THE ROLE OF THE REGENERATING PROTEIN FAMILY ON SKELETAL MUSCLE REGENERATION

By

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AbAm – Antibiotic / Antimycotic  ECM – Extracellular Matrix
BSA- Bovine Serum Albumin  EGF – Epidermal Growth Factor
C-type Lectins – Calcium-Dependent Lectins  ERK – Extracellular Signal-Regulated Kinases
C2C12 – Mouse Myoblast Cell Line  EXT – Multiple Exostoses
cAMP – Cyclic Adenosine Monophosphate  EXTL – Exostoses-Like Gene
CF – Cystic Fibrosis  FBS- Fetal Bovine Serum
CGMP – Cyclic Guanosine Monophosphate  FGF- Fibroblast Growth Factor
CNTF – Ciliary Neurotrophic Factor  IGF- Insulin-like Growth Factor
DHR – Down Hill Running  HGF- Hepatocyte Growth Factor
Dlk1 – Delta-Like 1 Homolog  HS – Horse Serum
DMD – Duchenne Muscular Dystrophy  IL – Interleukin
DMEM- Dulbecco’s Modified Eagles Medium  IRT – Immunoreactive Trypsinogen
DSS – Dextran Sodium Sulfate  ITS- Insulin Transferrin Selenium
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<td>JAK</td>
<td>Janus Kinase</td>
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<tr>
<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>mdx</td>
<td>Dystrophin Deficient Mice</td>
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<tr>
<td>Megf</td>
<td>Mouse Epidermal Growth Factor</td>
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<tr>
<td>MGF</td>
<td>Mechnano-Growth Factor</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>MRF</td>
<td>Myogenic Regulatory Factors</td>
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<tr>
<td>MyoD</td>
<td>Myogenic Determination Factor 1</td>
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<tr>
<td>MSC</td>
<td>Mouse Satellite Cell</td>
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<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
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<tr>
<td>Myf5</td>
<td>Myogenic Factor 5</td>
</tr>
<tr>
<td>NF-Kb</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
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<tr>
<td>PAP</td>
<td>Pancreatitis-Associated Protein</td>
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<tr>
<td>PAPep</td>
<td>Peptide Derivative of Human Reg IIIα</td>
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<tr>
<td>Pax</td>
<td>Paired Box Protein</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<tr>
<td>PSP</td>
<td>Pancreatic Stone Protein</td>
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<td>PTP</td>
<td>Pancreatic Thread Protein</td>
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<tr>
<td>Reg</td>
<td>Regenerating Protein Family</td>
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<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
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<td>TA</td>
<td>Tibialis Anterior Muscle</td>
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<td>TGF</td>
<td>Transforming Growth Factor</td>
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<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<td>VEGF</td>
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ABSTRACT

Skeletal muscle regeneration is dependent upon the influences of intrinsic and extrinsic factors that stimulate satellite cells. Regenerating proteins are upregulated at the onset of trauma or inflammation in the pancreas, gastrointestinal tract, liver, neural cells and other tissues. Studies have shown that Reg proteins have a mitogenic, anti-apoptotic and anti-inflammatory function in damaged tissues and is necessary for normal progression of regeneration. As skeletal muscle is also able to regenerate itself at a rapid rate, it seems highly likely that Reg proteins function to promote myogenesis in skeletal muscle regeneration. Therefore, the goal of our research was to characterize the expression of the Reg proteins and receptor in regenerating skeletal muscle and satellite cells, investigate the effect of exogenous Reg protein on myogenesis, and to examine direct Reg protein effect on satellite cell activity. To determine whether Reg proteins participate in skeletal muscle regeneration, mice were injected with marcaine in their tibialis anterior muscles to induce skeletal muscle damage. The gene expression analysis of undamaged and marcaine-damaged tibialis anterior muscles and mice satellite cells showed that Reg I, II, IIIα, IIIγ, IV and EXTL3 genes are present during skeletal muscle regeneration and satellite cells significantly express Reg I, IIIα, IIIγ and EXTL3. As Reg I and IIIα are most prevalent in vivo and in vitro respectively, we advocate these isoforms as the predominant candidates in skeletal muscle regeneration. To determine the effect of exogenous Reg protein on myogenesis, we performed gene expression and muscle morphometry analysis of Reg IIIα or PBS injected tibialis anterior muscles. Interestingly, our results indicate that the addition of Reg IIIα to damaged muscles
inhibited myogenesis. To determine the direct effect of Reg protein on myogenic stem cell activity, Reg proteins were added to mice satellite cells and C2C12 cells. Results from these studies were inconclusive due to the failure of known positive and negative controls. Overall, our studies suggest that Reg proteins contribute to skeletal muscle regeneration; however, as an overabundance of Reg IIIα in regenerating tissues may have inhibited myogenesis, it is imperative that other isoforms or lower concentrations be investigated.
CHAPTER 1

LITERATURE REVIEW

Skeletal Muscle

Skeletal muscle is a dynamic tissue that is important in numerous physiological processes. Skeletal muscle constitutes 30-40% of total body weight in an average human, making it one of the major organ systems. Over 600 skeletal muscles work together to perform essential functions such as locomotion, posture, respiration and thermoregulation (Powers and Howley 2004, Sherwood 2010). Damage to this system can impair movement, lead to loss of muscle mass and death in severe cases. Therefore, the exceptional regenerative ability of skeletal muscles has a profound impact on the continuation of these essential functions to maintain life.

Dysfunction of Skeletal Muscle

Muscular dystrophies, sarcopenia and muscle trauma are prime examples of the consequences of the loss of the regenerative capabilities of skeletal muscle. Muscular dystrophies are genetic diseases that cause mutations in proteins of the myonuclei, sarcomere, sarcolemma, extracellular matrix, basement membrane and enzymatic proteins (Amato and Russell 2008a). Necrosis of muscle tissue and infiltration by connective and fatty tissue leads to progressive loss of muscle function, leading to difficulties in locomotion, respiration and survival. The most prevalent form of muscular dystrophy is the X-linked Duchenne muscular dystrophy, and has a prevalence of 1 in
3,500 male births. Duchenne muscular dystrophy is characterized by progressive loss of muscle function, leading to dependence on ambulatory aides by their early teens and death in their late teens or early twenties. Mutations such as deletions, point mutations and duplications on the dystrophin gene causes altered dystrophin protein expression. Dystrophin is an essential myofibrillar protein that composes approximately 5% of sarcolemmal cytoskeletal protein and imparts structural stability by connecting the actin cytoskeleton to the extracellular matrix. An abnormal expression of dystrophin therefore results in decreased structural stability during muscle contraction, leading to increased susceptibility to muscle fiber damage and eventual muscle fiber necrosis (Fairclough, Bareja and Davies 2011). Serum creatine kinase levels, an indicator of muscle damage, are distinctly elevated at birth, then peak at approximately 3 years of age. Muscle biopsies in patients demonstrate variability in fiber size, scattered necrotic and regenerating muscle fibers and increased endomysial and perimysial connective tissue content. Numerous therapeutic methods including stem cell therapy, gene therapy and steroid therapy have been devised, but currently only corticosteroids have been shown as an effective therapeutic option in improving the quality of life for patients with Duchenne muscular dystrophy. Increased susceptibility to muscle damage leads to repeated degeneration and regeneration cycles until the regenerative capacity of satellite cells is exhausted and damaged muscle fibers are replaced by fibrotic and adipose tissues (Fairclough et al. 2011). Clearly, if the capacity of satellite cell regeneration was not exhausted or their intrinsic activity levels were significantly enhanced, it may prove to be an effective therapeutic method for muscular dystrophy treatment.
Sarcopenia is the progressive loss of muscle mass due to old age. Loss of muscle mass leads to overall loss of muscle function, compromising physical performance, independence, quality of life and survival. Some causes of sarcopenia include decreased levels of physical activity, alteration in endocrine function, inflammation and chronic diseases such as obesity and diabetes (Lynch 2011). Recent studies have shown that approximately 20% of the geriatric population in the United States is functionally disabled, with estimated sarcopenia-related health care costs in 2000 at approximately $18.5 billion, comprising a significant percentage of total healthcare expenditure at roughly 1.5% (Janssen et al. 2004). Janssen et al. speculated that a 10% reduction in sarcopenia prevalence could lower the economic burden by $1.1 billion a year, a reduction that is highly attainable with maintenance of skeletal muscle mass throughout life (Janssen et al. 2004). Some functional alterations in sarcopenic muscle include decreased maximum force production, a slowing of twitch response, higher prevalence of type I fibers, lower insulin sensitivity, reduced oxidative defense, poor mitochondrial function and infiltration by adipose and connective tissue (Ryall, Schertzer and Lynch 2008). Although exercise is highly effective in slowing the rate of muscle wasting, progressive muscle loss still occurs, indicating that intrinsic factors in skeletal muscle may be the primary contribution to sarcopenia. Ultimately, the rate of muscle catabolism overshadows the muscle’s anabolic capabilities leading to a cumulative state of muscle wasting (Narici and Maffulli 2010). Although controversial, some studies have demonstrated an age-dependent decrease in satellite cell content, which may decrease skeletal muscle regenerative capacity and ultimately cause an imbalance in the body’s
ability to maintain muscle mass (Snijders, Verdijk and van Loon 2009, Shefer et al. 2010, Kadi and Ponsot 2010, Koopman 2011). Changes in microvasculature, hormonal regulation and intracellular signaling cascades associated with aging can all disturb normal satellite cell activity, perturbing satellite cell activation, proliferation, differentiation and self-renewal (Russell and Leger 2011). Therefore, mitogenic factors that promote the regenerative and self-renewal function of satellite cells may alleviate problems associated with sarcopenia.

Traumatic skeletal muscle damage can be caused by exercise or crush injuries associated with automobile or construction accidents. Direct damage to skeletal muscles can cause fiber tearing, inflammation and myofiber necrosis. Specific mechanisms of skeletal muscle degeneration and regeneration will be covered in more detail in the following chapters.

**Experimental Models to Study Skeletal Muscle Regeneration In Vivo**

Experimental models have been vital in the investigation of skeletal muscle regeneration, whether it be the observation of the temporal progression of regeneration, importance of intrinsic and environmental factors, and determination of specific molecular and biochemical events. *In vivo* models allow investigation of the contribution of a complete physical system to skeletal muscle regeneration, while *in vitro* models allow specific isolation of cause and effect with fewer components involved.

Some of the first investigations into skeletal muscle regeneration have been in mammalian wound healing, where observations were broad and not specific to muscle at
the tissue level (Carlson 1970). An innovative method developed in 1952 by Studitsky of transplanting minced muscle back into damaged areas and observing regeneration reversed the theory at that time that skeletal muscle does not regenerate. This ischemic model of muscle regeneration also provided a method that isolated muscle tissue effects on regeneration more specifically than previous models being used (Carlson 2008). The initial stages are characterized by sarcoplasmic degeneration, formation of vasculature in the periphery of the minced mass and infiltration of inflammatory cells. Three distinctive zones appear in the minced mass at the onset of muscle regeneration: the central zone, the transitional zone and the peripheral zone. The central zone is characterized by a lack of vascularization and regeneration, the transitional zone contains sarcoplasmic fragments of the damaged fibers and shows initial stages of regeneration and some vascularization, while the peripheral zone has fully regenerated fibers. As time progresses, the central zone gradually minimizes in size as the peripheral zone grows, ultimately leading to fully regenerated fibers. Maturation and orientation of fibers, connective tissue development and full vascularization in the regenerated area follow. Although the fibers have technically been regenerated and replaced, the functionality of these regenerated muscle fibers is severely limited in size as well as by the increased connective tissue content (Carlson 1970). The muscle mince model was advantageous for histological studies to study the spatiotemporal correlation of revascularization and muscle regeneration, but was still not specific enough to eliminate the effects of non-muscle cells or tissues.

Free muscle grafting was developed next, where an intact muscle was completely removed from its bed by excising the tendons and all neurovascular connections, then
replaced by surgically reattaching the tendons. Similar to the minced muscle model, the
graft experiences an ischemic environment and therefore undergoes necrosis. Initiation
of vascularization and infiltration of inflammatory cells occur within a couple of days in
the rat, followed by the reinnervation of the graft, then myoblasts and early myotubes
form under the basal lamina and replace the damaged fibers. Reminiscent of the minced
muscle model, the functionality of the resulting regenerated muscle only reaches
approximately 30% of its original mass. However, if the nerves are left intact, the
necrotic process still occurs but the regeneration progression is accelerated and the
resulting regenerate resembles normal muscle, as well as developing 85-90% of its
original maximum contractile force (Carlson and Faulkner 1983). These studies
established that innervation is a crucial factor in the final functionality of the transplanted
muscle graft, as well as the development of an *in vivo* model that ultimately regenerates
muscle close to its original performance.

An adaptation of the free muscle graft model is cross-transplantation. The
transplantation of muscles from its original setting to a different environment allows
investigation of intrinsic and environmental factors on muscle regeneration. One of the
first cross-transplantation experiments was performed by transplanting slow and fast
muscles into their counterpart environments (Gutmann and Carlson 1975). The authors
observed that the transplanted muscles started to display the contractile and histochemical
properties of the environment rather than their original fiber types. While this study
utilized whole muscles, a previous study by Salafsky et al. used minced muscles of a
single fiber type and transplanted them into the environment of a different fiber type, and
saw that the regenerated fibers took on the characteristics of the environment (Salafsky 1973). When Salafsky transplanted minced dystrophic mice muscle into normal mice, the resulting regenerated muscle fibers achieved normal contraction characteristics, while normal minced muscle transplanted into dystrophic mice resulted in a dysfunctional regenerate (Salafsky 1971). To further investigate the importance of environmental factors on muscle regeneration, Carlson and Faulkner performed cross-transplantation between rats of different ages (Carlson and Faulkner 1989). While the transplanted muscles from old rats regenerated approximately 2 fold greater mass and 2.6 fold greater maximum contractile force in the young hosts than the old muscles autografted back into their original environments, the young muscles transplanted into old hosts faired significantly worse, with regenerated mass and maximum contractile force no different than the old muscles autografted back into their old hosts. In essence, the old muscles transplanted into young hosts regenerated as well as young muscles and the young muscles transplanted into old hosts regenerated as well as old muscles. These studies further contributed to the idea that the host environment determines the regenerative ability of skeletal muscle.

Parabiosis surgically connects the circulatory system of two individuals to study the humoral influences on a physiological characteristic. Parabiosis has been utilized to study obesity, diabetes, neurogenesis and skeletal muscle regeneration. The most common parabiotic technique used is a heterochronic parabiosis, where vascular anastomosis is performed between a young and old animal. To study the systemic factors affecting the activity of satellite cells, Conboy et al. generated parabiotic pairs consisting
of young and old mice. When muscles were injured in the old mice, the regeneration characteristics resembled those of the young mice, with successful myotube formation and scarce fibrosis formation. An important finding was that the nascent old satellite cells were the main contributors to this superior regeneration, with less than 0.1% contribution from the young counterparts. They concluded that the systemic factors of young animals promote proliferation and regeneration of skeletal muscles, and that aged cells retain their intrinsic proliferative capacity (Conboy et al. 2005). Parabiosis can be a powerful tool to determine systemic influence on skeletal muscle regeneration, without the complications that accompany muscle transplantation models. However, the increased stress placed on the animals and the exposure of factors from the original host can complicate observations.

The direct injection of myotoxic agents into muscle is a prevalent method to induce muscle damage. Local anesthetics, in particular, are popular agents that have been found to cause partial or full degeneration of muscle fibers. So far, all local anesthetics that have been examined cause muscle degeneration to some degree, including procaine, tetracaine, lidocaine, chloroprocaine, dibucaine, piperocaine, ropivacaine and marcaine. An injection of marcaine, a long-lasting piperidylidide derivative, causes local toxicity restricted to muscle fibers alone, without apparent effects on surrounding tissues. Among all the local anesthetics tests, marcaine causes the most severe muscle degeneration and is therefore widely adopted by those studying skeletal muscle regeneration (Foster and Carlson 1980, Benoit and Belt 1970, Zink and Graf 2004). Within minutes of local anesthetic injection, affected muscle fibers become hypercontracted, followed by
disruption of the sarcoplasmic reticulum, infiltration by macrophages and subsequent cell debris phagocytosis. Throughout the degeneration phase, the basal laminae remains intact, providing a theory that newly regenerated muscle fibers orient themselves by the basal laminae. The mechanism by which local anesthetics induce muscle degeneration is still under inspection, but main hypotheses circulate around the disruption of calcium homeostasis through stimulation of calcium release from the sarcoplasmic reticulum, decreased calcium permeability or mitochondrial calcium uptake inhibition. Benoit et al. observed that anesthetic and non-anesthetic agents that promote increased sarcoplasmic calcium levels induced a myonecrotic reaction, whereas tetrodotoxin, a local anesthetic with no effect on calcium homeostasis, failed to cause significant muscle degeneration (Benoit, Yagiela and Fort 1980). The variability of degeneration in local anesthetic injections can both be an advantage or a disadvantage – the comparison of undamaged and damaged areas adjacent to each other provide an intrinsic control for histological analysis, but one must take into account that pockets of undamaged fiber bundles exist within damaged areas for biochemical or molecular analysis. Injection of venom toxins from snakes, bees, tarantulas and bacteria cause degeneration patterns similar to local anesthetics, but the effects are not as specific to muscle fibers. Some toxins can cause vascular damage, which can ultimately alter the degeneration characteristic and introduce inconsistencies.

Other in vivo models for investigating skeletal muscle regeneration include transection, crush injury, thermal damage, denervation and loading/unloading models. These models suffer from inconsistencies in their degeneration characteristics as well,
however are much more applicable to real traumas experienced in human life (Carlson 2008).

Genetic models can also be utilized to study skeletal muscle regeneration. The *mdx* mouse model is perhaps the most extensively used as the animal model of Duchenne muscular dystrophy (DMD), where mice are deficient in dystrophin due to a mutation in the dystrophin gene (Chargé and Rudnicki 2004). Dystrophin is an important protein in maintaining the connection between the myofiber cytoskeleton to the extracellular matrix, stabilizing the membrane during contraction (Banks and Chamberlain 2008). The loss of structural stabilization during contraction makes *mdx* mice more susceptible to contraction-induced injury, which ultimately leads to myofiber necrosis. The skeletal muscles of *mdx* mice undergo continuous bouts of degeneration, inflammation and regeneration starting at 3 weeks of age (McGeachie et al. 1993, Partridge 2006). Limitations of the *mdx* mice model include a moderate 20% reduction in life span, no replacement of regenerated muscle fibers with fibrosis, only partial replacement with adipose cells, merely slight alterations in the myotendinous junctions, and no loss of mobility with age (Banks and Chamberlain 2008). All of these symptoms are relatively mild compared to Duchenne muscular dystrophy patients who experience a radical 70% reduction in life span accompanied by severe limitations in mobility and alterations to their skeletal muscle structure.
**Satellite Cells**

Adult mammalian skeletal muscle is composed of post-mitotic muscle fibers with multiple, peripherally located nuclei with a low nuclei replacement rate of ~1% per week in adult rat skeletal muscles (Schmalbruch and Lewis 2000). However, full regeneration of myofibers within 7 days post-muscle damage (Martinez et al. 2010) demonstrates the amazing regenerative capabilities of skeletal muscle, mainly due to the population of resident myogenic stem cells called satellite cells.

**History**

Satellite cells were first identified and named by Alexander Mauro in 1961 through electron microscopy of frog tibialis anterior muscles (Mauro 1961). He observed cells that were closely associated with skeletal muscle fibers that contained very little cytoplasm, causing the cell to assume the shape of its nuclei. Satellite cells were so named for their peripheral location under the basal lamina but above the sarcolemma, in contrast to myonuclei’s position within the sarcolemma. At this initial stage Mauro was unable to further investigate the function of these cells, however he did note that the incidence of satellite cells were much less frequent than normal myonuclei. Mauro then contacted other scientists and found that satellite cells were also found in the sartorius and ileofibularis muscles of the frog and the sartorius and tongue muscle of the rat (Mauro 1961). In accordance to skeletal muscle’s ability to regenerate itself after injury, Mauro hypothesized that these cells “are remnants from the embryonic development of the multinucleate muscle cell which results from the process of fusion of individual
myoblasts. Thus the satellite cells are merely dormant myoblasts that failed to fuse with
other myoblasts and are ready to recapitulate the embryonic development of skeletal
muscle fiber when the main multinucleate is damaged”. Discovery of satellite cells in
human, feline, canine, murine and fruit bat skeletal muscle closely followed in
subsequent years (Scharner and Zammit 2011). Further research showed that satellite
cells were rare in adult uninjured muscle of humans and bats, and cells were also more
prevalent in slow muscles, possibly indicating a fiber-type influence in satellite cell
characteristic. Initially, satellite cells as the source for new myonuclei in damaged
skeletal muscle remained a controversial issue, as many researchers still believed that
new myoblasts were produced by the dedifferentiation of existing myonuclei (Scharner
and Zammit 2011). Church et al. solidified the role of satellite cells when they observed
the survival of satellite cells in the crushed web muscles of the fruit bat (Church, Noronha
and Allbrook 1966). Not only did the satellite cells survive, but their subsequent
disappearance from areas of maximal damage corresponded with the appearance of
increasing numbers of proliferative myoblasts that eventually replaced the damaged
muscle fibers. When young rat tibialis anterior muscles were injected with
[3H]thymidine, Moss and Leblond saw that only satellite cells were labeled an hour post-
injection, confirming their suspicions that satellite cells were the source of new
myonuclei in skeletal muscle regeneration (Moss and Leblond 1971). 18 hours post-
[3H]thymidine injection, they observed that 5% of all nuclei labeled were myonuclei.
This trend increased with 50% of the total labeled nuclei being categorized as myonuclei
within 48 hours. They speculated that this was due to the division and fusion of
previously labeled satellite cells with damaged fibers, transforming into myonuclei. Isolation of individual myofibers allowed targeted seclusion of satellite cells in culture without the invasion of surrounding tissues and associated cells. Satellite cells on these isolated single myofibers subsequently gave rise to new myofibers which fused with existing fibers, proving that satellite cells locally resident on damaged myofibers gave rise to new myoblasts. The reappearance of satellite cells on the regenerated myotubes established their function as a myogenic stem cell by its ability to divide and differentiate into myogenic cells and self-renew for future regeneration. The authors also observed that not all satellite cells were incorporated as myonuclei. Satellite cells underwent repeated bouts of division, followed by the incorporation of a portion of their daughter cells as myonuclei. From these results the authors concluded that satellite cells were the source of new myonuclei in skeletal muscle growth of young animals. Following along the findings that satellite cell population decreases with age (Snow 1977), Schultz et al. established that satellite cells in non-growing adult muscle are quiescent when they observed a significant decrease in labeled satellite cells of adult mice (Schultz, Gibson and Champion 1978). Bischoff observed that approximately 32% of sublaminal nuclei in neonatal rats were satellite cells, which declines to approximately 5% at 2 months and onward (Bischoff 1994). Partridge and colleagues were one of the first groups to investigate the potential therapeutic application of implanting satellite cells into subjects with muscular dystrophies (Partridge, Grounds and Sloper 1978). When minced muscle was transplanted from the thymidine-treated rats into untreated littermates, the regenerating myonuclei in the littermate became labeled four to six days post-transplant.
They theorized that with further research and modifications, a transplantation of healthy satellite cells into patients with muscular dystrophies could promote muscle growth. These findings established satellite cells as skeletal muscle stem cells.

**Muscle Regeneration**

Skeletal muscle has a tremendous capacity for regeneration, whether it be of physical, chemical or genetic origins. Some examples of muscle damage of physical origin may include resistance training, crush injury, or freeze damage. Chemically-induced skeletal muscle damage can result from local anesthetics, biological toxins and other myotoxic agents. Genetic diseases such as Duchenne muscular dystrophy cause mutations in proteins of the myonuclei, sarcomere, sarcolemma, extracellular matrix, basement membrane and enzymatic proteins (Amato and Russell 2008b). Regardless of the source, muscle injury can lead to sarcomere disruption, extracellular matrix disturbance, connective tissue injury and increased capillary permeability; all can ultimately initiate muscle necrosis. Increased concentrations of muscle injury indicators such as creatine kinase, myoglobin, myosin, and inflammatory factors such as cytokines and neutrophils are among the numerous chemical changes observed (Clarkson and Hubal 2002).

Adult satellite cells are normally transcriptionally maintained in a mitotically and metabolically quiescent state. Numerous markers are present in quiescent satellite cells, including Pax7, M-Cadherin, CD34, caveolin-1, syndecan 3 and 4, and many more (Zammit 2008). The most notable of these factors is Pax7, a member of the paired-box
transcriptional regulators, implicated in determining the myogenic lineage of progenitor cells as well as the maintenance of satellite cell viability in postnatal muscle. When Seale et al. generated Pax7-null mice, they observed an absence of satellite cells in the postnatal skeletal muscles, as well as an inability to produce myoblasts in culture (Seale et al. 2000). Satellite cells underwent apoptosis soon after birth, causing these animals to grow abnormally, only reaching approximately 50% the body weight of wild-type littermates by day 7 and often failing to survive for longer than two weeks. Olguin & Olwin examined the effect of Pax7 regulation on satellite cell renewal, and saw that Pax7 was upregulated in cells that returned to quiescence. From this observation, they decided to induce the overexpression of Pax7 in proliferating cells and showed that satellite cells exited the cell cycle and returned to a dormant state (Olguin and Olwin 2004). The overexpression of Pax7 in proliferating cells downregulated MyoD and inhibited myogenin stimulation, resulting in myogenesis inhibition. These observations led to the theory that Pax7 expression is necessary for satellite cell self-renewal. However, the inactivation of Pax7 through conditional gene inactivation methods revealed that targeted inactivation of Pax7 in the tibialis anterior muscles of mice produced satellite cells that maintained their proliferative and self-renewal characteristics (Lepper, Conway and Fan 2009). Therefore, there may be a very limited window of Pax7 expression that preserves proper satellite cell survival and renewal in skeletal muscles. Pax7 is expressed in satellite cells throughout the proliferative stage and wanes once myoblasts begin to terminally differentiate. Much like Pax7, the Notch signaling pathway also has a crucial role in the maintenance of satellite cell quiescence. Quiescent satellite cells express
Notch3 in abundance, and an inhibition of Notch signaling completely eliminates postnatal satellite cells (Kuang and Rudnicki 2008, Conboy and Rando 2002).

Injury to skeletal muscle leads to the destruction of the sarcolemma and basal lamina, initiating the release of concentrated amounts of calcium (Belcastro, Shewchuk and Raj 1998). Calcium-activated enzymes (calpains) go into overdrive and instigate proteolysis of myofibril structures such as myosin, actin, desmin, titin, and troponin, which causes muscle fiber hyalinization and eventual lysis (Shi and Garry 2006, Ciciliot and Schiaffino 2010). Muscle necrosis leads to infiltration by inflammatory and phagocytic cells such as neutrophils and macrophages that scavenges tissue debris and release pro-inflammatory cytokines to promote inflammation (Ciciliot and Schiaffino 2010). Muscle damage also leads to the upregulation of nitric oxide synthase (NOS), which consequently causes increased production of NO (Anderson and Wozniak 2004). Overproduction of NO stimulates the activation of matrix metalloproteinase-2 (MMP2), a zinc-dependent endopeptidase that breaks down components of the extracellular matrix (Yamada et al. 2008). Degradation of the extracellular matrix allows the secretion of growth factors such as hepatocyte growth factor (HGF), insulin growth factor (IGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF). So far, HGF is the only growth factor shown to cause satellite cells to transition from the quiescent G0 phase to the G1 phase of the cell cycle, activating them (Tatsumi et al. 1998, Allen et al. 1995). HGF binds to the c-met receptor found on satellite cell plasma membranes and stimulates tyrosine kinase residues on the intracellular domain to become autophosphorylated, which promotes satellite cell activation and proliferation through the mitogen-activated
protein kinase (MAPK) pathway (Snijders et al. 2009, Basilico et al. 2008). NO itself may also activate satellite cells through the stimulation of gyanylate cyclase and the production of cyclic guanosine monophosphate (cGMP). The simultaneous expression of NO and cGMP upregulates follistatin, which has promising characteristics as another satellite cell activator (Pisconti et al. 2006). The Notch signaling pathway has also been implicated in the activation of satellite cells (Burdzińska, Gala and Paczek 2008). Muscle injury-inducing exercise protocols such as downhill running (DHR) and myofiber explantation induced Notch expression (Tsivitse et al. 2009, Conboy and Rando 2002), and Notch expression coincides with satellite cell activation in myofiber explant cultures and was shown to regulate the expression of the myogenic regulatory factors, as well as prevent satellite cell activation when Notch signaling was inhibited (Conboy and Rando 2002).

Myogenic regulatory factors (MRF) play an essential role in skeletal muscle regeneration. Members include MyoD, Myf5, Mrf4 and myogenin and belong to a family of helix-loop-helix transcription factors and have approximately 80% amino acid sequence identity with each other. MRF overexpression drives numerous nonmuscle cells such as fibroblasts, chondroblasts and epithelial cells into a myogenic lineage, indicating that MRF transcription factors are important in myogenic cell specification (Pownall, Gustafsson and Emerson 2002, Choi et al. 1990, Weintraub et al. 1991). None of the MRF family members are expressed until satellite cells receive activation signals. Once satellite cells are activated, expression of Myf5 and MyoD are upregulated (Chargé and Rudnicki 2004). The expression of either or both transcription factors are necessary
to commit satellite cells to the myogenic lineage, whereby satellite cells enter the proliferative stage. MyoD and Myf5 remain expressed during satellite cell expansion, then myogenin and Mrf4 are expressed once satellite cells enter the differentiation pathway and begin to fuse with one another or existing fibers (Palmer and Rudnicki 2002). Myf5 expression wanes at the onset of differentiation, while very low levels of MyoD expression remains (Yablonka-Reuveni et al. 2008). MyoD is essential in skeletal muscle regeneration as revealed by the interbreeding of MyoD deficient and mdx mice (Megeney et al. 1996). While the myopathic symptoms of mdx mice are mild, the interbreeding with MyoD mutant mice caused a marked decrease in survival and severe myopathy. Examination of MyoD mutant mice uncovered higher numbers of satellite cells that failed to proliferate or regenerate muscle fibers, leading to the conclusion that MyoD expression is essential in normal satellite cell progression through the regeneration program. MyoD also plays an important role in normal progression of satellite cells into the differentiation program, shown by a distinct reduction in myogenin expression and absence of Mrf4 expression in MyoD-null satellite cells (Ciciliot and Schiaffino 2010). Expression of myogenin appears to be required for satellite cells to undergo terminal differentiation, as satellite cells that fail to differentiate also fail to express myogenin and inhibition of myogenin translation also prevents differentiation (Funk, Ouellette and Wright 1991). The Wnt signaling pathway contributes to myotube differentiation (Burdzińska et al. 2008). When the Wnt signaling pathway is deliberately induced through agonists, upregulation of myogenin is observed (Tsivitse 2010).
As shown by Moss and Leblond in 1971, satellite cells are able to self-renew and repopulate the satellite cell pool for future muscle damage (Moss and Leblond 1971). A majority of activated satellite cells upregulate MyoD and proceeds through proliferation and differentiation stages of myogenesis to ultimately replenish damaged muscle fibers. However, approximately 20% of satellite cells downregulate MyoD and upregulate Pax7 during the proliferative stage, subsequently exiting the cell cycle and returning to a quiescent state and repopulating the satellite location under the basal lamina (Zammit et al. 2004). The Notch signaling pathway may be contributing at this stage as well, as Notch1 expression is upregulated in daughter cells that repopulate the satellite cell pool (Tsivitse 2010). A notable study by Collins et al. showed that transplanted muscle fibers with as few as seven associated satellite cells were able to produce more than 100 regenerated myofibers, and furthermore abundantly repopulated the satellite cell pool of radiation-ablated host muscles (Collins et al. 2005). Thus satellite cells have a tremendous ability for self-renewal and maintenance of the satellite cell pool, firmly establishing these cells as myogenic stem cells fundamental to skeletal muscle regeneration.

Skeletal muscle regeneration follows a distinctive timeline when injured by myonecrotic agents such as marcaine. Within hours of marcaine injection into skeletal muscle, disruption of the muscle fiber sarcolemma occurs, which increases permeability of the muscle fiber. The first 1 to 3 days are characterized by a high infiltration of mononuclear cells such as neutrophils and macrophages that phagocytize muscle tissue debris (Chargé and Rudnicki 2004, Jarvinen et al. 2008), which happen concurrently with
the activation and proliferation of satellite cells. Myogenic regulatory factors, transcription factors expressed during myogenesis, are expressed as satellite cells are activated. MyoD upregulation is seen within 12 hours of satellite cell activation, returns to baseline levels and then peaks around 3 days post-marcaine injection (Marsh et al. 1997, Zhao and Hoffman 2004). At this point, a portion of satellite cells self-renew by downregulating MyoD and upregulating Pax7, returning to their satellite position under the basal lamina for future muscle injury (Ono et al. 2012). Once satellite cells enter the differentiation program, they begin to express myogenin. Myogenin expression peaks around 3 to 4 days post-injury, where myoblasts entering the differentiation stage fuse to each other or damaged fibers to form myotubes (Jarvinen et al. 2008). Myogenin expression then gradually reaches baseline approximately 12 days later. Pax7, a transcription factor necessary for satellite cell viability and is expressed in quiescent satellite cells, is strongly upregulated within 24 hours of marcaine damage, steadily peaking at 4 days (Zhao and Hoffman 2004). Within 7 days post-injury, the majority of the damaged muscle fibers are replaced by regenerated fibers with centrally-located nuclei, in conjunction with myogenesis transcription factors returning to baseline. Thereafter the regenerated fibers increase in size and undergo maturation as mononuclear cell infiltration decreases (Martinez et al. 2010). Mature contractile components form in conjunction with a restoration of motor innervation, finishing skeletal muscle regeneration.
A schematic representing the contribution of satellite cells to muscle regeneration (Figure 1A) and their gene expression profile (Figure 1B) can be found in the figure below.

![Schematic Diagram of Satellite Cells](image)

**Figure 1.** Schematic Representing Contribution of Satellite Cells to Muscle Regeneration (Figure 1A) and their Gene Expression Profile (Figure 1B) (Shi and Garry 2006).

**Regulatory Factors**

The capacity for satellite cells to facilitate skeletal muscle regeneration is dependent upon regulatory factors that influence satellite cell activity. Numerous extrinsic and intrinsic factors including growth factors, hormones and nutrients regulate the proliferation and differentiation of satellite cells. Many factors known to alter
satellite cell activity are listed in the table below (Table 1.1), however the list is not comprehensive, with new factors being discovered regularly. The table was adapted from Rhoads et al. with modifications (Rhoads et al. 2009).
<table>
<thead>
<tr>
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<th>Differentiation</th>
<th>Reference</th>
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Table 1.1. Regulatory Factors that Affect Satellite Cell Activity.
Intracellular Signaling Pathways

The capacity for satellite cells to facilitate skeletal muscle regeneration is dependent upon numerous intracellular signaling pathways that influence satellite cell activity. Many pathways known to alter satellite cell activity are listed in the table below (Table 1.2), however the list is not comprehensive, with new pathways being shown to affect satellite cell activity.

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Table 1.2. Intracellular Signaling Pathways that Affect Satellite Cell Activity.

Experimental Models to Study Skeletal Muscle Regeneration In Vitro

Isolated satellite cells and the use of a murine myoblast cell line are the most common experimental models utilized to study skeletal muscle regeneration in vitro. The use of isolated satellite cells in culture allows a more specific observation of cause and effect without contributions from other cell types of surrounding tissues. The isolation technique triggers the activation of satellite cells due to the destruction of structural and cellular components of muscle fibers, and they proliferate and differentiate to regenerate skeletal muscle in culture. While the use of isolated satellite cells have been extremely useful in investigating the mechanisms of skeletal muscle regeneration, the model lacks a
significant involvement of the in vivo environment and introduces difficulties encountered in tissue culture methods. Due to this fact, results seen in isolated satellite cells can be challenging to demonstrate relevance in the in vivo skeletal muscle regeneration process (Zhao and Hoffman 2008). C2C12 cells are immortalized murine myoblast cell line derived from satellite cells that remains in a proliferative state as long as they are kept in a high serum environment (10-20% fetal bovine serum) and low cell confluency. Differentiation can be directly initiated by serum starvation (2% horse serum) or high cell confluency. Similar to satellite cells, C2C12 cells also experience the same limitations of a lack of an in vivo environment, but they also differ from satellite cells in regards to protein expression, regulatory systems and biochemical mechanisms. Grabowska et al. discovered that C2C12 cells exhibited lower expression of adhesion proteins, which may account for the difference in morphology between satellite cells and C2C12 cells (Grabowska et al. 2011). 25% of regulatory genes found in C2C12 cells were absent in satellite cells, offering a possible explanation for their immortal characteristic (Cornelison 2008).
Regenerating Protein Family

The capacity of satellite cells to effectively replace damaged muscle fibers depends upon their ability to respond to numerous stimuli, many of which have still not been fully characterized. One of these stimuli may be members of the regenerating protein family (Reg), a family of proteins that have increased expression in damaged or regenerating tissues including, but not limited to, the pancreas, liver, gastrointestinal tract, neural cells and the heart (Zhang, Ding and Lai 2003). The Reg family of proteins has a high sequence identity with the calcium-dependent lectin superfamily (C-type lectins), a family of extracellular calcium-dependent sugar binding proteins. Conserved domains include the carbohydrate binding domain, however the sugar-binding action does not seem to contribute to the mitogenic and anti-apoptotic function of Reg proteins (Drickamer 1993, Closa, Motoo and Iovanna 2007). Therefore, it has been speculated that the Reg family of proteins evolved from the same protein with the carbohydrate binding domain as C-type lectins, but the sugar binding property of the protein is no longer needed for its function. The mouse Reg protein genes span approximately 3 - 14 kilobase pairs and contain six exons connected by five introns, all located on chromosome 6 except for Reg IV, located on chromosome 3 (PubMed). The Reg proteins were first found in human pancreatic stones by De Caro et al. in 1979 and since then have been discovered in multiple damaged and regenerating tissue types (De Caro, Lohse and Sarles 1979). So far 4 members of this protein family have been found over multiple species including humans, rats, mice, hamsters, porcine and bovine. Regenerating protein expression is significantly increased in inflamed or regenerating
tissues, including diseased and cancerous tissues such as Alzheimer’s and hepatocellular carcinomas and organs suffering acute inflammatory responses such as pancreatitis.

Based on the primary structures of the proteins, the members have been classified into 4 classes: Reg I, II, III and IV; each class contain subclasses depending on species. So far, Reg I, Reg II, Reg IIIα, Reg IIIβ, Reg IIIδ, Reg IIIγ and Reg IV have been discovered and gene sequences isolated in mice.

**History**

**Reg I**

The Regenerating protein was first discovered by De Caro, Lohse and Sarles in 1979 when they extracted a 14 kD protein from human pancreatic stones (De Caro et al. 1979). Although the function of the protein was unknown at the time, they hypothesized that the protein may initiate protein precipitation and calcium formation within the pancreatic duct that ultimately leads to the formation of pancreatic stones. Corresponding to their assumed function, the protein was named Pancreatic Stone Protein (PSP). 4 years later PSP was found to be a strong inhibitor of calcium carbonate precipitation in pancreatic juice (Multigner et al. 1983). The following year in 1984, Keim et al. discovered a 16-17 kD protein in pancreatitis-induced rats that was absent in the control rats and named it Pancreatitis-associated protein (PAP) (Keim et al. 1984). A year later, Gross and colleagues isolated a 14 kD protein from the acid extracts of human pancreas and pancreatic secretions (Gross et al. 1985). This protein underwent a conformational change and transformed into a fibril structure between pH 5.4 and 9.2;
from these observations the protein was named Pancreatic Thread Protein. In 1988, Terazono and colleagues administered nicotinamide to 90% depancreatized rats to induce pancreatic islet regeneration (Terazono et al. 1988). In the process, they identified a gene that was solely expressed in regenerating pancreatic islets and corresponded with the increase in size of regenerating islets and a decrease in urinary glucose concentrations. From these findings they theorized that this gene was important in the regeneration of pancreatic islets and might prove crucial to diabetes treatment. Terazono et al. were also the first to coin the term “regenerating protein” (Reg Protein) to this gene product (Terazono et al. 1988). Several years later in 1990, Watanabe et al determined through nucleotide sequence comparisons that PSP, PAP and Reg Protein were all identical (Watanabe et al. 1990). Furthermore, Watanabe and colleagues detected the presence of the Reg Protein in the gastrointestinal tract and the kidney. From the early to late 1990’s, scientists discovered multiple isoforms under the Reg gene family. Due to the match in nucleotide sequences between PSP, PAP and Reg Protein, these were designated as Reg I (Reference). Moriiizumi et al. identified a novel human gene with a 91% homology to the Reg gene, and coined this isoform Reg Iβ while the original Reg gene was designated as Reg Iα (Moriiizumi et al. 1994). Northern blot analysis also showed that the Reg Iβ isoform was only found in the pancreas while the Reg Iα was found in the pancreas, kidney and stomach tissue.
Reg II

In 1993, Unno et al. isolated another novel Reg gene isoform that had a 56% gene sequence identity to the 5’-flanking sequence of Reg I (Unno et al. 1993). Unno et al. also noted that Reg II had a higher sequence identity to rat and human Reg I than mouse Reg I, hypothesizing that Reg II is a mouse homologue to rat and human Reg I. Chromosomal mapping studies showed that Reg I and Reg II were localized on completely different chromosomes, indicating that the two Reg genes are related yet function as distinct genes. Similar to Reg I, Reg II is expressed in hyperplastic islets but absent in normal islets. However, the overall expression of Reg II in the normal mouse pancreas is high. Perfetti et al. demonstrated that although mouse Reg II is strongly expressed in pancreatic tissues, the expression profile does not coincide with Reg I (Perfetti et al. 1996). They observed that while Reg I expression steadily decreased over 30 months, Reg II expression remained the same. From these results they hypothesized that the Reg gene family members may have evolved different functions specific to individual tissues types, while still maintaining some overlapping biological functions. Castellarin et al. correctly named the previously identified golden hamster Reg I as Reg II in 2007, showing that it had a 81% sequence identity with mouse Reg II (Castellarin et al. 2007). They also identified Reg II expression in multiple tissues including the stomach, pancreas, gastrointestinal tract, spleen, liver, muscle, lung, heart and brain. Reg II expression was highest in the pancreas and brain.
Reg III

Four subclasses of the Reg III gene have been discovered in mice: Reg IIIα, IIIβ, IIIδ and IIIγ. In 1994, the Suzuki group isolated a novel Reg gene that had a 42.2% amino acid sequence identity with Reg I (Suzuki et al. 1994). The most prominent distinction of this novel Reg gene, classified as Reg III, was it’s increased expression in regenerating pancreatic islets but weak expression in normal pancreatic islets. In 1997 Narushima et al. successfully identified mice Reg IIIα, Reg IIIβ and Reg IIIγ, all with sequence identities below 20% to mice Reg I (Narushima et al. 1997). Southern blot hybridization of the mouse genome with cDNA fragments of rat Reg III identified the 3 subclasses, all with 60 – 70% sequence homologies between the 3 subclasses. 3 years later, the same lab discovered yet another subclass with a sequence homology of 40-50% to the other members of the mouse Reg gene family (Abe et al. 2000). This novel gene had an amino acid sequence insertion found in all subclasses of mice Reg III, therefore the protein was determined to be a 4th subclass of Reg III and named Reg IIIδ.

Reg IV

In 2001, Hartupee et al. isolated a novel, 18kDa human protein with a sequence homology of 39% to Reg III, 38% to Reg Iα and 39% to Reg Iβ (Hartupee et al. 2001). All 6 cysteine residues present in all the Regenerating Protein Family members were conserved, however enough protein sequence differences were discovered to classify this novel gene into another class – Reg IV. Furthermore, Hartupee et al. identified Reg IV expression in intestinal mucosa samples of patients with or without inflammatory bowel
disease, with expression being significantly higher in patients suffering from inflammatory bowel disease. In 2010, Ho et al. discovered that although the human Reg IV protein is a member of the Reg protein family with homologous sequences to calcium-dependent (C-type) lectin-like domains, Reg IV is able to bind polysaccharides, mannan and heparin independent of calcium (Ho et al. 2010).

*Reg Receptor (EXTL3)*

Kobayashi et al. identified the Reg receptor protein in 2000 through the screening of the rat islet cDNA expression library for a rat Reg binding protein, and found a protein with a C-terminal sequence homology of 52% to multiple exostoses 2 (EXT2) and 40% to multiple exostoses 1 (EXT1), a family of genes that encode glycosyltransferases for heparan sulfate chain elongation (Kobayashi et al. 2000). However, the N-terminal region of this new receptor had no sequence homology to any of the exostoses (EXT) gene family members. Due to this difference, they named the receptor exostoses-like gene 3 (EXTL3). EXTL3 is mainly involved in heparan sulfate chain initiation, verified by the synthesis of longer heparan sulfate chains when silenced. The authors speculated that the silencing of EXTL3 inhibits heparan sulfate chain initiation, and the remaining substrates are recruited for excessive elongation (Busse et al. 2007). Additionally, unlike the other members of the EXT family, EXTL3 contains a membrane-spanning domain. Kobayashi et al. hypothesized that this transmembrane domain anchors EXTL3 to the cell membrane and facilitates Reg protein binding through its N-terminal region, establishing EXTL3 as a possible cell surface protein. They further confirmed EXTL3 as the Reg
receptor by generating a cell line that overexpressed EXTL3, and observed significantly increased BrdU incorporation in these cells with the addition of Reg protein (Kobayashi et al. 2000). Multiple pathways are thought to be activated by EXTL3 (Parikh, Stephan and Tzanakakis 2012), one of them being the NF-Kb pathway. Mizuno et al. saw that an overexpression of EXTL3 in human embryo kidney cells stimulated TNFα-induced activation of NF-Kb, and postulate a potential role of EXTL3 activation in tumorigenesis (Mizuno, Irie and Sato 2001). In a human pancreatic cell line, EXTL3 was shown to be the most likely candidate as a receptor for Human Proislet Peptide (HIP), or a derivative of human Reg IIIα (Levetan et al. 2008). When HIP was added to pancreatic cells in culture, it stimulated translocation of EXLT3 to the plasma membrane from the nucleus. This observation supported their hypothesis that HIP-induced islet neogenesis is facilitated through the EXTL3 receptor. EXTL3 is also expressed in keratinocytes, and was shown to severely inhibit Reg IIIα’s ability to induce cell proliferation when silenced (Lai et al. 2012). Similar results were seen in neural cells, were EXTL3 was shown to colocalize with Reg Iα in rat adrenal medulla cells and overexpression of EXTL3 caused a significant increase in neuronal outgrowth with the administration of Reg Iα (Acquatella-Tran Van Ba et al. 2012). Due to EXTL3’s apparent function as the Reg receptor across multiple tissues and species, it is highly likely that EXTL3 may also act as the Reg receptor in skeletal muscle regeneration.

Function
In Pancreas

After the initial discovery of Reg I protein in human pancreatic stones, numerous research was performed in an attempt to discern its specific function. Due to its original discovery in the pancreatic system, research into the role of Reg proteins in pancreatic diseases has been the most comprehensive. Multigner et al. observed a strong inhibitory effect of Reg I protein on the precipitation of supersaturated calcium carbonate solutions and hypothesized that Reg I protein may act to prevent pancreatic stone formation in supersaturated pancreatic juice (Multigner et al. 1983). Four years later, a group in Japan observed a corresponding relationship between the increased expression of rat Reg I and the increase in size of regenerating pancreatic islets in surgically depancreatized rats (Terazono et al. 1988). The same gene was also expressed in the hyperplastic islets of aurothioglucose-injected mice, indicating a possible role of Reg I in pancreatic cell regeneration, proliferation and maturation. To test that theory, Watanabe et al. administered exogenous rat Reg I protein to isolated pancreatic islets and 90% surgically depancreatized rats by intraperitoneal injections and analyzed the regeneration characteristics. Two months later, the diabetic indicators in the Reg I protein treated animals had improved drastically, with body weights and fasting plasma glucose levels typical to those of non-diabetic animals compared to the non-Reg I protein treated animals. While the isolated islets of the 90% depancreatized, non-Reg I treated rats showed severe degranulation of the β-cells and significantly reduced numbers of insulin-positive cells, the islets of Reg I-treated animals were consistently enlarged and contained approximately 2.5 fold greater mass of β-cells than control animals (non-depancreatized).
Furthermore, when Reg I proteins were added to isolated rat islets in culture, [3H]thymidine incorporation was stimulated in a dose-dependent manner. Watanabe et al. theorized that Reg I protein was inducing β-cell proliferation and regeneration, enough so that in just 60 days surgically induced diabetes was completely reversed (Watanabe et al. 1994b). When acute pancreatitis was chemically induced by sodium taurocholate injection in rats, the mRNA levels of Reg IIIα, Reg IIIβ and Reg IIIγ all increased significantly. When the three Reg III isoforms were inhibited in vivo by antisense oligodeoxyribonucleotides, they saw significant increases in serum amylase concentration, pancreatic edema, C-reactive protein and pancreatic inflammation compared to control animals. All of these results are consistent with an increase in the severity of pancreatitis, which suggests that the expression of the Reg III proteins provide a protective mechanism to reduce the inflammatory response (Zhang et al. 2004, Viterbo et al. 2008). This may be due to Reg IIIα’s ability to activate macrophages (Viterbo et al. 2008), which have been shown to have a protective mechanism against pancreatitis (Nakamichi et al. 2005).

In Gastrointestinal Tract

Watanabe et al. examined the expression of Reg I gene in numerous tumoral tissues, and found that colon and rectal cancers expressed Reg I at very high levels, while no expression was detected in any of the esophageal cancers they analyzed or in the nontumoral tissues of the colon, rectum or esophagus (Watanabe et al. 1994a). Kawanami et al. observed rat Reg I gene expression consistently throughout the entire
gastrointestinal tract, but the highest levels were found in the stomach, predominantly in highly proliferating endocrine cells. Indomethacin-induced inflammation of the gastric mucosa stimulated the expression of Reg I not only in these highly proliferating endocrine cells, but in other cell types of the gastric mucosa that may be contributing to regeneration (Kawanami et al. 1997). The intestinal villi of adult Reg I-knockout mice presented with an altered morphology of less densely packed and circular epithelial cells, and a reduction in the total number of proliferating cells (Ose et al. 2007). Fukui et al. investigated the direct effects of Reg I on gastric mucosal epithelial cells of rats in culture, and observed a significant dose-dependent increase in [H3]thymidine incorporation and cell growth when Reg I protein was added (Fukui et al. 1998). From these studies it was concluded that Reg I protein acts as a mitogenic factor in inflamed and regenerating gastric mucosa cells, and may play a pivotal role in early stimulation of cell proliferation. As mentioned earlier, Reg IV expression was significantly increased in the gastric mucosal tissues of patients with inflammatory intestinal diseases such as Crohn’s disease or ulcerative colitis, with no apparent expression profile specific to a disease (Hartupee et al. 2001). This infers that the inflammation or damage itself is upregulating Reg IV expression. Various gastric cancers were found to be expressing rat Reg Iα in vivo, while the addition of exogenous Reg Iα increased ERK1/2 activity and stimulated thymidine incorporation in gastric cancer cells in culture. Based on these findings, the authors speculated that gastric cancers may be releasing Reg Iα as an autocrine/paracrine factor to promote cell proliferation and development of malignancy (Kadowaki et al. 2002). Cash et al. reported Reg IIIγ’s antibacterial property in 2006
when they colonized microbiologically sterile mice with intestinal bacteria and saw a 31-fold increase in Reg IIIγ mRNA abundance. Further investigation showed that Reg IIIγ binds to Gram-positive intestinal bacteria and decreased their viability by more than 80%; the authors concluded that Reg IIIγ may contribute to “mucosal homeostasis in the face of changing microbial ecology” (Cash et al. 2006).

In Liver

Lasserre et al. first discovered a gene derived from the human Reg III gene and labeled it as Hepatocellular carcinoma-Intestine-Pancreas (HIP = human Reg IIIα), due to its increased expression in hepatocellular carcinoma, small intestine and pancreas (Lasserre et al. 1992). Due to the common embryonic origin of the liver, small intestine and pancreas, they theorized that HIP was an important factor in cellular proliferation and differentiation of these organs. To determine the function of Reg proteins in liver regeneration, transgenic mice with human Reg IIIα or mice Reg II gene upregulation specifically in hepatocytes were created (Simon et al. 2003, Lieu et al. 2006). After partial hepatectomy, Reg IIIα transgenic mice had higher BrdU incorporation in their hepatocytes, as well as heavier overall liver weights over a five-day recovery phase. The addition of Reg IIIα protein to wild-type hepatocytes also increased growth-factor mediated incorporation of BrdU in culture, affirming that Reg IIIα protein stimulates cell proliferation in hepatocytes in vivo and in vitro. The viability of Reg IIIα-transgenic hepatocytes stimulated to undergo cell death was significantly elevated, and the addition of Reg IIIα protein to wild-type hepatocytes increased cell survival by approximately
20%. These effects may be mediated through the cAMP/PKA pathway, confirmed by the increase in the growth-factor induced DNA synthesis in Reg IIIα-transgenic hepatocytes in the presence of forskolin (Simon et al. 2003). The same lab established that Reg IIIα-transgenic hepatocytes expressed indicators of synthesis phase (S phase) entry approximately 14 hours before wild-type hepatocytes, reinforcing the theory that Reg IIIα facilitates accelerated liver regeneration (Lieu et al. 2005). Mice Reg II was found to have similar mitogenic and anti-apoptotic properties as human Reg IIIα, with Reg II-knockout mice having significantly lower survival rates and delayed liver mass recovery than transgenic Reg IIIα or wild-type mice in response to chemically induced fulminant hepatitis or partial hepectomy. Further assessment of the Reg II-knockout mice revealed that they were much more susceptible to oxidative stress, confirmed by the low levels of reduced glutathione, a stabilizer of reactive oxygen species (Lieu et al. 2006). Human Reg IIIα’s antioxidant activity was further confirmed by Moniaux et al. when they observed the increased scavenging activity of reactive oxygen species by recombinant human Reg IIIα in a dose-dependent manner. Due to the stable and multifunctional nature of the recombinant human Reg IIIα protein, the authors have proposed and are in the process of testing the efficacy of this protein as a therapeutic drug to prevent acute liver failure (Moniaux et al. 2011).

In Neural Cells

Reg I proteins have also been found in normal and diseased neural tissue. In 1989, Ozturk et al. discovered low Reg I protein concentrations in the brain tissue of
normal patients, but much higher levels (50-100 fold higher) were observed in the brain tissues of Alzheimer and Down’s Syndrome patients (Ozturk et al. 1989). In 1997, Livesey et al. showed that rat Reg IIIβ (designated as rat Reg 2 in the literature) mRNA and protein expression was significantly increased in the regenerating motor and sensory neurons of animals with crushed sciatic nerves. They further investigated the function of rat Reg IIIβ in sciatic nerve regeneration by an inhibition of rat Reg IIIβ signaling by intraneural injection of an anti-Reg IIIβ polyclonal antiserum in vivo. They observed a significant reduction in the regeneration rate of axons, measured by the decreased distance of growth from the site of injury. They also observed a dose-dependent increase in BrdU incorporation with the addition of exogenous rat Reg IIIβ in vitro, but only in the presence of forskolin, an activator of adenylyl cyclase. This is consistent with other mitogenic factors of Schwann cells and is thought to be due to an increase in receptor expression through the activation of the cAMP pathway (Livesey et al. 1997b, Livesey et al. 1997a). A similar study by Namikawa et al. showed that Reg IIIγ mRNA and protein expression was significantly increased within 12 hours of injury and then peaks at 1 day in the crushed sciatic nerves of rats (Namikawa et al. 2005). Further investigation demonstrated that Reg IIIγ stimulated chemotaxis of macrophages in in vitro and in vivo models of nerve damage in rats, and ultimately promoted nerve regeneration (Namikawa et al. 2006). Duplan et al. found that Reg I protein was highly expressed during the pre-clinical stages of Alzheimer’s disease, inferring a possible analytical method to detect Alzheimer’s disease before the manifestation of clinical symptoms (Duplan et al. 2001). In cultured embryonic motorneurons, the addition or overexpression of rat Reg IIIβ
promoted motorneuron survival in a dose-dependent manner. To investigate whether Reg IIIβ was acting upon the ciliary neurotrophic factor (CNTF) survival pathway (a main signaling pathway that prevents motorneuron cell death) to promote cell survival, motorneurons were infected with Reg IIIβ-antisense adenovirus. They observed that the viability of these motorneurons significantly decreased when the CNTF pathway was activated compared to another neurotrophic pathway, indicating that Reg IIIβ expression was crucial for the CNTF-pathway to be effective at preventing cell death. Adding back exogenous Reg IIIβ rescues viability, further establishing Reg IIIβ as an important intermediate in the CNTF-pathway (Nishimune et al. 2000). The neurotrophic and neuroprotective function of Reg IIIβ may be useful for therapeutic treatments of spinal cord injuries and neurodegenerative diseases (Fang et al. 2011).

In Other Tissues

Decreased lung function from pulmonary edema and hypoxemia are very common complications to pancreatitis. Due to Reg proteins’ protective function in pancreatitis, whether Reg proteins impose a protective role to pancreatitis-associated pulmonary dysfunction was also investigated. The treatment of isolated rabbit lungs with recombinant rat Reg IIIβ protein prior to the induction of pulmonary distress significantly reduced the release of thromboxane (a powerful vasoconstrictor) in a dose-dependent manner, ultimately minimizing vasoconstriction and edema and confirming the protective role of Reg IIIβ in pancreatitis-associated lung damage (Heller et al. 1999). Following this study, Folch-Puy et al. investigated the possible systemic effect of pancreatitis-
induced rat Reg IIIβ protein secretion from the pancreas by injecting rat Reg IIIβ intravenously and analyzing lung and liver tissues. Contrary to the results seen by Heller et al. that showed rat Reg IIIβ as an anti-inflammatory, protective factor against lung dysfunction, the authors saw a significant stimulation of neutrophil infiltration and oxidative stress in the lungs. However, because the model utilized by Heller et al. does not accurately mimic the complexity of the systemic reaction to pancreatitis, it is possible that the secretion of Reg IIIβ systemically also induces a pro-inflammatory response in vivo. This theory was supported by further research that revealed a significant increase in circulatory TNFa (a potent pro-inflammatory factor) with Reg IIIβ administration. Significant increases in TNFa expression were observed in the hepatocytes of intravenous Reg IIIβ-administered animals, accounting for the source of increased plasma TNFa concentration. Although Reg IIIβ administration induced the overexpression of TNFa in the liver, the expression was not accompanied by liver inflammation (Folch-Puy et al. 2003). Reg I expression was found in human and rat infarcted hearts, with stronger transcriptional activation associated with the severity of myocardial damage (Kiji et al. 2005). Moreover, significantly increased expressions of Reg Iα, IIIα, IIIβ and IIIγ were observed in further studies of rat autoimmune myocarditis (Watanabe et al. 2008). PAPep, a peptide derivative of human Reg IIIα, significantly alleviated ocular inflammation of endotoxin-induced uveitis rats through an inhibition of the NF-Kb signaling pathway (Yang et al. 2011). Reg I protein expression was found to be highly upregulated in human seminoma testis compared to normal human and rat testis, with targeted expression in the seminiferous epithelium, where germ cells proliferate and
differentiate to generate spermatozoa (Nata et al. 2004, Mauro et al. 2008, Mauro et al. 2011). The authors hypothesized that Reg I may be crucial in the regulation of germ cell proliferation and differentiation, ultimately affecting spermatozoa formation.

In Disease Screening

Numerous studies have shown an increased expression or upregulation of Reg proteins in various inflammatory diseases. Chronic infection and inflammation of organs such as the pancreas, lungs, liver and the gastrointestinal tract are typical clinical symptoms of cystic fibrosis. Many studies have found that Reg I concentrations in the blood were significantly higher in neonates with cystic fibrosis, and possible applications for cystic fibrosis screening in newborns were proposed (Sarles et al. 1999, Sarles et al. 2005, Sommerburg et al. 2010). To test the validity of a Reg I test to determine cystic fibrosis, Vernooij-van Langen et al. executed an extensive screening for cystic fibrosis by analyzing the human Reg I protein concentration in more than 70,000 newborns from the middle and southeast Netherlands. They found that a combination of a well established CF screening method, immunoreactive Trypsinogen (IRT), in conjunction with a measurement of Reg I was a better indicator for CF compared to the IRT test alone (Vernooij-van Langen et al. 2012a, Vernooij-van Langen et al. 2012b).
Summary and Dissertation Objectives

Skeletal muscle is a major organ system that contributes to the maintenance of locomotion, posture, respiration and thermoregulation. Impairment of normal myogenic function can lead to decreased quality of life and loss of life. Some prime examples of dysfunctional skeletal muscle include genetic diseases such as muscular dystrophies, age-related loss of skeletal muscle mass or sarcopenia, and muscle trauma. The common factor among all of the examples of skeletal muscle dysfunction is their compromised skeletal muscle regeneration capabilities.

Skeletal muscle has a tremendous capacity for regeneration and this process is governed by numerous intrinsic and extrinsic factors. The capacity for skeletal muscle to regenerate is largely dependent upon the resident myogenic stem cell population, the satellite cell. Satellite cells were first discovered by Mauro in 1961 when he observed a cell that contained very little cytoplasm wedged between the basal lamina and sarcolemma of frog tibialis anterior muscles (Mauro 1961). He coined the term “satellite cell” on these cells based on their peripheral location and hypothesized that these cells were responsible for skeletal muscle regeneration after injury. The role of satellite cells as myogenic stem cells were soon confirmed by various scientists through muscle damage, [H3]thymidine labeling and transplantation studies (Church et al. 1966, Moss and Leblond 1971, Partridge et al. 1978). Satellite cell-mediate skeletal muscle regeneration occurs through the modulation of transcription factors, growth factors, inflammatory signals and intracellular signaling pathways. Modification in any of the
stimuli mentioned previously can drastically alter satellite cell activity and ultimately skeletal muscle regeneration.

Members of the Regeneration protein family are upregulated in damaged or regenerating tissues (Zhang et al. 2003). The Reg protein was first discovered in human pancreatic stones by De Caro et al. in 1979 and has subsequently been studied in extensively in the pancreas, gastrointestinal tract, liver, neural cells, the heart and many other tissues (De Caro et al. 1979). Some of the main functions of Reg proteins appear to be mitogenic, anti-apoptotic and anti-inflammatory in nature, with inhibition of Reg protein expression causing abnormal tissue regeneration and decreased cell survival. Addition of exogenous Reg protein can ameliorate experimentally-induced ailments such as diabetes and fulminant hepatitis (Watanabe et al. 1994a, Lieu et al. 2006), inferring to a possible therapeutic application for disease treatment.

Given the apparent necessity of the Regenerating proteins for normal regeneration of numerous damaged tissues and organs, it seems highly likely that the Regenerating proteins are also necessary for skeletal muscle regeneration, possibly by increasing satellite cell activity. Therefore, the first aim of this dissertation is to characterize the expression of the Regenerating protein family members and receptor protein in regenerating skeletal muscle and satellite cells. We hypothesize that multiple Reg gene classes are upregulated in damaged and regenerating skeletal muscles, with a significant portion of the expression originating from satellite cells. The second aim of this dissertation is to investigate the effect of exogenous Regenerating protein supplementation on skeletal muscle myogenesis. We hypothesize that in concurrence
with Reg protein’s mitogenic function in numerous other regenerating tissues, the addition of exogenous Reg protein will promote myogenesis and ultimately accelerate skeletal muscle regeneration. Our third aim of this dissertation is to examine the effect of Regenerating protein addition on cell activation, proliferation and differentiation in isolated satellite cells in vitro. We hypothesize that the direct addition of Reg proteins to satellite cells in culture will mediate satellite cell activation, hyper-proliferation and inhibit cell differentiation. Knowledge gained from these studies will provide valuable insight into skeletal muscle regeneration and fundamental knowledge of satellite cell activity and stimuli.
CHAPTER 2
THE EXPRESSION AND REGULATION OF THE REGENERATING PROTEIN FAMILY IN SKELETAL MUSCLE

Abstract

Regenerating proteins are upregulated in numerous damaged and regenerating tissues, but have yet to be explored in skeletal muscle. 3-6 month old female C57BL6 mice were injected with 50 ul of 7.5 mg/ml marcaine to induce muscle damage, or an equal volume of saline to serve as a control in their tibialis anterior muscles. The muscles were removed 0.5, 1, 2, 3, 4, 7 and 14 days later and analyzed for Reg I, II, IIIa, IIIb, IIIId, IIIg, IV and Reg receptor (EXTL3) gene expression. Reg I, II, IIIa, IIIg, IV and Reg receptor genes were expressed in regenerating tibialis anterior muscles, but Reg IIIb and IIIId expression were absent. Undamaged, damaged and regenerating tibialis anterior muscles were stained with all the Reg isoforms as well as the Reg receptor, with varied staining profiles across the timeline. Satellite cells were isolated from the same aged female C57BL6 mice and cultured for 2, 3, 4 and 7 days and analyzed for Reg I, II, IIIa, IIIb, IIIId, IIIg, IV and Reg receptor (EXTL3) gene expression. While Reg I, IIIa, IIIg and EXTL3 gene expression were upregulated in satellite cells, Reg II, IIIb and IIIId expression were absent. However, all Reg isoforms and EXTL3 were detected in the immunofluorescent staining of satellite cells. Taken together, these data suggest that multiple isoforms of Reg genes are upregulated during skeletal muscle regeneration and
Reg I, IIIa and IIIg may be directly acting upon satellite cells to stimulate myogenesis, possibly through it’s putative receptor, EXTL3.

**Introduction**

Skeletal muscle is a dynamic tissue that is important in numerous physiological processes. Muscular dystrophies, sarcopenia and trauma can cause significant damage to the skeletal muscle system and impair essential functions such as locomotion, posture, respiration and thermoregulation (Powers and Howley 2004, Sherwood 2010). Muscular dystrophies are genetic diseases that cause mutations of myogenic genes which ultimately lead to necrosis of muscle fibers and infiltration by connective and fatty tissue (Amato and Russell 2008a). Abnormal expression of myogenic genes results in decreased structural stability during muscle contraction, leading to increased susceptibility to muscle damage. Repeated degeneration and regeneration cycles exhaust the regenerative capacity of satellite cells; damaged fibers are then replaced by fibrotic and adipose tissues (Fairclough et al. 2011). Sarcopenia is the progressive loss of muscle mass due to old age, which may be caused by age-dependent changes in satellite cell content (Snijders et al. 2009, Shefer et al. 2010, Kadi and Ponsot 2010, Koopman 2011), hormonal regulation and intracellular signaling cascades (Russell and Leger 2011). Although exercise is highly effective in slowing muscle loss, the rate of muscle catabolism ultimately overshadows the muscle’s anabolic capabilities and results in a cumulative state of muscle wasting. However, Conboy et al. concluded through their parabiosis studies that satellite cells from aged animals retain their intrinsic proliferative activity, and that
systemic factors of aged individuals were responsible for the decreased regenerative capacity of sarcopenic muscle (Conboy et al. 2005). Clearly, an enhancement of the regenerative capacity of skeletal muscle by mitogenic factors that improve intrinsic myogenetic characteristics would alleviate some of these problems.

Members of the Regeneration protein family are abundantly upregulated in damaged, inflamed or regenerating tissues of the pancreas (De Caro et al. 1979, Keim et al. 1984, Terazono et al. 1988, Zhang et al. 2004, Viterbo et al. 2008), gastrointestinal tract (Hartupee et al. 2001, Watanabe et al. 1994a, Kawanami et al. 1997, Kadowaki et al. 2002), liver (Lasserre et al. 1992), neural cells (Ozturk et al. 1989, Livesey et al. 1997b, Livesey et al. 1997a, Duplan et al. 2001) and many other organs (Kiji et al. 2005, Watanabe et al. 2008, Mauro et al. 2008, Sarles et al. 2005, Sommerburg et al. 2010). Inhibition or gene manipulation of the various Reg protein isoforms in tissues have led to increased inflammation in chemically induced pancreatitis of Reg III inhibited rats (Zhang et al. 2004, Viterbo et al. 2008), reduced survival rates and delayed liver mass recovery in Reg II-knockout mice with chemically induced fulminant hepatitis or partial hepatectomy (Lieu et al. 2006), poor axon regeneration in the crushed sciatic nerves of rats with a concomitant inhibition of Reg IIIβ protein (Livesey et al. 1997b, Livesey et al. 1997a) as well as a significant decrease in cultured motor neuron viability with the infection of Reg IIIβ-antisense adenovirus (Nishimune et al. 2000). These studies infer a remarkable mitogenic, anti-apoptotic and anti-inflammatory function of Reg proteins in numerous damaged or regenerating tissues, and the presence of Reg proteins seems to be essential for normal progression of regeneration. This may also be the case in skeletal
muscle, where various physiological stimuli can have a significant consequence on skeletal muscle regeneration.

The direct injection of myotoxic agents into muscle is a prevalent method to induce muscle damage. Among them, direct intramuscular injections of marcaine is the most widely adopted in vivo model to study skeletal muscle regeneration, as marcaine causes severe local toxicity to muscle fibers but has minimal effects on surrounding tissues (Foster and Carlson 1980, Benoit and Belt 1970, Zink and Graf 2004). Within minutes of marcaine injection, affected muscle fibers become hypercontracted, followed by disruption of the sarcoplasmic reticulum, infiltration by mononuclear cells and subsequent cell debris phagocytosis. While some variability in degeneration exist with marcaine injection, the comparison of undamaged and damaged areas adjacent to one another provide an intrinsic control for histological analysis, which we will utilize in our immunofluorescent experiment. We are fully aware that pockets of undamaged fiber bundles exist within damaged areas that may affect our molecular analyses, however we concluded that enough and consistent damage was observed in the marcaine-injected tibialis anterior muscles to allow comparison with undamaged control muscles.

Study objectives were to determine the expression profile of Reg I, II, IIIα, IIIβ, IIIδ, IIIγ, IV and Reg receptor (EXTL3) in normal, damaged and regenerating skeletal muscle and isolated satellite cells to fully characterize the Reg protein system. A determination of whether a predominant Reg protein isoform exists during skeletal muscle regeneration and at what point in the timeline Reg proteins are most abundantly found in vivo and in vitro will shed light on the role of Reg proteins in myogenesis.
Materials and Methods

Animals

All animal care use was approved and supervised by the University of Arizona Institutional Animal Care and Use Committee. 3- to 6-month old female C57BL6 mice were purchased from Jackson labs and used for all tissue collection and primary satellite cell isolations. Standard rodent chow and water was made available to the animals on an ad libitum basis.

Treatments

3- to 6-month old female C57BL6 mice were anesthetized with a cocktail consisting of 100 mg/mL ketamine (Vedco) and 5 mg/mL diazepam (Hospira) in a 1:1 solution in a total volume of 50 μl or a 100 mg/kg ketamine and 10 mg/kg xylazine in phosphate-buffered saline solution (pH 7.4, Invitrogen) by intraperitoneal injections. The hind limbs of the mice were shaved and de-haired using a commercial chemical depilatory (Nair), then cleaned with 70% isopropyl alcohol. Using a 30-gauge hypodermic needle, the right tibialis anterior muscle was injected intramuscularly with 50 μl of 7.5 mg/ml marcaine solution by inserting the needle at the distal end of the tibialis anterior muscle, progressing to the proximal end of the muscle, then slowly expelling the marcaine solution as the needle was removed. This allows enhanced solution exposure to the full length of the muscle fiber. The left tibialis anterior muscle was injected in the
same manner with an equal volume of phosphate-buffered saline solution. Animals were placed on a heating pad and allowed to recover from anesthesia for 3 hours at 37°C.

To obtain positive control samples with significantly upregulated Regenerating protein expression, 3- to 6-month old female C57BL6 mice were given hourly intraperitoneal injections of 50 ug/kg cerulein (Sigma-Aldrich) in phosphate-buffered saline for 5 hours to induce acute pancreatitis. Another group of mice were given 2% weight/volume Dextran Sodium Sulfate (MP Biomedicals) in water ad libitum for 5 days to induce colitis.

**Tissue Collection**

At 12, 24, 48, 72, 96, 168 and 336 hours post-maracaine or phosphate-buffered saline injection, animals were sacrificed with CO2 asphyxiation and heart stick. Animals were fully submerged in 70% ethanol for sterilization, and manually skinned to allow exposure of the hind limbs. The tibialis anterior muscles were surgically removed by excision at the distal and proximal ends of the muscle and individually placed in foil pouches and snap-frozen in liquid nitrogen or embedded in OCT tissue-freezing media (Tissue Tex, Inc.) using 2-methylbutane pre-cooled in liquid nitrogen. Tissue samples were then transferred to the -80°C freezer for storage until further use.

Positive control mice with chemically-induced acute pancreatitis were sacrificed at 5, 24 and 72 hours post-final cerulein injection by cervical dislocation. The abdominal organs were exposed and the abdominal cavity was flooded with ice-cold RNeasy®
solution (Ambion®) to preserve pancreatic RNA. The pancreas was surgically removed and stored in RNAlater® solution (Ambion®) at 4°C.

Positive control mice with chemically-induced colitis were sacrificed at 0, 24 and 48 hours post-DSS solution by CO2 asphyxiation. The abdominal organs were exposed and the proximal and distal portions of the colon were removed and snap-frozen in individual foil pouches in liquid nitrogen, then stored at -80°C until further use.

**RNA Extraction from Tissues**

Marcaine-damaged or phosphate-buffered saline injected tibialis anterior muscles were placed in microcentrifuge tubes with 500 μl of ice-cold Trizol reagent. The samples were homogenized with a PowerGen 125 homogenizer (Fisher Scientific) for 15 second bursts three times on ice, or until samples were completely homogenized. 100 μl of chloroform was added, then vortexed for 20 seconds. Phase-lock gel tubes (5 Prime) were pre-spun at 1,500 x g for 30 seconds. Homogenized samples were added to the gel tubes and incubated at room temperature for 5 minutes, then centrifuged at 3,500 x g for 10 minutes at 4°C. The aqueous phase was aspirated into new 1.5 ml eppendorf tubes and an equal amount of isopropanol was added. Samples were mixed well by inversion and incubated on ice for 5 minutes, then centrifuged at 8,000 x g for 10 minutes at 4°C. The supernatant was discarded and pellets dried at room temperature for 2 minutes. The pellet was resuspended in 80% ethanol and transferred into a new eppendorf tube and centrifuged at 8,000 x g for 5 minutes at 4°C. Ethanol was aspirated, the tubes were then centrifuged for 5 seconds to allow aspiration of any residual ethanol. The RNA pellet
was dried at room temperature for 5 minutes, then resuspended in 100 μl of RNase-free water and stored on ice. Total RNA concentration was measured on a NanoDrop ND-1000 machine and software. RNA samples were stored at -80°C or processed immediately for RNA cleanup. RNA samples were cleaned using the QIAGEN RNeasy Mini Kit (QIAGEN) and concentrations remeasured on the NanoDrop ND-1000. Samples were stored at -80°C until further use.

Total RNA from positive control pancreas and colon samples were extracted and cleaned using the RNeasy Mini Kit (QIAGEN) and quantified by NanoDrop ND-1000. Samples were stored at -80°C until further use.

**Analysis of Gene Expression**

Two μg of total RNA was converted into complimentary DNA by reverse transcriptase using SuperScript™III First-Strand Synthesis for RT-PCR (Invitrogen). Real-time PCR were performed in a 25 μl total volume using iQ™ SYBR® Green Supermix (Bio-Rad) on an iQ™ 5 Real-Time PCR Detection System (Bio-Rad). Samples were analyzed in duplicates at 2 ng/ul and plasmid standards were diluted from 1e-6 to 1e-8 ng/ul for a 3-point standard curve. Fluorescence quantification was performed by the iQ™ 5 Real-Time PCR Detection System (Bio-Rad) and analyzed by the iQ™ 5 Optical System Software (Bio-Rad). The gene expression of the unknown samples were determined from the standard curve and adjusted for input RNA concentration.
Subcloning of Plasmid DNA Standards

Mouse Regenerating protein I, II, IIIα, IIIβ, IIIδ, IIIγ and IV primers were designed through sequence comparison in the Basic Local Alignment Search Tool (BLAST) library and using Invitrogen’s OligoPerfect™ Designer (Table 2.1, 2.2). Mouse EXTl3, Pax7, MyoD and Myogenin primer sequences were obtained through the literature (Table 2.1) and purchased through Eurofins MWG Operon. Primers were tested on a temperature gradient PCR of 58 – 62°C on a MyCycler™ thermocycler (Bio-Rad) and ran on a 2% agarose gel to select the optimum primer set and annealing temperature. Amplified PCR products of the correct sizes (Table 2.1) were extracted by excising from the agarose gel and isolated using the QIAquick™ Gel Extraction Kit (QIAGEN). Purified PCR products were transformed into pCR2.1-TOPO™ vectors (Invitrogen) and subcloned into One Shot™ Mach1™-T1™ Chemically Competent E. coli cells (Invitrogen). The transformed cells were grown overnight on ampicillin-supplemented agar plates pre-spread with 40 μl of 40 mg/ml X-gal (Fermentas) at 37°C for a minimum of 12 hours. Single white or light blue colonies were selected and grown overnight in 3 ml of ampicillin-supplemented agar medium and on ampicillin-supplemented agar plates at 37°C. The agar plates were sealed with Parafilm™ M (Pechiney Plastic Packaging) and stored at 4°C until further use. Plasmid DNA was isolated from the cultured ampicillin-supplemented agar medium using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) and quantified with NanoDrop ND-1000 spectrophotometer. 200 ng/ul of each sample was analyzed by the University of Arizona
Genetics Core sequencing facility (Tucson, AZ) to determine the correct insertion of the target sequence. Plasmid DNA standards were stored at -20°C until further use.

Once the correct insertion of the target sequence was verified by sequence analysis, single colonies from the previously stored agar plates were grown overnight in ampicillin-supplemented agar medium. 600 μl of the overnight culture was added to 400 μl of glycerol stock media and stored at -80°C until further use.
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Table 2.1. Forward and Reverse Mice Primer Sequences and Annealing Temperatures for Real Time PCR Analyses.

1Regenerating-islet derived (Reg); Exostoses-like gene 3 (EXTL3); Paired-box gene 7 (Pax7); Myogenic differentiation (MyoD)
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NM_009042  769 bp

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121  ctgagctgagactc actctctcct catctctcct ctctgtgct gtctcagctg
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**Mouse Reg2 mRNA**
NM_009043  692 bp

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**Mouse Reg3a mRNA**
NM_011259  837 bp

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NM_026328 1021 bp

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541  cggtctctgt gctctctgtg cttgctatatt acattatata actggtcatc
601  gtcctgacac aacctctccc acctcccctt acttcctctt ccctctggtat cggcatcttc
661  agtacaaacc ctgatagcag ccctaggggc atggtatatt acattatata actggtcatc
721  gtaaaaacac ttgagggagg cccagctctg cttccaggggc cagttgctacg aagccctgtct
781  gacagtttg gcctctgtgc ttcctctgttc atggaagagc cggctctacca
841  ggtgtcagag attagatgcc gggcctaggt ctttctctgc tctctctcttc aatgcatcttc
901  ctctcctctgc ctctcctctgc tctctctcttc aatgcatcttc
961  tgcactcttc gcctggtcttc aacagtttaa gtttggggcc caagaaacac
1021  c

Table 2.2. Nucleotide Sequence of Reg I, II, IIIα, IIIβ, IIIδ, IIIγ and IV. Yellow boxes denote forward primers, green boxes denote reverse primers.

Satellite Cell Isolation

Primary satellite cell isolations were performed according to Allen et al. (Allen et al. 1997) with modifications from Lees et al. (Lees, Rathbone and Booth 2006). 3 to 6 month old female C57BL6 mice were sacrificed by CO2 asphyxiation. The animals were bled out by a heart stick through the chest cavity, then fully submerged in 70% ethanol for sterilization. A small cut was made into the back skin to facilitate manual skinning of the mice to allow exposure of the hind limbs, fore limbs and back muscles. The muscles of the back, hind limb and forequarters were surgically removed in the laminar flow hood and placed in sterile phosphate-buffered saline (pH 7.4) on ice. The skeletal muscle tissues were cleaned of any connective or adipose tissues then manually minced with sterile scissors for 10 minutes. 50 ml conical tubes were filled with 5 grams of minced
skeletal muscle tissue, then filled to 50 ml with ice-cold PBS. The 50 ml conical tubes were centrifuged at 1,500 x g for 5 minutes, then the PBS was decanted. Sterile protease buffer (1.25 mg/ml Protease XIV (Sigma) in PBS) was added to a total of 50 ml in the conical and titrated with a 25 ml pipet 5 times, then digested in a 37°C water bath for 1 hour with intermittent agitation every 15 minutes. The tubes were then centrifuged at 1,500 x g for 5 minutes and the supernatant decanted. Sterile PBS warmed to 37°C was added to a total of 22.5 ml and titrated with a 10 ml pipet 5 times, then centrifuged at 500 x g for 10 minutes. Following centrifugation, the supernatant containing cells were decanted into new 50 ml conical tubes. The wash steps were repeated with 37°C PBS to a total of 17.5 ml and 12.5 ml, then centrifuged at 500 x g for 8 minutes and 1 minute, respectively. After each centrifugation step, the supernatants were collected in the new 50 ml conical tubes, then filtered using 100 μm Steriflip Filter Units (Millipore). The filtered cells were centrifuged at 1,500 x g for 5 minutes, and the supernatant removed. The cell pellets were resuspended in 30 ml of 37°C pre-plate media (Dulbecco’s Modified Eagle’s Medium (DMEM) + 10% Fetal Bovine Serum (FBS) + 1% Antibiotic/Anti-mycotic (AbAm)) and plated on uncoated 15 cm tissue culture dishes. The 15 cm plates were incubated for 2 hours in a sterile, humidified atmosphere of 5% CO2 at 37°C. Additional 15 cm tissue culture dishes were coated with poly-L-lysine (10 mg/ml diluted 1:100 in sterile PBS) for 5 minutes, then rinsed twice with sterile deionized water. The water was aspirated and the plates allowed to dry for approximately 20 minutes, then coated with fibronectin (10 μg/ml in sterile PBS) for 45 minutes. Fibronectin was aspirated and replaced with 30 ml of growth medium (DMEM + 20% FBS + 1% AbAm)
and placed in the 37°C incubator until use. Following 2 hours of pre-plate incubation, the cell-containing media was pipetted into a 50 ml conical tube and centrifuged at 1,500 x g for 5 minutes. The supernatant was removed and the pellet resuspended in 30 ml of 37°C growth media and seeded onto the poly-L-lysine and fibronectin coated 15 cm tissue culture dishes. Growth media was replaced every 24 hours.

**Immunohistochemistry**

Tibialis anterior muscles in OCT were cut into 10-micron-thick sections on a cryostat (Microm International) and fixed in 2% formaldehyde in PBS for 10 minutes. The sections were then blocked with 10% FBS and 1% Triton X-100 in PBS for one hour at room temperature. The sections were stained for Reg I (R&D Systems), Reg II (Santa Cruz Biotechnology), Reg IIIα (Santa Cruz Biotechnology), Reg IIIβ (R&D Systems), Reg IIIδ (Santa Cruz Biotechnology), Reg IIIγ (Abgent), Reg IV (R&D Systems) and EXTL3 (Santa Cruz Biotechnology) overnight at 4°C, then washed in PBS three times for 10 minutes each. Slides were stained with Alexa Fluor 594 or 488 donkey antigoat/rabbit/sheep secondary antibody (Invitrogen) for one hour at room temperature, subsequently washed in 0.2% Tween-20 in PBS and water, then incubated in DAPI for nuclear staining. For control slides, slides were incubated in secondary antibodies only. Images were taken with a Leica DM 5500B microscope and Image-Pro software.
**Immunocytochemistry**

Satellite cells were washed with ice-cold PBS, then fixed in 2% paraformaldehyde for 30 minutes on ice. The cells were then blocked in 1% BSA for one hour at room temperature. Cells were stained for Reg I (R&D Systems), Reg II (Santa Cruz Biotechnology), Reg IIIα (Santa Cruz Biotechnology), Reg IIIβ (R&D Systems), Reg IIIδ (Santa Cruz Biotechnology), Reg IIIγ (Abgent), Reg IV (R&D Systems), EXTL3 (Santa Cruz Biotechnology) and MyoD (Santa Cruz Biotechnology) overnight at 4°C, then washed with PBS three times. Cells were stained with Alexa Fluor 594 or 488 donkey anti-goat/rabbit/sheep secondary antibody (Invitrogen) for one hour at room temperature, then rewashed in PBS three times followed by two water washes. As a control, cells were incubated in secondary antibodies only. The cells were incubated in DAPI for nuclear staining, then imaged on a Leica DMI 4000 B microscope and Leica Application Suite software.

**Statistical Analysis**

Gene expression data were statistically analyzed using one-way analysis of variance (ANOVA) to compare expression between time points. Results were presented as the mean ± the standard error of the mean. Statistical significance was denoted by differing letters designated as significant when $P \leq 0.05$ and tended to be significant if $P \leq 0.10$. Measurements of gene expression from real-time PCR were normalized to input RNA, and any data with non-homogeneous variances were transformed using logarithmic 10 for statistical analysis.
Results

Gene Expression in Marcaine-Damaged Tibialis Anterior Muscles

Due to non-homogeneity among variances, data for Reg I, II, IIIα, IV, Pax7, MyoD and Myogenin were transformed using logarithmic 10 for statistical analysis; data were presented as the non-transformed mean ± SEM for Reg I, II, IIIα, IV, Pax7, MyoD and Myogenin while the Reg IIIα data were presented as the logarithmic 10 transformed mean ± SEM to allow better visualization of significance.

No statistical significance was found for the time course experiment of Reg I (Figure 2.1), II (Figure 2.2), IV (Figure 2.5) or EXTL3 (Figure 2.6) gene expression. The logarithmic 10 of average Reg IIIα RNA concentrations of marcaine-damaged tibialis anterior muscles over time were statistically significant between the undamaged control and the 0.5, 1, 2, 3, 4, 7 and 14 day time points (-42.53 vs. -25.05, -23.55, -20.85, -20.95, -16.68, -20.59 and -18.95 Log_{10}[fg/ng RNA]; \(P < 0.05\); Figure 2.3). Average Reg IIIγ RNA concentration was statistically higher at 0.5 day relative to the undamaged controls (-6.025 vs. -7.350 Log_{10}[fg/ng RNA]; \(P < 0.05\); Figure 2.4) and had a tendency for significance between the 1 day marcaine and undamaged control (-6.208 vs. -7.350 Log_{10}[fg/ng RNA]; \(P < 0.10\); Figure 2.4). Gene expression of Reg IIIβ and IIIδ were minimal in either the control or the marcaine-damaged tibialis anterior muscle samples over time. Gene expression levels of Pax7 (Figure 2.7A), MyoD (Figure 2.7B) and Myogenin (Figure 2.7C) were also presented to confirm appropriate myogenetic progression over the time course.
Figure 2.1. Reg I Gene Expression in Marcaine-Damaged Tibialis Anterior Muscles of Mice. Gene expression levels of Reg I in control and marcaine-damaged tibialis anterior muscle samples were measured over time as average RNA concentration (fg/ng) ± SEM ($P < 0.05$). Due to non-homogeneity among the variances, data were transformed using logarithmic 10 for statistical analysis; data presented are non-transformed means ± SEM. Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 2.2. Reg II Gene Expression in Marcaine-Damaged Tibialis Anterior Muscles of Mice. Gene expression levels of Reg II in control and marcaine-damaged tibialis anterior muscle samples were measured over time as average RNA concentration (fg/ng) ± SEM (P < 0.05). Due to non-homogeneity among the variances, data were transformed using logarithmic 10 for statistical analysis; data presented are non-transformed means ± SEM. Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 2.3. *Reg IIIα* Gene Expression in Marcaine-Damaged Tibialis Anterior Muscles of Mice. Gene expression levels of *Reg IIIα* in control and marcaine-damaged tibialis anterior muscle samples were measured over time as average RNA concentration (fg/ng) ± SEM (*P* < 0.05). Statistical significance was denoted by differing letters. Due to non-homogeneity among variances, data were transformed using logarithmic 10 for statistical analysis; data presented are transformed means ± SEM to allow better visualization of significance. Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 2.4. Reg IIIγ Gene Expression in Marcaine-Damaged Tibialis Anterior Muscles of Mice. Gene expression levels of Reg IIIγ in control and marcaine-damaged tibialis anterior muscle samples were measured over time as average RNA concentration (fg/ng) ± SEM (P < 0.05). Statistical significance was denoted by differing letters. Due to non-homogeneity among variances, data were transformed using logarithmic 10 for statistical analysis; data is presented as non-transformed mean ± SEM. Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 2.5. Reg IV Gene Expression in Marcaine-Damaged Tibialis Anterior Muscles of Mice. Gene expression levels of Reg IV in control and marcaine-damaged tibialis anterior muscle samples were measured over time as average RNA concentration (fg/ng) ± SEM ($P < 0.05$). Due to non-homogeneity among variances, data were transformed using logarithmic 10 for statistical analysis; data is presented as non-transformed mean ± SEM. Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 2.6. EXTL3 Gene Expression in Marcaine-Damaged Tibialis Anterior Muscles of Mice. Gene expression levels of EXTL3 in control and marcaine-damaged tibialis anterior muscle samples were measured over time as average RNA concentration (fg/ng) ± SEM ($P < 0.05$). Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 2.7. Pax7, MyoD and Myogenin Gene Expression in Marcaine-Damaged Tibialis Anterior Muscles of Mice. Gene expression levels of (A) Pax7, (B) MyoD and (C) Myogenin were measured in marcaine-damaged tibialis anterior muscle samples over time as average RNA concentration (fg/ng) ± SEM ($P < 0.05$). Statistical significance was denoted by differing letters. Due to non-homogeneity among variances, data were transformed using logarithmic 10 for statistical analysis; data is presented as non-transformed mean ± SEM. Measurements of gene expression from real-time PCR were normalized to input RNA.
Immunohistochemistry of Reg Protein Isoforms on Marcaine-Damaged Tibialis Anterior Muscles

Undamaged control muscle fibers were distinctly outlined with Reg I protein, but muscle fibers undergoing necrotic degeneration between 0.5 days to 2 days were no longer outlined with Reg I protein. The brightest Reg I protein staining was found at 3 days and 4 days post-marcaine induced muscle damage in the regenerating areas of muscle sections, where newly regenerated myofibers with centrally located nuclei were brightly stained (Figure 2.8). Undamaged control fibers were outlined and myonuclei were brightly stained with Reg II. Marcaine-damaged muscle fibers stained positive for Reg II, but the brightest staining was found in myofibers with centrally located nuclei at 4 days and 7 days (Figure 2.9). Overall, muscle fibers were weakly positive for Reg IIIα, with the brightest staining between at 1 day and 2 days post-marcaine damage, where muscle fibers are undergoing necrosis (Figure 2.10). Reg IIIβ protein staining was brightest in necrotic fibers between 0.5 and 2 days post-marcaine damage (Figure 2.11). Undamaged control muscles were very weakly stained with Reg IIIδ, while staining brightened from 0.5 to 2 days (Figure 2.12). Undamaged control fibers were brightly stained for Reg IIIγ, with a staining pattern similar to fiber type staining. Marcaine-damaged muscle fibers were positively stained for Reg IIIγ over time, but were most brightly stained at 1 day and 2 days post-marcaine damage within necrotic fibers (Figure 2.13). Undamaged control muscle fibers and myonuclei were brightly outlined with Reg IV. Reg IV protein staining was highest at 3 days and 4 days post-marcaine induced muscle damage, with the brightest staining on myofibers with centrally located nuclei.
(Figure 2.14). EXTL3 staining was similar to Reg IV staining, with the brightest staining at 3 days and 4 days in newly regenerated myofibers with centrally located nuclei (Figure 2.15).
Figure 2.8. Immunohistochemistry of (A) Reg I and (B) Composite of Reg I and DAPI on Control and Marcaine-Damaged Tibialis Anterior Muscles of Mice Over Time.
Figure 2.9. Immunohistochemistry of (A) Reg II and (B) Composite of Reg II and DAPI on Control and Marcaine-Damaged Tibialis Anterior Muscles of Mice Over Time.
Figure 2.10. Immunohistochemistry of (A) Reg IIIα and (B) Composite of Reg IIIα and DAPI on Control and Marcaine-Damaged Tibialis Anterior Muscles of Mice Over Time.
Figure 2.11. Immunohistochemistry of (A) Reg IIIβ and (B) Composite of Reg IIIβ and DAPI on Control and Marcaine-Damaged Tibialis Anterior Muscles of Mice Over Time.
Figure 2.12. Immunohistochemistry of (A) Reg IIIδ and (B) Composite of Reg IIIδ and DAPI on Control and Marcaine-Damaged Tibialis Anterior Muscles of Mice Over Time.
Figure 2.13. Immunohistochemistry of (A) Reg IIIγ and (B) Composite of Reg IIIγ and DAPI on Control and Marcaine-Damaged Tibialis Anterior Muscles of Mice Over Time.
Figure 2.14. Immunohistochemistry of (A) Reg IV and (B) Composite of Reg IV and DAPI on Control and Marcaine-Damaged Tibialis Anterior Muscles of Mice Over Time.
Figure 2.15. Immunohistochemistry of (A) EXTL3 and (B) Composite of EXTL3 and DAPI on Control and Marcaine-Damaged Tibialis Anterior Muscles of Mice Over Time.
Gene Expression in Isolated Mice Satellite Cells

Due to non-homogeneity among variances, data for Myogenin were transformed using logarithmic 10 for statistical analysis; data were presented as non-transformed means ± SEM. Average Reg I RNA concentrations tended to be significant between 2 days and 4 days (0.9312 vs. 0.0226 fg/ng RNA; $P < 0.10$; Figure 2.16) as well as between 2 days and 7 days (0.9312 vs. 0.0045 fg/ng RNA; $P < 0.10$; Figure 2.16) in isolated mice satellite cells. Average Reg III$\gamma$ gene expression tended to be significant between 2 days and 3 days (3.33e-3 vs. 1.30e-3 [fg/ng RNA]; $P < 0.10$; Figure 2.18) while average EXTL3 gene expression tended to be higher at 4 days compared to 3 days (0.192 vs. 0.229 fg/ng RNA; $P < 0.10$; Figure 2.19). Gene expression of Reg II, III$\beta$, III$\delta$ and IV were absent in mice satellite cells. Gene expression of Pax7, MyoD and Myogenin were also measured to confirm proper myogenesis progression (Figure 2.20).
Figure 2.16. Gene Expression of Reg I in Isolated Satellite Cells of Mice. Gene expression levels of Reg I were measured in isolated mice satellite cells over time as average RNA concentration (fg/ng) ± SEM ($P < 0.05$). Tendency for significance was denoted by differing letters. Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 2.17. Gene Expression of Reg IIIα in Isolated Satellite Cells of Mice. Gene expression levels of Reg IIIα were measured in isolated mice satellite cells over time as average RNA concentration (fg/ng) ± SEM ($P < 0.05$). Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 2.18. Gene Expression of Reg IIIγ in Isolated Satellite Cells of Mice. Gene expression levels of Reg IIIγ were measured in isolated mice satellite cells over time as average RNA concentration (fg/ng) ± SEM ($P < 0.10$). Tendency for significance was denoted by differing letters. Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 2.19. Gene Expression of EXTL3 in Isolated Satellite Cells of Mice. Gene expression levels of EXTL3 were measured in isolated mice satellite cells over time as average RNA concentration (fg/ng) ± SEM ($P < 0.10$). Tendency for significance was denoted by differing letters. Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 2.20. Gene Expression of Pax7, MyoD and Myogenin in Isolated Satellite Cells of Mice. Gene expression levels of (A) Pax7, (B) MyoD and (C) Myogenin were measured in isolated mice satellite cells as average RNA concentration (fg/ng) ± SEM ([A] $P < 0.05$; [B] $P < 0.005$; [C] $P < 0.001$). Statistical significance was denoted by differing letters. Due to non-homogeneity among variances, data for Myogenin were transformed using logarithmic 10 for statistical analysis; data is presented as non-transformed mean ± SEM. Measurements of gene expression from real-time PCR were normalized to input RNA.
**Immunocytochemistry of Isolated Mice Satellite Cells Over Time.**

Isolated mice satellite cells were positively stained for Reg I, II, IIIα, IIIβ, IIIδ, IIIγ and IV and all staining was localized to the cytoplasm of the cell. Fused myotubes at 72 and 96 hours had the brightest staining for Reg I (Figure 2.21), while no changes in expression patterns were observed over time for Reg II staining (Figure 2.22). Overall, satellite cells were weakly stained for Reg IIIα and over time, but staining seemed to noticeably dim after 2 days (Figure 2.23). Reg IIIβ staining seemed to peak 48 hours post-isolation, and from then on brighter staining corresponded with fused myotubes (Figure 2.24). Reg IIIδ staining was brightest at 3 days post-isolation, where fused myotubes correlated to stronger staining within the cytoplasm (Figure 2.25). Reg IIIγ staining remained constant over time, with no correlation of staining strength with differentiation (Figure 2.26). Finally, brighter Reg IV staining seemed to correspond with fused myotubes at 3 and 4 days post-isolation (Figure 2.27).
Figure 2.21. Immunocytochemistry of Reg I and DAPI Stained Mice Satellite Cells Over Time.
Figure 2.22. Immunocytochemistry of Reg II, MyoD and DAPI Stained Mice Satellite Cells Over Time.
Figure 2.23. Immunocytochemistry of Reg IIIα, MyoD and DAPI Stained Mice Satellite Cells Over Time.
Figure 2.24. Immunocytochemistry of Reg IIIβ, MyoD and DAPI Stained Mice Satellite Cells Over Time.
Figure 2.25. Immunocytochemistry of Reg IIIδ and DAPI Stained Mice Satellite Cells Over Time.
Figure 2.26. Immunocytochemistry of Reg IIIγ and DAPI Stained Mice Satellite Cells Over Time.
Figure 2.27. Immunocytochemistry of Reg IV, MyoD and DAPI Stained Mice Satellite Cells Over Time.
Discussion

The regenerative capacity of skeletal muscle is dependent upon the coordination of numerous extrinsic and intrinsic factors that control myogenesis. Presently there are hundreds of factors that are known to directly and indirectly affect skeletal muscle regeneration, with more being discovered frequently. Regenerating proteins are upregulated at the onset of damage and throughout the stages of repair in many tissue systems. Previous reports showed that the upregulation of rat Reg I expression coincides with an increase in the size of regenerating pancreatic islets (Terazono et al. 1988), rat Reg IIIβ expression was significantly increased in the regenerating motor and sensory neurons of animals with crushed sciatic nerves (Livesey et al. 1997a) and Reg I protein concentrations were 50-100 fold higher in the brain tissues of Alzheimer and Down’s Syndrome patients (Ozturk et al. 1989) while the inhibition of Reg IIIα, IIIβ and IIIγ expression increased the severity of pancreatitis (Zhang et al. 2004, Viterbo et al. 2008), Reg II-knockout mice have a significantly lower survival rate and delayed liver mass recovery in response to chemically induced fulminant hepatitis (Lieu et al. 2006) and Reg IIIβ inhibition resulted in a reduction in the rate of axon regeneration (Livesey et al. 1997b). These reports indicate a mitogenic, protective and anti-apoptotic role of Reg proteins in damaged and regenerating tissues. In accordance to these reports, it seems highly likely that Regenerating proteins would be expressed in inflamed and regenerating skeletal muscle as well and contribute to myogenesis.

Although no statistical significance was found, Reg I RNA concentration tended to be significantly increased at 0.5 days, which is consistent with the conclusion by Fukui
et al. that Reg I protein plays a pivotal role in early stimulation of cell proliferation (Fukui et al. 1998). mRNA abundance of Reg I was also the highest among all the isoforms, with the next highest RNA concentration at 10% that of Reg I. This may signify Reg I as the predominant Regenerating protein isoform in skeletal muscle, but the increased expression of the other isoforms in damaged muscle may imply they play a supportive role as well. Multiple Reg protein isoforms have been found to be highly expressed within the same tissue (Zhang et al. 2004, Viterbo et al. 2008, Ozturk et al. 1989, Livesey et al. 1997b, Livesey et al. 1997a) and there is no indication that the same cannot be true for skeletal muscle. Reg I protein staining was brightest at 4 days post-marcaine injection within myofibers with centrally located nuclei, which provides evidence that Reg I protein may be contributing mostly to the differentiation of newly regenerated myofibers. Satellite cell staining of Reg I support this evidence, as brighter staining was seen in fused myotubes at 3 days and 4 days post-isolation. Reg I gene expression tended to be upregulated at 2 days post-isolation in satellite cells, then gradually decreases from 3 to 7 days. This may indicate that Reg I gene expression stimulates early satellite cell proliferation and may ultimately promote skeletal muscle regeneration.

Overall Reg II RNA concentrations were 2 orders of magnitude smaller than Reg I, indicating that Reg II may not have a biological significance in skeletal muscle. Although statistical significance was absent between any of the time points, there was a trend of increasing Reg II gene expression only after 4 days post-marcaine injection, when the majority of damaged fibers have been replaced. Therefore Reg II may
contribute more in the later stages of regeneration during muscle fiber maturation. However, Reg II gene expression was absent in satellite cells, indicating that cells of non-satellite cell origin may be contributing to Reg II expression *in vivo*. Corresponding to these results, no changes in Reg II protein staining were observed in isolated satellite cells over time. This may indicate that at the onset of muscle damage, Reg II proteins are secreted by non-satellite cells and bind to satellite cells to stimulate their activity.

Reg IIIα expression was significantly higher between the control samples and all of the time points, however, the actual concentration of Reg IIIα RNA is 20 fold less than that of Reg I, suggesting a minimal biological significance; it is likely that Reg IIIα has no functionality in skeletal muscle regeneration. However, contrary to the *in vivo* results, Reg IIIα had the highest overall mRNA abundance in isolated satellite cells. This may indicate that Reg IIIα gene expression is highly localized solely within satellite cells, and mRNA abundance of Reg IIIα *in vivo* may be very low due to the absence of Reg IIIα gene expression in other cell types. Immunofluorescent staining of marcaine-damaged tibialis anterior muscles supports this evidence in that overall Reg IIIα staining is fairly weak across all time points except at 2 days when necrotic fibers are more brightly stained.

Reg IIIγ expression was statistically significant between the control and 0.5 day time point. While Reg IIIγ expression is a fold lower than Reg I, the expression patterns are very similar in that expression is upregulated at 0.5 days, then plateaus. Reg IIIγ gene expression was statistically higher 2 days post-isolation in satellite cells, and staining patterns remained constant in the immunofluorescently stained satellite cells over
time. Similar Reg IIIγ gene expression patterns were found by Namikawa et al. in the injured nerves of rats, where Reg IIIγ expression significantly increased within 12 hours and peaked at 1 day (Namikawa et al. 2005). They further demonstrated that nerve-injury stimulated Reg IIIγ expression resulted in macrophage chemotaxis, ultimately promoting nerve regeneration (Namikawa et al. 2006). Reg IIIγ may act in a similar manner in damaged skeletal muscles by recruiting macrophages to promote myogenesis.

Although no significance was found within the time course for Reg IV gene expression, there seems to be a higher trend of expression at earlier time points when muscle fibers are necrotic. The control muscle is distinctly outlined by Reg IV protein staining, and Reg IV gene expression is absent in satellite cells, indicating Reg IV may not play a pivotal role during skeletal muscle regeneration.

Although no statistical significance was found, there seems to be a higher trend of EXTL3 expression during times of early regeneration where necrotic fibers are being phagocytized and newly regenerated myofibers with centrally located nuclei are abundant. The immunofluorescent labeling of EXTL3 protein in marcaine-damaged muscle sections correlate with gene expression. EXTL3 may be working in conjunction with different Reg proteins; one Reg protein isoform (such as Reg I or Reg IIIγ) is significantly expressed at the onset of injury to promote early stimulation of cell proliferation and macrophage chemotaxis, which in turn stimulates the expression of EXTL3 during the late regenerative stages, where then other Reg protein isoforms (Reg II) are expressed and promote fiber maturation. EXTL3 gene expression is statistically
significant in satellite cells 4 days post-isolation, which approximately mimics the *in vivo* gene expression pattern as well.

Gene expression levels of Reg IIIβ and IIIδ were absent in either the control or marcaine-damaged tibialis anterior muscles as well as in isolated satellite cells, indicating that these Regenerating protein gene isoforms are likely not expressed by skeletal muscle. However, muscle sections and satellite cells stain positive for both protein isoforms, suggesting that these proteins are produced by non-myogenic cells and may be recruited by satellite cells at the onset of injury.

Taken together, these results support the hypothesis that multiple Reg protein isoforms are expressed in inflamed and regenerating skeletal muscle, and most likely contribute to skeletal muscle myogenesis. Among the isoforms, Reg I and IIIα are the most likely candidates as the predominant Reg protein isoform in skeletal muscle regeneration, as both are abundantly expressed in satellite cells. However, as Reg I gene expression is significantly higher *in vivo*, it is highly likely that non-satellite cells greatly contribute to Reg I gene expression. In contrast, Reg IIIα mRNA abundance is surprisingly low *in vivo*, suggesting that satellite cells may be the only source of Reg IIIα expression in damaged skeletal muscle. Future research should focus further on isolating the origin of Reg gene expression in damaged skeletal muscle, with emphasis on infiltrating cells and structural components such as connective tissue and vasculature.
CHAPTER 3
THE ROLE OF SUPPLEMENTED REGENERATING PROTEIN ON SKELETAL MUSCLE REGENERATION

Abstract

The addition of exogenous Reg proteins to damaged and regenerating pancreas, liver and neuronal tissue stimulated cell proliferation and viability, ultimately promoting accelerated regeneration in these tissues. As Reg gene expression is upregulated in regenerating skeletal muscle and satellite cells, the ability of exogenous Reg protein to accelerate skeletal muscle regeneration was investigated. 3-6 month old female C57BL6 mice were injected with 50 ul of 7.5 mg/ml marcaine in their tibialis anterior muscles. 24 hours later, 750 ng/ml of Reg IIIa protein or an equal volume of PBS was injected into the same tibialis anterior muscles without fully inserting the needle to the proximal end of the muscle to prevent further muscle damage. The muscles were removed 0.5, 1, 2, 3, 4, 7 and 14 days later and analyzed for EXTL3, Pax7, MyoD and Myogenin gene expression. The Reg protein or PBS treated tibialis anterior muscles at 4 days were also analyzed for muscle morphometry characteristics. EXTL3, Pax7 and Myogenin expression was significantly downregulated at 2 or 3 days in the Reg protein-treated muscle samples. Fiber number and fiber diameter in regenerating tibialis anterior muscles at 4 days were also significantly lower in the Reg-treated muscles. Taken together, our results suggest that the addition of exogenous Reg proteins to damaged tissue inhibited myogenesis and ultimately hindered skeletal muscle regeneration.
Introduction

Skeletal muscle has a tremendous capacity for regeneration and this process is governed by numerous intrinsic and extrinsic factors. The overexpression or supplementation of damaged skeletal muscle with factors such as nitric oxide, FGF, HGF, VEGF, IGF-I, estrogen and testosterone have shown to stimulate satellite cell activation, proliferation and differentiation, ultimately accelerating myogenesis (Table 1.1).

The addition or upregulation of Reg proteins to damaged or regenerating tissues have yielded remarkable results. Addition of exogenous rat Reg I protein to 90% depancreatized rats ameliorated surgically-induced diabetes through a 2.5 fold enlargement of β-cell mass within 2 months (Watanabe et al. 1994a), increased [H3]thymidine incorporation and cell growth of gastric mucosal epithelial cells and gastric cancer cells in culture (Fukui et al. 1998, Kadowaki et al. 2002), upregulation of hepatocyte Reg IIIα expression enhanced BrdU incorporation, increased liver weights in partially hepatectomized mice and improved cell survival by 20% (Simon et al. 2003, Lieu et al. 2006). In neural cells, Livesey et al. observed a dose-dependent increase in BrdU incorporation with the addition of exogenous rat Reg IIIβ protein in vitro in the presence of forskolin (Livesey et al. 1997b, Livesey et al. 1997a), while the viability of culture embryonic motoneurons was enhanced with the addition of rat Reg IIIβ protein (Nishimune et al. 2000). These studies highly suggest that Reg proteins have a mitogenic, anti-inflammatory and anti-apoptotic effect and the addition of exogenous Reg
proteins can further boost the original result or rescue the result in the absence of intrinsic Reg protein.

Study objectives were to determine the expression profile of EXTL3, Pax7, MyoD and Myogenin in Reg protein or PBS-treated, damaged skeletal muscles over time to investigate the myogenetic potential of exogenous Reg protein in skeletal muscle. Muscle morphometric analysis between the treatment groups will also be performed to examine whether the addition of exogenous Reg proteins ultimately accelerated skeletal muscle regeneration.
Materials and Methods

Animals

All animal care use was approved and supervised by the University of Arizona Institutional Animal Care and Use Committee. 3- to 6-month old female C57BL6 mice were purchased from Jackson labs and used for all tissue collection and primary satellite cell isolations. Standard rodent chow and water was made available to the animals on an ad libitum basis.

Treatments

3 to 6 month old female C57BL6 mice were anesthetized and hind limbs prepared for treatment according to the previous methods. Both the right and left tibialis anterior muscles were injected with 50 μl of 7.5 mg/ml marcaine solution as previously described in Chapter 2. The animals were allowed to recover for 24 hours, then the right tibialis anterior muscles were injected with 50 μl of 750 ng/ml Regenerating protein IIIα in phosphate-buffered saline by injecting the solution at the distal end of the muscle at the original marcaine injection site. The needle was not inserted fully to the proximal end to prevent further muscle damage. An equal volume of phosphate-buffered saline was injected in the same manner into the left tibialis anterior muscle. The animals were placed on a heating pad and allowed to recover from anesthesia for 3 hours at 37°C.

Tissue Collection
At 12, 24, 48, 72, 96, 168 and 336 hours post-Regenerating protein IIIα or PBS injection, animals were sacrificed with CO2 asphyxiation and heart stick. Animals were fully submerged in 70% ethanol for sterilization, and manually skinned to allow exposure of the hind limbs. The tibialis anterior muscles were surgically removed by excision at the distal and proximal ends of the muscle and individually placed in foil pouches and snap-frozen in liquid nitrogen or embedded in OCT tissue-freezing media (Tissue Tex, Inc.) using 2-methylbutane pre-cooled in liquid nitrogen. Tissue samples were then transferred to the -80°C freezer for storage until further use.

**RNA Extraction from Tissues**

The Regenerating protein IIIα-injected or PBS injected tibialis anterior muscles were placed in microcentrifuge tubes with 500 μl of ice-cold Trizol reagent. The samples were homogenized with a PowerGen 125 homogenizer (Fisher Scientific) for 15 second bursts three times on ice, or until the samples were completely homogenized. 100 μl of chloroform was added, then vortexed for 20 seconds. Phase-lock gel tubes (5 Prime) were pre-spun at 1,500 x g for 30 seconds. Homogenized samples were added to the gel tubes and incubated at room temperature for 5 minutes, then centrifuged at 3,500 x g for 10 minutes at 4°C. The aqueous phase was aspirated into new 1.5 ml eppendorf tubes and an equal amount of isopropanol was added. Samples were mixed well by inversion and incubated on ice for 5 minutes, then centrifuged at 8,000 x g for 10 minutes at 4°C. The supernatant was discarded and pellets dried at room temperature for 2 minutes. The pellet was resuspended in 80% ethanol and transferred into a new eppendorf tube and
centrifuged at 8,000 x g for 5 minutes at 4°C. Ethanol was aspirated, the tubes were then centrifuged for 5 seconds to allow aspiration of any residual ethanol. The RNA pellet was dried at room temperature for 5 minutes, then resuspended in 100 μl of RNase-free water and stored on ice. Total RNA concentration was measured on a NanoDrop ND-1000 machine and software. RNA samples were stored at -80°C or processed immediately for RNA cleanup. RNA samples were cleaned using the QIAGEN RNeasy Mini Kit (QIAGEN) and concentrations remeasured on the NanoDrop ND-1000. Samples were stored at -80°C until further use.

**Analysis of Gene Expression**

Two μg of total RNA was converted into complimentary DNA by reverse transcriptase using SuperScript™III First-Strand Synthesis for RT-PCR (Invitrogen). Real-time PCR were performed in a 25 μl total volume using iQ™ SYBR® Green Supermix (Bio-Rad) on an iQ™5 Real-Time PCR Detection System (Bio-Rad). Samples were analyzed in triplicates at 2 ng/ul and plasmid standards were diluted from 1e-5 to 1e-8 ng/ul for a 4-point standard curve. Fluorescence quantification was performed by the iQ™5 Real-Time PCR Detection System (Bio-Rad) and analyzed by the iQ™5 Optical System Software (Bio-Rad). The gene expression of the unknown samples were determined from the standard curve and adjusted for input RNA concentration.

**Subcloning of Plasmid DNA Standards**
Mouse EXTL3, Pax7, MyoD and Myogenin primer sequences were obtained through the literature (Table 2.1) and purchased through Eurofins MWG Operon. Primers were tested on a temperature gradient PCR of 58 – 62°C on a MyCycler™ thermocycler (Bio-Rad) and ran on a 2% agarose gel to confirm the optimum primer set and annealing temperature referenced in the literature. Amplified PCR products of the correct sizes (Table 2.1) were extracted by excising from the agarose gel and isolated using the QIAquick® Gel Extraction Kit (QIAGEN). Purified PCR products were transformed into pCR®2.1-TOPO® vectors (Invitrogen) and subcloned into One Shot® Mach1™-T1® Chemically Competent E. coli cells (Invitrogen). The transformed cells were grown overnight on ampicillin-supplemented agar plates pre-spread with 40 μl of 40 mg/ml X-gal (Fermentas) at 37°C for a minimum of 12 hours. Single white or light blue colonies were selected and grown overnight in 3 ml of ampicillin-supplemented agar medium and on ampicillin-supplemented agar plates at 37°C. The agar plates were sealed with Parafilm® M (Pechiney Plastic Packaging) and stored at 4°C until further use. Plasmid DNA was isolated from the cultured ampicillin-supplemented agar medium using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) and quantified with NanoDrop ND-1000 spectrophotometer. 200 ng/ul of each sample was analyzed by the University of Arizona Genetics Core sequencing facility (Tucson, AZ) to determine the correct insertion of the target sequence. Plasmid DNA standards were stored at -20°C until further use.

Once the correct insertion of the target sequence was verified by sequence analysis, single colonies from the previously stored agar plates were grown overnight in
ampicillin-supplemented agar medium. 600 μl of the overnight culture was added to 400 μl of glycerol stock media and stored at -80°C until further use.

**Immunohistochemistry**

Tibialis anterior muscles in OCT were cut into 10-micron-thick sections on a cryostat (Microm International) and fixed in 2% formaldehyde in PBS for 10 minutes. The sections were then blocked with 10% FBS and 1% Triton X-100 in PBS for one hour at room temperature. The sections were stained for Dystrophin (Santa Cruz Biotechnology) overnight at 4°C, then washed in PBS three times for 10 minutes each. Slides were stained with Alexa Fluor 594 anti-goat secondary antibody (Invitrogen) for one hour at room temperature, subsequently washed in 0.2% Tween-20 in PBS and water, then incubated in DAPI for nuclear staining. For control slides, slides were incubated in secondary antibodies only. Images were taken with a Leica DM 5500B microscope and Image-Pro software.

**Muscle Morphometry**

Images of Reg or PBS injected Dystrophin-stained tibialis anterior sections at 4 days were taken with a Leica DM 5500B microscope and Image-Pro software. Average fiber number for each treatment group was determined by designating a 400 x 400 μm area of interest and manually counting the number of regenerating fibers. The average fiber diameter for each treatment group was determined by measuring the fiber diameter
of 10 regenerating muscle fibers per image for 10 or 11 cross-sectional images of Reg or PBS-treated tibialis anterior muscles, respectively, with the ImageJ software.

**Statistical Analysis**

Gene expression data between the time points were statistically analyzed using two-way analysis of variance (ANOVA) while the muscle morphometry data was statistically analyzed using an unpaired t-test. Results were presented as the mean ± the standard error of the mean. Statistical significance was denoted by an asterisk (*) or differing letters designated as significant when $P \leq 0.05$ and tended to be significant if $P \leq 0.10$. Measurements of gene expression from real-time PCR were normalized to input RNA, and any data with non-homogeneous variances were transformed using logarithmic 10 for statistical analysis.
Results

Gene Expression of Reg IIIα or PBS-Injected Marcaine-Damaged Tibialis Anterior Muscles

Average EXTL3 RNA concentration tended to be significantly increased in the PBS-injected muscles at 3 days relative to the Reg IIIα-injected muscles (108.60 vs. 49.25 fg/ng EXTL3 RNA; \( P < 0.10 \); Figure 3.1). Average Pax7 RNA concentration was significantly increased in the PBS-injected muscles at 2 days relative to the Reg IIIα-injected muscles (0.246 vs. 0.150 fg/ng Pax7 RNA; \( P < 0.01 \); Figure 3.2). No statistical significance was found between the PBS or Reg IIIα-injected tibialis anterior muscles over time for the gene expression of MyoD (Figure 3.3). Average Myogenin RNA concentration was significantly increased in the PBS-injected muscles at 3 days relative to the Reg IIIα-injected muscles (1663 vs. 377 fg/ng Myogenin RNA; \( P < 0.001 \); Figure 3.4A).

Muscle Morphometry of Reg IIIα or PBS-Injected Tibialis Anterior Muscles

PBS-treated marcaine-damaged tibialis anterior muscles had significantly higher average fiber numbers than the Reg-treated muscle samples (45.57 vs. 39.64 fibers; \( P < 0.05 \); Figure 3.5A). Similar results were found for average fiber diameter, where PBS-treated marcaine-damaged tibialis anterior muscles had significantly higher average fiber diameters than the Reg-treated muscle samples (22.31 vs. 20.38 um; \( P < 0.001 \); Figure 3.5B).
Figure 3.1. Gene Expression of EXTL3 in Marcaine-Damaged Tibialis Anterior Muscles of Mice Injected with Reg IIIα Protein or PBS. Gene expression levels of EXTL3 in Reg protein or PBS injected marcaine-damaged tibialis anterior muscle samples were measured over time as average RNA concentration (fg/ng) ± SEM ($P < 0.10$). Tendency for significance between the Reg IIIa protein or PBS injected marcaine-damaged tibialis anterior muscles were denoted with an asterisk (*). Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 3.2. Gene Expression of Pax7 in Marcaine-Damaged Tibialis Anterior Muscles of Mice Injected with Reg IIIα Protein or PBS. Gene expression levels of Pax7 in Reg protein or PBS injected marcaine-damaged tibialis anterior muscle samples were measured over time as average RNA concentration (fg/ng) ± SEM ($P < 0.01$). Statistical significance between the Reg IIIα protein or PBS injected marcaine-damaged tibialis anterior muscles were denoted with an asterisk (*). Measurements of gene expression from real-time PCR were normalized to input RNA.
Gene Expression of MyoD in Marcaine-Damaged Tibialis Anterior Muscles of Mice Injected with Reg IIIα Protein or PBS. Gene expression levels of MyoD in Reg protein or PBS injected marcaine-damaged tibialis anterior muscle samples were measured over time as average RNA concentration (fg/ng) ± SEM ($P < 0.05$). Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 3.4. Gene Expression of Myogenin in Marcaine-Damaged Tibialis Anterior Muscles of Mice Injected with Reg III$\alpha$ Protein or PBS. Gene expression levels of Myogenin in Reg protein or PBS injected marcaine-damaged tibialis anterior muscle samples were measured over time as average RNA concentration (fg/ng) ± SEM ($P < 0.001$). Statistical significance between the Reg III$\alpha$ protein or PBS injected marcaine-damaged tibialis anterior muscles were denoted with an asterisk (*). Measurements of gene expression from real-time PCR were normalized to input RNA.
Figure 3.5. Muscle Morphometry Analysis of Reg or PBS-Treated Marcaine Damaged Tibialis Anterior Muscles of Mice. Measurements of (A) fiber number and (B) fiber diameter were presented as the average ± SEM ([A] $P < 0.05$; [B] $P < 0.001$). Statistical significance was denoted by differing letters.
Discussion

The tremendous capacity of skeletal muscle to regenerate is dependent upon numerous intrinsic and extrinsic factors. Numerous studies have found that supplementation with significantly higher amounts than what is normally found biologically can boost the rate of skeletal muscle regeneration. We have confirmed the presence of multiple Reg protein isoforms in regenerating skeletal muscle, and therefore hypothesized that supplementation with additional Reg proteins to regenerating muscles would promote accelerated myogenesis in vivo.

The consistent lower tendency of expression in the Reg-treated muscle samples may be noteworthy, in that the additional supplementation with Reg protein may be ultimately suppressing myogenesis. A closer look at the time points reveal that any significant difference in gene expression is seen at either the 2 or 3 day time points, a critical stage in myogenesis where the majority of satellite cells are switching from the proliferation to the differentiation pathway. An overabundance of Reg protein may be causing a negative feedback loop for EXTL3, inhibiting additional receptor production, which may ultimately suppress myogenesis. Pax7 upregulation is necessary for satellite cell renewal (Ono et al. 2012, Olguin and Olwin 2004), and a significantly decreased Pax7 expression in the Reg-treated muscles may suggest that overabundance of Reg proteins inhibits Pax7 expression and therefore restrains satellite cell renewal. However, if that were the case, MyoD expression in the Reg-treated muscle samples would be higher, indicating the promotion of proliferation in a larger proportion of satellite cells. As MyoD expression between the two treatment groups are almost identical with a slight
tendency for the Reg-treated muscles to have a lower MyoD expression, this does not lend evidence to the previous theory. As Pax7 and Myogenin expression are mutually exclusive, a decreased Pax7 expression in the Reg-treated muscles may suggest that these satellite cells are undergoing differentiation earlier; however, Myogenin expression of Reg-treated muscles do not correlate with this idea. In fact, Myogenin expression is significantly lower in the Reg-treated muscle samples at 3 days relative to the PBS-treated muscle samples, suggesting a reduction in satellite cell differentiation. Taken together, the gene expression profiles of EXTL3, Pax7, MyoD and Myogenin fail to correlate with one another, but individually they seem to suggest that supplementation with Reg IIIα protein inhibits myogenesis. Muscle morphometric analysis provides evidence to this theory, as both fiber numbers and fiber diameters were significantly lower in the Reg-treated tibialis anterior muscles than the PBS-treated muscles.

Overall, the data provides evidence that an overabundance of Reg IIIα protein to marcaine-damaged tibialis anterior muscles inhibited myogenesis, ultimately decelerating skeletal muscle regeneration. Further research should focus on the effect of other Reg protein isoform supplementation on skeletal muscle regeneration, as multiple Reg protein isoforms are expressed in damaged skeletal muscle and supplementation with other isoforms may in fact promote skeletal muscle regeneration.
CHAPTER 4
THE ROLE OF SUPPLEMENTED REGENERATING PROTEIN ON SATELLITE CELL ACTIVITY

Abstract

Skeletal muscle regeneration is predominantly dependent upon the manipulation of satellite cell activation, proliferation and differentiation by numerous intrinsic and extrinsic factors. As Reg proteins have a direct mitogenic effect on cells of the pancreas, liver, GI tract and nervous tissue, similar responses may be found with the addition of exogenous Reg proteins to satellite cells. 3-6 month old female C57BL6 mice were injected with 50 ul of 750 ng/ml Reg IIIa or an equal volume of PBS in their tibialis anterior muscles. 32 hours post-Reg IIIa injection, mice were injected intraperitoneally with 5 mg of BrdU per 100 g of body weight. 16 hours after BrdU injection, tibialis anterior muscles were excised and individually processed for satellite cell isolation. After 30 hours in culture, BrdU incorporation into satellite cells were analyzed by the DAB colorimetric assay. Results were inconclusive as positive and negative controls treatments failed to show any effect upon satellite cell activation. C2C12 cells purchased from Sigma-Aldrich or satellite cells isolated from 3-6 month old female C57BL6 mice were analyzed for cell proliferation and differentiation in the presence of increasing concentrations of Reg IIIa protein. Results were inconclusive as the positive and negative controls failed to show any effect upon satellite cell or C2C12 cell proliferation and differentiation. As all initial research on the direct effect of Reg proteins on satellite
and C2C12 cells were inconclusive due to the failure of known in vitro controls, future investigation should focus on resolving these in vitro issues to reveal the potential for Reg protein to alter satellite and C2C12 cell behavior.

**Introduction**

Satellite cells are a population of resident myogenic stem cells responsible for the amazing regenerative capabilities of skeletal muscle that allows full regeneration of myofibers within 7 days of muscle damage (Martinez et al. 2010). The discovery of satellite cells by Mauro in 1961 instigated the creation of a new branch of skeletal muscle regeneration research that ultimately confirmed these cells as the source of new myonuclei (Mauro 1961, Scharner and Zammit 2011, Church et al. 1966, Moss and Leblond 1971, Snow 1977, Schultz et al. 1978). Injury to skeletal muscle leads to the destruction of the sarcolemma and basal lamina, initiating the release of concentrated amounts of calcium (Belcastro et al. 1998). Calpains initiate myofibril proteolysis, muscle fiber hyalinization and eventual lysis, leading to the overproduction of nitric oxide, which activates matrix metalloproteinase-2 (MMP2), an endopeptidase that breaks down the extracellular matrix (Shi and Garry 2006, Ciciliot and Schiaffino 2010, Anderson and Wozniak 2004, Yamada et al. 2008). Degradation of the extracellular matrix allows the secretion of extrinsic and intrinsic growth factors and hormones that facilitate skeletal muscle regeneration by stimulating satellite cell activation, proliferation and differentiation. The overexpression or supplementation of numerous factors are
known to directly alter satellite cell activity and ultimately promote accelerated myogenesis (Table 1.1).

The beneficial effects of exogenous Regenerating proteins to damaged, inflamed and diseased tissues appears to work directly on proliferating or regenerating parenchymal cells of the target organ. Watanabe et al. found that Reg-I treated animals contained approximately 2.5 fold greater mass of β-cells than control animals, signifying that Reg I protein was inducing β-cell proliferation and regeneration (Watanabe et al. 1994a). Kawanami et al. observed the highest rat Reg I gene expression in the highly proliferative endocrine cells of the stomach (Kawanami et al. 1997), while tumors of the gastrointestinal tract and liver have shown significantly increased Reg protein expression (Watanabe et al. 1994a, Kadowaki et al. 2002, Lasserre et al. 1992) and are known to have considerable proliferative potential. These studies suggest that the addition of exogenous Regenerating protein would directly affect the proliferative potential of satellite cells, the only proliferating cells of skeletal muscle.

Isolated satellite cells and the use of a murine myoblast cell line (C2C12) derived from satellite cells are the most common experimental models utilized to study skeletal muscle regeneration *in vitro*. The use of these cells in culture allows a more specific observation of cause and effect without contributions from other cell types of surrounding tissues, and therefore is an ideal model to investigate direct Reg protein effects on skeletal muscle regeneration.

Study objectives were to determine the direct effect of exogenous Reg IIIa protein on satellite or C2C12 cell behavior in regards to activation, proliferation and
differentiation. The ability to manipulate myogenic stem cell activity with the addition of exogenous Reg proteins could reveal therapeutic uses to treat skeletal muscle dysfunction.
Materials and Methods

Satellite Cell Isolation

Primary satellite cell isolations were performed according to Allen et al. (Allen et al. 1997) with modifications from Lees et al. (Lees et al. 2006) in the same manner as in the previous chapters.

C2C12 Cell Culture

C2C12 cell line from mouse (91031101) was obtained from Sigma-Aldrich and stored in liquid nitrogen tanks until use. Vials of cells were placed in a 37°C water bath for 5 minutes then seeded onto 15 cm tissue-culture dishes at 1-2 million cells per dish in growth medium. Media was replaced every 24 hours. At 60-80% confluency, cells were passaged and reseeded onto 15 cm plates for cell expansion or plated onto experimental plates.

Satellite Cell Activation Assay

3-6 month old female C57BL6 mice were injected with 50 μl of 750 ng/ml Reg IIIα or PBS in their right or left tibialis anterior muscles, respectively. Another group of control mice were injected with 50 μl of 15 ng/ml HGF in PBS in their right tibialis anterior muscles. 32 hours after Reg IIIα or PBS injection, mice were injected intraperitoneally with 5 mg of BrdU per 100 g of body weight. 16 hours after BrdU injection, the tibialis anterior muscles were excised and individually processed for
satellite cell isolation as described above. After 30 hours in culture, BrdU incorporation into the satellite cells were analyzed by the diaminobenzidine (DAB) colorimetric assay. Cells were imaged on a Leica DMI 4000 B microscope using the Leica Application Suite software.

**Cell Proliferation Assay**

Satellite cell numbers were expanded by passaging fresh satellite cells at 72 hours, then culturing them in 10% FBS/DMEM pre-plate media for 48 hours in a 15 cm dish. Expanded satellite cells and C2C12 cells were then reseeded onto poly-L-lysine and fibronectin coated or uncoated 24-well plates at 10,000 cells per well respectively, and incubated in defined media (3.75 ng/ml Selenium, 1 μg/ml Linoleic Acid, 10⁻⁷ M Dexamethazone, 500 μg/ml Fetuin, 10⁻⁹ M Insulin, 50 ng/ml Fibronectin, 500 ng/ml Biotin, 100 ng/ml Vitamin E, 100 μg/ml Heparin, 1% PSN/AbAm, 0.5% Gentamycin, 25% McCoy’s 5A and 75% DMEM) for 24 hours, or reseeded onto poly-L-lysine and fibronectin coated 15 cm dishes. After further cell number expansion, cells from the 15 cm dishes were detached using TrypLE™ Select (Invitrogen 12563-029) and centrifuged at 1,500 x g for 5 minutes. Supernatant was removed and the cells resuspended in growth media and counted. 100,000 cells were aliquotted into microcentrifuge tubes and centrifuged at 1,000 x g for 5 minutes, then supernatants aspirated. The microcentrifuge tubes were then stored at -80°C until further use. 0 ng/ml, 30 ng/ml, 150 ng/ml and 750 ng/ml Regenerating protein IIIα, 10 ng/ml Hepatocyte Growth Factor (HGF) and 1 ng/ml Transforming Growth Factor Beta 1 (TGFβ1) in defined media were added to
experimental wells. Media was replaced every 24 hours. 24, 48 and 72 hours later, media was aspirated and the cells washed with ice-cold PBS. The plates were frozen at -80°C for a maximum of 4 weeks until further use.

CyQUANT cell proliferation assay kit (C7026) was purchased from Invitrogen. On the day of the experiment, the concentrated cell-lysis stock buffer was diluted 20-fold in water, then the CyQUANT GR working solution was diluted 400-fold into the diluted cell-lysis buffer. The prepared CyQUANT solution was protected from light and used within a few hours of preparation.

On the day of the cell proliferation assay, the frozen experimental plate and the microcentrifuge tubes containing the 100,000 satellite or C2C12 cells were thawed at room temperature for 10 minutes. The cell pellets were resuspended in 1 mL of the prepared CyQUANT solution and cell standards were made corresponding to cell numbers ranging from 50 to 50,000 cells per well. 500 μl of CyQUANT solution was added to each well containing satellite or C2C12 cell samples, and the experimental plate was incubated at room temperature for 2-5 minutes in the dark. The fluorescence of the experimental plate samples were measured on a SpectraMax spectrophotometer at an excitation maximum of 480 nm and emission maximum of 520 nm.

**C2C12 Cell Creatine Kinase Differentiation Assay**

C2C12 cells were expanded and reseeded onto poly-L-lysine and fibronectin coated 48-well experimental plates at 5,000 cells per well as described in the previous section. Cells were incubated in pre-plate media for 24 hours. 0 ng/ml, 30 ng/ml, 150
ng/ml and 750 ng/ml Regenerating 3a protein in differentiation media (DMEM + 2% Horse Serum (HS) + 1% AbAm) were added to experimental wells. Media was changed every 24 hours. At 0, 24, 48 and 72 hours post-Regenerating3a supplementation, experimental plates were rinsed with ice-cold PBS, then coated with pre-chilled 0.05 M glycylglycine (pH 6.75) and stored at -80°C until use. On the day of the assay, plates were thawed at room temperature for 15 minutes, then freshly made creatine kinase assay buffer (20 mM creatine phosphate, 20 mM glucose, 10 mM magnesium-acetate, 1 mM Adenosine Diphosphate (ADP), 10 mM Adenosine Monophoshate (AMP), 0.4 mM thio-NAD), 10 mM Dithiothreitol (DTT), 0.5 units/mL hexokinase, 1 unit/mL Glucose-6-Phosphate Dehydrogenase, in 0.1 M glycylglycine, pH 6.75) was added to each well. The plate was incubated at room temperature for 30 minutes then measured on a SpectraMax spectrophotometer at OD405nm. Measurements were repeated two times.

**Determination of Myogenic Differentiation Characteristics**

Satellite cells and C2C12 cells were expanded and reseeded onto poly-L-lysine and fibronectin coated or uncoated 48-well experimental plates at 5,000 cells per well, respectively. Cells were incubated in pre-plate media for 24 hours. 0 ng/ml, 30 ng/ml, 150 ng/ml, 750 ng/ml Regenerating protein IIIα, 10 ng/ml IGF-II and 10 ng/ml HGF in differentiation media (DMEM + 2% Horse Serum (HS) + 1% AbAm) were added to experimental wells. Media was changed every 24 hours. At 0, 24, 48 and 72 hours post-treatment, experimental plates were processed and stained for myogenin (Santa Cruz Biotechnology) for determination of cell fusion and DAPI for quantification of
myonuclei according to immunocytochemistry methods described in previous chapters. The cells were imaged on a Leica DMI 4000 B microscope and Leica Application Suite software.

**Statistical Analysis**

Data were statistically analyzed using analysis of variance (ANOVA) and the results were presented as the mean ± the standard error of the mean. Results were designated as significant when $P \leq 0.05$ and tended to be significant if $P \leq 0.10$. 
Results

Satellite Cell Activation

Results of the activation assay to determine Reg IIIα’s potential to activate satellite cells were inconclusive. No significance was observed between satellite cells isolated from Reg IIIα or PBS-injected tibialis anterior muscles, and the positive and negative control treatments failed to show any effect upon satellite cell activation.

Cell Proliferation

No Reg I, IIIα or IIIγ effect was seen on C2C12 proliferation at varying Reg protein concentrations over time (Figure 4.1). Cell numbers increased over time as expected of proliferating cells, and HGF (positive control) was the only factor to exert a significant effect on cell number. Results of the proliferation assay to determine Reg IIIα’s potential to increase satellite cell proliferation were inconclusive. The positive and negative controls failed to show any effect upon satellite cell proliferation and no obvious statistical significance was observed with increasing Reg IIIα concentrations.

Measurement of C2C12 Cell Differentiation by a Creatine Kinase Assay

No statistical significance was found between C2C12 cells treated with varying Reg I, IIIα or IIIγ protein concentrations over time (Figure 4.2). Absorbance at 405nm steadily increased over time for all treatments, as was expected of differentiating cells.
Measurement of C2C12 Cell Differentiation by Myogenic Differentiation Characteristics

The results of the measurement of cell differentiation characteristics to determine Reg IIIα’s potential to stimulate C2C12 cell differentiation were inconclusive. The positive and negative control treatments failed to show an effect on average nuclei quantity or average percent fused myonuclei, and no statistical significance was found between any of the varying Reg IIIα protein concentrations (Figure 4.3).

Measurement of Satellite Cell Differentiation by Total Nuclei Number and Fusion

The results of the measurement of cell differentiation characteristics to determine Reg IIIα’s potential to stimulate satellite cell differentiation were inconclusive. The positive and negative control treatments failed to show an effect on average nuclei quantity or average percent fused myonuclei, and no statistical significance was found between any of the varying Reg IIIα protein concentrations (Figure 4.4).
Figure 4.1. Proliferation Assays on C2C12 Cells Over Time. Proliferation assays on C2C12 cells incubated with varying concentrations of (A) Reg I, (B) Reg IIIα or (C) Reg IIIγ over time. Samples were measured as average cell number ± SEM ($P < 0.05$).
Figure 4.2. Measurement of Muscle Differentiation by a Creatine Kinase Assay. C2C12 cells were incubated with varying concentrations of (A) Reg I, (B) Reg IIIα or (C) Reg IIIγ over time. Creatine kinase activity was measured at 405 nm and results presented as mean $A_{405\text{nm}} \pm \text{SEM}$ ($P < 0.05$).
Figure 4.3. Examination of Myogenic Differentiation Characteristics in C2C12 Cells. Measurement of (A) average nuclei number and (B) average % fused myonuclei relative to varying concentrations of Reg IIIα protein over time in C2C12 cells. 10 ng/ml HGF and 1 ng/ml TGFb1 were included as positive and negative controls, respectively.
Figure 4.4. Examination of Myogenic Differentiation Characteristics in Isolated Mice Satellite Cells. Measurement of (A) average nuclei number and (B) average % fused myonuclei relative to varying concentrations of Reg IIIα protein over time in mouse satellite cells. 10 ng/ml HGF and 1 ng/ml TGFβ1 were included as positive and negative controls, respectively.
Discussion

The regenerative capacity of damaged skeletal muscle is coordinated by a variety of factors that stimulate or inhibit satellite cell activation, proliferation and differentiation to ultimately promote or hinder myogenesis. As was found in the previous chapters, multiple Reg protein isoforms are found in regenerating skeletal muscle and some evidence have been uncovered that suggest Reg IIIα protein’s inhibitory function on myogenesis. Therefore it was necessary to determine at which point in the regenerative process Reg proteins are acting upon satellite cells (i.e. activation, proliferation, differentiation) and whether an isoform variance could be found.

The satellite cell activation assay to discern Reg IIIα’s ability to activation satellite cells from a quiescent state was inconclusive. Known positive and negative activators of satellite cells failed to show any effect upon satellite cell activation, and therefore no inferences could be made from the treatment groups. Known positive controls for C2C12 proliferation showed a significant increase in total cell number, verifying C2C12’s ability to be stimulated by exogenous factors. However, although cell numbers increased over time as expected of proliferating cells, no significant changes in cell number was found between the Reg protein concentrations for any of the Reg protein isoforms. While this data suggests that none of the three Reg protein isoforms we tested affects myogenic stem cell proliferation, satellite cell proliferation assays do not correlate with this data, as the positive control failed to increase satellite cell proliferation. Due to known differences between C2C12 and satellite cells, one should be cautious in inferring that C2C12 cells function like satellite cells in vivo (Grabowska et al. 2011, Cornelison
and Wold 1997, Cornelison 2008) and therefore no conclusions can be made about Reg protein influence on satellite cell proliferation. Since no significant proliferative effect was found, we then performed a creatine kinase assay to determine whether the addition of exogenous Reg proteins promote cell differentiation. Although creatine kinase activity steadily increased which indicates cells were differentiating over time, no statistical significance was found between C2C12 cells treated with varying Reg I, III\(\alpha\) or III\(\gamma\) protein concentrations. While this data agrees with subsequent experiments where the degree of C2C12 or satellite cell differentiation was not statistically significant across varying Reg III\(\alpha\) protein concentrations according to nuclei numbers and cell fusion characteristics, positive and negative controls failed to promote or inhibit cell differentiation and therefore no inferences can be made from these experiments.

Future research should focus on resolving the \textit{in vitro} issues to allow the determination of conclusive results regarding Reg protein effect on satellite cell activation, proliferation and differentiation.
CHAPTER 5
SUMMARY AND FUTURE AIMS

Skeletal muscle is a dynamic tissue utilized in essential physiological functions such as locomotion, posture, respiration and thermoregulation (Powers and Howley 2004, Sherwood 2010). Damage to this system, therefore, severely limits normal function and the body relies on skeletal muscle’s tremendous capabilities of regeneration upon significant trauma. Skeletal muscle regeneration is fully dependent upon the coordinated influences of intrinsic and extrinsic factors that manipulate myogenesis by stimulating the activation, proliferation and differentiation of resident myogenic stem cells, the satellite cell. Satellite cells were first identified by Mauro in 1961 (Mauro 1961) and their self-renewal properties and ability to divide and differentiate into myogenic cells were established through subsequent research. Research in the following years quickly identified many regulatory factors that influence satellite cell activity, ultimately promoting or inhibiting skeletal muscle regeneration. A landmark study by Allen et al. identified HGF as a satellite cell activator, initiating a flurry of studies to further deduce the role of HGF in skeletal muscle regeneration (Allen et al. 1995). Further research showed that HGF also promotes satellite cell proliferation and inhibits differentiation (Miller et al. 2000). IGF-I and –II have both been found to increase both satellite cell proliferation and differentiation (Allen and Boxhorn 1989, Florini et al. 1996, Doumit et al. 1993), and the same has been observed for VEGF (Arsic et al. 2004, Christov et al. 2007). Myostatin, on the other hand, decreased both satellite cell proliferation and
differentiation (Joulia et al. 2003, McCroskery et al. 2003). While these are only a few of the plethora of factors that have been discovered to influence skeletal muscle regeneration, it is safe to conclude that many are yet to be found. However, even with the coordination of numerous factors, skeletal muscle regeneration can be compromised and the resulting consequences are great. Muscular dystrophy and sarcopenia are some of the outcomes of decreased myogenesis, and billions of dollars are spent annually in related health-care costs (Janssen et al. 2004). Mitogenic factors that promote the regenerative and self-renewal function of satellite cells have been found, but the therapeutic application of these factors are still being researched. Therefore, our goal was to determine whether a novel protein, the Regenerating protein family, is present in damaged skeletal muscle and could potentially promote myogenesis and possibly be a candidate for therapeutic purposes in the future.

The Regenerating proteins have a high sequence identity with the calcium-dependent lectin superfamily, a family of extracellular calcium-dependent sugar binding proteins. However, the Regenerating protein family members appear to be lacking the sugar-binding action and therefore it may no longer be needed for their function (Drickamer 1993, Closa et al. 2007). The Reg proteins were first found in human pancreatic stones by De Caro et al. in 1979 and since then multiple classes have been identified in a variety of damaged and regenerating tissues (De Caro et al. 1979). So far, four classes of the Regenerating protein family have been found, with the potential for the identification of additional classes or subclasses. Members of the Regenerating protein family are significantly upregulated at the onset of trauma or inflammation in the
pancreas (De Caro et al. 1979, Keim et al. 1984, Terazono et al. 1988, Zhang et al. 2004, Viterbo et al. 2008), gastrointestinal tract (Hartupee et al. 2001, Watanabe et al. 1994a, Kawanami et al. 1997, Kadowaki et al. 2002), liver (Lasserre et al. 1992), neural cells (Ozturk et al. 1989, Livesey et al. 1997b, Livesey et al. 1997a, Duplan et al. 2001) and other tissues (Kiji et al. 2005, Watanabe et al. 2008, Mauro et al. 2008, Sarles et al. 2005, Sommerburg et al. 2010). These findings suggest that Reg proteins are essential in the inflammatory or regenerative process of these tissues, and are further supported by studies in which the inhibition of these Reg proteins have led to increased inflammation, poor regeneration and decreased survival (Zhang et al. 2004, Viterbo et al. 2008, Lieu et al. 2006, Livesey et al. 1997b, Livesey et al. 1997a, Nishimune et al. 2000). In essence, these findings provide evidence that Reg proteins have a remarkable mitogenic, anti-apoptotic and anti-inflammatory function in numerous damaged or regenerating tissues necessary for normal progression of regeneration. As skeletal muscle is also able to regenerate itself at a surprisingly rapid rate (Martinez et al. 2010), it seems highly likely that Reg proteins function to promote myogenesis in skeletal muscle regeneration.

To determine whether Reg proteins participate in skeletal muscle regeneration, we performed gene expression analysis of undamaged, degenerating and regenerating tibialis anterior muscles as well as isolated mice satellite cells. Our results provide evidence that Reg I, II, IIIα, IIIγ and IV protein isoforms are present during skeletal muscle regeneration, with possible contributions to normal progression of myogenesis. Not only are multiple Reg protein classes expressed in regenerating skeletal muscle in vivo, but satellite cells themselves also significantly express Reg I, IIIα and IIIγ. Clearly, Reg I,
IIIα and IIIγ are the predominant candidates as participants in skeletal muscle regeneration. However, as Reg IIIγ mRNA abundance was significantly lower than Reg I or Reg IIIα, this infers that Reg IIIγ may not play a large a role as the other two isoforms. Nevertheless, as multiple isoforms have been found to be highly expressed within the same tissue (Zhang et al. 2004, Viterbo et al. 2008, Ozturk et al. 1989, Livesey et al. 1997b, Livesey et al. 1997a), it is possible that multiple isoforms may be working in conjunction with one another.

EXTL3 was identified as the Reg receptor protein by Kobayashi et al. in 2000 through the screening of the rat islet cDNA expression library for a rat Reg binding protein (Kobayashi et al. 2000). They found a protein with a C-terminal sequence homology of 40-50% to members of the multiple exostoses family (EXT), glycosyltransferases that elongate heparan sulfate chains. Unlike the other members of the EXT family, EXTL3 contains a membrane-spanning domain that is hypothesized to anchor the receptor to the cell membrane and facilitate Reg proteins binding. They further confirmed EXTL3 as the Reg receptor when they observed significantly increased BrdU incorporation in cells overexpressing EXLT3 with the addition of Reg proteins. Further research established EXTL3 as the Reg receptor when Reg IIIα’s ability to induce cell proliferation was inhibited in EXTL3-silenced keratinocytes (Lai et al. 2012), and overexpression of EXTL3 caused a significant increase in neuronal outgrowth with the administration of Reg Iα (Acquatella-Tran Van Ba et al. 2012). As EXTL3 functions as the Reg receptor across multiple tissues, it seemed highly likely that Reg proteins would act through EXTL3 in skeletal muscle as well.
Indeed, EXTL3 is expressed in both marcaine-damaged tibialis anterior muscles \textit{in vivo} and isolated satellite cells in culture. While no statistical significance was found over the time course, there seems to be a higher trend of expression during times of early regeneration \textit{in vivo} where newly regenerated myofibers with centrally located nuclei are abundant. Taken together with the Reg gene expression profiles, EXTL3 may be working in conjunction with multiple Reg protein classes at different times over myogenesis. Perhaps the upregulation of Reg I or Reg III$\gamma$ by muscle trauma promotes early stimulation of cell proliferation and macrophage chemotaxis, stimulates the expression of EXTL3 during the late regenerative stages, where then other less abundant Reg protein isoforms are expressed and promote fiber maturation. As there are still significant gaps in our knowledge of how EXTL3 facilitates the Reg protein effect, future research needs to be focused on uncovering the signaling pathways.

Given that endogenous Reg proteins have an apparent mitogenic, anti-apoptotic and anti-inflammatory function in damaged tissues, numerous studies were conducted to reveal the direct effects of adding exogenous Reg proteins to tissue regeneration. A radical study by Watanabe et al. showed that intraperitoneal injections of Reg I to surgically-induced diabetic rats alleviated diabetes in two months, while the non-Reg I treated animals showed severe degranulation of $\beta$-cells and significantly reduced numbers of insulin-positive cells (Watanabe et al. 1994a). Similar studies in the liver and the nervous system confirmed that the overabundance of Reg proteins accelerate regeneration (Simon et al. 2003, Livesey et al. 1997b). As significant expression of multiple Reg protein classes were found in regenerating skeletal muscle, we needed to
determine whether addition of exogenous Reg proteins would accelerate skeletal muscle regeneration.

Interestingly, our results suggest that the addition of Reg IIIα to damaged tibialis anterior muscles inhibited myogenesis. Although no statistical significance was found, the gene expression levels of EXTL3, Pax7 and Myogenin all tended to be lower in the Reg-treated muscles than the PBS-treated samples at certain time points, indicating a general decline in myogenesis. Muscle morphometric analysis correlated with this theory, in that both fiber number and fiber diameter were significantly lower in the Reg-treated muscle sections. One hypothesis is that the early peak in intramuscular Reg proteins stimulated the negative feedback loop and inhibited EXTL3 expression early in the myogenesis process, thereby delaying or dampening normal progress. Although controversial, some studies have found a correlation between the use of anti-inflammatory medication and hindrance of muscle regeneration after muscle injury (Almekinders and Gilbert 1986, Almekinders 1999). One study found that although the anti-inflammatory treated group showed significant improvements at earlier time points compared to the non-treated group, long-term functionality of the muscle was compromised (Mishra et al. 1995). They concluded that the suppression of edema and cell lysis by anti-inflammatory medication prevents continuous injury signals to be released, thereby promoting accelerated healing at the injury site. However, the prolonged inflammatory process is also necessary for long-term “cellular remodeling” and therefore a suppression of that process compromises the long-term functionality of muscle. As Reg proteins also have an anti-inflammatory and anti-apoptotic function, it is
possible that the addition of a large amount of protein may have inhibited the inflammatory process, thereby obstructing myogenesis. Studies focusing on the inflammatory response with the addition of Reg proteins in damaged skeletal muscle may help to reveal the answer.

To determine the direct effect of Reg protein on myogenic stem cell activity, Reg proteins were added to isolated mice satellite cells and C2C12 immortalized myoblast cells. Most of the results from these studies were inconclusive due to the failure of known positive or negative controls to impart an effect. However, HGF imparted a significant effect on C2C12 cell proliferation and therefore inferences could be made within this study. No significant Reg protein concentration effect was found over time for any of the Reg protein isoforms we studied (Reg I, Reg IIIα, Reg IIIγ). However, due to the consistent failure of the in vitro studies, we tentatively suggest that Reg proteins do not stimulate cell proliferation in C2C12 cells.

In conclusion, our research showed that regenerating skeletal muscles express multiple classes and subclasses of Reg protein and the Reg receptor (EXTL3), but further studies are needed to clearly determine the predominant Reg protein isoform of skeletal muscle. Despite some conflicting results in the supplementation study, the overall picture points to an anti-myogenetic function with an overabundance of Reg IIIα protein, possibly due to a negative feedback of the EXTL3 receptor or from the excess suppression of inflammation. Further research should examine the other Reg protein isoforms as pro-myogenetic factors, and thus possibly uncover an important factor that may be utilized for therapeutic purposes in the future.
### Table 5.1. Forward and Reverse Rat Primer Sequences and Annealing Temperatures for Real Time PCR Analyses.

1Regenerating-islet derived (Reg)

<table>
<thead>
<tr>
<th>Gene&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Forward</th>
<th>Reverse</th>
<th>°C</th>
<th>Length (bp)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Reg I&lt;sub&gt;α&lt;/sub&gt;</td>
<td>CCAGAAGGTTCCAATGCCTA</td>
<td>CAGAGAGGCCAGAAAGTTGC</td>
<td>60.0-64.0</td>
<td>153</td>
<td>Pubmed/Invitrogen</td>
</tr>
<tr>
<td>Reg III&lt;sub&gt;α&lt;/sub&gt;</td>
<td>GGTGACAGGCAGAGTGAAACA</td>
<td>GATGGTCTCCCCACTTCAGA</td>
<td>58.5</td>
<td>212</td>
<td>Pubmed/Invitrogen</td>
</tr>
<tr>
<td>Reg IIIβ</td>
<td>ACTCCATGACCCCACCCTTTG</td>
<td>TGACAAGCTGCCACAGAATC</td>
<td>58.0-66.0</td>
<td>136</td>
<td>Pubmed/Invitrogen</td>
</tr>
<tr>
<td>Reg IIIγ</td>
<td>AACAGAGGTGGATGGGAGTG</td>
<td>TCAGAAATCCTGAGGCTCGT</td>
<td>58.0-66.0</td>
<td>121</td>
<td>Pubmed/Invitrogen</td>
</tr>
<tr>
<td>Reg IV</td>
<td>CTGCATGACCCACAGAAGAA</td>
<td>TGCATCCCATTGTGTTCCAA</td>
<td>56.5-58.0</td>
<td>154</td>
<td>Pubmed/Invitrogen</td>
</tr>
</tbody>
</table>
**Rat RegIα mRNA**
NM_012641    776 bp

1  cccccccccaa cagacccctg tctcagcctg cagagattgt tgacttgcat cctaagcaga
61  agacagtgcct gacaagctca taagactcgg caaataaact atctctgcttt catgctgtat
121  ggtctttctct ccaagcccaag gccagaggcc gagaagagat ctaacatctg ccagagatcc
181  tgtgctagaa ggttcccaatg cctactacag cagactttatct cactctctgct cactcaggttt
241  atctctgggtc gagagcactc ttttttgcca gaacagataat tcagagttct tggctgctgtt
301  gctcagcagcg cggagacctgc cactctctggct tgtctgttac tactttcatgg aagaccattt
361  tggcaaatgc tgtgattggcc tccatgatcc caaaaaataat cgccgctggc agtcatccttga
421  tgggtctcttg tttctctaca aatcctgtaga ccttaacactt ccaatcgtgg
481  ctactggtga ctctgtgacat caaactcagat caaagtgcatttgagagata acagtgttga
541  tgcctccatca tcactttgctc gcagatctagt cctctgaaat ctagtcagctt cctttttctc
601  acagagccag cacaagaaaa actatggagat caaagtgaa acgaccactgct cttacaaag
661  caagactcag cccccccccaa cagactctagg cgcctgctgg cgcctgctgg
721  cagttccatt cttctgaaat ttttttttttta ccccccccct ccttactaag aaaaaaaa

**Rat RegIIα mRNA**
NM_172077     907 bp

1  atccccagatc actgcaaggg acagacctgc aaacgcagaga tggggctag taaagtgatat
61  cccctgagacac ctaggccaagag cagagagggccctctttggc cttggctcttg tggctacac
121  tctctggtgcc cggcctggctc tttctttgaaga gaaagatagtc gctcctcggc cactctgtttc
181  aactgtgctct gcgggctgcct acatactcctg tttctctttc agtgccagag tcagactgttca
241  cagagaacag cagccctctcag tttctctttc aacccagatgc gctcctgttc ttaagtttct
301  tactgtcata cctggctcag cagactctaga cctctgttagt cagactctgctg cctgctgtgtc
361  aagacacatc cagacacacct cttttcgaga gcagtgcgctg ctctgtgtct cttgctgcag
421  tccctggtgac cagccagaggt gaccaacaag ccacagacacac gattggttct ggtgtagctca
481  aacatggtggacta aaaaaacucca taaggtgacttg cggctgctgg gctcctgtatc cctgtgtttc
541  tatctctcact gcggatgggtgt ctcctctctcact gttaactcgg ctagcactgtc
601  aacactcagcttg agggtgggtgct cccctcgttct gttggtcagtct cttctctgtc gctcctgtttc
661  gcttcacagct gcttcacagct cttggtgcttg ccaggcctgtg cttggctgttg cttggctgttga
721  caagggagttggactagctc ctagacctgg gttggtcagtct cgctgttggctgttg cttggctgttga
781  tcgtgtttc ccttctcctcc cctgtttttctct cccctctcttctgctgtttc gttggtgattag
841  tttctcctcct cgcactctggagataataa atatctctct ttttttttttt aaaaaaaa
901  aaaaaaaaa
Table 5.2. Nucleotide Sequence of Rat Reg Iα, IIIα, IIIβ, IIIγ and IV.
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