CALPAINS IN SKELETAL MUSCLE: GENERATION OF AN INHIBITORY OVEREXPRESSION SYSTEM AND ANALYSIS OF DEGRADATION IN SIMULATED MICROGRAVITY

by

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DEDICATION

To my parents, Linda Grill and Dana Fisher,

for their never ending love and support

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ABSTRACT

Muscle atrophy is a serious side effect seen with extended time in space. Proteolytic degradation of specific muscle proteins leads to smaller, weaker muscles that are structurally more susceptible to damage. Calpains are proteases that specifically degrade target proteins of the myofibril, and have been implicated in many types of muscle atrophy. Calpain activity is regulated by a combination of activation by calcium and inhibition by calpastatin, its endogenous inhibitor. This dissertation describes the generation of a skeletal muscle-specific, doxycycline (Dox) controlled, calpastatin over-expression system in transgenic mice to regulate calpain activity. A dual construct system, the transactivator line utilizes an optimized tet-on system and a modified muscle creatine kinase promoter to create muscle specific expression of a tet transactivator. The second transgenic line, consisting of a bi-directional promoter centered on a tet responder element controlling both a luciferase reporter gene and a tagged calpastatin, is transcriptionally silent until activated by a dox induced transactivator molecule. Compound hemizygous mice showed high level, Dox dependent, muscle-specific overexpression of luciferase and transgenic calpastatin, demonstrating the effectiveness and flexibility of the tet-on system to provide a tightly regulated overexpression system in adult skeletal muscle. Consistent overexpression of calpastatin was hard to maintain, however, and not all of the proposed experiments could be achieved. Additional studies compared the degradation of hindlimb suspended mouse muscle proteins (weightlessness model) to those of ground control muscle proteins briefly incubated in Ca^{2+} (to initiate

calpain degradation). Four proteins known as targets for calpain degradation were selected for analysis. Degradation responses of myofibrillar proteins titin, nebulin, and troponin T in hindlimb suspension clearly mimicked that seen with the calcium incubations. The cytoskeletal calpain target protein, desmin, however did not respond the same to both treatments showing moderate degradation with calcium and no degradation with hindlimb suspension. These data suggest that myofibrillar calpain target proteins, but not necessarily cytoskeletal proteins, are rapidly targeted for degradation in hindlimb suspension in a manner similar to that induced by calcium, implicating calpain as a mediator of this degradation.

CHAPTER I

Introduction

Muscle Atrophy in Weightlessness

Extended time in space is known to cause several physiological problems. Muscle wasting is a prominent detriment with both short and long term effects. During extended space travel, muscles demonstrate a reduced functional capacity and increased fatigue (Greenleaf et al. 1989). These deleterious changes correspond with overall loss of muscle mass (Ilyina-Kakueva et al., 1976 and reviewed in Fitts et al., 2000) and conversion of muscle fiber type and are most dramatic in predominantly weight-bearing slow-twitch muscles like the soleus (Edgerton and Roy, 1996). In these muscles, atrophy is demonstrated by a 20-50 percent decrease in muscle fiber cross sectional area accompanied by a significant degradation of contractile versus cytoskeletal proteins (Fitts et al., 2000). Since these changes are attributed to reduced contractile activity, unloaded contractions, and a shortened working range, it is not surprising that similar muscle atrophy is also seen in disuse, denervation and several muscular disease states (Spencer et al., 1995, Widrick et al., 1997, Dupont-Versteegden et al., 1998, and Jankala et al., 1997). Muscle damage is an additional concern in these cases as the structural integrity of the muscles is decreased. This is problematic for astronauts when returning to the 1-g environment of Earth where weakened muscles sustain additional damage beyond the atrophy incurred during weightlessness (Riley et al., 1992).

Normal muscle homeostasis involves a complex balance of muscle synthesis and muscle degradation. Muscle atrophy occurs when there is an increase in degradation, a

decrease in synthesis, or a combination of the two. The specific loss of contractile proteins compared to cytoskeletal proteins in spaceflight, suggest that an increase in muscle degradation is the predominant cause of muscle loss. The two major proteolytic systems important for muscle degradation are the calpain proteases and the proteasome, or ubiquitin proteases (Tawa et al., 1997 and Goll et al., 2003). While the proteasome is well known for targeting monomeric proteins and peptide fragments (Tanaka, 1998), the calpains are known to cleave specific myofibrillar proteins of intact myofibrils (Goll et al, 1999 and 2003). These data suggest that calpain acts as the early rate-limiting step in muscle degradation and is a primary target for studies to prevent weightlessness induced muscle atrophy.

Calpains and Calpastatin

Calpains are calcium-dependent cysteine proteases that are found in every type of mammalian cell. Homologous proteins have also been found in chickens (Sorimachi et al., 1995 and Ishiura et al., 1978), *Caenorhabditis elegans* (Barnes and Hodgkin, 1996) *Drosophila melanogaster* (Theopold et al., 1995) and even a transmembrane form in plants (Lid et al., 2002). To date, there are fourteen identified mammalian calpains (Goll et al., 2003). These include the most common μ- and m-calpains (also known as calpain1 and calpain2) that are ubiquitously expressed, and several others which range in their expression and may be limited to only one or a few cell types. Most of these additional calpains have only been identified as DNA sequences or localized mRNAs and are not

well characterized yet. An exception to this is calpain3 or skeletal muscle specific (skm) calpain, who's mRNA is found at concentrations ten fold higher than the μ- and m-calpains in skeletal muscle (Sorimachi et al., 1993). Deficiencies in calpain3 have been shown to result in Type 2A Limb Girdle Muscular Dystrophy (Richard et al., 1995) and have been shown to perturb the IκBα/NF-κB pathway (Baghdiguian et al., 1999). The nuclear localization signal and cellular localization of calpain3 (Anderson et al., 1998 and Baghdiguian et al., 1999) as well as the lack of evidence that calpain3 has protease activity, suggest that tissue specific calpains may have additional or varied functions from their ubiquitous counterparts.

The μ- and m-calpains are the most well characterized calpains and are expressed in all cell types studied. These ubiquitous calpains are named for their relative micromolar ranging from 5-50μm, and millimolar ranging from 250-1000μm, concentrations of calcium required for activity (Cong et al., 1989). The values range from study to study, however demonstrated reduction of calcium requirement for activity by autolysis has been shown for each, bringing μ-calpain calcium activity requirement to 0.5-5μm (Saido et al., 1992) and m-calpain requirements to 50-200μm (Elce et al., 1997). Because these shortened forms of calpain have not been detected in normal tissue extracts (Edmunds et al., 1991), the primary autolysis is thought to generate a highly active calpain that is structurally less stable thereby restricting proteolysis by rapid degradation.

Although μ- and m-calpains are ubiquitous in nature and have roles in many different cellular processes, proteolysis by calpains is limited to a subset of molecules that are cleaved to specific, reproducible fragments. Cleavage of some molecules (such as fodrin and casein) is so consistent that their breakdown products are commonly used to assay calpain activity of different cell lysates (Saido et al., 1993; Thompson et al., 2000). This cleavage of substrates at specific inter-domain boundaries suggests modulation of function of targeted substrates instead of pure proteolysis and has earned calpain the title "biomodulator" (Suzuki and Sorimachi, 1998). Proteolysis by calpains is also restrictive, with different, limited target proteins specific to each cell type. In general calpains are associated with subcellular organelles. For example, calpains are commonly associated with plasma membranes, vesicles, and cytoskeletal components. They are also associated with cell type specific organelles such as myofibrils in muscle cells. Perturbation of calpain activity both *in vitro* and *in vivo* demonstrates its critical role in processes such as cell migration and attachments (Glading et al., 2002 and Dourdin et al., 2001), kinase regulation (Kishimoto et al., 1989), integrin signaling (Kulkarni et al., 2002 and Pfaff et al., 1999), cell division (Choi et al., 1997 and Mellgren et al. 1996), muscle cell fusion (Barnoy et al., 2000; Balcerzak et al., 1995; Dourdin et al., 1999; Schollmeyer 1986a), apoptosis (Lu et al., 2002 and Blomgren et al., 1999), and many other processes necessary for the development and survival of an organism. Recent studies have even linked calpains to regulation of caspases through complicated and not yet well-understood cross talk (Barnoy and Kosower, 2003 and Neumar et al., 2003).

Regulation of calpains is a complicated process that includes intracellular calcium concentration, autolytic activation, and cellular localization. The μ- and m-calpains are very similar in structure with each consisting of 2 subunits. The smaller 28kD subunit is common to both calpains, while the larger 80kD subunits are generated from different genes. The large subunits consist of 4 domains each when determined by amino acid sequence. The N-terminal domain is known as domain I and can undergo autolysis (Zimmerman and Schlaepfer, 1991). Domain II is the active site and contains the cysteine, histidine, and asparagine that make up the catalytic triad of the proteolytic region characteristic of this and other cysteine proteases (Arthur et al., 1995). Domain III and IV both contain calcium binding sites, but it is domain IV that has moderate calmodulin-like homology with predicted EF-hand calcium binding sites (Emori et al., 1986a and 1986b). This region of the large subunit is predicted to play a role in dimerization with the small 28kD subunit. The small subunit is also separated into functional domains known as V and VI. Domain V is very glycine rich with mid range hydrophobicity that originally suggested interactions with membranes (Imajoh et al., 1987). However, further amino acid sequence analysis showed a less ordered structure more likely to be involved in tethering calpain to other molecules (Goll et al., 2003). This domain, like the amino terminus of the large subunit, is thought to be involved in autolysis (McClelland et al., 1989). Domain VI is the calmodulin-like domain and contains several EF-hand calcium binding sites that are necessary for binding to the large subunit (Blanchard et al., 1997 and Lin et al., 1997).

Recent crystallographic analysis of m-calpain in the absence of calcium has revealed important structural data that differentially distinguishes the domain boundaries and provides new insights into their function (Strobl et al., 2000 and Hosfield et al., 1999). One such difference is the demonstration of five EF-hand calcium-binding sites in domain VI of the small subunit (Blanchard et al., 1997 and Lin et al., 1997) and corresponding sites in IV of the 80kD subunit (Hayfield et al., 1999). Identification of these sites classifies calpain as a penta EF-hand family member of proteins known to dimerize and have interactions with membranes (Maki et al., 1997). Crystallography studies of m-calpain have also shown that domain III contains highly conserved acidic residues that form salt bridges with corresponding lysine residues in domains II and III (Strobl et al., 2000 and Hosfield et al., 1999). These connections are kinetically disrupted by the addition of calcium allowing for calcium dependent conformational changes of the calpain molecule. Because the crystal studies of m-calpain were done in the absence of calcium, distance measurements of the amino acids that make up the catalytic triad also demonstrated that a conformational change of some kind is necessary to rotate the distanced cysteine residue into closer proximity to the histidine and asparagine for enzymatic activation (Arthur et al., 1995, Strobl et al., 2000 and Hosfield et al., 1999). Taken together these data suggest that a calcium induced electrostatic switch allows for a conformational change in domain II that assembles the catalytic triad, essentially activating the protease (Hosfield et al., 1999).

Calpain molecules also undergo a calcium dependent proteolytic autolysis of amino terminal ends of both μ- and m-calpains as well as the amino terminus of the small subunit (Suzuki et al., 1981, Cong et al., 1989, and McClelland et al., 1989). The calcium requirement for this autolysis is nearly the same as the requirement for proteolytic activity (Kuboki et al., 1987, Cong et al., 1989, and Zimmerman and Schlaepffer, 1991). This correlation, and evidence from other cysteine proteases suggested that calpain is normally found in an inactive proenzyme state that becomes active upon autolysis that removes a blocking polypeptide to provide accessibility to the active site (Mellgren 1987 and Suzuki et al., 1987). However, several recent studies have demonstrated proteolytic activity of both the unautolyzed μ- and m-calpains (reviewed in Goll et al., 2003). This opposition to the proenzyme theory was also corroborated by the crystallography analysis of m-calpain that shows the alpha helical N-terminus of the 80kD subunit does not physically block the active site (Strobl et al., 2000 and Hosfield et al., 1999). Analysis of the 28kD subunit also does not support the proenzyme theory for calpain. However, as mentioned earlier, autolysis of calpains does decrease the required calcium concentration for activation of the two ubiquitous types. Therefore, autolysis may play another role, possibly one that destabilizes the calpain proteases themselves. Studies using trypsin and chymotrypsin on full and autolyzed μ- and m-calpains demonstrate a corresponding conformational change of the molecules with autolysis that make the calpains much more susceptible to these broad range proteolytic degradations (Moldoveanu et al., 2002 and Thompson et al., 2003).

Calpains are also regulated by other molecules, most notably their ubiquitously expressed inhibitor, calpastatin. Calpastatin is a calpain specific inhibitor and does not inhibit or affect any other cysteine proteases. There are several forms of calpastatin due to alternative transcriptional start sites, alternative splice variants, and post-translational modifications. Even with all these differences, all calpastatins have in common one or more of the calpastatin inhibitory domains. Each of the inhibitory domains contains three highly homologous, systematically spaced subdomains known as A, B, and C. The central of these domains, B, is essential for calpain protease inhibition (Kawasaki et al., 1989) and interacts directly with the catalytically active Domain II of calpain (Todd et al., 2003), whereas the A and C domains block calpains ability to bind to cell membranes (Kawasaki et al., 1993). Further analysis of these two subdomains demonstrates that A binds specifically to domain IV of the calpain large subunit and C binds to domain VI of the small subunit (Takano et al., 1995 and Konno et al., 1997). Since binding prevents cell membrane interactions, this data reinforces the prediction that domains IV and VI of calpain have interactions with the membrane (Kawasaki et al., 1993).

There are currently four different classified homologs of calpastatin arising through the use of alternative start sites, with four additional variants generated by alternative splicing (reviewed in Goll et al., 2003). The most common, type III or prototypical, calpastatin isoform consists of all four of the aforementioned inhibitory domains and an amino terminal L-domain. Each inhibitory domain binds to and inhibits the proteolytic activity of calpains, thereby making this calpastatin isoform capable of

inhibiting four molecules of calpain (Maki et al., 1987). The functional role of the L-domain, however, is less well characterized and has not been shown to act directly on calpains. Having no homology to any other known gene, studies in cardiac myocytes have demonstrated a stabilizing role for the L domain in the regulation of L-type calcium channels (Hao et al., 2000 and Kameyama 1998). This maintenance of calcium channel activity was not only found to be reversible, but it could not be mimicked by synthetic calpain inhibitors (Seydl et al., 1995). Although it is not known whether the L domain interacts directly with the channel, this data suggests an L domain function that is independent of calpastatin's well-characterized inhibition of calpain proteolytic activity (Kameyama et al., 1998). Interestingly, there are three alternatively spliced variants of the prototypical calpastatin, all three of which modify or eliminate the L domain. One well-described form identified in human erythrocytes (Takano and Murachi, 1982) completely lacks both the L domain and the first inhibitory domain (Lee et al., 1992). Other splice variants remove only one or two exons from the L domain (Geesink et al., 1998 and Lee et al., 1992). Three additional types of calpastatins with different amino terminal sequences have thus far been identified. Type I and II calpastatins initiate translation at more 5' exons and each encode for their own XL domain as well as the L domain and all four inhibitory domains. Although not yet analyzed in protein form, type I transcripts have been identified in murine brain, liver, and testis (Takano et al., 2000). Type II calpastatin, first identified in bovine heart, has been purified and shown to contain 3 protein kinase A sites in its XL domain (Cong et al., 1998). Transcripts of this type II isoform have also been detected in murine skeletal and cardiac muscle and to a

lesser extent in other murine tissues (Takano et al., 2000). A fourth type of calpastatin identified in mouse testis initiates translation at a unique exon, 14t, located between the original exon14 and 15. This isoform contains part of inhibitory domain II and all of domains III and IV (Takano et al., 2000).

Inhibition of calpain activity by calpastatin is extremely important for specific regulation of calpain activity in many different cellular processes in several different cell types. Many studies have inhibited calpain function by injecting or transfecting full-length (Temm-Grove et al., 1999) or single inhibitory domains of calpastatin (Emori et al., 1988; Huang and Forsberg, 1998). It is well accepted from these studies that elevated levels of calpastatin will block calpain activity. However, studies looking at levels of calpain and calpastatin demonstrate that there is generally enough calpain to degrade all of its target molecules and enough calpastatin to prevent all of the aforementioned degradation (Thompson and Goll 2000). This demonstrates the critical role of tight regulation between these two molecules. In addition to the calcium activation and various calpastatin homologs, intracellular localization is thought to be intimately involved with activation and inhibition of calpain activity.

Calpains and calpastatin are often unevenly distributed within the cell. In both skeletal and cardiac muscle where most studies have been performed, calpains and calpastatin are generally localized to the Z disk of the myofibril (Goll et al., 1992 and Kumamoto et al., 1992). Calpain and calpastatin have even been found to co-precipitate

in extracts from L_8 myoblast cell cultures (Barnoy et al., 1999). Studies of dividing cells however, including L_8 myoblasts, place the localization of m-calpain at the plasma membrane (Schollmeyer, 1986b). This membrane localization was also reported during myoblast fusion (Schollmeyer, 1986a) and during mitosis in cultured PtK_1 cells (Schollmeyer, 1988) and platelets (Saido et al., 1993). This redistribution of calpain from a more centrally localized distribution to predominantly membrane localized was recently mimicked with calcium ionophore stimulation of COS 7 and LCLC203H cells (Gil-Parrado et al., 2003). These studies suggest a connection between activation of calpain and its translocation to the plasma membrane.

Since calpains are critical to so many cellular processes from early development to programmed cell death, it is sometimes difficult to study one specific aspect of calpain function. *In vitro* studies have proved useful for analyzing loss of function of calpain activity by use of inhibitors or low calcium media. These studies, while useful in demonstrating the absolute requirement for calpain proteolysis, are often limited to individual cell populations and stages of cellular development. *In vivo* studies however have proved difficult due to the multiple functions of calpains. Constitutive, long term activation or inhibition of calpain activity is likely to have pleiotropic effects such that it would be difficult to address specific experimental questions. Additionally, effects on early developmental roles may prevent or alter analysis of later non-developmental roles. This was seen with inhibition of calpains in skeletal muscle beginning at the onset of muscle cell differentiation, where adult fibers were abnormally small, albeit more

plentiful, due to defects in fusion (Tidball and Spencer, 2002). It is for these reasons that we decided to generate an inducible *in vivo* system for analyzing the role of calpains.

Expression Systems and Transgenic Mice

The advent of transgenic technologies has transformed our understanding of gene function during mammalian development (Gao et al., 1999; Capecchi, 2000).
One classic transgenic approach is to overexpress a protein of interest by randomly inserting the corresponding cDNA into the host genome. This approach can be used to express wildtype, mutated or truncated versions of the protein. Overexpression studies of this nature are quite useful for studying the effects of ectopic expression and, when mutated, may also affect the normal function of the protein. There are several limitations to these studies. Most notably, unregulated overexpression can have significant deleterious effects on cell growth and viability that may inhibit the generation of stable lines.

Another powerful approach to these *in vivo* studies is targeted gene inactivation through homologous recombination (Capecchi, 2001). These mice, termed "knockouts," lack all functional protein of the targeted gene and are widely used to determine the function of specific genes. However, because this process involves irreversible modifications to the genome, knowledge gained by their effects is often limited to the earliest developmental function required for viability of the embryo. These embryonic

lethal genes are often known or suspected to have additional roles in later development and/or in adulthood, roles that cannot be analyzed in these knockout mice. An additional, problem encountered with loss of function studies is redundancy. Many genes in mammals belong to families of homologous genes that were most likely generated by evolutionary gene duplication. The proteins from these genes, although often having individual roles and sometimes even different expression patterns, can frequently substitute for one another. This compensation of genes with overlapping function is known as "functional redundancy" and has limited the utility of knockout experiments. Perhaps the most well-known redundancy story comes from the myogenic regulatory family of basic helix loop helix (bHLH) transcription factors (Weintraub, 1993). This family consists of four closely related genes; myoD, myf-5, MRF4, and myogenin, that are all involved in the differentiation of muscle. When the myoD gene, the first of the family members to be discovered, was ablated, mice unexpectedly showed relatively normal muscle development (Rudnicki et al., 1992). Myf-5 is another myogenic determination gene that is upregulated in MyoD null mice, and knockout of the myf-5 locus similarly failed to produce a muscle phenotype (Braun et al., 1992 and Rudnicki et al., 1992). Mice homozygous null for MRF4 also developed relatively normal musculature but showed an upregulation of myogenin (Zhang et al., 1995). Myogenin null mice were the only one of the four family members to show neonatal lethality due to a muscle defect (Hasty et al., 1993 and Nabeshima et al., 1993) where cells were specified, but did not fully differentiate. It was not until mice null for both MyoD and

Myf5 were generated, that the expected phenotype of complete loss of skeletal muscle, including loss of myoblast pools, was seen (Rudnicki et al., 1993).

Although only the μ form of calpain has been knocked out thus far, the resulting mouse is viable with minor defects limited to reduced platelet aggregation and clot retraction (Azam et al., 2001). Because *in vitro* results suggest that μ- calpain has roles not only in multiple cell types, but also in many cellular processes including critical functions in cell proliferation, cleavage of cytoskeletal proteins and cell motility, the results from this knockout indicate a possible compensatory role by m-calpain. To further address the roles of μ- and m-calpains, two groups have generated knockout mice with loss of function of the small 28kD subunit. Since the small subunit is a functional part of both μ- and m-calpains, its loss should eliminate activity of both forms. These knockout mice had the more expected result of embryonic lethality at day 9 (Arthur et al., 2000) or day E10.5 (Zimmerman et al., 2000). Variability could be attributed to removal of different portions of the subunit gene, resulting in more or less "complete" gene disruption (Goll et al., 2003) or to strain variability. As with many loss of function studies, early lethality precludes analysis of the later developmental and physiological processes. To study the later functional roles of calpains, a more regulated *in vivo* system is needed.

Conditional gene targeting is one way to overcome early developmental lethality by combining site-specific recombination with stage specific or tissue specific regulation

(Sauer, 1998). After several modifications, there are two established systems for this that operate off of the same principle; Cre/loxP, from bacteriophage P1 (Sauer and Henderson, 1989) and Flp/FRT, from *Saccharomyces cerevisiae* (O'Gorman et al., 1991). To add this additional element of control requires the generation of two lines of transgenic mice. The conditional allele mouse line contains a "floxed" or "flrted" target gene where the normal gene is flanked by 34bp repeats (loxP or FRT recognition sites) that catalyze the site-specific recombination (reviewed in Lewandoski, 2001). For loss of function experiments, these recognition sites are generally inserted into introns so that normal gene expression is not affected until activation by Cre or Flp. The second mouse line contains the Cre or Flp cDNA, generally under control of a tissue specific promoter. When these effector proteins are expressed in compound transgenic mice, DNA contained between the repeats is deleted, traditionally in a manner to eliminate functional gene product. Because the deletion does not occur in the absence of Cre or Flp, independent stable conditional allele lines of mice can be generated and crossed to various stably maintained effector lines. This method also allows for the loss of function to occur in a limited space and time according to the tissue specific promoter driving the effector (Sauer, 1998 and Lewandoski, 2001). This technique has also been used for conditionally turning on a gene for overexpression studies. By introducing "floxed/flrted" junk DNA or a "floxed/flrted" polyadenylation signal in the 5' region of the gene, successful expression of the gene does not occur until this DNA is removed by the Cre/Flp recombinase (Sauer, 1998 and Gao et al., 1999). Although these methods may help to overcome the deleterious effects due to early gene targeting, selectivity of

gene ablation or activation is limited to the degree of specificity of the promoter driving CRE or Flp.

The use of cell specific promoters can also provide some degree of spatial and temporal regulation in traditional overexpression transgenics. By using a tissue specific promoter instead of a ubiquitous promoter, overexpression in restricted areas and developmental stages can be achieved, although even this approach is limited by the degree of specificity provided by the promoter used. Most skeletal muscle specific promoters, for example, are activated at the onset of skeletal muscle differentiation during embryogenesis, limiting their usefulness for studies in which induced protein expression is desired in adult muscles. An ideal system would allow for inducible overexpression with stringent temporal and spatial specificity that is continuously reversible (able to be turned on and off and back on again). This system would have minimal background expression so as not to create lethality issues when generating stable lines, but high levels of inducible expression when the transgene is induced. Lastly, the mode of induction should be rapid and yet have minimal effect on the mouse. With these criteria in mind, several inducible gene expression systems have been developed (reviewed in Gao et al., 1999 and Lewandoski, 2001).

Some of the first inducible systems involved stress responses. Use of the heat shock protein 68 (HSP-68) promoter allowed target gene induction to be induced with a temperature adjustment to 42°C. However, difficulties with this system included

pleiotropic effects of the heat shock itself as well as variability of expression amongst different tissues (Kothary et al., 1989). Another stress response system was developed as an interferon gamma (INF-γ) dependent inducible system (Kuhn et al., 1995). Direct treatment with INF-γ, a cytokine normally found at low levels in unstressed mice, initiates expression of the target transgene via the myxovirus resistance 1 (Mx1) promoter. Although induction is robust, high levels of INF-γ can adversely affect normal physiological function, most notably in the central nervous system (Popko et al., 1997). Heavy metals have also been used as inducers to regulate a modified metallothionein promoter from humans (Filmus et al., 1992). Animals induced with heavy metals however showed various toxic effects from the metals as well as very high levels of leaky expression in uninduced animals.

Problems with secondary effects of induction were overcome by incorporating inducible models from non-mammalian systems. The ecdysone inducible system utilizes the *Drosophila melanogaster* metamorphosis regulating steroid hormone, ecdysone (No et al., 1996). In this system, a modified ecdysone receptor combined with a retinoid X receptor is transgenically expressed off of a tissue specific promoter. A second construct consists of the target transgene under the control of the ecdysone response element. When activated by ecdysone, the ecdysone receptor complex binds to the ecdysone response element and initiates transcription of the target gene. Another hormone based system has been developed through modification of the yeast *Saccaromyces cerevisiae*UAS/GAL4 system (Ornitz et al., 1991). This modified system combines the

Gal4 transcriptional activator with a truncated and modified Ligand receptor binding domain of the progesterone receptor and a VP16 activation domain (GLVP; Wang et al., 1997 and Burcin et al., 1998). When expressed by a tissue specific promoter, the resulting GLVP protein is held in the cytoplasm by heat shock proteins 70 and 90 until administration of the synthetic steroid hormone and progesterone antagonist, RU486. This allows for translocation into the nucleus where the Gal4 can bind to the UAS (upstream activating sequence) and recruit transcriptional activators via the VP16 domain to initiate transcription of the target gene. This system is limited, however, to overexpression in the adult animal, or to topically applied hormone, as RU486 induces abortion of embryos (Wang et al., 1999).

One of the most promising inducible systems is the tetracycline controlled transcription activation system (Gossen and Bujard, 1992; Baron and Bujard, 2000). The tet system is based on the ability of tetracycline (or its analogue doxycycline [Dox]) to bind to and block transcriptional repression by the tetracycline repressor protein of *Escherichia coli*. For regulated expression in eukaryotic cells, a chimeric transactivator protein (tTA, tetracycline TransActivator) has been generated consisting of the tet repressor protein fused in frame to the transcriptional activation domain of the viral VP16 protein. The VP16 domain of tTA can activate transcription of a target promoter (the Tet Responsive Element; TRE), while the tet repressor domain confers sensitivity to tetracycline such that transcription is inhibited by administration of Dox. This "tet off" system has been modified through mutation of the tet repressor domain to create "tet-on",

in which the responding promoter is transcriptionally silent in the absence of Dox but becomes activated when Dox binds the reverse tetracycline transactivator (rtTA). Both tet-off and tet-on have now been used for regulated expression in a variety of cultured cell types, and more recently in transgenic mice (see, for example Furth et al., 1994; Efrat et al., 1995; Shin et al., 1999; Federov et al., 2001; Perl et al., 2002).

Early difficulties with this system included high background expression in cell culture and low induction levels in transgenic mice. Addition of a tetracycline-controlled transcriptional silencer (tTS) that binds to the TRE in the absence of Dox eliminates the cell culture background expression that is attributed to high copy number and lack of chromatin repression (Freundlieb et al., 1999 and Lamartina et al., 2002). tTS has also been used in transgenic mice to prevent leaky expression (Zhu et al., 2001), though leakiness is not frequently observed *in vivo*. Recent codon optimization of the reverse tet transactivator for improved expression in mammalian systems has further improved the efficacy of the tet inducible systems (Valencik and McDonald, 2001 and Wells et al., 1999). Novel mutations in the rtTA transactivator were recently generated, and one particular mutant improves transactivator stabilization, lowers background activity, and can be induced with lower concentrations of Dox when tested in transfected HeLa cells (Urlinger et al., 2000). In order to generate higher levels of tTA, an autoregulatory strategy has also been devised with the tet-off system by using the tet operator to drive its own expression, thereby leaving all the regulation to the presence or absence of Dox (Shockett et al., 1995). Attempts to assay and quantitate expression have led to

modifications of both parts of the system to include reporter genes. To assay transactivator expression, an rtTA IRES/GFP construct was tested in Chinese hamster ovary cells. Visible fluorescent marker corresponding to rtTA expression was observed when combined with the codon optimized rtTA sequence (Wells et al., 1999). Inclusion of a second reverse minimal CMV promoter creates a bicistronic responder construct and allows for co-expression of reporter constructs like GFP, luciferase, or ß-galactosidase with the target gene of interest (Baron et al, 1995).

Although several cell type-specific tetracycline regulated gene expression systems have been developed, an efficient method for inducible gene regulation in skeletal muscle has not been described. Chapter II of this dissertation describes the generation of a skeletal muscle-specific "tet-on" inducible system, using a modified muscle creatine kinase (MCK) gene promoter to drive expression of rtTA. Also described is the generation of a responder transgenic mouse line that can be induced to simultaneously express both the myc-his tagged full-length bovine heart (type II) calpastatin and the reporter gene, luciferase. Chapter III focuses on experiments looking at specific muscle proteins titin, nebulin, troponin T, and desmin and compares their degradation when incubated with calcium versus the degradation induced by hindlimb suspension simulated microgravity. These experiments emphasize the flexibility of a dual construct system, but also reiterate the potential drawbacks of transgenic expression as well as providing insight into the specific roles of calpain in muscle homeostasis.

CHAPTER II

Generation of a Tetracycline-Inducible System for Skeletal Muscle-Specific Over-Expression of Calpastatin in Transgenic Mice*

* Much of the work presented in this chapter was published in Grill, MA, Bales, MA, Fought, AN, Rosburg, KC, Munger, SJ and Antin PB. 2003. Tetracycline-inducible system for regulation of skeletal muscle-specific gene expression in transgenic mice. Transgenic Research 12:33-43. Use of this material is granted with kind permission of Kluwer Academic Publishers (see Appendix A).

Abstract

Tightly regulated control of over-expression is often necessary to study one aspect or time point of gene function and, in transgenesis, may help to avoid lethal effects and complications caused by ubiquitous over-expression. We have utilized the benefits of an optimized tet-on system and a modified muscle creatine kinase (MCK) promoter to generate a skeletal muscle-specific, doxycycline (Dox) controlled over-expression system in transgenic mice. A DNA construct was generated in which the codon optimized reverse tetracycline transactivator (rtTA) was placed under control of a skeletal muscle-specific version of the mouse MCK promoter. Transgenic mice containing this construct expressed rtTA almost exclusively in skeletal muscles. These mice were crossed to a second transgenic line containing a bi-directional promoter centered on a tet responder element (TRE) driving both a luciferase reporter gene and a tagged gene of interest; in this case the calpain inhibitor calpastatin. Compound hemizygous mice showed high level, Dox dependent, muscle-specific luciferase activity sometimes exceeding 10,000 fold over non-muscle tissues of the same mouse. Western and immunocytochemical analysis demonstrated similar Dox dependent muscle-specific induction of the tagged calpastatin protein. These findings demonstrate the effectiveness and flexibility of the tet-on system to provide a tightly regulated over-expression system in adult skeletal muscle. The MCKrtTA transgenic lines can be combined with other transgenic responder lines for skeletal muscle-specific over-expression of any target gene of interest.

Introduction

Skeletal muscles are comprised of multinucleated muscle fibers that function coordinately to enable body movement. During development, individual, specified myocytes align and fuse to form elongated, differentiated myotubes. Several studies have shown calpains to be required for this fusion. Early studies demonstrated a redistribution of m-calpain to the membrane in fusing L6 and L8 myoblasts that was not seen in the corresponding fusion incompetent fu-1 and M3A myoblast cell lines (Schollmeyer, 1986b). Additional experiments that block calpain activity via calpain specific inhibitors calpeptin (Ebisui et al., 1994) or leupepetin (Brustis et al., 1994) decrease myoblast fusion. Similar fusion reduction results were seen with calpain inhibition by calpastatin (Brustis et al., 1994 and Temm-Grove et al., 1999), calpain blocking antibodies (Brustis et al., 1994), and treatment with antisense oligodeoxyribonucleotide to m-calpain (Balcerzak et al., 1995). Opposite experiments performed by adding purified m-calpain (Brustis et al., 1994) or calpastatin antisense oligodeoxyribonucleotide (Balcerzak et al., 1998) to myoblasts induced earlier, faster fusion. Further experiments analyzing the levels of m-calpain and calpastatin demonstrate a decrease in calpastatin levels just prior to differentiation that allow for activation of m-calpain (Barnoy et al., 1996 and Barnoy et al., 2000). Several calpain target molecules have been identified as part of this fusion process including desmin, talin, and fibronectin (Dourdin et al., 1999). When taken together, these data present a clear role for tightly regulated control of m-calpain and calpastatin in the normal development of skeletal muscle.

There are three types of calpain found in skeletal muscle, the ubiquitous μ- and m-calpains, as well as a third skeletal muscle specific calpain, calpain3 (Sorimachi et al., 1989) [Several other ubiquitous isoforms have been described but their protein products have not yet been demonstrated in muscle (Sorimachi et al., 2001)]. Besides the critical role of calpains in development, analysis of calpains and calpastatins in certain diseases demonstrates their additional importance in more mature tissues. In Duchenne's muscular dystrophy, loss or mutation of the cytoskeletal protein dystrophin causes progressive muscle degradation (Hoffman et al., 1987) due to muscle cell membrane instability (Evrasti and Campbell 1991). Although not the primary cause of the disease, increased cellular calcium in dystrophin deficient cells has placed calpains as the probable downstream effector molecule causing necrotic cell death (Spencer et al., 1995 and Mariol and Segalat, 2001). Recent work by Spencer and Mellgren (2002) has demonstrated that blocking calpain activity with transgenic calpastatin overexpression reduces the level of necrotic death in dystrophic muscle. This data demonstrates a clear secondary role for calpains in the manifestation of Duchenne's muscular dystrophy. Another form of muscular dystrophy, limb girdle type 2A, has been directly attributed to mutations in calpain3 (Richard, et al., 1995). This, along with potential roles of calpain 9 in tumorigenesis (Yoshikawa et al., 2000 and Liu et al., 2000) and calpain 10 in insulin resistance and type II diabetes (Baier et al., 2000) are the only pathologies known to be directly caused by calpains. Examples of secondary "calpain-associated" pathologies like in Duchenne's muscular dystrophy are extensive and include roles in Alzheimer's disease

(Tsuji et al., 1998 and Grynspan et al., 1997), Multiple Sclerosis (Shields and Banik, 1999 and Shields et al., 1999) and post myocardial infarct damage (Sandmann et al., 2001; Yoshida et al., 1995; and Chen et al., 1998; additional diseases reviewed in Goll et al., 2003 and Huang and Wang, 2001). These implied roles and the likelihood of many yet to be discovered as the "new" calpains are localized and analyzed, demonstrate the need for better understanding of the regulation and potential mechanisms of the calpains.

This multitude of functions, plus the requirement for calpain in normal muscle development, reiterates the difficulty and importance of studying individual roles of calpains in specific cell types. For example, while it would be desirable to test the role of calpains in regulating muscle size in transgenic mice, standard knockout mice have shown minimal effects (μ- calpain, Azam et al., 2001) or are embryonic lethal (28kD subunit, Arthur et al., 2000 and Zimmerman et al 2000). Since multiple calpains are expressed in skeletal muscles, and they are also involved in a large number of cellular processes that would likely be disrupted by global gene inactivation or transgene expression, generation of a muscle-specific inducible gene expression system would provide a valuable means for addressing calpains role in muscle homeostasis and other questions about muscle physiology and function.

Here I describe a skeletal muscle-specific "tet-on" inducible system, using a modified muscle creatine kinase (MCK) gene promoter to drive expression of rtTA. MCK is normally expressed in both skeletal and cardiac muscle where it catalyzes the

regeneration of ATP from creatine phosphate, providing energy for contraction. The MCK promoter has been thoroughly analyzed in transgenic mice and control elements carefully mapped (Shield et al., 1996; Johnson et al., 1989). While MCK is normally expressed in all skeletal muscles and the heart, a 1256 nucleotide MCK promoter fragment in which three E box cis elements have been mutated (1256[3E mut] MCK) shows little or no activity in the tongue, diaphragm or heart (Shield et al., 1996 and Donoviel et al., 1996), while maintaining high levels of expression in appendicular skeletal muscles. This modified MCK promoter has been selected for use in the present study (referred to herein as MCK). Transgenic mouse lines containing the rtTA cDNA under control of this MCK promoter were generated and crossed to transgenic lines containing a bi-directional TRE driving luciferase expression in one direction and the calpain protease inhibitor calpastatin (bovine heart type II with c-myc and his_6tag), in the other direction. Results show that both luciferase and calpastatin expression are induced to high levels only in skeletal muscles after administration of Dox, while in the absence of Dox, transgene expression is undetectable. Because of the inherent flexibility of a dual construct system and the ability to cross the skeletal muscle-specific transactivator line to multiple transresponder (TRE) lines, this system will prove useful for analysis of a wide range of physiological questions relating to skeletal muscle development, physiology, and pathology.

Materials and Methods

Tet-on DNA Constructs

Both MCKrtTA and TRECSluc constructs were generated in a modified pUC18 plasmid that contained a large multiple cloning site with flanking Not I sites for excision ($pNNO_3$). All clones were sequenced and compared to original sequences after each PCR step.

For the MCKrtTA construct (full name: p1256[3EmutMCKrtTAβglobinIVS), the 1256[3Emut]MCK promoter was amplified from the p1256[3Emut]MCKCAT plasmid (gift of Stephen D. Hauschka, Donoviel et al., 1996) via PCR with 5' Spe I and 3' Pst I restriction sites at the ends of the primers. Primer sequences are 5': acggactagtccgGCTTGCATGCCCCACTACGG and 3': gagctgcagcTTTAGCTTCCTTAGCTCCTGAAAATCTC. Using these restriction enzymes, the amplified product was cloned into the poptrtTA plasmid (gift of John A. McDonald, Valencik and McDonald, 2001) just 5' to the optimized rtTA sequence. To optimize transgenic expression, the Hgh polyadenylation signal was replaced with a rabbit ß-globin polyadenylation signal that contains an intervening splice site (ß-globin IVS polyA, also from John A. McDonald) via 5' HindIII and 3' blunt end cloning.

A TRECSmychis$_6$βglobinIVS construct was generated by PCR amplifying full-length bovine heart calpastatin from pBHC2 (Cong et al., 1998) with additional

restriction sites 5' (EcoR I) and 3' (Xba I) added to primer ends. Primer sequences are 5': ccggaattcATGGCATTTGCAAGCTGG and 3': ctagtctaGACTTGTACTTTTTCCATCAGCTTTTGG and cloning that product into PGK 3.1B mychis (modified from pCDNA'3.1B, Invitrogen) using these restriction sites. Calpastatin with the 3' c-myc and his_6 tags was gel purified from a 3'Pme I partial digest (blunted) and 5' EcoR I digest and was used to clone into the Clontech pTRE vector (3' blunted Xba I, 5' EcoR I). Both TRECSmychis (5' Xho I, 3' BamH I) and the ß-globin IVS polyA (5' BamH I, 3' Hind III) were independently isolated by gel purification and cloned simultaneously into the $pNNO_3$ vector (5' Xho I, 3' Hind III).

In order to utilize the benefits of the bi-directional TRE with luciferase reporter gene, the construct containing the bi-directional TRE driving both the luciferase gene and the c-myc/his_6 tagged calpastatin cDNA was generated in the modified pUC18 plasmid, $pNNO_3$ (full name: biTRECSmychis$_6$βglobinIVSLucif). This was accomplished by isolating the TRE and the luciferase gene with SV40 Polyadenylation signal from the Clontech pBIL vector with 5' Aat II (blunted) and 3' Nhe I and cloning them into the $pNNO_3$ vector (5'BamH I, blunt, and 3' Xba I). Calpastatin myc/his with ß-globin IVS was amplified by PCR (from the pTRECSmychis$_6$βglobinIVS vector above) with an additional Hind III site 5', gel purified, and cloned non-directionally just 3' to the bi-directional TRE with Hind III. Primer sequences are: 5' ccaagcttATGGCATTTGCAAGCTGGT and 3'

GCGGTCCAACCACCAATCTCAAAGCTT. Diagnostic digests were used to isolate clones in the proper orientation.

An additional vector, pbiTREMCS$_{CN}$βglobinIVS was generated for future generation of TRE vectors. This construct has a multiple cloning site with several unique sites followed by the βglobin intervening sequence polyadenylation signal for driving a gene of interest in combination with the reporter gene luciferase. The construct retains the unique NotI excision sites on either end for isolating the DNA to be used to injection.

C2C12 Cell Culture Experiments

C2C12 cells were cultured in growth media (GM; DMEM plus 20% FBS and 5% chick embryo extract) at 60,000 cells per well on 6 well plates. Twenty-four hours after plating, cells were co-transfected in serum free DMEM for 6 hours with appropriate DNA: 0.66μg each of three DNA constructs (for Figure 2; pMCKrtTA, pBI-GL [Clontech] and ptTS [Clontech] and for Figure 3; MCKrtTA, and tTS [Clontech]) using 6μl each of Lipofectamine and Plus reagent per well added according to directions (Gibco/Invitrogen). Cells were allowed to recover in GM for 18 hours before being switched to differentiation media (DM; DMEM with 2% horse serum) for 72 hours. Induced cultures were then incubated in 1μg/ml Doxycycline in DM while uninduced cultures were given fresh DM for three (Figure 2) or one (Figure 3) additional day(s). Cells for Figure 2 were then fixed with 1% gluteraldehyde for 5 minutes, washed and incubated in a 1% X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside in dimethyl

formamide) KFeCN solution. Cells for Figure 3 were either harvested in lysis buffer, mixed with Tropix solutions A and B and analyzed for luciferase activity (Figure 3A); or fixed with 2% formaldehyde and immunolabeled (figure 3C) with a (1:500) mouse monoclonal antibody to His_6 (Santa Cruz Biotechnologies), followed by (1:500) Texas Red conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch).

Transgenic Animal Production

Animals were housed in compliance with the NIH Guide to Care of Laboratory Animals in AALAC accredited facilities at the Mayo Clinic, Scottsdale or at the University of Arizona. All mouse procedures were approved by the Institutional Animal Care and Use Committee. Thirty day-old female B6.CBAF1 females purchased from Jackson Labs (Bar Harbor, Maine) were superovulated with 5 IU of pregnant mare serum gonadotropin, followed 48 h later by 5 IU of human chorionic gonadotropin. Females were mated with 4-month-old C57BL/6J males, checked for vaginal plugs the morning following mating, sacrificed using CO_2 inhalation, and 1-cell embryos collected from the oviducts into Hepes media. The embryos were incubated in KSOM under mineral oil in a CO_2 incubator at 37° C. NotI excised linear DNA was microinjected into the male pronucleus of fertilized one-cell embryos, which were then implanted into the oviducts of 0.5 p.c. 36-day-old pseudopregnant CD1 females. Pups were weaned at 20 days of age, identified, and tail samples were collected for analysis. Founder mice were genotyped by PCR with DNA isolated from tail clippings (Clontech Nucleospin Tissue kit). Primers used to identify a 402bp band internal to the optimized rtTA of the MCKrtTA construct

are: 5' CATCTGCGGACTGGAAAAACAAC and 3' GCATCGGTAAACATCTGCTCAAAC. Primers used to identify a 576bp band internal to the luciferase gene of the TRECSluc construct are: 5' TCGTCCACAAACACAACTCCTCCG and 3' ACGCTTCCATCTTCCAGGGATACG. Primers to identify a 450bp band internal to collagen IV were often used to test the efficacy of the DNA isolations, primers are: 5' AGGCAGGTCAAGTTCTAGCG and 3' CAAGCATAGTGGTCCGAGTC. Transgenic founders were isolated and mated back to C57BL/6J wildtype mice to generate F1's for testing and subsequent breeding. For more detailed breeding information see Appendix C.

Doxycycline Inductions of transgenic mice

Experimental mice (which included compound hemizygous transgenics ([MCKrtTA X TRECSluc], TRECSluc only hemizygous transgenics, and wildtype mice) were separated into induced and uninduced subsets. For the first subset of experiments, induced mice were given 1mg/ml Doxycycline (Dox) in drinking water supplemented with 10mg/ml sucrose for palatability. Uninduced mice were given 10mg/ml sucrose drinking water without Dox. In the initial experiments, treated water was supplied for four to six days as the only water supply with fresh Dox/sucrose or sucrose only water changes every two days. Hindlimb suspension inductions were initiated three to four days before suspension and dox treatment was continued for ten to twelve days in suspension. Doxycycline was administered in three different ways in these experiments. 1) Dox/sucrose water at previously described concentrations 2) Doxycycline 21-day time

release pellets or corresponding placebo pellets (Innovative Research of America) were implanted subcutaneously with a trochar on the lateral side of the neck 3) 200mg/kg doxycycline food (with green dye used as an indicator of consumption) supplied as the only food source.

PCR and RT-PCR

Primers used to genotype MCKrtTA transgenic mice amplify a 402bp fragment of the reverse tet transactivator gene (rtTA). Primer sequences are 5': CATCTGCGGACTGGAAAAACAAC and 3': GCATCGGTAAACATCTGCTCAAAC. Primers used to genotype transgenic mice amplify a 567bp fragment of the luciferase gene. Primer sequences are 5': TCGTCCACAAACACAACTCCTCCG and 3': ACGCTTCCATCTTCCAGGGATACG. To ensure the quality and concentration of the DNA, a control PCR for Collagen IV was used to amplify a 450bp fragment. Primer sequences are 5': AGGCAGGTCAAGTTCTAGCG and 3': CAAGCATAGTGGTCCGAGTC. Genomic DNA was isolated from tails following the protocol for the Nucleospin tissue kit (Clontech). Total RNA for RT-PCR was isolated by rapid dissection of tissue and immediate immersion in liquid nitrogen. Samples were placed in RNA Stat 60 (TelTest "B"), put through one freeze thaw cycle and then homogenized. Chloroform was added and after vortex mixing the aqueous phase was removed to new tubes. RNA was precipitated, dried, and resuspended in RNase free water. 2μg of each RNA sample was DNase treated, incubated at 42° for one hour in an RT cocktail containing random hexamers, dNTPs, RNasin, DTT, RT buffer, Superscript

II (Invitrogen) and water, and then heat inactivated at 70°C for 15 minutes. All PCR protocols had the following 30 cycle program: 94°C - 30 sec, 55°C for luciferase and collagen IV, or 56.8°C for rtTA - 30 sec, and 72°C - 1 min after a 10 minute hot start at 94°C using Taq Gold (Perkin Elmer).[3]

Luciferase Assays

Tissues were rapidly dissected from mice and snap frozen in liquid nitrogen. Tissue fragments were then homogenized in 600μl ice cold lysis solution (Tropix luciferase assay kit) with 20 passages of a Dounce homogenizer, put through one freeze thaw cycle, spun 15 mins at 4°C and supernatant transferred to new tubes. 10μl of each sample (or 10μl of diluted sample for some high expressing tissues to maintain readings in linear range) was then mixed with 50μl solution A and 50μl solution B and immediately assayed in a Turner Designs TD 20/20 luminometer. The Luminometer settings were as follows: Delay = 2 seconds, Integration = 10 seconds, Repetitions = 2, and Sensitivity = 50.1% animal tissue or 59.8% for cell culture. In all cases, multiple readings were taken for each sample and averaged after normalizing to protein concentration (determined by Bradford assay using BioRad Protein Assay dye reagent compared to BSA standards).

β-galactosidase stain in embryos

Embryos were dissected from a 14 and 18 days post coitus pregnant females that had been fed Doxycycline water for 21 or 19 days respectively. After the tails were

removed for genotyping, embryos were fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 2 hours at 4°C. E14 embryos were stained in PBS containing 35mM potassium ferricyanide, 35mM potassium ferrocyanide, 1.5mM magnesium sulfate, and 1mg/ml X-gal (Sigma). E18 embryos were stained in the same staining solution supplemented with 0.2% sodium deoxycholate and 0.1% IGEPAL CA 630 (previously known as Nonidet P-40 both from Sigma). Embryos were stained at 37°C for 28 and 24 hours respectively.

β-galactosidase enzymatic activity assay

Quadricep muscle was isolated from 3 week old pups derived from the #9MCKrtTA X GFPTREßgal cross. Tails were isolated for genotyping but quadriceps samples from the entire litter were run. Protein was isolated and prepared as for luciferase assays, however, the Dual-Light (Tropix) kit was used to assay the activity of ß galactosidase. 25µl of Buffer A was added to 10µl of each sample followed by 100µl of Working Buffer B (Working Buffer B consisted of Galacton plus reagent diluted 1/100 in Buffer B). Samples were incubated for 30 minutes at room temperature before addition of 100µl of Accelerator II solution. Samples were read directly in tubes immediately after this addition for assaying β-galactosidase activity on the TD-20/20 Luminometer (Turner Designs). The Luminometer settings were as follows: Delay = 1 seconds, Integration = 10 seconds, Repetitions = 3, and Sensitivity = 47.9%.

Western Blots

50μg of the above prepared protein samples were run on an 8% SDS polyacrylamide gel. Protein was transferred to a nitrocellulose membrane, stained with PonceauS to check for transfer and blocked for 30 mins in 5% (w/v) milk in TBS-T. Blots were then incubated in 1:5000 dilution of polyclonal anti-myc antibody (Upstate Biotechnologies) or a 1:50,000 dilution of a calpastatin specific monoclonal antibody (generous gift of Darrel E. Goll) in blocking solution for 1 hour. After three washes in TBS-T (Tris buffered saline plus 0.1% Triton X-100), blots were incubated in either donkey anti-rabbit or donkey anti-mouse HRP conjugated secondary antibody (1:5000; Jackson Immunoresearch) in blocking solution for one hour. Blots were washed in TBS three times, soaked briefly in 1:1 Pierce Supersignal West Pico chemiluminescent substrate solutions, wrapped in plastic wrap and exposed to autoradiographic film for 2-10 seconds.

Immunohistochemistry

Gastrocnemius and gluteus muscles were extended, clamped, dissected, and frozen in liquid nitrogen. Samples were embedded in OCT, cryostat sectioned at 10μm and mounted to slides. Sections were rinsed in PBS, fixed for 10 mins in freshly prepared 2% formaldehyde, washed three times in PBS and blocked in 1% donkey serum/2% FBS in PBS for 30 minutes. After blocking, sections were incubated in a 1:100 dilution of rabbit polyclonal anti-myc primary antibody (Upstate Biotechnologies)(in sections for figure 5, 1:50 goat polyclonal anti-luciferase primary

antibody was also added at this step) in blocking solution cocktail for one hour. Sections were then washed three times and incubated for one hour in a 1:500 dilution of Cy3 conjugated donkey anti-rabbit IgG secondary antibody (Figure 5 also included a 1:500 dilution of Cy2 conjugated donkey anti-goat) in blocking solution (both secondaries from Jackson Immunoresearch). Samples were washed four additional times and coverslip mounted with Aqua PolyMount (Polysciences). Sections were viewed by epifluorescence on a Leica DMRE microscope.

Results

Tet-on inducible system

To generate a tightly regulated over-expression system specific to muscle, we have utilized the dual construct tet-on system. For the MCKrtTA transactivator constructs, the modified MCK promoter was used to drive skeletal muscle-specific expression of the codon-optimized reverse tetracycline transactivator (rtTA), consisting of the tetracycline receptor fused to the activation domain of VP16. The transresponder line, TRECSluc, is transcriptionally silent alone, but in the presence of both Dox and rtTA, transcription of luciferase and calpastatin is activated. This combination allows for over-expression that is not only restricted to skeletal muscle but also limited temporally by the administration of Dox (Figure 1).

Testing the tet-on system in cell culture

The efficacy of the transactivator construct was tested in C2C12 myoblasts. Myoblasts were co-transfected prior to differentiation with the MCKrtTA construct and Clontech's pBiGL (a bidirectional TRE construct driving β-galactosidase and luciferase) and allowed to recover before being switched to differentiation media. Once the cells had fused into myotubes, the plates were divided into doxycycline induced and control groups and treated accordingly for an additional 3 days to test the expression pattern and inducibility of our MCKrtTA construct. All cells were fixed and stained for β-galactosidase activity at the same time. Results of this experiment (Figure 2) show clear

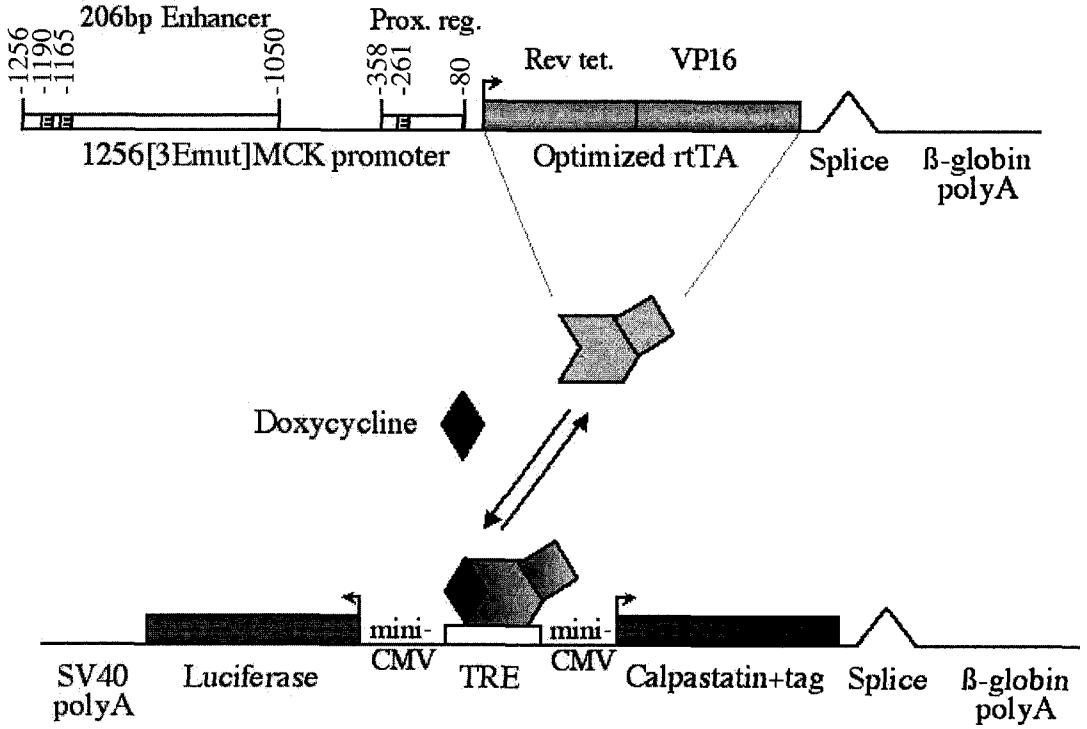


Figure 1: Schematic diagram showing constructs used for generating transgenic mice. The MCKrtTA construct (top) contains 1256 nucleotides of the mouse MCK promoter with mutations in 3 E boxes that abolish promoter activity in the heart but has no effect on the high level expression in appendicular skeletal muscle. This MCK promoter was cloned 5' to the codon-optimized rtTA, followed by the 3' end of the rabbit ß-globin gene containing an intron and a polyadenylation signal. This construct generates rtTA mRNA in a skeletal muscle-specific pattern. The construct (bottom) contains a central tetracycline response element (TRE) flanked by two oppositely oriented minimal CMV promoters driving the reporter gene luciferase in one direction and the tagged gene of interest (in this case, calpastatin with 3' myc and his_6 tags) in the other direction. The TRECMV promoters are activated upon binding of Dox-bound rtTA protein to the TRE, resulting in simultaneous expression of both the luciferase reporter gene and the tagged calpastatin gene.

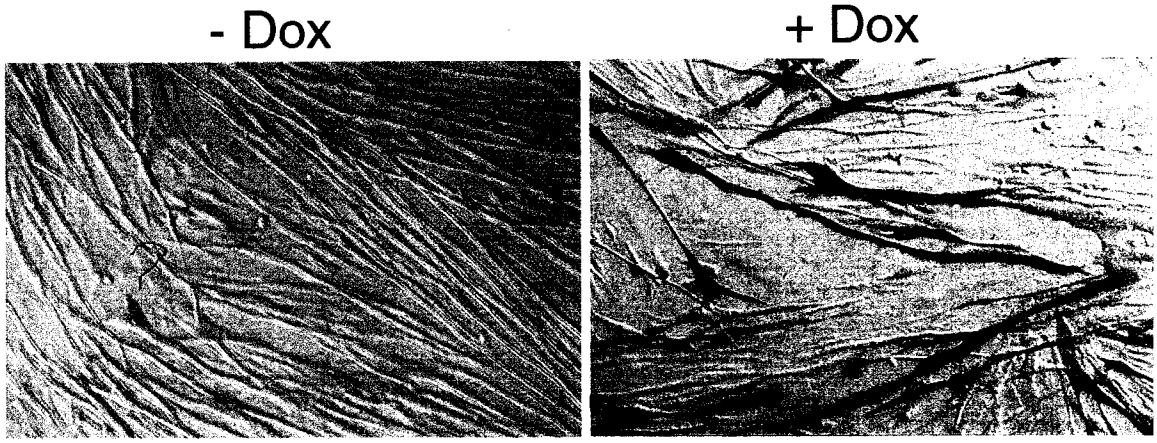


Figure 2: ß-galactosidase activity in transiently transfected C2C12 myotubes. β-galactosidase staining demonstrating the ability of rtTA protein expressed off the modified MCK promoter to drive expression of desired genes on the TRE construct in differentiated myotubes in a doxycycline dependent manner. Since cells are transiently transfected, only a small percentage of the cells will actually contain both constructs, about 18% of the cells in the doxycycline treated samples showed β-galactosidase activity whereas 0% of the untreated cells showed activity.

expression of β-galactosidase in the doxycycline treated cells and no expression in the untreated cells, confirming the ability of the MCKrtTA to activate the TRE in a doxycycline dependent manner. Although only a transient transfection, this experiment not only demonstrated the MCK promoter's transcriptional activity in differentiated myotubes but also the ability of the optimized rtTA combined with Dox to induce transcription of the TRE construct as compared to the absence of TRE activation in the absence of Dox.

To test the transactivator and transresponder constructs for the ability to direct muscle-specific inducible gene expression of calpastatin and luciferase, C2C12 myoblasts were transiently co-transfected with the MCKrtTA and TRECSluc plasmids along with a plasmid expressing a tetracycline-controlled transcriptional silencer (tTS). tTS binds to the TRE in the absence of Dox, abolishing background expression that is sometimes observed in transient co-transfection experiments in which high copy number and lack of chromatin repression are a concern (Freundlieb et al., 1999 and Lamartina et al., 2002). Twenty-four hours after transfection, cultures were switched to differentiation medium for three days, at which time the majority of cells had fused into myotubes. Plates were divided into Dox induced and control groups and treated accordingly for one additional day. Cells were then either harvested for luciferase activity or processed for immunocytochemical localization of the his_6 tagged calpastatin. As shown in Figure 3A, luciferase activity was at background levels in control (uninduced) cultures; but increased 600 fold following addition of Dox. Immunocytochemical localization of the his_6 tagged

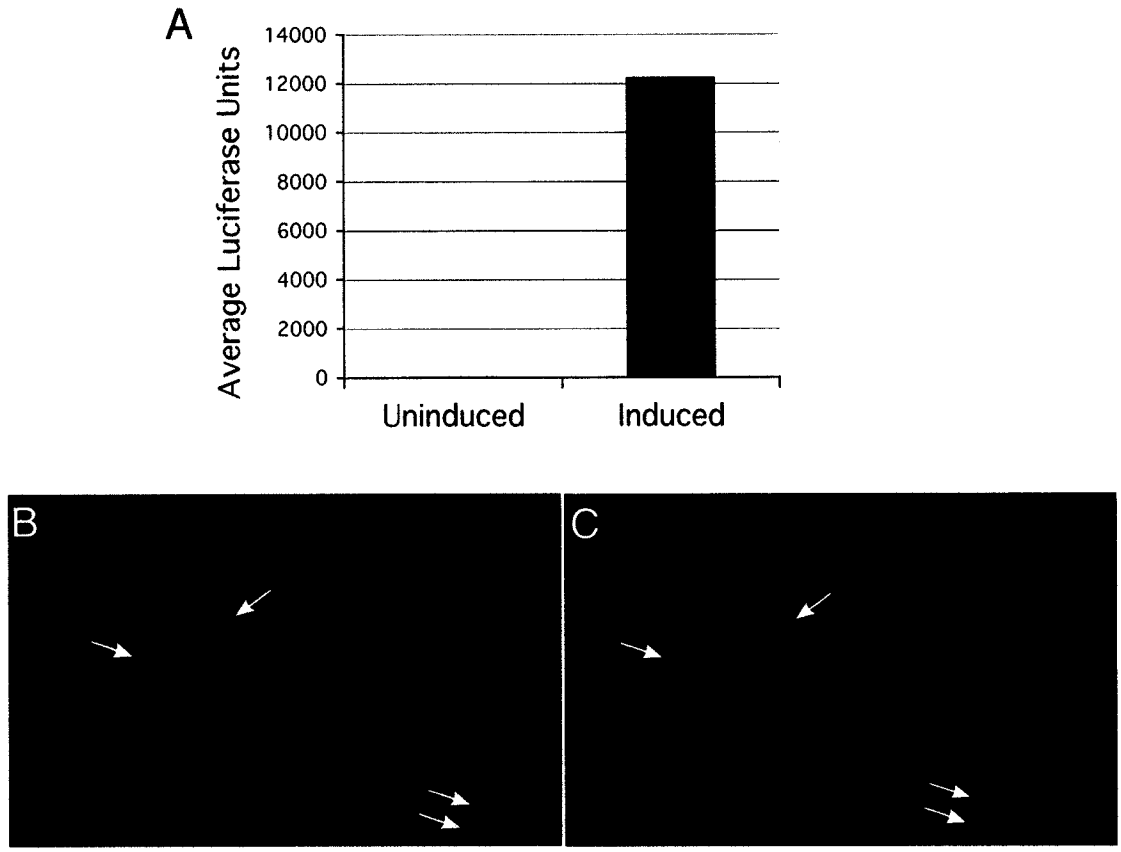


Figure 3: Inducible transgene expression in transiently transfected C2C12 myotubes. A) Luciferase assays from cell lysates of Dox-induced or uninduced cells co-transfected with MCKrtTA, and the tTs transcriptional silencer. Following addition of Dox, luciferase activities increase more than 600 fold (12,200 units versus 20 units – samples averaged over 4 independent experiments). B) Immunofluorescence localization of his_6 tagged calpastatin shows tightly regulated expression of the transgene only in differentiated myotubes. C) DAPI nuclear stain of the same field as B shows the corresponding nuclei, arrows indicate undifferentiated myoblasts that do not express calpastatin.

calpastatin (Figure 3C) showed that the calpastatin transgene was expressed at high levels in differentiated myotubes but was not detectable in the undifferentiated myoblasts in the culture (arrows, Figure 3B and C) or in any cells of cultures not treated with Dox (data not shown). These data demonstrate the capacity of this dual construct system to specifically over-express calpastatin and luciferase in a Dox dependent manner in cultured mammalian muscle cells.

Generating tet-on transgenic mice

Transgenic founder lines were generated by pronuclear injection of the MCKrtTA and TRECSluc constructs. Since a previous study demonstrated aberrant gene regulation when transactivator and transresponder constructs were co-injected into zygotes (Valencik and McDonald, 2001), independent transgenic mouse lines were generated and subsequently mated to each other or other established lines to generate experimental compound transgenic mice. Because generation of transgenic mice includes incorporation of the trangene into the mouse genome, and because each line is generated independently, background expression is not a general concern and so the repressor construct (tTS) was not used. F0 offspring were genotyped and mice positive for the MCKrtTA transactivator or TRECSluc transresponder construct were mated to wild type C57BL/6J mice to generate more mice to establish colonies of each line. A breakdown showing the number of mice required to generate the transactivator and transresponder lines is shown in Table I.

Table I: Generation of Transgenic Mice

	# Injected	# Born	#Founders	# Fertile	# Functional*
MCKrtTA	453	118	7	5	3
TRECSLuc	250	64	8	7	3**

* Functional refers to transgenic lines that express the rtTA or TRE lines that can be induced to express the calpastatin and luciferase

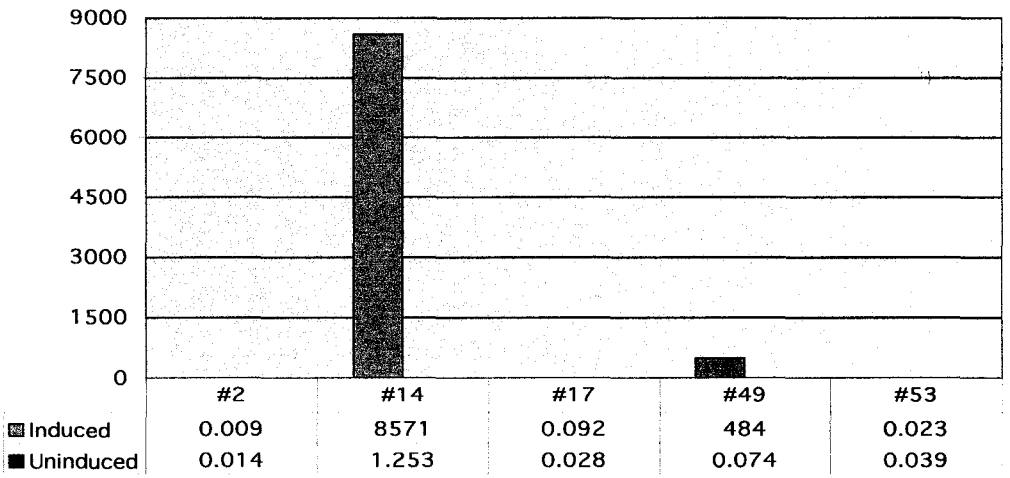
** One of these lines had background expression which proved detrimental to the health and fertility of the line

Testing the TRECSluc transgenic lines

Eight founders were identified by PCR and were outcrossed to wildtype C57BL/6J mice. RT-PCR analysis on F1 progeny was originally used to demonstrate lack of constitutive promoter activity in some of the original lines that were breeding well (data not shown). Since competent transactivator mice had not yet been generated and since transresponder lines do not have any testable expression alone, subsequent transgenic F1's were then crossed to a heart specific transactivator line, optrtTA (Valencik and McDonald, 2001). This transactivator line uses the mouse cardiac α-myosin heavy chain promoter (Gulick et al., 1991) to drive expression of the same codon optimized rtTA we used in our MCKrtTA constructs. Seven lines were successfully bred to this line and subsequent compound transgenic pups were induced with Dox and tested for induction. Three of the seven lines showed robust over-expression of the transgenes. Results of the luciferase assays (Figure 4A) and Western blot analysis (Figure 4B) performed on the first five of these lines are shown. Western blot analysis of these same samples using a monoclonal antibody (1F7, gift of Darrel E. Goll, Wei et al., 2002), epitope mapped to domain IV of the calpastatin protein, show similar results (Figure 4C). Some lines were not as quick to breed or did not produce double transgenic pups in their first two litters and were tested later with similar results. Table II summarizes the identification of transresponder mice and the results of testing for inducibility in the fertile lines. Antibody staining using a polyclonal antibody to the luciferase reporter protein and to the c-myc tag of the transgenic calpastatin on heart sections demonstrates the broad expression of both (Figure 5).

Figure 4: Demonstration of inducible over-expression of calpastatin and luciferase from two transgenic responder lines. A) Luciferase assays showing induced and uninduced levels of activity for 5 of the TRE lines tested. Transgenic lines #14 and #49 show dramatic induction with the addition of doxycycline (approximately 6,000 fold when compared to respective uninduced littermates). Three other lines are also shown that are not functional (typical for transgenic lines) and serve as additional controls. B) Western blot analysis detecting the c-myc tag on the transgenic calpastatin corroborates the luciferase data again showing induction in the doxycycline treated #14 and #49 transgenic lines. Bands correspond to typical sizes seen for calpastatin on western blots (145kD, 120kD, and 110kD, plus one additional smaller band) and are seen only in *induced* #14 and #49 mice. C) Western blot analysis detecting calpastatin with the monoclonal 1F7 antibody to the same tissue samples as in B demonstrates the myc stained overexpression protein is calpastatin.

A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Uninduced
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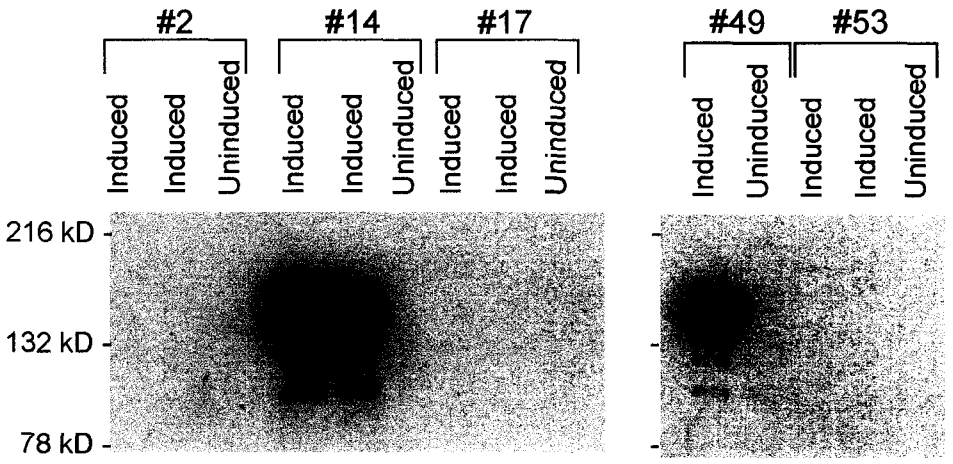
0.028
0.074
0.039

B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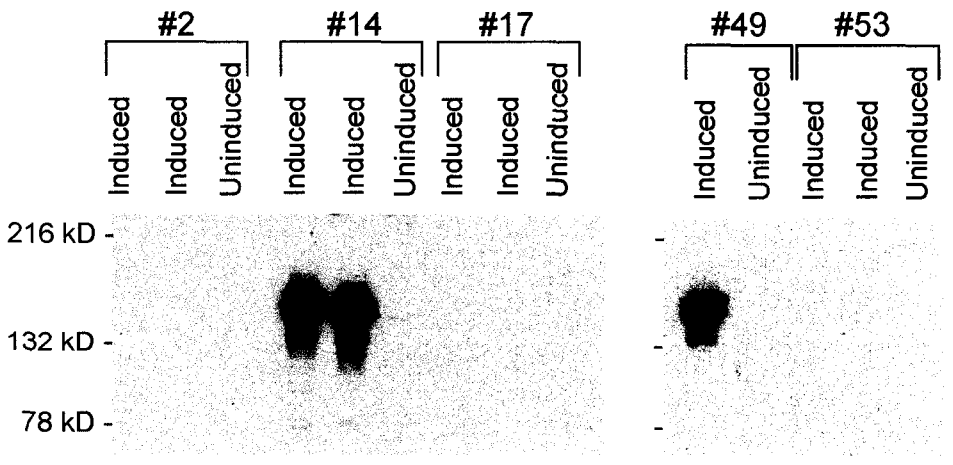
132 kD
78 kD

C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Induced
Uninduced
216 kD
132 kD
78 kD

Table II: Testing potential TRECSLuc lines

ID #	Sex	αMHC induction*	MCK induction*	Background	Breeding
#2	M	0	N/A	-	-
#3	M	N/A	0	-	-
#5	M	0	N/A	-	-
#8	F	N/A	100-10000	Minimal	Het X CBA
#14	M	8000	N/A	Some	Died
#17	F	0	N/A	-	-
#49	M	500	10-1000	Negligible	Het X CBA Het X Het
#53	F	0	N/A	-	-

*Inductions are fold over uninduced sibling controls
Ranges for MCK induction are from different muscles in multiple mice

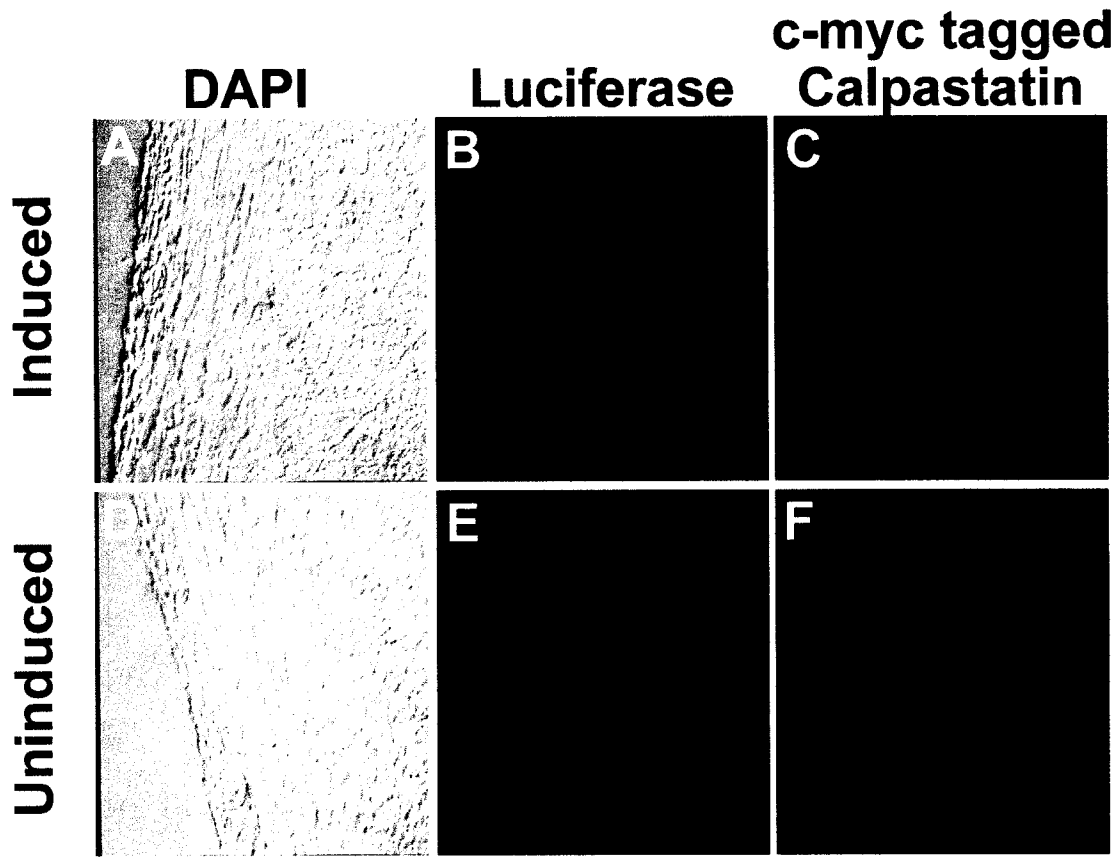


Figure 5: Luciferase and c-myc antibody staining in compound transgenic heart sections. A and D) DAPI overlaid phase image of stained sections shows nuclear stain and morphology of sections. B and C) Antibody staining of sectioned Dox induced compound transgenic heart tissue (transgenic line #14) shows luciferase (B) and transgenic calpastatin (C) staining throughout the myocardium. E and F) Lack of luciferase (E) and transgenic calpastatin (F) staining in an uninduced sibling compound transgenic mouse.

Testing the MCKrtTA transgenic lines

Two different sets of injections were necessary to generate functional transactivator lines expressing rtTA in a skeletal muscle specific pattern. F0 offspring were genotyped and mice positive for the MCKrtTA transactivator construct were mated to wild type C57BL/6J mice. At three weeks of age, a subset of transgene positive F1 progeny was assayed by RT-PCR for skeletal muscle-specific expression of rtTA. Figure 6 shows the expression profile of a typical transactivator line. A PCR product derived from rtTA RNA was observed in most skeletal muscles tested but not in many other tissue types, including the heart, liver, stomach, and kidneys. As previously reported for this modification of the MCK promoter, expression was not seen in the tongue or diaphragm (Shield et al., 1996; and Stephen Hauschka, personal communication). Of note, rtTA expression was also detected in the testis, a site of expression reported for many transgenes driven by tissue specific promoters.

On the first round of MCKrtTA injection, we did not yet have our own transresponder line to cross the mice to for functional analysis. The original two transactivator lines were therefore crossed to a previously described functional transresponder line. This α5-1 transresponder line (Valencik and McDonald, 2001) contains the bi-directional TRE driving expression of a truncated human α5 integrin subunit (Schreiner et al., 1989) and the reporter gene luciferase when induced with rtTA and doxycycline. Luciferase assays were done on a series of tissues from pups that were demonstrated to be double transgenic via PCR analysis. A full series of tissues including

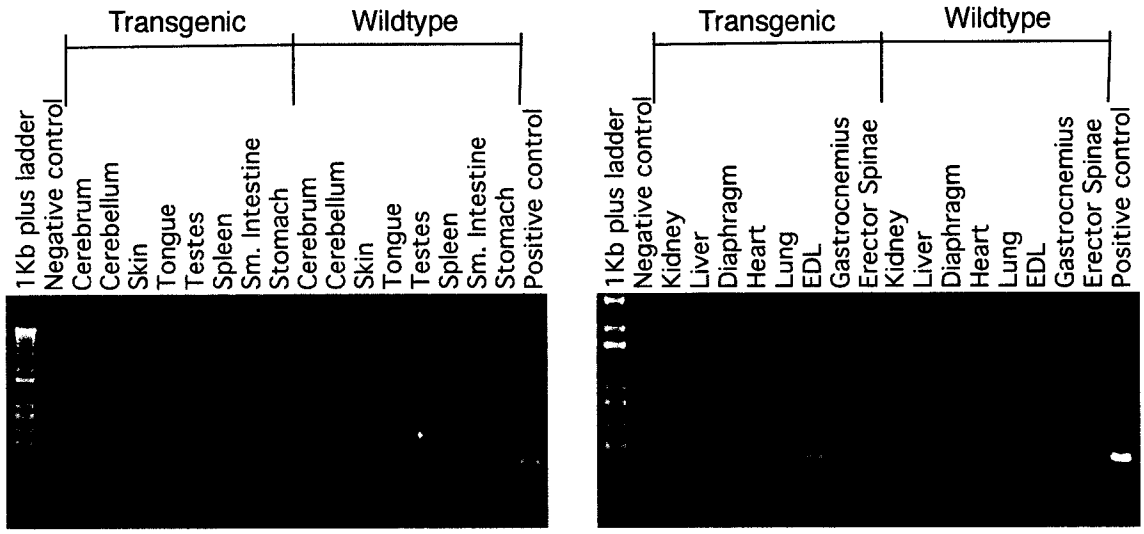


Figure 6: MCKrtTA transgenic mice express rtTA mRNA specifically in skeletal muscles. mRNA isolated from a panel of tissues from MCKrtTA transgenic and wildtype mice were subjected to RT-PCR using primers for rtTA. rtTA expression is observed in the skeletal muscles (EDL; gastrocnemius, and erector spinae muscles) of transgenic mice, but not in the heart or in any non-muscle tissues, except for the testes. Faint bands were sometimes observed in cerebrum and cerebellum samples. rtTA expression in wildtype mice was not observed in any tissue. Abbreviations: EDL, extensor digitorum longus; sm., small

four different skeletal muscles and thirteen additional tissues were tested on multiple double transgenic progeny (three and four induced pups respectively), but none showed any positive induction (data not shown). Because these mice did show RNA expression in a specific and appropriate pattern of muscle tissues, additional crosses were set up with a different transresponder line. F1 progeny from one of the MCKrtTA lines were crossed to a bi-directional transresponder line designed with two reporter genes, Green Fluorescent Protein (GFP) and β-galactosidase. Visual analysis of embryonic day 14 pups for GFP did not show fluorescence in the muscles, perhaps owing to difficulties involved in transmission of light through the skin. Embryos were then fixed and stained with X-gal to detect β-galactosidase activity. Again there was no evidence of over-expression in these pups. This experiment was repeated in embryonic day 18 pups to ensure pups were old enough to express the transgene with the same negative results. One additional set of pups was tested with an enzymatic assay for β-galactosidase activity in tissues from three-week-old pups. After these results also indicated that the mice were not producing functional transactivator (rtTA) protein, we eliminated these two lines and started with another round of injections to generate more transactivator lines.

Since two TRECSluc transresponder lines had already been shown to be inducible by the time the second round of MCKrtTA mice were ready to be tested, F1 progeny from three of the five (one F0, although testing positive for transgenic DNA did not produce any positive pups (0/34) while another F0 died giving birth to her first litter,

thereby preventing further analysis of both) new genetically positive transactivators were crossed to these transresponder lines. Resulting compound hemizygous offspring were assayed for luciferase activity in the presence or absence of Dox. Figure 7 compares luciferase activities from tissue extracts of an induced (mouse #111-13 in Table III) versus an uninduced compound transgenic mouse, and a wildtype sibling (hemizygous #8 X hemizygous #111MCKrtTA). Extremely high luciferase activities were observed in some Dox treated compound transgenic mice within appendicular skeletal muscles, whereas luciferase activities were undetectable in wild type and uninduced transgenic mice in any tissue assayed. Table IV summarizes the generation of the rtTA transgenic lines.

A more thorough analysis of luciferase activities in siblings from several independent crosses between three transactivator lines (#91, #102, and #111 MCKrtTA) and two transresponder lines (#49 and #8 TRECSluc) is shown in Table III. Variability in luciferase levels was observed between the two transresponder lines, with #8 often showing higher inducible gene expression than #49. Variable luciferase levels were also observed between different transactivator lines, indicating that careful selection of both transactivator and transresponder lines is important for optimal Dox-dependent transgene expression. As has been observed in other tet-on transgenic systems (Furth et al., 1994; Valencik and McDonald, 2001; Robertson et al., 2002), luciferase activities also varied between siblings carrying identical transgenes. For the #8 X #111MCKrtTA cross,

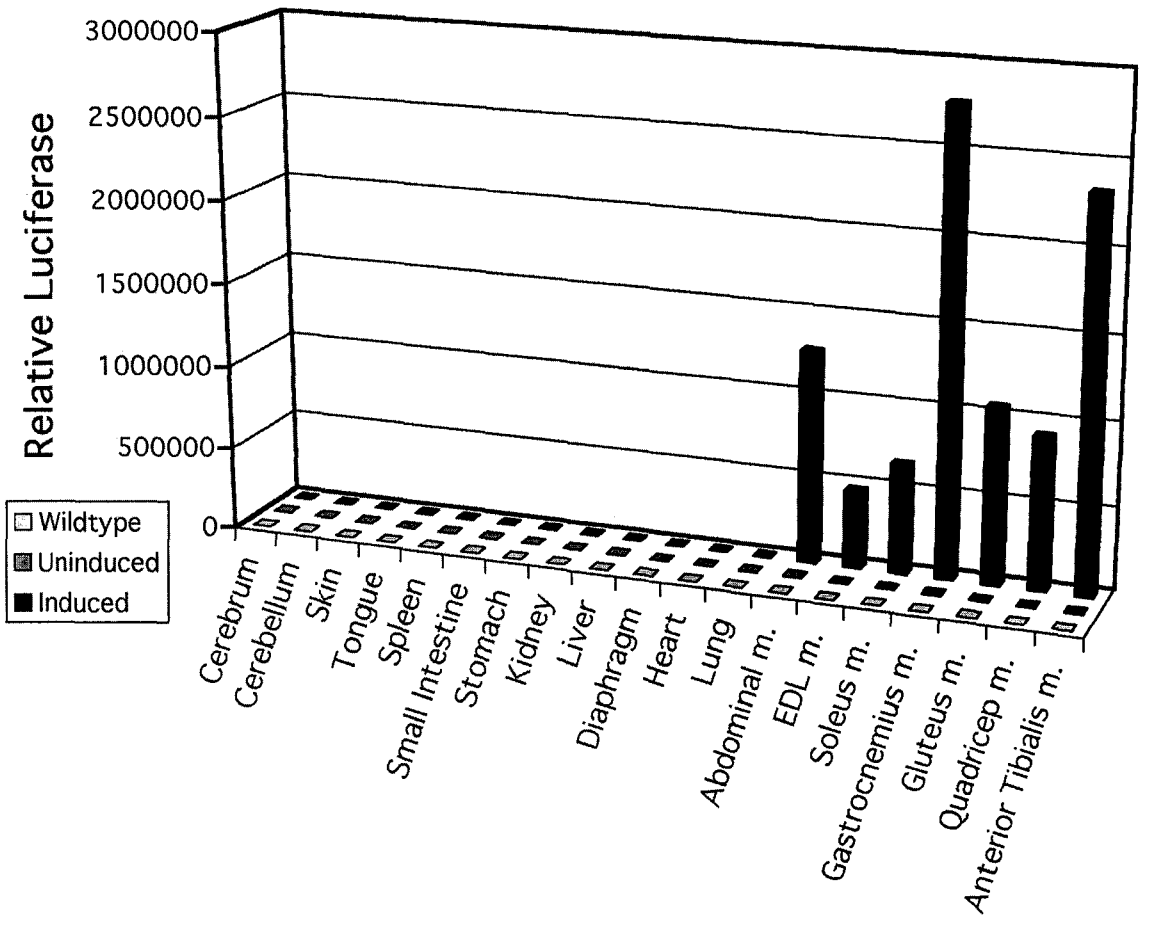


Figure 7: Luciferase assays demonstrate high level, muscle-specific inducibility of the construct by MCKrtTA *in vivo*. Extracts of tissues isolated from sibling control (uninduced) or Dox treated (induced) compound transgenic animals, or from a wildtype mouse, were assayed for luciferase activity. Extremely high luciferase levels were observed only in skeletal muscles of induced (Dox treated) transgenic mice.

Table III: Luciferase activity of induced compound transgenic mice

	Liver	Heart	Abs	EDL	Soleus	Gastroc	Gluteus	Quads	Ant. Tib.
#49 Responder									
#91-19	nd	nd	nd	14	2	nd	52	21	nd
#91-7	nd	nd	13	nm	nm	15	102	nm	nm
#91-20	nd	nd	547	26	22	540	2984	626	239
#102-2	nd	nd	50	38	nm	29	93	916	nm
#102-12	nd	nd	1993	336	27	367	836	167	277
#111-15	nd	nd	28	6	53	20	549	37	nd
#111-9	nd	nd	508	1579	nm	1926	12598	19336	nm
#8 Responder									
#111-10	nd	nd	nd	2	3	3	120	227	2
#111-4	nd	4	1373	2	2	514	80	76	29
#111-14	2	3	6	3426	1179	1445	521	157	545
#111-5	4	2	53142	63	5	5259	14562	1294	524
#111-17	nd	nd	10	141498	3061	1113	30537	10832	38438
#111-1	2	52	776	1941	25622	147601	50092	116143	47136
#111-2	19	7	906150	81500	17984	906200	1528694	4894992	16025
#111-13	145	109	1271642	465946	656290	2769166	1054432	911445	2326863

nm: not measured nd: not detectable (indicates value was within WT background range)
Abbreviations: Abs, abdominal; EDL, extensor digitorum longus; Gastroc, gastrocnemius; Quads, includes all four quadricep muscles; Ant. Tib., anterior tibialis

Table IV: Testing potential MCKrtTA lines

ID #	Sex	mRNA	Ability to induce	Background*	Breeding
#6	M	Yes	None	-	-
#9	F	Yes	None	-	-
#91	F	Not tested	Moderate/weak	No	Eliminated
#98	F	Not tested	Not germline	-	-
#102	F	Yes	Moderate	No	Homozygous line
#111	M	Yes	Variable	Sometimes	Homozygous** Het X Het
#114	F	Not tested	Died with first litter	-	-

*Background indicates expression in other tissues and may be caused by leakiness of the TRE

**Homozygous breeding is troublesome although individual homozygous mice are healthy and outcross well

shown in Table III, luciferase activities varied from almost undetectable to 2.7 million luciferase units in extracts of the same skeletal muscles from different mice. Mice showing the highest luciferase levels in skeletal muscles also showed low but detectable expression in the liver and heart.

Transgene expression was also assayed by western blot, using a myc antibody recognizing the carboxyl terminus myc-tag on the calpastatin transgenic protein. Consistent with results obtained using luciferase assays, myc-tagged calpastatin protein was detected at high levels in skeletal muscles of the induced mice (Figure 8). As reported previously (Cong et al, 1998), several bands corresponding to calpastatin were observed, including a doublet at 145kDa corresponding to full-length type II calpastatin protein, a slightly smaller 135kDa band, and an additional band of approximately 110kDa (although the predicted size of full-length calpastatin is approximately 78kDa, the protein runs anomalously on SDS-PAGE). Specific proteolysis, multiple translation initiation sites and alternative splicing of calpastatin mRNAs have all been reported and contribute to the multiple calpastatin protein sizes observed (Croall and Demartino, 1991; Cong *et al.*, 1998).

To examine the intracellular distribution of transgenic calpastatin protein, myc-tagged calpastatin was visualized by immunofluorescence within sections of skeletal muscles from induced and uninduced sibling animals. Consistent with luciferase and western blot results, myc-tagged calpastatin was detected within skeletal muscles from

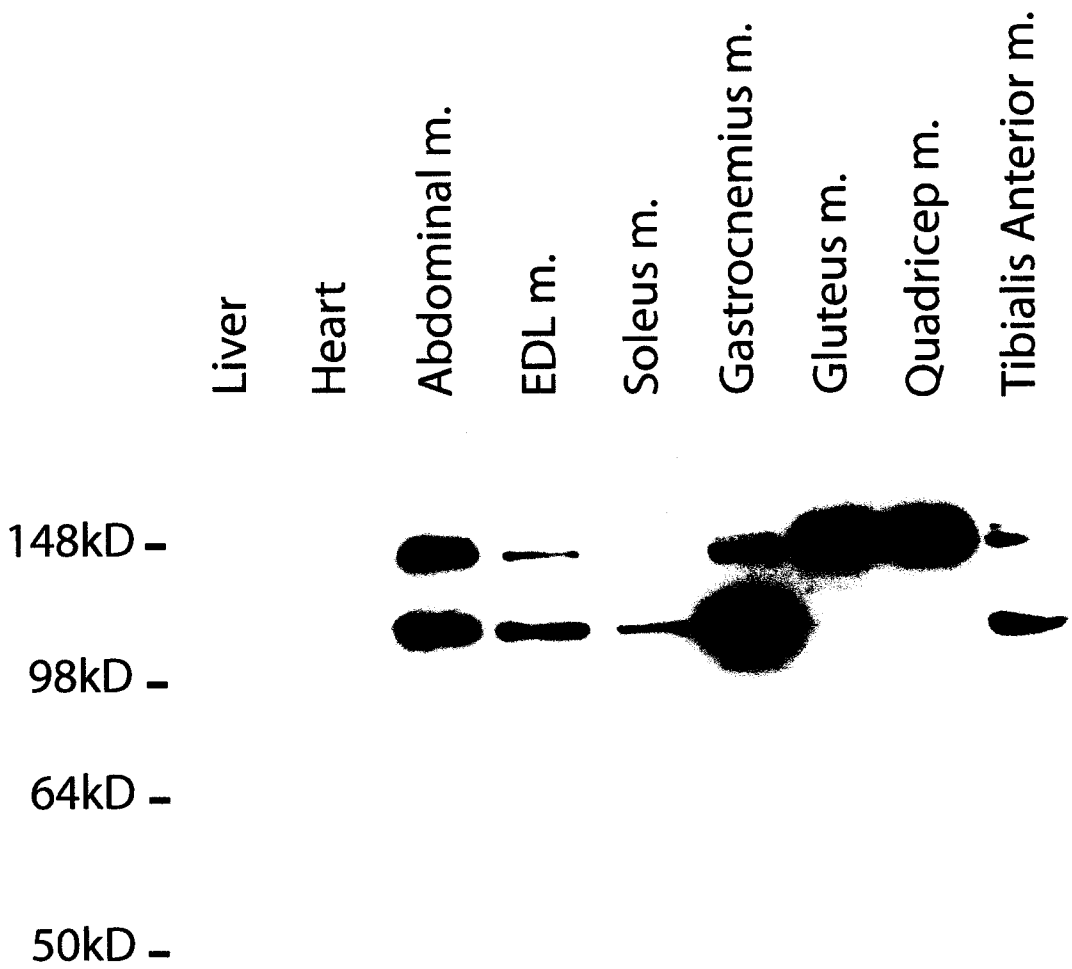


Figure 8: Western blot analysis demonstrates inducible muscle-specific expression of the calpastatin transgene. Polyclonal antibody to the c-myc tag on the transgenic calpastatin identifies multiple bands of sizes previously described for calpastatin protein in muscle tissues, while no expression is detected in non-muscle tissues. Band sizes include a doublet of 145kD and 135kD, plus a smaller band around 110kD. Sizes of the molecular weight markers used are indicated.

Dox treated animals (Figure 9). Staining was more intense in some fibers, but was widely distributed across the muscles examined.

Loss of transgene overexpression

Consistency of transgenic overexpression rapidly became a problem. While original test animals showed induction via luciferase assays, additional studies demonstrated that most of the induction levels seen with MCKrtTA/TRECSluc compound transgenics were not high enough to visualize the calpastatin on a western blot and were difficult or impossible to see with antibody staining on sections. One particular litter induced exceptionally well with some luciferase activity that went out of the range of our detection if samples were not diluted first. But even with this litter, there were some compound transgenics that barely induced at all and sibling mice from a litter born only a few weeks later did not produce any substantial luciferase activity. Table V summarizes the raw luciferase readings from the gastrocnemius muscles (a muscle that generally induced well when compared to other muscles) of all the MCKrtTA/TRECSluc compound transgenic, doxycycline induced mice. Variability within experiments on the same litter, between experiments of pups from the same parents, and among different crosses altogether but treated together demonstrate the lack of consistent inducibility in this system. Modifications in doxycycline delivery were also tried to more uniformly treat the mice, including subcutaneous insertion of time-release pellets for consistent drug delivery. No mice induced with this method. Doxycycline supplemented food was then used as the only food source available to the mice. This food was also dye labeled for

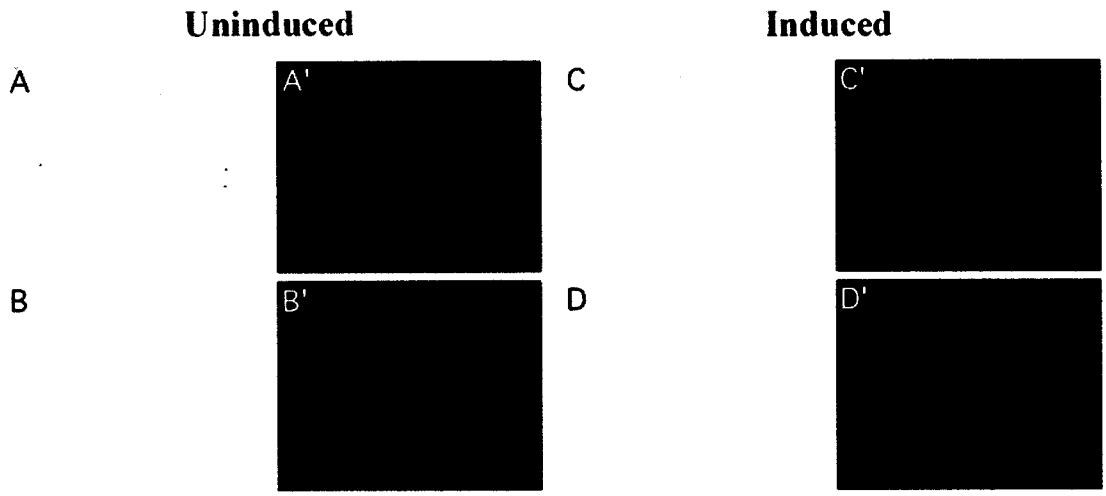


Figure 9: Immunocytochemical localization of transgenic calpastatin protein within skeletal muscles. Transgenic calpastatin protein was visualized by indirect immunofluorescence within skeletal muscles from induced and uninduced compound transgenic sibling animals (antibody to c-myc tag). Phase and corresponding fluorescence micrographs of transverse or longitudinal sections of gluteus muscles from uninduced (A, A' and B, B') or induced (C, C' and D, D') mice are shown. Myc-tagged calpastatin was only detected in fibers of muscle from the induced mouse. Some variability is noted in the intensity of staining between the fibers of the induced sections.

Table V: Summary of luciferase results from gastrocnemius muscles of Dox induced compound transgenic mice

	Cross	raw lucif induction	# induced mice	age (months)	method	days induced+HLS	generation
	49TRE X 102rtTA	0-115	5	1	food	6	F5(CBA) X F5
*	8TRE X 111rtTA	0-0	6	3.5	food	6	F4(CBA) X F4
	8TRE X 111rtTA	0-8	10	5	food	4+11 (2 on ground)	F4(CBA) X F4
	8TRE X 111rtTA	0-0	6	4	pellets	4+11	F4(CBA) X F4
	8TRE X 111rtTA	0-0	3	3.5	pellets	3+12	F4(CBA) X F4
	8TRE X 111rtTA	0-0	2	1	water	3+12	F4(CBA) X F4
	8TRE X 102rtTA	0.2-0.8	2	2	water	4.5	F3 X F2
	8TRE X 91rtTA	357-544	2	2	water	4.5	F3 X F2
*	8TRE X 111rtTA	0-49	3	2	water	4.5	F3 X F2
	8TRE X 111rtTA	19-out of range (>9999)	7	1	water	4.5	F3 X F2
**	49 X 111	0.3-0.5	3	3	water	2.5	F2 X F1
	49TRE X 102rtTA	2.0-65	2	2	water	2.5	F2 X F1
	49TRE X 111rtTA	2	1	3	water	5.5	F2 X F1
	49TRE X 102rtTA	39	1	2.5	water	5.5	F2 X F1
	49TRE X 91rtTA	0-35	2	1.5	water	5.5	F2 X F1
	49TRE X 111rtTA	483	1	1	water	6.5	F2 X F1
	49TRE X 102rtTA	11	1	1	water	6.5	F2 X F1
	49TRE X 91rtTA	2	1	1	water	6.5	F2 X F1

* Induction experiments enclosed in these boxes were perfomed on pups from the same parents

** Induction experiments enclosed in this box were performed on pups from the same sets of parents (see cross column)
Induction sets are also clustered together if mice were induced at the same time

further reassurance that the food was being ingested (coloring affected feces within 24 hours). No major induction was seen with this either. Even when going back to completely different lines (generally using #111MCKrtTA X #8 TRECSluc, switched to #102MCKrtTA X #49 TRECSluc), the induction levels seen with the one strong litter could not be replicated.

Upon realizing that the overexpression system was not working, experiments were designed to test the MCKrtTA lines. First, RT-PCR was performed to ensure that the transactivator mRNA was still being produced. Figure 10 shows the results from four independent mice that were assayed for rtTA mRNA. Both the #111 mice and the #102b mouse clearly showed muscle specific expression of transactivator (bands in gastrocnemius and quadriceps muscle, but not in heart or liver). Mouse #102a however showed very weak expression of transactivator even though a control PCR for collagen (data not shown) demonstrated good isolation of RNA for those PCR's. This data suggests that regulation at the mRNA level is possible and may be preventing expression in some of the mice, however many of the mice are still producing rtTA mRNA and none of them are inducing. Previous studies with two earlier generated transactivator lines also showed mRNA expression of rtTA but could never induce the responder, suggesting that inhibition of overexpression could be happening at another level. The second to last lane of Figure 10 reiterates this point. A sample of gastrocnemius muscle tissue was isolated from a compound transgenic (MCKrtTA and TRECSluc) mouse that had been induced for six days and showed no induction of reporter protein. A portion of this tissue was

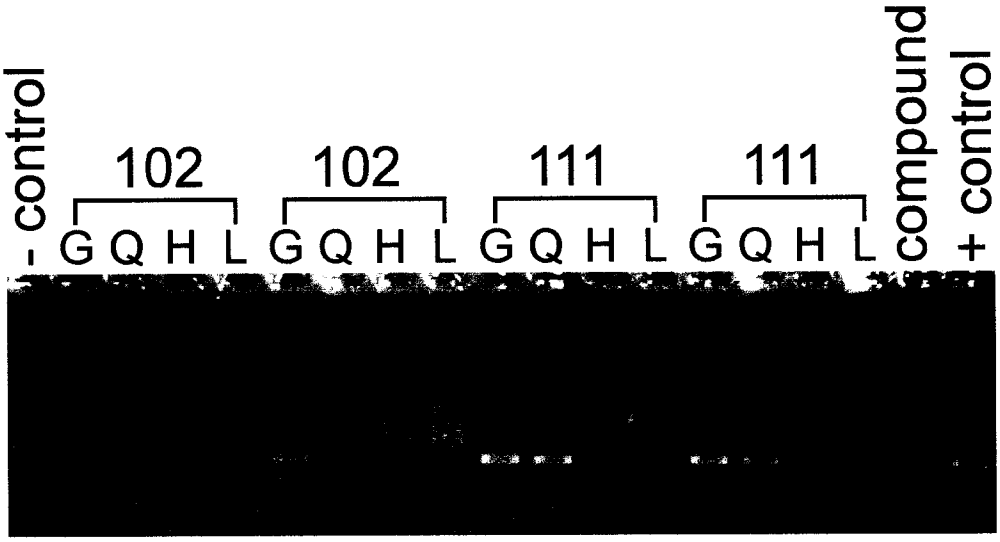


Figure 10: RT-PCR shows rtTA mRNA in later generation MCKrtTA mice and in compound transgenic induced muscle that did not overexpress luciferase. mRNA isolated from select tissues from two MCKrtTA #102 and two MCKrtTA #111 transgenic mice were subjected to RT-PCR using primers for rtTA. An additional gastrocnemius muscle sample was also run from an induced compound transgenic mouse (#111MCKrtTA X #8) that did not express luciferase or calpastatin. Although expression in #102a is weak, rtTA expression is observed in the skeletal muscles (gastrocnemius and quadricep) of all five transgenic mice, including the dox treated compound transgenic mouse that did not express luciferase or calpastatin. No expression was seen in the heart or liver samples. Abbreviations: G, gastrocnemius muscle; Q, quadriceps muscle, H, heart; L, lung.

used for RT-PCR and the results clearly demonstrate that rtTA mRNA is present, even in this mouse that did not functionally induce (compound lane in figure 10).

Discussion

Here we report a tet inducible system for gene expression within skeletal muscles of mice using a muscle-specific MCK promoter to drive expression of a modified rtTA cDNA in which the codon bias has been optimized for expression in mouse (Valencik and McDonald, 2001). Several of these transactivator lines were generated and crossed to transresponder lines containing the bi-directional TRE promoter driving luciferase and a myc- his_6-tagged cDNA encoding the calpain protease inhibitor calpastatin (TRECSluc). Transactivator and transresponder lines were crossed and analyzed for transgene expression following induction with Dox. Results show that high level, muscle-specific inducible expression can be achieved with this system.

Variability of transgene expression between lines of transgenic mice is a common observation in tetracycline regulatable systems, and more generally in transgenic mice (see, for example Furth et al., 1994; Dobie et al., 1997; Kistner et al., 1998; Valencik and McDonald, 2001; Robertson et al., 2002). During initial testing of our transactivator and transresponder lines, several genetically positive MCKrtTA transactivator lines failed to express the transgene, and several TRECSluc transresponder lines failed to show any Dox inducible gene expression after crossing with a functional transactivator line. After preliminary screening of multiple lines for transgene expression and Dox responsiveness, three MCKrtTA transactivator lines and two transresponder lines were selected and maintained. Compound transgenic mice resulting from crosses between these transactivator and transresponder lines showed Dox dependent, muscle-specific transgene

expression, although variability was observed between the different crosses. Variegated expression of transgenes has been reported between transgenic lines, between individual mice, between tissues and even between cells of the same tissues, and has been attributed to position effects relating to the site of transgene integration, number of copies inserted, and the size and origin of transgene DNA (Martin and Whitelaw, 1996). Methylation of transgenes has been reported in transgenic mice (Guy et al., 1997; Schumacher et al., 2000), and may represent the primary mechanism by which transgenes are inactivated. Transgenes often insert in multiple copies at the same integration site, and repeat induced gene silencing has been shown to reduce transgene expression through increased methylation and chromatin compaction at the insertion site (Garrick et al., 1998). Variable transgene expression has been overcome by the inclusion of locus-controlling regions such as those identified for the β-globin and CD2 loci (Grosveld et al., 1987; Festenstein et al., 1996), and by insertion of transactivator or transresponder constructs within larger DNA fragments such as YACs (Robertson et al., 2002). Our findings are consistent with many other transgenic studies and illustrate the importance of screening through transactivator and transresponder lines to identify those capable of high-level transgene expression.

Comparison of luciferase levels in offspring derived from multiple crosses also revealed large variability in Dox-dependent inducibility between siblings carrying identical transgenes. For the eight siblings of the #8TRECSluc X #111MCKrtTA cross shown in Table I, luciferase levels varied in gastrocnemius muscles from 3 to 2,769,111

relative luciferase units. Sibling variability has been observed in other studies using the tetracycline inducible system (Furth et al., 1994; Valencik and McDonald, 2001; Robertson et al., 2002) and may similarly result from selective methylation. Some evidence suggests that sibling variability may be strain dependent, possibly due to strain specific variations in heterochromatin modifiers (Opsahl et al., 2002). When a C57BL/6J transgenic line showing low and variable expression of Dox regulated LacZ was crossed with a CBA/Ca mouse, first generation mice were found to express a LacZ reporter at uniformly high levels (Robertson et al., 2002). Since we originally used mice of mixed C57BL/6J background, we backcrossed a generation of mice into the CBA background hoping to obtain more uniform transgene expression. These outcrosses however, failed to recover or normalize transgene expression.

Administration of Dox in drinking water represents another potential cause for the sibling variability observed in our study. Various methods have been used to help ensure continuous and uniform Dox administration, including oral gavage, intraperitoneal injection, inclusion in chow, and subcutaneous implantation of time-release pellets (Furth et al., 1994; Robertson et al., 2002). Although the persistent observations of sibling variability in multiple studies using all of these methods argues against differences in Dox uptake as the sole cause, we thought it may contribute to variability in some cases. Therefore, we tested levels of expression using the time-release pellets (Innovative Research of America) and the 200mg/kg doxycycline green dye chow (Bio-Serv). Neither of these methods produced better or more consistent overexpression results.

The 1256[3Emut] version of the MCK promoter was selected to drive expression of rtTA because it has been thoroughly tested in transgenic mice and shows very low or no expression in the heart and non-muscle tissues (Shield et al., 1996). Although the MCK gene promoter is activated at the onset of skeletal muscle cell differentiation, the Tet-on system is apparently not functional prior to E13.5 in mouse embryos (Federov et al., 2001). The MCKrtTA transactivator line should therefore be useful for muscle specific inducible gene expression from E13.5 onward. Previous transgenic analysis of the MCK promoter has, however, shown higher activity in fast fibers, with reduced but detectable activity in slow fibers (Johnson et al., 1989). This variability in MCK expression may be related to different mechanisms for energy utilization in fast versus slow fibers. Despite this heterogeneity, the MCK promoter has been used for many transgenic studies in which widespread transgene expression in skeletal muscles was required (see, for example Cordier et al., 2000; Naya et al., 2000; Larochelle et al., 2002). Generalized muscle-specific expression was observed, demonstrating that MCK promoter activity is sufficient to drive significant levels of transgene expression in the majority of muscle fibers.

In summary, we have shown that transgenic mouse lines in which the rtTA cDNA has been placed under control of the MCK promoter provide a valuable reagent for Dox dependent inducible gene expression in skeletal muscles of mice. Crossing of MCKrtTA transactivator lines with transresponder lines containing the Dox responsive bi-directional

TRE driving expression of luciferase and calpastatin showed high level, tightly regulated expression of both transgenes. Use of the MCKrtTA lines described in this study should therefore prove useful in combination with different transresponder lines for investigating a wide range of questions relating to skeletal muscle development, physiology and pathology.

CHAPTER III

Degradation of Skeletal Muscle Proteins: A Comparison of Calcium Inductions and Hindlimb Suspensions

Abstract

Extended time in space has been shown to elicit biochemical, physiological, and structural changes in skeletal muscle that result in overall atrophy of the muscles. Calpains are calcium activated neutral proteases that specifically degrade target proteins of the myofibril. This degradation is mediated, not only by calcium, but also by a tightly regulated balance between calpain and its inhibitor, calpastatin. Inhibition of calpain by calpastatin has been used in many studies to prevent muscle degradation. These data suggest that inhibition of calpains via overexpression of calpastatin could prevent the muscle degradation seen in microgravity models such as hindlimb suspension. Transgenic mice were generated to test this hypothesis, but as discussed in Chapter II of this dissertation, did not consistently or sufficiently overexpress calpastatin. Therefore correlation studies were performed to obtain data comparing the degradation of hindlimb suspended mouse myofibrillar proteins to those of ground control myofibrillar proteins briefly incubated in Ca^{2+}. Four proteins known as targets for calpain degradation (titin, nebulin, troponin T and desmin) were selected for analysis. Each of these proteins has specific well-defined degradation products that can be visualized with SDS-PAGE or western blot analysis. Lateral gastrocnemius muscle was isolated for all experiments for consistency. Tissue from 12 day hindlimb suspended mice was first compared to sibling ground control mice. The resulting pattern of degradation for titin, nebulin, troponin T, and desmin in hindlimb suspended animals was then compared to the degradation caused by tissue incubation in a 100mM calcium chloride solution for various amounts of time

ranging from no incubation to 15 minutes of incubation. Degradation responses of titin and nebulin in hindlimb suspension clearly mimicked that seen with the calcium incubations. Increase in degradation products of troponin T were also seen with hindlimb suspension and calcium incubations. Desmin protein degradation, however, did not occur in hindlimb suspension and was only moderately seen in the calcium incubations. Taken together these data suggest that calpain target molecules, titin, nebulin, and troponin T are all rapidly targeted for degradation in hindlimb suspension and that the degradation patterns correlate with those induced by calcium, implicating calpain as a mediator of this degradation.

Introduction

Skeletal muscles remain highly dynamic throughout adulthood, responding to external signals and stimuli to undergo atrophy, hypertrophy, repair and shifts in fiber type that result in altered contractile properties (Cameron-Smith, 2002). Maintenance of muscle size, in particular, is an active process requiring continuous muscle function, and is regulated by a balance of protein synthesis and protein degradation (Goll et al., 2003). There are two major systems currently thought to regulate the degradation aspect of muscle homeostasis, the calpains and the proteasome.

Calpains are calcium dependent neutral proteases found in all vertebrate cells. Originally discovered as calcium activated protease activity in muscle homogenates, calpains were noted for their ability to degrade sarcomeric Z-lines (Busch et al., 1972; Dayton et al., 1975). Since their discovery, significant effort has been directed towards isolating these calpains and characterizing the other elements of the calpain system to better understand how calpain activity is regulated (Ueda et al., 1998; Goll et al, 2003). In skeletal muscle, calpain targets include a limited set of structural proteins at the cell membrane and several select molecules of the Z-disk. Titin, nebulin, filamin, desmin, talin, troponin T, vimentin and fibronectin have all been shown to be cleaved by calpain *in vitro* (Dourdin et al., 1999; Robson et al., 1997) while other muscle molecules, such as myosin heavy chain and actin, are unaffected. The cleavage of myofibrillar components

specifically targets the Z-disk. While titin and nebulin are cleaved at sites of attachment in the Z-disk, the intermediate filament protein desmin is cleaved such that it disrupts the attachment of the Z-disk to the sarcolemma and releases Z-disk proteins such as α-actinin (Dayton et al., 1975 and 1976). Other myofibrillar components that are cleaved by calpain include troponin T and I as well as tropomyosin and C-protein, all of which contribute to the stability of the thin and thick filaments (Dayton et al., 1975 and Ho et al., 1994). This limited proteolysis, targeting structural proteins of fully assembled contractile units and cleaving them into defined protein fragments, suggests that calpain proteolysis is the initiator of normal myofibrillar protein degradation.

The proteasome is a large ubiquitous ATP- and ubiquitin-dependent proteolytic system. Involvement of this proteolytic system has also been implicated in skeletal muscle degradation (Solomon et al, 1998 and Mitch et al., 1999). However, unlike the calpain system, the proteasome cannot degrade cytoskeletal complexes or intact myofibrils (Solomon and Goldberg, 1996). Instead, it appears to have a major role in further degrading the released protein fragments (Solomon and Goldberg, 1996 and Sorimachi et al., 1994) as well as breaking the actin and myosin molecules down into smaller peptides or amino acids (Dayton et al., 1975; Eble et al., 1999; and Goll et al., 1999). Knowledge of the roles of these two proteolytic systems suggests a two-step model for turnover of myofibrillar proteins and implicates calpain as the early rate-limiting step of myofibrillar degradation.

Adult muscle undergoes a normal flux in protein turnover where muscle fiber proteins are continuously being degraded while simultaneous protein synthesis occurs at a similar rate to maintain a relatively constant muscle size. If calpain is the rate-limiting proteolytic system, increasing the activity of calpain should shift the balance toward excessive protein degradation, thereby decreasing myofibrillar protein stability and resulting in muscle atrophy. The opposite should also be true such that blocking calpain activity shifts the balance of protein to favor protein synthesis resulting in larger fibers and overall muscle hypertrophy.

Correlation between elevated calpastatin protein levels, and therefore reduced calpain activity, and increased muscle size have been demonstrated *in vivo* as a result of β-adrenergic receptor agonist addition to bovine systems (Parr et al., 1992). β-adrenergic receptors are G-protein linked receptors that, when stimulated, activate the cAMP pathway. Further evidence from our laboratory shows that calpastatin transcription is upregulated by constitutively active cAMP (Cong et al., 1998). Therefore, stimulation of β-adrenergic receptors can increase the amount of calpastatin that is being expressed in the treated muscle cells. This rise in calpastatin levels corresponds to an increase in myofibrillar size and overall skeletal muscle hypertrophy. This data suggests that increased calpastatin levels inhibit calpain activity, thereby reducing protein degradation rates to create an imbalance in muscle homeostasis that leads to larger muscle fibers.

Increases in calpain activity have the opposite response and show increased skeletal muscle protein degradation. Several studies have demonstrated myofibrillar degradation in postmortem tissue is increased with calcium incubation (Maruyama et al., 1981 and Delgado et al., 2001). Complimentary additional studies have demonstrated that this degradation can be mimicked by incubation with μ-calpain (Huff-Lonergan et al., 1996) and that partial prevention of this degradation can be seen with concentration dependent addition of calpastatin (Geesink and Koohmaraie, 1999).

Muscle atrophy is not limited to postmortem cases, but is a serious and potentially debilitating result of muscle disuse, extended time in space, old age, and diseases such as muscular dystrophy. In cases where it has been studied, muscle wasting correlates with a rise in calpain activity (Spencer and Tidball, 1992; Spencer et al., 1995). Although initiated by different causal factors, the resulting myofibril protein breakdown arises via a common response to muscle disuse. A secondary response by calpain has been implicated in several of these cases (Spencer and Mellgren, 2002; Dayton et al., 1979; and Arakawa et al, 1983). Cell culture studies using serum starved L_8 muscle cells overexpressing a dominant negative m-calpain or a calpastatin inhibitory domain reduced overall protein degradation by 30-63% (Huang and Forsberg 1998), again indicating a crucial role for calpains in muscle degradation.

Model systems such as hindlimb suspension, which create rapid loss of muscle mass, have been used to study weightlessness, disuse, and age related changes *in vivo*.

Hindlimb suspension is a well accepted, ground based model for simulated microgravity that closely mimics the deleterious effect of near zero gravity in skeletal muscle (Thomason and Booth, 1990 and Fitts et al., 1986). Several studies using this model have combined it with β-adrenergic receptor agonist (ß-agonist) treatment to lessen the simulated anti-gravity/disuse and aging effects. As would be expected with the downstream increase in calpastatin activity, muscle loss was offset by these treatments (Chopard et al, 2001; Linderman et al. 1994; and Apseloff et al., 1993). Even at physiological levels of clenbuterol (ß-agonist) treatment, modest atrophy reduction was seen in slow fiber type muscles during hindlimb suspension (Chen and Alway, 2000). These studies demonstrate that β-agonist addition can partially counteract the degradation-favored imbalance of muscle homeostasis and lessen the atrophy induced by this muscle-wasting model. Assuming that β-agonist restoration of muscle mass is mediated via an increase in calpastatin, it should also be possible to "rescue" atrophying muscle with direct addition of calpastatin to suppress heightened calpain activity.

Although mice have been generated to test this hypothesis, induction levels have been insufficient to perform the desired experiments. Therefore, to further test the role of calpain in myofibrillar degradation during hindlimb suspension, studies here look at direct targets of calpain in tissues from hindlimb suspended animals and tissue from ground control animals after brief calcium incubations. Analysis of titin, nebulin, and troponin T demonstrate the expected degradation with both methods, and support the

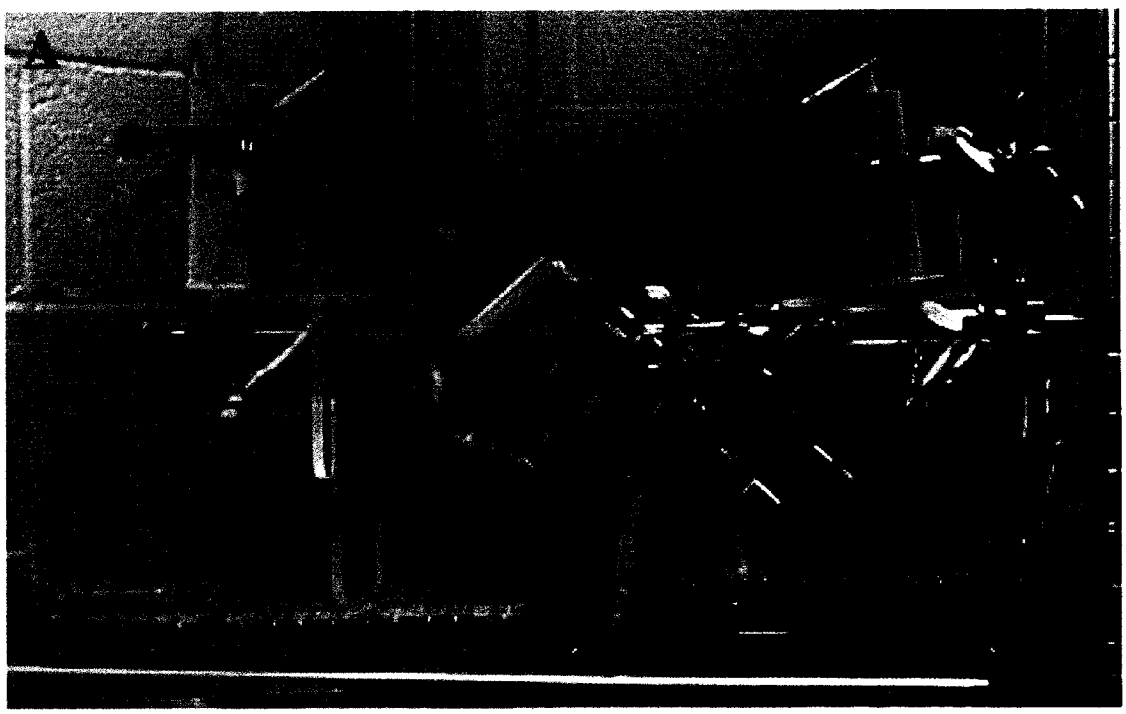
theory that early degradation is mediated by calpain degradation targeting specific proteins of the Z-disk.

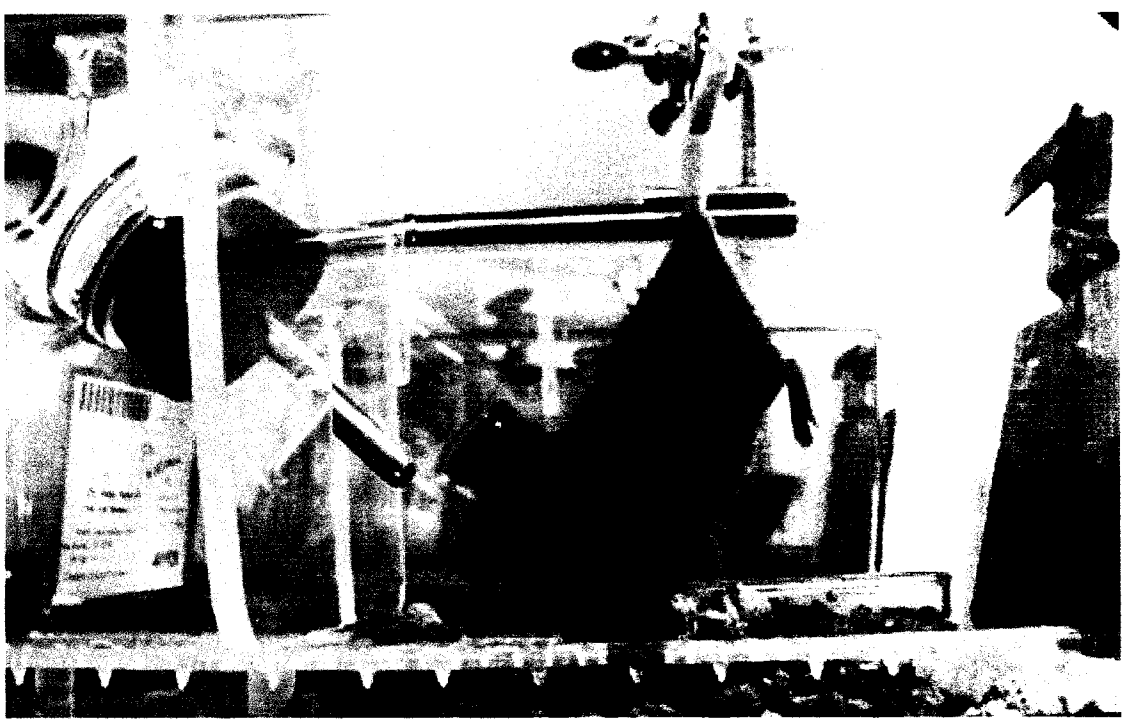
Materials and Methods

Hindlimb Suspensions

A 12cm piece of 20 gauge wire was bent to form a circular loop 1/3rd of the way in, and 2cm flaps folded up on each end. Mouse tails were cleaned with 100% EtOH and placed in a holder with the tail out. When the tail was dry, the wire was attached as close to the base of the tail as possible (loop more proximal) with medical adhesive tape (Johnson and Johnson) wrapped tightly around each section at least 8 times. Wire end tabs were then folded onto the tape ends to further secure them to the tail. The circular loop of the metal wire was placed on a swivel hook on 20lb test fishing line coming through a circular plexiglass "roof" of the suspension apparatus above the cage. The hind limbs of the mice were elevated 30+° so that weight could not be placed upon these limbs (Adjustments were made by adjusting the clamps holding the apparatus as well as via tension on the fishing line attached with an alligator clip at the top of a 50ml syringe mounted to the plexiglass roof). The mice were able to support themselves upon their forelimbs on a perforated floor. In this position, the mice were able to move in a 360° rotation with full access to food and water but were unable to touch the floor or sidewalls with their hindlimbs. Mouse water bottles were fitted with long rat sipper tubes, which were arranged to come through a custom cut plexiglass wall so the mice had access to water without access for climbing on the bottles. 60mM petri dish bottoms were glued to the corner of the fitted floor and were filled with crushed food so the mice could not stack and climb on the food. See Figure 11 for photographs. Hindlimb suspensions lasted

Figure 11: Hindlimb suspension model for microgravity. A) Hindlimb suspension cages were aligned on a four-tiered rack such that 12 hindlimb suspension and 10 ground controls could be housed on the same rack. B) Close up of an individual hindlimb suspended mouse to show detail. Mice were adjusted to about 45° in specially modified cages by a metal wire adhered to their tail so that their hindlimbs could not touch the ground or sidewalls of the cage.





twelve days, and mice were examined twice daily to check comfort status, suspension, food, and water. In early experiments mice were weighed daily on a specially constructed apparatus that maintained suspension and fit on the scale. The stress this induced in the mice eventually reduced the weightings to before, after, and once or twice during the suspensions. Also of note is the importance of age in mice selected for hindlimb suspension. Our first round of experiments was performed with young, four week old mice and did not show the typical body mass loss that should be seen with hindlimb suspension. To avoid this complication of developmental growth, all mice suspended for future experiments (and those included here) were at least 3.5 months old. A study looking at hindlimb suspension in young adult (8 month) and aged (22 month) rats indicated that an even more dramatic response is seen with older animals (Deschenes et al., 2001).

Tissue Collection

Mice were euthanized individually in a non-pressurized CO_2 chamber. Mice that were suspended were euthanized in a suspended state so as not to incur tears in the muscles or initiate any associated biochemical pathways. Multiple tissues were isolated and quickly frozen in labeled Eppendorf tubes in liquid nitrogen from each control and suspended mouse including heart and liver and a series of muscles from the right hind limb that included, medial and lateral gastrocnemius muscle, soleus muscle, extensor digitorum longus muscle (EDL), and anterior tibialis muscle. The left hind limb was skinned and cut off just proximal to halfway up the femur. The entire limb was placed dorsi-flexed in 15ml conical tube of freshly prepared 4% paraformaldehyde/PBS and kept

in 4°C for 24-36 hours before being equilibrated back into PBS and prepared for frozen or paraffin sections.

Tissue Sample Preparation

Approximately ten milligrams of the lateral gastrocnemius tissue was removed for analysis of each protein in SDS-PAGE or Western blot analysis. Samples subjected to calcium incubations were performed for varying time lengths from 30 seconds to 15 minutes in 100mM $CaCl_2$. The tissue section was then removed and rapid frozen in liquid nitrogen. All samples were then pulverized into a fine powder while submerged in liquid nitrogen. The powder was added to 1 ml of sample buffer (SDS-SB) containing a final concentration of 0.0625M Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.025% bromophenol blue, and freshly added 5% β-mercaptoethanol. The samples were then vortexed vigorously, snap frozen in liquid nitrogen, and stored at -80° C until used.

SDS-PAGE Analysis

Three gel systems were used to analyze the proteins of varying molecular mass. A 3% polyacrylamide gel without stacker was found to produce optimal resolution of the high molecular weight proteins, titin and nebulin. These gels were made using 3% fresh acrylamide (acrylamide: N, N'-bis-methylene acrylamide = 29:1 [wt/wt]), 15 % (vol/vol) glycerol, 1.5 M Tris-HCl (pH 8.8), 0.1% (wt/vol) SDS, 0.1% (wt/vol) ammonium persulfate (APS), and 0.16% (vol/vol) N'N'N'N'-tetramethylethylenediamine (TEMED). The addition of glycerol, fresh acrylamide, and fresh APS produced the clearest visibility of titin and nebulin. Prior to the addition of APS and TEMED, the solution was de-

gassed for 10 min to ensure polymerization after acrylamide preparation. The gels were allowed to polymerize overnight due to their low percentage. An 8% polyacrylamide gel with a 4% stacker was used to visualize desmin. The 8% gel consisted of 8% stock acrylamide solution (acrylamide: N,N'-bis-methylene acrylamide = 29:1 [wt/wt]), 1.5 M Tris-HCl (pH 8.8), 0.1 % (wt/vol) SDS, 0.1% (wt/vol) APS, and 0.06% TEMED. The 4% stacker contained 4% stock acrylamide solution, 1.0 M Tris-HCl (pH 6.8), 0.1% (wt/vol) SDS, 0.1% (wt/vol) APS, and 0.1% (vol/vol) TEMED. A 12% polyacrylamide gel was used to visualize troponin T. These gels were made using 12% stock acrylamide solution, 1.5 M Tris-HCl (pH 8.8), 0.1% (wt/vol) SDS, 0.1% (wt/vol) APS, and 0.04% TEMED. A 4% stacker gel was used over the 12% gel. The stacker contained the same solutions as the stacker previously described for the 8% gels.

The samples were prepared as previously described and then heated for 20 min. The optimal temperatures for titin and nebulin samples are 57°C and 65°C respectively (Granzier and Wang, 1993). Troponin T and desmin samples were also heated at 57°C. The samples were spun down to avoid loading extracellular components onto the gels. An amount of 50 microliters of each sample was immediately loaded onto the gels. The gels were run at 4°C in a running buffer consisting of tris base, glycine, and SDS. Titin gels were run for 5.5 hr at a constant voltage setting of 80V. Nebulin gels were run for 6.5 hr at 80V. Desmin gels were run at 50V for 30 min until samples entered the stacker and then the voltage was increased to 200V for 4.5 hr. Troponin T gels were run at 50 V for the first 30 min and then 200V for 7.5 hr. Titin and Nebulin gels were stained with

Coomassie Blue in a solution containing 0.1% (wt/vol) Coomassie brilliant blue R-250, 40% (vol/vol) methanol, and 10% (vol/vol) glacial acetic acid for 12 hours and destained with several changes of the same solution without the Coomassie brilliant blue R-250.

Western Blot Analysis

After electrophoresis gels were transferred to nitrocellulose membranes at a constant current setting of 90mA for 12 hrs at 4°C in a transfer buffer containing trizma base, glycine, and methanol. The nitrocellulose membranes were stained with PonceauS to check the transfer. The membranes were incubated in blocking solution containing 5% (wt/vol) nonfat dry milk in TBS-T (Tris-buffered saline with 0.1% Triton X-100). Blots were then incubated for 1 hr at room temperature in primary antibody diluted 1:1000 in blocking solution. Primary antibodies that were used in Western blotting included monoclonal anti-nebulin (NB2, Sigma), monoclonal anti-troponin T (JLT-12, Sigma), and polyclonal anti-desmin (Sigma). The blots were then washed 3 x 10 min in blocking solution. Then the membranes were incubated with secondary antibody diluted in blocking solution for 1 hr at room temperature. Secondary antibodies included donkey-anti-rabbit HRP (Jackson Immunoresearch) diluted 1:2000 for desmin blots, and donkey-anti-mouse HRP (Jackson Immunoresearch) diluted 1:1000 for nebulin and troponin-T blots. The blots were then washed 3 x 10 min in TBS and then exposed to a 1:1 mixture of Pierce Supersignal West Pico chemiluminescent substrate solutions for 5 min. The protein bands on the nitrocellulose membranes were detected with autoradiographic film.

Results

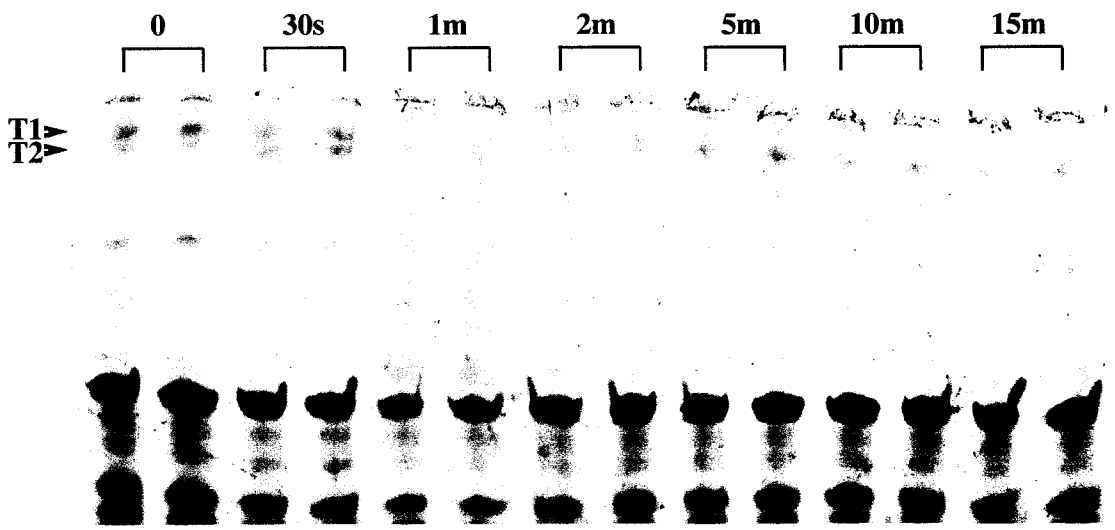
Titin

Demonstration of previously described calcium mediated T1 to T2 degradation is shown in Figure 12A via a Coomassie stained 3% polyacrylamide gel. Due to the relative instability of titin, whole muscle preparations were used to avoid any postmortem degradation of titin. A relaxing buffer (Granzier and Wang, 1993) was initially used to release titin from any myosin interactions, however this did not enhance T1/T2 resolution and so was not used. Samples were only heated to 57°C (Granzier and Wang, 1993) prior to loading to prevent smearing or disappearance of the titin T1 and T2 bands. Intact titin, T1, from mouse lateral gastrocmenimius muscle is clearly seen at ~3000kD with no calcium incubation (Figure 12A, 0 sec). The large T2 degradation product can also be seen in this sample at ~2400kD (as previously described in Suzuki et al., 1996). Western blot analysis was used to verify the positions of the T1 and T2 bands and to identify smaller degradation products of titin (data not shown). In this case, resolution of T1 and T2 was compromised by the sensitivity of the antibody to detect the many intermediary titins between T1 and T2, and therefore less informative then the Coomassie stained gels. Silver staining was also used to increase sensitivity of detection, however this method also converted T1/T2 into one smear (data not shown). Progressive degradation of T1 to T2 and eventual decrease of T2 to smaller degradation products is shown in duplicated lanes of tissues incubated with calcium for increased amounts of time ranging from 30 seconds to 15 minutes. This cleavage of titin from T1 to T2 occurs very rapidly but

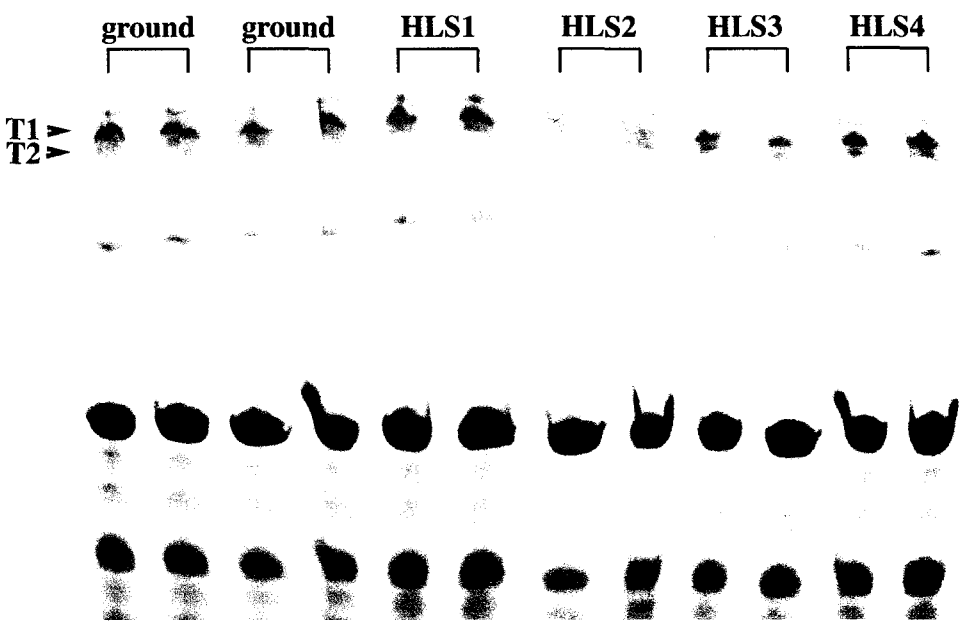
Figure 12: Calcium mediated and hindlimb suspension induced degradation of titin. A) Lateral gastrocnemius muscle samples were incubated in calcium for 30 sec, 1min, 2 min, 5 min, 10 min, or 15mins and run on a 3% polyacrylamide gel to compare titin T1 and T2 levels with unincubated sample. A gradual change can be seen where T1 is the prominent band at time 0 then T1 and T2 are almost even at 30sec, they become a smear at 1 and 2 minutes which is later resolved into a prominent T2 band that gradually disappears with increased incubation time. B) Lateral gastrocnemius muscle samples from two ground control mice are similarly compared to samples from four different hindlimb suspended mice. Ground control mice show dominant T1 bands whereas an increase in T2 can be seen in some of the hindlimb suspended mice (most notably in HLS3). Smearing like that seen in 1min calcium incubations was seen in HLS2, suggesting that hindlimb suspension causes degradation in titin similar to that seen with brief incubations in calcium. All samples run in duplicate.

TITIN

A Calcium Incubations



B Hindlimb suspensions



includes an intermediary period where T1, T2, and intermediary products appear as one large smear (previously described in Ho et al., 1997), suggesting titin is a susceptible target for calcium mediated degradation in a calpain specific cleavage pattern.

To analyze the role of calpains as the early step in weightlessness atrophy, several mice were subjected to 12 days of hindlimb suspension to mimic zero gravity in their hind limbs. Some sibling mice were maintained as ground controls and were placed in individual cages in the same animal room. Comparison of titin in two ground control versus four hindlimb suspended mice is visualized by Coomassie stain of a 3% polyacrylamide gel (Figure 12B) with duplicate lanes run for each mouse. Consistent with incubation in calcium, tissue from hindlimb suspended mice showed a decrease in the T1 intact titin. Hindlimb suspension mouse #1, #3 and #4 showed an increase in T2 when compared to the ground control mice, this early degradation is consistent with very brief calcium incubation times (Figure 12A, 30sec). Hindlimb suspension mouse #2 demonstrates a slightly more advanced stage of degradation where a decrease in T1 is replaced by the prominent smearing of T1, T2, and intermediary degradation products, corresponding to later calcium incubation around 2 minutes. Although the degree of degradation in the four hindlimb suspended mice shows some variability, the switch in prevalence from T1 to T2 and smaller in both systems suggests the degradation occurs via the same mechanism.

Nebulin

Nebulin is another major target of calpain proteolysis (Huff-Lonergan et al., 1996). Due to the large size of intact nebulin at 900kD, first analysis was performed with Coomassie staining of 3% SDS polyacrylamide gels (Figure 13). This analysis showed a faint but progressive decrease of the intact nebulin as well as a decrease of its varied degradation products at between 300 and 600kD (previously described in Ho et al., 1997) as time incubated in calcium increased from 0-15minutes (Figure 13). Samples were run in duplicate and loading consistency can be analyzed by comparison of myosin heavy chain (MHC).

Because these slight variations are difficult to analyze and may be complicated by smearing of nebulin as it is completely degraded to the lower molecular weight product (Ho et al., 1997), Western blot analysis was performed using a commercially available monoclonal antibody to nebulin (NB2, Sigma). A limited set of samples was run to allow for easier handling of the gels for transfer. Figure 14 A clearly shows diminished intensity of the primary 900kD band and also shows loss of nebulin degradation products not previously visualized with the Coomassie stained gels. Decreased intensity of all nebulin bands was also seen with hindlimb suspended mice when compared to the ground control mouse (Figure 14 B). Although not as profound as 10 minutes of calcium incubation, hindlimb suspension consistently showed decreased presence of all nebulin bands. Variations can be seen in the degree of loss of nebulin products in the different hindlimb suspended mice.

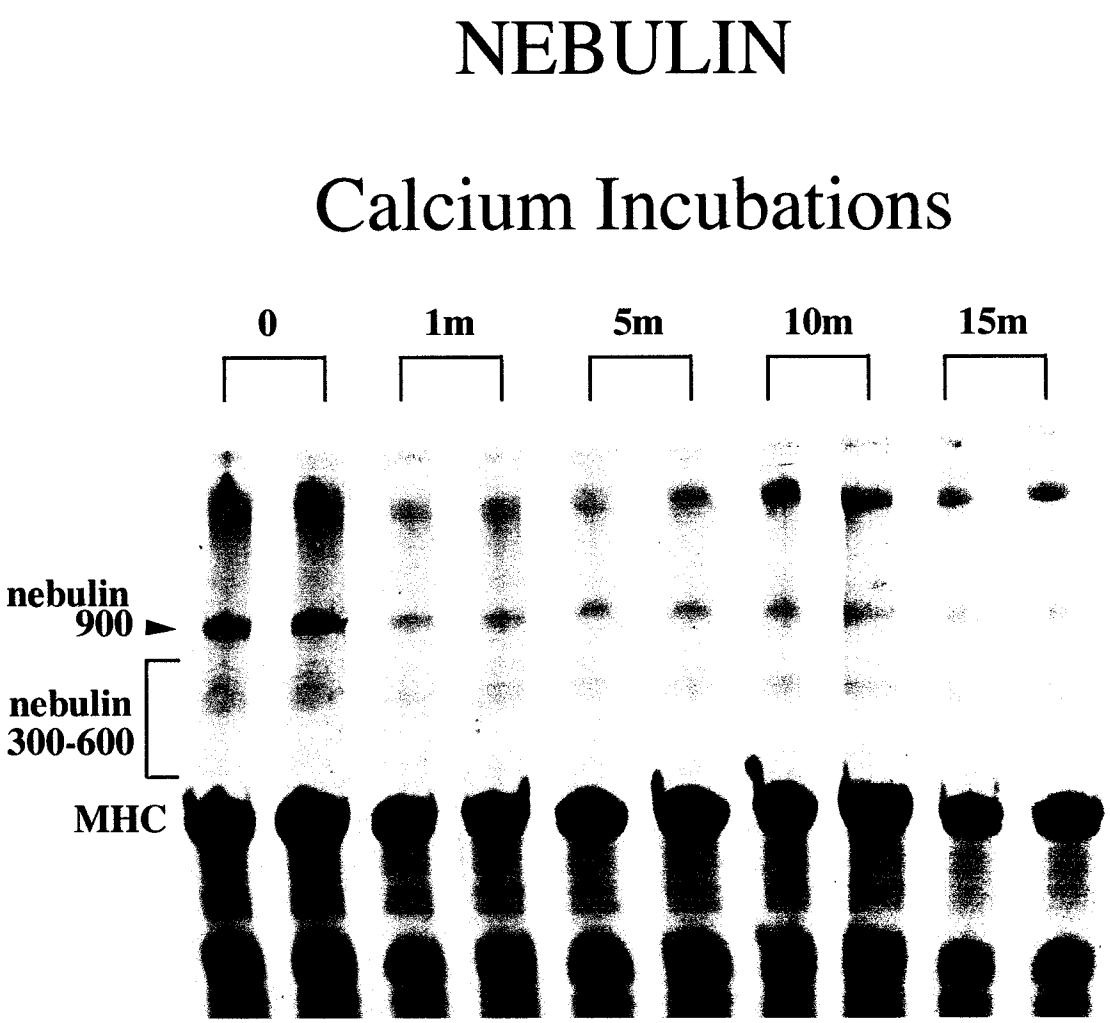


Figure 13: Calcium mediated degradation of nebulin. Lateral gastrocnemius muscle samples were incubated in calcium for 1min, 5min, 10min, or 15mins and run on a 3% polyacrylamide gel to compare nebulin degradation to unincubated sample. A gradual loss of the nebulin 900kD, as well as the intermediary degradation products that run between 300 and 600kD, can be seen over time. All samples run in duplicate.

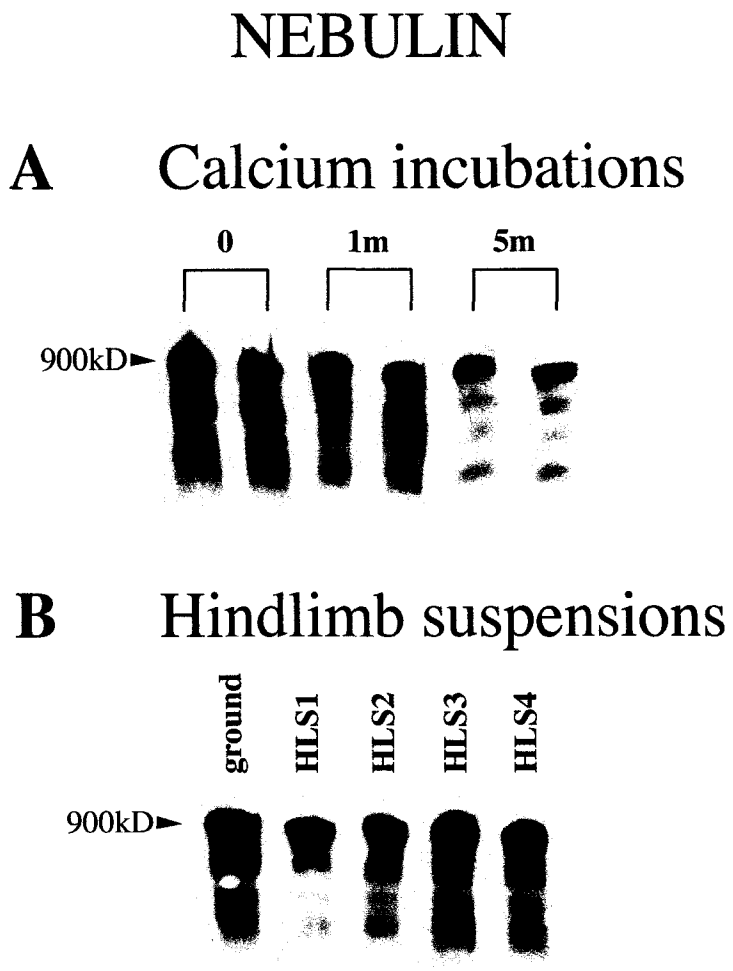


Figure 14: Western blot analysis of calcium mediated and hindlimb suspension induced degradation of nebulin. A) Lateral gastrocnemius muscle samples were incubated in calcium for 1 or 5 mins and run on a 3% polyacrylamide gel before being transferred for Western blot analysis. Anti-nebulin antibody recognizes the prominent 900kD nebulin as well as several degradation products between 300 and 600kD. Incubation with calcium shows a rapid decrease in all of these nebulin bands over time. Samples run in duplicate. B) Lateral gastrocnemius muscle sample from a ground control mouse was similarly compared to samples from four different hindlimb suspended mice. As seen with brief calcium incubation, most of the hindlimb suspended mice showed a decrease in all forms of nebulin when compared to the ground control, suggesting that hindlimb suspension causes degradation of nebulin similar to that seen with calcium incubations.

Troponin T

Troponin T has three isoforms that run tightly on SDS-PAGE at about 43kD (Ho et al., 1994 and Huff-Lonergan et al., 1996). Several specific degradation products can be seen ranging from 28-35kD. Western blot analysis with Troponin T specific monoclonal antibody, JLT-12, looks at calcium incubations of lateral gastrocnemius muscle to demonstrate the rapid transition through these degradation products (Figure 15A). While a significant proportion of the lower troponin T isoform remains, most troponin T gets serially degraded over the 0-15 minute calcium incubation time course. Four distinct bands of troponin T degradation products can clearly be identified. Figure 15B looks at the effect of hindlimb suspension on troponin T degradation. Samples from 5 different hindlimb suspended mice are compared to a sibling ground control mouse. Hindlimb suspended samples were run in duplicate like those of calcium incubations and clearly show increased degradation products when compared to the ground control tissue. While each of the mice have various degrees of each of these products, most of them demonstrate degradation that corresponds to between 1 and 10 minutes of calcium incubation.

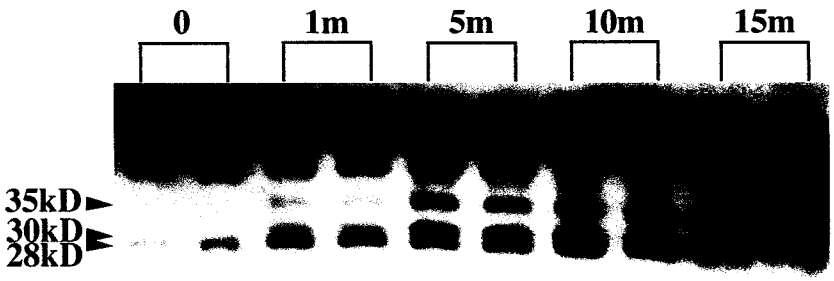
Desmin

In vitro studies of proteolytic degradation of desmin indicate that desmin is extremely susceptible to calpain cleavage (O'Shea et al., 1979 and Robson et al., 1997). Western blots using a polyclonal antibody to desmin on calcium incubated lateral gastrocnemius samples is shown in Figure 16A. Only a slight decrease is seen in the

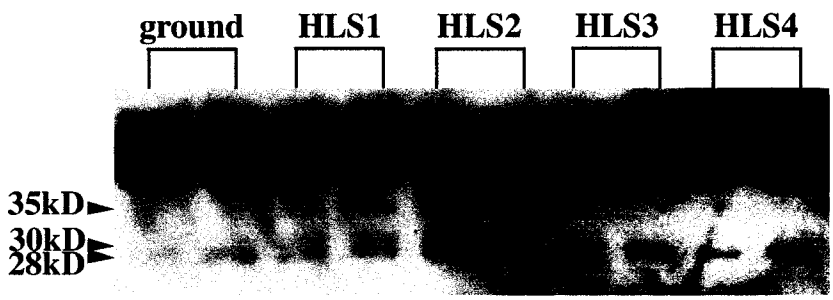
Figure 15: Western blot analysis of calcium mediated and hindlimb suspension induced degradation of troponin T. A) Lateral gastrocnemius muscle samples were incubated in calcium for 1min, 5min, 10min, or 15mins and run on a 12% polyacrylamide gel before being transferred for Western blot analysis. Anti-troponin T antibody recognizes the cluster of bands near 43kD representing the multiple isoforms of troponinT as well as the specific degradation products at 35kD, 30kD and 28kD. Incubation with calcium shows a gradual decrease in the intact isoforms that corresponds with a gradual increase in the smaller degradation products. B) Lateral gastrocnemius muscle samples from a ground control mouse and four different hindlimb suspended mice were similarly compared. Although a decrease in the 43kD cluster is not discernable, appearance of the 35 and 30kD bands can be seen in HLS1 and HLS2 and a more prominent 28kD band can be seen in HLS3 and HLS4 when compared to the ground control sample. The increase in these degradation products is similar to that seen with brief calcium incubations, and suggests that hindlimb suspension causes degradation of troponin T in a manner similar to that seen with calcium incubations. All samples run in duplicate.

TROPONIN T

A Calcium incubations



B Hindlimb suspensions



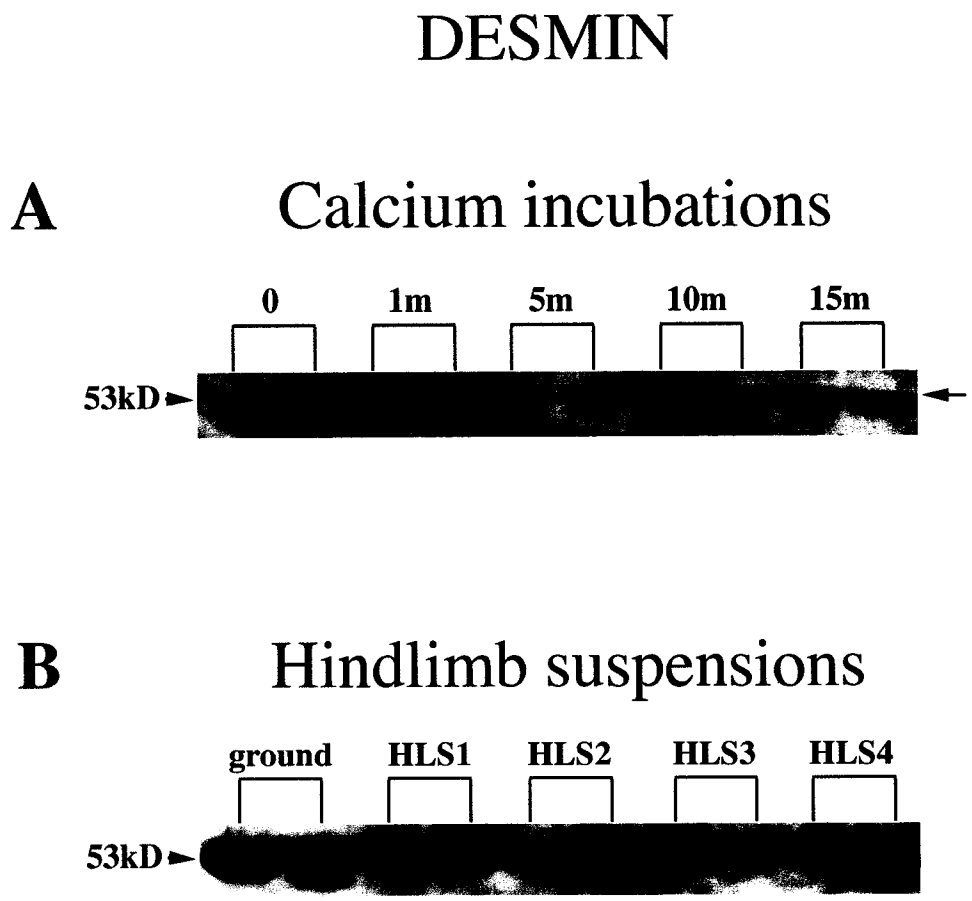


Figure 16: Western blot analysis of calcium mediated and hindlimb suspension induced degradation of desmin. A) Lateral gastrocnemius muscle samples were incubated in calcium for 1min, 5min, 10min, or 15mins and run on an 8% polyacrylamide gel before being transferred for Western blot analysis. Anti-desmin antibody recognizes a single band at 53kD. A slight decrease in desmin concentration can be seen with longer calcium incubations (5, 10, and 15min) as well as an additional, larger band in the 15min calcium incubation lanes (arrow). B) Lateral gastrocnemius muscle samples from a ground control mouse and four different hindlimb suspended mice were analyzed similarly and showed no noticeable change in desmin concentration. All samples run in duplicate.

53kD desmin band over time of calcium incubation up until 15 minutes, when a second, albeit larger, band appears. Aggregation of different degradation products or between intact desmin and degradation fragments has been described previously in postmortem sardine white dorsal muscle (Verrez-Bagnis et al., 1999) and could account for this unexpected result. Desmin from 4 different hindlimb suspended mice showed no detectable change when compared to the ground based littermate control (Figure 16B). These data suggest that desmin is not an early rapidly degraded target in hindlimb suspension induced muscle atrophy and is also relatively slow to degrade by calcium incubations when compared to the other proteins analyzed (titin, nebulin, and troponin T).

Discussion

In order to gain an understanding of myofibrillar degradation in weightlessness, we have utilized the hindlimb suspension model for microgravity induced muscle atrophy to look at degradation of specific muscle proteins. Studies shown here compare the degradation rates of four proteins in two different models of muscle degradation. All four proteins are known to be specifically cleaved by calpain into distinct characterized degradation products. In all experiments, lateral gastrocnemius muscle was isolated from ground dwelling mice and from 12 day hindlimb suspended mice and then compared by Coomassie blue stained SDS-PAGE and/or Western blot analysis. The resulting pattern of degradation for titin, nebulin, troponin T, and desmin in hindlimb suspended animals was compared to the degradation caused by tissue incubation in a 100mM calcium chloride solution for various amounts of time. Results suggest that titin, nebulin, and troponin T are rapidly degraded in hindlimb suspended mouse muscles in a pattern consistent with calcium treatment. Surprisingly, similar results were not obtained for the structural protein desmin, which had a slow degradation pattern with calcium incubations and no detectable change by hindlimb suspension. Overall these results suggest an early role for calpain-mediated degradation of the myofibrillar targets titin, nebulin, and troponin T in hindlimb suspension induced atrophy.

Titin (also known as connectin) is a large coiled string-like protein that spans one half the width of a sarcomere and forms elastic connections with thick filaments and the

Z-line to maintain mechanical stability of the muscle (Suzuki et al., 1996). Here we show degradation of this large structural protein from its intact T1 form to its primary degradation product T2 in both hindlimb suspension and calcium incubations. These data correlate with previous studies showing titin T1 to T2 degradation of postmortem tissue with the addition of calcium (Maruyama et al. 1976), which was later shown to be mediated by calpain proteolysis (Zeece et al., 1986 and Hu et al., 1989). The cleavage site that produces the T2 product is located in the I band (Kimura et al., 1992) and is important for connecting the thick filaments to the Z-disk and keeping them centered within the sarcomere (Suzuki et al., 1996). Taken together these data suggest that calpain mediated proteolysis of titin is a major contributor to the muscle atrophy induced by hindlimb suspension.

Nebulin is another structural protein of the myofibril that is targeted by calpain. Several studies suggest that a single nebulin molecule spans the entire length of the thin filament and is anchored by its C-terminus in the Z-disk (Wang, 1996). Several isoforms of nebulin exist that range in size, a variability that correlates proportionally to the length of the thin filaments in different muscles. These data, and the repetitious actin binding domain structure of nebulin, suggest that it may act as a protein ruler that binds to and regulates thin filament length (Labeit et al., 1991 and Labeit and Kolmerer 1995). Because of its multiple isoforms, nebulin is sometimes difficult to visualize as a defined band on SDS-PAGE. Here we show both SDS-PAGE and western blots to analyze the degradation of nebulin in hindlimb suspended mice compared to calcium incubated

muscle tissue. As with calpain mediated degradation (Taylor et al., 1995), western blot analysis of our hindlimb suspended mice and calcium incubated tissue shows rapid degradation of nebulin into a series of smaller polypeptides ranging from 300 to 600kD that progressively disappear with treatment. This data further implicates calpains in the direct degradation of nebulin during hindlimb suspension.

Troponins are a complex of three troponin subunits (C, I, and T) that function together to mediate actin/myosin interactions during muscle contractions. Troponin T, the elongated portion of the troponin complex, interacts with tropomyosin and actin and is thought to anchor troponin C and I dimers with the actin thin filament (Clark et al., 2002). A single troponin T gene encodes several isoforms, some of which include phosphorylation sites (Jin et al., 1992). These different isoforms lead to a cluster of bands on SDS-PAGE analysis that runs at about 43kD (Ho et al., 1994 and Huff-Lonergan et al., 1996). Calpain mediated degradation of Troponin T is identified by a rapid cleavage of the majority of these isoforms into distinct degradation products that run at 28, 30 and 35kD on SDS-PAGE. Here we demonstrate the generation of these same degradation products both with calcium incubation and with 12-day hindlimb suspension studies, suggesting that troponin T degradation during hindlimb suspension is mediated by calpain proteolysis.

Desmin is a small protein that polymerizes to form intermediate filaments that surround each Z-line and connect mofibrils to each other as well as to the cell membrane

(Lazarides, 1980) As a major component of the perisarcomeric compartment, desmin intermediate filaments are essential for the maintenance of structural and functional integrity (Capetanaki et al., 1997). Because cytoskeletal proteins play a crucial role in the resistance to imposed mechanical stress they have been proposed as likely targets for change during a modified functional demand such as hindlimb suspension. Six week hindlimb suspensions by Chopard et al. (2001) however, showed no change in desmin or α-actinin, despite the rapid loss of contractile proteins, myosin and actin. These results correspond with our finding that desmin shows no visible degradation upon two weeks of hindlimb suspension. In a similar study, Fitts et al (2000) showed that in microgravity induced adaptations of skeletal muscle, loss of contractile proteins is out of proportion compared to other cellular proteins. Calcium mediated degradation, like postmortem degradation studies, did however show loss of desmin. These findings suggest that cytoskeletal components such as desmin may be resistant to degradation while managing the structural forces applied to a living muscle, and may serve to protect the muscle from structural damage thereby allowing for adaptations like fiber type switching.

In summary, the rapid degradation of titin, nebulin and troponin T in these hindlimb suspension studies correlate with the degradation patterns of calcium and/or calpain mediated degradation and suggest that a calpain mediated pathway is initiated in hindlimb suspension that targets these molecules specifically. Loss of Z-disk attachments by titin and nebulin lead to the loss of attachment of both thick (myosin) and thin (actin) filaments respectively. These interactions are important not only for the structural

integrity of the myofibril, but also for its ability to contract. Additionally, loss of troponin T, part of the complex thought to regulate actin/myosin interactions, will further destabilize contraction essential interactions. On the other hand, lack of degradation of desmin suggests that some calpain-mediated degradation is prevented or blocked (perhaps by masking of the target proteins) to preserve the structural integrity of the overall muscle. This cytoskeletal protection would allow for maintenance of muscle fibers while rearrangement of proteins could occur to compensate for a modified functional demand. This could explain the ability, although not the mechanism, by which complete replacement of the myosin thick filaments from slow myosin heavy chain isoform to a fast myosin heavy chain isoform could occur as seen in several models of muscle disuse (Talmadge, 2000; Edgerton and Roy, 1996; and Ishihara et al., 1997).

Further studies that block calpain activity *in vivo*, either by the proposed transgenic calpastatin overexpression, injection of calpastatin, or transgenic overexpression of a dominant negative calpain, would be useful for demonstrating the role of calpain in weightlessness induced atrophy. As discussed in Chapter II, the tet-on transgenic mice generated for this overexpression did not produce consistent quantities of functional calpastatin to perform the desired studies. If they had, several experiments were planned to analyze the effects of blocking calpain-mediated degradation. In addition to degradation analysis of titin, nebulin, troponin T, and desmin (similar to those done here, only comparing HLS induced versus HLS uninduced versus ground control instead of calcium incubated samples), muscle from calpain inhibited mice would also be

analyzed by weight and cross sectional area of individual fibers. In both of these sets of experiments, reduction of hindlimb suspension-induced degradation by overexpression of calpastatin would demonstrate the critical role of calpain in mediating muscle atrophy. Muscle fiber type analysis experiments were also proposed to analyze the effects of blocking calpain activity on myosin heavy chain subtype switching. Analysis of midbelly cross sections of soleus muscle with antibodies that specifically recognize the slow (A4.951, ATCC CRL-2046) or fast (N3.36, ATCC CRL-2042) myosin heavy chain isoforms would be used to quantify the percentage of each fiber type in each experimental subgroup (HLS induced, HLS uninduced, and ground controls). Additionally, collaborative work could be done to look at the effects of calpain inhibition on changes in peak force generation in both hindlimb suspended and ground based mice. Preliminary data, like those shown here and our current knowledge of calpains in muscle degradation, suggests that these changes will be decreased or prevented by blocking calpain activity. The results of these and future experiments like them will aid in our understanding not only of weightlessness induced atrophy, but also of disuse and disease related atrophies as well as normal muscle homeostasis.

CONCLUSIONS

Four independent lines of transgenic mice were generated and maintained. Two of these lines are transgenic for the MCKrtTA transgene (#102, and #111). Of these two lines, only one (#102) can be stably maintained as a homozygous breeding colony. The #111 MCKrtTA line can generate homozygous mice, but they do not reproduce well. The other two lines contain the TRECSluc transgene (#8 and #49) and neither of these lines can be maintained as a homozygous line. Individual mice from the #49TRECSluc line can survive as homozygotes, and they will breed well to wildtype or heterozygote mice, but homozygous to homozygous crosses do not breed well. The #8TRECSluc line is not homozygous viable. Variability in homozygous viability is most likely due to the location of the inserted transgene rather than a byproduct of the transgene itself.

There is however some evidence that an rtTA transgene could be toxic to transgenic mice. Transcriptional squelching was first described in 1988 (Sadowski et al., 1988 and Triezenberg et al., 1988) as a titrating out of essential transcriptional machinery components by high levels of transactivator protein. This phenomenon results in reduced expression of other cellular genes often with a considerably cytotoxic effect. The weaker and more localized expression caused by tissue specific promoters should reduce this effect. However, as with many presumed cell specific promoters, high levels of expression were seen in the testes where it could affect reproduction in our lines. To get around this, scientists are now backtracking to make the system less efficient. Baron et

al. (1997) have used modifications to the VP16 component to reduce its potency and Strathdee et al. (1998) described modifications of the minimal promoters to drive rtTA or tTA expression combined with a bi-directional feedback loop system to maintain low expression in the uninduced state (Baron et al., 1995 and Shockett et al, 1995).

Leakiness in the TRECSluc lines could also cause problems with development and reproduction. Calpains are thought to be important for cell motility via cytoskeletal and membrane attachments, signal transduction pathways, regulation of gene expression, and apoptosis (reviewed in Goll et al., 2003). Evidence also suggests that calpains play a role in progression through both mitosis and meiosis. Recent studies have identified several calpains that are localized to the testes, and have speculated a role for calpains in spermatogenesis (Sultana et al., 2003 and Zhu et al., 2002). A unique splice variant of calpastatin has also been identified that is specifically transcribed in haploid germ cells of the testes (Li and Goldberg, 2000). Additionally, calpastatin treatment of spermatozoa acts as a meiotic inhibiting agent (Logothetou-Rell, 1995). Similarly, calpains undergo autolysis and relocalization in rat eggs that correlates with a meiotic increase in intracellular Ca2+ concentration (Malcov et al., 1997). Additional studies suggest a role for calpains in the calcium-mediated acrosome reaction that is required for sperm/egg membrane fusion events of fertilization (Li et al., 2000). Although the details of calpain's role in these cellular events is still being determined, it is plausible that even small amounts of leaky overexpression of calpastatin in the testes or ovaries of transgenic mice could affect fertility and growth.

Another form of toxicity is immune response. Latta-Mahieu et al. (2002) described a strong cellular and humoral immune response to intramuscular injection of either plasmid or adenoviral vectors containing the rtTA-M2 transcription factor. This immune response greatly reduced the duration of expression of the rtTA protein. There is also the potential of an immune response to the overexpressed transgene. Although calpastatin is normally expressed in all cells, high expression could evoke an immune response. Saulot et al. (2002) has shown that autoimmunity to inhibitory domain 1 of calpastatin occurs in both rheumatoid arthritis and in Systemic Lupus Erythematosis (SLE), and that this immune response correlates with vasculitis in SLE patients. Autoantibodies to other regions of calpastatin including inhibitory domains III and IV (Despres et al., 1995 and Schlosser et al. 1997) and an N-terminal fragment encompassing domain L and part of domain I (Lackner et al., 1998) have been identified in previous rheumatoid arthritis studies. The prevalence of this occurrence and of multiple autoantibodies generated to different regions of calpastatin suggests the possibility of eliciting an immune response with overexpression. Host immune response to rtTA or calpastatin could explain some of the sibling variability we have seen, but would not account for generational loss of transgenic expression. As described in the discussion section of Chapter II, loss of transgenic expression is most likely due to a combination of mouse C57/bl6 background and transgene methylation.

Ideally, the transgenic mice we generated would have consistently overexpressed the calpastatin transgene in an inducible manner. Hindlimb suspension studies to analyze the effect of inhibiting calpain during microgravity induced skeletal muscle atrophy were proposed. Experiments like those in Chapter III of this dissertation would have analyzed the direct effect of blocking calpain activity on the degradation of specific myofibrillar proteins titin, nebulin, and troponin T as well as the cytoskeletal desmin. We hypothesize that calpastatin overexpression would prevent or at least decrease the extent of titin, nebulin, and troponin T degradation, although longer suspensions may be required to better visualize these differences. Weight comparisons of individual muscles and measurement of cross-sectional area as well as number of fibers and localization of nuclei would also help to further analyze the effects of calpain inhibition on preventing muscle atrophy. Lastly, analysis of fiber type composition, particularly in the soleus muscle would be analyzed in cross section with fast and slow myosin heavy chain specific antibodies to see if calpastatin inhibition can prevent the slow to fast fiber type switching that is seen with extended periods of disuse.

Similar experiments to those proposed here were done by Tidball and Spencer (2002) with a traditional transgenic approach overexpressing calpastatin driven by the human skeletal muscle actin promoter. In these experiments, slow to fast fiber type switching that usually occurs in microgravity and simulated models was prevented. Degradation of muscle fibers was also decreased in the transgenic suspended animals when compared to non-transgenic suspended animals. Although some complications did

arise with early expression of calpastatin decreasing fusion in developing muscles leading to a higher number of fibers in a given cross sectional area, their experiments demonstrate a role for calpain in skeletal muscle degradation and fiber type switching of muscles in weightlessness simulation.

Despite the inability to perform the final studies proposed here, generation of the MCKrtTA mouse lines provides a valuable reagent for future muscle studies. These transactivator mice can be used in combination with any Tet transresponder line, to specifically overexpress a target gene. Because the MCK promoter restricts expression of the rtTA to skeletal muscle, specific muscular roles for proteins that have many cellular roles can be studied. Inherent to this tet-on system, use of this line will also provide doxycycline controlled temporal regulation of overexpression. Several requests have been made for these mice and a multitude of future experiments with their progeny is already underway.

APPENDIX A

Permission for Use of Published Material

Permission letter for use of copyrighted material from Kluwer Academic Publishers

Original letter indicating specific uses for copyrighted material

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28/10/2003

Re: Grill/Bales/Fought/Rosburg/Munger/Antin: *Transgenic Research* (2003), v.12, p.33-43

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Leslie van Mil

From: Joanna Lawrence
Sent: Wednesday, October 22, 2003 10:16 AM
To: Permissions NL/Europe
Subject: FW: Transgenic Research

ISSN: 0962-8819

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Joanna Lawrence
Senior Publishing Editor
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(B)
dissertation

Ms. Lawrence,

I am writing to request permission to use portions of the publication Grill, M. A., Bales, M. A., Fought, A. N., Rosburg, K. C., Munger, S. J., and Antin, P. B. (2003) Tetracycline-Inducible System for Regulation of Skeletal Muscle-Specific Gene Expression in Transgenic Mice, from Transgenic Research volume 12, pages 33-43, as a portion of my dissertation. A reference to the publication is clearly stated on the Chapter title page for the chapter containing the information. This dissertation is to be submitted in partial fulfillment of the requirement for my Doctor of Philosophy degree at the University of Arizona. The dissertation will be cataloged and microfilmed by University Microfilms Incorporated, Ann Arbor, Michigan, and will be available on demand for scholarly purposes. A letter stating your agreement would be greatly appreciated.

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Mischala A. Grill

****
Mischala Grill mischala@u.arizona.edu
Cell Biology and Anatomy phone: 520.626.6391
The University of Arizona fax: 520.626.6354
****

APPENDIX B

Research Animal Use Approval

#02-171 – "Colony Maintenance" IACUC Protocol Approval

#02-052 – "Mouse Hindlimb Unloading" IACUC Protocol Approval

Institutional Animal Care
and Use Committee

THE UNIVERSITY OF
ARIZONA®
TUCSON ARIZONA

P.O. Box 210101
Tucson, AZ 85721-0101

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 -- USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use.
The following listed proposal has been granted ***Final Approval*** according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:

#02-052 - "Mouse Hindlimb Unloading"

PRINCIPAL INVESTIGATOR/DEPARTMENT:

Parker B. Antin - Cell Biology & Anatomy

GRANTING AGENCY:

NBSRI

SUBMISSION DATE: **March 27, 2002**

APPROVAL DATE: **June 25, 2002** APPROVAL VALID THROUGH*: **June 24, 2005**

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: **June 27, 2002**

REVISIONS (if any):

MINORITY OPINIONS (if any):

Richard C. Powell

Richard C. Powell, PhD, MS
Vice President for Research

DATE: June 27, 2002

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.

Institutional Animal Care
and Use Committee

THE UNIVERSITY OF
ARIZONA.
TUCSON ARIZONA

P.O. Box 210101
Tucson, AZ 85721-0101

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 -- USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use. The following listed proposal has been granted ***Final Approval*** according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:

#02-171 - "Colony Maintenance"

PRINCIPAL INVESTIGATOR/DEPARTMENT:

Parker B. Antin - Cell Biology & Anatomy

GRANTING AGENCY:

NSBRI

SUBMISSION DATE: **September 23, 2002**

APPROVAL DATE: **December 11, 2002** APPROVAL VALID THROUGH*: **December 10, 2005**

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: **December 12, 2002**

REVISIONS (if any):

MINORITY OPINIONS (if any):

Richard C. Powell

Richard C. Powell, PhD, MS
Vice President for Research

DATE: December 12, 2002

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.

APPENDIX C

Breeding Logs for Maintained Transgenic Mouse Lines

Key

blacked out – outcrossed for experimental progeny

shaded – backcrossed to maintain the line

boxed – tissue harvested from this mouse

underlined – genetically positive for transgene, maintained

brown – litter died, documented to show trends in breeding pair

blue – indicates CBA outcross

#111 MCKrtTA breeding log

Male founder X C57/bl6J - stop 6/26/01

- Crossed to bl6 - 6 born 3/26 and 8 born 3/27 (8m, 5f, 1 died)
  - Positives (3 of 13): #2m, #5m, and #9f
- Again to bl6 8 born 4/17 (2 died early, 1m, 2f, and 3 more died) - tails 5/8 (#14-16)
  - Positives (1 of 3): #15f
- Again to bl6 - 4 born 5/6 (3m, 1f) and 7 born 5/9 (5m, 2f)- tails 5/30 (#17-27)
  - Positives (6 of 11): #18m, #19m, #21m, #22m, #24m, #27f
- Again to bl6 - 6 born 5/30 (1m, 5f) - tails 6/19 (#28-33)
- Positives (5 of 6): #28m, #29f, #31f, #32f, and #33f

F1 X F1 backcrosses - #18m X #29f and #19m X #31f

- #111/18 X #111/29 - 3 born 7/21 (2f, 1died) - tails 8/16 (#1-2)
  - Positives by PCR (2 of 3): #1f, #2f
- #111/19 X #111/31 - 3 born 7/21 (1m, 2f) - tails 8/16 (#1-3)
  - Positives by PCR (1 of 3): #1m
- #111/18 X #111/29 - 3 born 8/10 (1m 2f) - tails 9/5 (#3-5)
  - Positives by PCR (1 of 3): #4m
- #111/19 X #111/31 - 3 born 8/10 (2m 1f) - tails 9/5 (#4-6)
  - Positives by PCR(2 of 3): #5m, #6f (#111A cross)
- #111/18 X #111/29 - 10 born 8/30 (all died)
- #111/19 X #111/31 - 2 born 8/28 (1m,1f) - tails 9/10 (#7-8)
  - Positives by PCR (2 of 2): #7m, #8f
- #111/18 X #111/29 - 6 born 9/18 (2m,4f) - tails 10/9 (#6-11)
  - Positives by PCR (5 of 6): #6m, #7m, #8f, #10f, #11f (both crossed to #8TRECSluc)
- #111/19 X #111/31 - 13 born 10/1 (8m,5f) - tails 10/16 (#9-21)
  - Positives by PCR (12 of 13): #9m (#111A cross), #10m, #11m, #12m, #13m, #14m, #15m, #16m, #17f, #18f (to ßgal) #19f, #21f (both crossed to #8TRECSluc)
- #111/18 X #111/29 4 born (3m, 1f) 10/12 - tails 11/1 (#12-15)
  - Positives by PCR (3 of 4): #13m, #14m, #15f
- #111/19 X #111/31 - 10 born 11/20 (5m,4f) (#22-30)
  - Positives by PCR: #22m, #23m, #26m, #29f (crossed to 33 and kept with Todd), #30f
- #111/19 X #111/31 - 9 born 12/31 (5m,3f) – tails 1/22 (#31-38)
  - Positives by PCR (6 of 8): #33m(crossed to 29 and kept with Todd), #34m, #35m, #36f (111A), #37f, #38f

- #111/18 X #111/29 - no pups since 11/1

Backcrosses – should be homozygotes for transporting to Tucson
#111A cross is #111/19X31-9m X #111/19X31-6f (died giving birth 2/26) New female #111/19X31-36f
- 7 born (1m, 4f, 2 died) 3/22 – tails 4/2 (#1-5) – all positive by PCR

#111B cross ???

#111MCKrtTA X TRECSluc (#49 and #8) and biTREßgalGFP for testing
Cross #111/9f and #111/15f X TRECSluc #49F2-10m
- #111/9f X #49F2-10 TRECSlucm -8 born 6/11 (4m, 4f) - tails 7/3 (#1-8)
  - Double Positives: 4m and 6f (also kept #5m as WT control)
- #111/15f X #49F2-10 TRECSlucm - 8 born 6/14 (5m, 3f) - tails 7/3 (#1-8)
  - Double Positives: 5m and 6f (also kept #7f and #8f as controls)
- #111/9f X #49F2-10 TRECSlucm - 9 born 7/2 (4m, 3f, 2 died) - tails 7/24
  - Double Positives: #13f
- #111/15f X #49F2 - 5 born 7/26 (2m, 3f) - tails 8/14
  - Double Positives:
- #111/9f and 15f X #49F2 - 18 born 8/14

Cross #111/32 and #111/19X31-17 X biTREßgalGFP mouse
- #111/32 X GFPlacZ - 7 born 9/17 - tails 10/9 (#1-7)
  - All dissected 10/1
- #111/32 X GFPlacZ - 9 born 10/9 (4m, 4f) - tails 11/1 (#8-11) #12 died
  - Double Positives (2 of 8): #11m,#16f
- #111/19X31-18f X GFPlacZ – collected 9 embryos 2/12 (E13.5)
  - Double positives (all positive for rtTA):
- #111/19X31-17 X GFPlacZ – collected embryos 2/27 (E13.5)

Cross #111/19X31-19f and #111/19X31-21f X TRECSluc #8/19X24/1X2-7m
- 17 born 12/8 (11m, 6f) - tails 1/3 (#1-17)
  - Double Positives (8 of 17): #1m, #2m, #4m, #5m, #6m, #13f, #14f, #17f (#8m, #10m as controls)
- 17 born 12/28 (7m, 10f) - tails 1/22 (#18-34)
  - Double Positives (3 of 17): #21m, #25f, 27f (also kept #19m TRE only, and #24m WT as controls)
- 4 born and died
- 11 born 1/31 (4m, 7f) – tails 2/20 (#35-45)
  - Double positives(2 of 10): #41f and #45f
- 10 born 2/10 (5m,5f) – tails 3/4 (#46-55)

  - Double positives (2 of 10): #52 and#55 (also keep #51 as WT control)
- 3 born 3/3 mother sickly – all pups died
- 11 born 3/29

Cross #111/18X29-10f and #111/18X29-11f X TRECSluc #8/19X24-8m

- 8 born 12/19 (6m, 2f) - tails 1/8 (#1-8)
  - No Double Positives
- 9 born 4/1 – still need to be tailed

#111 MCKrtTA – Tucson breedings

- 111A original pan
  Pups 6/14 – 2m,1m, 2f

- Pan 1a – stopped 12/16
  Pups 7/23 – died
  Pups 8/25 – 2m, 4f
  Pups 9/29 – 5m, 1f
  Pups 10/24 – dead
  Pups 11/11 – 3m, 3m, 2f
- Panlb – 12/20 (pan3 10/18male X pan3 10/18 female)
  Pups 1/10 – 1m, 2m, 2f (#659-662) #659f, #662m
  Pups 1/31 - gone
  Pups 2/21 – 3m, 1f (#669-672) #669m, #670m
  Pups 3/18 – gone
  Pups 4/18
- Panlc – 4/2 (#669m X #659f)

- Pan 2a – pulled 12/3
  Pups 7/26 – gone
  Pups 8/16 – 4m, 2f
  Pups 9/8 – 2m, 2f
  Pups 9/30 – died
  Pups 10/28 – gone
- Pan 2b – 12/23(pan3 10/18 male X pan5 11/9 female) male died 2/20 give new male from panlb 1/10 – stopped 4/15
  Pups 1/11 – gone
  Pups 3/16 – dead

- Pan 3a – stopped 1/29
  Pups 7/25 – 1m, 4f (#46-50) - #46m, #47f, #48f, #49f, #50f

Pups 8/15 – 4m, 4f
Pups 9/8 – 3 runts (weaned from breeders 10/4)
Pups 9/30 – died
Pups 10/18 – 3m, 3m, 1f (to pan 1b, 2b, and 4b)
Pups 12/3 – 4PTS
Pups 1/13 – 2PTS

- Pan 3bHOMO – 3/27 (male exp. pan 4 and females exp. pan 2 – from orig 6/14 litter and pan3 8/15 litter
  Pups 4/17
  Pups 4/18

- Pan 4a – discard breeders 10/2/02
  Pups 7/25 – 1f (#51 = all +)
  Pups 8/25 – 2m
  Pups 9/18 – only 1, tossed with breeders
- Pan 4b – 12/23 (pan3 10/18 male X pan5 11/9 females (2)) – stopped 4/2
  n Pups 1/11 – gone
  n Pups 1/31 – gone
  o Pups 2/19 – gone
  n Pups 3/14 – gone

- Pan 5a – stopped 2/7 – good homozygous line
  Pups 7/25 – 3m, 1f, 1 died (#52-55) - #52m, #53m, #54m, #55f
  Pups 8/15 – gone
  Pups 9/4 – 2m, 5f
  Pups 9/26 – 3m, 4f
  Pups 11/9 – 3m, 4m, 3f (to pan 2b and 4b)
  Pups 12/13 – PTS
  Pups 1/17 – 3m, 3f (#663 – 668) #663m, #664m, #665m, #666f, #667f, #668f
- Pan 5bHOMO – 3/30 (male from exp. Pan7 and females from exp. pan 10 – from the 9/26 litter above)

- Pan 6a – #111 X CBA WT female
  Pups 8/3 – 2m, 1f (#60-62) - #60m
  Pups 8/24 – 1m, 1f (#63-64)
  Pups 9/4 – gone

Pups 9/22 – gone
Pups 10/13 – 4f (#163-166) – #164f, #166f

- Pan 6b – 10/23 (#60m from above X CBA WT female) – stopped 1/29
  Pups 11/22 – 3m, ?f (#653-655) no positives
  Pups 12/13 – 3m, ?f (#656-658) #656m, #658m
- Pan 6c – 4/2 (#658m, CBA WT female)
  Pups 4/25

#102 MCKrtTA breeding log

Female founder X C57/bl6J - stop 6/26/01

- Crossed to bl6 - 2 born 3/24 (2 died)
- Again to bl6 - 6 born 4/17 (2m, 5f) - tails 5/8 (#1-6)
  - Positives (3 of 6): #2m, #4f, and #6f
- Again to bl6 - 8 born 5/10 (5m, 3f) - tails 5/30 (#8-15)
  - Positives (5 of 8): #8m, #10m, #11m, #14f (malocclusion, sickly), and #15f
- Again to bl6 - 9 born 6/1 (4m, 5f) - tails 6/19 (#16-24)
  - Positives (3 of 9): #20f (backcrossed see below), #22f, and #23f

F1 X F1 backcrosses - #8m X #15f and #10m X #20f

- #102/8 X #102/15 - 8 born 7/18 (3m, 5f) - tails 8/7 (#1-8)
  - Positives by PCR(3 of 8): #2m (#102A cross), #4f (#102A cross), #5f
- #102/10 X #102/20 - 6 born 7/27 (6f) - tails 8/16 (#1-6)
  - Positives by PCR (2 of 6): #1f, #3f (crossed to #8TRECSluc)
- #102/8 X #102/15 - 5 born 8/12 (3m, 2f) - tails 9/5 (#9-13)
  - Positives by PCR (3 of 5): - #9m, #10f, #12f (crossed to #8TRECSluc)
- #102/10 X #102/20 - 8 born 8/19 (4m, 4f) - tails 9/10 (#7-14)
  - Positives by PCR (4 of 8): #7m, #8m, #9m, #12f (crossed to TRECSluc)
- #102/10 X #102/20 - 9 born 9/10 (5m, 4f) - tails 10/2 (#15-23)
  - Positives by PCR (3 of 9): #15m, #18m, #19m
- #102/10 X #102/20 - 9 born 10/19 (5m, 4f) - tails 11/13 (#24-32)
  - Positives by PCR (5 of 9): #24m, #27m, #28m, #31f (102B), #32f (102C)
- #102/10 X #102/20 - 9 born 12/3 (5m, 4f) - tails 1/3 (#33-41)
  - Positives by PCR (4 of 9): #36m, #37m, #39f, #40f
- #102/10 X #102/20 - 6 born 12/27 (4m, 2f) - tails 1/22 (#42-47)
  - Positives by PCR (3 of 6): 45m, 46f (crossed and left with Todd), 47f
- #102/10 X #102/20 – 9 born 2/13(4m,5f) – tails 3/5 (#48-56)
  - Positives by PCR (5 of 9): #49m, #53f, #54f, #55f, and #56f
- #102/10 X #102/20 – 12 born 3/26 (6m, 5f, 1died) - tails 4/2 (#57-67) ship with mother - she can be discarded after weaning
  - Positives by PCR (7 of 9): #57, #58, #59, #60, #61, #62, #63, #64, and #67

Backcrosses – should be homozygotes for transporting to Tucson
#102A cross is #102/8X15-2m X #102/8X15-4f

#102B cross is #102/8X15-9m X #102/10X20-31f

- 9 born 3/23 (7m, 2f) - tails 4/2 (#1-9) – all positive by PCR

#102C cross is #102/8X15-2m (rotated from A) X #102/10X20-32f
- 10 born 3/17 (9m, 1f) – tails 4/2 (#1-10) – all positive by PCR

#102MCKrtTA X TRECSluc (#49 and #8) for testing

Cross #102/4f and #102/6f X TRECSluc #49F2-9m
- #102/6f X #49F2-9 TRECSluc - 5 born 6/13 (2m, 3f) - tails 7/3 (#1-5)
  - Double Positives: #2f
- #102/4f X #49F2-9 TRECSluc - 3 born 6/12 (1m, 2f) - tails 7/3 (#1-3)
  - No Double Positives
- #102/4f X#49F2-9 TRECSluc - 9 born 7/2 (1m, 8f)- tails 7/24
  - Double Positives: #11f (also keep #10f TRE only as a control)
- #102/6f X #49F2-9 TRECSluc - 8 born 7/3 (6m, 2f) - tails 7/24
  - Double Positives: #12f
- #102/4f X #49F2-9 TRECSluc - 7 born 7/24 (2m, 5f) - tails 8/14
  - Double Positives:
- #102/4f and 6f X #49F2-9 TRECSluc - 18 born 8/14

Cross #102/8X15-12f and #102/10X20-12f X TRECSluc #8/19X24/1X2-5m
- 7 born 12/14 (2m, 5f) - tails 1/8 (#1-7)
  - Double Positives (2 of 7): #2m, #7f
- 7 born 1/30 (1m, 6f) – tails 2/20 (#8-14)
  - Double positives (2 of 7): #13f and #14f (also keep #12f WT)
- 5 born 3/22 (2m, 3f) - tails 4/2 (#15-19)
- Double positives (1 of 5): #17f

Cross #102/10X20-1f and #102/10X/20-3f X TRECSluc #8/19X24/1X2-2m – (4/1) male pulled to #8C cross will need to rotate male between these crosses)

#102 MCKrtTA – Tucson breedings

- Pan 1 – pulled 1/6/03
  - Pups 7/23 – died
  - Pups 8/13 – gone
  - Pups 9/3 – 3m, 3f
  - Pups 9/29 – 1m, 2f
  - Pups 10/17 – 1m, 2f (to pan 2b)
  - Pup 11/21 – 1m, 1f (#620-621) #620m, #621f

- Pan 2 –pulled 12/3
  Pups 7/25 – died
  Pups 8/16 – 5m, 4f
  Pups 9/27 –3m, 5f (several with cephalic and other defects – tossed litter)
- Pan 2b – 12/23 (pan3 10/28male X pan1 10/17 females (2)) – PTS after 3/31 litter
  n Pups 1/23 – 3m, 1f (#635-638) #635m, #636m, #637m, #638f
  n Pups 2/12 – 4m, 3f (#646-652) #646m,#647m,#648m,#649m,#650f,#651f,#652f
  o Pups 3/8 – 4m, 2f
  n Pups 3/18 – 4m, 3f
  o Pups 3/31 – 5m, 4f, 1maloclusion
- Pan 2c – 4/2 (#646m X #650& #651f)
  651 - Pups 4/23

- Pan 3 – stopped 1/22
  Pups 7/27 – 2m, 1f (#33-35) #33m, #34m, #35f
  Pups 8/16 – 3m, 6f
  Pups 9/10 – 4m, 3f
  Pups 10/5 – 2m, 5f
  Pups 10/28 – 2m, 2m (to pan 4b and 2b), 3f
  Pups 1/2 – 3m,1f (#624-627) #624m, #625m, #626m, #627f
- Pan 3b – 4/2 (#626m X #627f)
  627 - Pups 4/23 – gone

- Pan 4 – pulled 12/3
  Pups 7/25 – 4m, 3f (#36-43, no 42) - #36m, #37m, #38m, #39m, #40f, #41f, #43f
  Pups 9/8 – 2m, 3m, 2f
- Pan 4b – 12/23 (pan3 10/28 male X pan4 9/8 females (2)) – stopped 4/15
  n Pups 1/16 – gone
  o Pups 2/6 – 3m, 4f (#639-645) #639m,#640m,#641m,#642f,#643f,#644f,#645f
  o Pups 3/3 – 3m, 3f
  n Pups 3/14 – 1f
- Pan 4c – 4/2 (#639m X #642& #643f)
  643 - Pups 4/23

- Pan 5 – discard breeders 10/2/02
  Pups 7/27 – gone
  Pups 8/19 – 1m, 1f
  Pups 9/9 – 2m, 3f

- Pan 6 (het X CBA WT female)

Pups 8/2 – 1m, 3f (#56-59) - #56m
Pups 8/31 – 3m, 2f (#104-108) - #107f, #108f
Pups 9/24 – 4m, 6f (#153-162) - #153m, #159f, #161f, #162f
Pups 10/20 – gone
Pups 11/11 – 1m, 3f

- Pan 6b – 10/23 (#56m from above X CBAWT female)
  Pups 12/26 – 2m, 2f (#622-623) no positives
  Pups 1/17 – 3m, 4f (#628-634) #628m, #632f, #633f
  Pups 2/10 – died
- Pan 6c – 4/2 (#628m X CBA WT female)

#91 MCKrtTA breeding log

Female founder X C57/bl6J WT

- Crossed to bl6 - 7 born 3/24 - mixed with #98 so eliminated
- Again to bl6 - 9 born 4/16 (3m, 6f) - tails 5/8 (#1-9)
  - Positives (6 of 9): #1m, #2m, #5f, #6f, #7f, and #9f (backcrossed see below)
- Again to bl6 - 8 born 5/7 (1m, 3f, 4 died - even live ones Stephanie labeled as runts - hold off weaning but send tails)- tails 5/30 (#10-13)
  - Positives (2 of 4): #10m and #13f
- Again to bl6 - 8 born 5/28 (5m, 3f) - tails 6/19 (#14-21)
  - Positives (1 of 8): #15m

F1 XF1 backcrosses - #1m X #6f and #2m X #9f - Stopped 12/12/01

- #91/1 X #91/6 - 4 born 7/19 (all died)
- #91/2 X #91/9 - 7 born 7/18 (4m, 2f) - tails 8/7 (#1-6)
  - Positives by PCR (5 of 7): #1m (fat), #3m, #4m, #5f (91B), #6f
- #91/1 X #91/6 - 6 born 8/15 (2m, 4f) - tails 9/5 (#1-6)
  - Positives by PCR (4 of 6): #1m, #3f, #5f, #6f
- #91/2 X #91/9 - 7 born 8/9 (5m, 2f) - tails 9/5 (#7-13)
  - Positives by PCR (6 of 7): #7m, #9m, #10m (crossed with 20 and left with Todd), #11m (malocclusion), #12f, #13f (crossed to #8TRECSluc)
- #91/1 X #91/6 -8 born 9/17 (4m,4f) - tails 10/9 (#7-16)
  - Positives by PCR (7 of 8): #7m, #8m,#9m, #10m, #12f, #13f, #14f (crossed to #8TRECSluc)
- #91/2 X #91/9 - 4 born 10/4 (3m,1f) - tails 11/1 (#14-17) #16 died before weaning
  - Positives by PCR (2 of 3): #14m, #15m(#91A cross)
- #91/2 X #91/9 - 7 born 10/29 (2m,5f) - tails 11/20 (#18-22)
  - Positives by PCR (5 of 7): #18m, #19m, #20f (#91A cross), #21f, #24f
- #91/1 X #91/6 - 6 born 10/22 (4m,2f) -tails 11/13 (#15-20)
  - Positives by PCR: #15m, #16m #17m, #18m, #19f, #20f (crossed with 10 and left with Todd)
- #91/1 X #91/6 - 6 born 11/15 (6f) - tails 11/27 (#21-26)
  - Positives by PCR: #21f, #22f, #25f, #26f (died)
- #91/2 X #91/9 - 4 born 11/20 (#23-25) Don't look healthy #25 died
  - Positives by PCR: #23m, #24m (both died)
- #91/1 X #91/6 - 4 born 12/8 (2m, 2f) - tails 1/3 (#27-31) # 30 and #31 died
  - Positives by PCR (3 of 3): #27m, #28m, #29f (91B)

Backcrosses - should be homozygotes for transporting to Tucson
#91A cross is #91/2X9-15m X #91/2X9-20f

- 6 born 2/26 (2m, 3f, 1died)
  - Positives by PCR (5 of 5): #1m, #2m, #3f, #4f, #5f

#91B cross is #91/2X9-4m X #91/2X9-5f (died giving birth) new female #91/1X6-29f (4/1)

#91MCKrtTA X #49TRECSluc for testing

Cross #91/5f and #91/7f X TRECSluc #49F2-8m

- #91/7f X #49F2-8m - 8 born 6/12 (4m, 4f) - tails 7/3 (#1-8)
  - Double Positives: #4m and #7f
- #91/5f X #49F2-8m - 4 born - all died
- #91/5f X#49F2-8m - 5 born 7/9 (3m, 2f) - tails 7/31
  - Double Positives: #10m
- #91/5f and #91/7f X TRECSluc #49F2-8m - 7 born 8/2 – tails 8/21 (#14-20)
  - Double Positives: #17f, #19f, #20f
- #91/5f and #91/7f X biTRECSlus #49F2-8m – 3 born 9/10 (2m, 1f) – tails 9/10 (#21-23)
  - Double Positives: #23f
- #91/5f and #91/7f X biTRECSlus #49F2-8m – 3 born 9/10 (2m, 1f) and 5 born 9/13 (1m, 4f) – tails 10/2 (#24-31)
  - Double Positives: #29f
- #91/5f and #91/7f X biTRECSlus #49F2-8m – 2 born 10/2 (2m) – both died
- #91/5f and #91/7f X biTRECSlus #49F2-8m – 15 born 10/20 (8m, 7f) – tails 11/13 (#34-48)
  - NO KEEPERS – stop this cross

Cross #91/1X6-13f and #91/1X6-14f X TRECSluc #8/19X24/1X2-4m

- 9 born 12/17 (3m, 6f) - tails 1/8 (#1-9)
  - Double Positives (3 of 9): #2m, #3m, #7f (also kept #4f TRE control, #5f WT control)

Cross #91/2X9-13f X TRECSluc #8/19X24-5m

- 7 born 12/8 (3m, 3f, 1 died) - tails 1/3 (#1-6)
  - No Double Positives
- 7 born 12/17 (4m, 3f) - tails 1/8 (#7-13)
  - Double Positives (2 of 7): #7m, #13f (2/26)(can use with above cross, weaned same date – has controls)
- 2 born 12/31 (2m) - tails 1/22 (#14-15)
  - No Double Positives (0 of 2)
- 10 born 1/8 (5m, 5f) - tails 1/29 (#16-25)
  - Double Positives (4 of 10): #17m, # 20m, #21f, #22f (also kept #18m rtTA only and #23f WT as controls)
- 6 born 1/20 - died
- 10 born 1/30 (3m, 7f) – tails 2/20 (#26-35)
  - Double Positives (1 of 10): #31f NO KEEPERS

- 8 born 1/21 (5m, 1f) - tails 3/12 (#36-41)
  - Double Positives (1 of 8): #39 NO KEEPERS

*#8 TRECSluc note: 2/5/02 pulled #8/19X24-5m to the #8A cross so the #8/19X24/1X2-4m will rotate between these 2 sets of females

#91 MCKrtTA - Tucson breedings

Pan 1a – stopped 12/16
Pups 8/10 (labeled 6/8) – 1m, 2f, 1f
Pups 9/14 – 2m, 3m, 4f
Pups 10/7 – 3m, 5f
Pups 10/30 – 5m
Pups 11/23 – 3m, 2f
Pups 12/16 - PTS

- Pan 2a – discard after no pups 9/26
- Pan 2b – 9/26 (1m X 2f from pan1, 8/10 litter) – stopped 3/25
  Bl - Pups 10/15 – gone
  Bl - Pups 11/12 – gone
  Br - Pups 11/15 – 2m, 3m, 4f
  Bl - Pups 12/26 – gone
  Br - Pups 12/31 – 1m, 2f
  Bl - Pups 2/5 – 2m, 4f (#690-695) #691m, #693f, #694f, #695f (to p3c)
- Pan 2c – 4/3 (#691m X #693f & #694f)
  694 Pups 4/25 – died
  693 Pups 5/1 – 1m, 3f
  693 Pups 5/22 – 1f
  694 Pups 5/27 – 2m, 5f
  693 Pups 6/12 – 4m, 5f
  694 Pups 6/19 – 4m, 5f
  694 Pups 7/15 – 4m, 4f
  693 Pups 8/3 – 7f

- Pan 3a – discard after no live litters 10/23
  Pups 7/29 – gone
  Pups 9/27 – gone
  Pups 10/18 – gone
- Pan3b – 10/23 (1m X 2f of 9/14 pan1 pups) – 1 female died with 1st litter– stopped 3/25 let female wean litter
  Pups 11/14 – gone
  Pups 11/23 – gone

Pups 1/11 – 3m, 1f (#673-676) #674m
Pups 1/31 – 3m, 1f (#686-689) no positives
Pups 3/20 – gone

- Pan 3c – 4/3 (#674m X #695f & #701f) - 695f sore leg – PTS6/22
  695 Pups 4/25 – gone
  701 Pups 4/30 – 2f
  701 Pups 5/20 – 5m, 3f
  695 Pups 5/22 – 2m, 2f
  701 Pups 7/2 – 6m, 6f
  701 Pups 8/14

- Pan 4a – discard after no live litters 10/2/02
  Pups 8/17 – gone
- Pan 4b – 10/23 (1m X 2f of 9/14 pan1 pups) – stopped 3/25
  Pups ~11/14 – 7m, 5f
  o Pups 12/12 – 4m, 4f
  n Pups 12/31 – 4m, 4f (#709-712) 712f
  o Pups 1/13 – 5m, 4f (#677-685) #678m, #679m, #680m, #681m, #685f
  o Pups 2/7 – 4m, 5f (#696-704) #696m, #698m, #701f (to p3c), #703f, #704f
  n Pups 2/28 – 4m (#705-708) #706m, #707m, #708m
- Pan 4c – 4/3 (#706m X #703f & #704f)
  704 - Pups 4/25 – pups small – 1f
  703 Pups 5/1 – 1m, 1f small – PTS
  703 Pups 5/22 – 1m, 3f
  704 Pups 6/11 – 1m, 3f
  703 Pups 6/12 – 8m, 1f
  704 Pups 7/5 – 6m, 2f
  703 Pups 725 – 2m, 5f
  704 Pups 7/28 – 5m, 3f
  703 Pups 8/18
  704 Pups 8/19

- Pan 5a crossed to CBA WT- (female sick, euthanized 10/18 – tossed male too)
  Pups 8/3 – 4m, 2f (#65-70) - #65m, #67m, #68m, #69f, #70f
  Pups 10/10 – died
- Pan 5b – 4/2 (#65m X CBA WT) stopped 9/2
  Pups 4/25 – gone
  Pups 5/28 – gone
  Pups 6/16 – 2m, 3f
  Pups 7/8 – 4m, 3f
  Pups 7/29 – 4m, 3f

- Pan 6a – 9/26 (#65 X #69) – stopped 11/26
  Pups 10/15 – 2m, 1f (#208-210) - #209m, #210f

Pups 11/4 – 1m, 2f (#274-276) - #274m, #276f
Pups 12/2 - culled

- Pan 7a – 9/26 (#67 X #70) – stopped 11/26
  Pups 10/18 – 3m, 8f
  Pups 11/11 - 6m, 6f (#277-285) - #277m, #278m, #279m, #280m, #281m, #282m, #283f, #284f

- Pan 8a – 9/26 (m#68(CBAX91) X 1f pan1, 8/10 litter) – stopped 11/26
  Pups 10/19 – 1m, 4f (#269-273) - #270f, #272f
  Pups 11/11 – gone

#8 TRECSluc breeding log

Female founder X C57/bl6J WT
- Crossed to bl6 - 8 born 9/6 (6m, 2f) - weaned 9/25
  - Positives (1of 8): #6m
- Again to bl6 - 11 born 10/18 (8m, 3f) - weaned 11/6, ship 11/13 (#9-19)
  - Positives (4 of 11): #10m, #11m, #12m, and #19f
- Again to bl6 - 10 born 11/28 (5m, 5f) - tails 12/19 (#20-29)
  - Positives (2 of 10): #22m and #24m

F1 X F1 backcross - #8/19f X #8/24m - stopped 7/17
- #8/19 X #8/24 -8 born 3/12 (1m, 3f, 4 died) - tails 4/2
  - Positives by PCR: #1m and #2f(backcross below)
- #8/19 X #8/24 again - 2 born 4/4 (2m) - tails 4/17
  - Positives by PCR: #5m(to mck 91,then 8A) and #6m
- #8/19 X #8/24 again - 6 born 5/21 (2m, 3f, 1 died) - tails 6/14 (#7-9 and11-12)
  - Positives (5 of 5): #7m, #8m (to mck 111), #9f (#8A cross), #11f (to optrtTA, then 8B), and #12f (to optrtTA, then 8C)

F2 X F2 backcross #8/19X24-1m and #8/19X24-2f for more progeny
- 6 born 5/30 (6 died)
- 4 born 6/29 (4m) - tails 7/17
  - Positives by PCR (2 of 4): #2m (to mck 102, then 8C) and #4m(to mck 91)
- 7 born 8/27 (3m, 4f) - tails 9/10 (#5-11)
  - Positives by PCR (3 of 7): #5m (to mck102), #7m(to mck 111,then 8B), #8f (#8A cross)

Backcrosses – for transporting to Tucson
#8A cross is #8/19X24-5m X #8/19X24-9f and #8/19X24/1X2-8f

#8B cross is #8/19X24/1X2-7m X #8/19X24-11f

#8C cross is #8/19X24/1X2-2m (fat) X #8/19X24-12f

#8TRECSluc X optrtTA for testing
6/26/01 Cross #8F2-11f and #8F2-12f X optrtTA male (from Maria)
- #8F2-12 X optrtTA - 2 born 7/23(both died)
  #8F2-11 X optrtTA - 9 born 7/30 - tails 8/21 (#1-9)
  - Double positives (7 of 9): #1m, #3f, #4f, #6f, #7f, #8f, #9f
- #8F2-12 X optrtTA - 8 born 8/19 (6m, 2f) - tails 9/10 (#10-17)

- Double Positives (4 of 8): #10m, #13m, #15m, #16f
- #8F2 -11 X optrtTA - 11 born 9/13 (5m,6f) - tails 10/2 (#18-28)
  - Double Positives (8 of 11): #18m, #20m, #21m, #22m, #23f, #24f, #25f, #26f (no controls)
- #8F2 -12 X optrtTA - 13 born 9/21 (5m,8f) - tails 10/2 (#29-41)
  - Double Positives(7 of 13): #30m, #32m, #36f, #37f, #39f, #40f, #41f (also kept #35 even though rtTA didn't come up)
- #8F2 -12 X optrtTA - 5 born (4m, 1f)10/14 - tails 11/1 (#42-46)
  - Double Positives(1 of 5): #44m 2/26
- #8F2 -11 X optrtTA - 3 born 10/30 - tails 11/20 (#47-49)
  - Double Positives(2 of 3): #48f, #49f 2/26
- #8F2 -12 X optrtTA - 4 born 11/2 (2m, 2f) - tails 11/20 (#50-53)
  - Double Positives (1 of 4): #52f 2/26 (can group with above set – no controls)
- #8F2 -11 X optrtTA - 6 born 11/20 (2m, 4f) – tails 12/11 (#54-59)
  - Double Positives (1 of 6): #56f
- #8F2 -12 X optrtTA - 5 born 11/24 (3m, 2f) – tails 12/11(#60-64)
  - Double Positives (5 of 5): #60m, #61m, #62m, #63f, #64f (no controls)
- #8F2 -11 and #8F2 -12 X optrtTA - 5 born 12/31 (2m, 3f) tails 1/22 (#65-69)
  - No Double Positives (0 of 5)
- #8F2 -11 and #8F2 -12 X optrtTA - 6 born 1/21 (2m, 4f) – tails 2/5 (#70-75)
  - Double Positives (2 of 6): #70m ad #73f
- #8F2 -11 and #8F2 -12 X optrtTA – 4 born 2/13 (2m, 2f) – tails 2/5 (#76-79)
  - Double Positives (4 of 4): #76m, #77m, #78f, and #79f

#8 TRECSluc – Tucson breedings – line not doing well, pulled mice from outcrosses, need to check for rtTA contamination

- Pan 1a (8X91#2 male X WT CBA female)
  Pups 9/17 – 6m, 3f (#134-142) - #134m, #137m, #138m, #139m, #142f
  Pups 11/14 – 2m, 7f
- Pan 1b – 10/23 (#138 male X WT CBA female) – stopped 11/26 – keep female CBA to #49s
- Pan 1c – 4/3 (CBA WTm X #722f & #724f)
  722 - Pups 4/25

- Pan 2a (8X91#3 female X WT CBA male)
  Pups 9/14 – 2m, 3f (#122-126) - #122m, #123m, #125f, #126f
  Pups 10/28 – 1m, 1f
- Pan2b – 10/23 (#139 male X WT CBA 2 females) – stopped 3/25 let female wean litter
  Pups 11/19 – 6m, 3f

Pups 1/6 – 1m, 2f
Pups 1/27 – 3m, 4f (#735-741) #736m
Pups 2/19 – 3m, 6f
Pups 3/24

- Pan 2c – 4/25 (#736m X CBA WT 2 females)

- Pan 3a (8X91#20 male X CBA WT female)
  Pups 8/21 – 3m, 4f (#96-103, no 100) - #97m, #98m, #99f
  Pups 10/30 – 3m, 2f
- Pan 3b – 11/7 (#150m X CBA WT female) – stopped 3/25
  Pups 2/15 - gone

- Pan 4a (8X102#17 female and 8X111 #e25 female X CBA WT male) – pulled male on 10/23, to pan7b #49TRE line
  Pups 9/15 from #e25 – 4m (#118-121) - #120f, #121m
  Pups 10/26 from #e25 – 2m, 1f
  Pups 11/4

- Pan 5a (8X91#17 male X CBA WT female) – old, but doing really well, so leave – stopped 12/02
  Pups 8/3 – 3m, 1f (#76-79) - #76m, #79m
  Pups 8/25 – 3m, 5f (#80-87) - #80m, #82m, #83f
  Pups 9/19 – 6m, 4f (#143-152) - #144f, #145f, #146f, #150m, #152m
  Pups 10/13 – 2m, 7f (#190-198) - #190m, #192f, #194f, #196f, #198f
  Pups 11/6 – 2m, 2f

- Pan 6a (8X111 #52&55 females X CBA WT male)
  55f – Pups 9/12 – 3m, 2f (#113-117) - #114m, #116f, #117f
  52f – Pups 9/27 – 2m, 1f (#175-177) - #177m
  55f – Pups 10/3 – 3m, 4f (#178-184) - #181f
  53f – Pups 10/24 – 3m
- Pan 6b –11/7 (#92f & #120f) – stopped 1/29
  120f – Pups 11/14 – 2m

- Pan 7a (8X102 #14 & #8X111 e#11 females (2nd female added 9/26) X CBA WT male)
  Pups 8/31 – 3m (#93-95)
  e#111 Pups 10/14 – 3m, 6f (#199-207) - #199m, #200m, #203f, #205f, #207f
- Pan 7b – 11/7 (#125f & #126f) – stopped 4/2 – some rtTA contamination 3/3 and 3/7 - (these mice to Todd)
  #125 Pups 12/4 – 4m, 4f
  #126 Pups 12/9 – 7m, 6f
  #125 Pups 1/16 – 9m, 3f (#713-724) #715m, #718m, #720m, #722f, #724f

#126 Pups 1/21 – 5m, 5f (#725-734) #726m, #732f
#126 Pups 3/3 – 6m, 7f (#742-754) #745m, #747m, #748f, #749f, #750f
#125 Pups 3/7 – 6m, 5f (#755-765) #755m, #759m, #760m, #761f, #762f, #763f

- Pan 7c – 4/3 (#726m X CBA WT 2 females)
  Pups 4/28
  Pups 4/28

- Pan 8a (8XoptrtTA#77 male X CBA WT female)
  Pups 8/25 – 2m, 3f (#88-92) – #88m, #89m, #92f
  Pups 9/18 – 5m, 2f (#127-133) – #129m, #130m, #133f
  Pups 10/10 – 4m, 1f (#185-189) – #185m, #188m
  Pups 11/1 – 5m, 4f - PTS
- Pan 8b – 11/7 (#152m) – stopped 1/29
  Pups 11/25 – 4m, 2f
  Pups 12/17 – gone

- Pan 9a (#8X111#e18male X CBA WT female) – Female died, 10/11/02 – discard male as well
  Pups 9/26 - fostered to pan1 – died

- Pan 10a (male from 8A X CBA WT female) - toss male, Keep CBA female – hold, then to #49 pan 2b
  Pups 9/4 – gone

#49 TRECSluc

Male founder X C57/bl6J WT

- Crossed to WT black 6 - 5 born on 9/18 (2m, 3f) - weaned 10/9
  - Positives (0 of 5): No positives - PCR repeated 3X
- 2nd female - 5 born 9/24 (1m, 3f, 1 died) - weaned 10/16 (#6-9)
  - Positives (2 of 4): #6m and #7f
- Again to bl6 - 3 born 10/30 (2f, 1 died) - weaned 11/20 (#10-11)
  - Positives (1 of 2): #10f (died 6/28)
- Again to bl6 - 3 born 12/4 (1m, 2f) - tails 12/27-1/2 (#12-14)
  - Positives (1 of 3): #12m
- Again to bl6 - 7 born 12/24 (4m, 3f) - tails 1/15 (#15-21)
  - Litter culled
- Again to bl6 - 4 born 1/17 (2m, 2f) - tails 2/6 (#22-25)
  - Positives (0 of 4)
- Again to bl6 - 4 born 1/26 (3f, 1 died) - tails 3/20 (#26-28)
  - No positives (0 of 4)
- Again to bl6 - 2 born 3/15 (1m, 1f) - tail 4/2 (#29-30)
  - No positives (0 of 2)

F1 X F1 backcross #49/10f X#49/12m

- #49/10f X#49/12m - 7 born 3/18 (6m,1f) - tails 4/10 (#1-7)
  - Positives by PCR: #1m, #3m, #4m, and #6m
- #49/10 X #49/12 again - 7 born 4/9 (4m, 3f) - tails 5/4 (#8-14)
  - Positives by PCR (7 of 7): #8m, #9m, #10 (to mck mice), #11m (cross below), #12f, #13f, and #14f
- #49/10 X #49/12 again - 7 born 5/1 (2m, 3f, 2 died) - tails 5/22 (#15-16 and 18-20)
  - Positives by PCR (3 of 7): #15m(died), #19f (cross below), and #20f

F2 X F2 backcross #49F2 (10 X 12) pup: #49F212f and 14f X #49F3 11m (females scabbed replace)

- #49F311 X #49/12 and #49/14 - 4 born 9/18 (2m,2f) and 4 born 9/24 (1m,3f) - tails 10/9 (#1-8)
  - Positives by PCR (7 of 8): #1m, #3f, #4f (#49A cross), #5m, #6f, #7f, #8f (#49B cross) (some of these guys are over grooming hopefully better once they get crossed)

Replace females #49/10X12–19 X #49/10X12-11m – parents are looking pretty bad, scabs - stop after 3/27 wean

- #49F2-11 X #49F2-19 - 6 born 12/14 - all died
- #49F2-11 X #49F2-19 - 3 born 1/18 (2m,1f) – tails 2/5 (#9-11)

  - Positives by PCR (3 of 3): #9m, #10m, and #11f
- #49F2-11 X #49F2-19 – 2 born 2/8 (1m, 1f) – tails 3/4 (#12-13)
  - Positives by PCR (2 of 2): #12m and #13f
- #49F2-11 X #39F2-19 – 8 born 3/27(4m, 2f, 2 died) – tails 4/2 (#14-19)
- Positives by PCR (6 of 6): #14m, #15m, #16m, #17m, #18f, #19f

Backcrosses – for transporting to Tucson – should be homozygotes
#49A is #49/10X12/11X12-1m X #49/10X12/11X12-3f&4f (#3f bad sores –discarded 3/11)

- 8 born 2/26 – all died
- 8 born 3/4 – all died
- 4 born 3/25 – (3m, 1f) – tails 4/2
  - Positives by PCR (4 of 4): #1m, #2m, #3m, #4f

#49B is #49/10X12/11X12-5m X #49/10X12/11X12-7f&8f (one female died wit first litter 3/4)

- both females had pups – all pups and one mother died
- Plug 3/19

#49TRECSluc X optrtTA for testing

- 11/16 #49-7female X OptrtTA male - 7 born 11/26 (1m, 6f) - tails 12/14 (#1-7)
  - Double positives (2 of 7): #2f and #5f
- 12/11 #49-7female X OptrtTA – male killed female

#49 TRECSluc – Tucson breedings

- Pan 1a – 9/26 now with female previously of pan 4 – stopped 11/21
  Pups 10/23 – gone
  Pups 11/13 – gone
- Pan1b – 11/21 - #167m X CBA WT female (orig #8TRE) – stop 1/29 – move female to pan4b
  Pups 12/30 – toss 3m, 1f possibly from previous male
  Pups 1/21 – 4m, 2f (#781-786) #781m, #783m, #784m, #785f, #786f
  Pups 2/13 – 2m, 2f (#796-799) #796m
- Pan1c – 4/25 (#796m X CBA WT female)

- Pan 2a- female sick, separated for treatment – toss if no pups
  Pups 9/4 – gone
  Pups 9/26 – gone

- Pan 2b – 10/14 - male from 2a X CBA WT female (originally crossed with #8 line but separated 2 weeks) – found younger + male for this CBA female – see pan 8
- Pan 2C – 12/19 (#286m X CBAf) – stopped 1/29

- Pan 3a – stopped 11/21
  Pups 8/3 – dead
  Pups 8/20 – 1f runt weaned late (#112) - #112f
  Pups 9/12 – dead
  Pups10/25 – 4 fostered to #102 pan 1 - 2m, 2f (#286-289) - #286m, #287m, #288f, #289f
- Pan3b – 12/19 (#287m X #288f & #289f) – stopped 1/29
  Pups 1/21 – most gone, 1 left, fostered to pan6

- Pan 4a – switched out with female in pan1a see below
  Pups 9/7 – dead
  Pups 9/30 – dead
- Pan4b – 11/21 (#171m *HOMOZYGOTE* X CBA WT female X additional female from 1b on 1/29)
  - Pups 12/22 – 2m, 6f (#349-356) - #349m, #350m, #351f, #352f, #353f, #354f, #355f, #356f
  - Pups 1/21 – 4m, 2f
  - Pups 2/13
  - Pups 3/14 – gone
- Pan 4c – 4/3 (#350m X 2 CBAWT females)
  Pups 4/25

- Pan 5a – stopped 12/3
  Pups 7/27 – gone
  Pups 8/18 – gone
  Pups 10/18 – dead

- Pan 6a – stopped 11/21
  Pups 8/2 – 4m, 1f (#71-75)
  Pups 9/1 – 2m, 1f (#109-111) - #109m, #111f
  Pups 11/29 – gone
- Pan 6b – 11/21 - #172m X CBA WT female
  Pups 12/21 – 3m, 5f (#341-348) - #344f, #347f, #348f
  Pups 1/13 – 6m, 3f (#766-774) #766m, #769m, #771m, #774f
  Pups 2/5 – gone
- Pan 6c – 4/3 (CBA WT male X #347f & #348f)

- Pan 7a – (e#111 X ?dad? From pan 6)

Pups 10/14 – since mother is positive and most likely bred with positive father, keep and genotype pups

- 6m, 2f (#167-174) – #167m, #169m, #171m, #172m

- Pan7b – 10/25 (#111 female X CBA WT male (from TRE#8 pan4))
  Pups 11/29 – 6m, 4f (#331-340) - #334m, #335m, #336m, #338f, #340f
  Pups 12/26 – 7m, 5f (#357-368) - #357m, #360m, #361m, #362m, #365f, #368f
  Pups 1/16 – 3m, 3f (#775-780) #775m, #777m, #779f
  Pups 2/10 – 5m, 4f (#787-795) #788m, #791m, #792f, #793f, #795f
  Pups 3/3 – gone
- Pan 7c – 4/3 (CBA WT male X #365 & #368)
  368 - Pups 4/24

- Pan 8a – 10/22 – #109m X #112f and CBA WT female from pan2b – stopped 1/29
  CBA - Pups 11/5 fostered to #8 pan2 – 4m, 1f (#290-294) - #290m, #291m, #293m
  CBA - Pups 11/25 – died
  #112 - Pups 12/10 (7) fostered to #111 pan3 – 1f (#369) - #369f
  CBA - Pups 12/31 fostered to pan 7b
  #112 - Pups 1/17 fostered to pan 6b

Experimental breeding log

#8TRECSluc (1 gen CBA) X #111 MCKrtTA to generate experimental mice – all stopped 3/13

- Pan1 - #77(8) X 2 (111) females (pan3 8/15) – Br/het, Bl/het
  Br Pups 10/16 – 4m, 5f (#211-219) – no double positives
  Br Pups 11/6 – 4m, 7f (#242-252) – no double positives
  Br Pups 11/28 – 4m, 3f (#328-330& #370-373) – Double positives: #328f, #330f
  Bl Pups 12/2 – 6m, 2f (#412-419) – Double positive: #412m
  Bl Pups 1/12 – 7m, 1f (#520-527) – Double positives: #521m, #524m
  Br Pups 1/21 – 3m, 5f (#547-554) – Double positives: #547m, #548m
  Bl Pups 2/17 – 2m, 4f (#829-832) – Double positive: #829m
  Br Pups 2/27 – 5m, 3f (#926-933) – Double positive: #928f
  Br Pups 3/12 – PTS

- Pan2 - #80(8) X 2 (111) females (pan3 8/15) – n/Hom, o/Hom
  n Pups 10/20 – 1m, 2f (#225-227) – Double positive: #227f
  o Pups 10/28 – 3m, 3f (#228-230 & #233-235) – Double positives: #228f, #230f
  n Pups 11/12 – 5m, 5f (#295-304) – Double positives: #295m, #296m, #298m, #299m, #301f, #303f
  o Pups 11/18 – runts
  n Pups 12/31 – 3m, 6f (#474-482) – Double positives: #480f, #481f
  o Pups 1/16 – 4m, 4f (#528-535) – Double positives: #531m, #533f, #535f
  n Pups 1/23 – 2m, 7f (#555-563) – Double positives: #556m, #557f, #561f, #562f, #563f
  o Pups 2/19 – 3m, 7f (#847-850&865-870) – Double positives: #865f, #866f, #867f, #870m
  n Pups 2/22 – 5m, 5f (#871-880) – Double positives: #871m, #877f, #879f

- Pan3 - #82(8) X 2 (111) females (pan2 8/16) – n/Hom, o/het
  n Pups 10/19 – 1 runt
  o pups 10/28 – 2m, 6f (#231-232 & #236-241) – Double positive: #236f
  n Pups 11/8 – 1m, 3f (#253-256) – Double positive: #254f
  n Pups 11/30 – 6m, 4f (#374-383) – Double positives: #376m, #380f, #381f, #382f
  o Pups 1/9 – 5m, 3f (#493-500) – Double positive: #497m
  n Pups 1/21 – gone
  o Pups 2/1 – 4m, 5f (#581-589) – Double positives: #585f, #586f, #587f
  n Pups 2/12 – gone

o Pups 2/22 – 4m, 6f (#881-887&908-910) – Double positives: #883m, #884m
n Pups 3/7 = PTS

- Pan4 - 1 (111) male (pan1 6/14) X #79(8)f – male/Hom
  Pups 10/16 = 3m, 2f (#220-224) = Double positives: #220m, #223f
  Pups 11/8 – 5m, 2f (#257-263) – Double positives: #257m, #259m, #261m, #262f
  Pups 12/2 – 5m, 4f (#384-392) – Double positives: #384m, #387m, #388m, #389f, #390f, #392f
  Pups 12/26 – 3m, 5f (#466-473) – Double positives: #466m, #468m, #470f

- Pan5 - 1 (111) male (pan 1 6/14) X #83(8)f – male/het
  Pups 10/19 – gone
  Pups 11/8 – 1m, 4f (#264-268) – no double positives
  Pups 12/21 – 1m, 3f (#420-423) – Double positive: #422f
  Pups 1/13 – 1m, 1f (#536f-537m) - no double positives
  Pups 2/13 – 5m, 5f (#817-828) – Double positives: #817m, #823m,#826f

- Pan 6 – 1(111)m (pan5 9/26) X #116 and #117 (8)females – male/het
  #117 Pups 12/2 – 3m, 5f – (#404-411) – Double positives: #405m, #407f, #411f
  #116 Pups 12/25 = 9m, 1f (#456-465) = Double positives: #460m, #464m
  #117 Pups 1/23 – 1m, 3f (#577-580) – Double positives: #577m, #578f
  #116 Pups 2/17 – 2m, 3f (#833-837) – Double positive: #833m
  #117 Pups 3/11 – PTS

- Pan 7 – 1(111)m (pan5 9/26) X #144 and #99 (8)females – male/Hom
  #99 Pups 12/2 – 4m, 7f (#393-403) – Double positives: #395m, #399f, #402f
  #144 pups 12/4 - gone
  #144 Pups 12/23 = 6m, 3f (#427-435) = Double positives: #429m, #430m, #431m, #434f
  #99 Pups 12/25 – 4m, 4f (#448- 455) – Double positives: #448m, #450m, #452f
  #144 Pups 1/27 – 9m, 4f (#564-576) – Double positives: #565m, #569m, #570m, #572m, #574f, #575f, #576f
  #99 Pups 2/10 = 6m, 6f (#608-619) = Double positives: #608m, #615f, #616f, #619f
  #144 Pups 2/18 – 4m, 5f (#838-846) – Double positives: #839m, #840m, #842f, 3843f, #844f, #846f
  #99 Pups 3/1 = PTS

- Pan 8 - #122(8)m X 2(111)f (pan5 9/26) – n/het, o/het
  n Pups 11/20 – 5m, 3f (#305-312) – no double positives
  n Pups 12/12 – gone
  o Pups 12/13 – gone
  n Pups 1/2 - gone
  o Pups 1/8 – 2m, 5f (#501-507) – Double positives: #505f, #507f
  o Pups 2/3 – 4m, 6f (#598-607) – Double positives: #600m,#602m, #604f
  n Pups 2/23 – 5m, 3f (#911-918) – no double positives
  o Pups 3/6 – PTS

- Pan 9 – 1(111)m (pan5 9/26) X #145 and 146 (8)females – male/het
  #145 pups 11/28 – 4m, 4f (#313-320) – no double positives
  #146 Pups 11/28 – 4m, 3f (#321-327) – Double positives: #321m, #322m
  #145 pups 12/23 – 2m, 10f (#436-447) – Double positives: #436m #439f, #440f, #442f
  #145 1/10 – 8m, 4f (# 508-519) – Double positives: #513m, #516f, #518f
  #146 Pups 2/3 – 5m, 3f (#590-597) – Double positive: #592m
  #145 Pups 2/5 – 7m, 4f (#800-810) – Double positive: #804m, #806m, #808f, #810f
  #146 Pups 2/25 – 3m, 4f (#919-925) – Double positive: #923

- Pan 10 - #121(8)m X 2(111)f (pan5 9/26) – n/Hom, o/Hom
  n Pups 11/23 – gone
  o Pups 12/4 – runts
  n Pups 12/23 – 3m (#424-426) – Double positive: #424m
  o Pups 12/24 – 8m, 2f (#483-492) – Double positives: #485m, #486m, #488m, #490m, #491f
  n Pups 1/21 – 2m, 6f (#538-546) – Double positives: #538m, #541f, #543f, #544f, #545f, #546f
  o Pups 2/5 – 2m, 4f (#811-816) – Double positives: #811m, #814f, #815f
  n Pups 2/11 – gone
  n Pups 2/27 – PTS

#49TRECSluc X #102 MCKrtTA to generate experimental mice (all stopped and PTS 7/11)

- Pan 11 – 4/3 [#647m (102) X #351f & #352f (49)]
  352 Pups 4/24 – gone
  351 Pups 4/28 – gone
  351 Pups 5/21 – 3m, 4f

352 Pups 5/22 – 4m, 2f
352 pups 6/16 – PTS

- Pan 12 – 4/3 [#648m (102) X #353f & #354f (49)]
  353 Pups 4/28 – 2f (#888-889) – Double positives: #889f
  353 pups 6/12 – PTS

- Pan 13 – 4/3 [#649m (102) X #355f, & #356f (49)]
  356 Pups 4/28 2m, 3f (#890-894) – Double positives: #891m
  355 Pups 4/28 1m, 5f (#902-907) – Double positives: #903f, #905f, #906f, #907m

- Pan 14 – 4/3 [#640m (102) X #344f (49)]
  344 Pups 4/27 – 4m, 1f (#860-864) – Double Positives: #863m, #864m
  344 Pups 6/11 – PTS

- Pan 15 – 4/3 [#641m (102) X #338f & #340f (49)]
  340 Pups 4/25 – pups small 2f
  338 Pups 4/28 – 2m, 5f (#895-901) – Double positives: #895f, #897f, #898f
  338 Pups 6/11 – PTS
  340 Pups 6/17 – PTS

- Pan 16 – 4/3 [#349m (49) X #638f & #652f (102)]
  638 Pups 4/24 – 3m, 2f (#851-855) – Double Positives: #852f
  652 Pups 5/21 – 5m, 4f
  638 Pups 6/5 – PTS
  652 Pups 6/16 – PTS

- Pan 17 – 4/3 [#362m (49) X #644f & #645f (102)]
  644 Pups 4/24 – 2m, 2f (#856-859) – Double Positives: #856f , #859m
  645 Pups 4/29 – gone
  645 Pups 5/29 – PTS
  644 pups 6/4 – PTS

- Pan 18 – 4/25 #788m (#49) X #632f& 633f (102) – male died 5/27 – toss females after wean
  - 633 pups 5/21 – 5m, 4f
  - 632 Pups 5/22 – dead
  - 632 Pups 6/11 – PTS
  - 633 Pups 6/16 – PTS

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