FLIPPING THE SWITCH:
REGULATION OF PROLIFERATION AND DIFFERENTIATION IN
ADULT MUSCLE STEM CELLS

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by
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FLIPPING THE SWITCH:
REGULATION OF PROLIFERATION AND DIFFERENTIATION IN ADULT MUSCLE STEM CELLS

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FLIPPING THE SWITCH:
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Katie Lynn Capkovic Thompson

ABSTRACT

Adult muscle stem cells or satellite cells are the committed somatic stem cells responsible for maintenance and regeneration of adult skeletal muscle tissue. Skeletal muscle is capable of multiple rounds of complete regeneration due to the action of satellite cells. These cells are necessary for the muscle to respond to multiple stimuli including, periods of growth, injury, adaptation and aging. Additionally, in severe myopathic diseases such as Duchenne Muscular Dystrophy, the regenerative capacity of skeletal muscle is exhausted. The potential of stem cells in regenerative medicine lies in the ability to remove them from their natural niche, inducing them to proliferate in culture and placing functional cells back into a tissue environment. In order to achieve this it is important to better understand the mechanisms regulating the processes of proliferation and differentiation in satellite cells. I have addressed three important questions pertaining to the study of these functions in satellite cells. 1) Is it possible to differentiate between proliferating cells and cells that have recently committed to myogenic differentiation on the basis of their membrane raft components, specifically neural cell adhesion molecule expression? 2) Can separate domains of a single protein, syndecan-4, regulate proliferation and differentiation? 3) How can a secreted signal from differentiated skeletal muscle regulate satellite cell proliferation as a population?
Many signaling receptors, transducers, and effectors have been proposed to act in higher-order complexes associated with physically distinct areas of the plasma membrane known as membrane rafts. Membrane rafts and the signaling complexes formed within these regions have been reported to function in many adult stem cells. However, the components of membrane rafts had yet to be examined in satellite cells. To address this, we first isolated detergent-resistant membrane fractions from primary satellite cells, then analyzed their protein components using liquid chromatography-tandem mass spectrometry. Transmembrane and juxtamembrane components of adhesion-mediated signaling pathways made up the largest group of identified proteins; in particular, neural cell adhesion molecule (NCAM), a multifunctional cell-surface protein, was found to be localized at these regions. Immunohistochemical analysis revealed that not only is NCAM localized to these discrete areas of the plasma membrane, but it is also a very early marker of commitment to terminal differentiation in satellite cells. This finding is functionally relevant because it allows for the identification of differentiating cells from a heterogeneous population for subsequent biochemical analysis or downstream clinical applications.

Syndecan-4 is a type I plasma membrane glycoprotein that is expressed in satellite cells. While syndecan-4 appears to be dispensable for development, animals lacking syndecan-4 show multiple defects in muscle regeneration including decreased/delayed cell cycling, altered differentiation, and aberrant patterning of regenerated muscle. Due to the number of factors known to interact with syndecan-4, it has been difficult to identify the molecular basis of this pleiotropic phenotype. I demonstrate that re-expression of the full-length protein abrogates the \textit{in vitro} defects in proliferation and differentiation, while expression of syndecan-4 carrying inactivating mutations in intracellular domains selectively rescues different aspects of the null phenotype. In
particular, the phosphatidylinositol 4,5-biphosphate (PIP<sub>2</sub>) association motif of syndecan-4 appears to be required for efficient proliferation but dispensable for differentiation whereas the PDZ-binding motif of syndecan-4 is required for differentiation but not proliferation.

Satellite cell number as a proportion of muscle mass is tightly controlled in adult organisms. The average number of satellite cells per unit of muscle tissue is well conserved within sample types and after tissue regeneration. However, the signaling networks controlling satellite cell number have not been delineated. Using a transgenic mouse model, I found that overexpression of a transcription factor, TEAD1, leads to a 6.8 fold increase in the number of quiescent satellite cells. I show that this increase occurs non-cell autonomously and propose a model by which the differentiated muscle secretes a soluble, heat labile factor which signals to the satellite cell population to regulate the number of stem cells in the tissue.
CHAPTER 1. INTRODUCTION

**Adult Muscle Stem Cells**

Adult muscle stem cells or satellite cells are the committed somatic stem cells responsible for in vivo maintenance and regeneration of adult skeletal muscle tissue [1-4]. Quiescent satellite cells were first identified by Alexander Mauro using electron microscopy and thus named based on their position, satellite to the muscle fiber (Figure 1) [5]. Satellite cells reside between the basement membrane and the sarcolemma of mature muscle fibers and are physiologically characterized by their small size, high nuclear to cytosolic ratio, heterochromatinized DNA and minimal cellular metabolism [2, 6]. In addition to their small physical size satellite cells represent a very small portion of adult skeletal muscle mass. In mouse 2-7% of all adult skeletal muscle-associated nuclei belong to satellite cells [7, 8].

Despite the rarity of satellite cells, skeletal muscle is able to dynamically adapt to environmental changes, regenerate and grow as needed across the entire lifespan of the animal. Typically, in response to injury or disruption of the basal lamina the mitotically quiescent satellite cells become activated, migrate out from their niche and proliferate extensively [8]. The resulting muscle precursor cells, or myoblasts, eventually differentiate and fuse to existing post-mitotic muscle fibers or fuse to each other to form multinucleate myotubes [9]. A small fraction of satellite cells have been identified that do not undergo terminal differentiation, but instead retain the ability to proliferate and repopulate the original satellite cell niche [10].

The defining characteristics of a stem cell are self-renewal and potency. Interestingly, when satellite cells are cultured, the resulting cells can be induced to differentiate into fibroblasts, osteocytes or adipocytes [11]. Following treatment with
Figure 1. Satellite cell location within skeletal muscle. Skeletal muscle is composed of individual fiber bundles each surrounded by perimysium. Myofibers or muscle fibers are multinucleated muscle cells located within the fiber bundle. Each muscle fiber is surrounded by the basal lamina with the satellite cells nested between the basal lamina and the sarcolemma, muscle plasma membrane.

bone morphogenetic proteins (BMPs) or adipogenic inducers satellite cells will become osteocytes or adipocytes, respectively, suggesting some degree of plasticity within the mesenchymal lineage [12]. Only in the case of fibroblastic conversion has it been shown that satellite cells are capable of lineage conversion *in vivo* [13]. It remains to be seen if satellite cells can adopt adipogenic or osteogenic fates *in vivo*. Despite the ability to adopt divergent fates, during *in vivo* muscle regeneration the majority of satellite cells commit to the morphologically well-defined stages of myogenesis while a small side population remains undifferentiated and repopulates the satellite cell niche [10].

**Satellite Cell Activation and Proliferation**

Satellite cells are poised to receive extracellular signals from numerous sources including the adjacent myofiber, vasculature, motor neurons, immune cells and/or the existing satellite cell pool [2]. Growth factors, cytokines, neurotrophic factors and oxygen tension from these sources have all been shown to affect various stages of regeneration [2]. There are several factors reported to influence primary satellite cell activation from quiescence. However, only one growth factor, hepatocyte growth factor (HGF)[14, 15] and one signaling molecule nitric oxide (NO)[16], have been demonstrated to activate satellite cells [17]. The widely accepted mechanism for activation is the force-dependent (stretch) release of NO from the muscle fibers, which initiates HGF release from pockets within the extracellular matrix, allowing it to bind the HGF receptor, c-met, on the satellite cell surface [16-20]. Exogenously added HGF promotes activation while HGF-blocking antibodies prevent satellite cell activation [14] whereas blocking of NO using a nonspecific nitric oxide synthase (NOS) inhibitor (L-arginine methyl ester (L-NAME) or using NOS⁻/⁻ mice delays satellite cell activation[16]. Because the satellite cells are still able to activate without NO this data suggests that other mechanisms of satellite cell activation exist [17]. Conversely, myostatin, a member
of the TGFβ family, has been shown to negatively regulate the G1 to S phase progression of satellite cells and thus functions to maintain the quiescence of satellite cells [21]. Reducing or preventing function of myostatin by natural mutation, gene targeting or by blocking its signaling leads to doubling or quadrupling of skeletal muscle size [22, 23].

After activation and emergence from the basal lamina satellite cells are exposed to a number of growth factors. Many of these have been shown to influence proliferation including insulin-like growth factors 1 and 2 (IGF1/2)[24, 25], epidermal growth factor (EGF)[26], platelet derived growth factor (PDGF)[26, 27], interleukin-6 (IL-6)[28], leukemia inhibitory factor (LIF) [29, 30], androgens [31], transforming growth factor (TGFβ)[32] and fibroblast growth factors (FGFs)[2]. Of the approximately 20 known FGFs, about half have been shown to have mitogenic effects in muscle and act synergistically with HGF [2].

A number of other non-growth factor proteins have also been shown to influence proliferation. Both syndecan-3 and syndecan-4 are among the family of heparan sulfate proteoglycans required for signaling through different receptor tyrosine kinase receptors and are present on quiescent satellite cells [33]. Satellite cells deficient in syndecan-4 have defects in activation characterized by a delayed onset of proliferation [34]. In this way factors that modulate growth factor signaling are also capable of influencing proliferation.

**Satellite Cell Renewal**

A key property of adult stem cells is the ability to self-renew. Self-renewal assures that the resident stem cell pool is replenished and available for subsequent cycles of tissue repair or replacement. The lineage hierarchy of satellite cells is less defined than other resident stem cells: while there is general agreement that the majority of active muscle
regeneration is accomplished by satellite cells (syndecan-4+, Pax7+ cells beneath the basal lamina) there is evidence that satellite cell heterogeneity includes a more stem-like population of progenitor cells (reviewed in [3, 35]) and/or that cells outside the classical satellite cell position (dorsal aorta progenitors, side populations and hematopoetic stem cells) can themselves give rise to satellite cells [23, 36]. However, it remains unclear as to what extent and under what conditions these cells give rise to functional satellite cells. Direct evidence of satellite cell self-renewal was recently demonstrated by the transplantation of single fibers with their associated satellite cells from one mouse strain to another [10]. Using a genetic lineage marker, the donor satellite cells were shown to produce new satellite cells capable of repeating regeneration in the host.

The molecular mechanisms responsible for self-renewal have yet to be determined, but recent studies on asymmetric cell division suggest that these cell fate decisions (stem cell vs. progenitor) may occur through the combined actions of Wnt and Notch signaling [37-39]. Notch signaling activates downstream signaling molecule GSK-3β to modulate muscle stem cell proliferation versus Wnt signaling that inactivates GSK-3β to induce differentiation [13, 37, 40, 41].

**Satellite Cell Differentiation**

In order to completely regenerate after injury the pool of replacement myoblasts created by satellite cells must differentiate and fuse to create new myofibers. During this process the myoblasts irreversibly exit the cell cycle and express muscle specific transcription factors, cytoplasmic factors and structural genes. Through expression of these factors they become myocytes, which subsequently fuse to form the multinucleate muscle fibers. The transition between proliferation and differentiation is associated with
upregulation of genes that control both the shift to G_0 and the expression of muscle specific transcription factors, structural proteins, enzymes and membrane receptors [2].

The transition from proliferation to differentiation is regulated by cellular interactions with environmental factors such as growth and morphogenic factors that modulate cellular signaling pathways [2, 42]. Numerous _in vitro_ studies of cultured primary myoblasts and myogenic cell lines indicate this transition can be controlled by both serum and growth factor availability [43-45]. In MM14s, a myoblast cell line that was derived from adult primary myoblasts, proliferation is absolutely dependent on basic fibroblast growth factor (FGF2); in these cells withdrawal of FGF causes commitment to differentiation at the next G_1_ phase [43]. Conversely many factors that influence activation and/or proliferation such as FGF2 and myostatin inhibit differentiation. In the case of myostatin this is through down regulation of myogenic regulatory transcription factors [46].

Myogenic regulatory transcription factors (MRFs) belong to the basic helix loop helix (bHLH) family of transcription factors and play an integral role in skeletal muscle myogenesis [2]. Members of the MRF family (MyoD, Myf-5, myogenin and MRF4) are activated in turn to temporally regulate muscle specific gene expression through collaboration with other transcription factors of the myocyte enhancer binding factor-2 (MEF2) family [47]. MyoD and Myf-5 function early in progenitor specification. Myogenin and MRF4 have later expression and are regulators of differentiation [48]. Interestingly quiescent satellite cells do not express detectable levels of any MRFs. Upon activation cells express either MyoD or Myf5 and later, prior to differentiation, satellite cells eventually express both MyoD and Myf5 [18]. The ability of MyoD to initiate muscle specific expression program is so pervasive that ectopic expression in non-muscle cells is sufficient to initiate muscle differentiation [49].
NCAM Function in Myogenesis

Neural cell adhesion molecule (NCAM) is an adhesion molecule that functions as a signal transducing receptor molecule and interacts with numerous extracellular and intracellular proteins (reviewed in [50]). NCAM is widely expressed during early embryonic development. As development progresses, NCAM becomes primarily localized to nervous and muscular tissues [50]. In muscle, NCAM expression has been shown to increase during differentiation of developing myoblast and throughout regeneration of adult muscle [51, 52]. Of the members of the Ig superfamily of cell surface proteins NCAM was the first to be implicated in myogenesis. In muscle, NCAM exists in multiple isoforms all of which are encoded by a single gene and arise through alternative splicing [53]. Overexpression of specific NCAM isoforms in an immortalized myoblast cell line, C2C12s, has varied effects on fusion. The 140 kDa transmembrane isoform containing a muscle specific domain (MDS) increases myoblast fusion while the 120 kDa glycosylphosphatidylinositol-linked isoform lacking a MSD has no effect [54, 55]. Despite the effects of NCAM overexpression, loss-of-function mutations suggest overlapping roles for NCAM and other adhesion molecules in myogenesis. NCAM null mice produce no gross defects in skeletal muscle during development [56, 57]. Furthermore, primary myoblasts from NCAM null mice fuse at a similar rate as wildtype primary myoblasts in vitro [58]. Together this suggests that the increased fusion in NCAM overexpression studies maybe the result of increased overall adhesion and that other adhesion molecules are capable of compensating for the loss of NCAM [58].
Figure 2. Satellite cell response to injury. Satellite cells become activated, migrate out from the basal lamina and proliferate. A population of satellite cells reestablish the quiescent satellite cell pool through self-renewal. Satellite cells migrate to the damaged areas of muscle and either fuse to the damaged myofiber or to each other to produce new myofibers.

Syndecan-4

The molecular pathways that regulate each step of satellite cell myogenesis are the focus of a multitude of ongoing research in muscle stem cell biology. A summary of the morphological sequence of these events is outlined in Figure 2. The signaling molecule syndecan-4 has been implicated in several of these events in satellite cells yet the mechanistic role of syndecan-4 has not been defined in this cell type to date.

Syndecan-4 is one of four vertebrate syndecans, all of which are type I membrane-spanning proteins. They are divided into a short (28-34 aa) intracellular domain, a highly conserved single-pass transmembrane domain, and unique extracellular domains containing serine motifs that act as attachment sites for both heparan sulfate (HS) and chondroitin sulfate (CS) carbohydrate side chains (reviewed in [59]). Of the four syndecan family members, syndecan-3 and syndecan-4 are expressed in adult satellite cells [33]. Despite the homology between syndecan-3 and syndecan-4, unique skeletal muscle and satellite cell defects are observed in mice carrying homozygous deletions of these genes suggesting that these syndecans perform distinct functions in skeletal muscle [34]. Previously Dr. Cornelison has shown that syndecan-4 is necessary for many aspects of satellite cell mediated regeneration (activation, proliferation, and differentiation) both \textit{in vivo} and \textit{in vitro} [34]. Single fiber or mass cultured syndecan-4 null satellite cells have delayed activation and decreased proliferation compared to wild type. On myofibers they do not activate by 24 h nor do they proliferate before 48 h [34]. Cultured syndecan-4 null cells do eventually proliferate by 6 days, but fail to migrate effectively and appear in large clusters [34]. These cells also display decreased fusion and terminal differentiation, which may be explained by their reduced or mislocalized expression of myogenic regulatory factors, MyoD and myogenin [34]. Syndecan-4 null mice completely fail to regenerate after barium chloride induced injury and form unpatterned, nonfunctional fibers [34].
**Functional Domains of Syndecan-4**

Despite relatively little knowledge about syndecan-4 function in satellite cells, a considerable amount is known about syndecan-4 molecular interactions and function in other cell types. Let us first consider the make up of the syndecan protein (Figure 3). The cytoplasmic domains of syndecans are divided into two constant (C1 and C2) and one variable (V) domain (reviewed in [60][61]). The constant domains are 100% identical in all family members except for a single amino acid change in C1 of syndecan-2. While the intracellular domains of syndecans are very small (28-34 amino acids), they mediate interaction with a large number of different cellular factors via conserved elements in the C1 domain and a PDZ (postsynaptic density 95, disks large, zona occludens-1) binding motif in the C2 domain at the C-terminus. Syndecan-4 possesses a unique lysine-rich phosphatidylinositol 4, 5-biphosphate (PIP$_2$) binding motif in its V domain that is not found in the other family members, which facilitates syndecan-4-specific signaling interactions [62].

**Syndecan-4 Extracellular Interactions**

While syndecans are not generally thought to be independent receptors of cytokine/chemokine or matrix ligands; through their extracellular domain and HS chains syndecans interact with soluble factors including FGFs [63-65], transforming growth factor-β (TGF-β) family members [66-69]; and small chemokines such as SDF-1/CXCL12 [70] and RANTES/CCL5 [71]; transmembrane and membrane-bound signaling receptors such as integrins (reviewed in [72-75], ADAM (a disintegrin and metalloprotease)-12 [76, 77] and WNT receptors [78]; and extracellular matrix proteins such as laminins [79-84], tenascin [85, 86], collagen [87, 88] and fibronectin [89-91]. While some of these interactions are specific to only one syndecan (i.e., syndecan-4 is
the sole and obligate coreceptor for CXCR4-CXCL12 binding), many protein-protein
interactions (such as syndecan-integrin binding) are common among all four family
members, even though the four extracellular domains are each unique in their genomic
sequence.

**Syndecan-4 Intracellular Interactions**

The transmembrane and cytoplasmic domains of the four syndecans are much more
highly conserved than the extracellular domains. Conserved interactions sites for
Src/Fyn in the C1 domain and PDZ proteins in the C2 domain are common to all
syndecans (reviewed in [92]). Syndecan-4 has been shown to participate in signaling
interactions with elements of the cytoskeleton such as α-actinin [93], cortactin [64], and
tubulin [94]; PDZ domain-containing proteins such as syntenin [95, 96] CASK [96], and
syneclin [97]; signaling intermediates such as phosphatidylinositol 4,5-biphosphate
(PIP2) [62]; adaptor proteins such as paxillin and syndesmos [98]; and signaling effectors
such as c-Src [99], focal adhesion kinase [100] [101], protein kinase C (PKC) α [102-
104], PKCβ [105], PKCδ [106, 107], and PKCε [99], isoforms; dyamin [108]; and the
small G proteins Rho, Rac, and Cdc42 (reviewed in [109]). Signaling by/through
syndecan-4 has been shown to directly affect cell proliferation (reviewed in [96]),
differentiation [34, 99, 110-112], adhesion (reviewed in [61]) and migration [113-116] in
multiple cell systems.

**Syndecan-4 Multimerization and Translocation**

The mechanisms by which extracellular ligand engagement with syndecans is
transduced to the interior of the cell are not yet known. Current data suggests that
binding of the syndecan-4 ectodomain or heparan sulfate chains with extracellular
Figure 3. Syndecan-4 domain structure. A schematic view of syndecan, showing the core protein and glycosaminoglycan chains. HS refers to heparan sulfate chains; TM, transmembrane domain; C1 and C2 refer to conserved regions 1 and 2, and V refers to the variable region, of the cytoplasmic domain.

Adapted from Couchman, 2003
ligands leads to removal of an inhibitory phosphorylation on Ser183 (Ser179 of mouse syndecan-4) in the C1 intracellular domain, which produces a conformational change in the cytoplasmic domains [117]. This conformational change unmasks the lysine-rich PIP$_2$ binding site, and PIP$_2$ binding induces an additional conformational change and permits syndecan-4 dimerization [118]. The dimerized syndecan-4/PIP$_2$ then binds and activates protein kinase C-alpha (PKC$\alpha$) [103, 119, 120]. In other cells types [121] multimerized syndecan-4 (and possibly other proteins with which it is interacting) are relocalized into cholesterol- and sphingolipid-rich membrane rafts, which appear to be necessary for efficient signal transduction [96, 121]. The conformational change in the C2 region after desphosphorylation of Ser183/179 may also be required for binding of a subset of PDZ proteins, including syntenin, to the C-terminal PDZ-binding motif. Ser183/179 is phosphorylated by PKC$\delta$, but the specific phosphatase that activates syndecan-4 has not been identified [106].

**Syndecan-4 Proliferation Signaling**

One key activity of syndecan-4 is its role in transduction of pro-growth signals, as exemplified by its interactions with FGF2. Via its heparan sulfate side chains, syndecan-4 binds growth factors such as FGF2 cooperatively with their high-affinity receptors [122, 123]; it has also been shown to bind FGF2 independently of FGF receptor [124]. Downstream of cooperative growth factor binding, signaling through the receptor tyrosine kinase (RTK) following trans-autophosphorylation is well studied. The syndecan-4 cytoplasmic domain may also signal independently of RTKs to activate overlapping pathways such as MAP kinases [91].
**TEAD Family of Transcription Factors**

The vertebrate TEAD, also named TEF (transcription enhancer) genes encode a family of transcription factors that include TEAD-1 (NTEF-1/TEF-1), TEAD-2 (ETEF-1/TEF-4), TEAD-3 (DTEF-1/TEF-5), and TEAD-4 (RTEF-1/TEF-3). All TEAD family members contain an evolutionarily conserved 72-amino acid DNA binding domain that is found in human (TEF), fly (Scalloped), yeast (TEC1) and *Aspergillus* (AbaA), referred to as TEA (TEF-1, TEC, and AbaA) [125, 126]. TEA domain transcription factors, TEADs, have been shown to serve a regulatory role by binding to canonical MCAT elements (5’-CATTCC(T/A)-3’) located in the promoter/enhancer region of several cardiac, smooth, and skeletal muscle genes [126]. Although MCAT elements were originally identified in muscle they have been found in the promoter regions of several non-muscle genes [127]. Indeed most mammalian tissues express more than one TEAD protein during embryonic and adult life, several recent studies involving single or double TEAD gene inactivation (TEAD-1, TEAD-2, and TEAD-4) have shown that these transcription factors serve both unique and overlapping roles during embryonic development [128]. Further, gene inactivation of the TEADs leads to developmental deficiencies (TEADs-2, 4) and embryonic lethality (TEAD-1) [128-130]. Thus, the regulatory specificity of each TEAD protein during development and in adult tissues (including skeletal muscle) is likely due to their capacity to recognize a broad spectrum of DNA elements, accessibility to target elements, interactions with various tissue-specific co-activator/co-binding proteins, post-translational modifications, or subtle gradients in their expression levels [127].

**TEAD Protein Interactions**

TEAD proteins have been shown to interact with multiple transcription factors and co-factors for the regulation of muscle specific genes [127]. Co-factors that have recently been identified to interact with TEAD family members include SRC1 [131], TIF2 [131],
RAC3 [131], YAP65[132], TAZ [133], Vgl-2 [134], and Vgl-4[135]. Some co-factors such as Vgl-2 (also known as VITO1) are expressed in a tissue-specific manner in the differentiating somites and branchial arches during development and in skeletal muscle in the adult [134]. Other co-factors, such as Vgl-4, are not tissue specific and are expressed in multiple tissues including the heart, brain, kidney, small intestine, lung and placenta [135].

TEAD family members also interact with multiple transcription factors including SRF, MEF2, and Max [127, 134, 136, 137]. These factors have been shown to specifically control muscle-specific genes. Examples include, SRF and TEAD1 which synergistically activate the skeletal α-actin gene [136], whereas Max interacts with TEAD1 to regulate the expression of α-MHC in cardiomyocytes [137]. The combined action of TEAD with other transcription factors in multiple cell types highlights the ubiquitous role TEADs play in transcriptional regulation.

**TEAD1 Function in Skeletal Muscle**

In addition to the binding of MCAT elements that regulate many muscle specific genes, TEAD proteins have recently been shown to bind MEF2 and A/T-rich elements in the control regions of select muscle genes [138]. Importantly, TEAD1 binds to these elements during fast to slow skeletal muscle fiber type transitions. *In vivo* overexpression of TEAD1 has been shown to shift skeletal muscle towards a slower myosin heavy chain (MyHC) profile with a loss of fast type IIX MyHC in both fast and slow twitch muscles and an increase in type IIa and/or type I MyHC [139]. This observation is particularly interesting with reference to satellite cells as there is a known difference in satellite cell number between fiber types. To date no such mechanism has been identified that regulates the number of satellite cells in each fiber type or within whole skeletal muscle.
Overall the number of interactions between TEAD proteins, transcription factors, transcriptional co-activators and different DNA binding sites represent a broader than previously anticipated regulatory role for the TEAD proteins in genome-wide transcriptional regulation at all developmental stages. Thus, determining the mechanisms involved in TEAD transcriptional regulation remain an area of intense focus for additional research.

This introduction is a summary of the literature upon my entry into the field of the adult muscle stem cells known as satellite cells. The goal of my thesis is to provide new insights into the regulation of proliferation and differentiation of satellite cells. As such the following chapters discuss: how the expression of an adhesion molecule, NCAM, can distinguish proliferating satellite cells from differentiating satellite cells, how separate domains of a single protein, syndecan-4, can regulate proliferation and differentiation, and how the interpretation of a secreted signal can regulate satellite cell proliferation as a population.
References


CHAPTER 2. NEURAL CELL ADHESION MOLECULE (NCAM) MARKS ADULT MYogenic CELLS COMMITTED TO DIFFERENTIATION

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ABSTRACT

Although recent advances in broad-scale gene expression analysis have dramatically increased our knowledge of the repertoire of mRNAs present in multiple cell types, it has become increasingly clear that examination of the expression, localization, and associations of the encoded proteins will be critical for determining their functional significance. In particular, many signaling receptors, transducers, and effectors have been proposed to act in higher-order complexes associated with physically distinct areas of the plasma membrane. Adult muscle stem cells (satellite cells) must, upon injury, respond appropriately to a wide range of extracellular stimuli: the role of such signaling scaffolds is therefore a potentially important area of inquiry. To address this question, we first isolated detergent-resistant membrane fractions from primary satellite cells, then analyzed their component proteins using liquid chromatography-tandem mass spectrometry. Transmembrane and juxtamembrane components of adhesion-mediated signaling pathways made up the largest group of identified proteins; in particular, neural cell adhesion molecule (NCAM), a multifunctional cell-surface protein that has previously been associated with muscle regeneration, was significant. Immunohistochemical analysis revealed that not only is NCAM localized to discrete areas of the plasma membrane, it is also a very early marker of commitment to terminal differentiation. Using flow cytometry, we have sorted physically homogeneous myogenic cultures into proliferating and differentiating fractions based solely upon NCAM expression.
INTRODUCTION

Skeletal muscle is a terminally differentiated tissue consisting of ordered arrays of multinucleated, contractile myofibers. In vertebrates, skeletal muscle is formed during fetal and postnatal development by differentiation of previously specified myoblasts, which irreversibly exit the cell cycle and become myocytes. This transition is accompanied by changes in gene expression, growth factor responsiveness and structural protein production (reviewed in [1]). Upon differentiation myocytes subsequently fuse with each other or with existing myotubes to produce contractile syncytial myofibers [2].

In adult vertebrates myogenesis is believed to be primarily carried out by satellite cells, the somatic stem cells responsible for in vivo maintenance and regeneration of skeletal muscle tissue [3, 4]. These cells, which comprise a very small (1-6%) fraction of total muscle-associated nuclei, are defined anatomically by their position between the basement membrane and the sarcolemma of differentiated muscle fibers [3, 5, 6]. In response to injury, otherwise mitotically quiescent satellite cells become activated and proliferate extensively. The resulting population of adult myoblasts will then transit to the site of injury and differentiate into myocytes to replace the damaged myofibers, either by fusion with each other to form new muscle fibers or by fusing into existing post-mitotic muscle fibers [7, 8]. While the satellite cell compartment is repopulated following completion of a cycle of acute regeneration, it remains unclear what the exact cellular source(s) of these new quiescent cells may be: evidence exists for satellite cell self-renewal, either by asymmetric division [9] or stochastic events [10], as well as possible contributions from muscle-associated mesenchymal stem cell populations [9, 11].

The extracellular milieu encountered by newly-activated satellite cells requires that they detect and respond appropriately to a diverse array of rapidly changing stimuli. In addition to the damaged host muscle, local signaling sources would include
coincidentally damaged connective tissue, vasculature and nervous tissue, as well as infiltrating cells of the immune system [3]. Local extracellular signals would also be expected to vary with time after the initial injury. Thus, critical roles have been demonstrated for many soluble factors and matrix/adhesion molecules in the muscle tissue during satellite cell-mediated muscle repair [12-14], and there is a significant amount of ongoing investigation into the signaling pathways that function in satellite cells during regeneration.

An area that has not yet been addressed with respect to satellite cell signaling is the possible involvement of higher-order signaling complexes, such as those that have been proposed to assemble in membrane ‘rafts’. Membrane rafts, also known as lipid rafts, are small (10-200 nm), cholesterol and sphingolipid enriched membranes that function to compartmentalize cellular processes [15, 16]. These regions of the plasma membrane play important roles in intracellular protein transport, membrane fusion and transcytosis; they have also been proposed to act as platforms for assembly of signaling complexes, cell surface antigens and adhesion molecules. Cellular events commonly associated with membrane raft complexes include cell activation, polarization and signaling [17, 18]. In other adult stem cells (i.e., hematopoietic stem cells) membrane rafts are critical for cell cycle regulation and survival [19, 20], however very little is known about signaling pathways mediated by membrane rafts in satellite cells.

In this study, we attempted to isolate and characterize plasma membrane proteins expressed by primary mouse satellite cells, with the goal of prospectively identifying additional signaling pathways that may impinge upon satellite cell activity. Using liquid chromatography-tandem mass spectrometry, we identified classes of transmembrane and membrane-associated proteins present in freshly isolated murine satellite cells and enriched in detergent-resistant membrane domains. While surprisingly few of the expected transmembrane receptors were detected above the reliability
threshold, multiple proteins associated with adhesion-mediated signaling were identified. Several have not previously been connected with myogenesis, although many have; a significant subset have also been reported to act via membrane raft complexes. One such protein, neural cell adhesion molecule (NCAM), was found to be present and enriched in the detergent-resistant membrane fraction, and was selected for further study.

When examined in heterogeneous populations of adult myoblasts and myocytes, we found NCAM expression to be coincident with the earliest detectable markers of commitment to differentiation. In order to unequivocally differentiate between myoblasts and myocytes, it is common to assay for expression of differentiation-associated proteins such as myogenin, p21, or muscle structural proteins. However, all of these proteins are cytoplasmic or nuclear, and it would be desirable to determine cell status by assaying for a cell-surface epitope, allowing analysis of living unfixed cells. To date, no such marker has been reported. Here we show that by indirect immunohistochemistry, NCAM labels only non-proliferating cells that, based on their expression of differentiation markers including myogenin and muscle creatine kinase have committed to differentiation. We also use NCAM expression to separate a heterogeneous population of adult myoblasts into proliferating and differentiated fractions, as confirmed by expression of either proliferation or pro-myogenesis proteins. This molecular tool therefore represents a novel, non-terminal assay for the early identification and sorting of committed myocytes from heterogeneous populations derived from primary mouse satellite cells.

MATERIALS AND METHODS

Primary Satellite cell isolation and culture

Mouse satellite cells were isolated and cultured as described previously [21]. Briefly, muscle was dissected from the hind limbs of adult female mice (B6D2F1; Jackson labs)
between 80 and 130 d of age. Muscles from both legs were minced and digested in 400 U/ml collagenase type I (Worthington) diluted in Ham's F-12 medium (Invitrogen). The resulting cells were collected and pre-plated on gelatin-coated (0.66%) petri dishes in growth medium [Ham's F-12 (Gibco), 15% horse serum (Equitech) and penicillin/streptomycin (Gibco) supplemented with 0.5 nM rhFGF-2.] After 24 hrs, non-adherent cells (consisting primarily of myoblasts and residual red blood cells) were collected and replated on new gelatin-coated plates in growth medium supplemented with FGF-2; the original plates with the adherent cells (primarily fibroblasts) were discarded. After 72 hours total, the myoblasts had become adherent; they were washed and taken up with warmed PBS then replated as appropriate: cells to be expanded for an additional 2 days were replated on new gelatin-coated plates in growth medium supplemented with FGF2, and cells to be used for immunofluorescent staining were plated on glass coverslips coated with a thin layer of gelatin in growth or differentiation media as specified. Differentiation medium consisted of F-12 K (Gibco), 2% horse serum and penicillin/streptomycin.

**Detergent resistant membrane fractionation**

Primary satellite cells were isolated and cultured as above for 6 days in growth medium supplemented with FGF-2. The resulting subconfluent cells were washed with PBS, dissociated using collagenase, and harvested by centrifugation. The cell pellet was resuspended in 1 ml MNE (25 mM MES pH 6.5, 150 mM NaCl, 5 mM EDTA) containing 0.2% (v/v) Triton X-100 and 1x Roche Complete Protease Inhibitors. Cells were lysed with 10 strokes of a chilled Dounce homogenizer on ice then mixed with an equal volume (1 ml) of 80% (w/v) sucrose in MNE and placed into the bottom of an ultracentrifuge tube. Cell lysates were overlaid with 2 ml of 30% (w/v) sucrose followed by 1 ml of 5% (w/v) sucrose in MNE and ultracentrifuged at 38,800 rpm in a Sorvall TH-
660 rotor for 18 hours at 4°C. Fractions of 550 µl were collected from top to bottom and designated 1-9. Fraction(s) containing visible raft material were noted and processed for further analysis.

**Liquid chromatography-tandem mass spectrometry, sample preparation and data acquisition**

The protein concentration of each fraction was measured using a Pierce BCA assay. Protein samples (40 mg) in 80% acetone were incubated at -20°C for 2 hours then centrifuged for 20 minutes at 13,000rpm at 4°C. Acetone was removed and the pellet was dried. Protein pellets were re-suspended in 20 mL of buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 30 mM Tris-HCl pH 8.0. Cysteines were reduced and alkylated with 10 mM DTT (100 mM stock in Tris-HCl pH 8.0), and 40 mM IAA (200 mM stock in 100M Tris-HCl pH 8.0) respectively, using 1 hour incubations at RT for each. Remaining IAA was neutralized with an addition of DTT to 30mM. Urea concentrations for each sample were adjusted to 1 M with 100 mM Tris-HCl pH 8.0. Digestion was performed with 8.0 mL of a 0.2 mg/mL solution of sequencing grade-modified trypsin (Promega, Madison WI) in 50mM acetic acid to get a 1:25(w/w) ratio of trypsin to sample protein. pH was checked for near neutrality (pH 7.5-8) with indicator strips (adjustments with 100mM Tris pH 8.0), and the mix was then incubated at 37°C for 12 hours. Tryptic digests were lyophilized, and reconstituted in 30 mL 0.1% formic acid in water. Samples were analyzed on an LTQ ProteomeX linear ion trap LC-MS/MS instrument (Thermo Fisher, San Jose, CA). Sample was loaded onto a C18 packed nanospray column and eluted using an acetonitrile gradient (0-90% in 120 min). LC separation was performed using fused silica nanospray needles (360 mm outer diameter, 150 mm inner diameter; Polymicro Technologies), packed with “Magic C18” (100Å, 5 mm particles; Michrom Bioresources) in 100% methanol. Samples were analyzed in the data-dependent
positive acquisition mode. Following each full scan (400–2000 m/z), a data-dependent MS/MS scan for the three most intense parent ions was acquired. The nanospray column was held at ion sprays of 3.1 kV and a flow rate of 100 nL/min.

**Database searching and protein identification**

The National Center for Biotechnology Information (NCBI; ftp.ncbi.nih.gov/blast/) non-redundant protein database (as of June 2007) was used for querying all data. The FASTA database utilities and indexer of the BioWorks Rev 3.3, software was used to create a mouse database (keywords *Mus musculus*, and “mouse”) from the NCBI non-redundant database and to index it for trypsin cleavage with cysteine modified by carboxyamidomethylation (+57Da). Search parameters were set to include oxidation of methionine (+16) as a variable modification. Protein hits were filtered with the following criteria: peptide probability less than 0.001, XCorr values greater than 1.5, 2.0 and 2.5 for +1, +2, and +3 charged ions, respectively.

**Immunohistochemistry**

Primary satellite cells were amplified in culture under growth conditions for 4 days then plated on gelatin-coated glass coverslips. In time course studies cells were then cultured for 0, 12, 24, 48 or 72 h in differentiation medium; BrdU was added to a final concentration of 0.01 mM for 4 hrs before fixation and staining. Cells were fixed in 4% paraformaldehyde for 10 min at RT and blocked in 10% goat serum containing 1% NP-40. If anti-syndecan-4 was to be used an additional block in 10% Blokhen (Aves) was used. To detect BrdU incorporation, fixed cells were treated with 4 M hydrochloric acid for 10 min at RT. Primary antibodies used were rat anti-NCAM (Chemicon) at 1:100, chicken anti-syndecan-4 at 1:1500 [21], mouse anti-caveolin-3 (mAb 26, BD Biosciences) at 1:200, mouse anti-BrdU [22] at 1:20, and mouse anti-myogenin (F5D;
Developmental Studies Hybridoma Bank) neat. For GM1 detection Cholera Toxin Subunit B conjugated to Alexa 488 (Invitrogen) was used at 1:1000. Secondary antibodies conjugated to Alexa 488 or Alexa 594 (Invitrogen) were used at 1:500. Subtracting background, secondary only fluorescence, normalized all images (Slidebook, Intelligent Imaging Innovations).

For analysis of NCAM expression kinetics primary cells were plated onto coverslips in triplicate. For each time point, cells from five randomized 20x fields were counted. Total cell number was determined by DAPI-stained nuclei (or by brightfield images when BrdU was used). Errors bars represent the standard error of the mean for n=3.

**Scanning electron microscopy**

Correlative SEM was performed essentially as has been described [23]. Briefly, primary satellite cells grown on a gridded, gelatin-coated coverslips were fixed in 4% paraformaldehyde before imaging by immunofluorescence and noting the location of individual cells on the grid. Samples were then post-fixed in 2.5% glutaraldehyde, dehydrated in ethanol, critical point dried, and coated with a thin film of evaporated platinum. The previously identified cells were then identified and viewed on a Hitachi S4700 field emission SEM at the University of Missouri Electron Microscopy core facility.

**Fluorescence-activated cell sorting analysis**

MM14 myoblasts [24, 25] were amplified in culture under growth conditions, which for this satellite cell-derived line includes addition of supplementary FGF-2 every 12 hours to prevent commitment to terminal differentiation during the G1 phase of the cell cycle. To produce a heterogeneous population of proliferating and recently-committed cells, cultures that had previously been supplemented with FGF-2 were not given additional
FGF-2 for 12 hrs, leading to depletion of the available FGF2 and subsequent commitment of a fraction of the cells to differentiation. Cells were taken up with 0.05% collagenase and blocked in 10% normal goat serum for 45 min at 4°C. NCAM staining was performed using rat anti-NCAM (Chemicon) at 1:100 for 45 min at 4°C followed by secondary staining with anti-rat Alexa 488 (Invitrogen) at 1:500 for 30 min at 4°C. Cells were then sorted on a BD FACS DiVa®Vantage (Becton Dickinson, San Jose, CA) at the University of Missouri Cell and Immunobiology core facility; collection gates P1, P2 and P3 were defined as described in the text.

**Western blotting**

Cells from NCAM⁺ve and NCAM⁻ve sorted populations were lysed in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholic acid, 1 mM NaF, 1 mM sodium orthovanadate, 1 mg/ml Pepstatin A and 1x Roche Complete Protease Inhibitors) and protein concentration was determined using a Pierce BCA assay. Equal amounts (20 µg) of cell lysate were loaded in alternating lanes on an Invitrogen Bis-Tris 4-12% gradient gel and blotted onto PVDF. Each of the four blots were probed then stripped and reprobed with at least two antibodies, one predicted to be found in the NCAM⁺ve lysate and another predicted in the NCAM⁻ve lysate. Primary antibodies were: mouse anti-caveolin 3 (BD Transduction Laboratories) at 1:5000, goat anti-creatine kinase-M (Santa Cruz) at 1:200, mouse anti-myogenin (F5D; Developmental Studies Hybridoma Bank) at 1:50, rat anti-NCAM (Chemicon) at 1:200, rabbit anti-PCNA (Abcam) at 1:200, rabbit anti-phospho-histone H3 (Ser 10) (Santa Cruz) at 1:200, rabbit anti-phospho-GSK3β (inhibitory Ser 9, Cell Signaling) at 1:500. Primary antibody binding was detected with secondary antibodies conjugated to horseradish peroxidase [26] and detection of the signal was obtained using enhanced
chemiluminescence [26]. Total protein loading per lane was confirmed by staining two lanes from the same blot with Coomassie Brilliant blue G-250 (Fisher Scientific).

RESULTS AND DISCUSSION

Detergent-resistant membrane domain proteins of primary satellite cells

The light buoyant density and other physical properties of membrane raft domains facilitate their isolation based on insolubility in cold nonionic detergents [27]. To determine what proteins are partitioned into the detergent-resistant membrane domains (DRMs), we used cold Triton X-100 insolubility and flotation over a discontinuous sucrose gradient to isolate them from cultures amplified from primary murine satellite cells. We then used liquid chromatography-tandem mass spectrometry to identify the extracted proteins and construct a representative list of DRM-associated proteins of satellite cells. Table 1 includes 72 of the approximately 250 proteins identified. Many of them are present in or associated with membrane rafts of other cell types [18, 28], and/or have been previously described in muscle. Overall, satellite cell DRMs contained primarily signaling intermediates, adhesion and cytoskeletal proteins (Table 1).

While detergent-resistant membrane extraction has historically been used as a benchmark for membrane raft association [15, 27, 29], we note that more recent work would suggest that detergent-resistance can be a subjective approach because of its dependence on type and concentration of detergent, temperature and cell type [16, 29]. In spite of this caveat, it remains a straightforward and effective means of isolating individual membrane raft proteins and complexes [16] subject to later verification. Using this method, we identified many probable satellite cell raft proteins, several of which also form multicomponent signaling complexes with one another, suggesting the potential for functional organization within the DRMs.
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**Kinases and Phosphatases**

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* values are averages of original isoform assignment values
** coverage calculated from NCAM isoform consensus sequence
Table 1: Detergent-resistant membrane proteins identified by mass spectrometry. Table contains protein names, previously described raft association ("Y" refers to yes, the protein has been previously associated with rafts, "U" refers to unknown raft association, and "N/A" refers to not applicable), NCBI protein accession number and Gene ID, the number of unique tryptic peptides identified, and calculated p-value, cross-correlation, and percent coverage. Individual peptide sequence, mass, and charge data are available in Supplemental Figure 1. Prior raft associations were determined by searching the Harvester mouse database (http://harvester.fzk.de/harvester/) and combined searches in PubMed ("protein name" AND "raft").
Integrins β1 (fibronectin receptor β) and α6 were reliably represented in the DRM fractions (Table 1); microarray and RT-PCR analysis of primary satellite cells suggest that integrins α5 (fibronectin receptor α), α3 and α4 and are expressed by satellite cells as well (Cornelison and Olwin, in prep.) Integrins are ubiquitous transmembrane adhesion proteins known to be influenced by plasma membrane lipids; the functional activation of integrins can be linked to membrane raft localization [30, 31]. The lack of inherent catalytic activity requires integrins to be associated with other molecules for signaling activity. Both fibronectin-binding integrins and syndecan-4, a marker of satellite cells, are actively localized to membrane rafts and cooperate for the formation of vinculin-containing focal adhesions in vascular endothelial cells (Chapter 2, supplemental data)[32-34]. Detection of integrins in satellite cell DRMs raises the possibility that similar mechanisms of raft-based, adhesion-mediated signaling may apply in this cell type.

The ferlins are a family of proteins important for maintenance and repair of muscle membranes [35], particularly in the process of cellular fusion to form syncytial myofibers. In muscle, dysferlin interacts with other raft-localized proteins such as caveolin-3 and annexins for correct plasma membrane trafficking and repair, respectively [36-38]. Mutations in either dysferlin or caveolin-3 cause specific, non-Duchenne’s type muscular dystrophies [35, 39-41]. We detected myoferlin, dysferlin, and annexins A1, A2 and A5 with confidence in satellite cell DRMs (Table 1); while ferlins have been extensively characterized in myoblasts, this is the first indication that they are both expressed by primary adult satellite cells and potentially localized to membrane raft microdomains in this cell population. Further analysis of their expression and activity in these cells will provide useful information in disease models as well as nonpathogenic myogenesis.
We also detected multiple signaling molecules that, while not inserted into the plasma membrane, are known to be tethered to juxtamembrane cytoskeletal elements, particularly in membrane microdomains. Examples of these include multiple small G-proteins such as R-ras and Cdc42, the tyrosine kinases fyn and yes, and α- and β-catenin (Table 1). These data suggest that tightly-associated signaling complexes can be co-isolated in the DRM fractions used in this analysis. β-catenin in particular is a component of the canonical Wnt signaling pathway (reviewed in [42]) and acts as a transcriptional activator in synergy with Tcf/Lef family proteins (reviewed in [43]). Wnt signaling in satellite cells has been shown to act in initial specification and promotes both growth and differentiation [44-46]. Intriguingly, Wnt signaling through Frizzled has also recently been implicated in satellite cell transdifferentiation in vitro and in vivo [47]. Inactive β-catenin is localized at the plasma membrane by associations with the intracellular domains of cadherins, which act to regulate their activity [48]. Caveolin-1 expression inhibits Wnt/β-catenin/Lef-1 signaling by recruiting β-catenin to caveolae membrane microdomains [49], and it has been shown that inhibition of membrane raft structure by methyl-b-cyclodextrin affects myogenesis downstream of Wnt signaling [50, 51]. Thus, our isolation and identification of both α- and β-catenin further supports the hypothesis that DRM-based complexes may be key regulators of satellite cell activity.

**NCAM and membrane raft marker expression in proliferating and differentiating cultures**

Neural cell adhesion molecule (NCAM) was detected in satellite cell DRMs with high confidence, based on the large number of peptides retrieved, percent coverage and cross correlation value (Table 1). NCAM is an immunoglobulin superfamily adhesion molecule; its expression on human satellite cells was first observed in monoclonal antibody studies [52] while later work demonstrated that, in human [53] and rat [54],
NCAM is expressed on quiescent satellite cells and is maintained during proliferation and differentiation, through the formation of regenerated myofibers. It is also detected on the widely-used myoblast cell line C2C12 [55] where its expression is associated with the early stages of differentiation, concurrent with expression of T-type Ca\(^{2+}\) channels and inward rectifier K\(^{+}\) channels [56]. However, these studies and others [57] also established that NCAM is \textit{not} expressed by quiescent mouse satellite cells, and that its expression on satellite cells is heterogeneous during regeneration \textit{in vivo} [58, 59]. In spite of significant efforts to assign a function to NCAM in satellite cell physiology, its molecular role is still unclear.

NCAM has previously been found to localize and act in membrane rafts in C2C12 myoblasts as well as neuronal cells and various NCAM transfected cell lines [60-62]. To investigate the localization of NCAM in relation to known membrane raft markers in primary satellite cells, satellite cell cultures amplified for 96 hours after harvest from the mouse were either fixed (as a proliferating sample) or grown for an additional 48 hours under differentiating conditions then fixed. All samples were labeled with anti-NCAM and co-stained with either fluorescently labeled cholera toxin subunit B (CTB), anti-caveolin-3, or anti-syndecan-4 (Figure 1). CTB binds to ganglioside M1 (GM\(_{1}\)), a glycosphingolipid considered to be a specific marker of membrane rafts [29, 63]. Caveolin-3, or M-caveolin, is a muscle-specific isoform of caveolin that localizes to caveolae, a subset of membrane rafts, in the plasma membrane (reviewed in [64]). Syndecan-4, which has also been shown to associate with membrane rafts [34], is used as a marker of primary satellite cells [65].

Figure 1 A and B illustrate that GM\(_{1}\) and NCAM appear to identify distinct subpopulations of satellite cells: GM\(_{1}\) is prevalent in cells cultured under growth conditions that express little or no NCAM, while NCAM predominates in cultures that have been stimulated to differentiate, primarily on morphologically distinct, differentiating
Figure 1: Immunolocalization of membrane raft markers and NCAM in primary satellite cells. Satellite cell-derived cultures were induced to proliferate in culture for 4 days then either fixed (A, C, E) or placed in differentiation media for an additional 48 hrs (B, D, F) before fixing and staining. In proliferative cultures most cells did not express NCAM (red) (A, C, E). NCAM staining is observed in most cells after culture in differentiation media (B, D, F). CTB (cholera toxin subunit B) labels GM₁ (green) on cells in proliferative cultures and appears restricted to round cells adjacent to myotubes (A, B). Cav-3 (green) has the opposite expression pattern, appearing predominately on myotubes (C, D). Yellow indicates overlap between green and red staining. Both proliferating and differentiating cells express the satellite cell marker syndecan-4 (green) (E, F). Nuclei were stained with DAPI (blue) to identify all cells present. Bar = 100 μm.
myotubes that are also expressing caveolin-3 (Figure 1D and 1C). This coexpression is particularly intriguing in light of recent studies indicating caveolin-3 appears to mark terminally differentiated myotubes [66].

**Scanning electron microscopy of NCAM stained primary myocytes**

We next analyzed the surface architecture of NCAM-positive primary adult myocytes using scanning electron microscopy (SEM). Primary satellite cell cultures were induced to differentiate for 12 hours then fixed and immunostained for NCAM. The cells were imaged under fluorescence optics and their location on the coverslip recorded; the coverslips were then processed for SEM imaging. Figure 2 shows one myocyte sequentially imaged by immunofluorescence for NCAM and SEM; NCAM staining is punctate and overlaps with small structures at the plasma membrane (Figure 2C and 2D). Note that in figures 2B and 2D, the viewing plane is focused at the surface of the cell near the nucleus, thus NCAM staining on other portions of the cell is out of focus.

We speculate that NCAM localization to these domains may explain why NCAM staining is observed in comparatively large areas across the plasma membrane despite the extremely small size of membrane rafts. The identity and function of these structures remains to be determined.

**Time course of NCAM expression in differentiating myotubes**

Based on the expression of NCAM in proliferating and differentiating cultures we went on to test the potential for NCAM to be an early extracellular marker of commitment to myogenic differentiation. To test this we examined NCAM expression on primary mouse satellite cell cultures at fixed times after they were switched to differentiation medium. To measure proliferation the cells were incubated in BrdU for 4 hr prior to
Figure 2: Scanning electron microscopy of an NCAM-stained primary myocyte. SEM image (A) of a primary adult myocyte that had previously been imaged by indirect immunofluorescence using an antibody against NCAM (B). In higher magnification images, white arrows indicate small protrusions on the plasma membrane of the myocyte (C), while black arrows identify punctate NCAM staining that overlaps with these structures (D).
fixation and staining. We found that NCAM labels an expanding subpopulation of syndecan-4 positive cells over time in differentiation medium (Figure 3A). Prior to induction of differentiation, 10.7% of all cells were NCAM positive; 48 hours later almost all syndecan-4 positive cells were also NCAM positive, with NCAM expression on 97.9% of cells (Figure 3, Figure 4A). At 0 hours BrdU incorporation was 50.2%; after 24 hours in differentiation media BrdU incorporation was 1.1 % indicating a general exit from the cell cycle coincident with increasing incidence of NCAM expression (Figure 3B). Concurrent with NCAM expression, myogenin expression also increased throughout differentiation (Figure 3C and 4B). By 48 hours all NCAM positive cells were also myogenin positive. These results suggest NCAM is a proximal marker of commitment to myogenic differentiation.

**Facs sorting of NCAM⁺ and NCAM⁻ cells**

To test the utility and specificity of NCAM as an extracellular marker of differentiation, we separated a heterogeneous population of proliferating and recently-committed differentiating MM14 myoblasts by flow cytometry into two populations based on their NCAM immunoreactivity. MM14s are a satellite cell-derived cell line with morphology and gene expression very similar to primary satellite cells [24, 67]. They are dependent on FGF2 stimulation during the G1 phase of the cell cycle to prevent commitment to terminal differentiation [25]. The cells were amplified in culture under growth conditions then deprived of additional FGF2 for 12 hours before staining and sorting (Fig. 5A). Forward scatter and side scatter, measures of the size and granularity of cells, respectively, (Fig. 5B, left) were used to gate a physically homogenous population of small, mononucleated cells. Thus, sorted cells were either proliferating, or at such an early stage in myogenic differentiation that they had not begun to elongate.
Figure 3: NCAM immunolocalization in differentiating satellite cells. Primary satellite cells were amplified in culture for 4 days then switched to differentiation medium for 0, 12, 24, 48 and 72 hrs. Immunostaining for NCAM reveals an increasing subpopulation of NCAM^+ (red), syndecan-4^+ (green) satellite cells (A). Overlap of red and green fluorescence appears yellow. BrdU was added to differentiating cells 4 hrs before fixing. Lack of detectable BrdU (green) incorporation suggests that all NCAM^+ (red) cells have withdrawn from the cell cycle (B). Myogenin expression increases during differentiation; by 24 hrs most NCAM^+ (red) cells are also myogenin^+ (green) (C). Nuclei were stained with DAPI (blue) to identify all cells present. Bar = 100 µm.
Figure 4: Kinetics of NCAM expression, cell cycle exit and myogenin expression in differentiating satellite cells. After serum withdrawal the percentage of NCAM positive cells increases from 10.7 ± 3.5 % (mean ± SEM, n=3) at 0 hr to 96.4 ± 0.59 % at 72 hr (A). Over the same period the percentage of cells incorporating BrdU decreased to zero and myogenin positive cells increased to 93.9% (B). The percentage of syndecan-4 positive cells stayed roughly the same over the 72 hr time course (B).
and were thus morphologically indistinguishable from proliferative myoblasts. Figure 5B (right) shows the histogram of NCAM immunoreactivity for cells within the P1 gate; two clear peaks can be observed, with a small region of overlap between. Collection gates P2 and P3 were set to exclude this region of overlap, and NCAM negative and NCAM positive cells were sorted and collected for further analysis.

To determine the commitment status of the collected cell fractions we performed Western blots of lysates from the sorted cells with markers of proliferation and differentiation, sequentially (Fig. 5C). Lysates were assayed for protein concentration and 20 µg of total protein was run out in each lane. Phosphorylated histone H3 and PCNA, both markers of cycling cells, were detectable only in the NCAM negative fraction, consistent with actively proliferating, non-differentiating myoblasts. In contrast, the myogenic differentiation markers phospho-GSK3β, muscle creatine kinase (MCK), myogenin (myoG) and caveolin-3 (cav-3), as well as NCAM itself, were detected only in lysates from the NCAM positive fraction. We therefore conclude that NCAM is a marker of committed adult myocytes, which also has the technical advantage of providing a facile method for separating myoblasts from myocytes in a heterogeneous cell population.
Figure 5: Isolation of NCAM positive cells by fluorescence activated cell sorting. Commitment of a subpopulation of proliferating MM14 myoblasts was induced by 12 hours of FGF2 depletion, then cells were sorted based on NCAM expression (A). Forward scatter (FSC) and side scatter (SSC) gating (B, left) and the parameters used to separate populations of NCAM negative (-) and NCAM positive (+) cells are displayed (B, right). Twenty micrograms of total protein from each sample were resolved by SDS-PAGE and transferred to PVDF, followed by Western blotting (C). Blots from left to right: phospho-histone 3 (pH3), phospho-serine 9 glycogen synthase kinase 3β (pGSK3β), muscle creatine kinase (MCK), proliferating cell nuclear antigen (PCNA), myogenin (MyoG), caveolin-3 (CAV-3), neural cell adhesion molecule (NCAM). One set of lanes from the blot was stained with Coomassie blue to confirm equivalent loading (D).
**Conclusions and future directions**

Since the initial observation of NCAM expression on rat primary satellite cells, the role of NCAM during myogenesis has been elusive [12, 54]. In muscle, NCAM exists in multiple isoforms, which arise from alternative splicing of a single gene [68, 69]. NCAM is also subject to several forms of post-translational modification: polysialylation of residues in the immunoglobulin domains is commonly found during development and has recently been shown to increase membrane repulsion and to enhance directional migration in specific cell types [70, 71]. Conversely, O-linked glycosylation at the muscle specific domain (MSD) increases myoblast fusion [72]. In vitro overexpression of NCAM isoforms containing the MSD increases myoblast fusion, while the overexpression of NCAM isoforms lacking this domain has no effect [73-75]. Surprisingly, NCAM null mice display no gross defects in skeletal muscle during development, and primary myoblasts from these mice fuse at a similar rate as wild type in vitro [76]. Together, these data imply that the increased fusion noted in NCAM overexpression studies may be the result of increased overall adhesion, and that other adhesion molecules are capable of compensating for the loss of NCAM [12]. To this, the current study adds the suggestion that association of NCAM with complexes of adhesion and signaling molecules in specific, discrete physical microdomains and/or membrane structures could constitute an important aspect of NCAM regulation and activity. It also extends previous work on the heterogeneous expression of NCAM on activated mouse satellite cells by associating acquisition of NCAM expression with a change in cellular commitment status. Interestingly, we observe from the Western blots that while all three splice isoforms of NCAM (180kD, 140 kD, and 120 kD) appear to be present in the sorted cell lysates, the 120 kD form that has previously been associated with differentiation and enhanced fusion in cultured myocytes appears to be the most abundant.
This DRM proteomic study represents the first extraction and identification of putative membrane raft components from primary satellite cells. We find that satellite cell DRMs not only contain a multitude of established membrane raft-associated proteins and muscle specific proteins, but also show a strong potential to act as organizing scaffolds for higher-order, functional signaling complexes. Specific membrane raft components including GM\textsubscript{1}, caveolin-3 and NCAM are dynamically expressed during differentiation of satellite cells, suggesting that these cells may represent a useful new model system for the study of membrane raft dynamics. The specific factors isolated in this screen also point to intriguing new avenues of inquiry in satellite cell physiology.

Our analysis of NCAM expression in heterogeneous cultures of primary myoblasts and the satellite cell line MM14 indicates that it may be a proximal marker of the commitment to myogenic differentiation. Flow cytometry on the basis of cell-surface NCAM detection is therefore a promising new molecular tool, that will allow live cell sorting of heterogeneous primary cell populations into discrete populations for either biochemical analysis or, potentially, downstream clinical applications.
References


In other cell types Syndecan-4 has been shown to associate with the detergent resistant membrane fraction (DRM)\cite{1, 2} and has been shown to act as a scaffolding protein for higher-order adhesion and signaling complexes \cite{3}. Furthermore, many of the proteins previously shown to interact with Syndecan-4 in other systems are also associated with the DRM. Supplemental Figure 1 illustrates how Syndecan-4 is thought to cooperate with integrins and serve as an attachment point for the actin cytoskeleton during the formation of focal adhesions in endothelial cells. The formation of focal adhesions and other signaling ascribed to Syndecan-4 are thought to take place at specific areas of the cell membrane.

Here we expand on previous work showing that Syndecan-4 does indeed localize to the DRM in satellite cells and show this localization is necessary for Extracellular Regulated Kinase (ERK1/2) phosphorylation by using mass spectrometry to identify proteins that may interact with syndecan-4 and/or require Syndecan-4 for proper localization.

**Syndecan-4 is enriched in detergent resistant membrane microdomains.**

We used cold Triton X-100 insolubility and flotation over a discontinuous sucrose gradient to isolate membrane microdomains from wild type primary murine satellite cells. Supplemental Figure 2 demonstrates the insoluble raft fraction (2A) and a western blot.
Supplemental Figure 1
Schematic of Syndecan-4 Interactions

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of Syndecan-4 (Cornelison and Olwin, unpublished) (2B). Syndecan-4 is enriched at the interface of the 5% and 30% sucrose gradient consistent with its presence in the DRM. The multiple bands of Syndecan-4 in the low-density fractions correspond to Triton-X insoluble dimers typically observed in other cell types. The Syndecan-4 found in the high-density 40% fraction differs in mobility from the low-density Syndecan-4 likely resulting from differences in protein modification. This data indicates that Syndecan-4 is heterogeneously distributed over the plasma membrane and localizes with low-density membrane rafts of satellite cells.

**Syndecan-4 is clustered by growth factors and disrupted by cholesterol chelation in proliferating satellite cells.**

To further verify the subcellular localization of Syndecan-4, primary wild type satellite cells were isolated, briefly amplified in culture, and then starved of all serum and growth factors. Cells were then treated with FGF2 or HGF to induce Syndecan-4 multimerization and clustering and/or with methyl-β-cyclodextrin (MβCD), a cholesterol chelator to disrupt membrane rafts [4]. In endothelial cells MβCD has been shown to reduce clustering of Syndecan-4 at membrane raft regions [1]. We examined the localization of Syndecan-4 by immunostaining after treatments. In starved satellite cells Syndecan-4 staining is diffuse (Supplemental Figure 3A). However, when stimulated with either FGF2 or HGF Syndecan-4 rapidly (>15 min) redistributes to a punctate pattern across the membrane indicative of membrane rafts (Supplemental Figure 3B and C). This is consistent with observed syndecan-4 clustering seen with either FGF or a chimeric Fc Receptor-syndecan-4 molecule previously described [1, 5]. To block the redistribution of Syndecan-4 in response to FGF or HGF we used MβCD. When MβCD is
Supplemental Figure 2. Syndecan-4 localizes to detergent resistant membranes (DRM). (2A) Visible raft concentrates were associated at the 5-30% interface. (2B) All fractions and the insoluble pellet were separated on a 4-12% polyacrylamide gradient gel (Cornelison and Olwin unpublished). The blot was probed with chicken anti-Syndecan-4.
added, Syndecan-4 no longer appears punctate and is observed diffusely across the membrane (Supplemental Figure 3E and F). This data provides further support that Syndecan-4 is localized to membrane raft structures in satellite cells in response to growth factor stimulation.

Membrane raft disruption recapitulates signaling deficiencies of syndecan-4-/- satellite cells.

Syndecan-4-/- satellite cells fail to properly activate, proliferate, and differentiate in culture. We hypothesized that part of this phenotype is due to the inability to activate the signaling intermediates either directly downstream of growth factor receptor signaling and/or through direct Syndecan-4 signaling. Importantly, global tyrosine phosphorylation is not significantly changed in syndecan-4-/- satellite cells [6]. However, when stimulated with either FGF2 or HGF syndecan-4-/- cells show decreased phosphorylation (activation) of ERK1/2 MAP kinases compared to wild type cells (Supplemental Figure 4). This is significant because syndecan-4 has been shown to be capable of binding FGF2 and transiently stimulating ERK1/2 phosphorylation independently of growth factor receptors [7]. Thus the loss of Syndecan-4 potentially leads to unique defects in multiple signaling pathways, both Syndecan-4 growth factor receptor dependent and independent. To determine the requirement of membrane rafts in satellite cell signaling, we treated wild type satellite cells with MβCD and examined the cellular response to FGF2 and HGF stimulation (Supplemental Figure 4).

We found that raft disruption recapitulates the ERK1/2 signaling defect observed in syndecan-4-/- satellite cells. When using an antibody that specifically recognizes non-phosphorylated ERK1/2 (Supplemental Figure 4, lower panel) the Wt starved,
Supplemental Figure 3. Syndecan-4 localization in response to growth factor treatment and cholesterol chelation. Early passage (< 4) primary satellite cells were starved of growth factors (F-12 medium) for 16 h. Then either FGF-2 or HGF was added for 15 minutes followed by staining for syndecan-4 (GREEN) and DAPI (BLUE). A. Starved B. Starved + FGF C. Starved + HGF D. starved + MβCD E. starved + FGF + MβCD F. starved + HGF + MβCD.
FGF+MβCD and HGF+MβCD samples are non-reactive. This indicates that either all the ERK1/2 present is phosphorylated rendering it unreactive to the non-phosphorylated ERK1/2 antibody or that the levels of non-phosphorylated ERK are beneath the threshold of detection for this antibody under these conditions. Additionally, increased exposure times of the blot do not lead to the appearance of reactive bands in these lanes. It is interesting to note that in the syndecan-4⁻/⁻ cells we observed increased levels of non-phosphorylated ERK1/2 relative to wild type. This is consistent with other observations that without syndecan-4 in this cell type we see an increase in the protein levels of many intermediate signaling molecules such as, MEK1, Raf1, FAK, PKD and RSK1 (data not shown). It remains to be determined if this is a compensatory mechanism for the loss of syndecan-4 mediated signaling or if syndecan-4 is acting as a repressor of these molecules in the wild type satellite cells.


DRM extraction was carried out on syndecan-4⁻/⁻ cells as above (Supplemental Table 1 and supplemental Figure 2A) and the fraction(s) containing visible raft material were noted and processed for further analysis. Then using liquid chromatography-tandem mass spectrometry the extracted membrane raft proteins were identified (as in Chapter 2). Supplemental Table 1 includes 73 of the approximately 250 proteins identified in wild type satellite cells with good reliability. Many of them are present in or associated with membrane rafts of other cell types, and/or have been previously described in muscle [8, 9]. The majority of wild type DRM proteins were also identified in syndecan-4⁻/⁻ satellite cells (Supplemental Table 1).

An interesting observation of these preliminary results is that of the 7 kinases and phosphatases identified in wild type DRMs only one appears in the syndecan-4⁻/⁻
Supplemental Figure 4. ERK1/2 activation in wild type and syndecan-4-/- primary satellite cells. Satellite cells from wild type and syndecan-4-/- mice were cultured in medium without serum or other additives for 16 hours. The cells were then treated with FGF2, HGF and/or MβCD as indicated and western blotted for phospho ERK1/2. The same blot was then stripped and reprobed with anti-ERK1/2.
preparation. Similar to integrins Syndecan-4 itself does not contain an enzymatic domain and is dependent on the enzymatic activity of other proteins to convey signals. Future experiments will be necessary to determine if these proteins are responsible Syndecan-4 mediated signaling and without Syndecan-4 to serve as an anchoring point are no longer associated with the DRM.

Another interesting finding is that several of the Rab family of proteins are missing from the syndecan-4-/- preparation. Rab proteins are small GTPases known for their role in regulating SNARE function and coordinating consecutive stages of transport between organelles [10]. Given that Rabs and their effectors cluster into distinct functional domains it is not surprising that they separated into the DRM fraction. Previously, Syndecan-4 has been shown to regulate the distribution between membrane and cytosolic fractions of the GTPase-activating protein, p190RhoGAP, a protein important for the regulation of RhoA and focal adhesion formation [11]. Based on this role for Syndecan-4 it is easy to imagine how the Rab proteins may coordinate with Syndecan-4 to provide this function.

Future research will help to determine if the proteins missing from the syndecan-4-/- fraction actually interact with syndecan-4 and/or require syndecan-4 for proper localization. However, alternative methods will be needed to determine if membrane rafts of syndecan-4-/- cells are quantitatively different from wild type.
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**Supplemental Table 1. Wild type and syndecan-4⁻⁻⁻ membrane raft associated proteins identified by mass spectrometry.** The table contains protein names of all proteins identified in wild type DRM (same as non-supplemental data), previously described raft association ("Y" refers to yes, the protein has been previously associated with rafts, "U" refers to unknown raft association, and "N/A" refers to not applicable), syndecan-4⁻⁻⁻ satellite cell DRM, NCBI protein accession number and gene ID.
MATERIALS AND METHODS

Detergent resistant membrane fractionation

DRM fractionation was performed essentially as in Chapter 2. Primary satellite cells were isolated and amplified in cultured as above for 6 days in growth medium supplemented with FGF-2. The resulting cells were washed with PBS, dissociated using collagenase, and harvested by centrifugation. The cell pellet was resuspended in 1 ml MNE (25 mM MES pH 6.5, 150 mM NaCl, 5 mM EDTA) containing 0.2% (v/v) Triton X-100 and 1x Roche Complete Protease Inhibitors. Cells were lysed with 10 strokes of a chilled Dounce homogenizer on ice then mixed with an equal volume (1 ml) of 80% (w/v) sucrose in MNE and placed into the bottom of an ultracentrifuge tube. Cell lysates were overlaid with 2 ml of 30% (w/v) sucrose followed by 1 ml of 5% (w/v) sucrose in MNE and ultracentrifuged at 38,800 rpm in a Sorvall TH-660 rotor for 18 hours at 4°C. Fractions of 400 µl were collected from top to bottom and designated 1-12. Visible raft concentrates were associated with fractions 2 and 3. All fractions and the insoluble pellet were made up with Laemmli buffer and 15 µl of each were separated on a 4-12% polyacrylamide gradient gel. Each fraction makes up one lane in the gel. The separated proteins were blotted onto PVDF membrane and the membrane was probed with chicken anti-syndecan-4 at 1:1500 and detected by anti-chicken HRP (Promega) and enhanced chemiluminescence (Amersham.)

Cell culture, growth factor stimulation and cholesterol chelation

Early passage (< 4) primary satellite cells were plated on gelatin-coated glass coverslips and starved of serum and growth factors (F-12 medium) for 16 h. The medium was replaced with either starvation medium or starvation medium + 10 mM MβCD for 1 hour, then either 0.5 nM FGF-2 or 10 ng/ml rmHGF was added for 15 minutes followed by live
cell staining on ice. Cells were rinsed in cold PBS, blocked in 10% goat serum for 10 min, then incubated in chicken anti-syndecan-4 at 1:1500 [6] for 30 min. Cells were washed with PBS and incubated in chicken secondary antibody conjugated to Alexa 488 (Molecular Probes) at 1:500 for 10 min. After all staining was complete cells were fixed in 4% paraformaldehyde for 10 min, washed and mounted. Subtracting background, secondary only fluorescence, normalized all images (Slidebook, Intelligent Imaging Innovations).

**Western blotting**

Low-passage (<4) satellite cells from wild type and syndecan-4/- mice were digested and replated in F-12 medium without serum or other additions for 16 hours. Half of the plates were pretreated for 1 hour with 10 mM methyl-β-cyclodextrin (MβCD) (Sigma) and half were incubated with F-12 medium alone. The cells were then stimulated for 15' with 0.5 nM rhFGF-2 or 10 ng/ml rhHGF. Cells were harvested into lysis buffer and protein concentration was determined by BCA assay [12]. 20 mg of protein from each lysate was loaded on a 15% acrylamide gel, blotted and stained with anti-phospho ERK1/2 (Sigma) at 1:2000 dilution. The blot was then striped and reprobed with anti-ERK1/2 (Sigma) at 1:2000 dilution. Primary antibody binding was detected with secondary antibodies conjugated to horseradish peroxidase [12] and detection of the signal was obtained using the enhanced chemiluminescence [12].
References


CHAPTER 3. THE FUNCTIONS OF SYND ECAN-4 IN MUSCLE SATELLITE CELLS
ARE SEPARABLE AND DOMAIN-DEPENDENT

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ABSTRACT

Syndecan-4 is a type I plasma membrane glycoprotein that is expressed by all mononucleate skeletal muscle cells, including adult myoblasts (satellite cells). While syndecan-4 appears to be dispensable for prenatal and postnatal muscle specification and growth, animals lacking syndecan-4 show multiple defects in muscle regeneration. Due to the number of factors known to interact with syndecan-4 (integrins, receptor tyrosine kinases, G protein-coupled receptors, heparin-binding growth factors, components of the cytoskeleton, small G proteins, signaling adaptor molecules, and intracellular signaling proteins) it has been difficult to identify the molecular basis of this pleiotropic phenotype. We report here that re-expression of the full-length protein abrogates the in vitro defects in proliferation and differentiation, while expression of syndecan-4 carrying inactivating mutations in previously characterized intracellular domains selectively rescue different aspects of the null phenotype. In particular, the motif required for association with phosphatidylinositol 4,5-biphosphate (PIP₂) appears to be required for efficient proliferation but dispensable for differentiation. Alternatively, the PDZ-binding motif at the C-terminus is required for differentiation but not proliferation.
INTRODUCTION

Adult muscle stem cells (satellite cells) are the committed somatic stem cells responsible for *in vivo* maintenance and regeneration of adult skeletal muscle tissue (for review see [1-4]). Satellite cells are located between the external lamina and the sarcolemma of mature muscle fibers and are physiologically characterized by their small size, high nucleus to cytosol ratio, heterochromatinized DNA and minimal cellular metabolism [2, 5]. In mouse 2-7% of all adult skeletal muscle-associated nuclei belong to satellite cells [6, 7]. Through the action of satellite cells, skeletal muscle is able to dynamically adapt to environmental changes, regenerate, and grow as needed across the entire lifespan of the animal. After injury or disruption of the basal lamina, the mitotically quiescent satellite cells become activated, migrate out from their niche and proliferate extensively [7]. The resulting muscle precursor cells, or myoblasts, eventually differentiate and fuse to existing post-mitotic muscle fibers or to each other to form multinucleated myotubes [8].

Mice lacking the transmembrane glycoprotein syndecan-4 display defects in skeletal muscle regeneration [9]. These defects include delayed satellite activation, decreased/ delayed satellite cell cycling, decreased myoblast migration, decreased myogenic differentiation, and aberrant patterning of regenerated muscle *in vivo* [9].

Syndecan-4 belongs to a conserved family of heparan and chondroitin sulfate transmembrane proteins. Each syndecan molecule consists of a conserved single-pass transmembrane domain, a short cytoplasmic domain, and unique extracellular domain containing attachment sites for both heparan sulfate (HS) and chondroitin sulfate (CS) (reviewed in [10]). The cytoplasmic domains of syndecans are divided into two constant domains (C1 and C2) and one variable (V) domain (reviewed in [11] [12]). Through the intracellular domains, syndecans mediate interactions with a large number of different cellular factors. The C1 region serves as an anchor to the cytoskeleton through
interaction with proteins such as α-actinin [13], tubulin [14] and cortactin [14]. The C2 domain contains a PDZ binding motif known to bind at least four PDZ domain containing scaffolding proteins such as CASK [15, 16], synectin [17], synbindin [18] and syntenin [15, 19, 20].

The variable domain of syndecans is highly heterogeneous among the four family members ([21]). This domain has been extensively studied in syndecan-4 [21]. The V domain of syndecan-4 contains a PIP2 binding site important for syndecan-4 dimerization ([22]). This domain allows for two syndecan-4 molecules to be linked together by PIP2 in a twisted clamp formation [23-25]. Through PIP2 binding and dimerization syndecan-4 is capable of binding and activating PKCα [22][26-30]. These properties of syndecan-4 are regulated by phosphorylation of S183 near the N-terminus of the PIP2 binding motif[31]. Upon phosphorylation of the cytoplasmic tail binding of PIP2 oligomerization and activation of PKCα are reduced[32].

In nonmuscle cell types syndecan-4 is unique among syndecans in its localization to focal adhesions of adherent cells and is known to link extracellular matrix factors to cytoskeletal proteins. Some of these factors include fibronectin [33], ADAMs [34], laminin [35] tenascin [36, 37], collagen [38, 39] and integrins [40]. The heparan sulfate (HS) chains on the extracellular domain allow syndecan-4 to bind and interact with many soluble factors including FGF [29, 41], HGF [42] and TGFβ [43]. The heparan sulfate chains of syndecan-4 are known to mediate interactions between these soluble factors and their transmembrane receptor tyrosine kinases [44].

Based on the number of functions we previously observed for syndecan-4 in muscle satellite cells, we asked if these activities are separable on the basis of the protein-protein interactions occurring between syndecan-4 cytoplasmic domain motifs and cellular mediators of proliferation and differentiation. We report here that while
reconstitution of full-length syndecan-4 in primary satellite cells derived from syndecan-4^-/- mice will largely rescue both cellular defects, rescue is dependent on the integrity of the specific protein-protein interaction domain for each activity. We propose that syndecan-4 acts as a multi-functional scaffold for assembly of protein complexes required either to promote proliferation or to promote differentiation.

MATERIALS AND METHODS

Generation of mutant syndecan-4 expression vectors

The complete cDNA for murine syndecan-4 was cloned into pDsRed-monomer-N1 (Clontech) to encode a full-length protein tagged at the C-terminus with monomeric DsRed. Primer-mediated site-directed mutagenesis (QuickChange, Invitrogen) was used to introduce nucleotide changes to produce mutant syndecan-4 proteins as described below. Coding sequences were then subcloned in to pShuttleCMV (Stratagene) to generate recombinant pro-adenoviral plasmids using the AdEasy Adenoviral Vector System. HEK293 cells were transfected, and the recombinant adenovirus was passaged and amplified over three rounds of infection. Final-passage cells were lysed and the viral particles were titered; for the experiments described here recombinant adenovirus vectors were used at 10^7 pfu/ml.

Isolation and culture of primary mouse satellite cells

Primary satellite cells were isolated from adult (80-180 days of age) female mice according to our published protocols [9, 45]. Briefly, muscles are dissected in PBS, minced, and digested with 400 U/ml collagenase I (Worthington). The resulting slurry is filtered to remove debris, the cells are pelleted by centrifugation and plated in growth medium [Ham's F-12 (Gibco), 15% horse serum (Equitech) 100 U/ml penicillin/100 ug/ml
streptomycin (Gibco) and 0.5 nM recombinant human FGF2 (made in-house). After a 24-hour pre-plate to remove fibroblasts and other adherent cells, the purified adult myoblasts are cultured for an additional 72 hours before analysis. For adenoviral infections, cells were amplified in monoculture for 4 days then rinsed with Hams F-12 medium prior to infection. For adenoviral infection 5 µl of each virus was added to 2 ml F-12 and incubated on the cells for 20 minutes. Then 2.7 ml of growth medium plus 300 µl of FGF2 was added and cells were incubated 6 hours to overnight before adding another 5 ml of growth medium. Medium was then removed after 24 hours from start of transduction and cells are re-fed growth medium or differentiation medium [Ham's F-12, 5% horse serum and 100 U/ml penicillin/100 µg/ml streptomycin (Gibco)] as appropriate.

**Immunohistochemistry**

Primary satellite cells were amplified in culture under growth conditions and/or transduced with adenovirus then plated on gelatin-coated glass coverslips. Cells were fixed in 4% paraformaldehyde for 10 min and blocked in 10% goat serum containing 1% NP-40. Primary antibodies were incubated for 2 hrs at room temp or overnight at 4°C. Cells were washed 3 times in 1X PBS before addition of secondary Alexa 488 or Alexa 594 antibodies (Invitrogen) at 1:500. If anti-syndecan-4 was to be used cells were stained alive by rinsing with ice cold 1X PBS then blocked in 10% goat serum for 10 min. Anti-syndecan-4 was used at 1:750 and incubated on cells for 30 min before adding secondary Alexa 488 or Alexa 594 antibodies (Invitrogen) for 10 mins. The cells were then fixed in 4% paraformaldehyde. Primary antibodies used were chicken anti-syndecan-4 ([9]), rat anti-NCAM (Chemicon) at 1:100, rat anti-PH3 (Santa Cruz) at (1:500).
RESULTS

*Labeled syndecan-4 expressed from viral vectors is expressed and appropriately localized in syndecan-4−/− satellite cells.*

We addressed the potential role of syndecan-4 cytoplasmic domains in regulating the function of satellite cells by transducing *syndecan-4−/−* primary satellite cells with recombinant adenoviral vectors encoding either the full-length wild type sequence, or sequences harboring mutations to the PIP2 or PDZ motifs (Figure 1B). Shown in Figure 1A is a schematic depicting full-length endogenous syndecan-4 protein illustrating key domains. The extracellular region contains three attachment sites for HS glycosaminoglycan side chains. The single pass transmembrane domain is directly adjacent to the first of two conserved intracellular domains, C1. The variable (V) domain contains the PIP2 binding site and is followed by the second conserved region, C2, which contains the PDZ domain binding motif.

The significant body of work that exists describing the interactions of syndecan-4 in other cell types lead us to choose specific mutations for each domain. Through the use of GST-fusion and co-immunoprecipitation experiments several studies have established an interaction between syndecan-4, PIP2 and PKCα at the V domain [22, 28, 46]. However, *in vitro* experiments by, Horowitz et al, 1999, used surface plasmon resonance technology to show that the specific binding site, YKK, is necessary for PIP2 binding and that PIP2 binding is necessary for PKCα binding and activation [27]. Within the V domain, we performed the same mutation to the PIP2 binding motif. Three consecutive residues were mutated, YKK to LQQ; a mutation that removes the hydroxyl group of tyrosine and negates the positively charged lysines of this region [27].
Figure 1. Syndecan-4 schematic, adenoviral constructs and their expression in primary satellite cells.

A) Composition of syndecan-4 monomer, extracellular domain, containing attachments sites for heparan sulfate chains, single pass transmembrane domain and cytoplasmic domain, containing conserved C1 and C2 and variable domain V. Arrows from left to right indicate S183, PIP2 binding site YKK, PDZ binding domain EFYA. B) Schematic of adenoviral syndecan-4 cytoplasmic domain mutants used. From top to bottom: WT syndecan-4 fused to monomeric DsRed (S4DsRed), syndecan-4 containing cytoplasmic V domain YKK to LQQ mutation (S4PIP2), syndecan-4 cytoplasmic C2 domain deletion of carboxyl terminal A residue (S4PDZ) and monomeric DsRed (DsRed)(B). C-F) Syndecan-4+/− primary satellite cells transduced with adenoviral vectors: untransduced (C), S4DsRed (D), S4PIP2 (E) and S4PDZ (F).
Previous studies of the PDZ binding motif of syndecan-4 have identified several proteins capable of binding the C-terminal EFYA sequence [16-20]. Importantly, Grootjans et al., used syndecan-GST fusion constructs combined with ligand binding blots to determine that the last three amino acids were important for PDZ protein (syntenin) binding [20]. In these experiments they also showed that the C-terminal alanine was essential for syntenin binding [20]. In another study focused on determining consens sequences for all PDZ binding domains, Songyang et al., used surface plasmon resonance and PDZ domain-GST fusions to show the 0 and -2 positions confer binding specificity of the PDZ binding motif [47]. Therefore, for the PDZ binding motif mutation the C-terminal alanine was removed to abolish PDZ protein binding.

All exogenous syndecan-4 constructs were tagged with monomeric DsRed for monitoring both transduction and expression efficiencies of the adenoviral constructs. All constructs demonstrate robust infection of early-passage cells, with ~80% of mononuclear cells positive for DsRed at 6 days post harvest (Figure 1C-F). The transduced cells appear phenotypically identical to mock transduced syndecan-4 null cells (Figure 1C).

The PIP$_2$ association domain of syndecan-4 is required to promote satellite cell proliferation.

Previous cell culture studies on the role of the PIP$_2$ motif and syndecan-4 function indicated that PIP$_2$ binding led to increased PKC$\alpha$ interaction and activation in rat fat pad endothelial cells (RFPECs) [29]. In this cell type the PIP$_2$ motif was shown to be important for effective FGF2 mediated cell proliferation [29]. Therefore based on the observation that syndecan-4$^{-/-}$ cells do not proliferate efficiently we tested whether the PIP$_2$ binding motif was necessary for efficient satellite cell proliferation.

To determine the effect of the PIP$_2$ motif in satellite cells we transduced primary
cells with constructs containing wild type syndecan-4 (S4dsred), syndecan-4 with mutated PIP$_2$ (S4PIP$_2$), syndecan-4 with mutated PDZ (S4PDZ) and a dsred only control (Control). The adenovirally transduced satellite cells were immunolabeled with anti-phospho-histone H3 (PH3) to identify cells in late prophase. Figure 2A shows images of cells transduced with either control virus or syndecan-4 constructs and stained with anti-PH3. In the syndecan-4$^{-/-}$ satellite cells transduced with only the DsRed construct 6% of total cells are PH3 positive. Both the full length S4DsRed and the S4PDZ construct are capable of rescuing the proliferation defect of syndecan-4$^{-/-}$ satellite cells to levels found in wild-type cells (PH3+ 14.0 and 15.0 % of total nuclei, Figure 2B). However, S4PIP$_2$ expression only minimally reverts the phenotype with only 8.7% of the cells staining positive for PH3.

*The PDZ association domain of syndecan-4 is required to promote satellite cell differentiation.*

Differentiation of satellite cells into fully functional muscle fibers is a tightly controlled process requiring the production, assembly and reorganization of a large number of highly structural protein components including the actin cytoskeleton [2]. Previous studies describe a role for syndecan-4 in providing a structural linkage between the extracellular matrix and the cytoskeleton [21]. To assess the role of syndecan-4 in satellite cell differentiation we tested the ability of the each syndecan-4 construct to promote myogenic differentiation in cultured syndecan-4$^{-/-}$ primary satellite cells. Syndecan-4$^{-/-}$ cells were transduced as above then subjected to low serum conditions for 0, 12, 24, or 48 hours to induce myogenic differentiation. Levels of commitment to terminal differentiation were assessed by expression of NCAM, a marker of murine satellite cell differentiation.
Figure 2. Proliferation of **syndecan-4** null satellite cells is reduced without the **PIP\(_2\)** binding motif. A) Primary satellite cell derived cultures were transduced with wild type syndecan-4 (**S4DsRed**), control construct with dsred only (**Control**), syndecan-4 containing the **PIP\(_2\)** mutation (**S4PIP\(_2\)**), and syndecan-4 containing the PDZ mutation (**S4PDZ**), and stained with PH3 (green). S4DsRed and S4PDZ transduced cells have a significant increase in PH3 positive cells compared to DsRed and S4PIP\(_2\) cultures. B) Quantitation of PH3 positive DsRed positive cells counted as a percentage of total nuclei. Paired t-test P<0.05 *, P<0.005 **.
By 48 hours NCAM positive cells committed to differentiation (green), are clearly visible. We observed that either full-length S4DsRed or S4PIP₂ proteins are capable of reconstituting the majority (81.7 and 80.7% respectively) of the differentiation defect of the syndecan-4⁻/⁻ cells by 48 hours; compared to wild type satellite cells (97.9% NCAM⁺) by 48 hours (Figure 3B; [45]). However, the S4PDZ construct is only partially able to rescue differentiation (62.4%) at 48 hours compared to the control syndecan-4⁻/⁻ cells (45.5%). Cells were scored based on positive NCAM staining and DsRed expression (when appropriate) then compared to a percentage of total nuclei.
Figure 3. Differentiation of myoblasts is decreased in S4PDZ transduced cultures.  
A) Primary syndecan-4^- satellite cells were amplified in culture transduced with adenoviral constructs then switched to differentiation medium for 0, 12, 24, or 48 hrs. Immunostaining for NCAM reveals an increasing subpopulation of NCAM* (green) myocytes. 
B) A representative quantitation of NCAM positive cells in cells containing the different adenoviral vector constructs. Paired t-test to control (S4^-) at end of 48 hours where \( P<0.05 \) *, \( P<0.001 \) ***.
DISCUSSION

Of all the mammalian syndecans, syndecan-4 is the most ubiquitously expressed. While it is apparently dispensable for development it is necessary for proper regeneration of several tissues including dermis, vasculature, and muscle [9, 48]. Previously we have shown both single fiber and mass cultured syndecan-4 null satellite cells have delayed activation, decreased proliferation and decreased differentiation compared to wild type[9]. Here we have further defined the role for syndecan-4 in the regulation of these two mutually exclusive stages of commitment in satellite cells. Signals generated from either the PIP2 or PDZ binding motifs of syndecan-4 are responsible for transducing either proliferation or differentiation signals, respectively.

The PIP2 and PDZ binding domains of syndecan-4 have previously been characterized in other species and cell types [29, 34, 49]. In rat endothelial cells there is a decrease in FGF2 dependent proliferation of cells expressing the PIP2 mutant domain [29]. This decrease was attributed to the inability to activate PKCα [29]. Decreases in PKCα activation have been shown to negatively regulate cell proliferation in a variety of cell types [50-54]. In NIH3T3 fibroblasts, PKCα directly phosphorylates Raf1 triggering the MAPK signaling cascade leading to stimulation of proliferation [50].

Syndecan-4 null primary murine satellite cells are readily transduced by adenovirus and capable of expression of wild type and mutant syndecan-4 adenoviral constructs. When we examined proliferation of satellite cells transduced with the wild type and mutant constructs we observed increased proliferation of cells expressing the wild type and PDZ mutant forms of syndecan-4, but not the PIP2 mutant expressing cells. We hypothesize that it is necessary for syndecan-4 to bind and activate PKCα in order for robust proliferation in this cell type, and based on the similar requirement of FGF2 in satellite cells and endothelial cells, this action may be dependent on FGF2 signaling through syndecan-4.
We also examined the ability of the transduced fusion proteins to rescue the defects in differentiation of the syndecan-4 null satellite cells. When induced to differentiate we observed both the wild type and PIP₂ mutant proteins were capable of partially rescuing the differentiation defect but the PDZ motif mutant promoted only a slight increase in differentiation. This suggests the PDZ binding domain of syndecan-4 is necessary for efficient differentiation of myocytes. We hypothesize that the PDZ binding domain functions as part of a multi-protein complex essential for cytoskeletal arrangements during the differentiation process. Possible interacting partners in this process include PDZ proteins already identified to interact with syndecans in other cell type such as CASK/Lin2 [15, 16], Synectin [17], Synbindin [18] and Syntenin1/2 [15, 19, 20]. However, the role of these proteins in the differentiation of satellite cells has yet to be determined.

Our data demonstrates that syndecan-4 contributes to satellite cell function differentially through its cytoplasmic domains. We show that re-expression of the full-length protein abrogates the in vitro defects in proliferation and differentiation, while expression of syndecan-4 carrying inactivating mutations in intracellular domains selectively rescues different aspects of the null phenotype. With the functionality of these domains defined it further demonstrates the importance of syndecan-4 in regulation of satellite cell proliferation and differentiation. Biochemical characterization of the signaling pathways induced by each domain will be important for defining a molecular basis for these diverse functions.
References


CHAPTER 4. OVEREXPRESSION OF TEA DOMAIN FAMILY MEMBER 1 (TEAD1) DURING FETAL DEVELOPMENT RESULTS IN INCREASED ADULT MUSCLE STEM CELLS
ABSTRACT

Satellite cell number as a proportion of muscle mass is tightly controlled in adult organisms. The average number of satellite cells per unit of muscle tissue is well conserved between muscles of similar age, sex, and fiber type and after tissue regeneration. However, no mechanism for specifying this proportionality has been described. Using a transgenic mouse model, we show that muscle-specific overexpression of a single transcription factor, TEAD1, leads to a 6.8 fold increase in the number of quiescent satellite cells in adult skeletal muscle. This is both the first demonstration of a transcription factor-induced increase in satellite cell number and the first such effect caused by altered gene expression in cells other than the satellite cells themselves. Using conditioned media from differentiated primary myocytes derived from either wild type or TEAD1-overexpressing mice, we show that this increase occurs non-cell autonomously and propose a model by which the differentiated muscle secretes a soluble factor which promotes satellite cell proliferation in order to regulate the number of stem cells resident in the tissue.
INTRODUCTION

Skeletal muscle is a highly structured tissue comprised of long, cylindrical, multinucleated cells (myofibers). Each myofiber is composed of actin and myosin myofibrils, the components that make up the basic machinery necessary for muscle contraction, repeated in sarcomeres. Muscle is a highly adaptable tissue with the ability to adjust to changes in load bearing and usage, which results in adaptations in morphology, muscle fiber type and contractile properties [1]. While some of these changes do not require an increase in the number of nuclei present others, such as growth, repair, and hypertrophy require additional nuclei. Myogenic precursor cells, satellite cells, are the primary stem cell population that serves as the source of new myonuclei [2, 3].

Satellite cells were first identified in 1961 by Alexander Mauro who hypothesized that “satellite cells are merely dormant myoblasts that failed to fuse with other myoblasts and are ready to recapitulate the embryonic development of skeletal muscle fiber when the main multinucleate cell is damaged.” and that “… satellite cells are “wandering” cells that have penetrated the basement membrane and are lying underneath it ready to be mobilized into activity under the proper conditions” [4]. The current definition of satellite cells has been expanded yet they are still anatomically defined by their position between the basement membrane and the sarcolemma of differentiated muscle fibers. Satellite cells comprise a very small (2-7%) fraction of total muscle-associated nuclei [3, 5]. In response to injury or exercise, the otherwise mitotically quiescent satellite cells become activated, proliferate extensively and undergo self-renewal. The resulting population of adult myoblasts will then transit to the site of injury and differentiate into myocytes to replace the damaged myofibers, either by fusion with each other to form new muscle fibers or by fusing into existing post-mitotic muscle fibers [6].

Self-renewal is a key property of adult stem cells as it assures that the resident
stem cell pool is replenished and available for subsequent cycles of tissue repair or replacement. The general paradigm developed in other self-renewing tissues is that stem cells reside in highly structured 'niches' made up of other tissue-specific cell types that provide cues regulating stem cell activities, particularly proliferation. In well-studied systems such as the skin, there are specific temporal and spatial hierarchic relationships between stem cells in their niches and their differentiated progeny [7]. The stem cells themselves are rare, multipotent, and experience comparatively slow cycling. Their immediate progeny are precursor cells, which are more proliferative and are also capable of self-renewal but no longer multipotent. Precursor cells give rise to so-called 'transit amplifying' cells which are highly proliferative but lineage-restricted; and finally cells arise whose fate is to terminally differentiate into the component cells of the host tissue (reviewed in [8]). Proportional to tissue needs, stem cells may undergo asymmetric cell division, in which they generate one stem cell and one committed progenitor. Within this axis, cell proliferation, migration, differentiation, function, death, and removal are tightly regulated to maintain tissue homeostasis [8]. It is necessary to exercise dynamic, adaptive, and tightly regulated control over the relative size of the stem cell pool to ensure both efficient regeneration and optimal use of physiological resources.

The lineage hierarchy of satellite cells is less defined than other resident stem cells. Recently it has been shown that Pax7 positive satellite cells are the primary cell type responsible for muscle regeneration [9]. However, there is evidence that satellite cell heterogeneity includes a more stem-like population of progenitor cells (reviewed in [10-15]) and/or that cells outside the classical satellite cell position can themselves give rise to satellite cells [16-18].

Satellite cells arise as Pax3+/Pax7+ cells and are first detected beneath the newly formed basal lamina of myofibers during late myogenesis (late fetal/perinatal;
E17/birth in the mouse)[19-21]. Studies of satellite cell population dynamics in developing and mature muscles have determined that the number of satellite cells decreases with age. In rodent hindlimb muscle this number declines from 30% of total nuclei at a few weeks of age to less than 5% in the adult [22]. In the adult, there are differences in satellite cell number per fiber between fast-twitch and slow-twitch fibers. In this case, more are always associated with type-1 oxidative fibers and the soleus muscle [23]. Fiber type alone does not always provide an explanation for the differences in satellite cell number between different muscles. Interestingly, the frequency of satellite cell number on specific fiber types is different between the soleus and the EDL and appears to correspond more with the growth characteristics of each muscle [22]. Although the transcriptional and signaling networks controlling satellite cell number have not as yet been delineated, it is clear that satellite cell number is tightly controlled: average numbers of satellite cells per unit of muscle tissue are well conserved within sample types (based on species, sex, age, individual muscle and fiber type). An example of the degree of control is illustrated during injury. At first there is a significant increase in satellite cell number during the repair process, then the number steadily declines to previous steady state levels (2 to 7% of total myofiber nuclei) throughout the regeneration period [23].

Previous work has described a role for the TEA domain 1 transcription factor, TEAD1, on slow muscle gene expression, cardiac development and cardiac gene expression [24, 25]. TEAD1 belongs to a family of transcription factors (TEAD2, TEAD3, TEAD4) that bind to canonical MCAT elements located in the promoter regions of several cardiac, smooth, and skeletal muscle genes [26]. Although TEAD proteins are known to play key roles in muscle gene expression they also bind to promoter regions of non-muscle genes such as the SV40 enhancer [27]. While the TEAD genes are widely expressed they have distinct expression patterns during embryonic and adult life. The
TEAD proteins function in both activation and repression of different genes and are capable of binding to a variety of co-factors [28, 29]. Inactivation of TEAD1 genes in transgenic animals has shown that these transcription factors serve both overlapping and unique roles [30-33].

The expression of TEAD1 is first detected at embryonic day 8 and appears to have ubiquitous expression during embryogenesis [30, 34]. In the adult TEAD1 is present in most adult tissues but has increased expression in the kidney, skeletal muscle, heart and lung [34]. In mouse, when TEAD1 is knocked out using a transgenic strain containing a null mutation it causes embryonic lethality at day 11 due to heart malformations [30]. Conversely, overexpression of TEAD1 leads to age-dependent heart dysfunction and an increase in fetal heart gene transcripts [24]. Whereas TEAD1 overexpression on skeletal muscle leads to a shift towards a slower myosin heavy chain (MyHC) profile with a loss of fast type IIx/d MyHC in both fast and slow twitch muscles and an increase in slow type IIa and/or type I MyHC[25].

To investigate whether TEAD1 overexpression in differentiated skeletal muscle affects the resident stem cells of muscle, satellite cells, we used mice that express hemagglutinin (HA) tagged TEAD1 under the control of the muscle creatine kinase (MCK) promoter. The MCK promoter induces expression at fetal day 17 and restricts transgenic TEAD1 expression to skeletal and heart muscle [25]. Using these mice we find a significant increase in satellite cell number on freshly isolated myofibers from both the plantaris (fast-twitch) and soleus (slow-twitch) muscles of adult TEAD1 transgenic mice. This increase in cell number cannot be ascribed to increased hypertrophy of the myofibers or direct TEAD1 action in the satellite cells themselves. Instead we propose a mechanism by which TEAD1 overexpression in differentiated skeletal muscle leads to increased secretion of a soluble factor that stimulates satellite cell proliferation in a dose-dependent manner.
MATERIALS AND METHODS

Transgenic MCK-TEAD1 mice

For generation and screening of transgenic mice see [25]. Briefly, the mouse TEAD-1 cDNA containing an in-frame HA tag at its 5-end was subcloned at the 3-end of the Muscle Creatine Kinase (MCK) regulatory sequences. The HA-tagged TEAD-1 cDNA subcloned into pBSMCK (a generous gift from Dr. R. Kahn), which consisted of a 6.5 genomic region composed of 3.3 kb of the MCK promoter and enhancer 1, untranslated exon 1, 3 kb of intron 1, which included the enhancer 2 region, and the first 16 bp of exon 2 [35]. The TEAD-1 cDNA was followed by the SV40 polyadenylation signal. The transgene was released from the plasmid, gel-purified, and injected into fertilized eggs of BDF1 mice. Three independent mouse lines are shown here (Figure 1); Line 12, ln12, was used for all further experiments.

Muscle fiber isolation and culture

Plantaris and soleus muscles were dissected from the hind limbs of MCK-TEAD1 mice between 80 and 130 days of age. Muscles were digested in 400 U/ml collagenase type I (Worthington) diluted in Ham’s F-12 medium (Invitrogen). Individual muscle fibers were picked into fresh growth medium [Ham’s F-12 (Gibco), 15% horse serum (Equitech) and penicillin/streptomycin (Gibco) supplemented with 0.5 nM rhFGF-2 then either fixed immediately in 4% paraformaldehyde for 0 hr fibers or cultured at 37°C until the indicated time.

Primary satellite cell isolation and culture

Mouse satellite cells were isolated and cultured as described previously [5][36]. Briefly, all muscles from both legs were minced and digested in 400 U/ml collagenase type I (Worthington) diluted in Ham's F-12 medium (Invitrogen). The resulting cells were collected and pre-plated on gelatin-coated (0.66%) petri dishes in growth medium. After
24 h, non-adherent cells were collected and replated on new gelatin-coated plates in growth medium supplemented with FGF-2. After 72 h total, the myoblasts were washed and taken up with warmed PBS then replated to equal density: cells to be expanded for an additional 2 days were replated on new gelatin-coated plates in growth medium supplemented with FGF2, and cells to be used for immunofluorescent staining were plated on glass coverslips coated with a thin layer of gelatin in growth or differentiation media as specified. Differentiation medium consisted of F-12 K (Gibco), 5% horse serum and penicillin/streptomycin.

**Immunohistochemistry and analysis**

Muscle fibers were fixed and placed onto glass slides for immunohistochemical staining and analysis. Primary satellite cells were amplified in culture under growth conditions then plated on gelatin-coated glass coverslips. Cells were fixed in 4% paraformaldehyde for 10 min at RT and blocked in 10% goat serum containing 1% NP-40. If anti-syndecan-4 was to be used an additional block in 10% Blokhen (Aves) was used. Primary antibodies used were rat anti-NCAM (Chemicon) at 1:100, chicken anti-syndecan-4 at 1:1500 [37], mouse anti-Pax7 (Developmental Studies Hybridoma Bank) at 1:10, mouse anti-BrdU [38] at 1:20, mouse anti-myogenin (F5D; Developmental Studies Hybridoma Bank) neat and mouse anti-MyoD (MF20, Developmental Studies Hybridoma Bank). Secondary antibodies conjugated to Alexa 488 or Alexa 594 (Invitrogen) were used at 1:500. All images were background subtracted for secondary only fluorescence and normalized using Slidebook (Intelligent Imaging Innovations). For analysis of cell number on muscle fibers, consecutive images of individual fibers were taken at 10x and joined together using Photoshop, then imported into ImageJ software where they were measured for total length, width and volume. For analysis of cell number in cultured cells, cells from 10 randomized fields were counted per replicate with a minimum of 3
replicates per assay. Total cell number was determined by DAPI-stained nuclei using Metamorph image analysis software. Error bars are ± SEM.

Flow cytometry analysis

Satellite cells were isolated from hindlimb muscles as described above. Cells were further purified via a discontinuous Percoll gradient (D. Cornelison, unpublished). Cells were then transferred into 10 ml of dPBS in 15 ml conical tubes, and spun for 20 minutes at 4C. For muscle SP cell sorting cell pellets were resuspended in 1 ml of dPBS supplemented with 2 mM EDTA, 2% NGS, and 2% FBS and placed on ice for 20 minutes. Primary antibody concentrations were added as follows for 20 minutes on ice with mixing: chicken anti-Syndecan 4, 1:1500 [37]; mouse anti-ABCG2, 1:100 (eBioscience); rat anti-CXCR4 FITC conjugate, 1:500 (BD pharmingen); rat anti-Sca1 APC conjugate, 1:500 (eBioscience). Cells were spun down in a microcentrifuge for 10 minutes at 4C, washed in dPBS, spun again, and the cell pellet was resuspended in dPBS supplemented with 2 mM EDTA, 2% NGS, and 2% FBS containing secondary antibodies as follows for 15 minutes at 4C with mixing: Alexa Fluor 700 anti-mouse IgG, 1:500 (Molecular Probes); Alexa Fluor 564 anti-chicken IgY, 1:500 (Molecular Probes). Cells were then fixed in 4% paraformaldehyde for 1 hr, and refrigerated until subjected to flow cytometric analysis with a Cyan ADP flow cytometer (Beckman Coulter). Labeled non-specific isotype controls were utilized to determine gating parameters [Rat IgG2b FITC (eBioscience), Rat IgG2b APC (ebioscience), chicken preimmune sera (in house), mouse preimmune sera (in house)]. Single-labeled cells were used to establish compensation between channels. Data was analyzed with Summit 5.2 software (Beckman Coulter).
**Western blotting**

Cells from wild type or MCK-TEAD1 mice were lysed in modified Allen buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholic acid, 1 mM NaF, 1 mM sodium orthovanadate, 1 mg/ml Pepstatin A and 1x Roche Complete Protease Inhibitors). Protein quantitation was determined using a Bio-Rad protein quantitation assay. Equal amounts of cell lysate were loaded onto Invitrogen Bis-tris 4-12% gradient gels and blotted onto PVDF. Densitometry of individual bands was performed using Multi-gauge 3.0 image processing software (Fujifilm). Error bars are ± SEM.

**Conditioned media**

Primary cells were isolated as above and cultured for 5 days before the addition of differentiation media. Differentiation media was collected after 48 hours, filtered (.45um BDbiosciences), concentrated to 3x in a spin concentrator (3 kD cut off Millipore), and frozen at -80°C. For heat-denatured samples conditioned media was heated to 95°C for 20 min. before freezing. To test the effects of CM, satellite cells was isolated and cultured for 4 days, then split and plated onto gelatin-coated coverslips. On day 5 the cells were rinsed in 1x PBS before the addition of the conditioned media. After 24 hours the conditioned media was removed and the cells were fixed in 4% paraformaldehyde. Immunohistochemical staining and analysis was performed as above.
RESULTS

*Increased satellite cell number on myofibers of transgenic TEAD1 mice*

To compare satellite cell numbers between wild type and TEAD1-overexpressing muscle, myofibers were isolated from the plantaris and soleus of age- and sex-matched adult mice of each strain. The myofibers were harvested from both muscles and fixed immediately to ensure that all satellite cells would be under the basal lamina and not yet proliferating. Myofibers were collected from wild type and three independent TEAD1-overexpressing mouse lines (Ln4, Ln12, Ln14) and immunolabeled with Pax7 antibodies to identify individual satellite cells (plantaris shown in Figure 1A-D, green). A detailed description of the differential expression of TEAD1 in the 3 Ln mouse strains can be found in [25]. Although TEAD1 expression levels vary in the 3 lines a similar increase in satellite cell number is observed in each line (Figure 1A-D, and data not shown) Line12, which has a median level of expression, was used for all subsequent analysis.

There was a significant increase in the overall number of satellite cells per mm of muscle fiber on all fibers isolated from TEAD1-overexpressing mice (Arrows, Figure 1A-D and graph, Figure 1E). Because the myofibers were fixed immediately upon removal the identified satellite cells were quiescent, and remained under the basal lamina as shown by laminin staining in red (Figure 1A-D, bottom panels). Visual comparison of the hindlimbs of wild type and TEAD1-overexpressing mice shows an increase in the red appearing slow twitch fibers (due to an enhanced level of myoglobin) in the TEAD1-overexpressing hindlimb (Figure 1F)[25]. To determine if this previously described fast to slow fiber type transition in TEAD1-overexpressing mice is associated with an the increased satellite cell number, we assayed myofibers isolated from the predominantly fast twitch plantaris muscles (>90% type II fibers) and the predominantly slow twitch soleus (~60-70% type I fibers)[25]. We noted the characteristic 2-3 fold difference in
satellite cell number between the plantaris and soleus fibers in wild type, but within muscle types there was a significant increase in the satellite cell number in the transgenic samples (6.8X increase in the plantaris, 3.5x increase in the soleus, Figure 1E)[3].

To account for increases in satellite cell number per myofiber due to hypertrophy of the myofibers in the TEAD1 mouse, fiber diameters as well as length were measured and myofiber volume was calculated \( V = \pi r^2 L \) (Figure 1G-H). Despite a slight increase in myofiber diameter in the plantaris and a non-significant increase in the soleus myofibers (Figure 1G,) changes in the overall width or volume (Figure 1H) of the isolated fibers cannot account for the ~3.5 to 6.8 fold increase, respectively, in satellite cell number found in the TEAD1-overexpressing mice.

In vitro proliferation rates of satellite cells from TEAD1 mice are not altered

One possible explanation for the increase in satellite cell number could be that satellite cells from TEAD1-overexpressing mice proliferate more rapidly than wild type satellite cells. To determine the proliferative capacity of the satellite cells, we quantified satellite cell numbers on myofibers in single fiber culture over a 48-hour span (Figure 2A). In myofibers isolated from both the plantaris and the soleus, the initial numbers of satellite cells are increased in the transgenic. However, after 48 hours numbers of both wild type and transgenic satellite cells have expanded comparably (Figure 2A).

We also examined the proliferation rate of satellite cells in monoculture. With an initial time 0, starting 4 days after isolation we show the proliferation curves of the TEAD1-overexpressing and wild type cultures are parallel, with the initial higher satellite
Figure 1. Increased satellite cell number in MCK-TEAD1 mouse lines.

A-D) Freshly isolated plantaris myofibers stained for the presence of satellite cells using the Pax7 antibodies. A) Wild type, B) Ln 4 MCK-TEAD1 C) Ln 12 MCK-TEAD1 and D) Ln 14 MCK-TEAD1. Arrows denote locations of Pax7+ satellite cells. Boxes below Figure 1A-D are selected enlargements of individual satellite cells (anti-Pax7, green) demonstrating their location underneath the basal lamina (anti-laminin, red) and a merged image containing Pax7, laminin, and nuclear staining (DAPI, blue).

E) Quantification of satellite cell number per mm of myofiber length from the fast-twitch plantaris muscle and slow-twitch soleus muscle of wild type and TEAD1-overexpressing mice. F) Image of hindlimb segments from wild type (left) and TEAD1-overexpressing mice (right) showing changed (darker) appearance of the transgenic muscle. G and H) Comparative analysis of the average width (G) and volume (H) of plantaris and soleus myofibers. n= total number of fibers from ≥ 3 mice. Error bars represent the SEM. Paired Student t-test between wild type and TEAD1 for each muscle type. P< 0.001 ***.

Acknowledgements Figure 1. A-H) Katie Capkovic; E, G, A) Trenton Keel; All mice generated by Richard Tsika [25].
cell counts that characterize the TEAD1-overexpressing mice leading to a higher overall final count after 60 hours in culture (Figure 2B). We also measured the amount of proliferating cells after 4 days in culture by propidium iodide (PI) staining analyzed by flow cytometry. We observed an average of 25% and 24% of cells in S/M phase in wild type and TEAD1-overexpressing mice, respectively. A representative run is shown in Figure 2C and D. By all measures tested, no significant difference in proliferation between genotypes was detected. We therefore conclude that simple acceleration of the cell cycle does not appear to be responsible for the expanded number of satellite cells.

**Satellite progenitor cells (satellite-SP) are expanded proportionately to quiescent satellite cells**

Similar to other adult tissues, including bone marrow, heart and lung, skeletal muscle has been shown to possess side population (SP) cells. SP cells are subpopulation of cells identifiable by their ability to efflux Hoechst dye through the membrane receptor, ABCG2, in which the majority of tissue-specific progenitor cells reside [39]. Here we identify satellite stem cell precursors and satellite cells by cell-surface antigens using flow cytometry. Syndecan-4 expression marks all satellite cells, and syndecan-4, ABCG2, and Sca1 coexpression marks at least one satellite stem cell population referred to as satellite-SP [39]. To determine if increased numbers of satellite cells in the TEAD1-overexpressing muscle is due to an increase in the number of progenitor cells, we compared satellite cell number versus satellite-SP number in samples of freshly isolated mononucleate cells from each genotype (Figure 2C). We found no significant increase in the proportion of satellite-SP cells to satellite cells in the TEAD1-overexpressing sample (Figure 2M). While the overall pool of cells present in the TEAD1 is enlarged, this result would not be consistent with an increase in the satellite progenitor pool as the cause for the satellite cell hyperplasia we observe.
Figure 2. Satellite cells from wild type and TEAD1 mice have similar proliferative capacities and SP progenitor cell populations.

Myofibers and their associated satellite cells were cultured then fixed and stained as in Figure 1a. **A**) Quantification of satellite cell number on isolated myofibers in single fiber culture conditions at 0, 24 and 48 hours.  **B**) Quantification of cell number in satellite cell derived myoblasts tissue culture conditions over 60 hours.  **C**) Myoblasts isolated from wild type and TEAD1-overexpressing mice were cultured for four days then stained with propidium iodide (PI) and counted by flow cytometry.  **E-M**) Flow cytometric analysis of satellite and satellite-SP cell populations isolated from TEAD1 and wild type hindlimbs.  **E, F**) Scatter plots showing gating (dark box) of cells analyzed in G-L.  **G, H**) Cells staining above threshold for syndecan-4 and gated (R1) for analysis in I-L.  **I, J**) Cells from R1 staining above threshold for Sca1 and gated for analysis in K, L.  **K, L**) Cells staining above background for syndecan-4, Sca-1, and ABCG2, which experimentally
define the satellite-SP population. M) Comparison of normalized Sca$^+\text{ABCG2}^+$ cell populations as a percentage of the sorted Syndecan4$^+$ satellite cells. Error bars represent the SEM. Paired Student t-test between wild type and TEAD1 for each muscle type. P< 0.001 ***. Acknowledgements Figure 2. A, B) Katie Capkovic; A) Trenton Keel; A) Juan Ji; C) D. Cornelison; E-M) Brian Thompson; All mice generated by Richard Tsika [25].
**TEAD1 proliferating satellite cell-derived myoblasts are not distinguishable from wild type**

One possible explanation for the change in satellite cell number could be attributed to innate differences in the satellite cell population from the TEAD1 mice. To this end proliferating satellite cells from wild type and TEAD1-overexpressing hindlimb muscles were examined for expression of satellite cell markers and the myogenic regulatory transcription factor, MyoD. Both Syndecan-4 and Pax7 are widely used markers of quiescent and recently activated satellite cells [37, 40]. There is no significant difference in the fraction of total cells expressing either Syndecan-4 or Pax7 between the genotypes under proliferation conditions *in vitro* (Figure 3A-F). Satellite cells from both genotypes also express MyoD equally at this stage in culture (Figure 3G-J).

Western blot analysis of cultured satellite cells from both genotypes shows that neither endogenous TEAD-1 driven from its own promoter nor exogenous HA-tagged TEAD-1 driven from the exogenous MCK promoter is expressed significantly in proliferating cells (Figure 3K). While very low levels of TEAD1 expression are occasionally observed, this is most likely due to the presence of a minor background population of precociously differentiated satellite cells present in the cell culture even at day 4 (data not shown).

**Recapitulation of satellite cell hyperplasia in vitro requires the onset of differentiation and subsequent transgene expression**

In order to examine the effects of differentiation, cultured satellite cell derived myoblasts were induced to differentiate by low serum media. After 48 hours under differentiation-promoting conditions, we observed high levels of transgenic TEAD1
Figure 3. Satellite cell derived myoblasts are identical by means of biomarkers and regulatory protein states. A-J) Micrographs of cultured satellite cells from wild type (A-C, G-H) and TEAD1 (D-F, I-J) mice stained for syndecan-4 (A and D), Pax7 (B and E) and a merged image with DAPI (C and F). G-J) Cultured satellite cells stained for MyoD (G and I) and merged image with MyoD and DAPI (H and J). No appreciable differences in cell staining or morphology were seen in any of the micrographs. K) Western blot analysis of satellite cell lysates from two independent wild type and TEAD1 satellite cell preparations assayed for TEAD1, and internal control IP90. Acknowledgements Figure 3. A-K) Katie Capkovic; All mice generated by Richard Tsika [25].
expression in differentiating myocytes as shown by detection of the HA tag (Figure 4A-D). We noted concurrent increases in the expression of the differentiation-specific marker, NCAM [36] (Figure 4A). Note that the nuclear expression of HA-TEAD1 is only present in differentiated, NCAM positive cells (Figure 4A-D). Western blot analysis of differentiated myocytes from both genotypes demonstrates the expression of TEAD1 in wild type cells and the additional expression of HA-TEAD1 in the transgenic cells (Figure 4E).

When TEAD1-overexpressing myocytes were cultured in differentiation conditions, we noted a pronounced increase in the number of BrdU+ cells present in the culture when compared to the wild type cells under identical conditions (Figure 4F and G). This would suggest that an increased number of satellite cells were foregoing differentiation in the TEAD1 background and remaining in a proliferative state. To further demonstrate the commitment state of these cells, we also examined the number of Pax7 positive cells (Figure 4H and I). Interestingly, we found a significant increase in Pax7+ cells in the TEAD1 background. When quantified and normalized (3 independent experiments), the overall number of Pax7+ cells in TEAD-1 transgenic cultures was ~40% higher than in wild type under differentiation conditions (Figure 4J).

**A soluble, diffusible factor is responsible for the increase in proliferative cells under differentiation conditions**

The increase in the number of BrdU+ and Pax7+ cells in cell populations derived from the TEAD1-overexpressing mice under differentiation conditions suggests that the TEAD1-expressing myocytes are signaling to the non-differentiated population of myoblasts in the mixed culture. To attempt to identify the mechanism for this increase, we collected and concentrated differentiated cell conditioned media from equal numbers
Figure 4. Differentiated cells express TEAD1 and contain an enlarged population of replicating and Pax7+ cells.

A-D) Images of TEAD1 myocytes during differentiation conditions. A) Anti-NCAM staining, B) anti-HA (TEAD1) staining, C) DAPI, and D) merged. Note that only NCAM+ cells are HA-TEAD1 positive.

E) Western blot analysis of two sets of differentiating myocytes from wild type and MCK-TEAD1 mice.

F-G) Comparative images of wild type (F) and TEAD1 (G) differentiating cells, demonstrating an enlarged pool of BrdU+ cells (green) in the TEAD1 mice. Some BrdU+ cells are labeled with arrows.

H-I, J-L) Comparative images of wild type (H, K) and TEAD1 (I-L) differentiating cells demonstrating an enlarged pool of Pax7+ cells (green) in the TEAD1 mice.

J) Quantification of Pax7+ cells after 48 hours of differentiation. K-L) Isolated image of only the Pax7+ cells. Error bars represent the SEM. Student t-test between wild type and TEAD1 P< 0.05 *.

Acknowledgements Figure 4. A-L) Katie Capkovic; J) Trenton Keel; All mice generated by Richard Tsika [25].

*
of differentiating wild type and TEAD1-overexpressing myocytes. As shown in Figure 5A-D, the addition of concentrated conditioned media (Wt or TEAD1) results in an increase in the number of Pax7+ and BrdU+ cells in both wild type and TEAD1 transgenic differentiating cultures. The conditioned media isolated from the TEAD1-overexpressing cells led to a dramatic increase in the number of proliferating (Ki67+, 30%) and proliferation-competent (Pax7+, 33%) cells compared to wild type conditioned media (Figure 5D and E). To distinguish between a potential heat labile protein factor and other potential secreted molecules, TEAD1 conditioned media was heated at 95°C for 20 minutes prior to addition to wild type cells. Heating the conditioned media led to a significant decrease in the number of proliferating cells compared to cultures treated with nonadenatured CM (Figure 5F). These data support the hypothesis that differentiated myocytes produce a soluble diffusible factor (most likely a protein) that signals to local myoblasts and either promotes proliferation or inhibits terminal differentiation. The amplification of this effect using CM derived from TEAD-1 overexpressing myocytes, together with our observations of satellite cell hyperplasia in these transgenic mice, suggest that the secreted factor is either directly or indirectly downstream of TEAD1 transcriptional activity.
Figure 5. A soluble, diffusible factor from TEAD1 myocytes is sufficient to increase the proliferative Pax7⁺/Ki67⁺ pool of cells under differentiation conditions.

Conditioned (low-serum) media isolated after 48 hours of differentiation was collected, concentrated to 3X, and added to proliferating wild type satellite cells. (A) Wild type cells with 1X wild type conditioned media; (B) wild type cells with 3X wild type conditioned media; (C) wild type cells with 3X TEAD1 conditioned media. Cells are stained for NCAM (red) to mark differentiating myocytes and Pax7 (green) to identify proliferation-competent myoblasts. (D) The percentage of Pax7⁺ cells normalized to the number present in cultures with wild type, 1X conditioned media (CM). (E) The percentage of Ki67⁺ (proliferating) cells normalized to the number present in cultures with wild type 1X conditioned media. (F) The percentage of Ki67⁺ (proliferating) wild type cells present in cultures with heat-treated 3X TEAD1 conditioned media. Error bars represent the SEM. Student t-test between wild type 1X and 3xWt or 3xTg P< 0.05 * P< 0.01 **. Acknowledgements Figure 5. A-D) Katie Capkovic; D-F) Brian Thompson; All mice generated by Richard Tsika [25].
Figure 6. A model of signaling to induce the proportion of proliferating satellite cell derived myocytes.

Q represents quiescent satellite cells under the basal lamina. P represents proliferation competent myoblasts derived from satellite cells. D represents the differentiated skeletal muscle cells. Following the black arrows from the bottom, quiescent satellite cells (Q) give rise to proliferation competent myoblasts (P). Once a sufficient pool of proliferative myoblasts (P) is created, the myoblasts either commit to differentiation (D) (right black arrow) or return to quiescence (Q) (downward black arrow). Green arrows and lines indicate actions that increase the number of the P cells. Red arrows or lines indicate actions that decrease the number of P cells. A) A model of known signaling pathways that regulate the number of P cells. B) A model of how differentiated skeletal muscle induces a soluble signal that acts on the P cells to induce proliferation or inhibit differentiation. Acknowledgements Figure 6. Katie Capkovic; D. Cornelison; Brian Thompson; Richard Tsika.
DISCUSSION

Our study provides evidence that in vivo overexpression of a transcription factor, TEAD1, leads to an up to 6.8-fold increase in quiescent satellite cell number. We found that the increase in satellite cell number was consistent on muscle fibers isolated from either predominantly type I soleus muscle, or type II plantaris muscle. While it is known that TEAD1 overexpression in skeletal muscle results in a shift towards a slower myosin heavy chain profile and that slow muscle fibers contain more satellite cells, the changes in muscle fiber type alone are not adequate to account for the drastic changes in satellite cell number.

One possible explanation for the change in satellite cell number could be attributed to innate differences in the satellite cell population from the TEAD1 mice. To this end we examined the expression of satellite cell markers, syndecan-4 and Pax7, as well as expression of MRFs and found no differences in phenotype of the TEAD1 cells. Additionally, we examined the ability of the primary cells to proliferate both on extracted myofibers and in culture and found no differences between genotypes. In fact, by all methods we used in this study, we could not observe any differences in TEAD1 proliferating satellite cells; they appeared completely identical to wild type. This finding is not surprising given that TEAD1 is not expressed during proliferation in the wild type or transgenic satellite cells.

TEAD1 is expressed in differentiated muscle endogenously; we have also exploited a transgenic mouse line in which TEAD1 is overexpressed in differentiated myocytes and myofibers [25]. Importantly, when satellite cells differentiate and become myocytes they no longer express the satellite cell marker Pax7. When we examined the differentiating cultures derived from TEAD1 satellite cells we observed more proliferating and Pax7 positive cells. Based on the fact that the proliferating cells do not express TEAD we hypothesized that the differentiated cells were signaling to the remaining
population of undifferentiated satellite cells. To test this hypothesis we used conditioned media from the TEAD1 differentiated satellite cells and placed it on wild type cells entering differentiation. Interestingly, the wild type satellite cells responded as if they were transgenic cells and we saw an increase in proliferating and Pax7 positive cells. This demonstrated that a soluble factor from the TEAD1 differentiated cells was signaling to the wild type satellite cells to inhibit differentiation and allow more cells to remain Pax7 positive. This phenotype was attenuated when the conditioned media was heated before addition to differentiating cells suggesting that a heat labile, soluble factor is responsible for the increase proliferation during times of differentiation.

One candidate mediator that impinges on molecular signaling pathways already known to be involved in mediating stem cell divisions and self-renewal is GSK3α/β. The Rudnicki group has shown that stimulation with Wnt7a (which inhibits GSK3) increases the probability of undergoing asymmetric stem cell division [10], while Brack et al. [2] have recently shown that a temporal balance (crosstalk) between Notch (GSK-3β activation) and Wnt (GSK-3β inactivation) signaling via GSK-3β modulates muscle stem cell proliferation (Notch) versus differentiation (Wnt). In our mouse model, the persistent increase in total TEAD1 protein (endogenous TEAD1 and transgenic HA-TEAD1) resulted in the constitutive activation of GSK-3α/β and decreased nuclear β-catenin in adult skeletal muscle [25]. We also found activation of GSK-3α/β in isolated differentiated satellite cells obtained from the TEAD1 transgenic mice [25]. However, when we examined the downstream targets of Notch and Wnt signaling, β-catenin or Hes1 respectively, in the satellite cells from TEAD1 mice we found no change in the nuclear expression of these proteins (data not shown). Together with data indicating that the progenitor SP cell population is also increased illustrates that these factors are unlikely to be the mediators of the increased cell number.
Recent research in mammalian embryonic and adult stem cells has implicated the mammalian equivalent of the Hippo signaling pathway in the regulation of progenitor cell number and organ size [41-43]. The Hippo signaling pathway, originally identified in Drosophila, consists of several serine/threonine kinases (Mst1/2, Lats1/2) that phosphorylate the transcription co-activator YAP causing it to be sequestered in the cytoplasm [43]. Yap has been shown to interact with at least five families of transcription cofactors including the TEAD family of proteins [41]. Recently, Yap function through TEAD has been shown to be an important regulator of proliferation and fate in several stem/progenitor cell populations including embryonic [44], neuronal [41], and skin [45]. Importantly, in all cases Yap/TEAD appear to function through direct transcriptional regulation (cell autonomously) and a secreted signaling factor downstream of Yap/TEAD has yet to be identified.

Many factors have been identified that regulate myoblast cell proliferation [3]. Figure 6A outlines a model of known signaling pathways that increase the number of proliferating satellite cell derived myoblasts. In this model the quiescent satellite cells (Q) give rise to proliferation competent myoblasts (P) (upward black arrow). Once a sufficient pool of myoblasts (P) is created, the myoblasts either commit to differentiation (right black arrow) or return to quiescence (downward black arrow). These decisions are actively regulated by cell signaling events in response to (usually) secreted factors from multiple potential sources; in this model stimuli that promote expansion of the proliferation-competent myoblast compartment are shown in green and stimuli that decrease the number of proliferation-competent myoblasts are shown in red. External factors such as insulin-like growth factors I and II (IGF-1, 2) increase satellite cell derived myoblast proliferation in vitro and in vivo (Figure 6A, downward green arrow); this eventually leads to increased muscle mass when the enhanced pool of myoblasts differentiate [46]. Conversely, members of the transforming growth factor-β (TGF-β)
family that inhibit satellite cell proliferation [3, 47] (Figure 6A, downward red line) lead to decreased muscle mass after differentiation. When the TGF-β family member myostatin is reduced or deleted in skeletal muscle by natural mutation or gene targeting, satellite cell proliferation is enhanced and differentiation leads to doubling or quadrupling of skeletal muscle size [48]. Interestingly, in these and other examples of this type of regulation the satellite cell population always remains proportional to the differentiated muscle mass. Thus, external factors are necessary to regulate myoblasts proliferation in vivo but are unlikely to disrupt the satellite cell number to muscle mass proportionality.

It is also likely that satellite cells themselves are capable of regulating their own proliferation and differentiation (Figure 6A, red and green curved arrows from P). Many well-characterized satellite cell mitogens are expressed by activated satellite cells (FGF2[49] HGF[50]), suggesting the potential for autocrine or paracrine modulation of satellite cell proliferation. In culture primary satellite cells will proliferate until their numbers increase to a point were they come into increased contact with one another, then many begin to differentiate[51]; however the switch from proliferation can also occur in cells spread sparsely on the plate when mitogens such as FGF2 or serum are removed [52]. Because the tendency for satellite cell-derived myoblasts to proliferate or differentiate is largely dependent on extrinsic factors (i.e., autocrine expression of growth factors will not prevent differentiation in the absence of serum), it seems unlikely that regulation of proliferating cell population size requires only signals derived from that cell population.

Through genetic labeling experiments we also know that the proliferating myoblasts are capable of returning to quiescence [53]. However, because satellite cells have only been reported returning to quiescence in vivo and a method for the in vitro study of quiescence has not yet been developed, a mechanism for this type of regulation has not been identified (Figure 6A, curved red arrow denoted by “?”). However, several
in vivo examples exist where increasing the number of proliferation-competent myoblasts during growth or after injury cells also increases the size of the quiescent satellite cell pool at later times [54, 55]. For example, MyoD−/− mice develop normally, but have defects in muscle regeneration; the satellite cells are not able to commit to differentiation resulting in an increase in the number of proliferating progenitor cells present [54]. This appears to lead to an increase in the number of quiescent satellite cells [54]. A similar situation is found in the syndecan-3−/− mice. These mice have satellite cells that are differentiation-defective, leading to an increase in the number of proliferation-competent myoblasts [55, 56]. As regeneration completes, fewer cells make the transition to differentiation and the comparatively large number of proliferating myoblasts leads to an increased number of cells when they return to quiescence [55].

We propose a model where quiescent satellite cell number is regulated in proportion to the differentiated skeletal muscle, via modulation of the size of the proliferation-competent myoblast pool either during growth or after injury (the only times such a receptive population exists) (Figure 6B). In this model the differentiated muscle secretes a titratable signal, which is received by the proliferating myoblasts. This signal either induces proliferation or inhibits differentiation, both of which lead to an increase in the proliferation-competent myoblast compartment. This signal would be expected to increase with additional muscle mass, thereby increasing the proliferation of the responsive myoblasts cells (Figure 6B, green arrow). Alternatively, if signaling from the differentiated muscles were reduced, such as in injury, the inhibition of differentiation would be lifted and more differentiated muscle would be created (Figure 6B, green line). This model is supported by our TEAD1 overexpression data; TEAD1 is only expressed in the differentiated muscle making it unlikely that the myoblasts themselves are regulating their proportionality. We also show using conditioned media experiments that a secreted molecule is released from the TEAD1-overexpressing myocytes, and that this
molecule acts on proliferation competent myoblasts of either genotype to maintain their proliferation even if serum is removed. Based on our model this myocyte-specific factor could be expressed and acting at any stage during development as well as during regeneration. Our next goal is to identify the soluble factor and test candidate molecules to establish the molecular pathway between TEAD1 transcription activity and proportionate regulation of muscle stem cell number.
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Adult skeletal muscle exhibits remarkable plasticity in its ability to adapt and change to physiological demands. These adaptations result in morphological changes to muscle fibers as well as their contractile properties. In muscle growth, repair, and hypertrophy, when additional myonuclei are required to serve the increased volume of cytoplasm in each myofiber, they are recruited from muscle satellite cells, the resident stem cell of skeletal muscle. Since their original description by Alexander Mauro in 1961 [1] a growing understanding of the 'life cycle' of a satellite cell has emerged. The currently accepted paradigm is that satellite cells are first activated by hepatocyte growth factor (HGF)[2] following its release due to stretch and/or injury. The activated satellite cells then proliferate to produce a population of replacement myoblasts; it is believed that during this process some progenitor cells are reserved to repopulate the satellite cell pool. At some point, the myoblasts will differentiate into myocytes, permanently exiting the cell cycle and either fusing to each other or to existing fibers, to produce fully functional multinucleated myofibers. A delicate network of regulation of signal transduction pathways, transcription factors as well as adhesion and extracellular matrix factors have been shown to play important roles during muscle stem cell homeostasis and regeneration. Although remarkable progress has been made in recent years toward understanding the overall process of muscle regeneration many factors and molecular mechanisms are still being identified. In this dissertation I illustrate my unique contributions to this field of satellite cell biology, which are highlighted here beginning with the role of signaling molecule syndecan-4.

Syndecan-4 is a transmembrane glycoprotein necessary for many aspects of satellite cell mediated regeneration (activation, proliferation, and differentiation) both in
vitro and in vivo [3, 4]. Due to the large number of factors known to interact with syndecan-4, it has been difficult to identify a molecular basis for its pleiotropic phenotype. The extracellular domain of syndecan-4 along with the heparan sulfate glycosaminoglycan side chains contains binding sites for soluble growth factors, integrins, small chemokines, transmembrane- and membrane-bound receptors, and extracellular matrix proteins [10]. The small intracellular domain, consisting of two domains conserved among syndecan family members as well as a unique V domain, has been called “one of the most overworked small proteins among the signaling giants”[10], due to the disproportionately large number of proteins it interacts with and cellular processes it affects. The variable domain contains a binding site for the signaling intermediate phosphatidylinositol 4,5-biphosphate (PIP$_2$); PIP$_2$ binding induces a conformational change in syndecan-4 that permits its multimerization and transit to membrane raft regions of the cell where it interacts with and activates PKC$\alpha$ [11, 12]. The second conserved region, C2, contains a PDZ-protein binding site for interaction with PDZ domain containing cytoskeletal scaffolding proteins including syntenin [13], CASK [14], and synectin [15].

Based on the number of functions previously observed for syndecan-4 in satellite cells, we asked if these activities were separable on the basis of protein-protein interactions occurring between syndecan-4 cytoplasmic domain motifs and cytosolic cell mediators of cell proliferation and cell differentiation. By reconstituting syndecan-4 expression, we demonstrated that the motif required for association with PIP$_2$ appears to be required for efficient proliferation but is dispensable for differentiation, whereas the PDZ domain-binding motif is required for differentiation and not proliferation. Overall these results suggests that the formation of distinct multiprotein complexes involving syndecan-4 may be critical for pro-growth and pro-differentiation signaling in primary
satellite cells. Future research in the Cornelison lab will help identify the specific factors that interact with the separable domains of syndecan-4 in primary satellite cells.

Formation of higher-order signaling complexes, such as those that have been proposed to assemble in membrane “rafts”, may also be an aspect of syndecan-4 specific signaling in satellite cells. These regions of the plasma membrane play important roles in intracellular protein transport, membrane fusion, and transcytosis, serving as platforms for assembly of signaling complexes. Syndecan-4 localizes to detergent resistant membrane fractions (largely associated with membrane rafts) in primary satellite cells and clusters in response to growth factor stimulation; growth factor signaling (possibly through syndecan-4) is also decreased when membrane rafts are disrupted. We describe here a decrease in specific proteins associated with membrane rafts in the absence of syndecan-4 based on identification by liquid chromatography tandem mass spectrometry (LC MS/MS). A large group of transmembrane and juxtamembrane components of adhesion-mediated signaling pathways were identified including neural cell adhesion molecule (NCAM). NCAM, a multifunctional cell adhesion protein, had been previously associated with muscle regeneration and myoblast fusion [16]. We revealed that not only is NCAM localized to discrete areas of the plasma membrane but it is also an early marker of commitment to terminal differentiation in primary mouse satellite cells. This finding allows for sorting of live satellite cells based on their membrane expression of NCAM, providing a new tool for use in determining cell commitment stages of myoblasts in cultured systems.

Previous work in the lab of our collaborator, Dr. Richard Tsika, has focused on the role of the transcription factor TEAD1 on slow muscle gene expression, cardiac development, and cardiac gene expression [17, 18]. Dr. Tsika has shown that overexpression of TEAD1 is skeletal muscle results in shift towards a slower myosin
heavy chain (MyHC) profile with a loss of fast type IIx/d MyHC in both fast and slow twitch muscles. The satellite cell number to muscle mass ratio is highly conserved varying only between species, age, sex and muscle fiber type. Since the TEAD1 overexpressing mouse contains alterations in fiber composition we wanted to examine the effects of its overexpression in the satellite cell population.

We found that the overexpression of TEAD1 causes up to a 6.8 fold increase in the number of quiescent satellite cells present on myofibers. This increase in satellite cell number was not due to TEAD1 dependent muscle fiber hypertrophy or fiber type switching. Proliferating satellite cells from TEAD1 overexpressing mice are phenotypically identical to wild type cells under *in vitro* conditions, as TEAD1 expression is only upregulated upon the initiation of cell differentiation. The result of TEAD1 overexpression during differentiation is a pronounced increase in the number of proliferating satellite cells that persists in the differentiating cultures. Through conditioned media experiments we found that media from TEAD1 overexpressing differentiated cells contained a heat labile soluble factor capable of increasing satellite cell proliferation. This is both the first demonstration of a transcription factor-induced increase in satellite cell number and the first such effect caused by altered gene expression in cells other than the satellite cells themselves. We propose a model in which either the differentiated myocytes or whole muscle is capable of releasing a soluble factor which signals to the undifferentiated population of satellite cells maintaining a proliferative state. This model could apply to satellite cells and their precursors during development as well as through periods of growth and regeneration. This is the first proposed model for the regulation of satellite cell number in proportion to skeletal muscle mass. The potential benefits of increased numbers of satellite cells and satellite cell precursors during muscle regeneration and repair has yet to be determined.
The progression of satellite cells from quiescent cells to functional components of mature muscles is critical for muscle regeneration. During this multistage process cells must pass through a proliferative stage and either return to quiescence (to repopulate the satellite cell niche) or differentiate. For in vivo satellite cells the transition from a proliferating myoblast to a differentiating myocytes is permanent. Therefore, it is necessary for this transition to be tightly regulated to ensure both efficient regeneration and optimal use of physiological resources. Each cell must interpret extracellular cues from many sources including muscle fibers, satellite cells, immune cells, vasculature and neurons. The cells must then respond to these extracellular cues based on the complement of proteins present in the intracellular environment.

We now know that a heterogeneous mixture of proliferating and differentiating satellite cells can be divided into two separate stages based on the extracellular expression of NCAM, that a single protein, syndecan-4, can mediate different responses to extracellular signaling based on interactions occurring at the intracellular domains and that a secreted molecule can signal to and regulate the transition from proliferation to differentiation. Future research will focus how global changes in cell state are regulated and maintained.
References


VITA

I was born in Kirkwood, Missouri in St. Louis County. As a child my interest in biology was illustrated at young age; I would break open plants and seedpods to see what was inside. I remember always wanting to know, what they were made of? What where their smallest parts? As I continued through secondary education Mrs. Franks biology class furthered my interest in the life sciences. In high school I was lucky enough to have several excellent chemistry teachers who gave me a strong base of knowledge in the subject as well as the opportunity to take college level courses in high school.

Due to my background in math and chemistry I entered college with the planned major of chemical engineering. By my sophomore year I decided that my true interests lie in biology. I continued to pursue biology and graduated in 2004 with a Bachelor of Science from the University of Missouri. After one year as a research technician in 2005, I entered into graduate school at the University of Missouri. In 2011, I will earn a Doctor of Philosophy at the University of Missouri in Biological Sciences in the lab of Dr. D Cornelison.