ROLE OF LEK1 IN SKELETAL MYOGENESIS

By

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To all scientists who work for world peace and human happiness.

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Satellite cells are skeletal muscle stem cells that play a major role in postnatal muscle growth and repair. In healthy muscles, most satellite cells are in an inactive quiescent state and localized between the sarcolemma and basal lamina of the muscle fibers. Upon stimulation, such as muscle injury or disease, they become activated and muscle regeneration occurs via activation, proliferation, differentiation, and central localization of satellite cells. In spite of their active recruitment during muscle regeneration, the absolute number of quiescent satellite cells remains unchanged, suggesting the maintenance of satellite cell pool by the self-renewal. In mouse, a novel centromere protein LEK1 significantly affects cellular proliferation and differentiation, however, its role depends on the cell type and developmental stage. In C2C12 myoblasts, ectopic expression of dominant inhibitory C-terminus portions of LEK1 protein significantly stimulated proliferation and inhibited differentiation, suggesting the inhibitory and stimulatory roles of LEK1 in myoblast proliferation and differentiation, respectively. On the other hand, the effects of dominant inhibitory LEK1 on myoblast differentiation depend on its size in *MyoD*-treated C3H10T1/2 cells. Smaller LEK1 mutant (22 kDa) had no effect while larger ones (28 and 47 kDa) significantly inhibited differentiation, implicating that there may be an important domain between 22 and 28 kDa of C-terminus most LEK1 for myoblast

differentiation. During muscle regeneration, expression of LEK1 protein was elevated in the most heavily damaged sites during the proliferation phase of muscle regeneration and maximal in the sarcoplasm of regenerated muscle bed during the differentiation phase. This observation supports the notion that LEK1 inhibits myoblast proliferation and stimulates differentiation. Interestingly, virtually all LEK1 proteins translocated to the central nuclei while some of them were found with the myonuclei in the satellite cell niche in the fiber growth phase. These data implicate that LEK1 may play multiple roles during muscle regeneration, including inhibition of proliferation, stimulation of differentiation, central translocation of myonuclei, self-renewal, and maintenance of a quiescence state of satellite cells.

CHAPTER 1
LITERATURE REVIEW

Introduction

Human and animal activity requires movement via skeletal muscle contraction. Function of respiratory muscles is necessary to maintain their lives. The major function of skeletal muscle is to cause the movement of limb and organs. However, skeletal muscle alone cannot achieve this goal. For voluntary contraction, signal generated in the brain is transmitted to skeletal muscle via central nervous system, motor neuron, and excitation-contraction (EC) coupling. Then, force generated by skeletal muscle is transmitted to the tendon and bone. Skeletal muscle function depends on many physiological factors and it is impaired when even one of these factors does not function. Therefore, it is not surprising that skeletal muscle function highly depends on muscle mass (Reed et al., 1991; Harris, 1997; Elkin et al, 2000), bone mass, and bone strength (Schiebl, et al., 1996). These variables are deteriorated by aging (Frontea et al, 1991; Jubrias et al., 1997), muscle disuse (Selsby et al, 2007), disease (Emery, 2002), injury (Dodd et al, 2005), or microgravity exposure (Martin et al, 1988). To maintain the functional muscle mass, muscle growth and repair are necessary. This role is mainly played by muscle stem cells (i.e., satellite cells) with help of other molecules. In this section, skeletal muscle structure/function, satellite cell biology, and the role of a novel centomere protein in skeletal myogenesis will be briefly reviewed and discussed.

Skeletal Muscle Biology

Muscle Structure

Skeletal muscle fibers are attached to the bone via the tendon and transmit force generated by their contraction. A number of fibers are innervated by a single motor neuron and the combination is called a motor unit. Most muscles have hundreds of motor units. Each fiber

consists of a number of myofibrils which are surrounded by sarcoplasmic reticulum (SR). SR releases Ca^{2+} when stimulated and becomes wider and shorter during muscle contraction. The transverse tubule (T-tubule) resides perpendicular to the longitudinal axis of muscle fibers and encircles myofibrils at regular intervals. In mammalian skeletal muscle cells, it lies at the junction of the A- and I-bands. SR is dilated on both sides of the T-tubule (i.e., terminal cisternae) and a T-tubule and both sides of terminal cisternae form the triad (McComas, 1996).

Force generation (measured as the maximal tetanic tension) is directly proportional to the sum of the cross-sectional areas (CSA) of all the muscle fibers within a muscle (i.e., physiological CSA) and also affected by the angle of fiber insertion relative to force generation axis (i.e., pennation angle). The pennation angle varies among muscle types and ranges from 0° to approximately 30° (Lieber, 1992). When the fiber orientation is parallel to the force generation axis (i.e., fusiform), all the force is transmitted to the tendon. On the other hand, when fibers are inserted into the tendon with pennation angle, only a portion of generated force is transmitted. Two pennation angles are formed when fibers are inserted into both sides of the tendon (i.e., bipennate). Some muscles are unipinnate while the others are bipinnate. The advantage of pennation angle is that it allows the muscle to increase physiological CSA by packing the greater number of fibers. In a bipennate muscle, the amount of tendon movement is greater than that of muscle fiber shortening during muscle contraction (Gans and Gaunt, 1991).

Myofibrils are arranged in parallel and composed of thousands of sarcomeres. Myofibrils contain thick and thin filaments, also referred to as myosin and actin filaments, respectively. Individual myosin molecules spontaneously aggregate with each other to form a double-helical rod of myosin filaments. A pair of globular heads are projected from a myosin molecule to form a cross bridge. These heads of the myosin molecule are separated by 60° of rotation from those

projected from the next myosin molecule (McComas, 1996). Two light chains are associated with each myosin head and believed to affect the action of myosin heads. The pair of myosin heads are called subfragment 1 (S-1) and are linked to the myosin molecule via a neck region called subfragment 2 (S-2). A head portion including S-1 and S-2 is called heavy meromyosin (HMM) and a remaining tail portion is called light meromyosin (LMM). Each myosin filament is surrounded by a hexagonal array of actin filaments. Each actin filament is composed of a number of actin monomers arranged in a long helical structure. This structure creates a long groove along with it into which tropomyosin filaments fit. Each tropomyosin molecule spans approximately seven actin molecules. A complex of three polypeptides (i.e., troponin T, C, and I) is also located on every seven actin monomers. Troponin T attaches the troponin complex to tropomyosin while troponin I indirectly inhibits acto-myosin interaction by binding to actin in the absence of calcium ion (Lieber, 1992). When cytosolic calcium level is raised, troponin C captures four calcium ions and undergoes conformational change to move tropomyosin away from actins, exposing actins to myosin heads and thus promoting muscle contraction (McComas, 1996). These thick and thin filaments are longitudinally arranged and partially overlapped with each other in a resting muscle. In the regions without acto-myosin overlap, actin and myosin filaments exhibit light (i.e., I-band) and dark (i.e., H-zone) appearance, respectively. The overlapped region is the darkest and the sum of this region and the H-zones forms the A-band. The central portion of the H-zone is called the M-region where myosin filaments are cross-linked and regular spacing between them is maintained. Another filament protein titins longitudinally stabilize myosins by linking them to the Z-disks. Actin filaments are strengthened by nebulins which resides in parallel to actins. Finally, intermediate filaments link Z-disks longitudinally and transversely, keeping all the myofibrils in register (McComas, 1996).

Muscle Fiber Types

Skeletal muscle is composed of thousands of fibers (McComas, 1996) and most mammalian muscles contain multiple fiber types (Lieber, 1992). Muscle fibers exhibit four different myosin isoforms and can be classified in terms of contractile and metabolic properties as well as their color (Zierath and Hawley, 2004). Slow-contracting fibers are called slow-twitch or type I fibers while fast-contracting fibers are designated as fast-twitch or type II fibers. Type II fibers are further categorized into type IIa, IIx (or IId), and IIb fibers based on their contractile and metabolic properties (Schiaffino and Reggiani, 1994). Type I fibers are characterized by higher content of mitochondria and myoglobin with higher capacity of aerobic metabolism than type II fibers. Therefore, type I fibers can produce energy without lactate accumulation and are less fatigable (Zierath and Hawley, 2004). In contrast, type II fibers utilize glycolytic pathway for energy production and are more fatigable. Type I and type IIa fibers are more reddish and called red fibers due to the higher content of myoglobin while type IIx and IIb fibers are more pale and called white fibers (Zierath and Hawley, 2004). Interestingly, the fiber type depends on a motor neuron attached. When motor neurons attached to fast- and slow-twitch fibers are surgically exchanged via cross-reinnervation, they are converted to slow- and fast-twitch fibers, respectively (Buller et al., 1960a).

Skeletal Muscle Function

Skeletal muscle functions via contraction, which is achieved via intention to move, activation of central nervous system (CNS) and motor neuron, and EC coupling. When a signal in form of action potential (AP) is transmitted to an axon terminal of a motor neuron, voltage-dependent calcium channels open and calcium ions enter into the motor neuron, which causes exocytosis of acetylcholine (ACh)-containing vesicles releasing ACh into the synaptic cleft at the neuromuscular junction (Dodge and Rahamimoff, 1967). ACh diffuses across the synaptic cleft

and binds to the ACh receptor (ACh-R) on the motor end plate of muscle. When two ACh molecules bind to ACh-R, the receptor opens the ion channel allowing Na^+ to enter and K^+ to leave the muscle cell. The resulting depolarization of sarcolemma propagates in both directions and reaches the deeper portion of the fiber via T-tubules. This stimulates SR and Ca^{2+} is released into the cytosol via unknown mechanisms (Lieber, 1992). It has been suggested that the voltage sensitive dihydropyridine (DHP) channel in the T-tubule membrane (Fosset et al., 1983) senses the APs propagated through sarcolamma and transmits the signal to the ryanodine receptor (RYR). Then, the DHP channel unplugs the RYR channel (Chandler et al., 1976) or lifts the top of the RYR (Rios and Gonzalez, 1991), allowing Ca^{2+} to be released from SR into the cytosol of muscle cells. Once the cytosolic calcium level is elevated, myosin heads bind to actins and muscle contraction is triggered via the troponin-tropomyosin interaction. This conversion of electrical stimulus to mechanical response is called EC coupling (McComas, 1996).

The sliding filament hypothesis has been well accepted as the muscle contraction mechanism. According to this theory, the opposing actin filaments slide along myosin filaments and pulled toward each other, shortening the H-zone and I-band (Huxley and Niedergerke, 1954). Furthermore, it has been proposed that the level of force production depends on the amount of cross-bridge interactions caused by the overlap between myosin and actin filaments. This hypothesis was based on the relationship of sarcomere length with tension in frog muscle fibers. Over a certain range, tension development was proportional to the degree of the overlap while extreme shortening caused decreased tension. In the latter case, the opposing actin filaments overlap each other and may interfere with the cross-bridge mechanism (Gordon et al, 1966a). On the other hand, several mechanisms have been proposed for the myosin cross-bridge cycle. The common factor among these theories is that adenosine-5'-triphosphate (ATP) on

myosin head is required to detach an actin from an acto-myosin complex (Lymn and Taylor, 1971; Huxley and Simmons, 1971; Rayment et al., 1993). The action order of one of the well explained mechanisms is 1) ATP binds to the acto-myosin complex; 2) the actin dissociates from the myosin-ATP complex; 3) ATP is hydrolyzed on the myosin; 4) the actin associates with the myosin-ADP-Pi complex; 5) myosin cross bridge generates force during power stroke (Lymn and Taylor, 1971).

Satellite Cell Biology

Myogenesis

Satellite cells are skeletal muscle stem cells that are responsible for postnatal muscle growth and repair. They are localized between the sarcolemma and basal lamina of the muscle fibers (Mauro, 1961). Although other stem cell types, such as bone marrow cells, can give rise to myonuclei of regenerating myofibers (Gussoni et al., 1999), satellite cells are the main sources of myonuclei (Moss and Leblond, 1971). Satellite cells are metabolically more active with abundant cytoplasm in young muscles but become inactive as animals get older (Schultz, 1976). In mice, the percentage of satellite cell nuclei in total myonuclei (i.e., the sum of satellite cell nuclei and myonuclei) is approximately 30% at birth but decreased to about 5% after 2 months (Snow, 1977; Bischoff, 1994). Decline of the percentage with age (Campion et al., 1981) indicates fusion of satellite cells for postnatal muscle growth (Gibson and Schultz, 1983). On the other hand, the absolute number of quiescent satellite cells remains unchanged during muscle regeneration (Gibson and Schultz, 1983), suggesting the maintenance of satellite cell pool by the self-renewal. The percentage of satellite cell nuclei in total myonuclei is affected by the species, age, fiber type, disease, and exercise. It is much higher in soleus muscles of rats than those of mice (Snow, 1977; Gibson and Schultz, 1983). In both mouse and rat soleus muscles, it is significantly reduced as they get older (Snow, 1977; Gibson and Schultz, 1983). In both humans

and rats, it is higher in slow- than fast-twitch fibers (Schmalbruch and Hellhammer, 1977; Kadi et al., 2006). In human skeletal muscles, Duchenne's muscular dystrophy (DMD) patients have higher satellite cell content than control patients (Watkins and Cullen, 1988). Finally, resistance training significantly increases the satellite cell number in human skeletal muscles (Kadi and Thornell, 2000). In healthy muscle, most satellite cells are in an inactive quiescent state. Upon stimulation, such as muscle injury or disease, they become activated and enter the cell cycle. Muscle repair is a multi-step process including muscle degeneration and regeneration. During degeneration, phagocytes (i.e., neutrophils and macrophages) invade injured muscle within 6 and 48 hours, respectively. Neutrophils ingest cellular debris while macrophages ingest neutrophils. (Orimo et al., 1991; Fielding et al., 1993; Charge and Rudnicki, 2004). The muscle degeneration phase is followed by the regeneration phase. Activated satellite cells expand in number via cell division (Hawke and Garry, 2001). Once satellite cells are activated and proliferate, they are often referred to as “myogenic precursor cells” or MPCs (Charge and Rudnicki, 2004). A majority of MPCs exhibit rapid proliferation and become fusion-competent myoblasts (Conboy et al., 2003). They may fuse with either existing damaged fibers (i.e., hypertrophy) or other MPCs (i.e., hyperplasia). However, skeletal muscle regeneration is achieved mostly by hypertrophy (Hawke and Garry, 2001). It has been suggested that a slowly dividing population of MPCs returns to the quiescent sate, replenishing the satellite cell pool. These cells lose the expression of MyoD but maintain that of paired-box transcription factor, Pax7 (Zammit et al., 2004). Following fusion, nuclei of myoblasts translocate to the central portion of myofibers and provide fibers with new myonuclei for muscle fiber growth. After the fibers grow and obtain the sufficient size, myonuclei move to the periphery of the fibers, which are now indistinguishable from other undamaged fibers (Charge and Rudnicki, 2004).

Myogenic Regulatory Factors

Satellite cell myogenesis requires muscle gene transcription to be activated, which is primarily mediated by the MyoD family transcription factors called myogenic regulatory factors (MRFs). Four members of this family (i.e., MyoD, Myf5, myogenin, MRF4) play major roles in the regulation of muscle genes. Upon activation of satellite cells, either *MyoD* or *Myf5* genes are expressed first and then *MyoD* and *Myf5* genes are coexpressed. Subsequently, *myogenin* gene is expressed as cells proceed along the myogenic lineage toward terminal differentiation (Cornelison and World, 1997). In general, MyoD and Myf5 serve as myogenic specification factors while myogenin mediates terminal differentiation by activating late muscle genes (Gerber et al., 1997). MRF4 plays a dual role in specification and differentiation (Penn et al., 2004). However, previous studies using MRF knockout mice suggest that roles of MRFs are both specific and redundant (Penn et al., 2004). *MyoD* knockout mice are viable and exhibit close to normal phenotype with upregulated Myf5 expression (Rudnicki et al., 1992) while *Myf5* knockout mice show perturbed muscle regeneration with moderately impaired proliferation of satellite cells (Gayraud et al., 2007). In contrast, *myogenin* knockout mice are devoid of skeletal muscle and die immediately after birth (Hasty et al., 1993). *MRF4-myogenin* double knockout mice are deficient in the fiber size but sufficient in the fiber number compared to those of wild type (WT). Both *MyoD-Myf5* (Rudnicki et al., 1993) and *MyoD-MRF4* (Rawls et al., 1998) double knockout mice exhibit severe muscle deficiency similar to that of *myogenin* knockout mice. Finally, *MyoD-myogenin-MRF4* triple knockout mice maintain a number of myoblasts but were deficient in skeletal muscle (Valdez et al., 2000). Integrated interpretation of these evidences is very difficult. However, it is likely that MRFs play both specific and compensatory roles in transcription of muscle genes. MRFs possess the basic helix-loop-helix (bHLH) domain in which two α-helices are connected by a loop. This structural motif is responsible for

dimerization and DNA binding (Shirakata et al., 1993). Each MRF dimerizes with a E protein (i.e., E12 or E47) and then binds to an E-box DNA consensus sequence CANNTG, stimulating muscle gene transcription (Chanoine et al, 2004; Heidt et al., 2007). However, the precise mechanisms for recognizing their target genes remain unclear. Interestingly, MRFs interact with each other. For instance, MyoD binds to the E-box in the *myogenin* promoter and activates transcription of *myogenin* (Heidt et al., 2007). Therefore, MyoD is required for proper *myogenin* expression. In addition to MRFs, a second family of muscle-specific transcription factors, myocyte enhancer factor-2 (MEF2) family also regulate muscle gene expression by associating with MRF-E protein heterodimmers. MEF2 can associate with only MRFs dimerized with E proteins and alanine and threonine in the center of MRFs mediate this interaction (Black and Olson, 1998; Nava and Olson, 1999). MyoD and Myf5 are 10-fold more efficient than myogenin at activating transcriptionally silent genes. Functional specificity of MRFs may depend on the structural difference in the transactivation domains of MRFs to form a muscle gene activator complex (MAC) (Chanoine et al, 2004). Acetylation, phosphorylation, and chromatin remodeling regulate the transcriptional activity of MyoD. Histone acetyltransferases (HATs) acetylate MyoD as well as nucleosomal histones around E-boxes, which promotes DNA binding and transcriptional activity of MyoD (Puri and Sartorelli, 2000; Chanoine et al, 2004). Twist, a bHLH protein, prevents HAT-induced stimulation of transcriptional activity of MyoD by binding to HATs (Hamamori et al., 1999). Histone deacetylases (HDACs) deacetylate MyoD and inhibit expression of MyoD-dependent muscle-specific genes in undifferentiated skeletal muscle (Mal et al, 2001). Phosphorylation of MyoD by Mos protein (encoded by proto-oncogene c-Mos) also stimulates the transcriptional activity of MyoD by promoting binding of MyoD to E12 during proliferation (Pelpel et al., 2000). Furthermore, MyoD can remodel chromatin at the binding sites

in the muscle gene enhancers to activate transcriptionally silent loci (Gerber et al., 1997). In contrast, chromatin remodeling enzymes prevent MRFs from accessing DNA (Chanoine et al., 2004). Thus, activation of the muscle transcriptional program via MRFs is highly regulated by posttranslational mechanisms.

Roles and Expression Pattern of Pax7 in Myogenesis

Several satellite cell markers have been identified to study satellite cell biology (Hawke and Garry, 2001). Among them, Pax7, c-Met, and M-cadherin are expressed both in quiescent and proliferating satellite cells (Cornelison and Wold, 1997; Seale et al., 2000). The fact that approximately 5% of total myonuclei in adult skeletal muscle are satellite cell nuclei (Snow, 1977; Bischoff, 1994) and about 5% of total myonuclei also express transcription factor Pax7 in a quiescent state (Seale et al., 2000) indicates Pax7 is an appropriate marker of quiescent satellite cells. In *Pax7* knockout mice, skeletal muscles lack satellite cells and animals normally die within 2 weeks after birth with deficiency in muscle growth (Seale et al, 2000), suggesting the importance of Pax7 in the maintenance of satellite cell pool (Buckingham, 2006). Pax7 is also thought of as a marker of proliferating satellite cell since it is expressed in a majority of mitotically active cells but its expression is reduced as the cells transcribe *myogenin* and differentiate (Halevy et al., 2004). During skeletal muscle regeneration, Pax7 is widely expressed in regenerating fibers and even in some central nuclei (Luque et al., 1996; Seale et al., 2000). The number and characteristics of satellite cells in regenerating muscle become comparable to those of WT within 30 days following injury-induced skeletal muscle regeneration (Luque et al., 1996). Recent studies have implicated that Pax7 plays a role for myogenic specification, maintenance of quiescence, and survival of satellite cells (Seale et al., 2000; Olguin and Olwin, 2004; Oustanina et al., 2004). Most activated satellite cells express Pax7, MyoD, and Myf5 (Zammit et al., 2002; Zammit et al., 2006) while Pax7 is downregulated as myoblasts

differentiate (Seale et al, 2000; Zammit et al., 2004). Indeed, cells do not differentiate in the presence of Pax7 (Zammit et al., 2006). It has been suggested that Pax7 promotes the entry of myoblasts into the myogenic program by stimulating transcription of *MyoD* (Buckingham, 2007) and regulating *Myf 5* (McKinnell et al., 2008). Pax7 can elevate MyoD expression (Zammit et al., 2006) and turns on the myogenic gene program via chromatin modifications in satellite cell-derived myoblasts. It associates with histone methyltransferase (HMT). The Pax7-HMT complex then binds to Myf5 and tri-methylates histone H3 lysine of surrounding chromatin (McKinnell et al., 2008). Therefore, it is believed that *Pax7* is genetically upstream of *MyoD* and *Myf5* (Zammit et al., 2006; Buckingham, 2007) in a majority of activated satellite cells. On the other hand, a minority of activated satellite cells maintain Pax7 but lose MyoD (Zammit et al., 2006). Pax7 is upregulated in cells that lack MyoD (Seale et al, 2000), exit the cell cycle, and escape from differentiation (Olguin and Olwin, 2004). In addition, overexpression of Pax7 downregulates MyoD, prevents myogenin induction, promotes cell cycle exit (Olguin and Olwin, 2004), and slows both proliferation and differentiation in myoblasts (McFarlane et al., 2008). Therefore, it has been suggested that Pax7 helps these cells return to quiescence (Olguin and Olwin, 2004; Zammit et al., 2006). Finally, Pax7 may also play an anti-apoptotic role since the expression of dominant negative Pax7 mutant protein destroys satellite cells (Oustanina et al., 2004; Relaix et al., 2006). In summary, Pax7 may turn on the myogenic gene program by stimulating transcription of *MyoD* and associating with *Myf 5* in satellite cells. It maintains proliferation, prevents precocious differentiation, and promotes survival of satellite cells. On the other hand, in a minority of activated satellite cells, it may promote the self-renewal of satellite cells by inhibiting proliferation and differentiation via downregulation of MyoD and myogenin.

Myogenic Capacity of Satellite Cells

One of the best methods to estimate the myogenic capacity of satellite cells is transplantation of cells into regenerating host skeletal muscles. The myogenic capacity almost entirely depends on the environment of satellite cells, which has been best shown by single fiber transplantation (Collins et al., 2005). Satellite cells reside in the stem cell niche between the sarcolemma and basal lamina in healthy muscles. When this environment is kept in single fiber transplantation, a single satellite cell of the transplanted fiber generated 27 myofibers on the average in the host muscle. In contrast, when satellite cells immediately after isolation from skeletal muscle were transplanted, each satellite cell produced approximately 1/1,000 of myofibers (Montarras et al., 2005). Furthermore, when isolated satellite cells were expanded with 3 passages, only 1/10,000 of myofibers were generated (Ikemoto et al., 2007). It is plausible that this is due to a enzymatic treatment of cells since cell expansion without passage did not further deteriorate the myogenic capability of satellite cells (Ikemoto et al., 2007). These studies demonstrate that a quiescent condition with an appropriate environment is extremely important for satellite cells to exert their myogenic capability during skeletal muscle regeneration.

LEK Family Proteins

Centromere Proteins

During mitosis, the mitotic spindle attaches to the kinetochore on the centromere and pulls the chromosome apart (i.e., chromosome segregation). The kinetochore remains assembled from the G2 phase to the end of cell division (Bomont et al., 2005). The kinetochore consists of more than 50 proteins (Hauf and Watanabe, 2004) including centromere proteins (CENPs). The inner plate of the kinetochore contains CENP-A, -B, -C, -G, -H, and -I and plays a role in kinetochore assembly while the outer plate possesses CENP-E and –F and contributes to microtubule attachment and dynamics (Bomont et al., 2005). The kinetochore assembly links the

centromere to the spindle and the onset of anaphase is held until all the chromosomes attach to the microtubules at the kinetochore. Thus, failure to form centromere-kinetochore-spindle complex results in mitotic arrest (Cleaveland et al., 2003) and kinetochore defect results in choromosome missegregation and aneuploidy (i.e., the abnormal number of chromosomes in daughter cells), suggesting an important role of the kinetochore in separation of sister chromatids (Hauf and Watanabe, 2004).

LEK Family Proteins

Centromere protein-F (CENP-F) or mitosin was discovered as a kinetocore-associated protein whose expression is cell cycle dependent (Rattner at al., 1993). *CENP-F* genes are well conserved among many species and proteins are called CENP-F/mitosin in human, LEK1 in mouse, and cardiomyogenic factor 1 (CMF1) in chicken. Approximately 40% of the amino acids of these proteins are composed of leucine (L), glutamic acid (E), and lysine (K). Therefore, these proteins are called LEK family proteins (Goodwin et al., 1999). The C-terminus domains of these proteins are well conserved (Zhu et al., 1995; Hussein and Taylor, 2002; Redkar et al, 2002; Ashe et al., 2004) and contain an atypical retinoblastoma (Rb)-binding domain, nuclear localization sequence (NLS), bHLH domain, and leucine zippers (Goodwin et al., 1999; Ashe et al., 2004). These LEK family proteins bind to a cell cycle inhibitor, protein Rb or pRb (Zhu et al., 1995; Redkar et al., 2002; Ash et al., 2004), suggesting a potential role of LEK family proteins in cell cycle progression. During development, expression of LEK1 and CMF1 proteins are elevated in the early proliferation phase while it is reduced in the later differentiation phase (Goowin at al, 1999; Dees et al., 2000). In contrast, CENP-F is not well studied during development. CNEP-F is not expressed in the G1 phase of the cell cycle while LEK1 and CMF1 are expressed throughout the cell cycle.

CNEP-F/Mitosin

While CENP-F is not expressed in the G1 phase, it is localized in the nucleus during the S-phase. It is assembled into the kinetochore in the late G2 phase and degraded at the end of mitosis (Liao et al., 1995). CENP-F assembles earlier than other known transient kinetochore proteins in the late G2 phase and thus may play a role in the initial steps of kinetochore assembly (Bomont et al., 2005). CNEP-F is required for M-phase progression and inhibition of its expression causes mitotic arrest (Bomont et al., 2005). CENP-F interacts with mammalian nuclear distribution element (NudE) homologues (i.e., Ndel-1, Nde-1) which bind to Lis 1 and dynein. Cytoplasmic dynein transports cellular cargo by walking along microtubules towards the cell center (i.e., minus end) and plays a role in transportation of chromosome, mitotic spindle, and other organnelles. The interaction among CENP-F, NudE, Lis 1, and dynein is critical for chromosome alignment and segregation. Disruption of these components results in mal-alignment/orientation of choromosome (Vergnolle and Taylor, 2007). Importantly, CENP-F maintains the microtubule-kinetochore tension and attachment (Bomont et al., 2005).

LEK1 Protein Structure

The **s**tructure of LEK1 is very similar to that of CENP-F and characterized by the well conserved C-terminus domain containing the Rb-binding domain, NLS, and bHLH domain (Figure 3-1). Among these, the Rb-binding domain plays an important role in regulation of cellular proliferation and differentiation since pRb inhibits the cell cycle. NLS is responsible for protein targeting to the nucleus. The bHLH domain is a dimeric protein structural motif in which two α-helices are connected by a loop and one of the helices contains a DNA-binding region. Computer analysis has shown that the structure of this protein is mostly α-helices with heptad amino acid repeats except the globular C-terminus portion. The α-helices are separated by turns

and the repeats are composed of hydrophobic and hydrophilic amino acids. Many of these α-helices are leucine zippers and leucine resides in the 4th position of the heptad. Thus, leucine resides every 7 amino acids and one leucine binds to the other per 2 turns (Goodwin et al., 1999). The known function of leucine zipper includes protein dimerization and DNA binding. It is predicted that α-helices of this protein fold into four coiled coils. LEK1 also possesses a spectrin repeat in the N-termius portion which is three-helix bundle structure and coordinates cytoskeletal interactions (Djinovic-Carugo et al., 2002).

Subcellular Localization of LEK1

LEK1 protein is posttranslationally cleaved into larger N- and smaller C-terminus portions. The N-terminus portion of LEK1 (nLEK1) has cytoplasmic distribution while the C-terminus portion (cLEK1) is targeted to the nucleus (Ashe et al., 2004; Soukoulis et al., 2005; Pooley et al., 2006). Nuclear cLEK1 is further cleaved into approximately 50 and 60 kDa fragments in 23A2 myoblasts when the cell cycle is arrested by stimulation of MAPK signaling. When the MAPK signaling pathway is stimulated in mitotically active myoblasts, cells exit the cell cycle and LEK1 protein translocates from the cytosol to the nucleus (Reed et al., 2007). On the other hand, cytosolic nLEK1 is colocalized with NudE, Lis1, and dynein and disruption of nLEK1 results in abnormal cell shape and microtubule organization, suggesting a role of nLEK1 in chromosome alignment and segregation (Soukoulis et al., 2005). nLEK also interacts with synaptosomal-associated protein of 25 kDa (SNAP-25) and plays a role in recycling of transport vesicles that mediate trafficking of proteins between plasma membrane and organelles (Pooley et al., 2006; Söllner et al., 1993).

LEK1 and Cell Cycle

The role of LEK1 protein in cellular proliferation has been suggested by several *in vivo* and *in vitro* studies. However, a significant discrepancy exists among studies due to the difference in the cell type or developmental stage of the cells. These suggestions were based on the localization of LEK1 protein in certain conditions, the dominant inhibitory effects of LEK1 mutant proteins on proliferation, or the effects of LEK1 depletion on cell cycle progression using morpholino oligomers to disrupt translation of LEK1 mRNA in certain cell types. In satellite cells and myoblasts, the evidence suggests that LEK1 suppresses cell cycle progression. In the section of mouse tibialis anterior muscle (TA), LEK1 was localized with satellite cell marker Pax7 in the nucleus of quiescent satellite cells as well as other myonuclei (Reed et al., 2007). In 23A2 myoblasts and primary culture of satellite cells, LEK1 was cytosolic when the cells were mitotically active. However, it was translocated from the cytosol to the nucleus when these cells were mitotically inactive (Reed et al., 2007). This study indicates that LEK1 suppress proliferation in these cell types. In the dominant inhibitory studies, DNA constructs containing various sizes of genes encoding cLEK1 were introduced into the cells so that the cells express LEK1 mutants which interact with the same elements as endogenous LEK1 protein (e.g., pRb). These mutants can compete with WT LEK1 protein and inhibit some of its function (i.e., dominant inhibitory effects). The ectopic expression of cLEK1 (85 kDa) significantly reduced incorporation of 5-bromo-2-deoxyuridine (BrdU) in COS-7 fibroblasts (Evans et al., 2007). In other words, competitive inhibition of endogenous LEK1 reduced proliferation in this cell type, suggesting that LEK1 protein promotes proliferation in COS-7 cells. In other studies supporting this notion, LEK1 was localized in the nuclei in proliferating C2C12 mouse muscle myoblasts (Goodwin et al, 1999) and its expression was elevated in embryonic and neonatal mouse heart during the proliferation phase (Dees et al., 2005; Goodwin et al., 1999). In addition, blockage of

LEK1 expression using morpholino oligomers reduced proliferation in HL1 cardiac cells (Dees et al., 2005), NIH3T3 mouse embryonic fibroblasts, and C2C12 myoblasts (Ashe et al., 2004). These *in vitro* and *in vivo* studies suggest that LEK1 promotes proliferation in these cell types and the developmental stages of cardiomyocytes. In summary, the role of LEK1 protein in cellular proliferation highly depends on the cell type and the developmental stage of the cells.

LEK1 and Cellular Differentiation

The role of LEK1 in cellular differentiation also depends on the cell type and the developmental stage of cells. In cardiomyocytes differentiated from embryonic stem (ES) cells, deletion of pRb, ectopic expression of dominant inhibitory cLEK1 (ΔLEK1), or combination of both almost completely blocked differentiation (Papadimou et al., 2005), suggesting that LEK1-pRb binding is necessary for differentiation of embryonic cardiomyocytes. Indeed, overexpression of pRb rescued the differentiation-deficit ΔLEK1- expressing *Rb* knockout cells. This study implicates that the LEK1-pRb interaction promotes differentiation and the presence of ΔLEK1-pRb complex does not interfere with differentiation when the amount of LEK1-Rb complex is sufficient in this cell type and the developmental stage. In contrast, LEK1 expression was reduced during the differentiation phase (after 7 days of birth) in neonatal mouse heart (Dees et al., 2005) and the ectopic expression of ΔLEK1 (165 kDa) significantly promoted differentiation in C2C12 myoblasts (Goodwin et al, 1999), suggesting that LEK1 negatively regulates differentiation in these cell types. This discrepancy may be due to the difference in the cell type, developmental stage of cells, or the size of ΔLEK expressed in the cells (i.e., 7 kDa vs. 165 kDa).

LEK1 and pRb

Like other LEK family proteins, LEK1 protein binds to pRb via the Rb-binding domain (Goodwin et al., 1999). Through this site, exogenous cLEK1 also binds to pRb (Ashe et al., 2004). pRb is a member of the Rb proteins (p107, p130, and pRb) which are also referred to as the pocket proteins (Chen et al., 2008). pRb is a tumor suppressor protein and cell cycle inhibitor (Murphree and Benedict, 1984). Tumorigenesis occurs in *Rb*-mutant mice and more than one-third of all human tumors are caused by the absence or mutation of pRb (Weinberg, 1992). Deletion of pRb results in the shorter G1 phase and the longer S phase (Classon et al., 2000). Although the precise mechanisms of pRb-mediated cell cycle inhibition remain to be elucidated, it has been suggested that it occurs due to inhibition of cell cycle regulatory gene expression via the interaction of pRb with E2F transcription factors and active repression of the cell cycle via chromatin modifying factors (Trimarchi and Lees, 2002). E2F transcription factor family can affect cell cycle progression (Blais & Dynlacht, 2004). The family consists of transcriptional activators (i.e., E2F1, E2F2, E2F3a) and repressors (E2F3b, E2F4, E2F5, E2F6, E2F7, and E2F8). E2Fs become functional when bound to DNA-binding protein partners (i.e., DP1 and DP2). Although various roles of E2Fs (e.g., roles in proliferation, differentiation, apoptosis, tumor suppression, or development) have been suggested, elucidation of the precise mechanisms is challenging due to the cross-regulation among E2Fs. The E2F transcriptional activators stimulate expression of cell cycle regulatory genes by binding to DNA and mutation of E2F1-3 completely blocks proliferation. When a cyclin binds to a cyclin-dependent kinase (cdk), the cdk is activated and phosporylates its substrate protein, such as pRb (Trimarchi and Lees, 2002). The phosphorylated form of pRb (i.e., inactive form) does not bind to E2Fs (Classon and Harlow, 2002), allowing free E2Fs to bind DNA and stimulate E2F-responsive gene expression. In contrast, pRb is hypophosphorylated (i.e., active form) during the G0 (i.e., quiescent) and early

G1 phase of cell cycle. Hypophosphorylated pRb binds to a E2F–DP dimer and inhibits E2F-mediated stimulation of cell cycle progression (Trimarchi and Lees, 2002). For active repression by chromatin modifying factors, the pRb-E2F–DP complex recruits factors which influence chromatin structure, including HDACs and histone methyltransferase SUV39H1. Chromatins are not condensed during the interphase of the cell cycle (i.e., G1, S, and G2 phase). However, HDACs bind to pRb-E2F-DP complex on chromatin and deacetylate lysine 9 of histone H3, stimulating nucleosome packing and inhibiting transcription of E2F-responsive genes. Subsequently, SUV39H1-heterochromatin protein 1 (HP1) complex replaces HDACs and methylates lysine 9 of histone H3, which modifies the histone tail and silences gene expression (Trimarchi and Lees, 2002). As described earlier, the LEK1-pRb interaction is necessary and LEK1 or pRb alone is not sufficient to induce differentiation in cardiomyocytes derived from ES cells (Papadimou et al., 2005). The ΔLEK1-mediated blockage of differentiation may have been caused by stimulation or elongation of cell cycle progression. However, this study did not determine the dominant inhibitory effects of ΔLEK1 on cellular proliferation. On the other hand, LEK1 depletion by morpholino antisense oligomers that disrupt translation of LEK1 mRNA reduced the cell number in NIH3T3 fibroblasts and C2C12 myoblasts and delayed cell cycle progression in NIH 3T3 cells (Ashe et al., 2004). This study suggested that the LEK1-pRb interaction allows free E2Fs to bind DNA, stimulating expression of E2F-responsive genes to promote cell cycle progression or inhibit terminal differentiation (Ashe et al., 2004).

CHAPTER 2
MATERIALS AND METHODS

Gene Cloning

The C-terminus of LEK1 was amplified by polymerase chain reaction (PCR) using mouse CENP-F cDNA (Open Biosystems). The 3' primer (CGGGATCCCCTTCCAGAACCCT GAGTGG) and the 5' primers (CG***GGATCC***AAAGCTTCAGGCAAGAGGC, CG***GGATCC***CT CGAGAAAGCCAAGGAGATATTAG, or GC***GGATCC***CTGCAGAATTCCCACAAGAG) were used to yield cDNA fragments for 597bp, 760bp, and 1273bp, respectively. A BamHI restriction site (bolded italic letters) was included in each primer. Fragments were separated through agarose gel and purified by the gel cleanup kit (Perfectprep® Gel Cleanup, Eppendorf North America). Purified cDNAs were digested with BamHI at 37°C for 90 minutes, and inserted into BamHI-treated pAcGFP1-C1 vectors (Clonetech) to generate fusion proteins of cLEK1 and green fluorescent protein (GFP). Insert orientation and reading frame fidelity were determined by DNA sequencing.

Cell Culture

C2C12 mouse skeletal muscle myoblast cells were cultured in the high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% v/v penicillin/streptomycin (P/S), and 0.2% v/v gnetamycin at 37°C with 5% CO_2 and 95% O_2. C3H10T1/2 mouse embryo fibroblast cells were cultured in the Basal Medium Eagle (BME) supplemented with 10% FBS, 1% v/v L-glutamine, 1% v/v P/S, and 0.2% v/v gnetamycin at 37°C with 5% CO_2 and 95% O_2. Differentiation was induced by culture in the low glucose DMEM supplemented with 2% horse serum, 1% v/v P/S, and 0.2% v/v gnetamycin at 37°C with 5% CO_2 and 95% O_2. All culture media, supplements, and sera were purchased from Invitrogen, Carlsbad, CA.

Transfections of DNA constructs

C2C12 myoblast and C3H10T1/2 fibroblast cells were transfected by calcium phosphate precipitate (Jordan and Wurm, 2004). C2C12 cells received DNA precipitate containing the pAcGFP1-C1 vector (3.3µg/ml) and GFP-cLEK1 DNA constructs (3.3µg/ml). Following the incubation for 5 hours at 37°C and 5% CO_2 and 95% O_2, cells were washed by phosphate buffered saline (PBS) and cultured in the new growth medium for 24 h at 37°C. Then, cells were used for western blot analysis and immunocytochemistry. For the Dual-Luciferase Reporter Assay, C3H10T1/2 cells received pAcGFP1-C1 control vector (158 ng/ml), vector-cLEK DNA constructs (158 ng/ml), pEM *MyoD* (79 ng/ml), a *Renilla* luciferase reporter plasmid (pRL-tk, 16 ng/ml), and internal response element of the quail troponin I gene (TnI-luc, 316 ng/ml). Following incubation for 5 hours at 37°C with 5% CO_2 and 95% O_2, cells were washed with PBS and cultured in the differentiation-permissive medium for 2 days at 37°C with 5% CO_2 and 95% O_2 prior to cell lysis and measurement of luciferase and *Renilla* luciferase activities (Dual-Luciferase® Reporter Assay System E1960, Promega, Madison, WI).

Western Blot Analysis

C2C12 cells were washed twice with ice-cold Tris-Buffered Saline (TBS) and then lysed in 4X SDS-PAGE sample buffer (250 mM Tris pH 6.8, 8% SDS, 40% glycerol, 0.4% β-mercaptoethanol) containing 1mM PMSF, 1mM NaF, and 1mM $Na_4P_2O_7$. Lysates were transferred to 1.5 ml microcentrifuge tubes (Fisher Scientific), heated at 95°C for 5 minutes, cooled on ice for 5 minutes, sonicated, cooled on ice for another 5 minutes, and centrifuged at 13,000 rpm for 20 seconds. Supernatant was transferred to new microcentrifuge tubes and stored at – 20°C until used. Equal amounts of protein were electrophoretically separated through polyacrylamide gels and transferred to nitrocellulose membranes. The blots were incubated with

5% nonfat dry milk in Tris-Buffered Saline Tween-20 (TBST: 10mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) overnight at 4°C to block nonspecific binding sites. Primary antibody (mouse anti-GFP, Santa Cruz Biotechnology) was diluted 1 : 500 in blocking solution and incubated with the blots for 60 minutes at room temperature. The blots were washed three times with TBST for 5 minutes each. Then, the blots were incubated with donkey peroxidase-conjugated anti-mouse IgG (Vector Laboratoeis, 1: 5,000) in blocking solution for 45 minutes at room temperature and washed three times with TBST. Visualization of protein bands was accomplished by chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ) and exposure to X-ray films (XAR-5, Kodak).

Immunocytochemistry

For detection of a proliferation marker Ki67, C2C12 cells were fixed with 4% paraformaldehyde (PFA) in PBS for 10 min, permeabilized with 0.25% Triton-X 100 in PBS for 20 min, and blocked with 5% horse serum in PBS for 60 minutes. For Ki67 immunostaining, fixed cells were incubated with rabbit anti-Ki67 (Abcam, 1:1000) in 5% horse serum in PBS overnight at 4°C. After three 5-minute washes with PBS, the cells were incubated with Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen, 1: 200) for 45 minutes. For detection of myosin heavy chain (MyHC), fixed C2C12 cells were incubated with mouse monoclonal anti-sarcomeric myosin antibody (MF20, Developmental Studies Hybridoma Bank, 1:5) in 5% horse serum in PBS for 60 minutes at room temperature. After three 5-minute washes with PBS, the cells were incubated with Alexa Fluor 568 goat anti-mouse IgG (Invitrogen, 1:200) in 5% horse serum in PBS for 45 min. For all assays, cells were incubated with Hoechst 33245 (1ug/ml) in PBS for 10 min. Representative photomicrographs were captured at 200X with a Nikon epifluorescent

microscope equipped with a Nikon DM1200F digital camera and NIS-Elements AR2.3 software. Images were adjusted for the intensity and contrast with Adobe Photoshop CS.

Bupivacaine Injection

All procedures and experiments were under the approval of the Institutional Animal Care and Use Committee at the University of Florida. Six-week-old male C57BL/6 mice were housed in a 12:12-h light-dark photoperiod in an environmentally controlled room and fed *ad libitum* throughout the experimental periods. Animals were randomly divided into 6 groups of 2 animals each. Bupivacaine (Marcaine®, 5mg/ml, 70 µL) was injected into the left TA. Control animals received saline (70 µL). Injection was performed at both the proximal and distal TA. Animals were then housed in cages for appropriate period until sacrificed by CO_2 inhalation. For the control group, mice were sacrificed by CO_2 inhalation 24 hours after bupivacaine injection. For the bupivacaine-treated groups, mice were sacrificed at 1, 3, 5, 7, and 10 days post-injection. Right and left TA muscles were embedded in the optimal cutting temperature (Sakura Tissue-Tek OCT Compound, Sakura Finetek USA, Torrance, CA) and frozen in isopentane cooled on dry ice.

Immunohistochemistry

Ten micrometer cryosecitons were collected onto glass slides (Fisherbrand Superfrost Microscope Slides, Fisher Scientific). Samples were blocked with 5% goat serum in PBS for 20 min and incubated with primary antibodies in blocking solution for 60 minutes. Primary antibodies used were rabbit polyclonal anti-LEK1 (1:200, Cocalico Biologicals, Reamstown, PA), mouse monoclonal anti-dystrophin (1:500, Abcam), and mouse monoclonal anti-Pax7 (1:50, R & D Systems, Mineapolis, MN). After extensive washing with PBS, tissues were incubated with secondary antibodies in blocking solution for 45 minutes. Secondary antibodies included Alexa Fluor 568 donkey anti-rabbit IgG (1:200, Invitrogen) and Alexafluor 594 donkey

anti-mouse IgG (1:200, Invitrogen). Following fixation with 4% PFA for 10 minutes, Hoechst 33245 (1ug/ml) in blocking solution was applied for 10 minutes at room temperature. Photomicrographs were captured at 100X, 300X, and 600X with a Nikon DMX1200F digital camera and NIS-Elements software.

Statistics

All the data were compared to appropriate controls and each other by One Way ANOVA. Data were presented as Means ± SEM. Treatments were considered significantly different when $P \leq 0.05$. The Tukey HSD test was used for a Post-hoc test. For Ki67 immunostaining and the Dual Luciferase Reporter Assay, experiments were duplicated and triplicated, respectively.

CHAPTER 3
RESULTS

Expression of LEK1 Mutants

Objective

Previous studies have shown that LEK1 protein plays a role in chromosome segregation, recycle of transport vesicles, cellular proliferation, and differentiation (Goodwin et al., 1999; Papadimou et al., 2005; Soukoulis et al., 2005; Pooley et al., 2006; Evans et al., 2007). However, the effects of LEK1 protein on cellular proliferation and differentiation have been controversy because different cell types and developmental stages of the cells have been used among these studies. These studies have determined the expression level of LEK1 during development, localization of LEK1 protein, the effects of LEK1 depletion on cell cycle progression, the dominant inhibitory effects of ΔLEK1 on proliferation and differentiation in certain cell types and developmental stages. To delineate the role of LEK1 protein in proliferation and differentiation in myoblasts, the current study was designed to determine the effects of dominant inhibitory ΔLEK1 containing the Rb-binding domain and NLS on myoblast proliferation and differentiation. The objective of the experiments was to express GFP-ΔLEK1 fusion proteins in C2C12 myoblasts.

Creation of LEK1 Expression Plasmids

cDNA fragments for 597bp, 760bp, and 1273bp of cLEK1 were amplified from mouse CENP-F cDNA. After separated and purified through agarose gel electrophoresis, the cDNA fragments were ligated into pAcGFP-C1 expression vectors to generate 22, 28, and 47 kDa of ΔLEK1 with 26 kDa of GFP. These sizes were selected because the focus of this study was on the effects of the functional domains in the C-terminus most portions of endogenous LEK1 protein fragment, including the atypical Rb-binding domain and NLS. LEK1 is

posttranslationally cleaved into the cytosolic N- and nuclear C- terminus portions and then the latter is further cleaved into approximately 60 and 50 kDa fragments where the 50 kDa fragment is the C-terminus most portion of endogenous LEK1 fragment (Figure 3-1). As expected, DNA sequencing confirmed the appropriate insert orientation and reading frame fidelity of the plasmids.

Expression of GFP- LEK1 Mutant Fusion Proteins

The plasmids containing pAcGFP1-C1 vector and ΔLEK1 were transfected into C2C12 myoblasts by calcium phosphate precipitate formation (Jordan and Wurm, 2004). Following incubation of the cells for 5 hours and culture in the new growth medium for 24 hours, cells were appropriately treated for the Western blot analysis using anti-GFP primary antibody and appropriate secondary antibody. As expected, bands of GFP-ΔLEK1 fusion proteins were detected at approximately 48, 54, and 73 kDa (Figure 3-2). The results were appropriate because GFP is approximately 26 kDa and ΔLEK1 mutants were 22, 28, and 47 kDa.

Dominant Inhibitory Effects of LEK1 Mutants on Myoblast Proliferation

Objective

The role of LEK1 protein in cellular proliferation highly depends on the cell type and developmental stage of the cells and thus controversy. In addition, the dominant inhibitory effects of ΔLEK1 may depend on the size of LEK1 mutant. The objective of the experiments was to determine the dominant inhibitory effects of various sizes of GFP-ΔLEK1 fusion proteins containing the Rb-binding domain and NLS on proliferation in C2C12 myoblasts.

The LEK1 Mutants Stimulate Myoblast Proliferation

Following transfection of C2C12 myoblasts with the plasmids containing genes encoding GFP (control) and GFP-ΔLEK1 fusion proteins, incubation in the growth medium with

precipitate for 5 hours, and culture in the new growth medium for 24 hours, moderately confluent C2C12 myoblasts were fixed, permeabilized, blocked, and immunostained for a proliferation marker Ki67 using rabbit anti-Ki67 primary antibody and appropriate secondary antibody. The percentage of Ki67 positive cells among GFP-positive cells was determined. Surprisingly, nearly 100% of GFP-positive cells were also Ki67 positive in the ΔLEK1-treated groups. In contrast, only half of the of GFP-positive cells in the control group expressed Ki67 (Figure 3-3, 3-4, and 3-5). The results showed that ectopic expression of dominant inhibitory GFP-ΔLEK1 significantly stimulates myoblast proliferation ($P < 0.05$).

Dominant Inhibitory Effects of LEK1 Mutants on Myoblast Differentiation

Objective

The role of LEK1 protein in cellular differentiation may also depends on the cell type, developmental stage, or size of dominant negative ΔLEK1. Ectopic expression of the smaller ΔLEK1 (7 kDa) almost completely blocked differentiation in embryonic cardiomyocytes (Papadimou et al., 2005) while expression of the larger ΔLEK1 (165 kDa) accelerated differentiation in C2C12 myoblasts (Goodwin et al., 1999). The objective of the experiments was to determine the effects of GFP-ΔLEK1 on differentiation in myoblasts using immunostaining for MyHC in C2C12 myoblasts and the Dual Luciferase Reporter Assay in *MyoD*-treated C3H10T1/2 fibroblasts. The latter is a well established experimental approach. In various fibroblasts including C3H10T1/2 cells, ectopic expression of MyoD converts them to myoblasts and induces differentiation (Tapscott et al., 1988).

The LEK1 Mutants Suppress Myoblast Differentiation

Following transfection of C2C12 myoblasts with the plasmids containing genes encoding GFP (control) and GFP-ΔLEK1 fusion proteins, incubation in the growth medium with

precipitates for 5 hours, culture in the new growth medium for 24 hours, and culture in the differentiation-permissive medium for 48 hours, fixed C2C12 cells were incubated with mouse monoclonal anti-sarcomeric myosin primary antibody and appropriate secondary antibody. In control cells, some GFP-positive cells differentiated and expressed MyHC (Figure 3-6). In contrast, all GFP-positive cells in the ΔLEK1-treated groups failed to express MyHC (Figure 3-6 and 3-7). Thus, ΔLEK1 completely blocked differentiation in C2C12 myoblasts.

To further clarify the effects of ΔLEK1 on myoblast differentiation, C3H10T1/2 fibroblasts were treated with *MyoD* and the Dual Luciferase Reporter Assay was performed. Following transient transfection with pEM *MyoD*, TnI-luc, pRL-tk, pAcGFP-ΔLEK1, and pAcGFP1-C1 plasmids (control), incubation in the growth medium with precipitates for 5 hours, and culture in the differentiation-permissive medium for 48 hours, C3H10T1/2 fibroblasts was lysed and the luciferase activities were determined. The *Renilla* luciferase activities showed the transfection efficiency was very high in all treatment groups (data not shown). The smallest GFP-ΔLEK1 (48 kDa) did not affect differentiation of *MyoD*-treated C3H10T1/2 fibroblasts. In contrast, the larger GFP-ΔLEK1 (54 and 73 kDa) significantly inhibited differentiation ($P < 0.05$).

In summary, GFP-ΔLEK1 proteins completely blocked differentiation in C2C12 myoblasts. However, in *MyoD*-treated C3H10T1/2 fibroblasts, only the larger GFP-ΔLEK1 (54 and 73 kDa) inhibited differentiation. The size of the smallest GFP-ΔLEK1 fusion protein (48 kDa) was not sufficient to suppress differentiation in *MyoD*-treated C3H10T1/2 fibroblasts.

Establishment of a Muscle Regeneration Model

Objective

Morphological changes during skeletal muscle regeneration are best characterized following severe damage of muscle fibers (Charge and Rudnicki, 2004). Following muscle damage, muscle regeneration occurs via activation, proliferation, and differentiation of satellite cells (Hawke and Garry, 2001; Charge and Rudnicki, 2004). To induce muscle degeneration, various models have been employed, such as crushing, lacerating, mincing, exposing to extreme temperature/strain, or injecting myotoxin (Carlson and Faulkner, 1983; Classon, 1986; Armstrong et al., 1991; Marlow et al., 1996; Menetrey et al., 2000; Plant et al., 2006). Among them, the myotoxin models have been well established. The advantage of using myotoxin is that consistent muscle damage can be obtained, which allows direct comparison among treatment groups (Plant et al., 2006). However, the effects of myotoxin on structural and functional muscle damage can vary among species. For instance, the effects of bupivacaine injection on skeletal muscle function are much less in mice than those in rats (Rosenblatt, 1992; Holmes et al., 2002). In addition, the type and concentration of myotoxin, dosage, muscle type, gender, and age may cause the difference in the level of muscle damage and regeneration. Therefore, to compare the results to the literature, it is very important to establish a skeletal muscle regeneration model using a specific method, species, muscle type, gender, and age. The objective of the experiments was to establish a skeletal muscle regeneration model following a submaximal dosage of bupivacaine injection into TA of adult male mice.

Muscle Regeneration Model

Saline (70 μL) or 5% bupivacaine (5mg/ml, 70 μL) was injected into TA of 6 week-old C57BL/6 male mice. Mice were fed *ad libitum* and housed in an environmentally controlled room throughout the experimental periods. For the control group, mice were sacrificed by CO_2

inhalation 1 day after bupivacaine injection. For the bupivacaine-treated groups, they were sacrificed at 1, 3, 5, 7, and 10 days post-injection. Muscles were removed and cryosectioned. Fixed samples were immunostained for dystrophin and Hoechst to identify myofiber membranes and myonuclei, respectively. In control samples, muscle fiber membranes were intact and Hoechst-staining was observed in the periphery of myofibers (Figure 3-9). In the bupivacaine-treated samples, progressive fiber damage was observed for the first 3 days. The muscle membranes began to be degraded and Hoechst staining was concentrated in the injured areas on Day 1 (Figure 3-9). On Day 3, large cavities were formed in the heavily injured sites. In agreement with the previous study, the fiber damage was maximal 3 days after bupivacaine injection into adult male C57BL mice (Plant et al., 2006). Hoechst-staining was concentrated within the injured cavities on Day 3. From Day 5 to 7, progressive fiber repair was observed. On Day 5, damaged muscle fibers were significantly repaired and the cavity size was decreased while Hoechst-staining was still concentrated within the injured sites (Figure 3-10). On Day 7, muscle damage was mostly repaired and the cavity size was further reduced (Figure 3-11). Finally, on Day 10, myofibers were almost completely repaired and a single or multiple myonuclei were centrally located in each fiber (Figure 3-11). These results completely agree with the previous studies (Charge and Rudnicki, 2004; Plant et al., 2006; Arsic et al., 2008; Epting et al., 2008).

Role of LEK1 in Muscle Regeneration

Objective

The roles of LEK1 protein in chromosome segregation, recycle of transport vesicles, embryonic development, cellular proliferation, and differentiation have been suggested by several studies. However, no study has shown the role of LEK1 in skeletal muscle regeneration following muscle injury despite that activation, proliferation, and differentiation of satellite cells

are the key factors in myogenesis (Charge and Rudnicki, 2004). The objective of the experiments was to determine the expression pattern of LEK1 protein during bupivacaine-induced skeletal muscle regeneration using a well established model. Muscle sections were immunostained for LEK1 protein. It is well known that proliferation of activated satellite cell and macrophage influx occurs during injury-induced skeletal muscle degeneration and regeneration (Charge, 2004; Fielding et al., 1993; Orimo et al., 1991). However, 4', 6'-diamidino-2-phenylindole 2HCl (DAPI) stains macrophage as well as DNA (Browne et al., 2002). In this study, muscle sections were stained with Hoechst which is structurally and functionally virtually identical to DAPI as a fluorescent stain (Buys and van der Veen, 1982; Bernheim and Miglierina, 1989). Therefore, it was expected that myonuclei, nuclei of satellite cells, and macrophages be stained with Hoechst. To distinguish the nuclei of activated satellite cells from other myonuclei and macrophages, samples were immunostained for a satellite cell marker Pax7 which is expressed both by quiescent and by activated satellite cells (Zammit et al., 2004).

Expression Pattern of LEK1

In control samples, LEK1 expression was minimal (Figure 3-12). However, as expected, some LEK1 molecules and Hoechst staining were identified in the satellite positions (Figure 3-22). On the other hand, progressive expression of LEK1 protein was mainly seen in the heavily degenerating or regenerating sites up to Day 5 in myotoxin-treated samples. On Day 1, LEK1 began to be expressed. Although its expression level was very low (Figure 3-21), LEK1 completely overlapped with Hoechst staining in the degenerating sites (Figure 3-12). On Day 3, muscle damage was maximal and LEK1 expression was significantly elevated and overlapped with Hoechst staining in the cavities of the most heavily damaged sites (Figure 3-13). On Day 5, muscle damage was significantly repaired and LEK1 expression reached its peak in the sarcoplasm of regenerating myofibers while Hoechst staining was still concentrated in the

injured sites (Figure 3-13 and 3-21). On Day 7, damaged myofibers were mostly repaired and LEK1 expression was significantly reduced (Figure 3-14 and 3-21). Finally, on Day 10, LEK1 was localized in the center of myofibers with Hoechst staining (Figure 3-14 and 3-20). Importantly, some LEK1 molecules were identified with Hoechst staining in the satellite positions on Day 10 (Figure 3-20).

Expression Pattern of Pax7

In control sample, Pax7 expression was minimal. However, as expected, some Pax7 expression was identified with LEK1 and Hoechst staining (Figure 3-16). In bupivacaine-treated samples, progressive expression of Pax7 was observed up to Day 3. On Day 1, Pax7 was slightly elevated and co-localized with LEK1 although most LEK1 molecules were not co-localized with Pax7 (Figure 3-16). On Day 3, Pax7 expression was maximal (Figure 3-17) when myofibers were most heavily damaged (Figure 3-13). Surprisingly, Pax7 and LEK1 molecules were completely overlapped with each other. On Day 5, Pax7 expression was dramatically reduced when muscle damage was significantly repaired (Figure 3-17). On Day 7, little Pax7 expression was identified (Figure 3-18). Finally, on Day 10, Pax7 was undetectable (Figure 3-18).

In summary, satellite cell marker Pax7 completely overlapped with LEK1 and Hoechst staining throughout the experimental period both in intact and bupivacaine-treated samples (except Day 10 when Pax7 was undetectable). In intact muscles, although expression of Pax7 and LEK1 was very limited, Pax7 was detected with LEK1 and Hoechst (Figure 3-16) which were also observed in the satellite position (Figure 3-22). In bupivacaine-treated samples, the level of Pax7 expression and Hoechst staining were proportional to that of muscle injury, reaching their peaks on Day 3 (Figure 3-17 and Figure 3-21). During this stage, LEK1 and Pax7 completely overlapped with each other (Figure 3-17). When muscle damage began to be repaired on Day 5 (Figure 3-13 and 3-21), Pax7 was dramatically reduced (Figure 3-17) while LEK1

expression reached its peak in regenerating muscle bed (Figure 3-13 and 3-21). As fiber damage was further repaired, LEK1 expression was significantly reduced (Figure 3-14 and 3-21) and Pax7 were hardly detectable (Figure 3-18). When central nuclei were formed on Day 10, a majority of LEK1 translocated from the sarcoplasm to the center of myofibers (Figure 3-20 and 3-22) and Pax7 became completely undetectable (Figure 3-18). On the other hand, a minority of LEK1 were identified with Hoechst staining in the satellite position again (Figure 3-22).

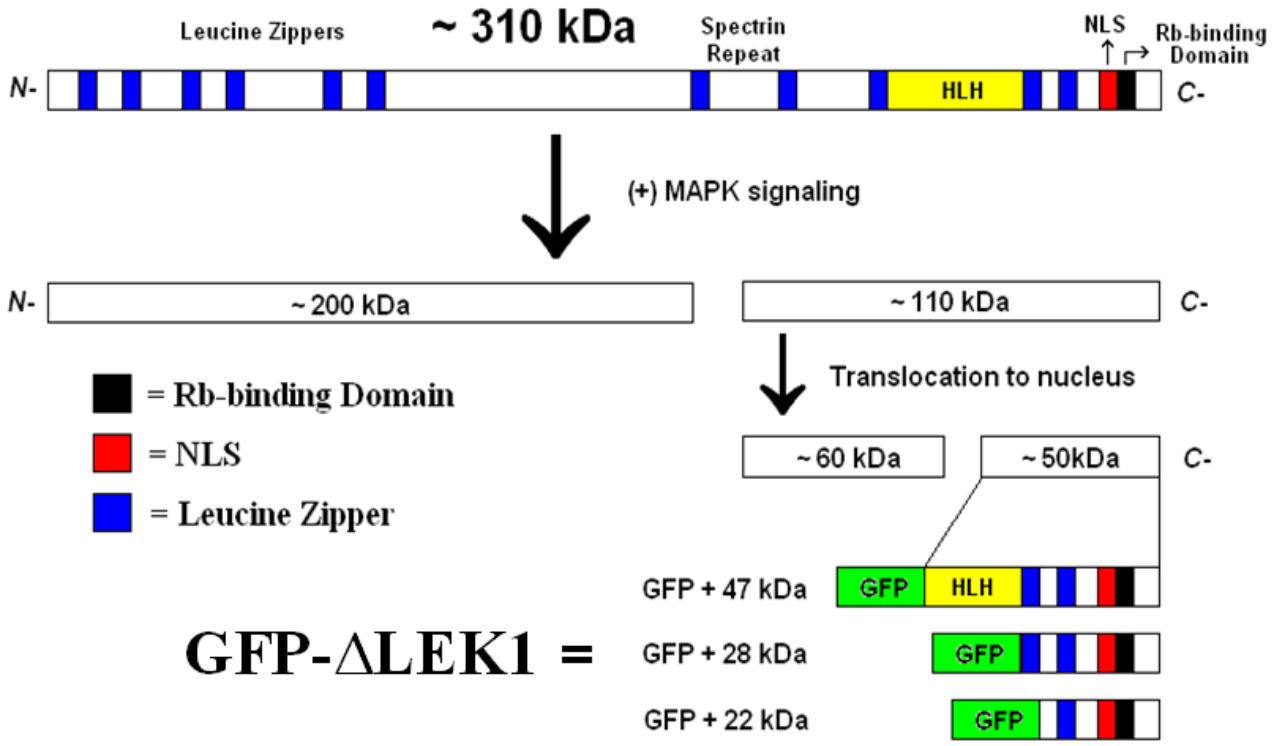


Figure 3-1. Structure of full length of LEK1 protein and its posttranslational cleavage products. LEK1 protein is cleaved into the N- and C-terminus portions. In response to stimulation of MAKP signaling, the C-terminus portion is further cleaved into two fragments. DNA constructs with various length of C-terminus LEK1 gene were created to determine the dominant inhibitory effects of GFP-ΔLEK1 fusion proteins on cellular proliferation and differentiation. HLH, NLS, and ΔLEK1 indicate the basic helix-loop-helix domain, nuclear localization sequence, and C-terminus LEK1 mutant, respectively.

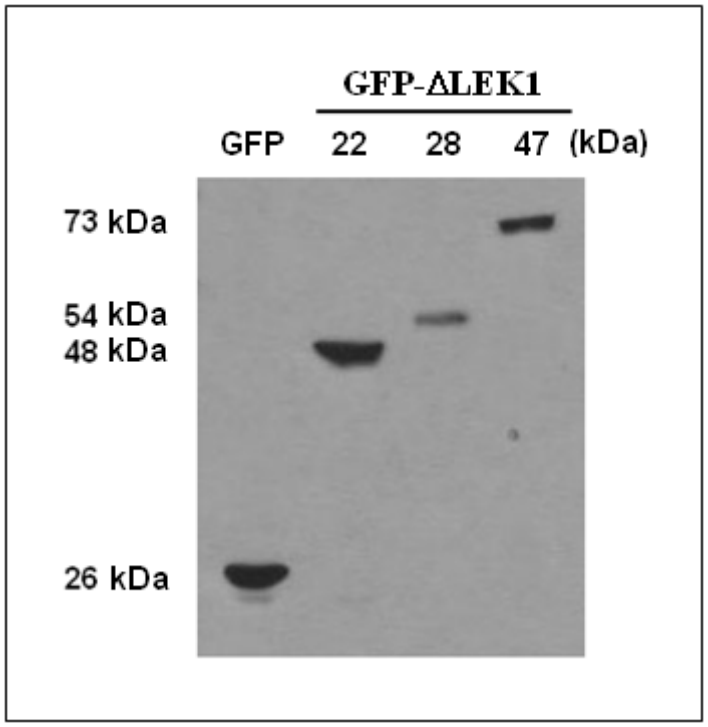


Figure 3-2. Westernblot of GFP-ΔLEK1 fusion proteins in C2C12 myoblasts. As expected, the fusion proteins were detected at approximately 48, 54, and 73 kDa. C2C12 myoblats were transfected with DNA precipitates containing pAcGFP1-C1 vector or GFP-ΔLEK1 gene constructs to generate 22, 28, and 47 kDa of ΔLEK1 with 26 kDa of GFP. Following incubation with precipitates for 5 hours, culture in the new growth medium for 24 hours, wash with TBS, cell lysis, sonication, and centrifugation, supernatant of samples was electrophorectically separated through polyacrylamide gels and transferred to nitrocellulose membrane. The blots were blocked with 5% milk in TBST and incubated with anti-GFP antibody in blocking solution for 60 minutes and donkey peroxidase-conjugated anti-mouse IgG in blocking solution for 45 minutes. ΔLEK1 indicates C-terminus LEK1 mutant.

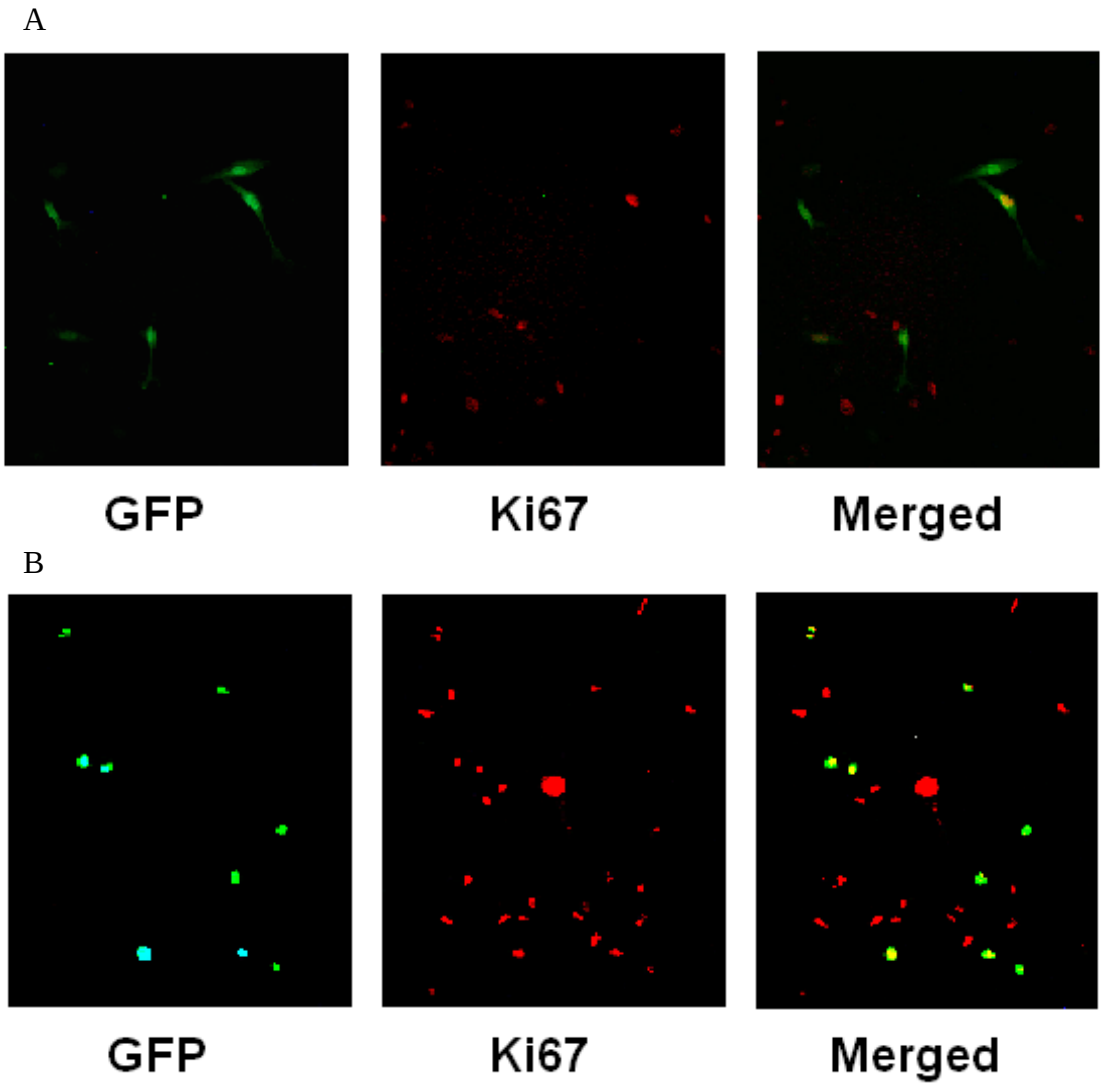


Figure 3-3. Expression of proliferation marker Ki67 in control and transfected C2C12 myoblasts. A) Cells transfected by the control vector pAcGFP-C1. B) Cells transfected by the DNA construct encoding GFP-ΔLEK1 (22 kDa) fusion protein. Ectopic expression of GFP-ΔLEK1 (22 kDa) stimulated proliferation of myoblasts. Cells were transfected with DNA precipitates containing pAcGFP1-C1 vector or GFP-ΔLEK1 gene construct. Following incubation with precipitates for 5 hours, culture in the new growth medium for 24 hours, cells were fixed, permeabilized, blocked, and immunostained for Ki67. ΔLEK1 indicates C-terminus LEK1 mutant. Representative photomicrographs at 200X are shown.

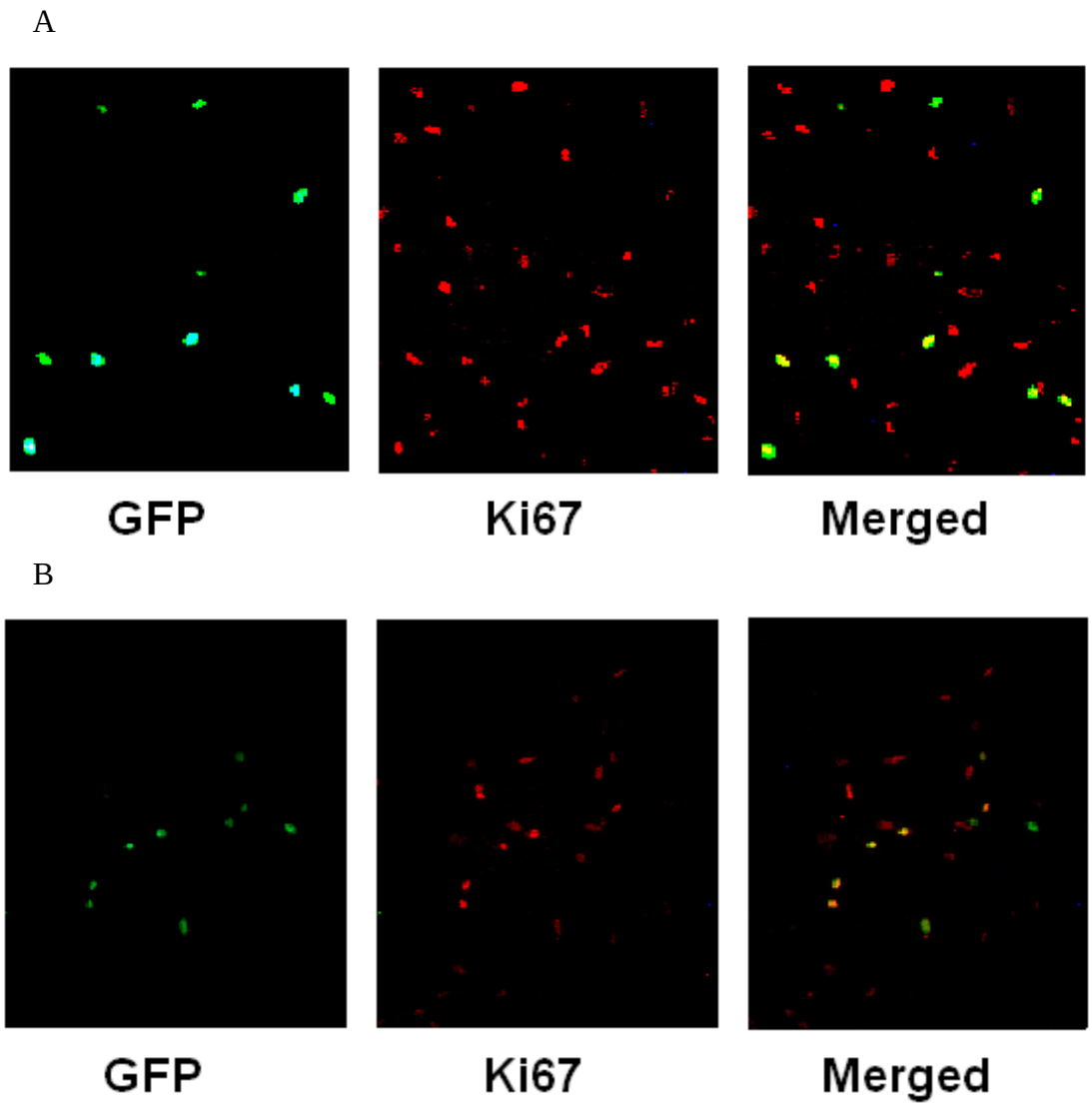


Figure 3-4. Expression of proliferation marker Ki67 in transfected C2C12 myoblasts. A) Cells transfected by the DNA construct encoding GFP-ΔLEK1 (28 kDa) fusion protein. B) Cells transfected by the DNA construct encoding GFP-ΔLEK1 (47 kDa) fusion protein. Ectopic expression of GFP-ΔLEK1 (28 or 47 kDa) fusion proteins stimulated proliferation of myoblasts. Cells were transfected with DNA precipitates containing GFP-ΔLEK1 gene constructs. Following incubation with precipitates for 5 hours, culture in the new growth medium for 24 hours, cells were fixed, permeabilized, blocked, and immunostained for Ki67. ΔLEK1 indicates C-terminus LEK1 mutant. Representative photomicrographs at 200X are shown.

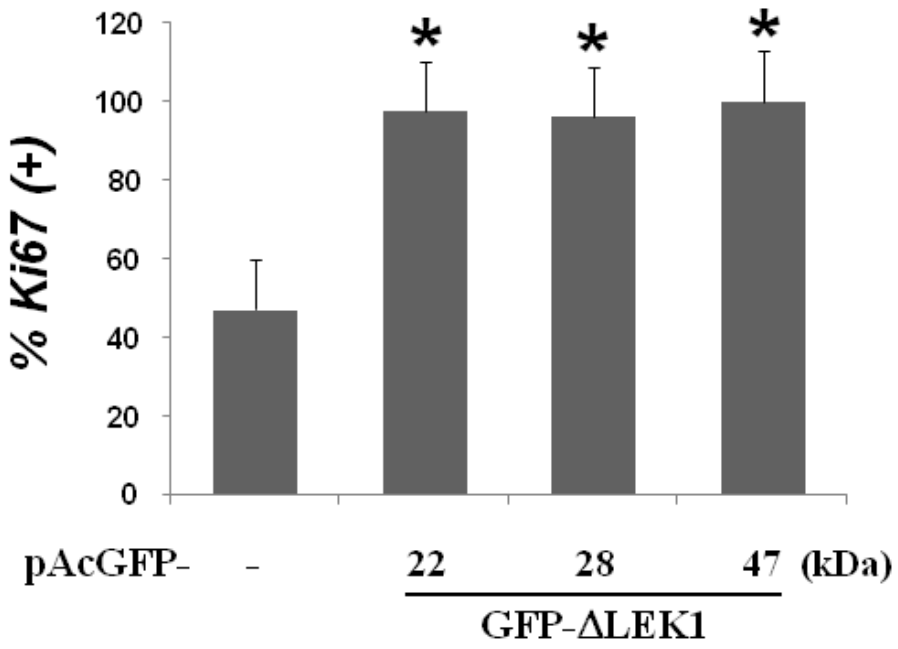
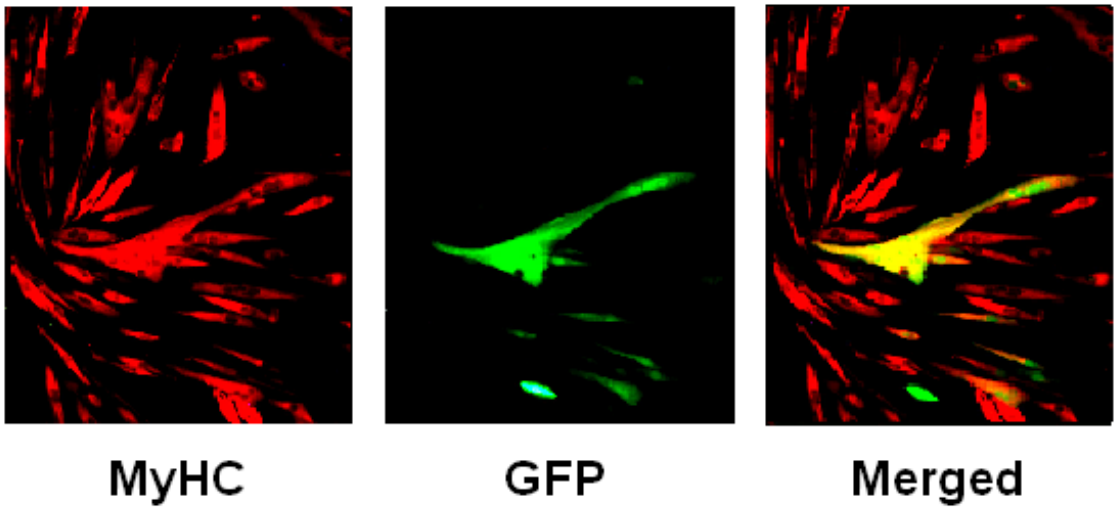


Figure 3-5. Summary graph of Expression of proliferation marker Ki67 in C2C12 myoblasts. Ectopic expression of GFP-ΔLEK1 (22, 28 or 47 kDa) fusion proteins doubled proliferation of myoblasts. Cells were transfected with DNA precipitates containing pAcGFP-C1 vector or GFP-ΔLEK1 gene constructs to generate 22, 28, or 47 kDa ΔLEK1 with GFP. Following incubation with precipitates for 5 hours, culture in the new growth medium for 24 hours, cells were fixed, permeabilized, blocked, and immunostained for Ki67. The percentage of Ki67-positive cells among GFP-positive cells were calculated. ΔLEK1 indicates C-terminus LEK1 mutant (22, 28, or 47 kDa). Experiments were duplicated. The Asterisk * indicates a significant difference ($P < 0.05$).

A



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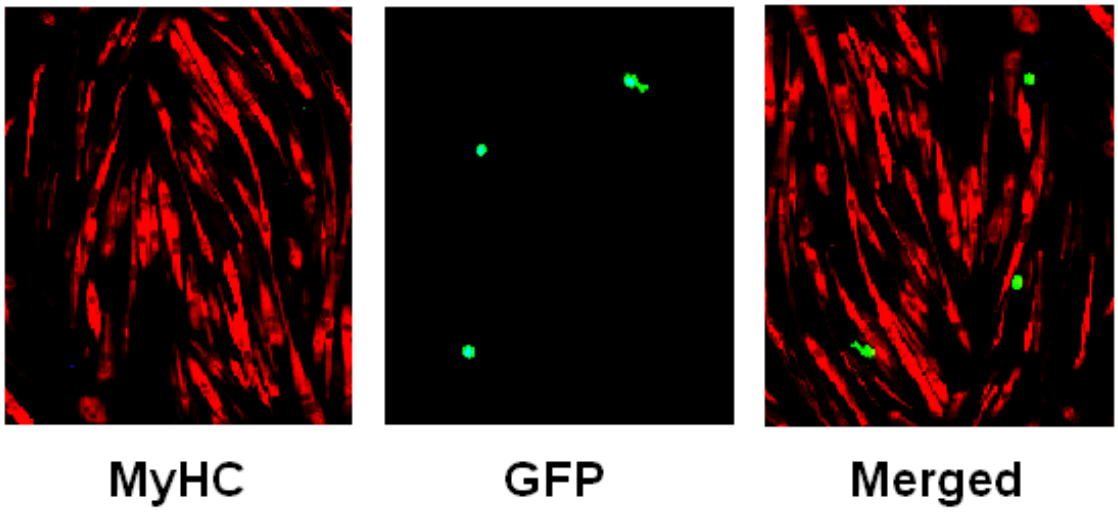


Figure 3-6. Expression of differentiation marker MyHC in control and transfected C2C12 myoblasts. A) Cells transfected by the control vector. B) Cells transfected by the DNA construct encoding GFP-ΔLEK1 (22 kDa) fusion protein. Ectopic expression of GFP-ΔLEK1 (22 kDa) fusion proteins inhibited differentiation of myoblasts. Cells were transfected with DNA precipitates containing pAcGFP-C1 vector or GFP-ΔLEK1 gene construct. Following incubation with precipitates for 5 hours, culture in the new growth medium for 24 hours, and culture in the differentiation-permissive medium for 48 hours, cells were fixed, permeabilized, blocked, and immunostained for MyHC. Representative photomicrographs at 200X are shown.

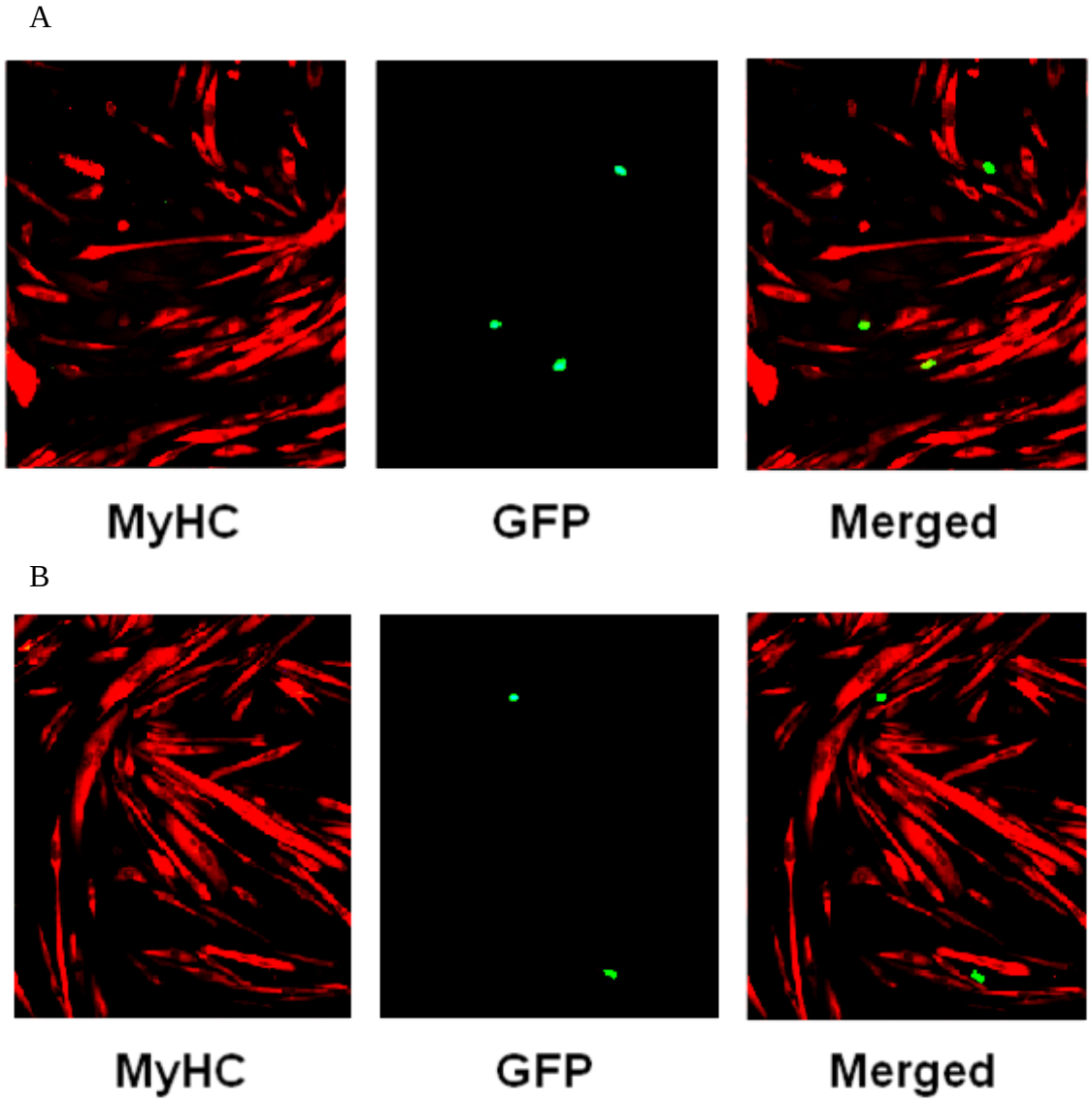


Figure 3-7. Expression of differentiation marker MyHC in transfected C2C12 myoblasts. A) Cells transfected by the DNA construct encoding GFP-ΔLEK1 (28 kDa) fusion protein. B) Cells transfected by the DNA construct encoding GFP-ΔLEK1 (47 kDa). Ectopic expression of GFP-ΔLEK1 (28 kDa or 47 kDa) inhibited differentiation of myoblasts. Cells were transfected with DNA precipitates containing GFP-ΔLEK1 gene constructs. Following incubation with precipitates for 5 hours, culture in the new growth medium for 24 hours, and culture in the differentiation-permissive medium for 48 hours, cells were fixed, permeabilized, blocked, and immunostained for MyHC. Representative photomicrographs at 200X are shown.

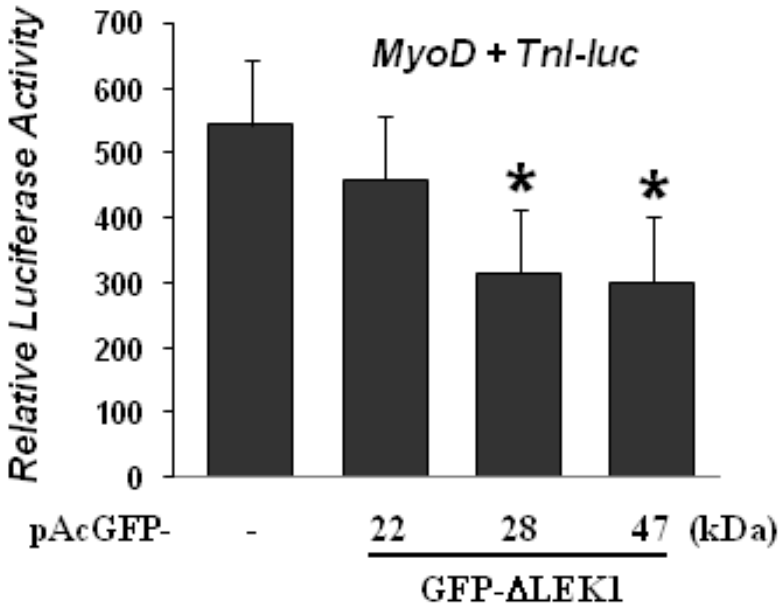
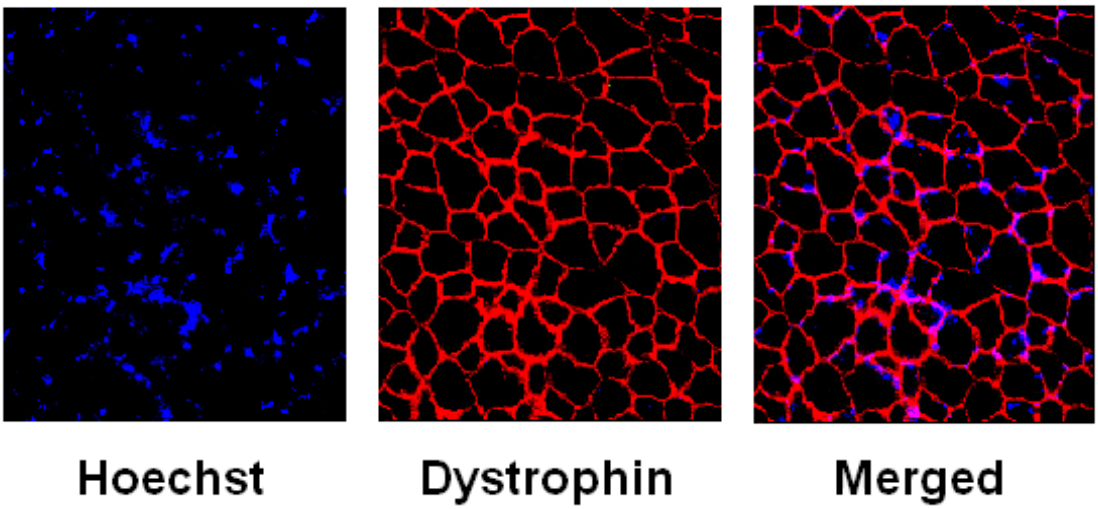


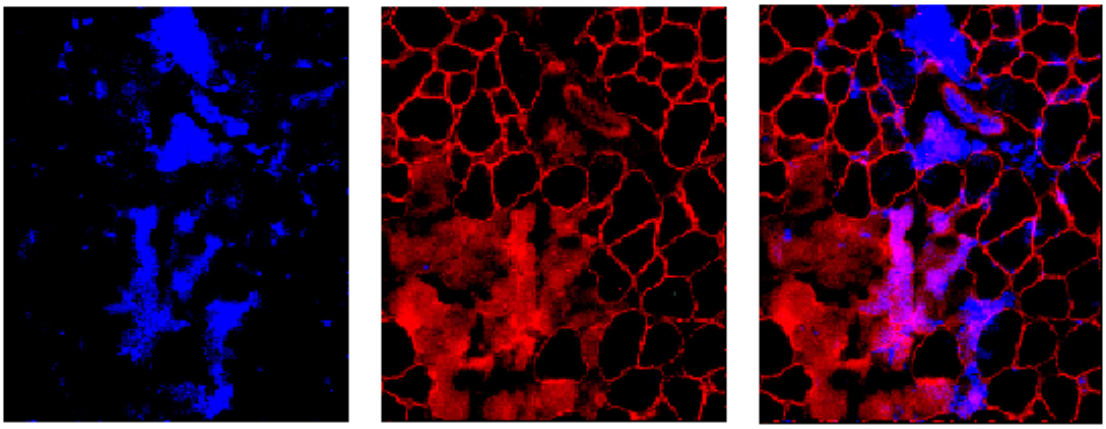
Figure 3-8. Estimation of myoblast differentiation by the Dual Luciferase Reporter Assay. The smallest GFP-ΔLEK1 (22 kDa) did not affect differentiation in *MyoD*-treated C3H10T1/2 fibroblasts. In contrast, the larger GFP-ΔLEK1 (28 or 47 kDa) significantly inhibited differentiation. In addition to pAcGFP-C1 vector or GFP-ΔLEK1 plasmids, 10T1/2 fibroblasts received *MyoD*, TnI-luc, and pRL-tk. Following incubation with precipitates for 5 hours and culture in the differentiation-permissive medium for 48 hours, cells were lysed and the luciferase activities were measured by the Dual-Luciferase Reporter Assay System. ΔLEK1 indicates C-terminus LEK1 mutant (22, 28, or 47 kDa). Asterisk * indicates a significant difference ($P < 0.05$).

A



Hoechst Dystrophin Merged

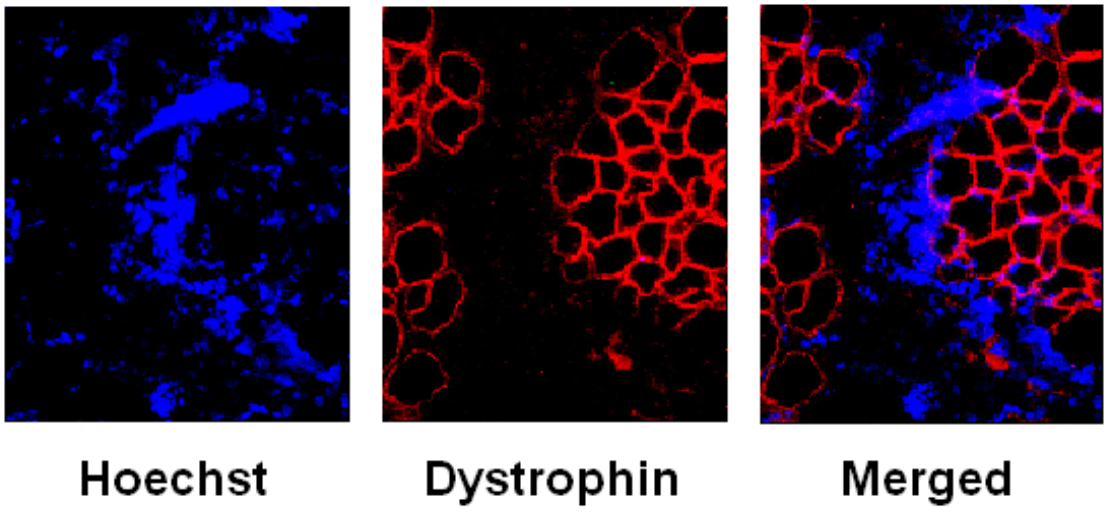
B



Hoechst Dystrophin Merged

Figure 3-9. Dystrophin and Hoechst staining on Day 1. A) Control muscle sections. B) Bupivacaine-treated muscle sections. Bupivacaine treatment induced skeletal muscle degeneration within a day. Saline or bupivacaine was injected into mouse TA muscles. Animals were sacrificed 24 hours later. Muscles were cryosectioned and immunostained for Hoechst and dystrophin. Representative photomicrographs at 300X are shown.

A



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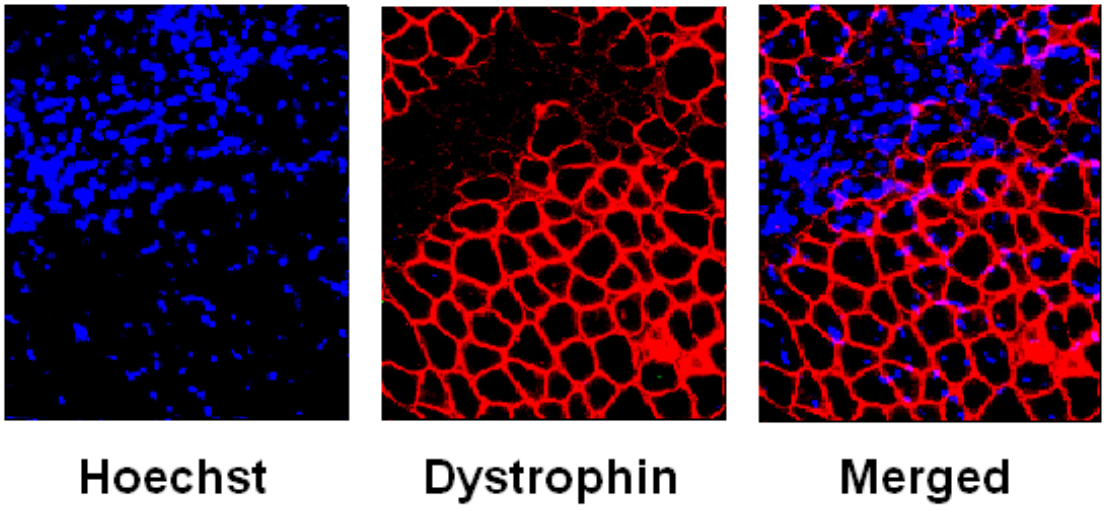


Figure 3-10. Dystrophin and Hoechst staining on Day 3 and 5. A) Bupivacaine-treated muscle sections on Day 3. B) Bupivacaine-treated muscle sections on Day 5. Bupivacaine-induced muscle damage was maximal on Day 3. Damaged myofibers began to be repaired within 5 days. Influx of activated satellite cells and/or macrophages was seen in the injured sites in both Day 3 and 5. Bupivacaine was injected into mouse TA muscles. Animals were sacrificed 3 and 5 days later. Muscles were cryosectioned and immunostained for Hoechst and dystrophin. Representative photomicrographs at 300X are shown.

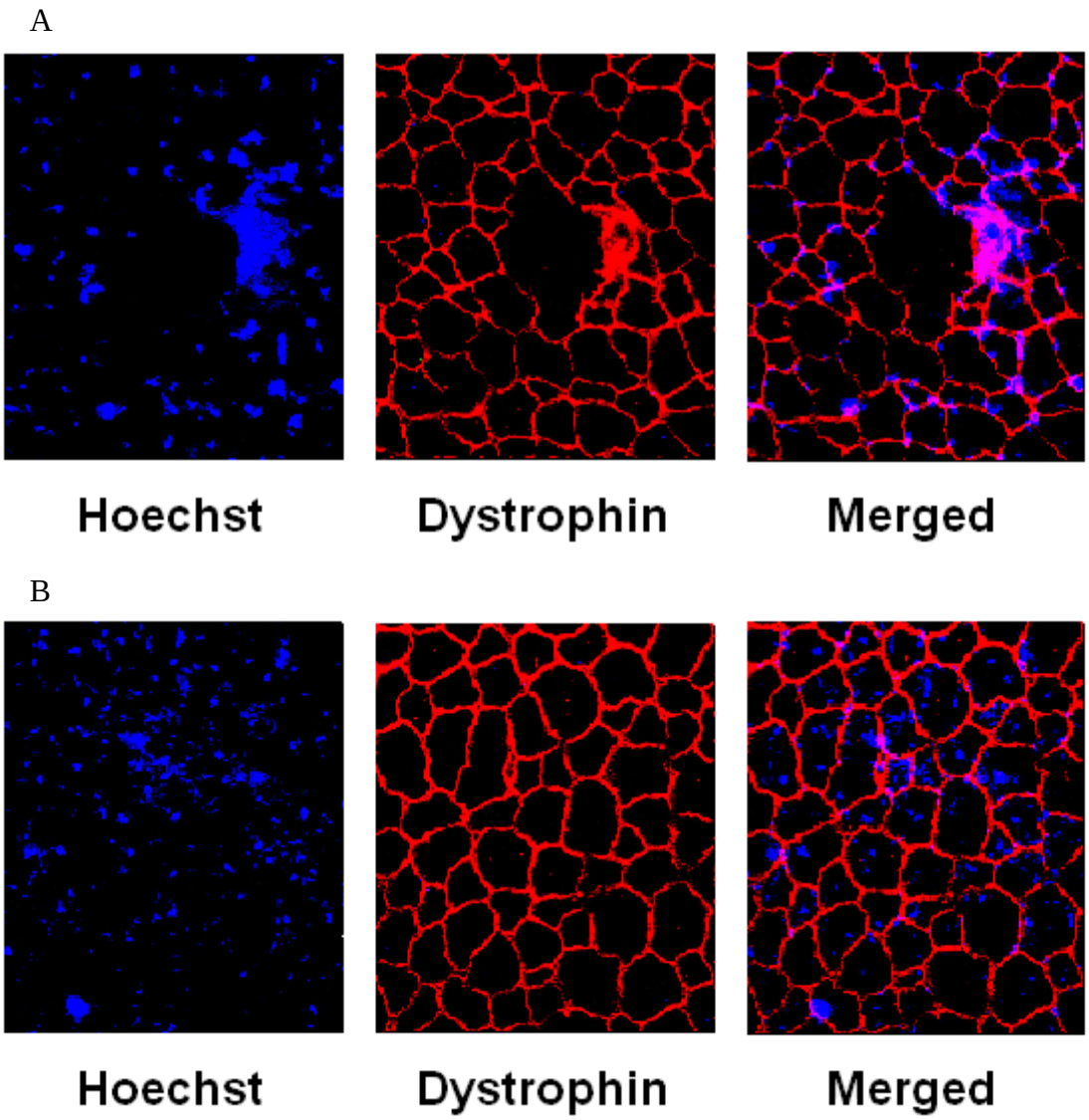


Figure 3-11. Dystrophin and Hoechst staining on Day 7 and 10. A) Bupivacaine-treated muscle sections on Day 7. B) Bupivacaine-treated muscle sections on Day 10. Most fiber damage was repaired within 7 days following the bupivacaine treatment. Hoechst staining was removed from the injured site on Day 7 and translocated to the center of myofiber on Day 10. Bupivacaine was injected into mouse TA muscles. Animals were sacrificed 7 and 10 days later. Muscles were cryosectioned and immunostained for Hoechst and dystrophin. Representative photomicrographs at 300X are shown.

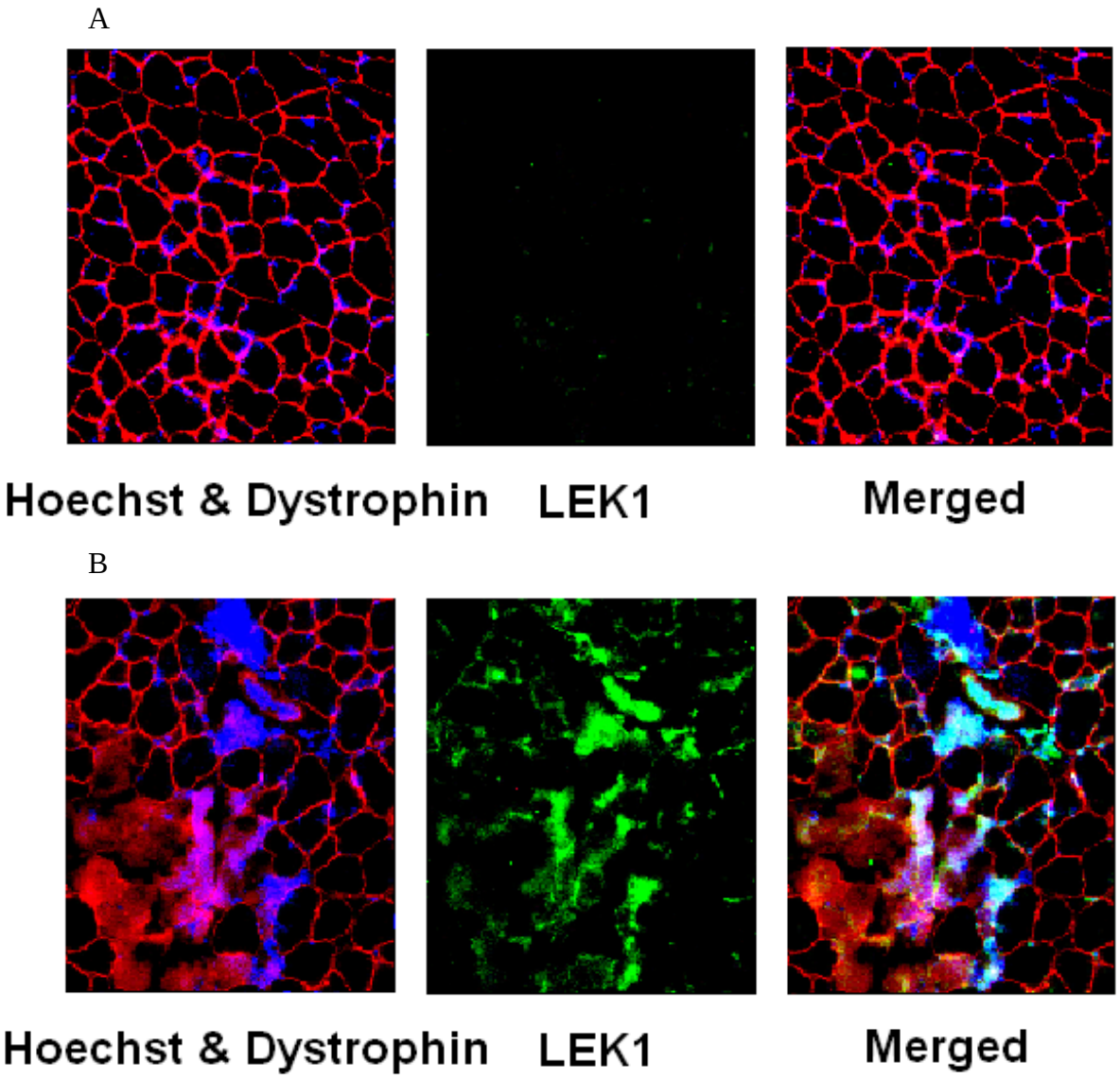


Figure 3-12. Dystrophin, Hoechst, and LEK1 staining on Day 1. A) Control muscle sections. B) Bupivacaine-treated muscle sections. LEK1 protein was expressed in the damaged area within a day following the bupivacaine treatment. LEK1 expression overlapped with Hoechst staining. Bupivacaine was injected into mouse TA muscles. Animals were sacrificed 24 hours later. Muscles were cryosectioned and immunostained for Hoechst, dystrophin, and LEK1. Representative photomicrographs at 300X are shown.

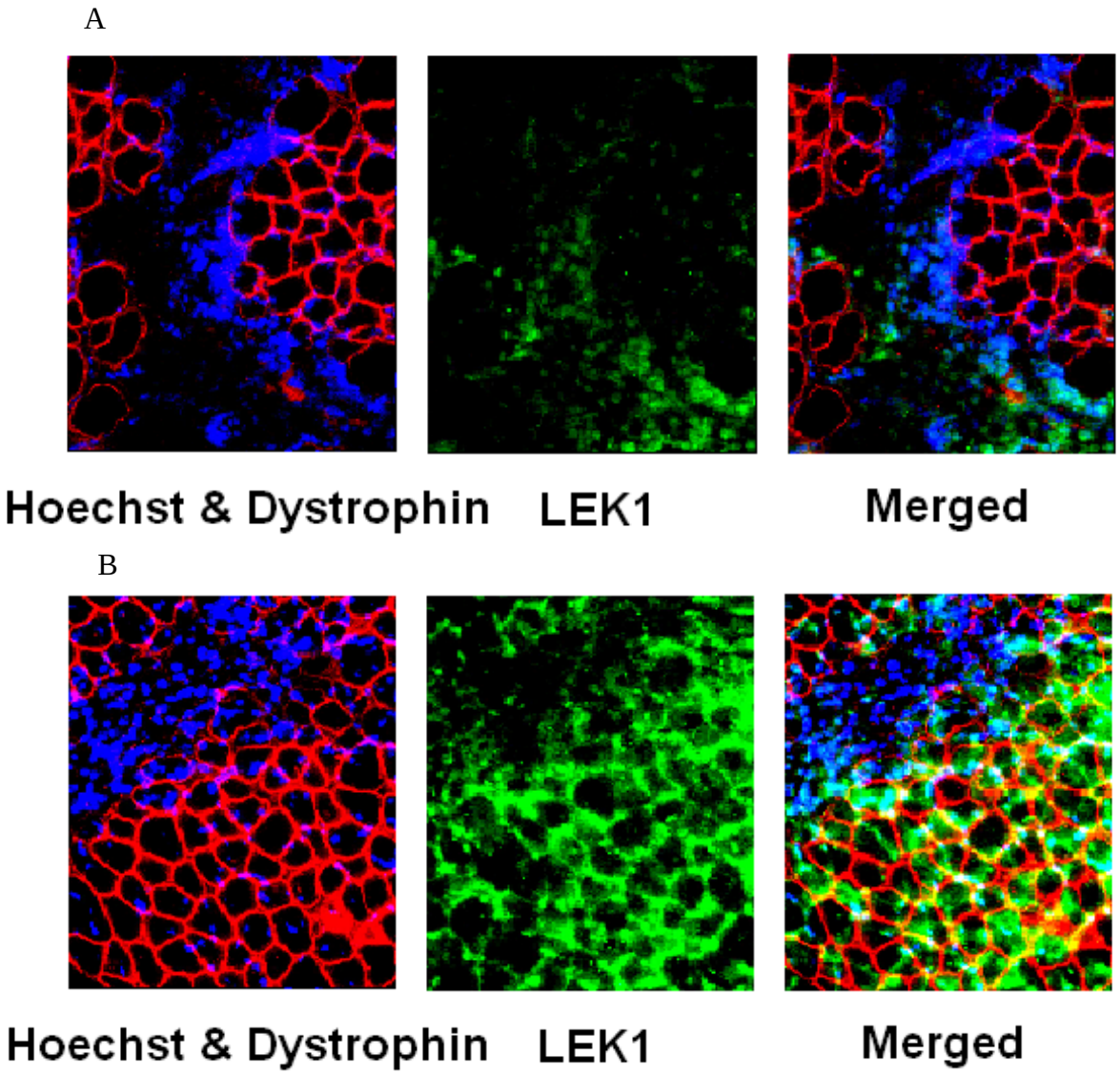


Figure 3-13. Dystrophin, Hoechst, and LEK1 staining on Day 3 and 5. A) Bupivacaine-treated muscle sections on Day 3. B) Bupivacaine-treated muscle sections on Day 5. LEK1 was progressively expressed up to Day 5. The LEK1 expression was at its peak on Day 5 and translocated from the injured cavity to regenerating muscle bed. Bupivacaine was injected into mouse TA muscles. Animals were sacrificed 3 and 5 days later. Muscles were cryosectioned and immunostained for Hoechst, dystrophin, and LEK1. Representative photomicrographs at 300X are shown.

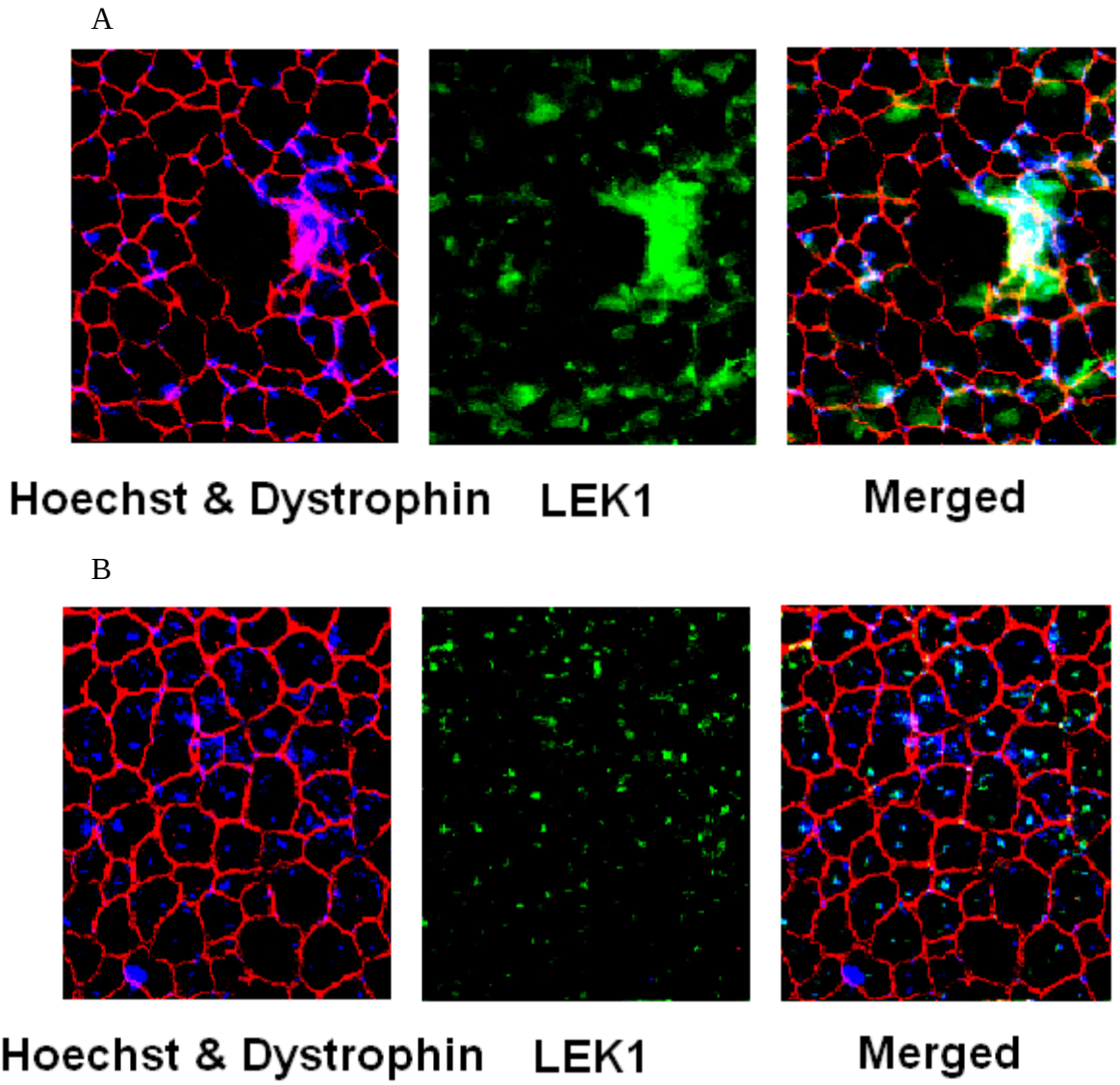


Figure 3-14. Dystrophin, Hoechst, and LEK1 staining on Day 7 and 10. A) Bupivacaine-treated muscle sections on Day 7. B) Bupivacaine-treated muscle sections on Day 10. Damaged myofibers were mostly repaired and LEK1 expression was reduced on Day 7. Myofibers were almost completely repaired and both myonuclei and LEK1 translocated to the center of myofibers on Day 10. Bupivacaine was injected into mouse TA muscles. Animals were sacrificed 7 and 10 days later. Muscles were cryosectioned and immunostained for Hoechst, dystrophin, and LEK1. Representative photomicrographs at 300X are shown.

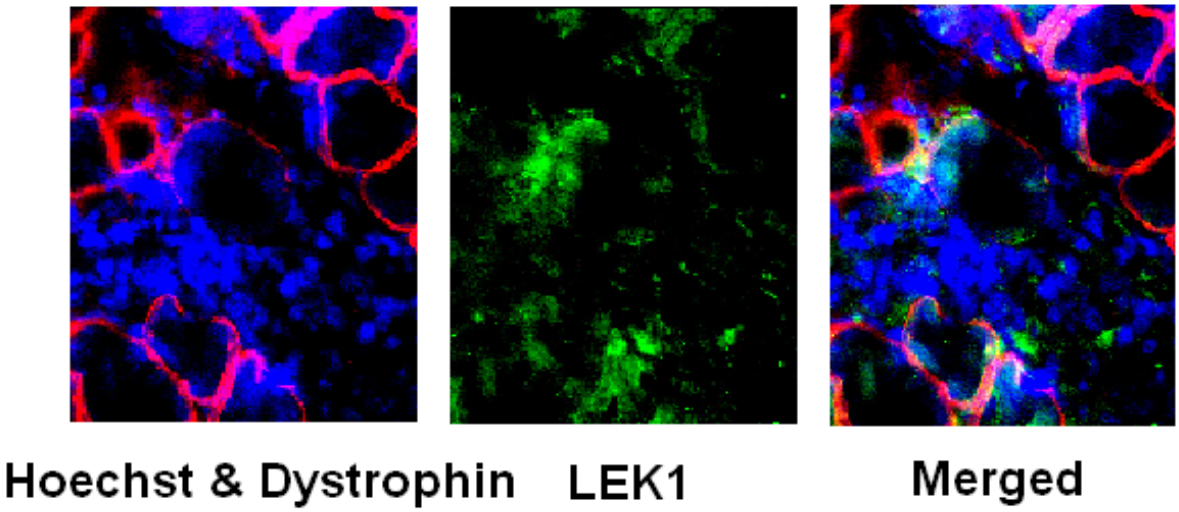


Figure 3-15. Presence of LEK1 in the mononuclear satellite position in the cavities of most heavily damaged site on Day 3. LEK1 expression overlapped with Hoechst staining. LEK1 also completely overlapped satellite cell marker Pax7 (Figure 3-17). Bupivacaine was injected into mouse TA muscles. Animals were sacrificed 3 days later. Muscles were cryosectioned and immunostained for Hoechst, dystrophin, and LEK1. Representative photomicrographs at 600X are shown.

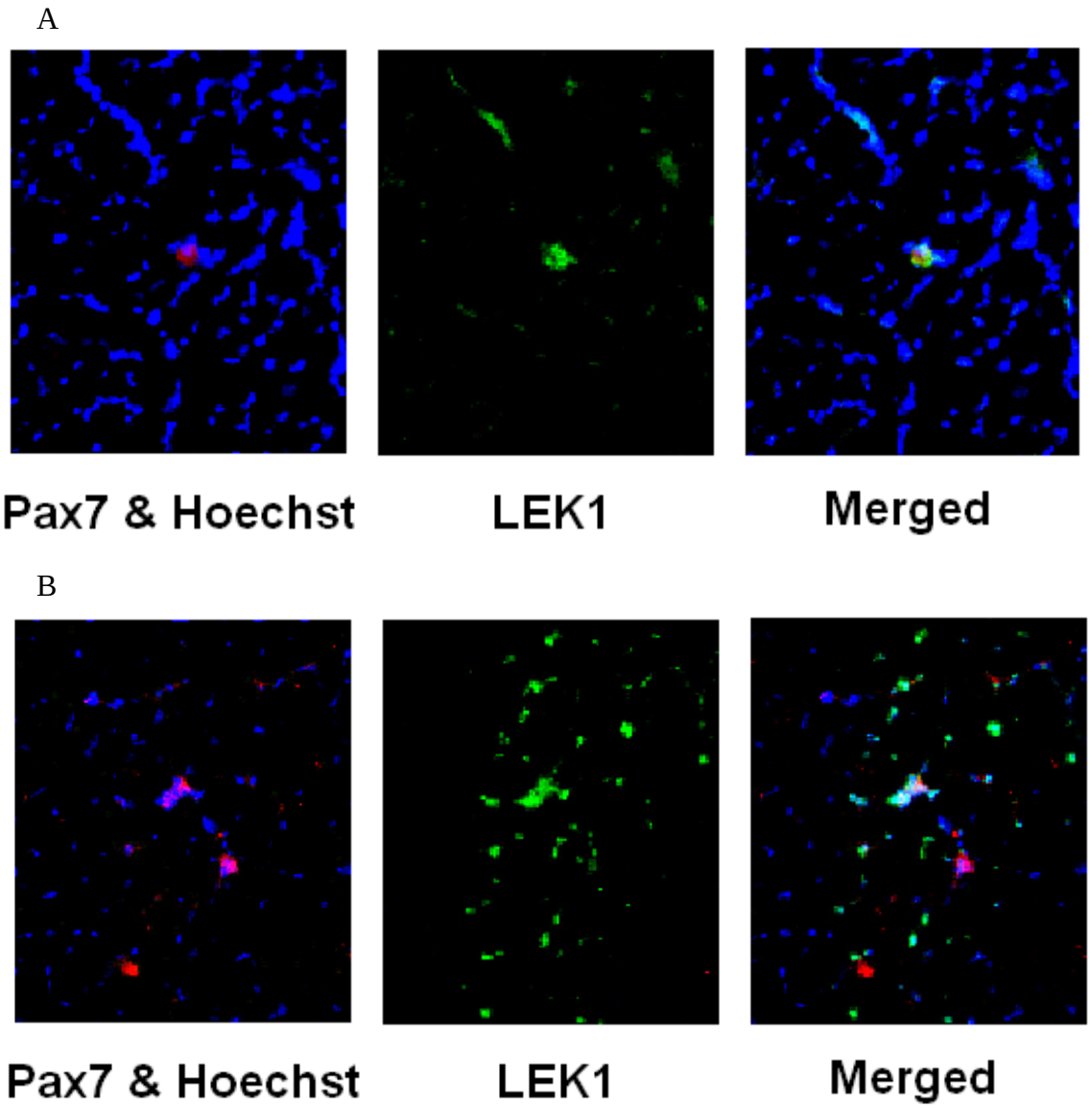


Figure 3-16. Pax7, Hoechst, and LEK1 staining on Day 1. A) Control muscle sections. B) Bupivacaine-treated muscle sections. In intact muscles, expression of Pax7 and LEK1 was very limited. In bupivacaine-treated samples, Pax7 level was slightly elevated on Day 1. In both control and bupivacaine-treated samples, Pax7 overlapped LEK1 and Hoechst staining. Bupivacaine was injected into mouse TA muscles. Animals were sacrificed 24 hours later. Muscles were cryosectioned and immunostained for Pax 7, Hoechst, and LEK1. Representative photomicrographs at 300X are shown.

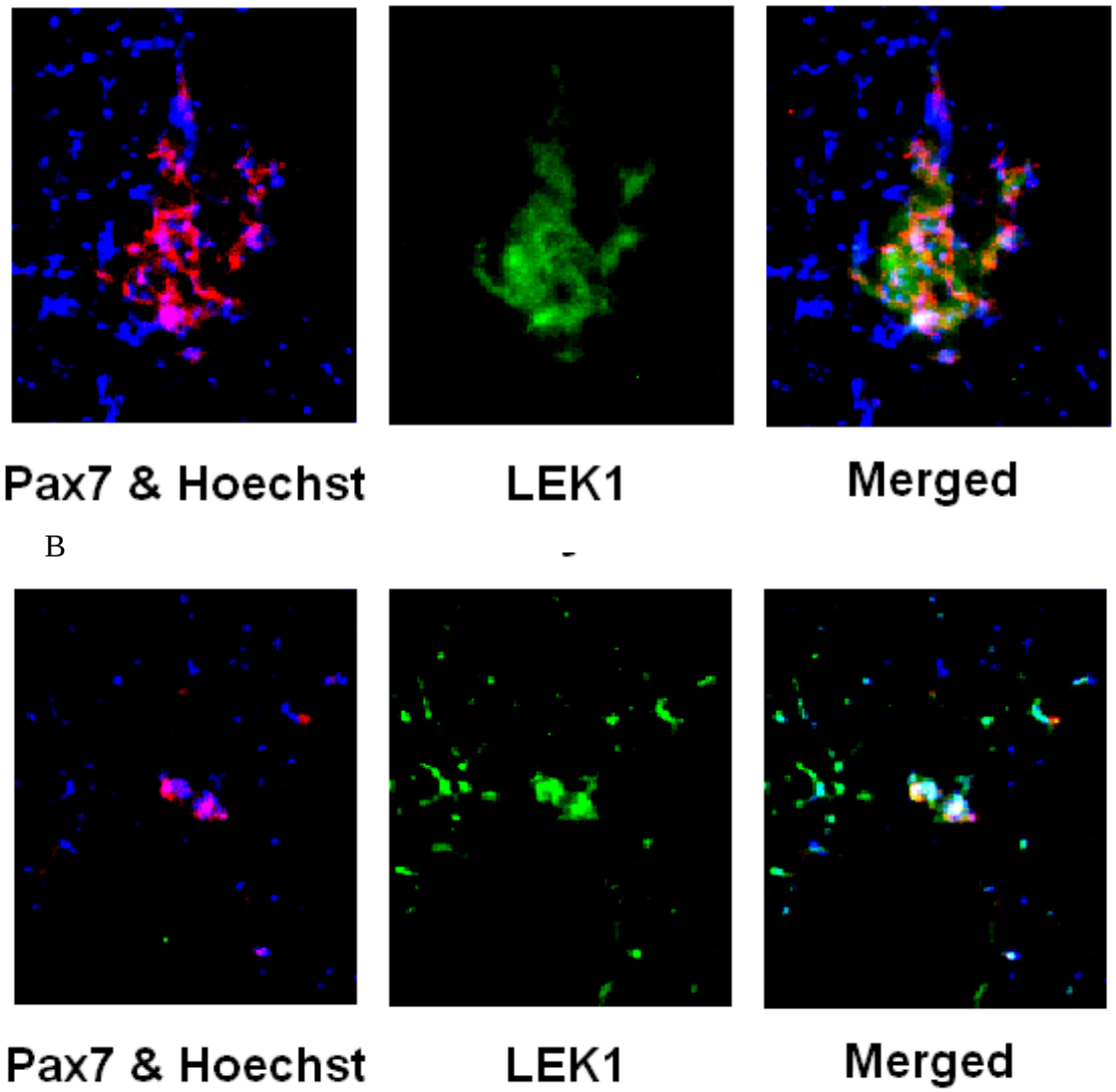


Figure 3-17. Pax7, Hoechst, and LEK1 staining on Day 3 and 5. A) Bupivacaine-treated muscle sections on Day 3. B) Bupivacaine-treated muscle sections on Day 5. Pax7 expression was at its peak and completely overlapped with LEK1 on Day 3. As damaged fibers were repaired, Pax7 expression was reduced while LEK1 reached its peak on Day 5 (Figure 3-13 and 3-21). Bupivacaine was injected into mouse TA muscles. Animals were sacrificed 3 and 5 days later. Muscles were cryosectioned and immunostained for Pax 7, Hoechst, and LEK1. Representative photomicrographs at 300X are shown.

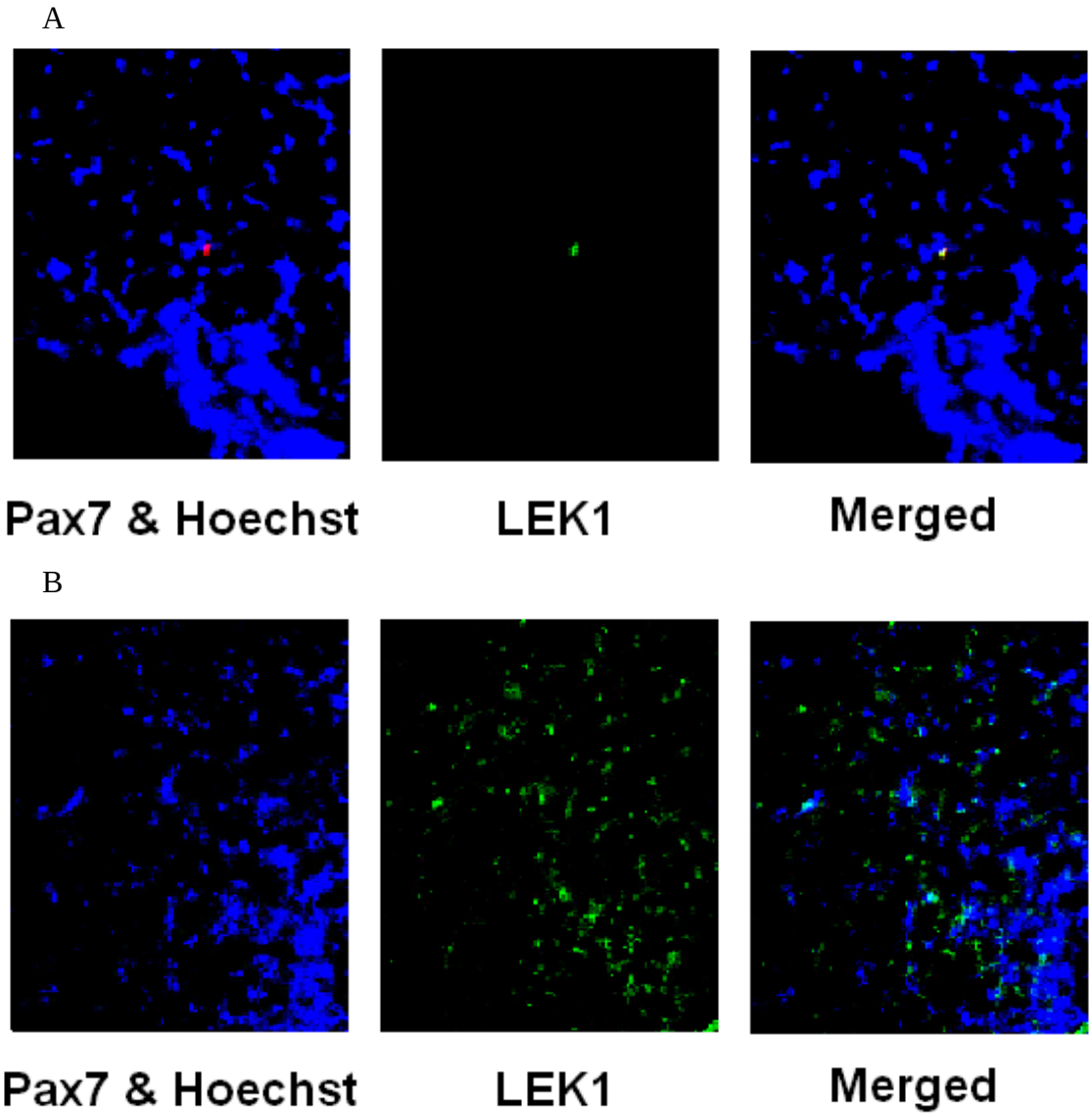


Figure 3-18. Pax7, Hoechst, and LEK1 staining on Day 7 and 10. A) Bupivacaine-treated muscle sections on Day 7. B) Bupivacaine-treated muscle sections on Day 10. Pax7 expression was hardly detectable on Day 7 and completely undetectable on Day 10. Bupivacaine was injected into mouse TA muscles. Animals were sacrificed 7 and 10 days later. Muscles were cryosectioned and immunostained for Pax 7, Hoechst, and LEK1. Representative photomicrographs at 300X are shown.

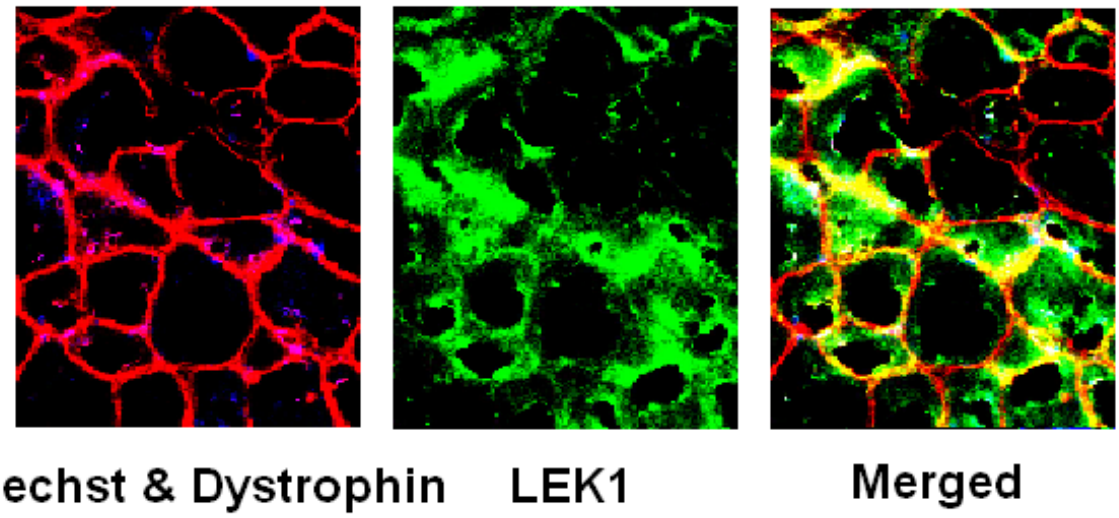


Figure 3-19. Presence of LEK1 in the sarcoplasm of regenerated muscle bed on Day 5. As damaged fibers began to be repaired, LEK1 reached its peak (Figure 3-13 and 3-21). Bupivacaine was injected into mouse TA muscles. Animals were sacrificed 5 days later. TA muscles were cryosectioned and immunostained for Hoechst, dystrophin, and LEK1. Representative photomicrographs at 600X are shown.

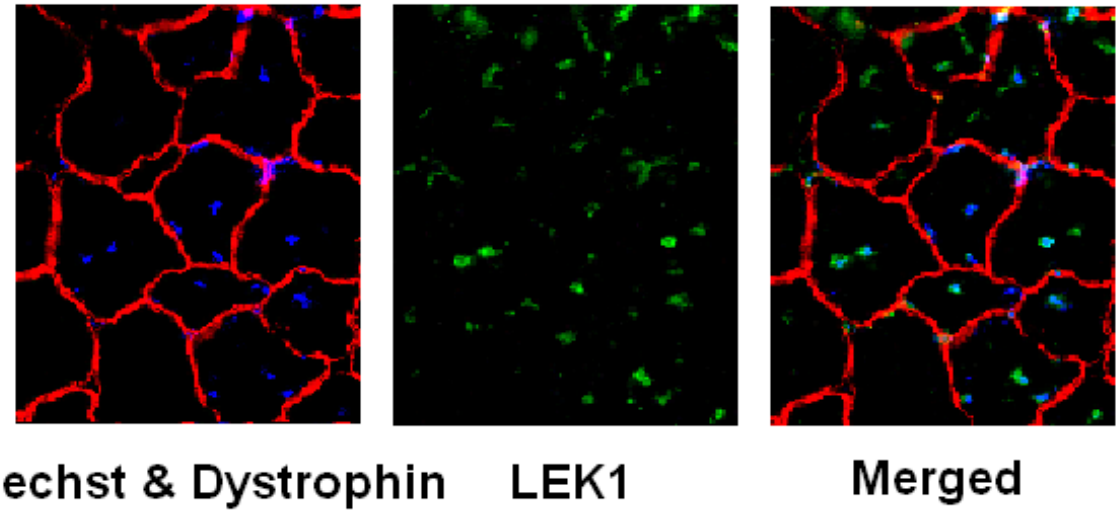


Figure 3-20. Translocation of LEK1 to central nuclei on Day 10 (see also Figure 3-14 and 3-22). Bupivacaine was injected into mouse TA muscles. Animals were sacrificed 10 days later. TA muscles were cryosectioned and immunostained for Hoechst, dystrophin, and LEK1. Representative photomicrographs at 600X are shown.

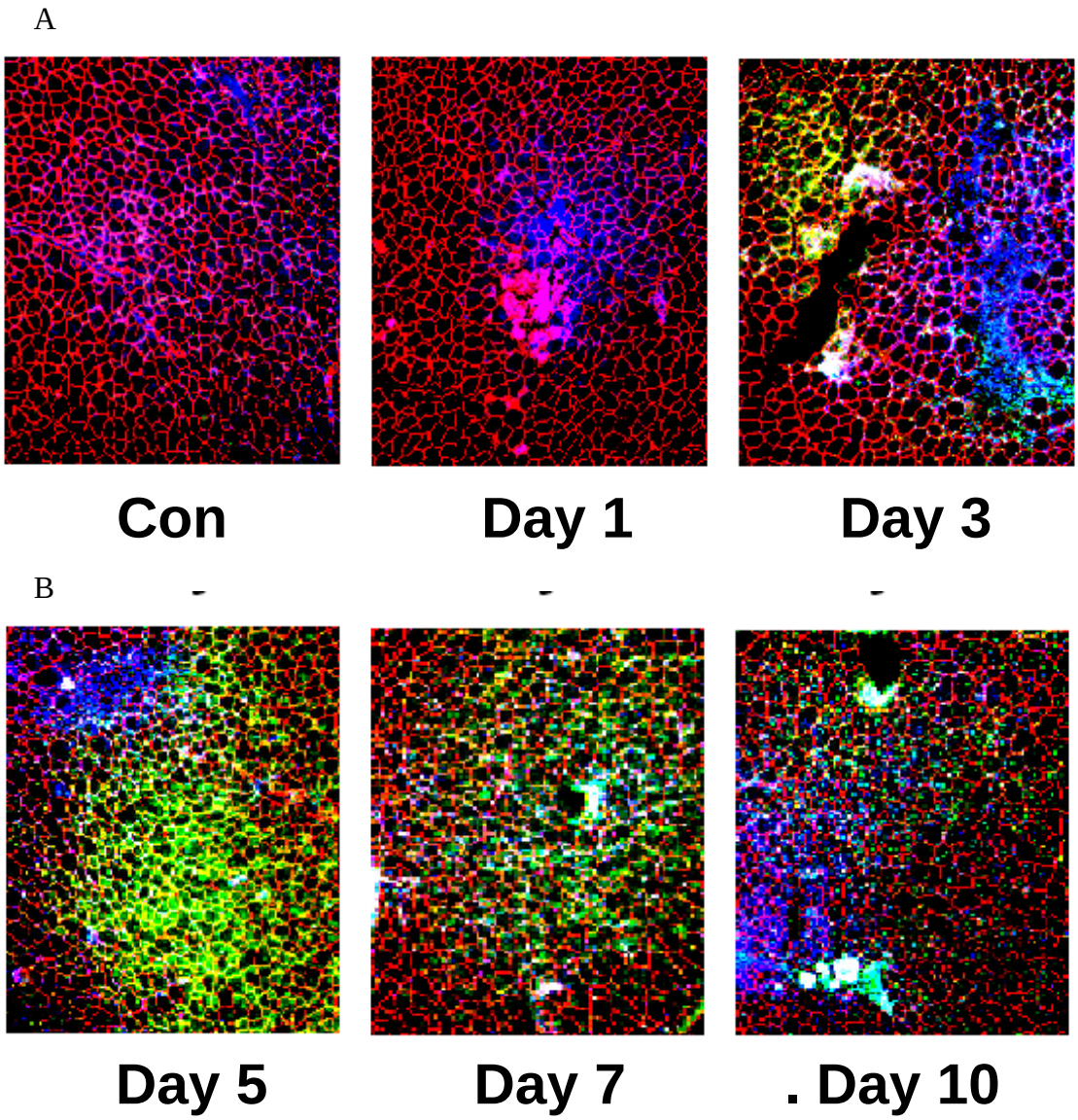
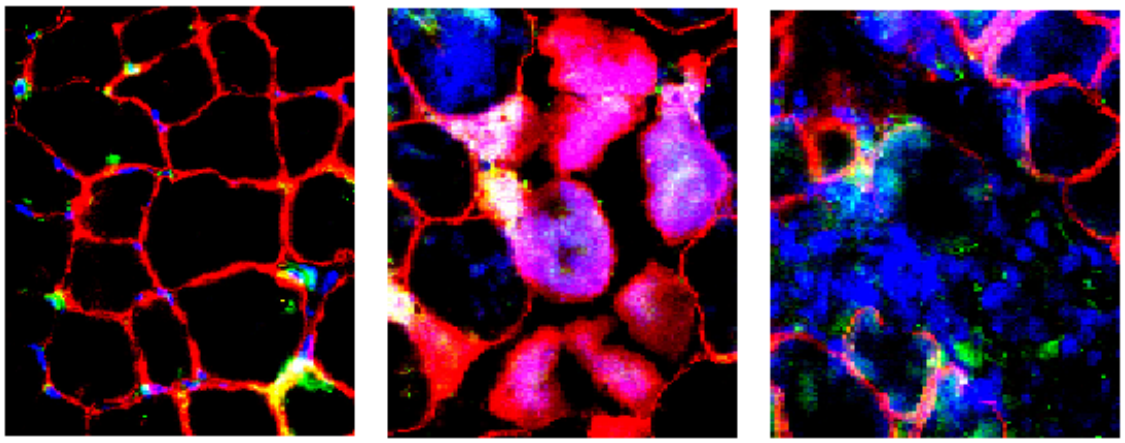


Figure 3-21. Dystrophin, Hoechst, and LEK1 staining throughout the experiment (X100). A) Control and bupivacaine-treated muscle sections on Day 1 and 3. B) Bupivacaine-treated muscle sections on Day 5, 7, and 10. Muscle damage and Hoechst staining reached their peaks on Day 3. In contrast, LEK1 expression reached its peak on Day 5. As damaged fibers were further repaired on Day 7, LEK1 was reduced and then translocated from muscle bed to central nuclei on Day 10 (see also Figure 3-22). Saline or bupivacaine was injected into mouse TA muscles. Animals were sacrificed 1, 3, 5, 7 and 10 days later. Muscles were cryosectioned and immunostained for dystrophin, Hoechst, and LEK1. Representative photomicrographs at 100X are shown.

A



Con Day 1 Day 3

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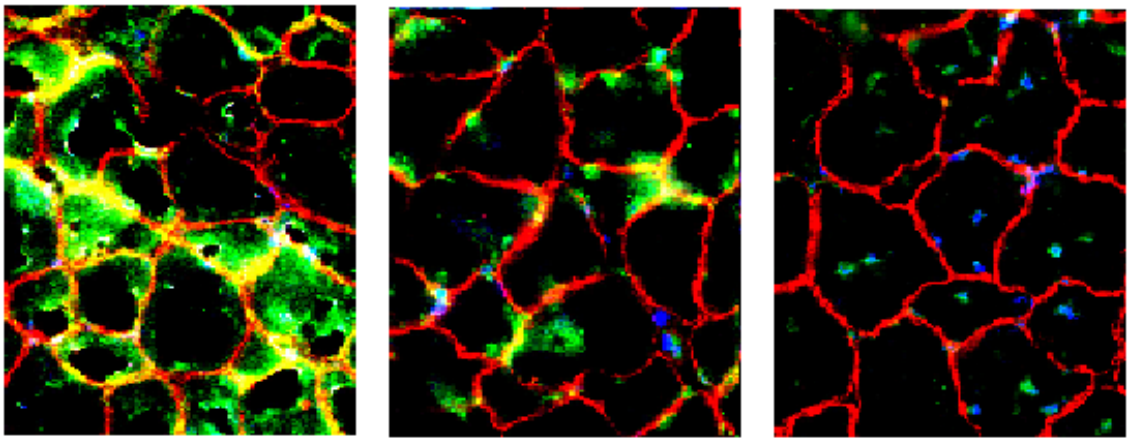

Day 5 Day 7 Day 10

Figure 3-22. Dystrophin, Hoechst, and LEK1 staining throughout the experiment (X600). A) Control and bupivacaine-treated muscle sections on Day 1 and 3. B) Bupivacaine-treated muscle sections on Day 5, 7, and 10. LEK1 with Hoechst staining was identified in the satellite position in control and bupivacaine-treated muscle on Day 10. Muscle damage and Hoechst staining reached their peaks on Day 3. LEK1 was maximal on Day 5. LEK1 was reduced on Day 7 and translocated from muscle bed to central nuclei on Day 10. Saline or bupivacaine was injected into mouse TA muscles. Animals were sacrificed 1, 3, 5, 7 and 10 days later. Muscles were cryosectioned and immunostained for dystrophin, Hoechst, and LEK1. Representative photomicrographs at 600X are shown.

CHAPTER 4
DISCUSSION

Dominant Inhibitory LEK1 Mutants

LEK1 protein is posttranslationally cleaved into the cytoplasmic N- and nuclear C-terminus portions (Ashe et al., 2004; Soukoulis et al., 2005; Pooley et al., 2006). The latter is further cleaved into approximately 60 and 50 kDa fragments in response to MAPK signaling (Reed et al., 2007). Among these, the C-terminus most portion of LEK1 (~ 50kDa) contains several well-known functional domains, such as the Rb-binding domain or NLS (Goodwin et al., 1999; Ashe et al., 2004). Therefore, competitive inhibition of these domains of endogenous LEK1 would reveal the function of these domains in various biological conditions. A dominant negative protein inhibits the function of wild type protein by interacting with the same target as the wild type protein. In this study, various sizes of exogenous GFP-ΔLEK fusion proteins were expressed in C2C12 myoblasts following transfection to determine the effects of C-terminus most 22, 28, and 47 kDa of LEK1 proteins on myoblast proliferation and differentiation (Figure 3-1 and 3-2). Theoretically, these LEK1 mutant proteins compete with endogenous LEK1 for binding to pRb and thus inhibit LEK1-pRb interaction. Since pRb is known as a cell cycle inhibitor and LEK1-pRb interaction is critical for differentiation in a certain cell type (Papadimou et al., 2005), this experimental approach is an ideal to study the function of cLEK1 in myoblast proliferation and differentiation. DNA sequencing and the Western blot analysis showed that ligation and transfection were successfully performed and the plasmids were ready to be used for the proliferation and differentiation assays (Figure 3-2).

The LEK1 Mutants Promote Myoblast Proliferation

The role of LEK1 protein in cellular proliferation have been implicated by several studies based on localization, dominant inhibitory effects of ΔLEK, and the level of LEK1 protein in

various cell types and conditions (Goodwin et al., 1999; Ashe et al., 2004; Dees et al., 2005; Reed et al., 2007; Evans et al., 2007). These studies have shown that its role depends on the cell type and developmental stage.

In mouse TA section, LEK1 protein was localized in the nucleus of satellite cells. In primary culture of satellite cells, LEK1 and E2F5 were found in the nucleus when cells were mitotically inactive. When these cells were activated, LEK1 and E2F5 changed their localization to the cytosol. Furthermore, when 23A2 myoblasts were mitotically arrested by MAPK signaling, LEK1, pRb, and E2F5 translocated from the cytosol to the nucleus (Reed et al., 2007). pRb is a cell cycle inhibitor (Murphree and Benedict, 1984) and E2F5 is one of the transcriptional repressor E2Fs which are necessary for cell cycle exit (Gaubatz et al., 2000). Therefore, via an unknown mechanism, LEK1, pRb, and E2F5 may work in concert to induce the cell cycle arrest. LEK1 binds to all pocket protein members including pRb (Ashe et al., 2004). Although pRb does not bind to E2F5, it may indirectly interact with E2F5 via LEK1 (Reed, 2006) or cross-regulation of E2Fs (Trimachi and Lees, 2002). In response to MAPK signaling, phosphorylated extracellular signal-regulated kinases (pERK1/2), LEK1, pRb, and E2F5 may form a complex with ERK1/2 at nLEK1 and then LEK1 is cleaved into nLEK1 and cLEK1, producing pERK1/2-nLEK1 and pRb-cLEK1-E2F5 complexes. Then, a pERK1/2-nLEK1 complex stays in the cytosol while a pRb-cLEK1-E2F5 complex is targeted to the nucleus (Reed, 2006; Reed et al., 2007). E2F5 does not possess NLS (Chen et al., 2008) while LEK1 and pRb do (Efthymiadis et al., 1997; Goodwin et al, 1999). Therefore, E2F5 may be shuttled to the nucleus by LEK1 or the LEK1-pRb complex when myoblasts are mitotically arrested (Reed, 2006; Reed et al., 2007). LEK1-pRb interaction is necessary for differentiation in embryonic cardiomyocytes (Papadimou et al., 2005). In myoblasts, proliferation and

differentiation are mutually exclusive and cells can differentiate only after the cell cycle exit. Thus, LEK1-pRb interaction may directly or indirectly affect the activity of E2F5 to induce the cell cycle arrest prior to differentiation. This hypothesis is strongly supported by the study using a *Rb* knockout cell line. (Papadimou et al., 2005). In this study, differentiation was almost completely blocked by the absence of pRb, presence of ΔLEK1 (7 kDa), and combination of both. Both endogenous LEK1 and exogenous ΔLEK1 bind to all Rb family pocket proteins (Ashe et al., 2004). In the presence of exogenous ΔLEK1 and endogenous pocket proteins, ΔLEK1 competes with endogenous LEK1 for binding to p107, p130, and pRb and less LEK1-pocket protein complex should be formed. In *Rb* knockout cells, neither LEK1-pRb nor ΔLEK1-pRb complex is formed. This also occurs in the presence of exogenous ΔLEK1 and absence of pRb. These findings indicate that reduced interaction of LEK1 with p107, p130 and/or pRb blocked differentiation. However, absence of pRb alone was sufficient to block differentiation. Therefore, simultaneous expression of LEK1 and pRb rather than p107 and p130 is required for differentiation in cardiomyocytes differentiated from ES cells. Importantly, ectopic expression of pRb completely rescued the ΔLEK1-expressing *Rb* knockout cells and the cells fully differentiated. In this condition, overexpression of pRb should have increased the formation of both LEK1-pRb and ΔLEK1-Rb complexes. Since both LEK1 and ΔLEK1 proteins were expressed in the cells, the expression level of exogenous pRb must have been strong enough so that the sufficient amount of LEK1-pRb complex was formed to rescue the differentiation-deficit cells. In myoblasts, cells must exit the cell cycle to terminally differentiate. Therefore, the LEK1-pRb interaction may induce the cell cycle arrest prior to differentiation. When this is the case, the ΔLEK1-pRb interaction may promote proliferation by reducing the amount of LEK1-pRb complex via competitive inhibition. The results of the current study showed that competitive

inhibition of LEK1 by ectopic expression of ΔLEK1 doubled expression of proliferation marker Ki67 in C2C12 myoblasts (Figure 3-3, 3-4, and 3-5), suggesting that LEK1 induces the cell cycle arrest prior to differentiation in myoblasts, possibly via its interaction with pRb and E2F5. On the other hand, it has been reported that LEK1 may promote proliferation in C2C12 myoblasts (Goodwin et al., 1999; Ashe et al., 2004), NIH3T3 fibroblasts (Ashe et al., 2004), COS-7 fibroblasts (Evans et al., 2007), and embryonic/neonatal heart (Goodwin et al., 1999; Dees et al., 2005). In embryonic and neonatal cardiomyocytes, LEK1 protein expression was high during the mitotically active developmental stage and low during the differentiation stage (i.e., several days after birth) (Goodwin et al., 1999; Dees et al., 2005). In proliferating C2C12 myoblasts, LEK1 protein was found more in the nucleus than the cytosol (Goodwin et al., 1999). In COS-7 fibroblasts, ectopic expression of ΔLEK1 (85 kDa) significantly reduced BrdU incorporation (Evans et al., 2007). Finally, deletion of LEK1 protein using morpholino antisense oligomers resulted in the significantly lower number of cells compared to control in C2C12 myoblasts and NIH3T3 fibroblasts and the reduced number of phospho-histone H3-positive cells in NIH3T3 fibroblasts (Ashe et al., 2004).

It is likely that the disagreement among these studies is due to the different cell types, developmental stages, size of ΔLEK1, and experimental approaches to determine the effects of LEK1 expression on cellular proliferation. For instance, deletion and competitive inhibition of LEK1 protein are not the same biological phenomena. To determine the effects of the LEK1-pRb interaction on cellular proliferation or differentiation, the latter experimental approach is more useful. Cardiomyocytes differentiated from ES cells (*in vitro*) are also biologically different from embryonic/neonatal heart (*in vivo*). Finally, ectopic expression of different sizes of ΔLEK1 (7 kDa vs. 85 kDa) may cause different results. Although it has been suggested that the LEK1-pRb

interaction allows free activator E2Fs to bind DNA stimulating expression of E2F-responsive genes to promote proliferation or inhibit terminal differentiation (Ashe et al., 2004), this hypothesis was based on the study without direct manipulation of pRb expression level or competitive inhibition of LEK1-pRb binding.

Rb pocket proteins are known as coordinators of cellular proliferation and differentiation (Goodrich et al., 1991; Kobayashi et al., 1998). Although the precise mechanism is unclear, it has been suggested that hypophosphorylated pRb binds to E2Fs, repressing and promoting transcription of genes required for proliferation and differentiation, respectively (Nevins et al., 1992). Therefore, pRb-mediated repression of proliferation and promotion of differentiation depends on the phosphorylation status of pRb which is regulated by cdks. However, it also depends on acetylation of pRb by HAT, such as p300 or CBP (Khidr and Chen, 2006). Most pRb are hypophosphorylated in quiescent cells and phosphorylated in proliferating cells (DeCaprio et al., 1989). pRb is activated by dephosphorylation in the G0/1 phase of the cell cycle and inactivated by phosphorylation during the late G1-, S-, G2-, and M-phase (DeCaprio et al., 1989; Buchkovich et al., 1989; Khidr and Chen, 2006). Abundance of growth factors stimulates cyclin D1-3 expression and the cyclin-cdk interaction, which promotes the initial phosphorylation of pRb (Trimarchi and Lees et al., 2002). Accumulation of cyclin E with cdk2 promotes the subsequent phosphorylation of pRb allowing free activator-E2Fs to stimulate transcription of cell cycle genes, which is necessary to pass through the restriction point (i.e., G1 phase checkpoint) of the G1 phase. Once cells go through the restriction point, they enter the S-phase. On the other hand, when cellular conditions are not appropriate to enter the S-phase (e.g., lack of growth factors), proliferation is inhibited and cells may enter the quiescent G0 phase. Inhibitory role of pRb in cellular proliferation depends on availability of active form of pRb before the restriction

point of the G1 phase. Overexpression of hypophosphorylated pRb in the early G1 phase inhibits the S-phase entry while that in the late G1 phase has no effect on proliferation (Goodrich et al., 1991). Thus, pRb-mediated inhibition of cellular proliferation requires hypophosphorylated pRb before the restriction point. Hypophosphorylated pRb binds to an activator E2F-DP complex, which prevents E2F-mediated transcriptional activation of cell cycle genes. One of the suggested mechanisms is that pRb blocks the activation domain of E2F by binding to it (Flemington et al., 1993). The other postulation is that the pRb binds to the E2F–DP complex and recruits chromatin modifying factors, such as HDAC and SUV39H1. HDAC binds to the pRb-E2F-DP complex and deacetylate histone H3, which promotes formation of tightly packed heterochromatin preventing transcription of E2F-responsive genes. Subsequently, SUV39H1 binds to HP1 and methylates histone H3, which silences gene expression by modifying the histone tail (Trimarchi and Lees, 2002). It has further been postulated that repressor E2Fs occupy the E2F response promoters when pRb sequesters activator E2Fs (Frolov and Dyson, 2004). Thus, pRb-mediated cell cycle arrest via these mechanisms requires hypophosphorylated pRb at appropriate timing (i.e., prior to the restriction point). In addition, acetylation of pRb is required for pRb-mediated cell cycle exit and differentiation (Nguyen et al., 2004). In summary, current study suggests that LEK1 protein is a potential inhibitor of cell cycle progression when bound to pRb and that pRb-mediated cell cycle arrest requires the LEK1-pRb interaction.

The LEK1 Mutants Suppress Myoblast Differentiation

The effects of LEK1 protein on cellular differentiation also depends on the cell types, size of ΔLEK1, and developmental stages of cells and the opposite roles of LEK1 protein have been implicated by previous studies (Goodwin et al., 1999; Dees et al., 2005; Papadimou et al., 2005). As described earlier the LEK1-pRb interaction is necessary for differentiation in

embryonic cardiomyocytes (Papadimou et al., 2005). On the other hand, ectopic expression of ΔLEK (165 kDa) stimulated differentiation in C2C12 myoblasts (Goodwin, et al., 1999) and the LEK1 protein level was low during the differentiation phase (i.e., several days after birth) in neonatal mouse heart (Goodwin, et al., 1999; Dees et al., 2005). The disagreement among these studies may be due to the different size of ΔLEK used (i.e., 7 vs. 165 kDa), cell types, and developmental stages.

The role of pRb in cellular differentiation is tissue and cell type specific (Khidr and Chen, 2006). During myoblast differentiation, the level of pRb is elevated (Coppola et al., 1990) and MoyD-mediated transcriptional activation of myogenic genes is stimulated (Gu et al., 1993). *Rb* knockout mice die before birth and newborn mice with reduced expression of pRb exhibit high apoptosis rates and skeletal muscle defects (Zacksenhaus et al., 1996). Inactivation of pRb inhibits differentiation of myoblasts and promotes the cell cycle reentry of differentiated myotubes with reduced expression of MyHC and extremely elevated cyclin A/B and cdk2 (Novitch et al., 1996). Thus, active pRb is necessary for myoblast differentiation (Schneider et al., 1994). However, pRb-mediated skeletal muscle differentiation depends on acetylation of pRb by HAT (e.g., p300 or CBP) and activity of a novel protein, E1A-like inhibitor of differentiation 1 (EID1). Acetylation of pRb is necessary for pRb-mediated cell cycle exit and differentiation (Nguyen et al., 2004) and differentiation inhibitor EID1 need to be degraded prior to muscle differentiation (Krutzfeldt et al., 2005). EID1 is expressed in adult cardiac and skeletal muscle cells (McLellan et al., 2000). EID1 interacts with pRb via its Rb-binding domain, inhibits the HAT activity of p300/CBP, and suppresses MyoD-dependent transcription of muscle-specific genes (McLellan et al., 2000; Bavner et al., 2002; Ji et al., 2003; Khidr and Chen, 2006). Furthermore, disruption of the EID1-pRb interaction potentiates MyoD-dependent muscle gene

transcription (McLellan et al., 2000). Finally, proteasome-dependent degradation of EID1 is required for muscle differentiation (Krutzfeldt et al., 2005), which is promoted by p300/CBP-mediated acetylation of pRb (Nguyen et al., 2004). In C2 myoblasts, pRb depletion stimulates proliferation and inhibits differentiation with elevated E2F1 and cyclin D1 and reduced MyoD, myogenin, and MyHC (Kobayashi et al., 1998). Expression of dominant inhibitory pRb also promotes proliferation and prevents differentiation in C2C12 myoblasts (Li et al., 2000). Thus, pRb is necessary for terminal differentiation in myoblasts. However, without interaction of LEK1, pRb alone is not sufficient to induce differentiation in cardiomyocytes (Papadimou et al., 2005), suggesting that the LEK1-pRb interaction as well as acetylation of pRb is required for differentiation. Although both LEK1 and EID1 bind to pRb, they do so in the opposite situations. The LEK1-pRb interaction promotes differentiation while the EID1-pRb binding inhibits pRb acetylation and differentiation. Thus, LEK1 and EID1 don't have to compete for binding to pRb. Finally, LEK1 and myogenin are coexpressed in one of the daughter cells following satellite cell division while Pax7 and E2F5 are expressed in the other daughter cell (Quellette, 2007), suggesting that LEK1 is required for the cell to differentiate following asymmetric cell division.

In the current study, the dominant inhibitory ΔLEK1 inhibited cellular differentiation both in C2C12 and in *MyoD*-treated C3H10T1/2 cells. In C2C12 myoblasts, ΔLEK (22, 28, and 47 kDa) completely blocked MyHC expression (Figure 3-6 and 3-7). However, in *MyoD*-treated C3H10T1/2 cells, only two of the three ΔLEK (28 and 47 kDa) reduced the level of differentiation marker protein, troponin-I (Figure 3-8). These results suggest that ΔLEK reduces the LEK1-pRb interaction by competing with LEK1 for binding to pRb and inhibits differentiation in myoblasts. Since only 28 and 47 kDa of ΔLEK1 inhibited differentiation in *MyoD*-treated C3H10T1/2 cells, there may be an important domain between 22 and 28 kDa of C-

terminus most LEK1 to inhibit myoblast differentiation. In summary, this study suggests that LEK1 is required for pRb-mediated cell cycle arrest and differentiation in myoblasts.

Establishment of a Muscle Regeneration Model

In response to certain stimuli, satellite cells are activated and skeletal muscle regeneration occurs via activation, proliferation, differentiation, and central localization of satellite cell nuclei (Hawke and Garry, 2001; Charge and Rudnicki, 2004). Since morphological changes during muscle degeneration and regeneration are best characterized following muscle damage (Charge and Rudnicki, 2004), various models such as myotoxin injection or dystrophin-lacking *mdx* mice have been employed. However, features during muscle damage and repair can differ among models and species. For instance, the effects of bupivacaine on muscle damage vary between rats and mice (Holmes et al., 2002; Rosenblatt, 1992). Injection of different myotoxin results in the different degree of muscle damage and repair (Plant et al, 2006). In the *mdx* mice model, there is a report that Pax7 can be expressed in some central nuclei of hindlimb muscle (Seale et al., 2000) despite that another study has shown satellite cells cannot differentiate in the presence of Pax7 in the single myofiber model (Zammit et al., 2006). The damage and regeneration level would also depend on the dosage of myotoxin. Thus, it is important to establish a model which produces a consistent level of muscle degeneration and regeneration in a certain species. In this study, a submaximal dosage (70µl) of 5% bupivacaine was used to induce consistent muscle damage in skeletal muscles of adult male mice (Plant et al., 2006).

In intact adult skeletal muscles, dystrophin staining showed that the integrity of myofiber membrane was maintained. In the bupivacaine-treated samples, fiber necrosis occurred within 24 hours, which was characterized by fused dystrophin (Figure 3-9). The level of muscle damage was maximal on Day 3, creating large cavities (Figure 3-10 and 3-21). Damaged fibers

were significantly repaired on Day 5 (Figure 3-10 and 3-21) and mostly repaired on Day 7 (Figure 3-11 and 3-21). On Day 10, the integrity of myofiber membrane was almost completely recovered and most fibers were centrally nucleated (Figure 3-11). Interestingly, some myofibers had multiple central nuclei, which agrees with the studies using the freeze- and cardiotoxin-induced muscle injury models (Sachidanandan et al., 2002; Epting et al., 2008). Considering the role of central nuclei in fiber growth via gene expression (Charge and Rudnicki, 2004), it is not surprising that some myofibers possess multiple central nuclei in the fiber growth phase of muscle regeneration. All of these results agree with the literature (Charge and Rudnicki, 2004; Plant et al., 2006; Arsic et al., 2008; Epting et al., 2008) and thus the bupivacaine-induced skeletal muscle regeneration model in male mice was well established in this study.

Since Hoechst was used for staining of myonuclei in this study and a similar fluorescent stain DAPI has been used as a marker of macrophages (Browne et al., 2002), it is important to consider the role of macrophage in muscle regeneration. Although the precise role of macrophage in muscle regeneration is unknown (Tidball et al., 1999), it has been believed that macrophages promote muscle regeneration and has been considered as a promising therapeutic tool for muscle diseases. Macrophages and activated satellite cells are recruited to the damaged sites of skeletal muscle within a few days following muscle injury (Fielding et al., 1993; Orimo et al., 1991). One of the known roles of macrophage is to ingest apoptotic nuclei (Esashi et al., 2003). However, more importantly, macrophage and satellite cell cross-stimulate their chemotactic activity and attract each other. Furthermore, macrophages accelerate satellite cell proliferation in a dose-dependent manner *in vitro* (Chazaud et al., 2003). For convenience in the following discussion, individual experimental periods are referred to as the degeneration (Day 1), proliferation (Day 3), differentiation (Day 5), late differentiation (Day 7), and fiber growth (Day

10) phases. This is due to initiation of myofiber degeneration on Day 1, maximal Pax7 expression in injured sites on Day 3, initiation of fiber repair on Day 5, near complete fiber repair on Day 7, and central nuclei on Day 10.

Limited Expression of LEK1 in Healthy Adult Skeletal Muscle

In the intact adult skeletal muscles, Hoechst staining was in the periphery and expression of Pax7 was nearly undetectable (Figure 3-12 and 3-16). LEK1 expression was also minimal in agreement with the previous studies (Goodwin et al., 1999; Dees et al., 2005). However, some Pax7 and LEK1 expression was observed with Hoechst staining in the satellite position (Figure 3-22). Since the inflammatory response does not have to be considered in the intact muscles, Hoechst staining indicates the myonuclei. This observation agrees with the evidences in three different conditions (i.e., in quiescent satellite cells *in vivo*, primary culture of mitotically inactive satellite cells, and mitotically inactive 23A2 myoblasts) and supports the notion that LEK1 may play a role in maintenance of a quiescent state of satellite cells (Reed et al., 2007; Quellette, 2007).

Limited Role of LEK1 in the Degeneration Phase

During the degeneration phase (i.e., Day 1), myofibers began to be destroyed and Hoechst staining was concentrated in the injured sites (Figure 3-12). Since Pax7 was hardly detectable (Figure 3-16), Hoechst staining was mostly due to macrophage invasion, indicating that macrophage influx occured prior to satellite cell recruitment to the injured sites. This observation agrees with the evidences that macrophage influx occurs within 48 hours following skeletal muscle injury (Orimo et al., 1991; Fielding et al., 1993; Charge and Rudnicki, 2004). Although the level of LEK1 protein was low (Figure 3-21), it virtually 100% overlapped with Hoechst staining within degenerating muscle bed (Figure 3-12). Finally, rare expression of Pax7

overlapped with that of LEK1 (Figure 3-16). Because of its low expression level, it was concluded that LEK1 does not play much role during the degeneration period.

Presence of LEK1 in the Mononuclear Satellite Position

In agreement with the literature (Plant et al., 2006), muscle damage was maximal and the large cavities were formed during the proliferation phase (i.e., Day 3). Hoechst staining and LEK1 expression were significantly elevated (Figure 3-21) and concentrated in the cavities (Figure 3-13). Pax7 expression was maximal (Figure 3-17), indicating maximal recruitment and proliferation of satellite cells. This observation agrees with the study in which Pax7 was widely expressed in regenerating muscles of *mdx* mice (Seale et al, 2000). Importantly, Pax7 and LEK1 completely overlapped with each other, suggesting that LEK1 was present in the mononuclear satellite position in the injured cavities (Figure 3-13 and 3-17). In contrast, approximately half of Hoechst staining was Pax7-positive (Figure 3-17). However, the amount of macrophages seemed to be unchanged since Hoechst staining doubled (Figure 3-21), which supports the suggestion that macrophages stimulate satellite cell proliferation (Chazaud et al., 2003). Although LEK1 protein expression was elevated on Day 3, its maximal expression was observed in the differentiation phase (i.e., Day 5). Throughout the experimental period (i.e., Day 1-10), the level of muscle damage and repair differed among individual myofibers. For instance, central nuclei were observed in some myofibers on Day 5 and 7 although most myofibers were centrally nucleated on Day 10 (Figure 3-13 and Figure 3-14). These early regenerating myofibers must have already been in the differentiation phase on Day 3. Therefore, it is likely that LEK1 expression was moderately increased for pRb-mediated cell cycle arrest and differentiation of satellite cells in these myofibers.

Presence of LEK1 in the Sarcoplasm of Regenerated Muscle Fibers

During the differentiation phase (i.e., Day 5), damaged myofibers were significantly repaired with the decrease in the cavity size and even some central nucleated fibers were observed (Figure 3-13). Hoechst staining was reduced by approximately 50% (Figure 3-21) and concentrated in the injured cavity (Figure 3-13). Pax7 expression was very low (Figure 3-17), which is an indication of satellite cell differentiation. Following the cell cycle exit, a majority of satellite cells lose Pax7 (Zammit et al., 2006) and differentiate (Conboy et al., 2003). The cells expressing Pax7 cannot differentiate because it downregulates MyoD and prevents myogenin induction (Olguin and Olwin, 2004). On the other hand, a minority of satellite cells lose MyoD, maintain Pax7, and return to the quiescence (Zammit et al., 2004; Zammit et al., 2006). Thus, the small number of satellite cells expressing Pax7 on Day 5 (Figure 3-17) may have re-obtained a quiescent state in the subsequent phase (Olguin and Olwin, 2004), which should have occurred since the absolute number of quiescent satellite cells remains unchanged following muscle regeneration (Gibson and Schultz, 1983). LEK1 expression was maximal on Day 5. LEK1 was mainly expressed in the sarcoplasm of regenerated muscle bed rather than the regenerating cavity and the integrity of dystrophin was well recovered in these regions (Figure 3-13), indicating that satellite cells shifted from the proliferation to differentiation phase with maximal LEK1 expression. This observation suggests that LEK1 was maximally expressed to induce cell cycle arrest and differentiation of satellite cells possibly via the pRb-LEK1 interaction, which is supported by the results of Ki67 staining (Figure 3-3, 3-4, and 3-5), differentiation assays (Figure 3-6, 3-7, and 3-8), and the study by Papadimou et al. (2005).

The LEK1 Protein Expression Overlaps the Pax7 Domain

During the late differentiation phase (i.e., Day 7), the cavity size further decreased and myofibers were mostly repaired (Figure 3-14 and 3-21). Hoechst staining was mostly in the

periphery. Although little Pax7 expression was observed (Figure 3-18), it overlapped LEK1. Indeed, Pax7 overlapped LEK1 throughout the experiments both in the intact and bupivacaine-treated samples (Figure 3-16, 3-17, and 3-18), implicating the critical role of LEK1 protein in satellite cell proliferation and differentiation during muscle regeneration. LEK1 expression was also significantly reduced (Figure 3-14 and 3-21), suggesting that most satellite cells committed to the myogenic lineage had already differentiated and the role of LEK1 in LEK1-mediated differentiation was reduced.

The LEK1 Protein Translocates to Central Nuclei

In the fiber growth phase (i.e., Day 10), myofibers were almost completely repaired. A single or multiple myonuclei were observed in the center of most myofibers (Figure 3-14), indicating that most myofibers shifted to the fiber growth phase. However, some myonuclei were found in the satellite cell niche with LEK1 expression (Figure 3-20 and 3-22). LEK1 expression was further reduced and translocated to central nuclei (Figure 3-20 and 3-22). Finally, Pax7 was undetectable (Figure 3-18). In most muscle regeneration studies, only a single central nucleus per fiber section has been shown following injury-induced skeletal muscle regeneration (Chargé and Rudnicki, 2004; Plant et al., 2006). However, some studies have shown that multiple central nuclei exist during the fiber growth phase of muscle regeneration (Arsic et al., 2008; Epting et al., 2008). Considering the function of central myonuclei, multiple central nuclei per fiber section should be more efficient to induce gene expression for maximal fiber growth. The absence of Pax7 in the fiber growth phase agrees with the evidence that satellite cell-derived myoblasts cannot differentiate in the presence of Pax7 (Zammit et al., 2006). However, in one study, Pax7 was observed in some central nuclei of the hindlimb muscle of *mdx* mice (Seale et al., 2000). This may be due to the difference in the muscle regeneration model since few other studies have shown the same result. In skeletal muscles lacking dystrophin, degeneration and

regeneration process repeatedly occur and thus satellite cells are continuously recruited. In this condition, replenishment of satellite cell pool becomes more serious task for muscle cells. Expression of Pax7 in some central nuclei of *mdx* mouse skeletal muscle may be related to the repetitive degeneration and regeneration cycle. However, in the other study, endogenous Pax7 was not detected in the central nuclei of *mdx* mouse TA muscle (Seale et al., 2004). Therefore, expression of endogenous Pax7 in central nuclei of *mdx* mouse skeletal muscle must be rare, if any. In contrast, delivery of exogenous Pax7 can generate Pax7-positive central nuclei in *mdx* mice (Seale et al., 2004). The simultaneous translocation of myonuclei and LEK1 proteins to the center of myofiber implicates that LEK1 plays a role in central translocation of the myonuclei and/or maintenance of their central localization. Finally, LEK1 expression with Hoechst staining in the satellite cell niche suggests that LEK1 may play a role in the self-renewal and/or the maintenance of a quiescence state of satellite cells.

CHAPTER 5
SUMMARY AND CONCLUSIONS

Competitive inhibition of LEK1 by certain sizes of its C-terminus mutants significantly stimulated proliferation and inhibited differentiation in myoblasts. Since these mutants contain the atypical Rb-binding domain, it was postulated that LEK1 protein inhibits proliferation and stimulates differentiation by interacting with pRb in myoblasts. During skeletal muscle degeneration and regeneration, LEK1 expression was very low in the degeneration phase, suggesting that LEK1 does not play much role during muscle degeneration. Maximal expression of LEK1 in the differentiation phase implicates its role in cell cycle arrest and differentiation of satellite cells possibly via the pRb-LEK1 interaction. Furthermore, LEK1 may play a role in central translocation of myonuclei and/or its central localization. Finally, localization of LEK1 protein in the satellite cell niche of intact myofibers and myotoxin-injured fiber following damage repair leaves the possibility that LEK1 is involved in the maintenance of a quiescent state and/or the self-renewal satellite cells. Thus, LEK1 may play various roles in the proliferation, differentiation, and fiber growth phase of skeletal muscle regeneration. To my knowledge, this is the first finding that LEK1 is involved in injury-induced skeletal muscle regeneration.

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BIOGRAPHICAL SKETCH

Shigeharu Tsuda was born in Osaka, Japan, in 1968. Since he met his uncle Yoshinori Ikeda at the age of 10, he has been thinking about the value/meaning of existence of the universe. The concept of “universe” later encompassed known/unknown physical/nonphysical entities/phenomena, such as galaxy or mind, in him. As one of the entities on the earth, Shige believed that human lives have priceless value and began to care about the world peace and human happiness, especially after he graduated from Tenri University in 1991. Shige encountered excess greed of human for money in the investment bank, Sanyo Securities Inc. and then learned the beauty of human happiness in the United Sports Club XAX in Japan. The latter experience made him decide to study medicine in the U.S., to contribute to human happiness. Indiana University taught him the ideality in the American academia. University of Florida taught him the reality as well as the ideality as a scientist. Now, Shige is convinced that he can realize the purpose of his life “contribution to human happiness via medicine” only when both the reality and ideality are synchronized. However, the ideality is always superior to the reality in him as a human as well as a scientist. Shige obtained M.S. in Kinesiology at Indiana University in December, 2003 and is finishing another M.S. in Animal Science at University of Florida in August, 2008.