) preferentially bound the neuromuscular junction (NMJ) while *Ricinus communis* agglutinin (RCA) stained both the NMJ and the extrasynaptic sarcolemma in a non-discriminating manner [43]. This characteristic staining of the NMJ by DBA held across multiple vertebrates (rabbit, hamster, chick and frog [faintly but selectively]) [43]. Lectins have also been previously used to identify
the role various glycans on αDG play in acetylcholine receptor (AChR) clustering at the NMJ and to identify dystrophin as a member of the DGC [17, 44]. In 1988, Scott et. al. also found that a lectin from *Vicia villosa* (VVA-B4 isolectin) preferentially bound the NMJ and that this specificity held across many non-human species [45].

Furthermore, Scott et. al. concluded that the carbohydrate modification restricted to the NMJ was *N*-acetyl-D-galactosamine (GalNAc) dependent (an observation noted by Sanes and Cheney in 1982 as well) [45]. This conclusion was based on the GalNAc specificity of the lectins which preferentially bind the NMJ. This specificity implies three different scenarios (or a combination therein) for the restriction of GalNAc residues to the NMJ: proteins with GalNAc modifications may be preferentially synthesized at the NMJ and therefore remain there; proteins may also be synthesized ubiquitously within the cell but targeted for transport specifically to the NMJ; alternatively the GalNAc modifications may be synthesized universally but be masked by sialic acid extrasynaptically and preferentially unmasked by the removal of sialic acid at the NMJ.

*Significance of current work*

Some current innovative therapies for DMD, and other related muscular dystrophies, center around redistributing restricted NMJ type glycosylation extrasynaptically in an effort to concomitantly redistribute utrophin (and associated proteins), rescuing the dystrophic phenotype. It is known that cell surface proteins, αDG specifically, modified with GalNAc residues are normally restricted, along with associated UGCs, to the NMJ. It has been posited therefore that changing glycosylation of sarcolemmal αDG may provide a mechanism to increase sarcolemmal utrophin usage and ultimately lead to the rescue of the dystrophic phenotype. Related work by
Martin’s group has shown that increasing WFA reactivity of αDG can rescue the dystrophic pathology in mdx mice [46] and as such validates the theory in an animal model.

It is noteworthy, however, that differences in glycosylation patterns have been noted between humans and other mammals, specifically mice. The goal of the current work is to develop the necessary methodology to rigorously and thoroughly characterize muscle glycosylation. Initial work on murine cells will provide the proof of principle to transition to human cells. The ultimate goals are to detect any differences in glycosylation between DMD and wild type human cells, identify a glycoform of human αDG which promotes laminin binding and utrophin use, and ultimately the development of a high throughput screen to identify small molecules that will alter human muscle cell glycoprotein glycosylation to improve muscle cell function. The goal of these studies is to provide the potential for novel types of therapy in DMD.
Figure 1-1: Various forms of mammalian glycosylation are depicted on different protein and lipid backbones. Note that the vast majority of glycosylation is present on the exterior surface of the cell membrane. The focus of the current work is changes in glycosylation of glycoproteins. Image modified from [47].
Figure 1-2: The dystrophin-glycoprotein complex and the homologous utrophin-glycoprotein complex

A) The dystrophin-glycoprotein complex (DGC) acts to anchor the muscle fiber to the ECM. Dystrophin connects the actin filaments to the transmembrane βDG. αDG noncovalently binds βDG and completes the interaction with the ECM via glycan-dependent interactions with laminin. B) At the NMJ dystrophin is substituted by its protein homologue utrophin creating the utrophin-glycoprotein complex (UGC). It is important to note that αDG glycosylation correlates with its cellular localization and protein association; αDG associated with the DGC binds the lectin WGA while αDG bound to the UGC preferentially binds the lectin WFA. Image modified from [48].

Figure 1-2: A) Extra-synaptic sarcolemma
B) Synaptic sarcolemma (NMJ)
CHAPTER TWO

Changes in glycosylation during murine myoblast differentiation
INTRODUCTION

Cells undergoing differentiation are known to change biochemically and morphologically. One biochemical change during differentiation is a change in glycosylation of cell surface glycoproteins. While this concomitant change during differentiation has been studied in other cell types (including T cell [49, 50], B cells [51], and embryonic stem cells [52]), very little is understood about changes in glycosylation during myoblast differentiation. While the reactivity of the lectin WFA and the specific glycan-reactive mAbs IIH6 and VIA-4 change during differentiation of myoblasts into myotubes [53, 54], a global profile of changes in glycosylation is still lacking. As glycosylation of cell surface proteins plays a significant role in determining how cells interact with their environment, this limited knowledge on muscle cell glycosylation represents a gap in current muscle biology research.

Multiple methods can be utilized to determine changes in glycosylation. An effective and straightforward method is to detect changes in glycosylation via changes in the reactivity of lectins known to bind specific sugars. The extensive literature pool on lectin characteristics and their sugar specificities allows for the compilation of a comprehensive panel of lectins for glycan detection. By including lectins known to have a variety of sugar specificities it is possible to determine lectin reactivities and thus create a global profile of changes in glycosylation of surface glycoproteins during differentiation. Other means of analyzing changes in glycosylation include carbohydrate structure analysis via mass spectrometry [55] and lectin microarray [56, 57].

As with all protein-substrate interactions, lectin reactivity is dependent upon proper glycan epitope presentation. While sugar moiety specificity is known for most lectins, it is noteworthy that some lectins bind multiple sugars [58] and reactivity with specific glycan types
(N-, O-, O-mannosyl glycans) is unknown for most lectins on muscle cells. For this reason it is important to map lectin glycan epitopes in order to thoroughly understand how specific glycans decorate specific sites on muscle cell proteins.

As glycosylation is an enzymatic process (see Ch.1), lectin epitopes can be mapped by inhibiting the processing of specific glycans. Enzymatic processing can be blocked one of two ways: 1) through the use of pharmacologic inhibitors or 2) via the inhibition of enzymatic expression via siRNA. Pharmacologic inhibitors are an easy screening method as media need only be supplemented appropriately; siRNA treatment allows for the confirmation of changes seen due to pharmacologic inhibitors and for the inhibition of enzymes lacking pharmacologic inhibitors. Ultimately, changes in lectin reactivity following glycan processing inhibition allow for the determination of the glycans required for specific lectin epitopes.

In the current work, a panel of twelve lectin binding assays was developed to profile global changes in glycosylation during myoblast differentiation and to map lectin binding epitopes. Data is presented that demonstrates 1) an increase in GalNAc modifications and asialo core 1 O-glycans during C2C12 myoblast differentiation; and 2) lectin reactivity, even amongst those known to recognize GalNAc, is dependent upon different types of glycan modifications. These findings present novel conclusions regarding muscle cell glycosylation and glycan presentation which can have significant impacts on ongoing research aimed at modifying glycosylation of muscle fibers.
MATERIALS AND METHODS

Cell culture

C2C12 murine myoblasts were purchased from the American Tissue Culture Collection (ATCC) and cultured in growth media (GM, Dulbecco’s Modified Eagle Medium [DMEM] supplemented with Hepes, sodium pyruvate, Glutamax [Invitrogen] and 10% fetal bovine serum [FBS, Hyclone]). For passage myoblasts were rinsed with phosphate buffered solution (PBS), trypsinized, centrifuged and resuspended in GM. A T-75 flask was initially plated with 150,000 cells, maintained at 37°C and 5% CO$_2$ and not allowed to reach more than 70% confluency. Myoblasts utilized in experiments were between passages 3 and 10. Myotubes were formed via myoblast differentiation by substituting 10% FBS from growth media with 2% equine serum (ES, Hyclone) for 48 hours.

Lectin binding assays and time course

C2C12 cells were plated at a density of 1500 cells per well in a 96-well tissue culture plate (96 WP, Thermo Fisher Scientific) in 100μl growth media. Cells were grown to confluency for three days. On the third day myoblast plates were washed twice with ice cold PBS and then fixed overnight (O/N) in 100μl 2% paraformaldehyde/PBS at 4°C. Myotube plates were washed once with 100μl differentiation media (DM) on the third day and then incubated for 2, 4 or 7 days in 100μl DM; DM was refreshed every 48 hours for 4 and 7 day time points. Myotubes were fixed in the same manner as myoblasts. After fixing plates, wells were washed with 100μl PBS via multichannel pipette. Plates were then blocked in 200μl/well with 1% bovine serum albumin (BSA, Gemini BioProducts) supplemented PBS (PBA) for 1 hour at room temperature.
PBA was removed and wells were incubated with varying concentrations of biotin-conjugated lectins or an equivalent concentration of biotin-conjugated BSA (Vector Laboratories) in PBA. Lectins and BSA controls were incubated at 4°C O/N and then washed three times with 100μl PBS supplemented with 0.1% Tween-20 and 1% BSA (PBSTB). Plates were then incubated with 1μg/ml streptavidin-horseradish peroxidase (SA-HRP) in PBSTB for 1 hour at RT. Plates were then washed three times with 100μl PBSTB followed by three washes with 100μl PBS. Plates were incubated with 100μl 3,3′,5,5′-tetramethylbenzidine (TMB, Thermo Fisher Scientific) for five minutes at RT. The HRP reaction was quenched with 100μl 2M sulfuric acid. Plates were read at 450 nm on a Bio-Rad Benchmark Plus Microplate Spectrophotometer via Microplate Manager v5.2 software.

All biotin-conjugated lectins (*Wisteria floribunda* agglutinin [WFA], *Vicia villosa* isolecit [VVA-B4], soybean agglutinin [SBA], *Dolichos biflorus* agglutinin [DBA], wheat germ agglutinin [WGA], *Sambucus negra* agglutinin [SNA], *Maackia amurensis* isolecit [MAA II], Jackfruit lectin [or Jacalin, Jac], peanut agglutinin [PNA], *Concavalin A* [ConA], and *Phaseolus vulgaris* leucoagglutinin [PHA-L]) were purchased from Vector Laboratories with the exception of *Helix pomatia* agglutinin [HPA] which was purchased from Sigma Aldrich.

*Treatment with lobeline*

Cells were plated at the above mentioned concentration in a 96 WP and grown to confluency for three days. At day three myoblasts were differentiated with 100μl DM supplemented with either varying concentrations of lobeline HCl (Sigma Aldrich) or vehicle control dimethyl sulfoxide (DMSO). After 48 hours in supplemented DM, plates were fixed as above. WFA reactivity was detected per the protocol mentioned above for lectin binding assays.
Complex N-Glycan inhibitor treatment

Cells were plated in either 96-well plates for spectrophotometric detection or 6-well plates for detection by flow cytometry of lectin binding. At confluence, cells were treated with 2mM dexoymannojirimycin (DMNJ) or vehicle control in DM for 72 hours. Spectrophotometric detection of lectin binding was performed as mentioned above. Flow cytometry was performed by lifting cells in warmed PBS/EDTA pH 8.0. Cells were then collected and incubated in either biotin-conjugated BSA (control) or PHA-L. Bound lectin was detected by streptavidin-FITC (SA-FITC) on a FACScan flow cytometer (Beckon Dickinson). Results were analyzed using CellQuest Pro v6.0 software.
RESULTS

Previous work by other groups had shown that increasing reactivity of αDG with *Wisteria floribunda* agglutinin (WFA) could cause a redistribution of utrophin and related sarcolemmal proteins to rescue the dystrophic phenotype in *mdx* mice [46]. Recently, our group created a high throughput screen (HTS) utilizing resources at the Molecular Screening Shared Resource (MSSR) of the California Nanosystems Institute (CNSI) to identify small molecule compounds which would increase biotin-conjugated WFA (bWFA) reactivity of C2C12 myoblasts *in vitro* [59]. In the HTS, a 384 well plate binding assay utilizing enhanced chemiluminescent was developed for detection of increased bWFA binding to C2C12 cells [59]. My first goal was to convert this binding assay to a format that could be utilized more easily in our laboratory: a 96 well plate (96 WP) format utilizing monochromatic spectrophotometry for the detection of WFA binding.

First, WFA reactivity on C2C12 cells was standardized in a 96 WP to determine the optimal dilution of bWFA for detection of binding. C2C12 cells were plated in a 96 WP, grown for three days and either fixed as myoblasts or differentiated for an additional 2 days and fixed as myotubes. Fixed cells were blocked in PBA and then incubated with varying concentrations between 3.2 and 320nM bWFA. The linear range for reactivity of bWFA reactivity was determined to be between the concentrations of 3.2 and 32nM (Figure 2-1). It is important to note that myotubes differentiated for 48 hours were between 1.5-2 fold more bWFA reactive compared to myoblasts when accounting for nonspecific background (Figure 2-1).

Having standardized bWFA reactivity, I confirmed the findings from the HTS published by our group using the 96 WP format [59]. Previously we found that differentiation of C2C12 myoblasts in differentiation media (DM) supplemented with 100μM lobeline caused a 2-fold
increase in reactivity of bWFA in a 384 WP detected by chemiluminescence [59]. Detection by chemiluminescence is more sensitive than spectrophotometry based on the monochromatic absorbance of an enzymatic byproduct. To confirm these findings in a 96 WP format, C2C12 cells were differentiated with either 100μM lobeline or DMSO vehicle control. Binding of bWFA to myotubes increased following lobeline treatment (Figure 2-2) as we had seen in the 384 WP format [59]. Thus it was demonstrated that the substrate TMB was sensitive enough to detect changes in lectin binding in this assay format.

Since TMB was sensitive enough to detect changes in lectin binding, I utilized a panel of lectins to perform a more comprehensive profile of changes in murine myoblast and myotube glycosylation (Table 2-1). Multiple lectins recognizing GalNAc were included as these have been used previously to characterize GalNAc modifications in muscle [40, 43, 45, 46, 60-62]; in addition it has been demonstrated that various GalNAc binding lectins recognize GalNAc modifications in different positions within a sugar chain with varying affinities [63]. Lectins recognizing other sugars (sialic acid, core 1 O-glycans, and varying N-glycan types) were included to provide a wide range of lectin specificities to define a large variety of changes in glycosylation.

Based on the standardization of bWFA reactivity, binding of all biotin-conjugated lectins was standardized using 5 concentrations on both myoblasts and myotubes differentiated for 48 hours. Of the five GalNAc recognizing lectins, binding of three (WFA, VVA-B4 and HPA) increased at least 1.5 fold following 2 days of differentiation (Figure 2-3A, B&D). Strikingly, VVA-B4 binding increased by at least 3 fold (Figure 2-3B). It is noteworthy that following 2 days in differentiation, binding of SBA or DBA, two other lectins which bind GalNAc, did not increase (Figure 2-3C&E). This suggests that specificity may exist as to which GalNAc moieties
are increasingly accessible for binding following differentiation. Interestingly reactivity of PNA, which recognizes asialo core 1 O-glycans, increased significantly (2-7 fold) following differentiation (Figure 2-3J). Jacalin, which recognizes sialylated core 1 O-glycans, on the other hand did not increase in reactivity following 2 days of differentiation (Figure 2-3I). In contrast to the notable changes in WFA, VVA-B4, HPA and PNA no changes were observed in the binding of the three sialic acid recognizing lectins WGA, SNA, and MAA II. Additionally, no significant increase was seen in the binding of lectins recognizing specific N-glycan types, ConA and PHA-L.

As significant differences were seen in lectin reactivity following 2 days of differentiation (Figure 2-3), I next determined changes in lectin reactivity after 7 days of differentiation. I observed a significant increase in VVA-B4 binding (+150%) after a 7 day period in differentiation media (Figure 2-4A); similar changes were seen in reactivity of all other GalNAc binding lectins: WFA increased 20%, SBA increased about 150%, HPA increased over 100% and strikingly DBA binding increased 400% following differentiation while showing no binding greater then nonspecific background to undifferentiated myoblasts. It is important to note that no changes were seen in SBA or DBA reactivity after 2 days of differentiation either during the initial standardization of concentrations (Figure 2-3C) or during the following time course (data not shown). This suggests a delayed onset in increased availability of certain GalNAc moieties as detected by SBA and DBA.

In contrast to significant increases seen in GalNAc binding lectins, no change was seen in binding activity of the sialic acid binding lectins WGA and SNA over the longer time course. For example SNA reactivity did not change during the 7 day time course (Figure 2-4B). Binding of Jacalin, which recognizes, sialylated core 1 O-glycans, did not increase after 7 days of
differentiation (Figure 2-4C). Interestingly, PNA binding to asialo core 1 O-glycans increased slightly following seven days in differentiation media (Figure 2-4C). Thus, following seven days of differentiation, C2C12 myotubes increase reactivity of GalNAc modifications and asialo core 1 O-glycans on cell surface proteins.

In order to better understand what glycans decorate αDG and the role the specific glycans play in muscle cell function, I started mapping lectin binding epitopes to specific glycan types. I first assessed the requirement of complex N-glycans for lectin reactivity. Complex N-glycans result from enzymatic processing of glycoprotein precursors in the late ER and the Golgi complex. In the late ER, N-glycan precursor structures are transferred en bloc to asparagine residues of protein backbones (Figure 2-5A). N-glycans are then further modified as proteins are processed through the Golgi complex. Enzymatic processing in the Golgi complex creates oligomannose structures from the N-glycan precursor structure known as high mannose type N-glycans. Further processing of high mannose structures by the enzyme mannosidases I and II trims back mannose residues and allows for the addition of other saccharide units, a process which creates two other N-glycan types: hybrid and complex. The pharmacologic inhibitor deoxymannojirimycin (DMNJ) prevents the creation of hybrid and complex type N-glycans by inhibiting mannosidase I. Thus, the dependence of any biochemical interaction upon hybrid and complex type N-glycans can be determined by treating cells with DMNJ.

ConA recognizes high mannose type N-glycans [64] while PHA-L binds lactosamine repeats of complex N-glycans [65]. Accordingly, ConA and PHA-L reactivities can detect appropriate processing of N-glycans and as such also act as a control for DMNJ treatment. As the processing of complex N-glycans is inhibited by DMNJ, a decrease in PHA-L binding following DMNJ treatment is expected as well as a concomitant increase in ConA as the
inhibition of mannosidase I allows for increased presentation of high mannose N-glycans at the cell surface.

In mapping lectin epitopes on hybrid and complex type N-glycans, C2C12 myoblasts were differentiated for 72 hours in the presence of 2mM DMNJ. On average a decrease of at least 75% in PHA-L binding was observed following DMNJ treatment while an increase of 50% or more in ConA binding was seen concomitantly (Figure 2-5B). Inhibition of complex N-glycan processing was also confirmed via flow cytometry as treatment with DMNJ reduced PHA-L binding to background levels (Figure 2-5C). Consequently, differentiation of C2C12 myoblasts with 2mM DMNJ for 72 hours was sufficient to inhibit the creation of hybrid and complex type N-glycans.

Since creation of complex type N-glycans was inhibited by DMNJ, I determined which lectins required complex N-glycans for binding. C2C12 cells were treated with DMNJ as above and any changes in lectin binding were analyzed as described above. Previous work in our group had demonstrated that WFA reactivity in C2C12 cells was dependent upon complex N-glycans [59]. I was able to confirm this finding using the 96 WP binding assay (Figure 2-5D). I observed that treatment with DMNJ caused a 60% decrease in WFA reactivity (Figure 2-5D). Interestingly, not all GalNAc recognizing lectins were dependent upon complex N-glycans for reactivity. While treatment with DMNJ caused a decrease in WFA reactivity and a 35% reduction in binding of SBA, reactivity of VVA-B4, HPA or DBA, three other GalNAc binding lectins, was not reduced (Figure 2-5D). DMNJ treatment further demonstrated that recognition of sialic acid in C2C12 cells by WGA and MAA II is independent of complex N-glycans (Figure 2-5E). However, 50% of binding by SNA is complex N-glycan dependent (Figure 2-5E).
In summary, this work depicts novel findings regarding changes in muscle glycosylation during differentiation. An increase in GalNAc modifications reactivity on C2C12 cells occurred following differentiation; increase in reactivity of the lectins WFA, VVA-B4 and HPA was detected after only 2 days of differentiation, while increases were not detected by SBA or DBA until after 7 days of differentiation. An increase of PNA reactivity was also could be detected as early as 2 days of differentiation while no changes were noted in binding of WGA, MAA II, SNA or Jac. Lectin epitopes were also partially mapped and demonstrated the dependence of WFA and SNA binding on complex N-glycans.
DISCUSSION

The current work describes the first comprehensive profile of changes in glycosylation during myoblast differentiation. I have shown that monochromatic spectrophotometry is sensitive enough to detect changes in lectin reactivity on C2C12 myoblasts and myotubes. Importantly, significant increases were seen in GalNAc lectin reactivities following 7 days of differentiation as well as significant increases in PNA reactivity after 2 days of differentiation. Furthermore, lectin dependence upon complex N-glycans was determined. Strikingly not all GalNAc binding lectins were complex N-glycan dependent. Changes in lectin reactivity as well as initial mapping of lectin epitopes to sugar types pose many unanswered questions.

Changes detected in lectin reactivity demonstrate that glycosylation of sarcolemmal proteins changes significantly following differentiation (Fig. 2-3,-4). Multiple mechanisms could be causing the significant increases in GalNAc and asialo core 1 reactivity following myoblast differentiation. Increased reactivity could result from increased modification of glycoproteins with GalNAc residues and asialo core 1 glycans if the amount of protein per cell surface area unit does not change following differentiation. Elevated sarcolemmal retention or recycling of GalNAc and asialo core 1 glycan modified surface glycoproteins could also explain the increase. As PNA reactivity also increased concomitantly with GalNAc binding lectins, increased expression or activity of sialidases, a class of enzymes which remove terminal sialic acid residues from glycan chains, could potentially unmask previously internal saccharide residues. In this manner, increased sialidase activity could increase both PNA and GalNAc lectin reactivity as sialic acid can be added to both core 1 O-glycans and GalNAc residues, and sialic acid removal would allow binding to residues previously internal to terminal sialic acids.
A good question raised by the increase in PNA and GalNAc lectin reactivity surrounds the biological significance of the increase: why do myoblasts increase reactivity during differentiation? While it has been demonstrated that asialo core 1 O-glycans play a role in modulating C2C12 myoblast fusion and differentiation [66], a thorough search of the literature demonstrates that no direct observation or research has occurred regarding GalNAc modifications in myoblast differentiation. Perhaps increased reactivity of sarcolemmal glycoprotein GalNAc residues is required for fusion and terminal differentiation into myotubes. It has been reported that a GalNAc binding lectin secreted by embryonic chick muscle cells is developmentally regulated during differentiation. It could be possible that these two events are linked. Multimerization of the GalNAc lectin could provide a connecting bridge between differentiating, highly GalNAc modified myoblasts by binding GalNAc residues on adjacent cells. In this way differentiating cells could be linked, ultimately contributing to fusion of cell membrane and formation of mature myotubes. The significance of increased GalNAc reactivity during differentiation remains to be deciphered.

Lectin epitope mapping provided further insight into the increase seen in GalNAc reactivity. While it was shown that GalNAc reactivity increased for all five GalNAc lectins following a week of differentiation (Figure 2-4), treatment with the mannosidase I inhibitor demonstrated differences in lectin dependence upon complex N-glycans for reactivity (Figure 2-5). This is significant as it suggests that whatever mechanism drives increased GalNAc reactivity is operating independent of specific glycan type. Further epitope mapping will allow for a better understanding of the breadth of glycan types modified with increased GalNAc reactivity.

Previous work on muscle biology had utilized GalNAc binding lectins in a somewhat indiscriminate manner to identify the NMJ [43, 45] and roles that glycosylation play in
sarcolemmal protein function [5, 40, 46]. My data show, however, that these various lectins do not bind GalNAc residues on the same glycan type. While a significant amount of WFA reactivity and a portion of SBA reactivity is dependent upon complex N-glycans (Figure 2-5 and [59]), reactivity of the other three GalNAc binding lectins (VVA-B4, HPA and DBA) were not (Figure 2-5). In order for a more congruent understanding of the role that various glycan structures play in muscle function, a more comprehensive knowledge base must be established regarding the epitopes of the lectins utilized. Further lectin epitope mapping will allow for glycoform identification via specific glycan structure analysis and lectin reactivities.

Future studies relating to this work will allow for further characterization of changes in glycosylation during myoblast differentiation. Analysis of glycan structures via iDAWG mass spectrometry [55] will allow for the discernment of changes in abundance of specific glycan arrangements and linkages following differentiation, while lectin microarray [56, 57] will allow for the evaluation of changes in even more lectin reactivities. Ultimately, analysis of the muscle glycotranscriptome during differentiation will allow for identification of changes in glycan related substrates, enzymes and transporters [67]. This wealth of information will allow for a comprehensive profile of changes in glycosylation during myoblast differentiation. Most importantly, these studies will provide the proof of principle for related work on human cells in identifying changes in glycosylation which increase laminin binding, utrophin recruitment and muscle function.
<table>
<thead>
<tr>
<th>CATEGORY</th>
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<th>SUGAR SPECIFICITY</th>
<th>REASON FOR INCLUSION</th>
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<td>Used to demonstrate specificity of GalNAc moiety for NMJ [45] and in human biopsies [60]</td>
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<td>Included in studies surround lectin-NMJ specificity [43, 60-62]</td>
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<tr>
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<td>Lectin used to first determine specificity for NMJ [43] and in studies on human biopsies [60-62]</td>
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<td>Jac</td>
<td>Sialylated Core 1 O-glycans</td>
<td>Core 1 O-glycans have been identified to have a role in formation of αDG associated complexes [44]</td>
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<td>Asialo Core 1 O-glycans</td>
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<td>High mannose N-Glycans</td>
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<tr>
<td></td>
<td>PHA</td>
<td>Complex N-glycans</td>
<td>Detection of complex N-glycans [65, 71]</td>
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Figure 2-1: Standardized concentration curves of biotin-conjugated WFA (bWFA) on C2C12 myoblasts and myotubes are shown. Myotubes bind approximately 1.5-2 fold more bWFA than myoblasts. Values represent averages of triplicates ±SD minus background non-specific binding of bBSA control.
Figure 2-2: Dose response curve of bWFA binding on C2C12 myotubes following treatment with lobeline (black bars) or vehicle control (0, DMSO, gray bars). Cells were treated as they were differentiated for 48 hours. 100μM lobeline increases bWFA binding 2 fold as compared to untreated controls. bBSA is utilized as a control for non-specific binding of bWFA. Values shown are averages of triplicates ±SD.
FIGURE 2-3: LECTIN PANEL CONCENTRATION RANGES

A: *Wistera floribunda* agglutinin (WFA) (GalNAc)

B: *Vicia villosa* isoelectin (VVA-B4) (GalNAc)

C: Soybean agglutinin (SBA) (GalNAc)
G: *Sambucus nigra* agglutinin (SNA) (Sialic Acid)

![Graphs showing the absorbance of Myoblasts, Myotubes, and Ratio against Concentration (nM) for BSA and concentrations of 0.75, 1.5, 4.5, 15, and 45 nM.](image)

H: *Maackia amurensis* isolectin (MAA II) (Sialic Acid)

![Graphs showing the absorbance of Myoblasts, Myotubes, and Ratio against Concentration (nM) for BSA and concentrations of 0.75, 1.5, 4.5, 15, and 45 nM.](image)

I: Jackfruit lectin (Jac) (Sialylated Core 1 O-Glycans)

![Graphs showing the absorbance of Myoblasts, Myotubes, and Ratio against Concentration (nM) for BSA and concentrations of 0.75, 1.5, 4.5, 15, and 45 nM.](image)
FIGURE 2-3: Standardized concentration curves for the 12 lectins included in the panel are shown for both myoblasts and myotubes differentiated for 48 hours. Ratios of myotube binding to myoblast binding are shown, accounting for nonspecific binding. White bars represent bBSA
controls. The lectins HPA and DBA lack ratios for two lectin concentrations as no binding was seen above background levels. The panels are: A) *Wisteria floribunda* agglutinin (WFA; GalNAc); B) *Vicia villosa* isolectin (VVA-B4; GalNAc); C) Soybean agglutinin (SBA; GalNAc); D) *Helix pomatia* agglutinin (HPA; GalNAc); E) *Dolichos biflorus* agglutinin (DBA; GalNAc); F) Wheat germ agglutinin (WGA; sialic acid [GlcNAc]); G) *Sambucus nigra* agglutinin (SNA; α-2,6-linked sialic acid); H) *Maackia amurensis* isolectin (MAA II; α-2,3-linked sialic acid); I) Jackfruit lectin (Jac; sialylated core 1 O-glycans); J) Peanut agglutinin (PNA; asialo core 1O-glycans); K) *Concanavalin* agglutinin (ConA; High mannose N-glycans); L) *Phaseolus vulgaris* leucagglutinin (PHA-L; complex N-glycans).
Figure 2-4: C2C12 Lectin Reactivity Time Course

A) Reactivity of VVA-B4 over a time course of 7 days of differentiation is shown. Myoblasts and myotubes differentiated for either 2, 4, or 7 days were fixed, blocked and then probed with the lectin. An increase of over 150% was noted following 7 days of differentiation. Similar increases were seen in the reactivity of other GalNAc binding lectins. B) Reactivity of Jac over the 7 day time course is depicted. No change was seen following 7 days; similar results
were seen in the reactivity of WGA and SNA. C) Percent increase over day 0 (fixed, confluent myoblasts) are depicted for all lectins. Increases were seen in all GalNAc binding lectins while no increase was noted in sialic binding lectins.
FIGURE 2-5: PHARMACOLOGICAL INHIBITION OF COMPLEX N-GLYCANS

A

B

Controls for DMNJ Treatment

Absorbance

PHA (450uM) ConA (75pM)

C

PHA-L Binding

D

Change in Reactivity of GalNAc binding lectins

Percent Change

Lectin (Concentration)

E

Change in Reactivity of Sialic Acid binding lectins

Percent Change

Lectin (Concentration)
FIGURE 2-5: A) The enzymatic processes involved in N-glycan creation are depicted (modified from [72]). Three various types of N-glycans are created via these processes: high mannose (recognized by the lectin ConA), hybrid, and complex (both recognized by the lectin PHA-L). Use of the pharmacological inhibitor DMNJ prevents the process of high mannose N-glycans to hybrid and complex N-glycans and thus allows for the evaluation of the role of complex and hybrid type N-glycans in cellular function. B) Treatment of C2C12 cells with DMNJ inhibits the creation of complex N-glycans. Following 72 hours of treatment with DMNJ, C2C12 bind 75% less PHA-L and 50% more ConA demonstrating efficacy of the inhibitor. C) DMNJ treatment is confirmed by flow cytometry. Cells treated with DMNJ bind less bPHA-L as detected by SA-FITC when compared to control treated cells. D) Binding of the included GalNAc lectins is not equally inhibited by DMNJ treatment. WFA binding decreased substantially (~65%) and SBA decreased 35% following treatment while other GalNAc binding lectins (VVA-B4, HPA and DBA) did not decrease. E) DMNJ treatment impacted binding of sialic acid recognizing lectins differently. WGA and MAA II saw no change in reactivity following DMNJ treatment while SNA binding decreased 50%.
Chapter Three

Future Directions and Conclusions
While many avenues exist for potential therapeutics for DMD, one current focus is the manipulation of sarcolemmal protein glycosylation. It has been theorized that increasing glycosylation associated with the utrophin-glycoprotein complex (UGC) to the entire sarcolemma will redistribute the complex and compensate for the loss of the dystrophin-glycoprotein complex (DGC) which occurs in DMD. While the transgenic overexpression of an enzyme responsible for UGC associated glycosylation has been shown to rescue the DMD phenotype in mice [46], it is important to note that a comprehensive knowledge base of muscle glycosylation is lacking. This is a significant deficit if ongoing and future research is aimed at changing myofiber glycosylation, especially since what little knowledge is known about muscle glycosylation implies that there may be striking differences between human and non-human species [43, 45, 60, 62, 73-75]. Thus, the current work aimed at profiling changes in glycosylation during murine myoblast differentiation to provide a proof of principle for characterizing glycosylation in human muscle.

My research employed a panel of 12 lectins to note changes in glycosylation during and following differentiation of C2C12 murine myoblasts. Lectins reactive with a breadth of sugars were included in order to provide for a comprehensive profile of changes. Five lectins reactive with the residue GalNAc were included as they have been used somewhat indiscriminately either for lectin histochemistry or biochemical techniques. Lectins recognizing different sialic acid linkages, as well as O- and N-glycans were also included to provide a fuller picture of any changes that might occur. Lectins were utilized to probe glycan reactivity of both myoblasts as well as myotubes differentiated for varying amounts of time. As early as two days after differentiation, changes were already noticeable in binding levels. PNA binding increased between 2-7 fold while WFA, VVA-B4 and HPA binding increased between 1.5-3 fold.
Interestingly, following seven days of differentiation, reactivity of all GalNAc binding lectins was increased over day 0.

These changes demonstrate that as myoblasts are differentiating, proteins being sent to the cell surface are glycosylated differently compared with those present prior to differentiation. Multiple mechanisms could be behind the increase in reactivity of PNA and the five GalNAc binding lectins. An increase in PNA reactivity following differentiation has been shown to correlate with the expression of skeletal NCAM, a requisite for differentiation into functional myotubes [66]. Perhaps an increase in GalNAc modified proteins at the sarcolemma facilitates myotube development by facilitating membrane fusion of neighboring myoblasts; in a manner similar to increased PNA reactivity, perhaps increased GalNAc reactivity represents specific glycoforms of proteins requisite in mature myotube function.

Significant changes were noted in lectin reactivity of murine myoblasts during differentiation in the current work. This represents the first profiling of these changes. This is significant as current potential therapies aim to manipulate glycosylation. Strikingly, no such profile has been performed on human myoblasts which is even more concerning as significant differences have been noted between human and non-human glycosylation patterns. Therefore, literature surrounding differences in glycosylation between human myoblasts and myotubes, and wild type and diseased state muscle is lacking. Future work will 1) profile human cells for changes in glycosylation during differentiation as well as differences between wild type and diseased muscle, 2) allow for the identification of αDG glycoforms associated with the UGC and 3) ultimately lead to a high throughput screen to identify small molecule candidates that improve muscle function by upregulating utrophin retention at the cell surface and increasing laminin binding.
Elucidating the mechanism behind changes in glycosylation

In the current work significant changes were seen in reactivity of GalNAc modifications on C2C12 myotubes following 7 days in differentiation media. Similarly a marked increase was noted in PNA reactivity following 2 days of differentiation. As described, a multipronged approach will be utilized to further elucidate the changes in glycosylation observed following differentiation. MS, lectin microarray and glycotranscriptome analysis will help broaden the knowledge base surrounding post differentiation changes in glycosylation.

iDAWG glycan analysis via MS will allow for elucidation of changes in the abundance of specific glycan structures and linkages. Changes in specific glycan linkages could provide significant insight as lectins known to recognize the same monosaccharide have been shown to have varying affinities depending upon the monosaccharide linkages [63]. Therefore, changes in specific linkages could confirm previous data regarding increased lectin binding. Our group has utilized iDAWG MS previously to identify quantitative changes in glycosylation of C2C12 myotubes following treatment with lobeline [59], thus iDAWG provides a robust means to detect these changes in glycan structure.

Further elucidation of the mechanism behind increased PNA and GalNAc lectin binding will involve glycan analysis via lectin microarray [56, 57]. Lectin microarray will allow for a more extensive profile of glycosylation changes as more lectins are included in the array than could be possible to evaluate via the binding assays described in Chapter 2. The array will provide a means of validating findings from my current work as well as discovery of changes not yet noted. Lectins identified as changing in reactivity following differentiation will then be further evaluated in our laboratory regarding specificity and glycan type dependence.
Changes in enzymes driving increased lectin reactivity will be determined by utilizing glycotranscriptome analysis [67]. Glycotranscriptome analysis provides a rigorous and precise means of detecting changes in glycosyltransferase and glycosidase transcript levels [67]. The precision of this analysis allows for detection of changes as small as a 20% fluctuation in myotube transcript levels as compared to those of undifferentiated myoblasts. This is significant as even a 20% change in transcript level of glycosyltransferases and glycosidases can have a profound impact on glycosylation due to their enzymatic activity. Once significant changes in enzyme transcript levels have been determined, siRNA knockdown of identified glycosyltransferases and/or glycosidases will allow for the further elucidation of the mechanism involved in increased lectin reactivity.

MS glycan analysis, lectin microarray reactivity and changes in glycotranscriptome levels will allow for a global perspective regarding changes in glycosylation. Utilizing multiple detection techniques provides the most thorough and robust means of understanding the changes occurring as myoblasts differentiate. While a glycan related transcript may increase during differentiation, its effects may not be seen at the cell surface in the form of altered glycan expression due to the many variables in the enzymatic process of creating glycans (availability of protein backbone, substrate donor pool etc.). Therefore, glycan analysis via iDAWG MS provides us with that specific knowledge. Observations regarding changes in glycan structures are significant as not all changes may be detected by lectin reactivity. None of the lectins in the current panel are mammalian lectins and therefore do not represent endogenous mammalian receptors for glycans. As such specific changes in glycosylation may not be detectable by changes in reactivity of the twelve specific plant lectins included in the panel. In this manner the three pronged approach provides a the advantage that changes noted utilizing a given technique
can potentially be confirmed or rationalized via findings from the other two techniques. Ultimately the comprehensive profile allows for an understanding of changes in C2C12 glycosylation during differentiation.

*Further lectin epitope mapping*

The pharmacological inhibitor DMNJ was utilized in the current work to determine the role of complex N-glycans in lectin reactivity. A different pharmacological inhibitor, benzyl-α-GalNAc, will allow for the elucidation of the role of O-glycans in lectin reactivity. [76, 77]. As the diminution of PHA binding provided a control for DMNJ treatment, changes in PNA reactivity, that binds asialo core 1 O-glycans (Table 2-1), will provide a control for benzyl-α-GalNAc activity. Treatment of myoblasts with benzyl-α-GalNAc will establish the requirement of core 1 O-glycans in the reactivity of the lectins in the current panel as pharmacological inhibition of O-glycan processing may impact creation and processing of other glycans. Glycan structures other than core 1 O-glycans that bear lectin epitopes under normal conditions may be absent or improperly processed due to a lack of O-glycosylation.

siRNA will also be employed in determining the role of other glycan structures in lectin reactivity for two reasons; 1) siRNA knockdown is required for enzymes lacking pharmacological inhibitors, such as those involved in O-mannosyl glycan creation; and 2) siRNA knockdown of enzymes inhibited pharmacologically in previous experiments will allow for validation of these findings. Currently six enzymes have been identified for siRNA knockdown in order to determine their role in lectin reactivity.

Our group has shown that activity of the enzymes POMT1/T2, which transfer the requisite first mannose residue for O-mannosyl glycans (Figure 3-1), is not required for WFA
reactivity [59]. Repetition of this siRNA knockdown will assist in determining the lectin binding epitopes on O-mannosyl sugars. POMGnT is involved in the creation of the GlcNAc-Gal-Sialic Acid branch known to exist on O-mannosyl glycans of αDG (Figure 3-1) [12] and as such, may be involved in the creation of epitopes required by sialic acid binding lectins included in the current panel. siRNA knockdown of POMGnT will allow for further narrowing of the location of the epitope necessary for lectins shown to be dependent upon POMT1/T2 activity for binding. Lectins which require POMT1/T2 but not POMGnT activity must bind epitopes on either of the other two branches on O-mannosyl glycans (Figure 3-1). Activity of the enzyme LARGE modifies αDG by creating one of the other two branches on O-mannosyl glycans, a xylose and glucuronic acid repeat glycosylaminoglycan (GAG; Figure 3-1) [38], while the activity of a homologous protein LARGE2 has been shown to more effectively modify αDG though its precise activity has not been elucidated [78]. The role of this GAG in lectin reactivity will be determined via siRNA knockdown as well.

Enzymes not involved in the formation of O-mannosyl sugars will also be examined as αDG alone is highly glycosylated (Figure 3-1). Much of the research regarding Galgt2 activity utilized increased WFA binding as a readout, however no determination has been made of the exact glycan structure which is increasingly modified in the overexpression system Martin’s group utilizes [40, 46]. As such siRNA knockdown of Galgt2 will determine lectin reactivity created by this enzyme. Also, confirmation of findings from pharmacological inhibition will be performed via siRNA knockdown of mannosidase I and core 1 β1,3-Galactosyltransferase, the enzymes inhibited by DMNJ and benzyl-α-GalNAc respectively.

The impact of either pharmacological inhibitors or siRNA knockdown of specific enzymes will be analyzed using multiple techniques. Lectin binding assays on treated cells, as
performed previously, will allow for a quantitative analysis of changes in lectin reactivity. Furthermore, lectin precipitations from treated whole cell lysates will allow for the determination of specific proteins bound by lectins. In this way specific proteins could be identified as lectin receptors. Selective glycoforms of αDG could be identified by immunoprecipitation (IP) of αDG with a glycosylation independent antibody specific to the core polypeptide of αDG. Probing with various lectins for reactivity following treatment and IP would allow for the visualization of glycoform specificity. These means of detection will allow for the formation of a holistic picture regarding epitopes required for lectin binding of C2C12 myotubes and for the potential identification of glycoforms of αDG.

*Why is research on murine cells not enough?*

The *mdx* mouse model has been utilized to elucidate the pathological components of DMD since its creation in 1984 [79]. However, it is important to note that differences in morphology and pathology are known to exist between the *mdx* mouse and human DMD patients [80]. Boys suffering from DMD undergo significant muscle degeneration followed by fiber necrosis and physical disability [80]. *mdx* mice, however, are not as clinically debilitated; the regeneration mechanism is known to compensate for the fiber degeneration and necrosis and therefore muscular hypertrophy results [80]. These differences in pathological progression are significant. Understanding the underlying mechanism by which the *mdx* phenotype is milder could facilitate treatments for human patients. More importantly, however, these differences present serious challenges when evaluating potential pharmaceutics in treatment of DMD as effects seen in mice may not translate perfectly to human patients.
*mdx* mice are different from humans suffering from DMD both morphologically and biochemically as many differences exist between human glycosylation and that of mice. Around the same time that lectins were utilized to detect the NMJ in rodent [43], other groups utilized lectins to determine changes in muscle membrane characteristics of human biopsy samples [60, 62, 73, 74]. Striking differences can be noted by comparing observations from these studies. For example, it is known that a 370kDa protein reactive with the galactose residue binding lectin RCA-I that is present in mouse muscle is missing in human DMD samples [73] but upon examining *mdx* biopsies this protein is still present [74]. VVA-B4 reactivity, which is punctate and restricted to the NMJ of rodent biopsies [43, 45], is not restricted to the NMJ in human skeletal muscle biopsies [45, 60]. DBA and SBA are also known to selectively bind the NMJ in rodent, however, in human samples no binding of the lectin is found, punctate or indiscriminate [62]. Thus, significant differences can be seen in the glycosylation of non-human and human muscle tissue.

Of note is the worsened DMD phenotype in the *mdx/cmah* double knockout mouse model. As described above the pathology of *mdx* mice does not match human pathological progression perfectly. One known difference between humans and the rest of the animal kingdom is the loss of the enzyme cytidine monophosphate-sialic acid hydroxylase (CMAH) in humans [75]. CMAH is required for the creation of the sugar N-glycolylneuraminic acid (NeuGc), a form of sialic acid found in all other animals but absent in all human cells due to the loss of CMAH activity [75]. Humans therefore are only capable of producing the sialic acid N-acetylneuraminic acid (NeuAc) while non-human species, including mice, are known to produce both NeuAc and NeuGc. Interestingly, a mouse model with pathological progression more relevant to human pathology was created by introducing a human type *cmah* knockout into *mdx*
mice to create a mouse model lacking both dystrophin expression and the ability to produce NeuGc [81]. While this single difference in sugars does not account for all the differences between the mouse model and the human disease DMD, it does reinforce the differences that exist in glycosylation between humans and mice and the impact these differences could have in development of novel treatments.

As striking differences have been noted between murine and human muscle glycosylation and little research has been performed on human muscle cells undergoing differentiation, the ultimate goal of the present work is to provide the proof of principle for future work with human cells. Work on human cells would evaluate differences in glycosylation across two dimensions: changes as myoblast differentiate into myotubes; and differences between wt and DMD cells. As to date, no quantitative research has been performed regarding differences in glycosylation across either of these dimensions.

Future research on human myotube glycosylation will be heavily grounded in the core resources of the Center for Duchenne Muscular Dystrophy (CDMD) here at UCLA. Core B of the CDMD represents a high throughput screening core which, in collaboration with the MSSR of the CNSI, allows for the screening of small molecule compounds on human fibroblast and myoblast cell lines. Of specific interest are the human-myoblast like cells as well as the inducible directly reprogrammable myotubes (iDRMs). To date, the CDMD core has created 20 different human cell lines for testing consisting of wild type parent-Duchenne patient pairs. These cell lines provide the unique opportunity to quantitatively detect changes in glycosylation of human muscle cells.

Differences in glycosylation will be evaluated via the same multipronged approach used for C2C12 murine cells. Lectin reactivity, glycan analysis via MS, and glycotranscriptome
analysis will allow for a thorough understanding in changes in glycosylation resulting from differentiation as well as differences noted between diseased and wild type myotubes. Evaluation of lectin reactive glycoforms of αDG will also play a significant role in human cell work. One of the main aims of future work will be to determine if there is a human glycoform with specificity for the UGC over DGC as is the case in mice where αDG in the UGC preferentially binds WFA. Identification of a lectin-glycoform specific relationship will allow for the identification of novel therapeutics by HTS.

Work in our group previously utilized a lectin binding assay to perform a HTS of the Prestwick library in order to identify compounds which increase WFA reactivity of C2C12 myoblasts [59]. In this work lead candidates were identified in the HTS and then validated in the laboratory for dose-response activity, cytotoxicity and biochemical effect. A similar HTS will be performed on human skeletal muscle myotubes upon identification of an appropriate readout lectin. This HTS will allow for the screening of the approximately 1200 compounds in the Prestwick library as well as the 100,000 additional compounds available for testing at the CNSI MSSR facility. The ultimate goal will be to identify a small molecule compound which upregulates utrophin usage at the sarcolemma, increases laminin binding of αDG and improves muscle cell function. This research will provide many avenues for the development of novel therapeutics for DMD.
**FIGURE 3-1: PUTATIVE LECTIN BINDING EPITOPES**

**A**

- **αDG** is highly glycosylated bearing three glycan types: `-N-glycans,
- mucin type O-glycans, LARGE modified O-mannosyl glycans (detailed in B). As αDG is highly glycosylated it is decorated with possible binding sites for multiple of the lectins in the current panel.

**B**

- O-mannosyl glycans, along with the requisite enzymes for branch extension,
are detailed as they have been extensively studied. Multiple putative sites for lectin binding exist on O-mannosyl sugars
REFERENCES


