REGULATION OF STORE-OPERATED CALCIUM CHANNEL BY MITSUGUMIN29 IN SKELETAL MUSCLE AGING

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ABSTRACT OF THE DISSERTATION

REGULATION OF STORE-OPERATED CALCIUM CHANNEL BY MITSUGUMIN29 IN SKELETAL MUSCLE AGING

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The study of store-operated Ca^{2+} channel entry (SOCE) and its role in muscle contractility in young and aged skeletal muscle necessitates a thorough knowledge of the Ca^{2+} signaling from the sarcoplasmic reticulum (SR) that activates SOCE. Yet, all of the molecular components involved have yet to be fully elucidated, as neither T-tubule voltage sensors, nor SR ryanodine receptor Ca^{2+} channels, together or independently, are necessary or sufficient for the establishment of a close association between the T-tubule and SR membranes. Therefore, other protein components must be involved for the formation of triad junctional complexes.

Mitsugumin29 (MG29), a protein localized to the triad junction, may function as a structural component involved in the coupling between the SR and T-tubule, as abnormalities in both T-tubule and SR membranes have been reported in *mg29(-/-)* mice. In addition, muscles from these mice share many morphological and functional characteristics with muscle from aged mice,

including increased susceptibility to fatigue, defective SR Ca^{2+} release and defective SOC function. Either of these may be responsible for the altered Ca^{2+} signaling in skeletal muscle during exercise and aging.

Our data suggests that SOCE is not merely important for skeletal muscle function in aging; but, it is also required for maintenance of Ca^{2+} signaling during repetitive stimulation under intensive muscle activity (i.e., fatigue) in the healthy state. In addition, we propose that SOCE diminishes with age, contributing to the age-associated muscle weakness. Finally, we find that while SOCE is a functional marker of muscle performance in aging, MG29 is a molecular marker, as SOCE is compromised in aged wild type mice through the decreased expression of MG29. Therefore, *mg29(-/-)* mice can serve as an appropriate model for the study of skeletal muscle aging. Through this project, we have begun to understand the physiological function of SOCE and MG29 and their contribution to muscle contractility in both young and aged mice.

“No one can make you feel inferior without your consent.”

Eleanor Roosevelt

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Chapter 1. Introduction

Mammalian skeletal muscles are composed of many myofibers, each of which is composed of consecutively smaller structures. The fibers have an abundance of connective tissue, acting as an extracellular matrix (ECM) protective barrier (Figure 1). The epimysium is the tough superficial fascia that covers the entire surface of the muscle. The epimysium is a collection of collagen fibers tightly woven into bundles, and it is connected to the underlying layer of connective tissue, known as the perimysium. This deeper layer of fascia divides the muscle into bundles, or fascicles, of fibers. It is in this region that the blood vessels and intramuscular nerve branches are found. The deepest ECM layer is the endomysium, which envelops each individual muscle fiber in a dense network of collagen fibers. The endomysium also serves as the outer layer of the sarcolemma, or muscle cell membrane (Guyton and Hall, 2000; McMahon et al, 2001; MacIntosh et al, 2006).

Each muscle fiber actually contains hundreds to thousands of myofibrils, which are composed of thick and thin filaments. The thick filaments, or myosin filaments, are made of six polypeptide chains. One pair of heavy chains forms the tail of the myosin structure. The remaining two pairs – light chains – form the two globular myosin heads. The myosin heads have both actin-binding sites necessary for cross-bridge formation and an ATPase moiety (Costanzo, 2002).

The thin filaments are made up of a complex of three proteins: actin, tropomyosin, and troponin. Actin contains the myosin-binding sites, sites which

are covered by tropomyosin when the muscle is at rest. The complex is made up of three globular proteins: troponin T, which attaches the troponin complex to tropomyosin; troponin I, which, along with tropomyosin, inhibits interaction between actin and myosin when the muscle is at rest; and troponin C. Troponin C is bound by Ca^{2+} when the intracellular Ca^{2+} concentration ($[Ca^{2+}]$) increases. This, in turn, produces a conformational change in the complex that moves tropomyosin so that actin and myosin can interact and from cross-bridges so the muscle can contract (Guyton and Hall, 2000; Costanzo, 2002).

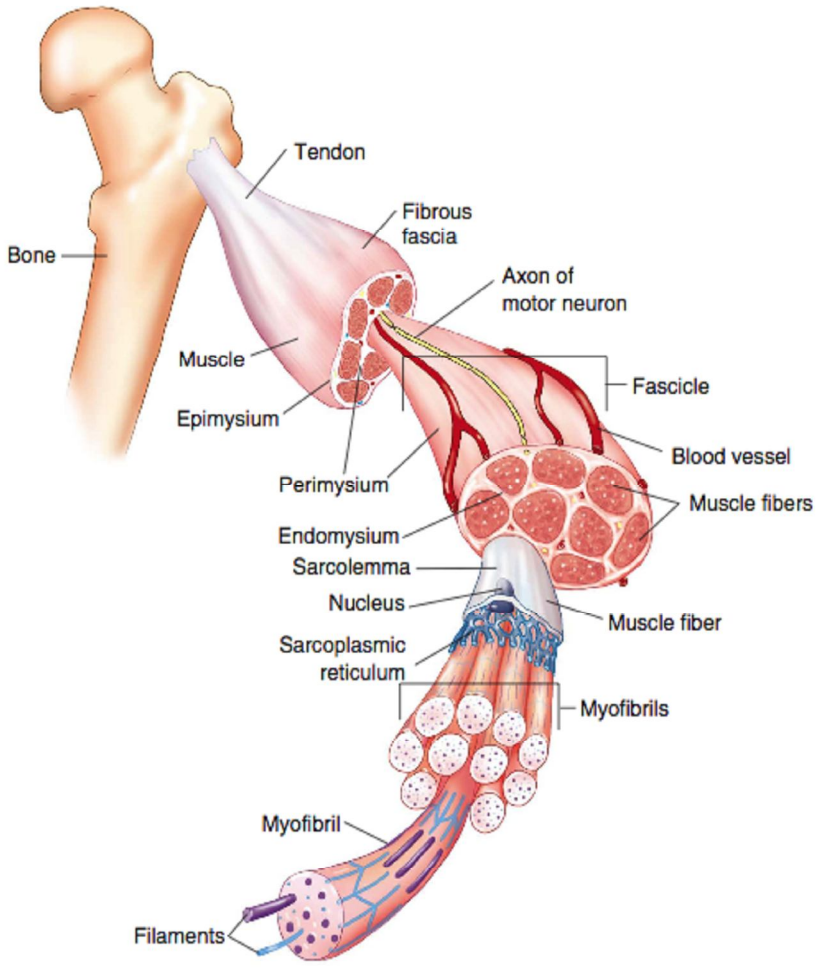


Figure 1. Layers of connective tissue in skeletal muscle (Mosby Inc., 2006).

The basic contractile unit of the muscle is the sarcomere (Figure 2). At its center is the A band, where the thick and thin filaments can overlap and potentially form cross-bridges. The A band is bisected by the M line, which contains electron-dense proteins that link the thick filaments together. The H zones, also at the center of the A band, include the M line and the bare zones, which are the areas with no thin filaments and is therefore where no cross-bridges can form. At either end of the A band is the I band, which contains the thin filaments and Z lines, which delineate the ends of the sarcomere (Guyton and Hall, 2000; Costanzo, 2002).

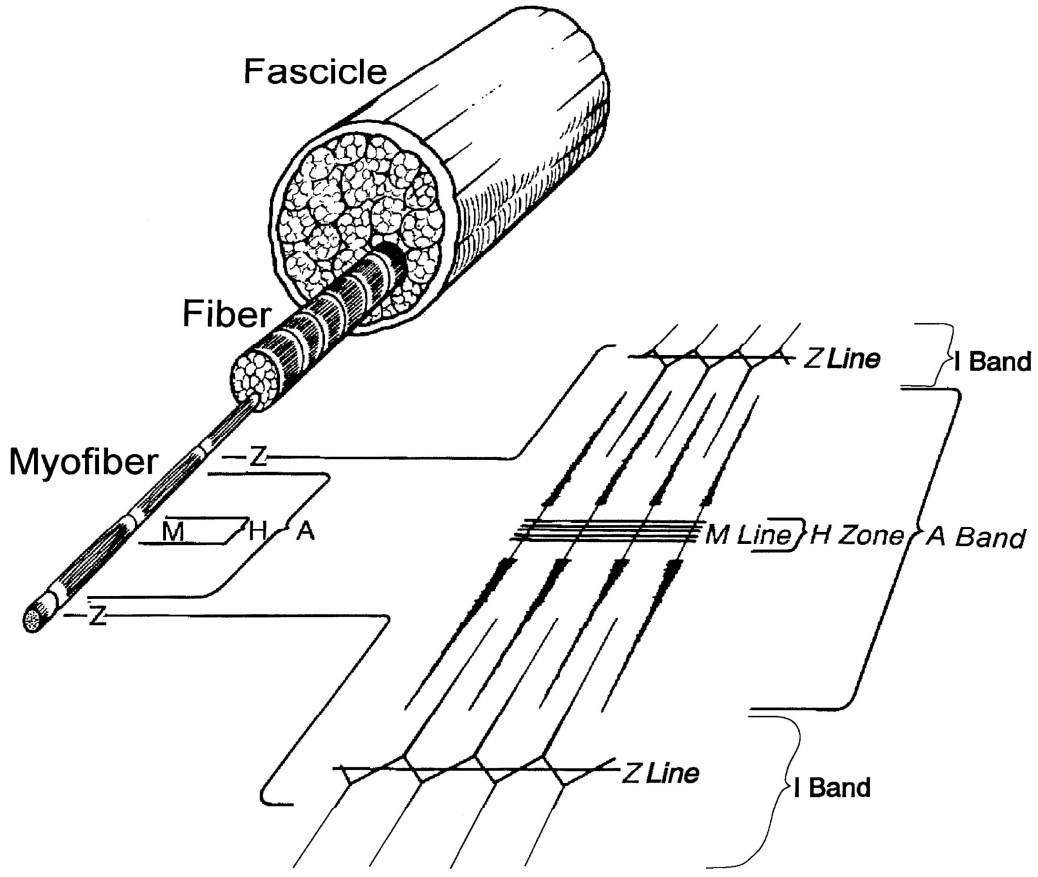


Figure 2. Structure of skeletal muscle fiber (Garrett and Best, 2000).

Chapter 2. Excitation-contraction coupling

Excitation-contraction (E-C) coupling in skeletal muscle is the process by which an action potential ultimately triggers the contraction of the sarcomere myofilaments. Essential to this process is the release of Ca^{2+} from the sarcoplamic reticulum (SR) intercellular Ca^{2+} reservoir into the cytoplasm. The transverse tubular (T-tubule) invagination of the sarcolemma touches the terminal cisternae of the SR (Figure 3) – forming the "triad junction" – which enables direct physical coupling between T-tubule voltage sensors, or dihydropyridine receptors (DHPRs), and SR Ca^{2+} release channels, the ryanodine receptors (RyRs) (Figure 4). This physical interaction allows direct relay of the depolarization signal, which induces voltage-induced Ca^{2+} release (VICR) from the SR (Shimuta et al, 1998; Nagaraj et al, 2000; Kurebayahi and Ogawa, 2001; Islam et al, 2002; Pan et al, 2002; Launikonis et al, 2003; Collett and Ma, 2004; Hirata et al, 2006).

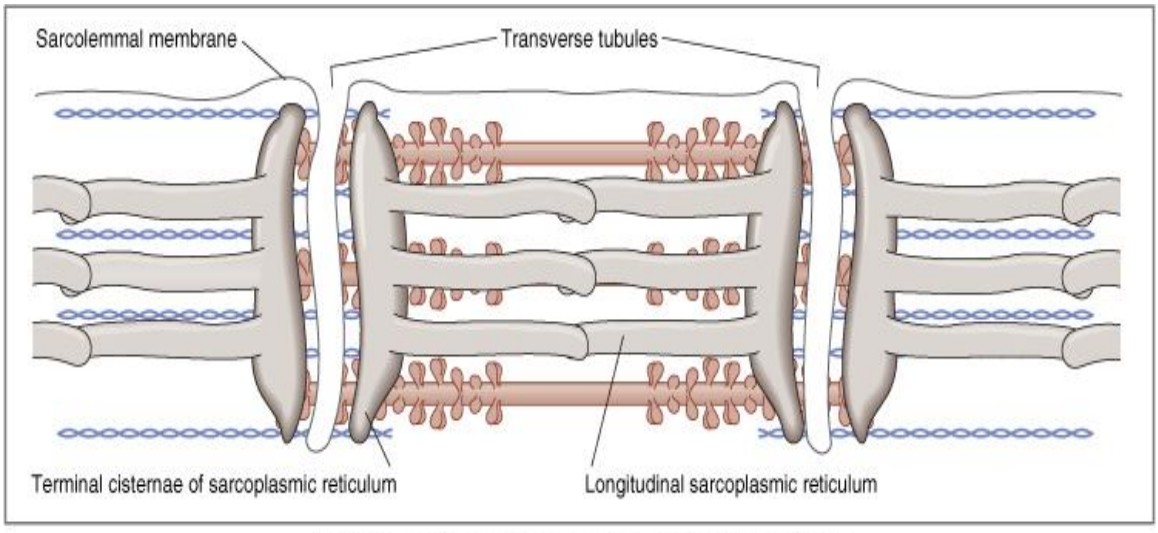


Figure 3. Ultrastructure of skeletal muscle (Costanzo, 2002).

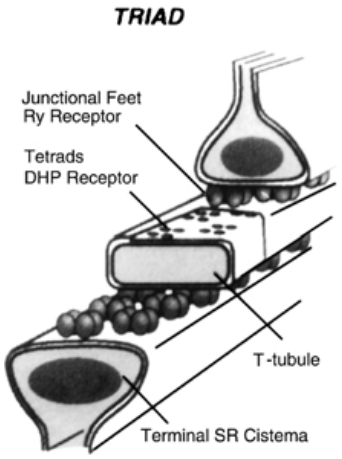


Figure 4. Structure of the triad junction (Caputo, 2001). Action potential travels down T-tubule invaginations to DHPRs coupled to RyR SR Ca^{2+} channels, triggering Ca^{2+} release into the sarcoplasm.

Similar junctional membrane complexes exist in cardiac myocytes as the "dyad junction" (Rayns et al, 1968), in smooth muscle and immature striated muscle as "peripheral coupling" (Flucher et al, 1992), and in neurons as "subsurface cisternae" (Verkhratsky et al, 1996). Many investigators involved in current E-C coupling research examine the molecular and structural components that cause the close interaction of the T-tubule and SR, and study the roles of accessory proteins that may interact with DHPRs or RyRs and thereby modulate the VICR process.

The RyR Ca^{2+} release channel mediates Ca^{2+} mobilization from the SR and is crucial to the process of E-C coupling. Three RyR isoforms have been identified. RyR1 is the primary isoform in skeletal muscle; RyR2 is the major isoform in cardiac muscle; and RyR3 is more ubiquitously expressed. RyR1

interacts with several proteins, including the DHPR, triadin and FK506-binding protein (FKBP12). Dissociation of the interaction between FKBP12 and RyR1 by such ligands as FK506 or rapamycin, can result in increased RyR1 sensitivity, causing RyR1 activation by lower concentrations of either caffeine or Ca^{2+}. Consequently, it has been suggested that FKBP12 plays a key role in the function of the RyR1 (Missiaen et al., 2000; Murayama and Ogawa, 2002; Shin et al, 2002).

The other major protein that facilitates E-C coupling is the DHPR located in the T-tubules. DHPR is a multi-subunit protein with the principal 175 kDa α_1-subunit forming the L-type Ca^{2+}-channel pore and functioning as the voltage-sensing unit. In addition, there are the 143/27 kDa α_2/δ-subunit complex, the 54 kDa β-subunit, and the 30 kDa γ-subunit (Ryan et al, 2000). Unlike cardiac muscle, it is believed that DHPR does not support Ca^{2+} influx in skeletal muscle under normal physiological conditions. Therefore, the same Ca^{2+} channels that activate RyR1 are not thought to concomitantly serve as Ca^{2+} conductors into the skeletal muscle cell (O'Brian et al, 2002; Tanabe et al, 1990; Tanabe et al, 1991). Skeletal muscle contractions are, therefore, primarily triggered by VICR from the SR, and amplified by Ca^{2+}-induced Ca^{2+} release (CICR), as neighboring RyR1s not physically interacting with DHPRs are activated by Ca^{2+} released through RyR1s that are directly coupled to DHPRs (Islam et al, 2002; Pan et al, 2002; Weigl et al, 2003).

In addition the Ca^{2+} released from the SR via RyR1, another means of intracellular Ca^{2+} release is mediated by the inositol 1,4,5-trisphosphate (IP_3) cascade (Berridge, 1993), in which receptor activation induces phosphatidylinositol 4,5-bisphosphate hydrolysis, generating IP_3 and diacylglycerol – the former functioning as an intracellular Ca^{2+} release channel and the latter as an activator of protein kinase C (Powell et al, 2001). Within skeletal muscle are many of the molecular components of this cascade, and some of the enzymes involved have been found to be localized to the triad junction (Carrasco et al, 1988). In addition, Suarez-Isla reported the existence of IP_3-sensitive Ca^{2+} channels in the SR (Suarez-Isla et al, 1988).

Chapter 3. Store-operated Ca^{2+} Entry

As skeletal muscle E-C coupling does not involve significant entry of extracellular Ca^{2+} into muscle cells, other mechanisms must be present in skeletal muscle to replenish the internal Ca^{2+} stores after they are depleted in the course of normal muscle physiological function. Instead of voltage-related entry of Ca^{2+}, Dr. James Putney advocated another mechanism for the Ca^{2+} to enter the cytosol. He proposed that the emptying of internal Ca^{2+} stores is linked to a "store-operated" extracellular Ca^{2+} influx through cation channels in the cell membranes of nonexcitable cells. This Ca^{2+} influx would replenish the intracellular stores and, during prolonged stimulation, prevents the intracellular $[Ca^{2+}]$ from dropping to basal levels (Putney, 1986). It was consequently suggested that the amount of Ca^{2+} in the stores controlled the degree of Ca^{2+} influx in this process that would come to be known as store-operated Ca^{2+} entry (SOCE) or capacitative Ca^{2+} entry (CCE).

The key feature of SOCE is that it is activated, exclusively, by the fall in the SR store Ca^{2+} content and not by the resulting increase in cytoplasmic $[Ca^{2+}]$. Ca^{2+} influx does not occur when stores are full. As the stores lose Ca^{2+}, a signal is sent to the cell membrane that results in the opening of the store-operated channels (SOCs) and the subsequent entry extracellular Ca^{2+} (Missiaen et al, 2000; Weigl L et al, 2003; Parekh and Putney, 2005; Singaravelu K, et al, 2006; Smyth et al, 2006; Parekh, 2007).

A. I_{CRAC}

Evidence supporting the SOCE hypothesis was provided by electrophysiological studies in mast cells in a work that established that Ca^{2+} store depletion activated a Ca^{2+} current called Ca^{2+} release-activated Ca^{2+} current or I_{CRAC}. I_{CRAC}, a non-voltage activated, inwardly rectifying current with low conductance, is notable for its remarkable Ca^{2+}-selectivity and is found in several cell types, including hematopoietic cells (Parekh and Putney, 2005; Soboloff et al, 2006; Smyth et al, 2006). Though I_{CRAC} is not the only store-operated current the has been experimentally resolved, it was the first store-operated Ca^{2+} current to be described and is a popular model for studying SOCE. Consequently, studies of I_{CRAC} have heavily influenced our understanding of all store-operated Ca^{2+} currents (Parekh and Putney, 2005; Smyth et al, 2006). I_{CRAC} is thought to be mediated, in part, by ion channels of the transient receptor potential-canonical (TRPC) family, which have been reported to couple to both the IP_3 receptor and the RyR intracellular Ca^{2+} channels (Weigl et al, 2003). Recent studies have provided significant insight into the molecular machinery involved in SOCE, which will be discussed at length later (Section B).

A variety of factors that couple Ca^{2+} store depletion to Ca^{2+} entry have been reported in non-excitable cells, as well as in excitable cells (Weigl et al, 2003). Since any channel that can be shown to exhibit Ca^{2+} store-dependent activity can be referred to as a SOC, SOCE may occur via a family of Ca^{2+}-permeable channels with different properties in different cell types.

Experimentally, entry through SOCs can be distinguished from other modes of Ca^{2+} entry by employing protocols that achieve store Ca^{2+} depletion independently of activation of other signaling pathways (Parekh and Putney, 2005; Smyth et al, 2006).

There are several methods for depleting intracellular Ca^{2+} stores (Table 1). Store depletion can be triggered by an increase in the IP_3 level or by another Ca^{2+}-releasing signal preceding Ca^{2+} release from the stores. Application of the Ca^{2+} ionophore, ionomycin, permeabilizes internal cellular membranes, releasing Ca^{2+}. Dialyzing the cytoplasm with high concentrations Ca^{2+} buffers, such as ethylene glycol tetraacetic acid (EGTA) or 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) (BAPTA), chelates any Ca^{2+} that leaks from the stores to prevent store refilling, thus leading to passive depletion of the store. This can also be accomplished by exposing the cell to sarcoplasmic/ endoplasmic reticulum Ca^{2+}-ATPase (SERCA) to inhibitors, such as thapsigargin (TG) or cyclopiazonic acid (CPA) to prevent P-type ATPases from refilling the stores. Loading a membrane-permeable metal Ca^{2+} chelator like *N*,*N*,*N*',*N*'-tetrakis (2-pyridylmethyl) ethylene diamine (TPEN) directly into the stores lowers the level of free Ca^{2+} in the store without changing total store Ca^{2+} concentration. All these pharmacological manipulations lead to a significant reduction in intracellular Ca^{2+} storage, leading to activation of SOCE (Parekh and Putney, 2005).

Table 1. Biophysical properties of SOCs (Parekh and Putney, 2005)

Current	Conductance	Selectivity	Permeability Ratio	Activation	Cell Type
I_{CRAC}	0.02 pS; 110 Ca^{2+}	Ba^{2+}>$Ca^{2+}Sr^{2+}$	Ca^{2+}:Na^{+} 1,000:1	RA	Mast cell
				IP_3	RBL-1/-2H3
				TG	Jurkat T cells
				EGTA/ BAPTA	Hepatocytes
				Ionomycin	Dendritic cells
				TPEN	Megakaryocytes MDCK cells
I_{SOC}	11 pS; 10 Ca^{2+}	Ca^{2+}>Na^{+}	Ca^{2+}:Na^{+} >10:1	RA	Endothelia
	1 pS; 100 Ca^{2+}	Ca^{2+} = Ba^{2+}>>K^{+}	Ca^{2+}:K^{+} 1,000:1	RA	A431 epidermal cells
				IP_3	
				TG	
	2 pS; 160 Ca^{2+}	Ba^{2+}>Ca^{2+}	?	BAPTA	A431 epidermal cells
				TG	
	2.7 pS; 90 Ca^{2+}	Ca^{2+} = Ba^{2+} = Na^{+}	Ca^{2+}:Na^{+}:K^{+} 1:1:1	TG	Aortic myocytes
				BAPTA-AM	
	2.3 pS; 1.5 Ca^{2+}	Ca^{2+}>Na^{+}	Ca^{2+}:Na^{+} 50:1	CPA	Portal vein myocytes
				BAPTA-AM	
				Caffeine	
	5.4 pS; 20 Ca^{2+}	?	?	CPA	Pulmonary artery myocytes
	0.7 pS; 90 Ca^{2+}	?	?	Sp	Mesangial cells
	43 pS; 1.3 Ca^{2+}	Na^{+}, K^{+}>Ca^{2+}	Ca^{2+}:Na^{+} 1:13	RA	Pancreatic acinar cells

Since SOC activation relies exclusively on store reduction, the Ca^{2+} store must be able to communicate its reduction to SOCs within the plasma membrane in some way. Among the many proposed mechanisms are two general hypotheses for the activation mechanism of various SOCE pathways. The first focuses on the action of a diffusible messenger released from the SR to activate SOCs upon SR Ca^{2+} depletion (Randriamampita and Tsien, 1993; Rzigalinski et al, 1999). The second involves a coupling between ER or SR membrane proteins with SOCs in the sarcolemma such that certain components of the molecular machinery for SOCE interact to relay the store depletion signal from the SR to the sarcolemma (Irvine 1990; Weigl et al, 2003; Singaravelu et al, 2006; Smyth et al, 2006).

SOCE provides the crucial link between extracellular Ca^{2+} and intracellular Ca^{2+} storage, serving essential roles in processes such as proliferation, exocytosis, apoptosis, muscle development, muscle fatigue, aging, immunodeficiency, and motility (Pan et al, 2002; Collet and Ma, 2004; Singaravelu et al, 2006). Extracellular Ca^{2+} entry via SOCs is coupled to a retrograde opening of the channels that is a factor of the SR calcium content and RyR conformational changes. Thus, SOCE not only provides the ideal mechanism for refilling of intracellular Ca^{2+} stores; it also provides skeletal muscles with the Ca^{2+} required during intense exercise and fatigue when the intracellular stores of Ca^{2+} become depleted. In fact, dysfunctional SOCs may contribute to muscle contractility, fatigue, exercise intolerance, and aging (Pan et al, 2002; Zhao et al,

2005; Brotto et al, 2007; Zhao et al, 2008; Stiber et al, 2008). While cellular mechanistic studies of the regulatory function of SOCE have been hindered, recent breakthroughs have began to elucidate the molecular machinery for SOCE in a number of tissues.

B. Molecular Components of SOCs

Stromal interaction molecule (STIM) in Drosophila has been identified as an essential component of SOCE (Vig et al, 2006). Roos and colleagues performed a large-scale RNAi screen of 170 genes and reported that only STIM was deemed necessary for SOCE and activation of I_{CRAC} (Roos et al, 2005). Liou and colleagues, in a parallel study, targeted 2304 proteins, and identified two mammalian STIM homologues, STIM1 and STIM2 (Liou et al, 2005; Smyth et al, 2006). Treatment of cells with siRNA targeting STIM1 abolished SOCE, but had no apparent effect on either the intracellular SR Ca^{2+} content or the resting cytosolic Ca^{2+} concentration. Ablation of STIM2 only reduced SOCE. Thus, STIM1 appears to be a crucial link between the SR and the SOCs in the sarcolemma (Smyth et al, 2006; Stiber et al, 2008).

STIM1 is a phosphoprotein containing a single transmembrane-spanning domain. It also has several conserved domains: a sterile-alpha motif, a coiled coil region, and an EF-hand domain. As it seems unlikely that a channel protein would contain only one transmembrane domain, STIM1 is thought to be the SR sensing component of the I_{CRAC} pathway rather than the CRAC channel itself. Its

C-terminal Ca^{2+}-binding motif may be crucial to this function (Smyth et al, 2006), as Zhao and colleagues (Zhao et al, 2005) were the first to demonstrate that STIM1-dependent SOCE in muscle provides a mechanism that sustains cytosolic Ca^{2+} concentration increases, both to preserve contractility during repeated contractions and to activate Ca^{2+}-dependent signaling events that trigger muscle development and neurostimulation-associated remodeling. Stiber and colleagues later showed that neonatal mice lacking functional STIM1-dependent SOC died from a perinatal myopathy and that STIM1 haploinsufficiency in adult mice increased their susceptibility to fatigue, likely resulting from ineffective sensing of store depletion with repetitive stimulation (Stiber et al, 2008).

A second protein component of I_{CRAC} was identified by genetic linkage in a human population with severe combined immunodeficiency, characterized by the lack of functional CRAC channels (Jousset et al, 2007). Named “Orai” after Greek mythological characters, three family members – Orai1, Orai2 and Orai3 – are expressed in various mammalian tissues and are predicted to be membrane-spanning proteins with four transmembrane domains (Smyth et al, 2006). Initially, only Orai1, also known as CRACM1, was shown to be required for SOCE and I_{CRAC} (Soboloff et al, 2006; Stiber et al, 2008).

Unlike STIM1, Orai1 apparently resides only at the plasma membrane. With the exception of the transmembrane segments, it has no other readily identifiable modular domains (Smyth et al, 2006). As observed for STIM1, knockdown of Orai1 drastically reduced both SOCE and I_{CRAC}. Co-expression of

STIM1 and Orai1, but not expression of either protein alone, reconstituted SOCE and generated massive CRAC currents (Soboloff et al, 2006). Mutagenesis studies showed Orai1 to be the CRAC channel, as Orai1 point mutations transformed it from a Ca^{2+}-selective, inwardly rectifying channel into an outwardly rectifying channel permeable to monovalent cations (Jousset et al, 2007).

Both Orai2 and Orai3 also form Ca^{2+}-selective channels with the characteristics of I_{CRAC} when co-expressed with STIM1. However, co-expression with STIM2 resulted in both store-dependent and store-independent gating (Stiber et al, 2008). Mercer and colleagues (Mercer et al, 2006) reported that Orai2 properties are comparable to those of Orai1, in that Orai2 overexpression inhibited SOCE in HEK293 cells, whereas its coexpression with STIM1 resulted in amplified SOCE and I_{CRAC}, though the magnitude of the currents with Orai2 were somewhat less than for Orai1. Orai3 overexpression restored Ca^{2+} entry in cells where endogenous Orai1 expression had been ablated, demonstrating that Orai3 is able to function in the Ca^{2+} permeation pathway. Thus, in transient transfection experiments, the rank order of efficacy of Orai family members as SOCs appears to be Orai1>Orai2>Orai3 (Smyth et al, 2006).

During store depletion, STIM1 redistributes into distinct structures, or punctae, which aggregate underneath the sarcolemma within 10-15 nm of the SOCs (Vig et al, 2006; Stiber et al, 2008). Punctae formation precedes the activation of CRAC channels by several seconds, consistent with a causal role of

STIM1 in activation of SOCs. The STIM-Orai interaction restricts Ca^{2+} influx to specific regions of the sarcolemma located 10 nm away from the SR (Figure 5). This cellular structure resembles the synaptic cleft and creates a diffusion barrier that prevents the escape of Ca^{2+} ions from the cleft (Jousset et al, 2007).

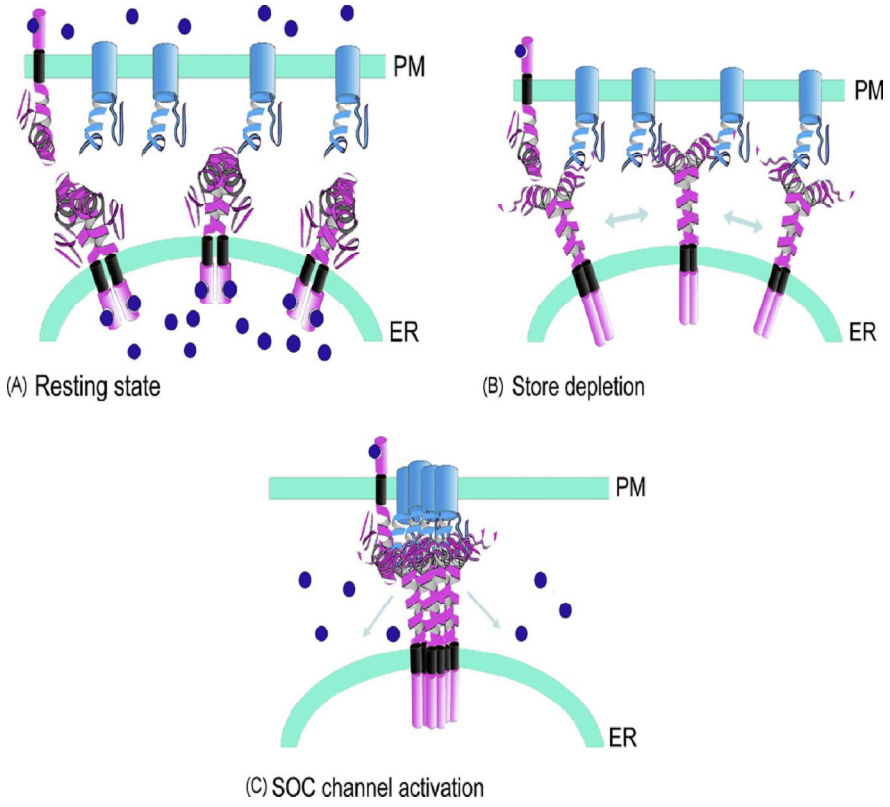


Figure 5. Proposed model for SOC formation via the STIM1/Orai1 interaction. (A) At rest, binding of Ca^{2+} (dark blue circles) in the store lumen maintains the extracellular region of STIM1 (pink). (B) Store depletion results in aggregation of STIM1 dimers within the SR to specific sites beneath the sarcolemma. (C) Aggregation of STIM1 in the store causes clusters of Orai1 (light blue) and cytoplasmic STIM1 to form punctae in the sarcolemma that couple the store and sarcolemma, establishing functional SOCs (Dziadek and Johnstone, 2007).

Chapter 4. Hypotheses and Specific Aims

Skeletal muscle Ca^{2+} entry via SOCs provides additional Ca^{2+} for refilling the SR. Additionally, SOCs may play roles in such condition as hypoxia, aging, and dystrophies. The study of SOC function and its role in muscle contractility and aging necessitates a thorough knowledge of the Ca^{2+} signaling from the SR that activates the SOCs.

Yet, all of the molecular components involved have yet to be fully elucidated, as neither DHPRs nor RyRs, together or independently, are necessary or sufficient for the establishment of a close association between the T-tubule and SR membranes. In both DHPR and RyR knockout models, triad junctions appear intact; and co-expression of DHPR and RyR in heterologous systems does not bring about apposition of the sarcolemma and SR membranes. Therefore, other protein components must be involved for the formation of triad junctional complexes.

MG29 is a protein localized to the triad junction, and abnormalities in both T-tubule and SR membranes have been reported in *mg29(-/-)* mice (Nishi et al, 1998; Takeshima et al, 1998; Nishi et al, 1999; Nishi et al, 2000; Takeshima et al, 2000). In addition, skeletal muscle from both aged and *mg29(-/-)* mice exhibit increased susceptibility to fatigue, defective SR Ca^{2+} release and defective SOCE function. Either of these may be responsible for the altered Ca^{2+} signaling in skeletal muscle during exercise and aging.

Taken together, these findings prompt three questions: 1) Does SOCE contribute to muscle contraction in the healthy condition? 2) Does SOCE in skeletal muscle change with aging? 3) What role does MG29 play in SOCE regulation? We therefore tested three hypotheses: 1) SOCE is required for maintenance of Ca^{2+} signaling during repetitive stimulation under intensive muscle activity (i.e., fatigue); 2) SOCE diminishes with age, thereby contributing to age-associated muscle weakness; and 3) while SOCE is a functional marker of muscle performance in aging, MG29 is a molecular marker; therefore, *mg29(-/-)* mice can serve as an appropriate model for the study of skeletal muscle aging. Thus, the specific aims of the project are: 1) to understand the physiological function of SOCE and its contribution to muscle contractility in both adult and aged mice and 2) to lay the groundwork for future assessment of the function of MG29 in SOCE by optimizing a method of acutely ablating the protein's expression in specific wild type mice muscles and restoring its expression in specific *mg29(-/-)* mice muscles. Together, the two proposed aims will provide a better understanding of the physiological importance of both MG29 and SOCE.

Chapter 5. Does SOCE contribute to muscle contraction in the healthy condition?

Ca^{2+} ions are important second messengers in many cellular signal transduction pathways (Clapham, 1995; Berridge, 1997; Berridge et al, 1998; Weigl et al, 2003). There are two principle sources of Ca^{2+} in the cell: channels in the sarcolemma that open to allow external Ca^{2+} to flow into the cell, and SR internal stores that release Ca^{2+} into the cytosol (Inesi, 1981; Franzini-Armstrong and Jorgensen, 1994). The junctional membrane complexes between the sarcolemma and SR present in skeletal muscle cells provide an effective mechanism for cross talk between Ca^{2+}-dependent channels and transporters on the sarcolemma and Ca^{2+} release channels on the store (Pozzan et al, 1994; Berridge, 1998).

Controversy exists over whether external Ca^{2+} is necessary for adult mammalian skeletal muscle E-C coupling (Payne, 2004a). Experiments in which muscles have been bathed in solutions from which external Ca^{2+} have been removed or to which Ca^{2+} channel blockers have been added have been reported to persist in E-C coupling and contraction (Armstrong et al, 1972; Tanabe et al, 1990; Dulhunty, 1992; Rios and Pizarro, 1991). Meledi and coworkers found that SR Ca^{2+} release was functional under similar conditions (Meledi et al, 1984).

However, changes in the concentration of extracellular Ca^{2+} have been reported to affect muscle contractility. Decreases in the concentration are associated with increased inactivation of slow contractures produced by long depolarizations (Luttgau and Spiecker, 1979; Cota and Stefani, 1981) and slowed

recovery from inactivation (Luttgau et al, 1987). In addition Barrett and colleagues reported that buffering extracellular Ca^{2+} with high concentrations of Ca^{2+} chelator EGTA prevented muscle contraction (Barrett and Barrett, 1978). It has therefore been suggested that while extracellular Ca^{2+} may not be essential to elicit muscle contraction, it does function as a modulator of muscle contraction (Dulhunty and Gage, 1988).

These reports lead to our first question: does SOCE contribute to muscle contraction in the healthy condition? Though extracellular Ca^{2+} entry is not a requisite for muscle contraction, we believe it is vital for maintaining Ca^{2+} homeostasis when muscle is repetitively stimulated. Consequently, we propose that SOCE is required for maintenance of Ca^{2+} signaling during intense muscle activity.

A. Experimental Protocols

a. Muscle Preparation (Brotto et al, 2004)

Intact extensor digitorum longus (EDL) and soleus (SOL) muscles of wild type (*WT*) male mice, 8-10 weeks of age, were surgically removed from tendon to tendon by blunt dissection and immediately placed in a dissecting dish containing a modified bicarbonate Ringer solution with the following compositions (mM):

	NaCl	KCl	$CaCl_2$	NaH_2PO_4	$MgCl_2$	Glucose	EGTA
Ca^{2+}	135	5	2.5	0.4	0.5	10	---
0 Ca^{2+}	135	5	---	0.4	0.5	10	0.1

The pH was adjusted to between 7.4 and 7.55 with 0.5-M $NaHCO_3$, followed by the addition of fetal bovine serum (to 0.2%) to increase viability of the dissected muscle (Huisamen et al, 1994). The solution was continuously aerated with a gas mixture consisting of 95% O_2 and 5% CO_2. EDL and SOL muscles were mounted vertically between two Radnoti (Monrovea, CA, USA) stimulating platinum electrodes and immersed in a 20 mL bathing chamber containing the incubation medium. Via the tendons, the muscles were suspended from movable isometric force transducers above the chambers and secured to the base of the tissue support within the chambers. The analog output of the force transducer was digitized, stored and analyzed with PowerLab Software (Colorado Springs, CO, USA). For each muscle, the resting tension and the stimulatory voltage were

provided by a Grass S8800 digital stimulator (West Warwick, RI, USA) and were adjusted to produce a maximal isometric tetanic force (T_{max}).

b. *Intact Muscle Protocol* (Brotto et al, 2002)

After initial T_{max} determination, the muscles were subjected to a protocol of intermittent fatigue in the incubation medium containing: 2.5 mM Ca or zero Ca and/or 5 mM $NiCl_2$, 1 mm $NiCl_2$, 10 μM nifedipine, or 25 μM bromoenol lactone (BEL). The intact muscles were allowed a 20-minute equilibration, during which time they were stimulated with pairs of alternating high (that produced T_{max}) and low (that produced 1/2 T_{max}) frequency pulse-trains administered with a periodicity of 1 minute. The results indicate the relative contributions from the contractile proteins (T_{max}) and from the SR (1/2 T_{max}) to the stimulation.

Following equilibration, the muscles were subjected to a 5-minute intermittent fatiguing stimulation protocol consisting only of the high frequency tetanic trains (that produced T_{max}) administered at a 1-s periodicity (50% duty cycle). Thereafter, the periodicity of the stimulation was returned to 1-min intervals of alternating high and low frequency stimulation and the muscles were allowed to recover for 20 min. All force data were normalized as the absolute force.

c. *Intracellular Ca^{2+} Measurements*

For quantitative measurements of intracellular [Ca^{2+}], flexor digitorum brevis (FDB) muscle fibers were enzymatically isolated in a 0 Ca^{2+} Tyrode solution containing 2 mg/mL type I collagenase for 2 hours in a shaking incubator at 37 °C, before being transferred to a 0 Ca^{2+} Tyrode solution without collagenase and gently triturated with a pipette. The fibers were then loaded with 5 μM Fura-2-AM for 40 minutes, after which the Fura-2 AM was washed off. As fiber motion artifacts are associated with intracellular Ca^{2+} release, 20 μM *N*-benzyl-*p*-toluene sulfonamide (Sigma-Aldrich, St. Louis, MO), a specific myosin II inhibitor, was then applied for 20 minutes. A dual-wavelength (excitation at 340 nm and 380 nm) PTI spectrofluorometer (Photon Technology International, Birmingham, NJ) was used to determine the kinetic changes of caffeine and ryanodine (C/R)-induced intracellular Ca^{2+} transients.

d. *Manganese Quenching*

FDB fibers were loaded with Fura-2/AM fluorescent Ca^{2+} indicator. SR Ca^{2+} depletion with caffeine and ryanodine (C/R), with and without 25 μM BEL, was followed by the addition of 0.5 mM $MnCl_2$ (with and without 25 μM BEL) to the extracellular medium. The Mn^{2+} quenching of Fura-2 fluorescence was measured at 360 nm. The decay of Fura-2AM fluorescence upon Mn^{2+} addition was expressed as percent decrease in Fura-2AM fluorescence.

B. Results

In the first series of experiments, we investigated the effects of extracellular Ca^{2+} entry via SOCE in *WT* young skeletal muscles by removing Ca^{2+} from the extracellular solution (Figures 6 and 7). Following the removal of extracellular Ca^{2+}, a decrease in the high frequency force was observed, while the low frequency component was not significantly altered, suggesting that during a high frequency tetanic contraction enough Ca^{2+} is lost from the SR to trigger entry of Ca^{2+} via SOCE. In multiple experiments we observed full recovery of contractile force upon restoration of 2.5 mM Ca^{2+} in the extracellular medium (n=8/10). Notice the recovery phase is rapid, suggesting that the effect of extracellular Ca^{2+} may be related to direct shuttling of Ca^{2+} across the sarcolemmal membrane by a pore-conduction mechanism rather than passive diffusion.

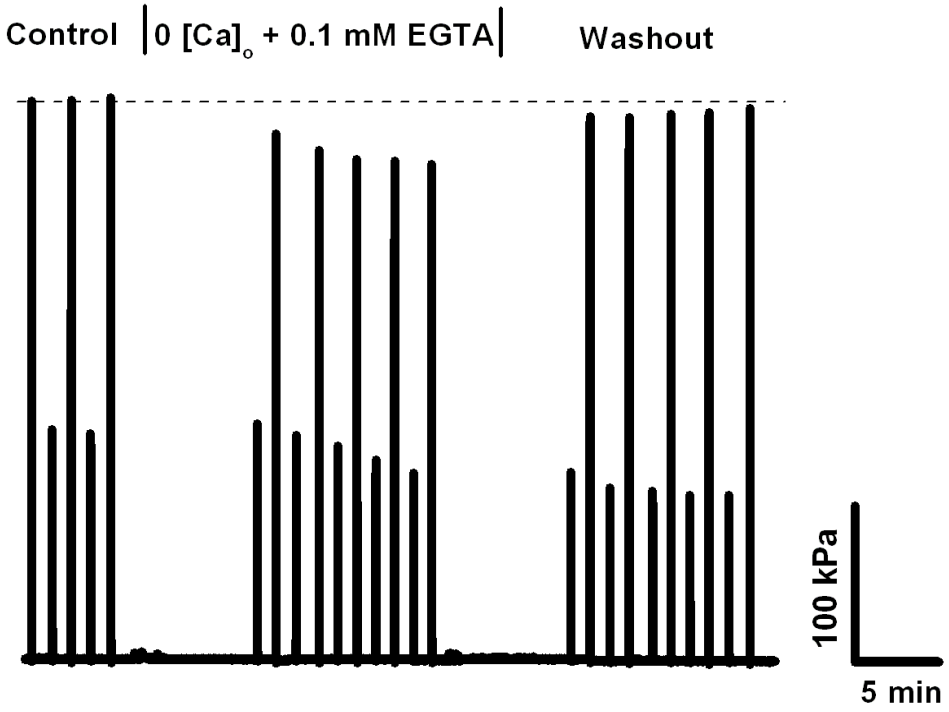


Figure 6. Removal of extracellular Ca^{2+} reduces tetanic contractile force in skeletal muscle. An intact EDL muscle was electrically stimulated with low and high frequency in a bath solution with either 2.5 mM extracellular Ca^{2+} (control) or 0 extracellular Ca^{2+} + 0.1 mM EGTA. Fast recovery upon return of preparation to 2.5 mM extracellular Ca^{2+} is observed.

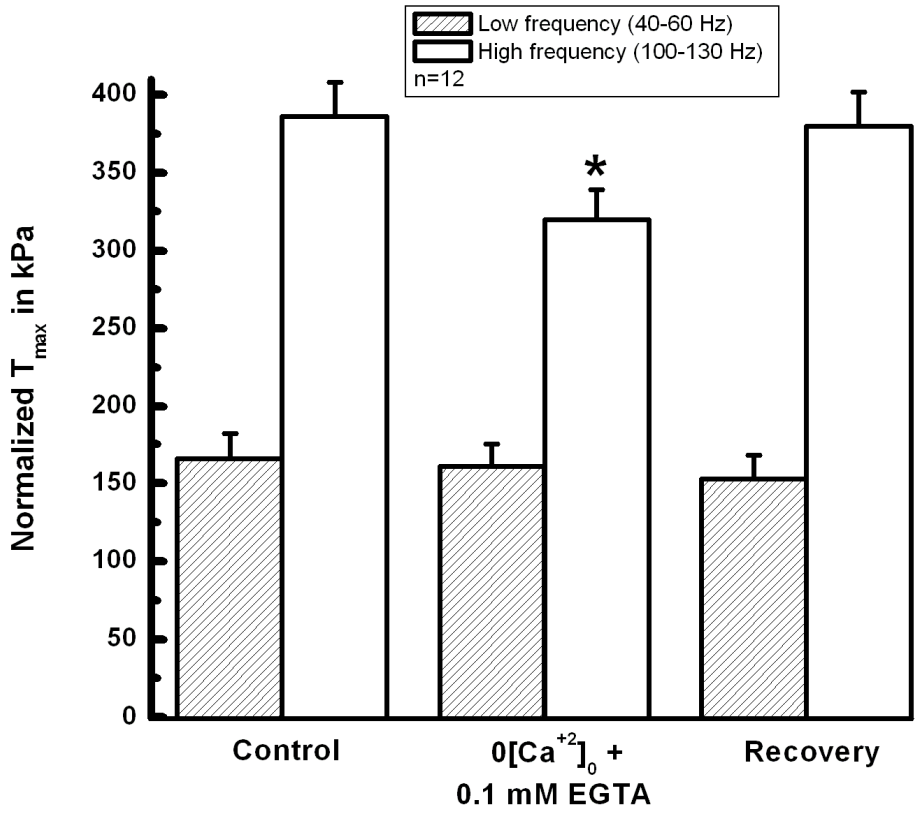


Figure 7. Data summary for the effects of 0 extracellular Ca^{2+} on EDL muscle at 25^{o} C (n = 8, $p < 0.01$).

To establish whether the effect of extracellular Ca^{2+} entry on contractility of young skeletal muscle was mediated by the L-type Ca^{2+} channel, we incubated the EDL and SOL muscles with nifedipine (10 μM). As shown in Figure 8, there was no significant decrease in contractility observed with up to 13 minutes of exposure to nifedipine, a finding in agreement with previous reports from Miller and colleagues (Miller et al, 1989a; Miller et al, 1989b).

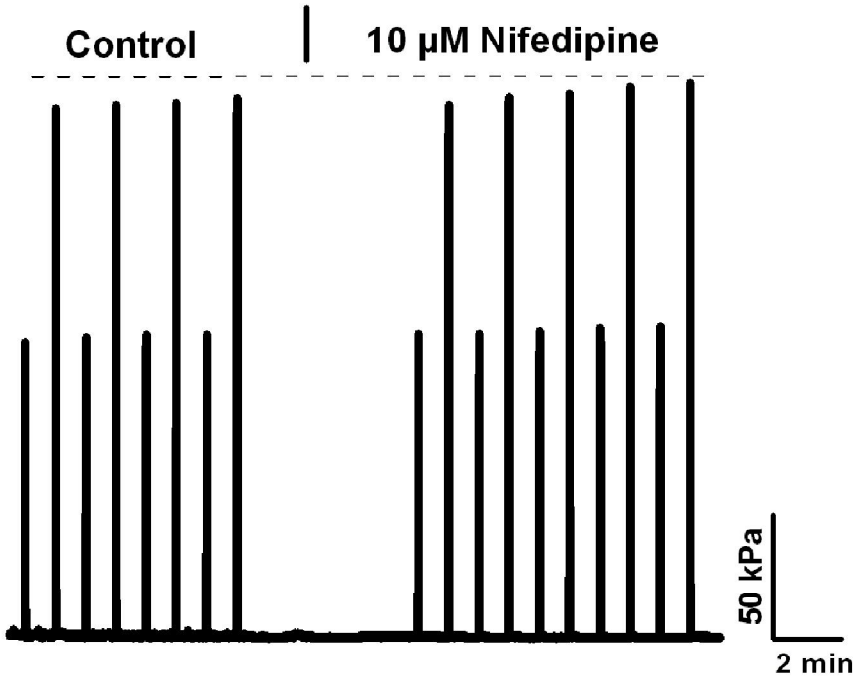


Figure 8. Nifedipine does not reduce tetanic force in young skeletal muscle. Nifedipine does not affect contractile force of intact soleus muscles generated by either low- or high-frequency stimulation. Experiment was performed with a bath solution containing 2.5 mM extracellular Ca^{2+} (control) or 2.5 mM extracellular Ca^{2+} + 10 µM nifedipine.

The next series of experiments was designed to inhibit extracellular Ca^{2+} entry through addition of $NiCl_2$ in the extracellular solution. As shown in Figures 9 and 10, addition of 5 mM Ni to an isolated EDL muscle results in a nearly instantaneous drop in the contractile force, with this inhibitory effect continuing to develop with prolonged exposure to Ni. We found that Ni has more pronounced effects on the high frequency stimulation as compared to the low frequency of stimulation, likely suggestion that under conditions of higher Ca^{2+} demand, SOCE exerts a more prominent role. The effect of Ni was completely reversible after washout in the majority of the experiments, suggesting that observed effects are not secondary to unspecific chemical effects.

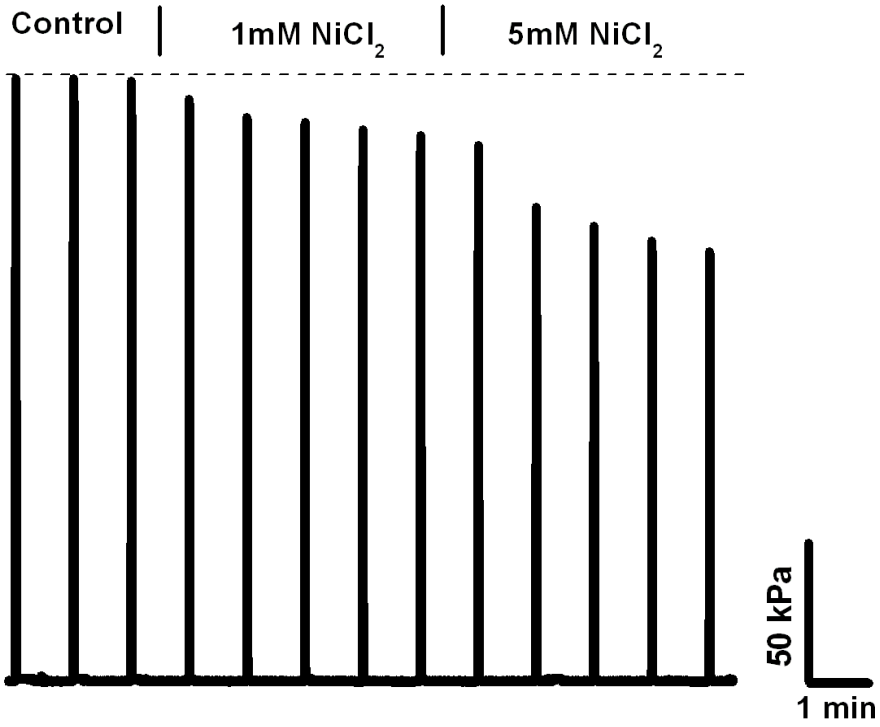


Figure 9. Extracellular Ni reduces tetanic force in skeletal muscle. An intact EDL muscle was electrically stimulated with low frequency and high-frequency in a bath solution with 2.5 mM extracellular Ca^{2+} (control), flowed by the addition of 1 and 5 mM $NiCl_2$. Ni inhibits force generated with high- but not low-frequency stimulation. Washout of Ni leads to ~95% of control forces (see Figure 10).

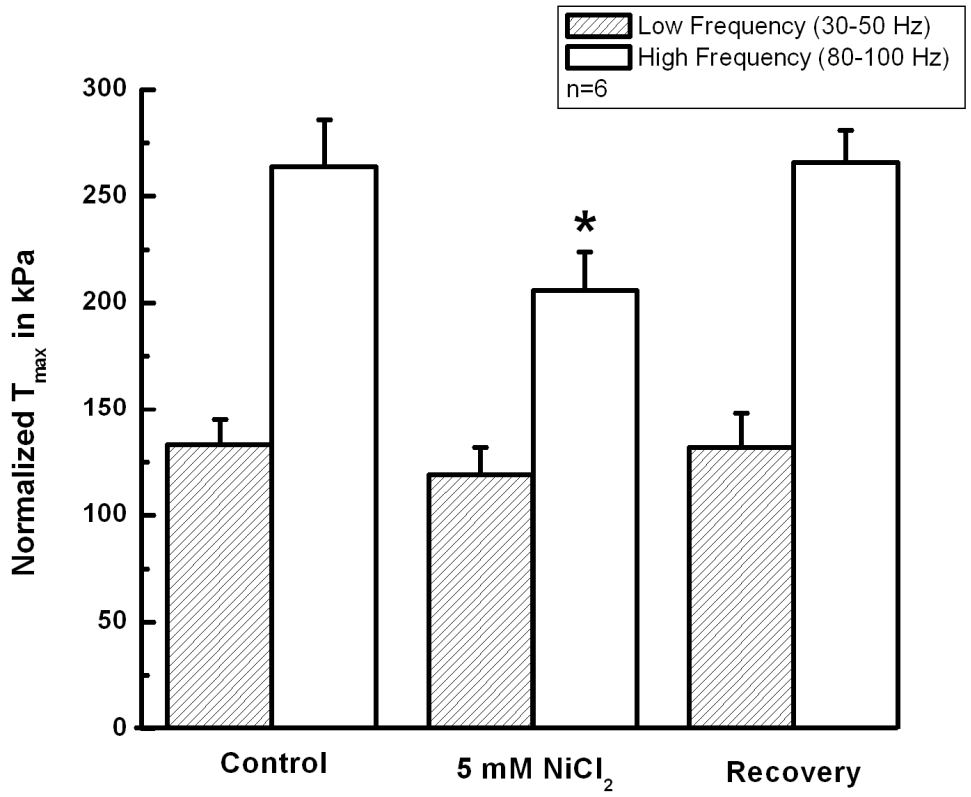


Figure 10. Summary of the Ni effects on young EDL muscle (n = 9, * p < 0.01).

Since the aforementioned experiments were performed at 25°C, and physiological mechanisms display obvious dependence on temperature, we replicated experiments performed at room temperature at the physiological temperature of 37°C to establish the extent to which Ca^{2+} entry effects contraction of working muscle. These experiments revealed that a similar effect of extracellular Ca^{2+} entry on contractility can be observed at physiological temperatures, even when only 1 mM Ni was used (Figure 11).

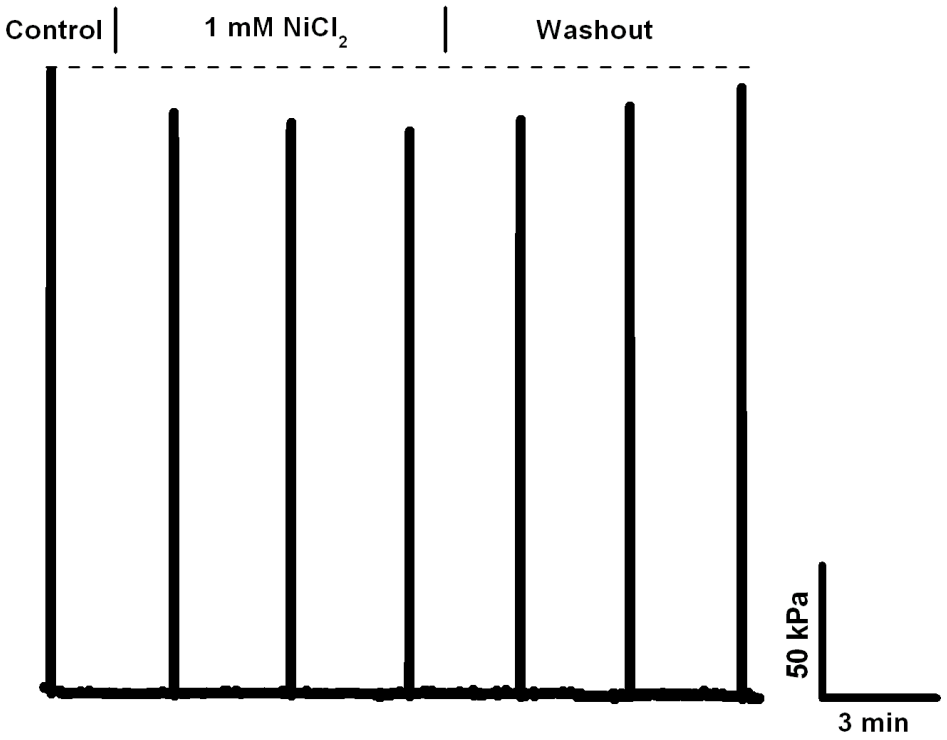


Figure 11. Effects of Ni on contractility of skeletal muscle at 37°C. Addition of 1 mM Ni to an intact SOL muscle leads to significant inhibition of the tetanic force at 37^{o} C. To prevent rundown, muscle was electrically stimulated (130 Hz) every 3 minutes. Washout of Ni leads to complete recovery of force. n = 3.

To complement our intact muscle contractility work, we also conducted Ca^{2+} imaging studies in enzymatically dissociated FDB cells to evaluate the macroscopic SOCE property of intact muscle fibers. Our data with young FDB cells show that 25 μM BEL can significantly inhibit SOCE activation elicited by caffeine/ryanodine treatment (Figure 12), suggesting that BEL can inhibit both RyR-1 dependent and RyR1-independent SOCE pathways. We also performed contractility experiments and found that BEL can significantly decrease the high-frequency force of young EDL muscle (Figure 13).

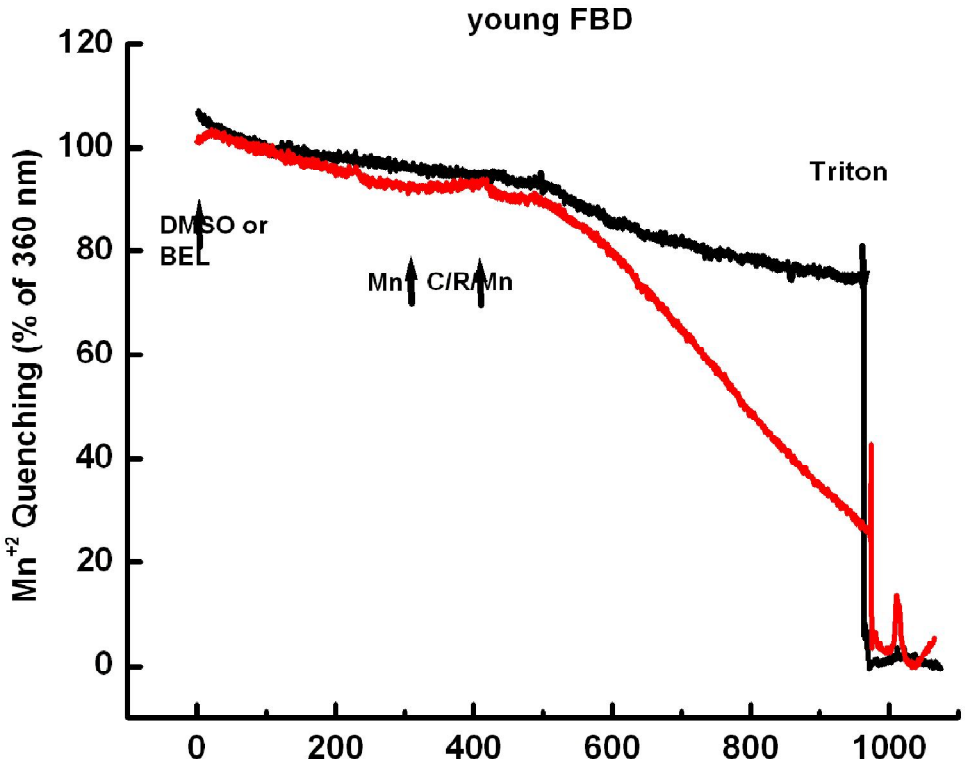


Figure 12. BEL reduced SOCE in young skeletal muscle. SOCE was monitored with the Mn^{+2} quenching technique. BEL significantly inhibits SOCE in young FDB muscle fibers (black) as compared to muscle fibers treated with DMSO (red, control) (n=5).

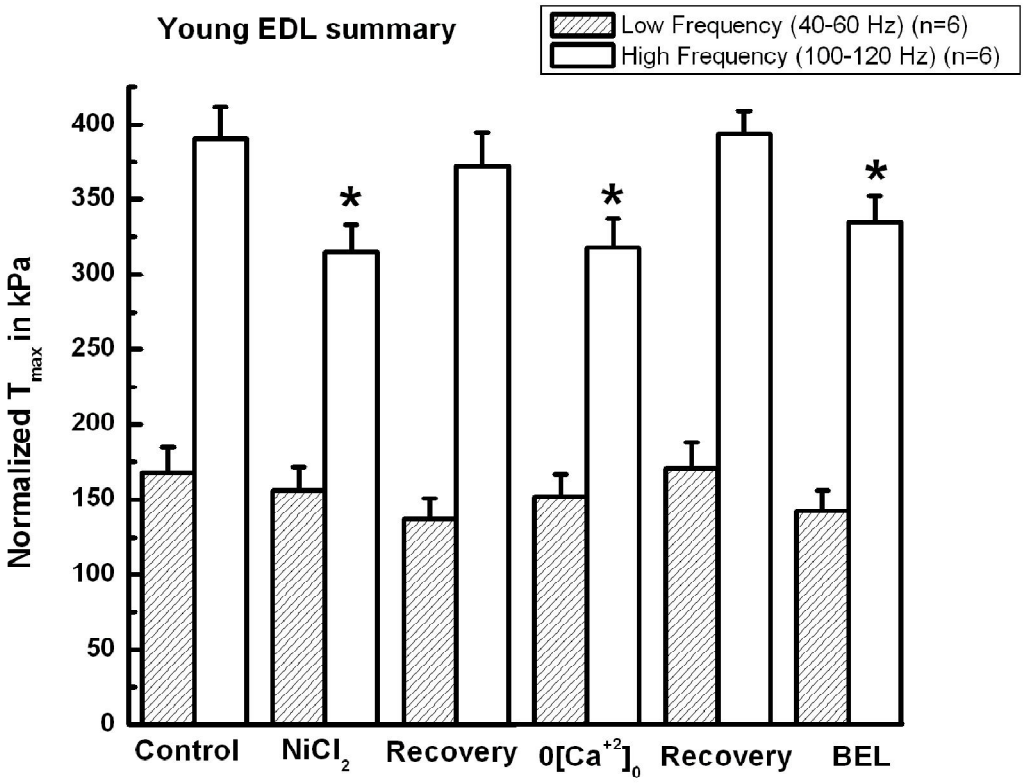


Figure 13. Summary of reduced tetanic force in young skeletal muscle. Maximal tetanic force was significantly inhibited by ~ 10% in young EDL muscles ($p < 0.05$). Data is the average of 6-8 intact muscle preparations.

C. Discussion

This work demonstrates that extracellular Ca^{2+} entry contributes to skeletal muscle contractility, as the muscle force generated is reduced when Ca^{2+} entry is blocked by a variety of methodology. The prevailing opinion has been that extracellular Ca^{2+} is not required for muscle contraction as the Ca^{2+} released from the SR is sufficient to elicit contraction. However, it has been previously reported that increased muscle activity does involve entry of extracellular Ca^{2+} (Vanterpool et al, 2005; Zhao et al, 2005). Therefore, the reductions in force exhibited by the muscles from the young mice could be attributed to the onset of muscle fatigue.

Fatigue is defined as a reversible decrease in the isometric contractile force in response to an increase in the frequency of stimulation; or, as any acute impairment in the muscle's ability to exert force, even when the task at hand can still be successfully performed (Brotto et al, 2000; Allman and Rice, 2002). There are several theories about the mechanisms of fatigue. Included are the sodium theory, which proposes that ionic concentration changes in the muscle cell cause action potential propagation failure; activation of Ca^{2+} proteases, which leads to a rise in intracellular Ca^{2+}, activation of Ca^{2+} proteases and subsequent cleavage of essential E-C coupling related proteins; the reactive oxygen species (ROS) theory, which states that increased muscle activity causes increased mitochondrial respiration, yielding a net increase in superoxide,

hydrogen peroxide and free radicals that alter protein function (Brotto et al, 2001, Brotto et al, 2007); and the lactic acid/acidosis theory.

During muscle contraction, ATP allows cross-bridge cycling between the actin and myosin filaments, resulting in force production. With repeated contractions, the phosphocreatine stores in muscle are used to resynthesize and maintain ATP concentrations. As the number of contractions increases, the [phosphocreatine] decreases, and other fuels must be utilized to generate ATP. The glycolytic pathway is activated, causing the formation of pyruvate and ATP. If all of the pyruvate produced cannot be oxidized by the mitochondria, the remainder is converted to lactate in the myoplasm. It has generally been accepted that this accumulation of lactate is directly associated with the production of H^+ ions, leading to a fall in intramuscular pH or acidosis, and, subsequently, to a decline of muscle force generation, or the development of fatigue (Robergs et al, 2004; Kristensen et al, 2005; Cairns, 2006). During high-intensity exercise, the intramuscular accumulation of lactic acid has long been considered one of the most important factors in the development of fatigue. However, within the last 25 years, evidence has suggested that accumulated lactate and acidosis have little detrimental effect on muscle performance. (Cairns, 2006).

The presence of 15 mM lactate has been reported to cause no reduction in depolarization-induced Ca^{2+} release from the SR, while 30 mM lactate, comparable to that occurring during strenuous anaerobic exercise, reduced Ca^{2+}

release by less than 10% (Stackhouse et al, 2001). This data suggests that lactate plays only a small role in the production of fatigue. In addition, during some fatigue protocols on in mammalian fibers, there was virtually no change of intramuscular pH, casting doubt on the impact of acidosis on muscle performance (Cairns, 2006).

As per our previous work (Brotto et al, 2001; Brotto et al, 2002; Brotto et al, 2004; Brotto et al, 2007), we designed our experiments to mimic the physiological conditions under which muscles normally work. Our data suggest SOCE is involved in the normal contractile function of skeletal muscle. Further, SOCE also plays a crucial role, not only in providing additional Ca^{2+} to replenish the SR, but also when muscles demonstrate a greater demand for Ca^{2+}, such as during exercise and with aging (Zhao et al, 2005; Brotto et al, 2007; Zhao et al, 2008). This led us to question whether SOCE functionality changes with age.

Chapter 6. Does SOCE in skeletal muscle change with aging?

"Sarcopenia", the Greek term for "poverty of flesh", was first used by Rosenberg to describe age-related changes in skeletal muscle (Rosenberg, 1989; Evans and Rosenberg, 1991). Typically, it manifests as a loss of muscle mass, decreased strength, and reduced aerobic activity; but, it is also associated with age-related losses in bone mineral, reduced basal metabolic rate and increased body fat content (Dutta and Hadley, 1995; Forbes and Reina, 1970; Evans and Campbell, 1993; Johnston et al, 2008; Doherty, 2003). The term not only describes the age-related musculoskeletal changes, but also encompass the effects of alterations in neural innervations, hormonal state, and dietary intake, all of which may contribute to the skeletal muscle atrophy and weakness associated with sarcopenia, ultimately diminishing the quality of life for those affected (Schneider and Guralnik, 1990; Roubenoff and Hughes, 2000; Navarro et al, 2001; Roubenoff, 2001; Moreland et al, 2004).

Janssen and colleagues reported that loss in skeletal muscle mass begins in approximately the 4th decade of life, progressing at a rate of 0.5-1% each year (Janssen et al, 2000). Novak reported that concomitant with the age-associated muscle mass decrease is an increase in body fat (Novak, 1972; Parise and Yarasheski, 2000). This, of course, is associated with an increased risk for developing chronic disorders, such as hypercholesterolemia, atherosclerosis, insulin resistance, type II diabetes, and hypertension (Borkan et al, 1983; Depres et al, 1990).

While a reduction in the level of physical activity among the elderly certainly contributes to the decrease in muscle mass and increase in body fat (Bortz, 1982), the underlying mechanisms of sarcopenia have not been fully elucidated. It has been suggested that disruption of intracellular Ca^{2+} homeostasis is thought to play a crucial role, as muscle atrophy can only partially explain the loss of force during the aging of skeletal muscles (Delbono, 2002; Payne et al, 2004; Weisleder et al, 2006). Fraysse and colleagues (Fraysse et al, 2006) have reported reduced permeability of sarcolemma to divalent cations in aged skeletal muscle. In addition, reduced SOCE was identified in both aged neuronal cells (Vanterpool et al, 2005) and in aged fibroblasts (Papazafiri and Kletsas, 2003). Zhao and colleagues (Zhao et al, 2008) used muscles from aged mice as a model for sarcopenia, since aged mice display decreased skeletal muscle mass and reduced contractility in a fashion similar to aged human skeletal muscles (Barton-Davis et al, 1998; Pagala et al, 1998; Hamrick et al, 2006; Rader and Faulkner, 2006). In addition, Weisleder and colleagues reported an association between muscle aging and the development of a segregated SR Ca^{2+} pool that uncouples from the normal E–C coupling machinery. Therefore, we tested our hypothesis that defective SOCE contributes to muscle weakness associated with the aging process.

A. Experimental Protocols

All experimental protocols utilized were the same as those used to evaluate the muscles taken from the 8-10 week-old mice, except *WT* male mice, 27-30 months of age, were used, instead, and nifedipine was not added to the bathing solution during the Intact Muscle Protocol.

B. Results

Expanding our contractile measurements from young muscles to aged muscle preparations, we found the expected decrease in specific contractile force in aged skeletal muscle (Figure 14). However, we also identified remarkable differences in sensitivity of aged muscle to inhibition of extracellular Ca^{2+} entry. First, as illustrated in Figure 15, there is a negligible effect of Ni on the high-frequency tetanic force in aged muscles. Second, we found that neither removing extracellular Ca^{2+} (Figure 16), nor addition of Ni (Figure 17) had any apparent short-term significant impact on the acute contractile function of aged skeletal muscle. Finally, the addition of BEL significantly attenuated force generation in ages muscle (Figure 18), while inhibiting SOCE in the aged samples (Figure 19).

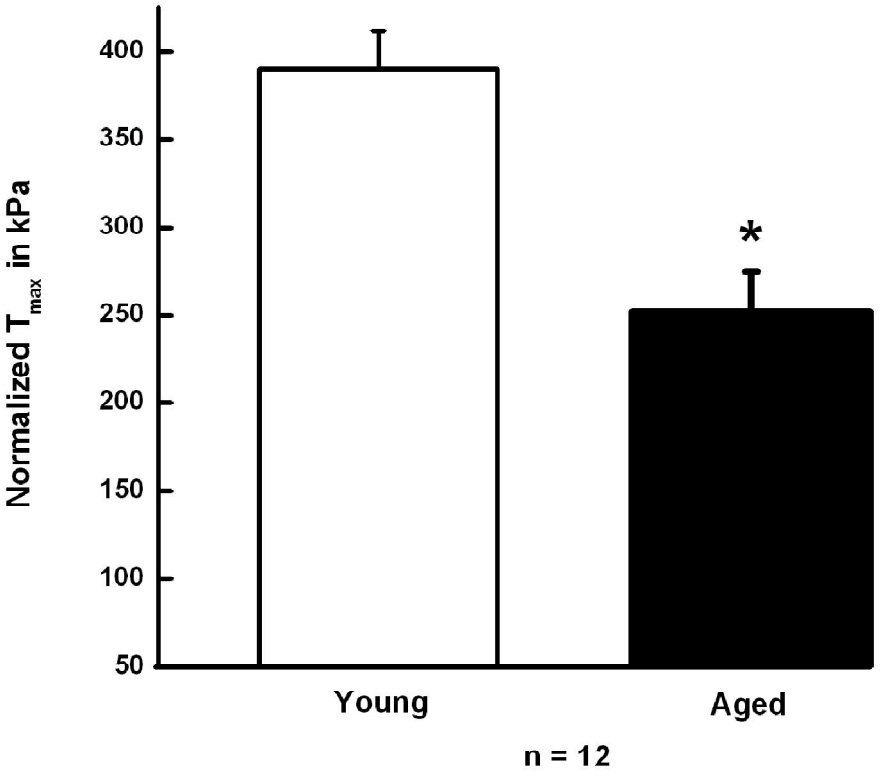


Figure 14. Reduced specific force in aged skeletal muscle is associated with altered extracellular Ca^{2+} dependence. Maximal tetanic force normalized to the cross sectional area in young (white) and aged (black) EDL muscles (n = 12, * $p < 0.001$).

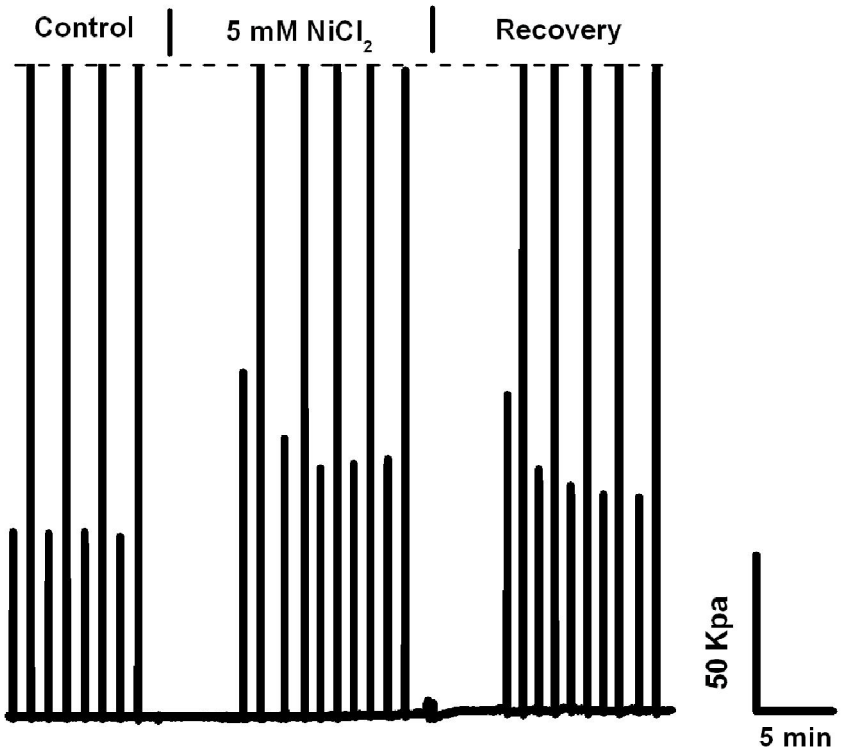


Figure 15. Intact EDL muscle from aged mice subjected to Ni treatment. Ni had no effect on the force generation of aged skeletal muscle at high frequency.

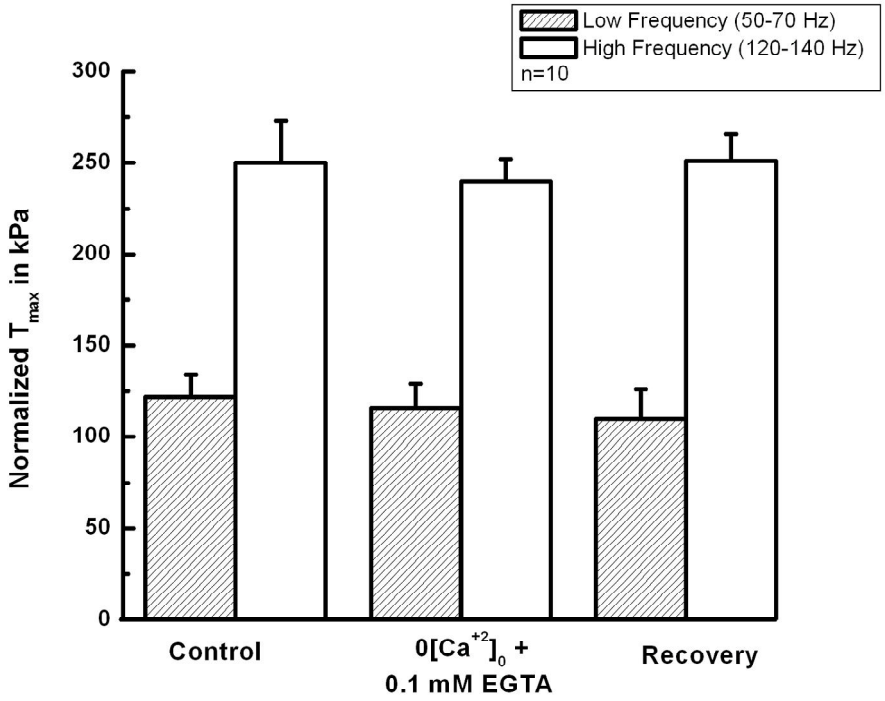


Figure 16. Summary data for the effects of 0 extracellular Ca^{2+} and respective washout each treatment (n = 10).

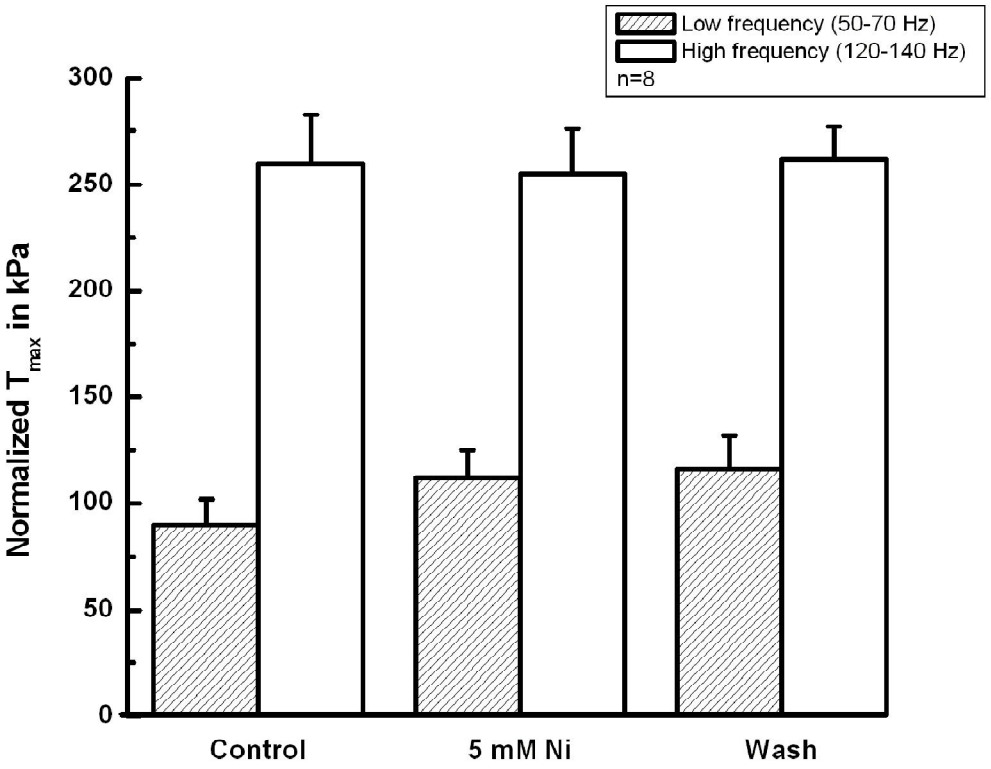


Figure 17. Summary data for the effects of Ni and respective washout after treatment (n = 8).

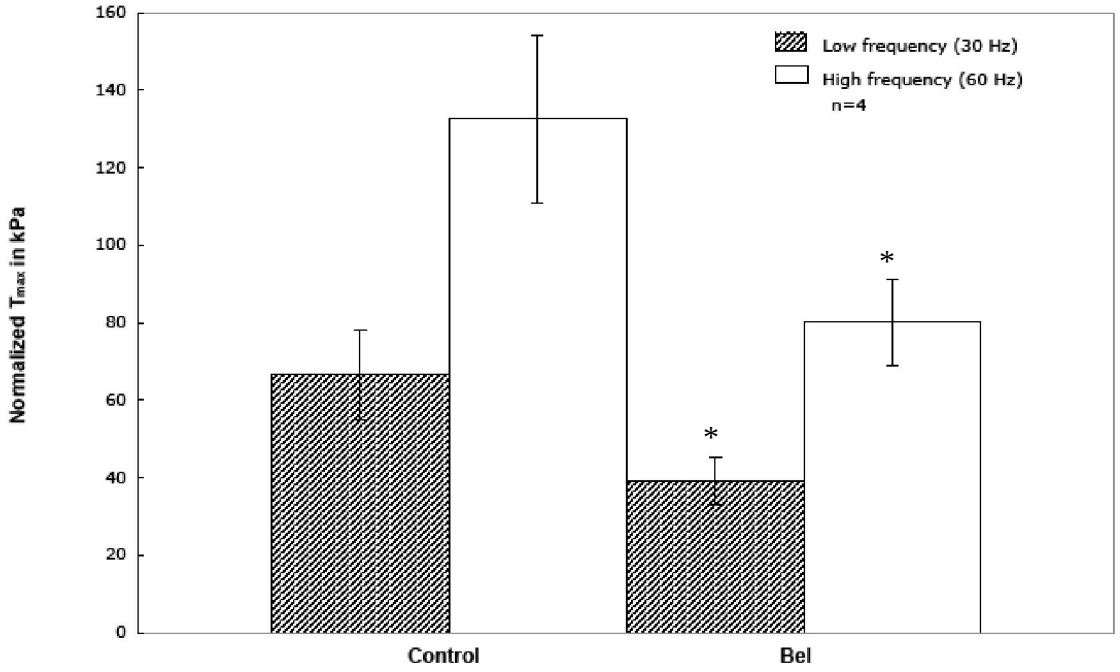


Figure 18. Summary data of BEL effects on aged skeletal muscle. BEL significantly reduces force generation in aged skeletal muscles ($p < 0.01$, n=4).

C. Discussion

The human aging process is associated with a significant decline in neuromuscular function and performance, generally termed as sarcopenia (Dutta and Hadley, 1995; Doherty et al, 1993; Grimby and Saltin, 1983; Roos et al, 1997; Vandervoort, 2002). For the elderly population, this means a potential loss of autonomy and a reduced quality of life (Johnston et al, 2008). For skeletal muscle, this can manifest as age-associated weakness and muscle atrophy, and that atrophy is believed to be limited to a specific muscle fiber type.

Skeletal muscle is composed of two main fiber types. Type II fast-twitch fibers have low oxidative capacity, high glycolytic potential and fast twitch response; and; type I slow-twitch fibers have high oxidative capacity, capillary density and myoglobin content (Gollnick and Hodgson, 1986). Most human skeletal muscle is composed of both types, and the force demanded of the muscle determines the pattern of fiber recruitment (Evans and Campbell, 1993). As skeletal muscle mass and fiber type composition are two of the most important determinants of muscle strength, significant age-related muscle mass decline and fiber-type transition could adversely affect muscle strength (Korhonen et al, 2006).

Larsson (Larsson, 1983) reported an increase in the percentage of fast-twitch fibers in men as a function of age. Both Sato and Grimby reported no such change in fiber type (Sato et al, 1984; Grimby et al, 1984). Further, Lexell and colleagues concluded that muscle mass loss was due to decreases in both

fiber area and in the total fiber number (Lexell et al, 1983). After further investigation, Lexall reported that an age-related selective atrophy of fast-twitch fibers was, in fact, responsible for the majority of the muscle mass loss (Lexell, 1995). These findings were supported by similar reports from Trappa and colleagues (Trappe et al, 1995; Ryan et al, 2000).

It is believed that this selective fiber-type loss causes a transition towards the prevalence of type I fibers, resulting in an overall decrease in force-generating capacity (Korhonen et al, 2006), as Trappe and colleagues later reported that peak force appreciably decreases in fast-twitch fibers, but not in slow-twitch fibers, with age (Trappe et al, 2003), indicating that with the type II muscle atrophy is a concomitant decrease in muscle fiber quality, meaning this selective muscle fiber atrophy is detrimental to overall muscle strength (Johnston et al, 2008). However, while half of the decrease in muscular strength during aging can be explained by this muscle atrophy, the reasons for the other half remain elusive.

In addition to the effects of selective fiber-type atrophy, altered function of certain triad junction proteins has been shown to contribute to disrupted Ca^{2+} homeostasis in aged skeletal muscle (Delbono et al, 1995; Margreth et al, 1999). This would certainly contribute to the reduction in muscle force generation. Payne and Delbono have suggested that cumulative uncoupling of the VICR process may actually contribute to the age-associated muscle changes (Payne and Delbono, 2004b).

Renganathan and colleagues developed this hypothesis of E-C uncoupling and sarcopenia, after demonstrating that the main functional consequence of such uncoupling is a signal transduction failure – the transduction of T-tubule depolarization into a proper Ca^{2+} release signal – leading to the weakened mechanical response and muscle strength deficit (Renganathan et al, 1997). The molecular basis for the uncoupling is an age-related reduction in the number T-tubule voltage sensors and a consequential increase in the percentage of RyR1s being uncoupled from DHPRs (Delbono et al, 1995). This seems especially probable, as a physical linkage between a subpopulation of RyR1s in the SR with the T-tubule DHPRs has been reported (Block et al, 1988; Brandt et al, 1990). Further, others have reported a reduction in DHPR-sensitive Ca^{2+} currents in both aged human and aged murine skeletal muscle, the effects of which were a significant reduction in the Ca^{2+} available for triggering the required mechanical responses (Damiani et al, 1996; Delbono et al, 1995; Renganathan et al, 1997).

Alterations in the capacity to maintain normal cellular homeostasis, due to E-C uncoupling, or even due to neuronal alterations, oxidative stress, or post-translational protein modification (Navarro et al, 2001) may, therefore, underlie the age-associated reduction in muscle fiber function characteristic of the aging process. Weisleder and colleagues presented evidence suggesting triad junction protein MG29 may act as a sentinel against the effects of age on skeletal muscle Ca^{2+} homeostasis (Weisleder et al, 2006). Therefore, the logical next step in elucidating this uncoupling mechanism is to determine the involvement of MG29.

Chapter 7. What role does MG29 play in SOCE regulation?

Although the triad junction is essential for cross talk between DHPRs and RyRs, neither of these two proteins, together or independently, is necessary or sufficient for the establishment of a close association between the T-tubule and SR membranes. In both DHPR and RyR knockout models, triad junctions appear intact; and co-expression of DHPR and RyR in heterologous systems does not bring about apposition of sarcolemma and SR membranes. Therefore, other protein components must be involved for the anchoring and biogenesis of the T-tubule to SR membranes (Yoshida et al, 2001; Komazaki et al, 2002; Nishi et al, 2002; Minamisawa et al, 2004; Hirata et al, 2006).

Takeshima and colleagues identified the 29 kDa transmembrane protein MG29 in the skeletal muscle triad junction (Takeshima et al, 1998). The primary structure of MG29 indicates that it is a member of the synaptophysin protein family, whose members are known to be important transmembrane proteins on synaptic vesicles (Johnston et al, 1989; McMahon et al, 1996). It was found that MG29 is abundantly expressed in skeletal muscle, appearing during SR development prior to T-tubule formation, and is localized to the triad junction in mature cells, indicating that the protein may be involved the development of the SR-sarcolemma communication network (Kawasaki et al, 1999; Brandt and Caswell 1999; Nishi et al, 1999).

Nishi and colleagues reported that *mg29(-/-)* mice subsequently generated displayed no obvious health or reproductive abnormalities, nor were

there defects in motor coordination or ability. Yet, electron microscopy studies indicated that the SR networks of these mice, similar to that of aged skeletal muscle, were ill-formed, having either vacuolated, fragmented, or tubular SR structures, leading to misalignment of the triad junctions. The T-tubules were either swollen or absent from the A-I junction altogether. The muscles exhibited an irregular force-frequency relationship, as compared to the *WT* controls (Nishi et al, 1999).

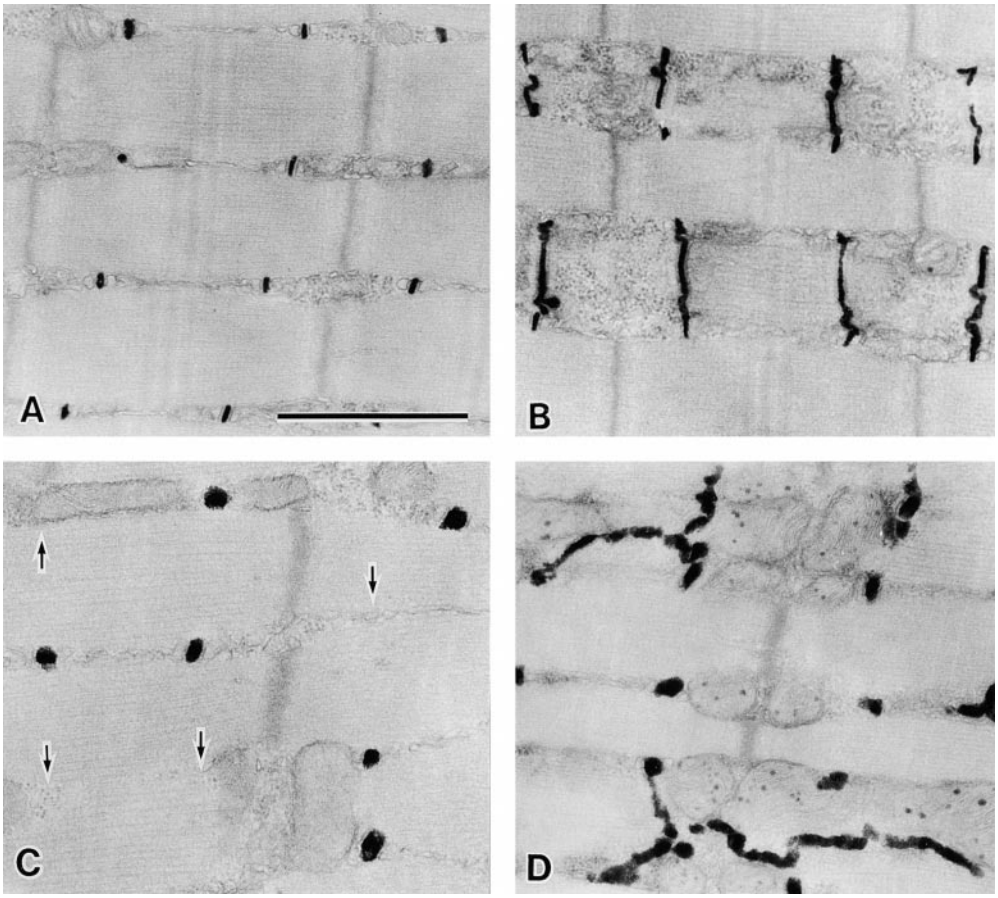


Figure 19. Abnormal SR and T-tubule formation in *mg29(-/-)* mice (Nishi et al, 1999). The first row depicts wild type SR networks (A) and T-tubule orientation (B). The second row shows SR networks (C) T-tubule alignment in *mg29(-/-)*.

The abnormal triad junction structure and defective muscle function of the *mg29(-/-)* mice adversely affect the muscles' Ca^{2+} homeostasis, resulting in increased susceptibility to fatigue, as with aged skeletal muscle. Nagaraj and colleagues compared the contractile properties of muscle types before and after a fatigue protocol using the fast-twitch EDL and the slow-twitch SOL from *WT* and *mg29(-/-)* mice. The EDL and SOL from the *mg29(-/-)* mice were more susceptible to fatigue, both fatiguing more and recovering less than the *WT* control muscles (Nagaraj et al, 2000). Confirmation was provided when Pan and colleagues demonstrated increased fatigue susceptibility of SOL muscles in both the presence and absence of extracellular Ca^{2+}, along with dysfunctional SOCs in *mg29(-/-)* skeletal muscle (Pan et al, 2002).

These studies indicate that both *mg29(-/-)* muscle and aged skeletal muscle exhibit both reduced SOCE and increased susceptibility to fatigue. In addition, Weisleder and colleagues report a decrease in MG29 expression as a function of age (Weisleder et al, 2006). The functional similarities, coupled with the morphological parallels lead to our hypothesis that not only does MG29 contribute to SOCE functionality in muscle physiology; but, also that, while SOCE serves as a functional marker, MG29 can serve as a molecular marker for skeletal muscle aging.

A. Development of Unique Methodologies

a. Gene Silencing

In 1998, Fire and colleagues found that the injection of double-stranded (ds) RNA into *Caenorhabditis elegans* led to efficient gene silencing, which has been referred to as RNA interference (RNAi) (Fire et al, 1998). RNAi is a sequence-specific gene silencing process that occurs at the posttranscriptional level (Dykxhoorn et al, 2003; Meister and Tuschlm, 2004). The effectors of RNAi are short (21–28 nucleotides) ds RNA molecules, called small interfering RNAs (siRNAs), processed from longer precursors by the ribonuclease, Dicer (Bernstein et al, 2001). The antisense strand of the siRNA serves as a template for the RNA-induced silencing complex (RISC) to recognize and cleave a complementary messenger RNA (mRNA), which is then rapidly degraded (Figure 10) (Hammond et al, 2000; Elbashir et al, 2001a; Elbashir et al, 2001b; Martinez et al, 2002; Sandy et al, 2005). It has since been demonstrated that short (<30 bp) synthetic dsRNAs can trigger sequence-specific knockdown of gene expression without inducing an interferon response in cultured mammalian cells (Elbashir et al, 2001a). This discovery propelled the development of RNAi as a powerful reverse genetics tool for the functional study of mammalian genes (Sharp PA, 2001; Mittal, 2004; Hannon et al, 2004).

siRNAs can be constitutively transcribed as stem–loop precursors by the Pol III polymerase, and stable cell lines can be established in which the siRNA is expressed in a constitutive manner (Brummelkamp et al, 2002a; Donze and

Picard, 2002; Miyagishi and Taira, 2002; Yu et al, 2002). The siRNA cassette can also be inserted into a viral vector. Use of viral vectors for siRNA gene delivery allows the transfection of cells that are otherwise difficult to transfect, such as primary cell cultures and skeletal muscle cells (Brummelkamp et al, 2002b; Rubinson et al, 2003; Stewart et al, 2003).

b. Gene Delivery

In vivo gene delivery methods can generally be classified into two categories: non-viral and viral. Non-viral systems can be further classified into two categories: synthetic vectors, which include cationic lipids and polymers, and the direct injection of naked plasmid DNA or DNA-peptide conjugates (Liu et al, 2001; Donà et al, 2003; McMahon and Wells, 2004). Several genetically modified viral vectors, including retrovirus, herpes simplex virus, Epstein–Barr virus, adenovirus (AV), and adeno-associated virus (AAV), have been tested for gene delivery into muscle cells. Many induce an immune response against intrinsic viral antigens, leading to serious toxicity to the host and making successive treatment difficult (Liu et al, 2001). Viral proteins, particularly the adenoviral capsid, stimulate the innate immune system and cause inflammation and loss of the vector DNA within 24 hours of injection (McMahon and Wells, 2004). AAV, so far, has not been associated with any particular disease, is capable of transfecting non-proliferating muscle fibers, and can sustain longer periods of transgene expression than other viral vectors (Donà et al, 2003). However, the gene size that can be inserted into the AAV genome without

disabling gene transfer is 4.8 kb, maximally (Liu et al, 2001). In addition, the time and expense required to obtain the viral titer necessary for its effectiveness in skeletal muscle is another challenge, resulting in efforts to optimize non-viral gene delivery systems.

Of the non-viral methods, injection of naked plasmid DNA is the most straightforward. The circular ds DNA is easily manipulated and can be produced from bacterial culture in large quantities. Purification of long sequences of plasmid DNA is easily accomplished, the DNA can be refrigerated for long periods of time, and there is no likelihood of exogenous proteins, being expressed in eukaryotic cells (Liu et al, 2001; Donà et al, 2003; McMahon and Wells, 2004; Jang et al, 2004).

Gene transfer by injection of naked plasmid DNA to the skeletal muscle was reported by Budker and colleagues (Budker et al, 1998), who found that plasmid DNA can be delivered to and expressed in the hindlimb muscles of rats. Direct injection of nude DNA inside muscle tissue is the easiest approach, but it is also the technique with the poorest efficiency, precluding its widespread use (Donà et al, 2003; Jang et al, 2004). Plasmid-based gene transfer has several advantages, as well as disadvantages, for therapeutic gene transfer into skeletal muscle fibers compared to viral vector-mediated gene transfer.

The major advantages include a very large insert capacity and the relatively favorable cost-effectiveness of its production (Molnar et al, 2004). However, another limitation is the great variability in the level of foreign gene expression,

which may impair the clinical development of therapeutic intramuscular DNA administration (Mir et al, 1999). Not only is the transfection efficiency of naked DNA by intramuscular injection is relatively low, particularly in large animals, establishing transgene expression only at the injection site (Liu et al, 2001; Mathiesen, 1999; Memunni et al, 2002; Mir et al, 1999; Andre and Mir, 2004), but plasmid DNA would have to be administered systemically in order to achieve effective gene therapy for these diseases (Liu et al, 2001).

However, recent experiments in animals indicate that the efficiency of plasmid-based gene transfer can be substantially enhanced by a technique, named electrotransfer, sonoporation, or electroporation (EP). This process permits one to target a specific tissue and strongly decreases interindividual variability, which is one of the largest restrictions to the use of intramuscular naked DNA (Mathiesen, 1999; Mir et al, 1999; Andre and Mir, 2004; Molnar et al, 2004). The application of EP techniques to plasmid DNA gene transfer has yielded dramatic improvements in reported transfection efficiencies in muscle ranging from 10- to 1000-fold (McMahon and Wells, 2004). More recently, EP has been applied in vivo to enhance the uptake of DNA, including plasmids, into cells. This was found to enhance markedly the transfection efficiency of cells, including muscle fibers, by plasmid-based gene transfer (Molnar et al, 2004).

At its essence, EP is the application of an electric field to a biological cell. The external electric field induces a change in the resting potential of the cell, the value of which depends on the cell shape, media conductivity, and cell

radius, among other factors (Andre and Mir, 2004). If the field strength exceeds a certain threshold value, this can lead to the generation of transient, hydrophilic, inverted pores in the cell membrane (EP) (Figure 21), or to fusion of adjacent cells (electrofusion) (Kotnik et el, 1998; Mathiesen, 1999; Gehl, 2003).

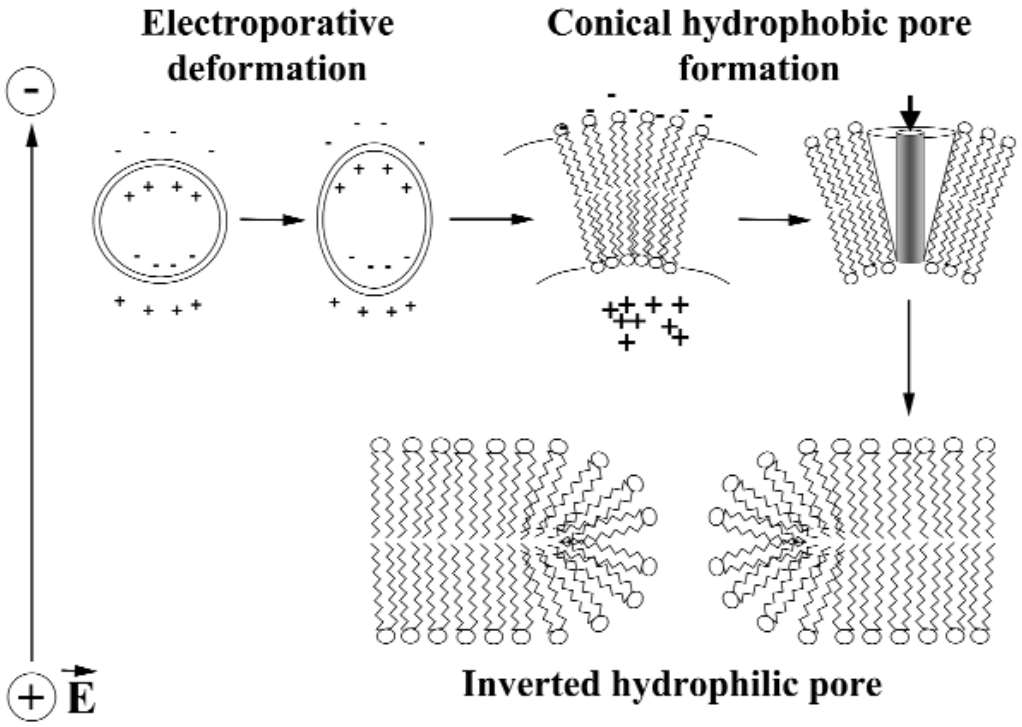


Figure 20. Model for EP-induced pore formation (Neumann et al, 1999; Soliari et al, 2000).

It has been proposed that in vivo plasmid electrotransfer to cells consists of two components. The first, EP, is thought to be a necessary prerequisite for DNA transfer into the cell, the second component (Bureau et al, 2000; McMahon

et al, 2001). It is believed that by optimizing the electrical parameters, it is possible to achieve excellent levels of cell permeabilization that are compatible with cell survival (Mir et al, 1999).

Neumann and colleagues published the first demonstration that DNA could be introduced into living cells by means of electric pulses in 1982 (Neumann et al, 1982). The second paper describing the successful transfer of DNA to eukaryotic cells in vitro was published by Potter in 1984 (Potter et al, 1984). The use of electric pulses for cell EP has since been used to introduce foreign DNA into prokaryotic and eukaryotic cells in vitro and to transfect melanoma cells, brain tumor cells, liver cells, and mouse skeletal muscle in vivo (Table 2) (Mir et al, 1999; Mathiesen, 1999).

Table 2. In vivo therapeutic gene delivery by means of DNA EP (Memunni et al, 2002; Andre and Mir, 2004).

Target	Genes	Tissues	Animals
Analgesia	proopiomelanocortin	Intrathecal space	Rat
Cancer	IL-2; IL-18; GM-CSF; CpG containing DNA; full TRH-2 or epitopes; diphtheria toxin; HSV TK; TIMP; p53; bcl-xs; MBD-2; Flk-1 VEGF receptor; Stat3 variant; K1-5; K1-3-HAS; endostatin	Muscle, tumor, skin, liver	Mouse, rat
Atherosclerosis	IL-12; Human plasma platelet-activating factor acetylhydrolase	Muscle	Mouse
Ischemia	IL-10; IL-18; hVEGF-A and hVEGF-B; protein-disulfide isomerase	Muscle; right hippocampus	Mouse, rat
Myocarditis	IL1-ra; IL-10	Muscle	Mouse, rat
Anemia	Dimeric erythropoietin fusion protein	Muscle, skin	Mouse, rat
Diabetes	IL-4; insulin precursors; IGF-1	Muscle	Mouse
Neuropathy	Neurothrophin3	Muscle	Mouse
Hemophilia B	Factor IX	Muscle	Mouse, dog
Muscular Dystrophy	Dystrophin or minidystrophin; laminin a2; GA-binding protein	Muscle	Mouse
Neurodegeneration	Cardiothrophin	Muscle	Mouse
Arthritis	IL-10; proopiomelanocortin; soluble TNF receptor	Muscle	Mouse, rat
Gastric disorders	Gastrin	Muscle	Mouse, rat
Kidney regeneration	HGF	Muscle	Rat
Liver regeneration	HGF	Muscle	Mouse, rat
Muscle regeneration	IGF-1	Muscle	Mouse
Ocular diseases	Human tissue plasminogen activator	Corneal endothelium	Rat
Bone formation	BMP-4	Muscle	Mouse

Although electrical stimulation of muscle tissue enhances DNA transfer by inducing pore formation in muscle fibers, the continuous, non-fenestrated ECM connective tissue exhibits a low permeability to solute and large macromolecules, acting as a barrier against intravascular gene delivery. Quite simply, it prevents the necessary contact between the injected DNA and the muscle fiber. (Liu et al, 2001). Consequently, enzymatic permeabilization of the ECM could facilitate DNA diffusion, and thus increase the number of muscle fibers entering into contact with the injected DNA (McMahon et al, 2001; Memunni et al, 2002).

Specific enzymes such as hyaluronidase (HYAse) and collagenase, break down components of the ECM, and are used for muscle dissociation for the preparation of primary myoblast cultures. In 1984, HYAse was used to improve delivery of $CaCl_2$ plasmid DNA precipitates to the liver (Dubensky et al, 1984). Memunni and colleagues tested the effects of preinjecting the enzymes, collagenase, HYAse, and elastase, on gene electrotransfer. They reported a slight increase in expression mediated by collagenase that was not statistically significant, a 4-fold increase in expression mediated by HYAse, and no additive effect in mice pretreated with a mixture of the three enzymes. Thus, their findings, and those of others, indicate that pretreatment with HYAse can lead to an increase in transgene expression that is greater than that observed with other ECM-degrading enzymes (Memunni et al, 2002).

To be more specific about the mechanism involved, HYAse catalyzes the hydrolysis of the β-(1–4) linkage of hyaluronic acid, a ubiquitous constituent of

the ECM, leading to its depolymerization and causing a temporary decrease in the viscosity in the connective tissue (Memunni et al, 2002). Clinically, it is a medical preparation of highly purified bovine testicular enzyme used clinically to minimize tissue damage following nutrition solution extravasation, to reduce myocardial ischemic injury, to reduce edema caused by the rejection of transplanted organs, and in applications related to the formulation of local anesthetics. Consequently, there is no pathology associated with HYAse pretreatment, and it has been shown to both increase transfection efficiency and prolong transgene expression in skeletal muscle (McMahon et al, 2001; Memunni et al, 2002).

EP also precludes the use of viral vectors, reducing both safety problems and cost, and avoiding the problems caused by antibody formation against the viral vector. Also, as previously mentioned, plasmid DNA has stimulatory effects on the mammalian immune system that depend on the presence of unmethylated CpG dinucleotides in the prokaryotic DNA. It has been proposed that the EP of plasmid probably avoids the endosomal pathway by directly introducing the plasmid into the cytoplasm of the cell, reducing the pool of unmethylated CpG dinucleotides available for stimulation (McMahon et al, 2001).

For skeletal muscle tissues, EP provides drastically increased, localized, and sustained expression of target genes in the target muscle (Jang et al, 2004). Electrotransfer is achievable in different muscles of various species, including primates, indicating wide applicability (Mir et al, 1999). Consequently, a naked

plasmid DNA vector coupled to EP can be considered one of the best gene transfer strategies for skeletal muscle (Jang et al, 2004). Thus, we adapted an EP approach to deliver plasmids that can modulate the expression of MG29 into viable mouse muscle *in vivo*.

B. Experimental Protocols

a. *Plasmid Construction*

Initial screening experiments for RNAi probes to suppress MG29 were conducted using several synthetic siRNA oligonucleotides targeting conserved regions within the mouse *mg29* cDNA. Using a MG29-specific antibody, Western blot analysis of MG29 expression in human embryonic kidney (HEK) cells revealed one probe to be highly effective at silencing the expression MG29. This probe was used for further studies by cloning the synthesized oligonucleotides that targeted this conserved sequence in mouse *mg29* cDNA: 5'GATCCGGGTTGTACCAGGTCCAGTATTCAAGAGATACTGGACCTGGTACAACCTTTT TTGGCGCGCCG3' into the *Bam*HI and *Eco*RI restriction sites of the Lenti-RFP plasmid (GenScript Corporation, Piscataway, NJ) for synthesis of the siRNA probe, Lenti-RFP-MG29siRNA. The control plasmid used was the Lenti-RFP construct. In addition, an expression cassette for MG29 was inserted into the pCMS-mRFP plasmid (Clontech, Palo Alto, CA) using the *Xba*I and *Eco*RI restriction sites to generate the pCMS-MG29-mRFP over-expression plasmid. The control plasmid used was the pCMS-mRFP construct.

b. *Electroporation*

C57BL mice (2-5 months in age) were anesthetized with ketamine (1 mg/10 g body weight)/ xylazine (0.1 mg/10 g body weight). HYAse (10 μL of 2 mg/mL) was dissolved in PBS and injected subcutaneously into the footpads of each mouse. One hour after the HYAse injections, 30 μg naked plasmid DNA in PBS was injected into the center of each FDB muscle – the control plasmid in one FDB and the siRNA plasmid in the opposite FDB. After 15 minutes, 0.5-inch Millennia needle electrodes (UPC Medical Supplies, Inc., San Gabriel, CA, USA) were positioned subcutaneously at the proximal and distal tendons of the FBD muscles. The EP protocols used was 20 consecutive 100 V/cm pulses of 20 ms duration and 100 ms pulse interval were delivered using an ECM 830 square-wave pulse generator (BTX, San Diego, CA, USA). Mice were humanely sacrificed after 28 days and evaluated.

C. Results

In this final experiment, MG29 expression was acutely silenced in wild type FDB muscle, in order to determine its affect on SOCE function. Though western blot in the HEK cells show complete MG29 ablation (Figure 22), there was only decreased MG29 expression in the FBD muscle (Figure 23). The time for silencing this gene *in vivo* requires further optimization. In parallel studies, we also conducted experiments with restoration of MG29 into the *mg29(-/-)* muscle. For this purpose, we used EP to deliver the MG29 cDNA into the FDB muscle.

Western blot assay revealed that ample expression of MG29 can be detected within 1 day after electroporation, and the protein contained to increase up to 2 weeks following transfection (Figure 24). These studies show the feasibility for our future functional studies in correlating the expression of MG29 with the function of SOCE in skeletal muscle. These studies are the project of separate graduate students in Dr. Ma's laboratory.

Figure 21. Western blot of decreased MG29 expression in HEK cells. Top panel shows the MG29 expression. Lane 1 contains the HEK control; lane 2 contains HEK cells transfected with the MG29 expression plasmid; lanes 3 and 4 each contain a MG29siRNA probe. The bottom panel shows equal gel loading, demonstrated by α-actin expression.

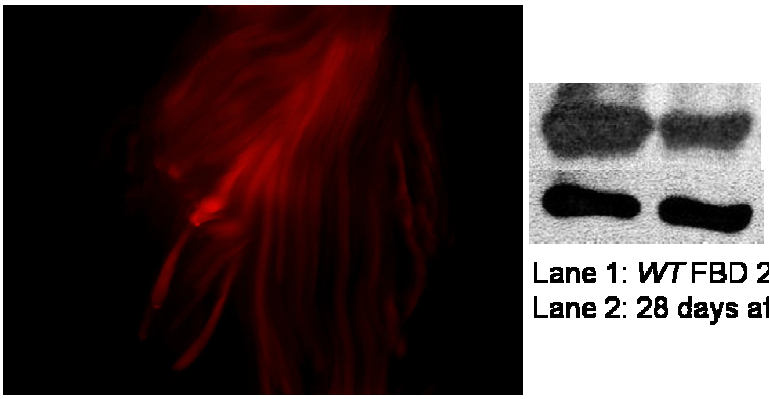
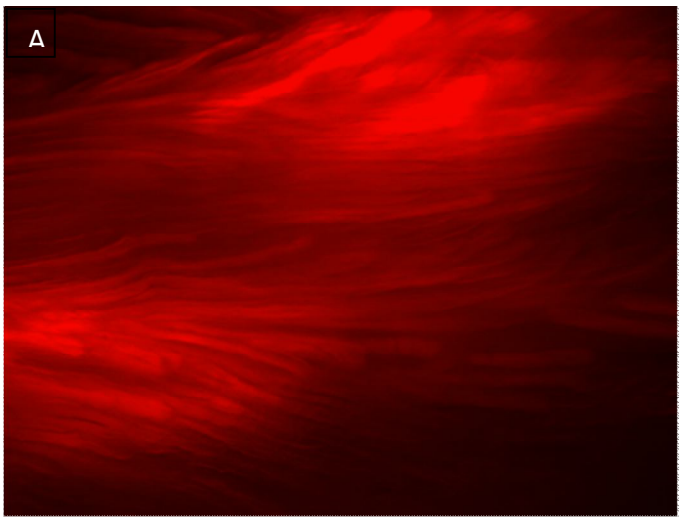


Figure 22. EP efficiency and western blot of decreased MG29 expression in *WT* FDB. Top panel is the *WT* muscle electroporated with a RFP expression plasmid in one FDB (A) and with a MG29siRNA probe in the opposite FDB (B). Equal gel loading is demonstrated by α-actin expression.



B

Lane 1: *WT* FBD control
Lane 2: 1 day after EP in *mg29(-/-)*
Lane 3: *mg29(-/-)* control
Lane 4: 7 days after EP in *mg29(-/-)*
Lane 5: 11 days after EP in *mg29(-/-)*

Figure 23. EP efficiency and western blot of increased MG29 expression in *mg29(-/-)* FBD. A) Efficiency of MG29 expression in intact FDB muscle. B) Western blot showing MG29 expression 1, 7, and 11 days after EP (lanes 2, 4, and 5, respectively), with *WT* and *mg29(-/-)* muscle samples (lanes 1 and 3, respectively) as controls. Equal gel loading is indicated by the α-actin expression levels.

D. Discussion

We obtained reduced expression of MG29 in the muscle fibers in which MG29 had been targeted by the siRNA probe, despite the fact that MG29 expression was not completely ablated. Though overexpression of MG29 was obtained as early as 1 day after EP, the few transfected muscle fibers subsequently isolated proved too fragile to survive the intracellular Ca^{2+} measurements and Mn^{2+} quenching experiments. Still, our results verify the viability of functional characterization of SOCE as a function of MG29 expression.

The morphological and functional irregularities previously described suggest that MG29 is crucial for the development of the triad junction and for successful E-C coupling. Additionally, the muscle fibers isolated from young *mg29(-/-*) mice displayed significantly compromised SOCE, mirroring the decline in SOCE observed in aged wild type muscle fibers. In fact, Weisleder and colleagues reported that both aged and *mg29(-/-*) muscle fibers display a segregated Ca^{2+} store functionally uncoupled from the normal depolarization process and that MG29 expression decreases as a function of age (Weisleder et al, 2006).

Changes in the triad junction architecture could result in a reduction in the Ca^{2+} available for contraction, which would result in a reduction in the contractile force generated. Any increase in the spacing between T-tubules and SR, would disrupt the coupling of DHPR and RyR1. Such an alteration in E-C uncoupling could be caused by the reduced expression of MG29 that occurs with aging.

In conclusion, we have shown that SOCE is the principal mechanism of extracellular Ca^{2+} entry in young healthy skeletal muscle and demonstrated that SOCE is compromised in aged skeletal muscles, which also exhibits a decreased MG29 expression, when compared to younger muscles. This reduced MG29 expression and resulting SOCE dysfunction contributes to the force decline during aging not explained by muscle atrophy. This suggests that while SOCE is a functional marker of muscle contractility and aging, MG29 is a molecular marker, supporting our proposal that *mg29(-/-)* mice can serve as a model for the study of skeletal muscle aging.

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List of Abbreviations

1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) (BAPTA)
Adeno-associated virus (AAV)
Adenovirus (AV)
Barium ions (Ba^{2+})
Bromoenol lactone (BEL)
Calcium ions (Ca^{2+})
Calcium concentration ($[Ca^{2+}]$)
Calcium release-activated Ca^{2+} current (I_{CRAC})
Capacitative Ca^{2+} entry (CCE)
Cyclopiazonic acid (CPA)
Dihydropyridine receptors (DHPRs)
Double-stranded (ds)
Extensor digitorum brevis (EDL)
Ethylene glycol tetraacetic acid (EGTA)
Electroporation (EP)
Excitation-contraction (E-C)
Extracellular matrix (ECM)
Flexor digitorum brevis (FDB)
FK506-binding protein (FKBP12)
Hyaluronidase (HYAse)
Inositol triphosphate (IP_3)
Potassium ions (K^+)
Maximal isometric tetanic force (T_{max})
Messenger RNA (mRNA)
Mitsugumin29 (MG29)
N,N,N',N'-tetrakis (2-pyridylmethyl) ethylene diamine (TPEN)
Sodium ions (Na^+)
Receptor agonist (RA)
RNA interference (RNAi)
RNA-induced silencing complex (RISC)
Ryanodine receptors (RyRs)
Sarcoplasmic reticulum (SR)
Sarcoplasmic/ endoplasmic reticulum Ca^{2+}-ATPase (SERCA)
Small interfering RNAs (siRNAs)
Soleus (SOL)
Spontaneous (Sp)
Strontium ions (Sr^{2+})
Store-operated Ca^{2+} entry (SOCE)
Store-operated channels (SOCs)
Stromal interaction molecule (STIM)
Thapsigargin (TG)

Transient receptor potential-canonical (TRPC)
Transverse tubular (T-tubule)
Voltage-induced Ca2+ release (VICR)
Wild Type (WT)

Angela M. Thornton

Curriculum Vitae

Education

08/1989 – 05/1994	Bachelor of Arts – History, Minor – American Studies; The University of Alabama, Tuscaloosa, AL
08/1989 – 05/1995	Bachelor of Science – Chemistry; The University of Alabama, Tuscaloosa, AL
01/2000 – 08/2001	Master of Science – Chemical Engineering; The University of Alabama, Tuscaloosa, AL
09/2001 – 10/2008	Doctor of Philosophy – Biomedical Engineering & Physiology and Integrative Biology; Rutgers, The State University of New Jersey; New Brunswick, NJ
07/2008 – 05/2012	Doctor of Osteopathic Medicine; Lincoln Memorial University DeBusk College of Osteopathic Medicine; Harrogate, TN

Employment

01/1991 – 05/1991	Cashier/Server, Wipplestix Restaurant; Tuscaloosa, AL
01/1992 – 12/1992	Copy Editor, The Crimson White, University of Alabama; Tuscaloosa, AL
08/1992 – 05/1995	Computer Laboratory Monitor, University of Alabama; Tuscaloosa, AL
07/1995 – 05/1996	Administrative Manager, The Electronics Boutique; Montgomery, AL
05/1996 – 06/1997	Organic Chemist, Alabama Department of Environmental Management; Montgomery, AL
07/1996 – 10/1996	Loader, United Parcel Service; Montgomery, AL
09/1997 – 01/1998	Medical Records Clerk, Druid City Hospital; Tuscaloosa, AL
01/1998 – 08/1999	Engineering Cooperative Education Participant, Universal Oil Products; Chickasaw, AL
01/1999 – 05/1999	Cashier, Rite Aid Pharmacy; Tuscaloosa, AL
02/2000 – 05/2000	Student Safety Monitor, University of Alabama Police Department; Tuscaloosa, AL
05/2000 – 08/2000	Loader, United Parcel Service; Tuscaloosa, AL
08/2003 – 05/2007	Graduate Advisor, Rutgers College Department of Residence Life; New Brunswick, NJ

Undergraduate Research Experience

01/1993 – 05/1993	Research Assistant, University of Alabama Biology Department; Tuscaloosa, AL
01/1995 – 05/1995	Research Assistant, University of Alabama Geology Department; Tuscaloosa, AL
01/1995 – 05/1995	Research Assistant, University of Alabama Chemistry Department; Tuscaloosa, AL

08/1997 – 05/1999 Research Scholar, University of Alabama Civil and Environmental Engineering Department; Tuscaloosa, AL

09/1999 – 12/1999 Research Assistant, University of Alabama Chemical Engineering Department; Tuscaloosa, AL

Graduate Research Experience

01/2000 – 08/2001 Master's Thesis: "Synthesis and characterization of pH-sensitive hydrogels for their potential use in the targeted delivery of cardiovascular drugs," Chemical Engineering Department, University of Alabama; Tuscaloosa, AL

06/2002 – 08/2002 Research Intern, Center for Engineering in Medicine: "Synthesis and characterization of pH-sensitive influenza hemagglutinin fusion domain," Boston's Shriners Hospital; Boston, MA

08/2002 – 08/2003 Research Assistantship: "Design, expression and characterization of environmentally-sensitive Viral Protein Linear (VPL) Motors as nanorobotic components," Biomedical Engineering Department, Rutgers University; Piscataway, NJ

06/2003 – 08/2003 Research Intern, Center for Engineering in Medicine: "Biomolecular Nanorobotics: Generation of intrinsically fluorescent viral protein linear motors through site-directed mutagenesis and error-prone polymerase chain reaction," Boston's Shriners Hospital; Boston, MA

10/2003 – 10/2008 Doctoral Research Project: "Relationship of mitsugumin 29-deficiency to store-operated calcium channel dysfunction in skeletal muscle cells." Biomedical Engineering Department, Physiology and Integrative Biology Department, Rutgers University, UMDNJ/ RWJMS Graduate School of Biomedical Sciences; Piscataway, NJ

Teaching Experience

08/2000 – 12/2000 Graded homework, proctored exams, lectured for heat transfer course (CHE 306), Chemical Engineering Department, University of Alabama; Tuscaloosa, AL

01/2001 – 05/2001 Recitation and tutoring for undergraduate basic chemical engineering laboratory (CHE 319), Chemical Engineering Department, University of Alabama; Tuscaloosa, AL

05/2001 – 07/2001 Supervised heat transfer experiments, proctored final exam for undergraduate summer chemical engineering unit operations laboratory (CHE 320), Chemical Engineering Department, University of Alabama; Tuscaloosa, AL

09/2002 – 12/2002 Designed gene transfer laboratory, assisted in development of protein quantification laboratory for junior-level

	biomedical engineering course, Biomedical Engineering Department, Rutgers University; Piscataway, NJ
01/2003 – 05/2003	Developed and taught gene transfer laboratory for undergraduate biomedical measurements and analysis laboratory (14:125:315), Biomedical Engineering Department, Rutgers University; Piscataway, NJ

Publications

AM Thornton and CS Brazel. "Design of pH-Sensitive Materials for On/Off Release of Thrombolytic and Anticoagulant Drugs," E Mathiowitz, S Mallapragada, B Narasimhan, R Korsmeyer, Eds. *Cardiovascular Biomaterials*, Materials Research Society, Pittsburgh, December 2000.

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