**Annual Report**  
**Regional Research Project NC-131**  

**2003**

**Project Title:** Molecular Mechanisms Regulating Skeletal Muscle Growth and Differentiation

**Project Period:** 10-1-02 to 9-30-2003

### Institutional and Project Leaders

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**COOPERATING AGENCIES AND PRINCIPAL LEADERS**

*Denotes voting member of the Technical Committee*
PROGRESS OF THE WORK AND PRINCIPAL ACCOMPLISHMENTS

Objective 1: Characterize the signal transduction pathways that regulate skeletal muscle growth and differentiation.

The role of hepatocyte growth factor (HGF) and nitric oxide (NO) on activation of satellite cells in passively stretched muscle has been examined at the Arizona Station. A hind limb suspension model was used in which the vastus, adductor, and gracilis muscles were stretched for periods of 0 to 2 hours. Satellite cell activation was measured by injecting the rats with 5-bromo-2'-uridine (BrdU) following stretch and then harvesting (16 hours later) and culturing the satellite cells (30 hours). The BrdU labeling indicated passage of the cells through the cell cycle and nuclear division. Stretching muscle caused satellite cell activation. There was also an increase in the mature form of HGF in stretched muscles. Extracts of stretched muscle activated satellite cells in vitro, and antibodies against HGF neutralized this effect. In addition injection of an inhibitor of nitric oxide synthase (L-NAME) blocked activation of satellite cells when injected in vivo or added to satellite cells in culture. In contrast the enantiomer D-NAME was inactive in both assays. Stretched muscle has elevated nitric oxide synthase activity. It was concluded that stretching muscle causes the liberation of HGF that subsequently activates satellite cells. Muscle stretch also appears to activate nitric oxide synthase.

The Illinois Station has investigated the changes in protein/myonuclei ratios during hypertrophy and atrophy through the mechanisms of apoptosis. Through a stretch-induced model of hypertrophy in the ALD muscle of both young and old chickens and subsequent atrophy after release of stretch, a rapid reduction in muscle mass and myonuclei number was observed. Changes in caspase activity were indicative of apoptosis. Interestingly, apoptotic myonuclei were not uniformly distributed throughout the myofiber or muscle. Individual fibers or clusters of fibers had localized concentrations of apoptotic nuclei suggesting that a mechanism for selecting nuclei for apoptosis may be functioning.

The growth of most tissues is markedly depressed as a result of zinc deficiency by uncharacterized mechanisms that clearly involve the insulin-like growth factor (IGF) system. The Illinois Station investigated the mechanism by which zinc (Zn2+) maintains IGF-II in an active form by directly regulating IGF-II binding to IGF-binding proteins (IGFBPs) and the type 1 IGF receptor (IGF-1R). The specificity of Zn2+ effects was confirmed by using other cations that can (Cd2+ and Au3+) or cannot (La3+) mimic Zn2+ actions. Human fibroblasts, glioblastoma cells and murine myoblasts were used to determine the kinetics of IGF-II binding to cell surface IGFBP-3, IGFBP-5 and the IGF-1R, respectively. Zn2+, Cd2+ and Au3+, but not La3+, decreased total binding and the affinity for [125I]-IGF-II association with IGFBP-3 and IGFBP-5. These effects were a result of lowered rate of ligand association without affecting rate of dissociation. In contrast, Zn2+ enhanced [125I]-IGF-II binding to the IGF-1R by enhancing the rate of ligand association and decreasing the rate of dissociation. Previous work had shown that Zn2+ acts at physiological levels to alter IGF binding. Together with the current work, these findings imply that Zn2+ acts in vivo to prevent secreted IGF-II from binding to IGFBP-3 and IGFBP-5, thus maintaining IGF-II in an ‘active state’; i.e. readily available for IGF-1R association.

Skeletal muscles exhibit atrophy following periods of reduced weight bearing. The Indiana Station has examined the ability of sonic hedge-hog (Shh) and insulin-like growth factor (IGF-I) to stimulate
muscle hypertrophy using a mouse *in vivo* electroporation approach. Additional studies examined the ability of these anti-atrophic proteins to inhibit disuse atrophy when over-expressed in skeletal muscle. Gastrocnemius muscle was injected with plasmid DNA (20 µg of growth factor plasmid and 20 µg of control lac-Z). Contralateral limbs were injected with only lac-Z plasmid DNA. Mice were euthanized at 7, 14 and 30 d after electroporation and muscles were collected and processed. Ectopic expression of IGF-I and/or Shh was found within the gastrocnemius/soleus muscle, and it significantly stimulated muscle fiber hypertrophy and increased muscle size. In addition, ectopic expression of IGF-1 and/or Shh within the gastrocnemius/soleus muscle attenuates the loss of muscle fiber area, muscle mass and muscle mass density that normally occurs during disuse muscle atrophy. During this type of atrophy, there is an increased expression of various fibroblast growth factors (FGF) in those fibers resistant to atrophy. Members of the FGF family are involved in muscle hypertrophy, however, their effects on muscle atrophy are not known. Therefore, it was hypothesized that changes in FGF signaling could alter muscle atrophy. Using the aforementioned approach, gastrocnemius and soleus muscles of mice were injected with plasmid DNA containing a reporter gene construct (lac-Z) and 30 µg of plasmid DNA encoding fibroblast growth factor receptor 1 (FGFR-1). After electroporation, mice were randomly assigned to hindlimb suspension (HS) for 7 or 14 d. Another group was suspended for 7 d, and then allowed to recover for 7 d. After 7 d of suspension, positive fibers were 18.1 % larger in FGFR-1 injected muscles than contralateral controls. Similarly, positive fibers in FGFR-1 treated muscles of animals suspended 14 d were 60.1% larger than contralateral muscles. In animals allowed to recover, fibers remained 29.4 % larger in FGFR-1 injected muscles than controls. These data suggest that fibers expressing FGFR-1 failed to experience atrophy and exhibited improved muscle recovery, suggesting that elements of FGF signaling may mediate changes in protein metabolism during disuse atrophy.

Continued efforts to understand this model suggest that the calpain protease system and the ubiquitin proteosome pathway may be involved. Hepatocyte growth factor (HGF) is a growth factor that activates quiescent satellite cells and can modulate their ability to proliferate and differentiate in culture and in skeletal muscle tissue. In an attempt to study the mechanism by which HGF modulates muscle growth via changes in satellite cell activity, the Indiana Station generated an expression construct to produce an epitope-tagged HGF protein. Reverse transcription-polymerase chain reaction (RT-PCR) was used to clone HGF cDNA from mouse liver total RNA. The cDNA was subcloned into the pcDNA3.1 V5-His-TOPO expression vector and used to transfect C2C12 myoblasts. Immunoblots containing conditioned medium from myoblasts stably expressing rHGF showed that the recombinant HGF could be detected using antibodies against HGF or the V5 epitope. Moreover, passing conditioned medium through a His tag column removed the majority of immuno-reactive recombinant protein. Furthermore, conditioned medium from transfected cells stimulated proliferation in a manner similar to adding a commercially available recombinant HGF to the medium. Finally, immuno-neutralization of conditioned media using an anti-HGF antibody eliminated this proliferative effect in culture. It was concluded that the recombinant epitope-tagged HGF is biologically active when expressed in C2C12 myoblasts. This construct provides a valuable reagent for defining further the role of HGF in controlling satellite cell activation.

The Kansas Station evaluated effects of a 5% ground flaxseed supplement (flax), a rich source of α-linolenic acid (αLA), and a trenbolone acetate and estradiol-17β implant (Revalor-S) on circulating IGF-I and muscle IGF-I mRNA. Sixteen crossbred yearling steers (initial BW = 397 kg) were randomly assigned to one of four treatments: 1) flax/implant, 2) non-flax/implant, 3) flax/non-
implant and 4) non-flax/non-implant. Blood serum was collected on d 0 (before implant or flax addition), 14 and 28, and used in subsequent analyses of circulating IGF-I. Biopsy samples were obtained from the longissimus muscle on d 0, 14, and 28. Total RNA was isolated from the muscle samples and real-time quantitative-PCR was used to assess relative differences in IGF-I mRNA. Flax supplementation had no effect (P > 0.10) on circulating IGF-I levels. Following implantation, sera from implanted steers had 52 and 84% greater (P < 0.05) IGF-I levels than sera from non-implanted steers on d 14 and 28, respectively. On d 28, local muscle IGF-I mRNA levels increased 2.4-fold (P < 0.01) in biopsy samples obtained from implanted compared to non-implanted steers. Biopsy samples from non-flax cattle had 4.4-fold higher (P < 0.01) levels of IGF-I mRNA than those from flax cattle on d 28. To determine if a component of flax, α-linolenic acid (αLA), was directly responsible for IGF-I mRNA down-regulation, primary cultures of bovine satellite cells, from implanted and non-implanted steers, were incubated in two concentrations of αLA (10 nM and 1 μM). An implant x dose interaction (P < 0.05) was observed for IGF-I mRNA concentrations in satellite cells cultured for 72 h with αLA. Satellite cells from non-implanted steers had similar (P > 0.10) IGF-I mRNA concentration regardless of the level of αLA exposure. However, satellite cells from implanted steers exposed to 10 nM and 1 μM αLA had 2.5 and 2.0-fold greater IGF-I mRNA levels, respectively, than cells from implanted steers that were not exposed to αLA (P < 0.05). Administration of a Revalor-S implant increased circulating IGF-I and local muscle IGF-I mRNA concentrations in finishing cattle. However, muscle IGF-I mRNA levels were reduced by flax supplementation. Muscle cell culture experiments suggested αLA was not responsible for the IGF-I mRNA down-regulation. Since αLA appears not to be responsible for the down-regulation of IGF-I mRNA levels observed in flax-fed steers other components abundant in flaxseed are currently being investigated. In addition to being a rich source of αLA, flax is also very abundant in the phytoestrogen, secoisolariciresinol diglycoside (SDG). The SDG component is a precursor for two mammalian lignins, enterolactone (EL) and enterodiol (ED). The supplementation of SDG to rats results in reduced circulating IGF-I. Preliminary results suggest that exposure (48 h) of both L6 and C2C12 cells to EL and ED dramatically reduces thymidine incorporation at 72 h in culture. It is hypothesized that the mammalian lignins EL and ED may be affecting the IGF system in muscle and thus, is the component responsible for IGF-I mRNA down-regulation in flax-fed steers.

Research has shown that progestins can elicit an anti-proliferative effect on cells through a non-genomic mechanism. These non-genomic mechanisms are classified as an action by which the progestin does not work through the typical pathway involving transcriptional regulation by the progesterone receptor. Previous results from the Kansas Station have shown the addition of melengestrol acetate (MGA), a synthetic progestin, to cultured bovine muscle satellite cells resulted in a dose-dependent decrease in DNA synthesis rate. Progesterone (P4) was found to have no significant effect on DNA synthesis. The purpose of these experiments was to investigate the effects of MGA and P4 (0, 10 pM, 10 nM) on insulin-like growth factor-I (IGF-I) and myogenin mRNA abundance in cultured, proliferating satellite cells. At 72 h, total RNA was isolated from the cells and reverse transcribed for cDNA synthesis. Real-time quantitative-PCR was performed on the cDNA to estimate mRNA abundance. Melengestrol acetate addition (10 nM) increased (2.2-fold, P < 0.05) IGF-I mRNA abundance, but did not alter (P > 0.05) the level of myogenin mRNA. Cells treated with P4 (10 nM) had similar (P > 0.05) IGF-I mRNA levels, but myogenin mRNA abundance was increased (2.5-fold, P < 0.05). Even though MGA decreased proliferation in previous experiments, IGF-I mRNA abundance was increased in the presence of MGA. These results show that MGA and P4 may be working through either a genomic or non-genomic mechanism to effect factors that impact
both proliferation and differentiation. Further research is needed to determine the actual mechanism through which these progestins work in non-target tissues such as skeletal muscle.

L-Carnitine supplementation has been shown to increase circulating IGF-I in gestating sows, as well as to affect circulating concentrations and hepatic messenger RNA (mRNA) levels of IGF-system members in rats. The IGF system influences fetal muscle growth and development. The objective of experiments conducted at the Kansas Station was to determine the effects of L-carnitine on embryonic cell proliferation and IGF-system component’s mRNA levels in cultured porcine embryonic myoblasts (PEM) that were isolated from fetuses at mid-gestation from sows fed a common gestation diet with either a 50 g top dress of 0 (control, n= 6) or 100 mg L-carnitine (n=6). Proliferation of PEM was evaluated in two cell culture assays (24, 48, 72 or 36, 48, 60, 72 h post-plating). Real-time quantitative-PCR was used to determine mRNA levels of IGF and IGFBP in the muscle cultures (second assay) at 96 h post-plating. In both assays, the number of cells/cm² did not differ (P > 0.05) from sows fed either diet but the number of cells/cm² increased (P < 0.05) between each evaluated time-period. There tended to be a treatment x time interaction (P = 0.15) for number of doublings and separation of the means indicated a difference (P < 0.05) between 36 and 48 h for PEM isolated from dams fed L-carnitine compared to controls. To determine the direct effects of L-carnitine, PEM were incubated with L-carnitine (n=4) at differing concentrations (3.125, 6.25, 12.5, 25, 50, and 100 µM). No proliferation differences were detected (P > 0.05) between L-carnitine concentrations, indicating that the action of L-carnitine for increased muscle cell proliferation is regulated through an indirect mechanism. There was no difference (P > 0.05) between feeding treatments for the expression of IGF-I or IGFBP-5. However, PEM isolated from sows fed L-carnitine had decreased (P < 0.05) mRNA levels of IGF-II, IGFBP-3, and myogenin (61, 59, and 67%, respectively) compared to controls. These data suggest alterations in mRNA levels of IGF-II and myogenin could be affecting muscle cell proliferation and differentiation. These results increase our understanding of how L-carnitine may play a role in muscle growth and development in pigs.

Transmission of information from the extracellular surface to the nucleus involves induction of kinase signaling events. Multiple growth factors that alter myogenesis invoke kinase modules comprised of MEKK1, MEK and MAPK. The most extensively studied of these three-tiered signaling systems are those that culminate in p38, Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK1/2) activity. Previously, the Pennsylvania Station has demonstrated that Raf kinase, a MEKK family member, affects muscle formation in both a positive and negative manner. Low-level Raf activity promotes muscle gene expression while high intensity Raf/ERK activity leads to a severe reduction in myogenesis. Coincident with the loss of muscle formation in response to elevated Raf action is the up-regulation of TGF-β1 and GDF-8. To determine the involvement of TGF-β1 as an autocrine contributor to the disruption of myogenesis, a soluble inhibitor of TGF-β (βSRI) was added to the culture medium. Supplementation of the culture medium with βSRI did not reinstate the differentiation program to the Raf-repressive myoblasts. The cells remained mononucleated and failed to direct substantial amounts of muscle-specific reporter gene activity. Thus, it was concluded that TGF-β1 is not entirely responsible for the Raf-imposed block to myogenesis. The involvement of GDF-8 remains unclear. Treatment of 23A2 myoblasts with GDF-8 minimally inhibits muscle reporter gene activity and only weakly stimulates Smad-reporter gene activity. These results would suggest 23A2 mouse myoblasts are nonresponsive to concentrations of GDF-8 that are sufficient to inhibit myogenesis in other cell types. Future studies will more closely examine this issue.
Repression of muscle gene expression by activated Raf occurs through both MEK-dependent and independent signaling events. To clarify the existence of MEK/ERK independent Raf signaling, a mutant Raf protein deficient in MEK interactions (RafT481A) was transiently transfected in MyoD-converted fibroblasts at the Pennsylvania Station. After 48 hours in differentiation medium, the cells were lysed and muscle reporter gene activity measured. Robust activation of muscle reporter genes was found in C3H10T1/2 cells transfected with MyoD-only. Coexpression of activated Raf or RafT481A with MyoD resulted in an 80% reduction in MyoD directed reporter gene activity. These results support previous data indicating the presence of a MEK-independent signaling module for Raf. The identity of components of this atypical axis remains unknown. To isolate proteins of the MEK-independent module, Raf T481A was fused in frame with the Gal4 DNA binding domain and used to screen a human skeletal muscle cDNA library in yeast. To date, 27 clones have been isolated that interact with the Raf bait protein. The identity and interaction specificity of the various clones will be determined.

Insulin-like growth factor binding proteins (IGFBPs) have been shown to affect proliferation of several cell types via insulin-like growth factor (IGF)-dependent and IGF-independent mechanisms. The goal of a study by the Minnesota Station was to determine if levels of IGFBP-2, -3, -4 and -5 mRNA changed during differentiation of cultured porcine embryonic myogenic cells. Total RNA was isolated from muscle cultures at various stages of differentiation and Northern blots of this RNA were incubated with P32-labeled cDNA probes specific for individual IGFBPs. Fusion, myogenin mRNA, and creatine phosphokinase activity were used as markers of differentiation. The level of IGFBP-3 mRNA in differentiating cultures (120 h in culture) was only one-third of the level in myogenin negative, nonfused cultures (72 h in culture) (P<.05, n=4). In contrast, the level of IGFBP-3 mRNA in extensively fused cultures (144 h in culture) was increased by three-fold as compared to the level in myogenin negative, non-fused cultures (P<.05, n=4) and approximately 7-fold as compared to the 120 h cultures (P<.05, n=4). No significant change in the level of IGFBP-5 mRNA was observed during differentiation of myogenic cultures. IGFBP-2 mRNA levels were not significantly different at 72, 96 and 120 h, but at 144 h IGFBP-2 mRNA level was increased three-fold as compared to non-fused cultures (72 h) (P<.05, n=4). IGFBP-4 mRNA was not detectable on Northern blots of total RNA from porcine myogenic cultures at any stage of differentiation. Changes in IGFBP-3 and IGFBP-2 mRNA levels are associated with differentiation of embryonic porcine myogenic cells in culture and this may indicate that these IGFBPs play a role in differentiation of these cells.

Insulin-like growth factor binding protein (IGFBP-3) is produced by cultured porcine embryonic myogenic cell (PEMC) cultures and is secreted into the medium. Levels of secreted IGFBP-3 and IGFBP-3 mRNA are significantly reduced during differentiation and increase after differentiation is complete, suggesting that IGFBP-3 may play some role in myogenesis and/or in changes in myogenic cell proliferation that accompany differentiation. IGFBP-3 reportedly may either suppress or stimulate proliferation of cultured cells depending on cell type. Additionally, IGFBP-3 has been shown to affect proliferation via both IGF-dependent and IGF-independent mechanisms in some cell types but not all. Currently, the effect, if any, of IGFBP-3 on myogenic cell proliferation is not known. Consequently, the goal of a study by the Minnesota Station was to assess the IGF-I dependent and IGF-I-independent actions of recombinant porcine IGFBP-3 on proliferation of cultured porcine myogenic cells. To facilitate these investigations, porcine IGFBP-3 was expressed in the baculovirus system, purified and characterized. An antibody to the expressed recombinant porcine
IGFBP-3 (rpIGFBP-3) has been produced and characterized, and it neutralizes the biological activity of porcine IGFBP-3. RpIGFBP-3 suppresses IGF-I-stimulated proliferation of porcine embryonic myogenic cells in a concentration dependent manner with equimolar concentrations of IGF-I and rpIGFBP-3 resulting in complete suppression of IGF-I-stimulated proliferation. RpIGFBP-3 also suppresses Long-R3-IGF-I-stimulated proliferation of PEMC, indicating that rpIGFBP-3 possesses IGF-independent activity in this cell system. These data establish that IGFBP-3 has the potential to affect proliferation of embryonic porcine myogenic cells during critical periods of muscle development that may impact ultimate muscle mass achievable postnatally.

Both transforming growth factor (TGF) β and growth and development factor (GDF)-8 (myostatin) affect muscle differentiation by suppressing proliferation and differentiation of myogenic cells. In contrast insulin-like growth factors (IGF) stimulate both proliferation and differentiation of myogenic cells. Experiments at the Minnesota Station showed that treatment of porcine embryonic myogenic cell (PEMC) cultures with either TGF-β1 or GDF-8 suppressed proliferation and increased production of IGFBP-3 protein and mRNA (P<0.005). An anti-IGFBP-3 antibody that neutralizes the biological activity of IGFBP-3 reduced the ability of either TGF-β1, or GDF-8 to suppress PEMC proliferation (P<0.005). However, this antibody did not affect proliferation rate in the presence of both TGF-β1 and GDF-8. These data show that IGFBP-3 plays a role in mediating the activity of either TGF-β1 or GDF-8 alone but not when both TGF-β1 and GDF-8 are present. In contrast to findings in T47D breast cancer cells, treatment of PEMC cultures with IGFBP-3 did not result in increased levels of phosphosmad-2. Since TGF β and GDF-8 are believed to play a significant role in regulating proliferation and differentiation of myogenic cells, the current data showing that IGFBP-3 plays a role in mediating the activity of these growth factors in muscle cell cultures and strongly suggest that IGFBP-3 also may be involved in regulating these processes in myogenic cells.

Insulin-like growth factor (IGF)-I stimulates both proliferation and differentiation of myogenic precursor cells. In vivo, IGFs are bound to one of the members of a family of six high-affinity IGF binding proteins (IGFBP 1-6) that regulate their biological activity. One of these binding proteins, IGFBP-3, affects cell proliferation via both IGF-dependent and IGF-independent mechanisms and it has generally been shown to suppress proliferation of cultured cells; however, it also may stimulate proliferation depending upon the cell type and the assay conditions. Work at the Minnesota Station demonstrated that cultured porcine embryonic myogenic cells produce IGFBP-3 and the level of the latter drops significantly immediately prior to differentiation. Additionally, IGFBP-3 suppresses both IGF-I and Long-R3-IGF-I-stimulated proliferation of embryonic porcine myogenic cells. In this study the effects of recombinant porcine IGFBP-3 (rpIGFBP-3) on IGF-I- and Long-R3-IGF-I-stimulated proliferation and differentiation of the L6 myogenic cell line were examined. L6 cells potentially provide a good model for studying the actions of IGFBP-3 on muscle because they contain no non-muscle cells and they do not produce detectable levels of IGFBP-3. rpIGFBP-3 suppresses both IGF-I and Long-R3-IGF-I-stimulated proliferation of L6 cells, indicating that it suppresses proliferation via both IGF-dependent and IGF-independent mechanisms. Similarly, rpIGFBP-3 suppresses IGF-I-stimulated differentiation of L6 cells. In contrast, however, rpIGFBP-3 does not suppress Long-R3-IGF-I-stimulated differentiation. This suggests that rpIGFBP-3 does not have IGF-independent effects on L6 cell differentiation. The data shows that rpIGFBP-3 is unable to increase the level of phosphosmad-2 in L6 cultures and suggests that IGFBP-3 does not bind to the type II TGF β receptor in these cells as it reportedly does in some other cell types.
The Minnesota Station has used a muscle biopsy technique in conjunction with real-time PCR analysis to examine the time course of changes in muscle IGF-I, IGFBP-3, myostatin and hepatocyte growth factor (HGF) mRNA in the longissimus muscles of Revalor-S implanted and non-implanted steers. Biopsies were obtained on d 0, 7, 12 and 26 after implantation. Administration of a Revalor-S implant increased (P < 0.01) ADG and improved (P < 0.05) feed efficiency, 36 and 34 %, respectively, as compared to steers that received no implant during the 26 d trial. Daily dry matter intake was not different (P > 0.15) between non-implanted and implanted steers. Steers receiving the Revalor-S implant had increased (P < 0.001) circulating IGF-I concentrations as compared to non-implanted steers. The muscles of steers receiving the Revalor-S implant contained increased (P < 0.001) IGF-I mRNA levels as compared to muscles of non-implanted steers over the 26-d duration of the study. Muscle IGF-I mRNA levels in implanted steers were increased (P < 0.003) relative to d 0 concentrations on d 7, 12 (101% and 128%, respectively) and by d 26 muscle mRNA levels were more than 3 times (P < 0.0001) those in the muscles of the same steers on d 0. There was no treatment effect on the level of muscle IGFBP-3, myostatin, or HGF mRNA at any time point; however, levels of IGFBP-3, myostatin and HGF mRNA did increase with time on feed. Based on current and previous studies, it was hypothesized that the increased IGF-I level in muscle of implanted steers by d 7 of implantation stimulates satellite cell proliferation and maintains a high number of proliferating satellite cells at a point in the growth curve where satellite cell numbers and activity are normally dropping off. This would prolong the period of rapid muscle growth resulting in the observed increased rate and efficiency of muscle deposition in implanted steers.

Androgenic and estrogenic steroids enhance muscle growth in a number of species including humans; however, the mechanism by which anabolic steroids enhance muscle growth is not known. The goal of a study at the Minnesota Station was to determine if treatment of bovine satellite cell (BSC) cultures with 17β-estradiol (E2) or trenbolone (a synthetic androgen) directly affects proliferation rate or level of muscle mRNA for estrogen receptor (ER)-α, androgen receptor, and growth factors that have been shown to affect muscle growth (insulin-like growth factor (IGF)-I, insulin-like growth factor binding protein (IGFBP)-3, and myostatin). Castrated male cattle (steers) provide a particularly good model system in which to study the effects of anabolic steroids on muscle growth because they respond dramatically to treatment with both estrogens and androgens. BSC cultures were established from the semimembranosus muscles and then treated for 48 hours with various concentrations of 17β-estradiol or trenbolone ranging from 0.001 nM to 10 nM. IGF-I mRNA levels in proliferating BSC cultures were significantly increased at 0.01 (1.9 times control values, p < 0.02) and at 0.1, 1 and 10 nM E2 (2.9, 3.5 and 3.5 times control values, respectively, p < 0.0001). Additionally both 1 and 10 nM trenbolone increased IGF-I mRNA levels to 1.7 times control values (p < 0.02). ER-α mRNA was detectable in BSC cultures, and levels were increased (2.3 times control levels, p < 0.001) in cultures treated with 0.001 nM E2 but not in cultures treated with higher concentrations of E2. Androgen receptor mRNA levels also were increased (1.5 times control levels, p < 0.02) in cultures treated with 0.001 nM trenbolone but not by treatment with higher concentrations of trenbolone. Levels of IGFBP-3 were increased (1.4 times control values, p < 0.02) by treatment with 0.001 nM E2 but not by treatment with high concentrations of E2. Myostatin mRNAs were not increased by any concentration of either of the steroids. Although, levels of IGF-I mRNA were 10 times greater (p < 0.02) in fused BSC cultures than in proliferating cultures, treatment of fused cultures for 48 hours with 10 nM E2 increased IGF-I mRNA levels (2.5 times control levels, p < 0.02). Both E2 and trenbolone increased 3H-thymidine incorporation rate (1.5 times control levels, p < 0.001) in BSC cultures in media containing serum from which IGFBP-3 had been removed by anti-
IGFBP-3 affinity chromatography. In summary, treatment of BSC cultures with either E2 or trenbolone increased IGF-I mRNA level and proliferation rate, thus, establishing that these steroids have direct anabolic effects on cells present in the BSC culture.

The effects of sub-therapeutic antimicrobial supplementation and weaning on serum levels of IGF-I and insulin-like growth factor binding proteins (IGFBP-2, -3 and -4) were determined in crossbred weanling pigs at the Minnesota Station. At weaning, pigs were allotted to a diet containing 21.8% crude protein and 1.15% lysine with or without Aureozol (100 g/ton of Aureomycin™ chlortetracycline, 100 g/ton of sulfathiazole and 50 g/ton penicillin) for 4 wk. Insulin-like growth factor binding protein (IGFBP) and insulin-like growth factor-I (IGF-I) analyses were performed on blood samples that were drawn weekly. Weaning decreased serum IGFBP-3 levels in both control and Aureozol-treated groups on d 6 and d 14 (P < 0.05) relative to pre-weaning levels. IGFBP-3 values returned to pre-weaning levels by d 21. Although the circulating levels of both the 43-kDa and the 39-kDa glycosylation variants of IGFBP-3 were affected by weaning, the level of the 39-kDa IGFBP-3 was affected relatively more than that of the 43-kDa IGFBP-3 (P < 0.05). As compared to circulating IGFBP-3 levels in control pigs, Aureozol-treated pigs had significantly higher circulating IGFBP-3 levels on d 21 (43%, P < 0.05) and d 27 (46%, P < 0.05). In direct contrast to the effect of weaning on serum IGFBP-3 level, serum IGFBP-2 levels increased on d 6 and d 14 after weaning (P < 0.05) and decreased to pre-weaning levels by d 21. IGFBP-2 levels continued to decline and were less than pre-weaning levels by d 27 (P < 0.05). Aureozol treatment had no effect on serum IGFBP-2 levels at any time. Serum levels of non-glycosylated IGFBP-4 were not affected by either weaning or Aureozol supplementation. Weaning decreased circulating IGF-I concentration on d 6 in both control and Aureozol-treated pigs (76 and 73%, respectively, P < 0.05) and on d 14 (62%, P < 0.05) and d 21 (32%, P < 0.05) in control pigs. Aureozol-supplemented pigs had higher serum IGF-I concentrations than control pigs on d 14 (82%, P < 0.05), d 21 (55%, P < 0.05) and d 27 (36%, P < 0.05). The Aureozol fed pigs had a 14.2% increase in body weight gain (P < 0.05) and a 59.6% increase in average daily gain (P < 0.05) compared with pigs fed the control diet. Both Aureozol-supplementation and weaning cause changes in serum IGFBP levels and IGF-I concentrations; these factors may be involved in regulating rate and efficiency of growth.

The effect of early posthatch starvation on myonuclear apoptosis was examined in chickens at the North Carolina Station. Male broiler chickens were kept with or without feed for the first 3-d posthatch. Subsequently, all chickens were provided feed for an additional 4 days. Chickens were killed at 3- and 7-d posthatch, and the pectoralis thoracicus was harvested, fixed and embedded in paraffin. Muscle sections were labeled with the terminal deoxynucleotidyl transferase histochemical staining technique to identify apoptotic nuclei. At 3- and 7-d posthatch, there was a significantly (P < 0.05) smaller myofiber cross-sectional area for the nonfed compared with the fed chickens. A larger proportion (P < 0.05) of apoptotic nuclei relative to total nuclei was observed in the nonfed compared to the fed chickens killed at 3-d posthatch, but the proportion of apoptotic nuclei relative to total nuclei did not differ (P > 0.05) between the nonfed and fed chickens at 7-d posthatch. It appears that apoptosis is a mechanism contributing to the smaller myofiber size observed when feed is not provided early posthatch.

Myofiber growth is dependent upon the contribution of new nuclei from the mitotically active satellite cell population. The objective of a North Carolina Station study was to examine satellite cell mitotic activity in conjunction with different nutritional paradigms during the early posthatch
period. Turkey poultts were provided a standard turkey starter diet; the starter diet top-dressed with a hydrated low-fat, highly digestible protein and carbohydrate nutritional hatchling supplement, Oasis; the starter diet top-dressed with Solka-floc dyed green; or no food for the first 3 d posthatch. All birds were fed a standard starter diet during the experimental period. 5-Bromo-2'-deoxyuridine (BrdU) was continuously infused into all treatments (n = 5 all groups) between hatch and 3 d of age. A second group of identically treated poults housed in separate pens was continuously infused with BrdU between 2 and 9 d of age. Mitotically active satellite cells were identified in the pectoralis thoracicus and quantitated using BrdU immunohistochemistry in combination with computer-based image analysis. Satellite cell mitotic activity was significantly higher (P less than or equal to 0.05) in the birds fed a standard starter diet compared to all other treatments at 3 d posthatch. However, there were no (P greater than or equal to 0.05) differences in satellite cell mitotic activity among treatments at 9 d posthatch. The results of the current study suggest that any improvements in meat yield through early nutritional supplementation do not appear to occur through a satellite cell pathway and that there is no compensatory response in the satellite cell population following refeeding after early posthatch starvation.

The effects of neonatal nutrition on calpain message levels were determined by the North Carolina Station. Male broiler chickens (Ross) were provided a standard starter diet top-dressed with Oasis' nutritional supplement (fed; Novus International, St. Louis, MO, USA), or feed was withheld for the first 3 days posthatch. Subsequently, the standard starter diet was provided to all chickens between 3 and 7 days posthatch. RNA was extracted from the Pectoralis thoracicus, and skeletal muscle-specific n-calpain-1 (p94) calpain, mu-calpain, and m-calpain expression was evaluated using quantitative Northern analysis. Early posthatch starvation did not (P > 0.05) affect calpain mRNA levels on each day examined. Similarly, there were no (P > 0.05) changes in mu-calpain or m-calpain mRNA levels between 0 and 7 days posthatch in fed birds. However, p94 calpain mRNA levels were significantly (P < 0.05) lower at 7 days posthatch compared to 0 or 2 days posthatch. Therefore, in the early posthatch chicken, it appears that the calpain system may not be affected by the presence of oral nutrition, but there is an age-related down regulation of p94 calpain mRNA expression.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme in the glycolytic pathway, and it is a popular internal standard for northern blot analysis. The North Carolina Station examined GAPDH expression early in life when feed is either provided or withheld. Male broiler chickens were provided a standard starter diet plus Oasis nutritional supplement (fed group; Novus International, St. Louis, MO, USA) or feed was withheld for the first 3 d posthatch. Subsequently, the standard starter diet was provided to all chickens between 3 and 7 d posthatch. RNA was extracted from the pectoralis thoracicus, and GAPDH expression was evaluated with quantitative northern analysis. GAPDH expression was significantly (P < 0.05) higher in the fed than in the feed withheld group at 3 d posthatch, suggesting that nutritional manipulations can alter GAPDH transcription. Similarly, GAPDH mRNA levels were significantly (P < 0.05) higher at 7 d posthatch compared with all younger animals, suggesting that GAPDH is developmentally up-regulated with advancing age. GAPDH expression changes with age and nutrition status in the early posthatch chick, suggesting that GAPDH is not a proper internal standard for muscle studies using quantitative northern analysis.

Replication-defective retroviral vectors are efficient vehicles for the delivery of exogenous genes, and they may be used in the generation of transgenic animals. The replication-defective retroviral SNTZ vector carrying the lacZ gene with a nuclear localized signal was injected into the subgerminal cavity
of freshly laid eggs in a study by the North Carolina Station. Subsequently, the eggs were allowed to hatch, and the chickens were screened for the lacZ gene by using the polymerase chain reaction. Eight of 15 male chickens that survived to sexual maturity contained the lacZ gene in their semen. Subsequently, these males were mated with wild-type female chickens. From one of the eight lacZ-positive G(0) males, two lacZ-positive male chickens were produced from a total of 224 G(1) progeny for a germline transmission rate of 0.89%. Both G(1) male chickens carrying the lacZ gene were mated with wild-type female chickens and 46.5% of the G(2) progeny contained the lacZ gene, a finding consistent with the expected Mendelian 50% ratio for a heterozygous dominant allele. The product of the lacZ gene, nuclear localized beta-galactosidase, was expressed in primary myoblast cultures derived from G(2) chickens, and it was also expressed in whole G(2) chicken embryos. Thus development of a protein expressing transgenic chicken has been achieved for the first time.

Chickens do not possess the necessary enzymes to efficiently hydrolyze lactose into glucose and galactose. The bacterial enzyme beta-galactosidase can convert lactose into glucose and galactose. Transgenic chickens that carry the E. coli lacZ gene and express beta-galactosidase could potentially utilize lactose as an energy source. The objective of a North Carolina Station study was to determine the ability of the transgenic chicken small intestinal mucosa to hydrolyze lactose into glucose and galactose. Lactase activity was examined in the intestinal mucosa from wild-type chickens and two lines of chickens that carry the lacZ gene and express P-galactosidase. Lactase activity was significantly higher in both transgenic lines compared with wild type birds (P < 0.05). The presence of the beta-galactosidase enzyme was revealed by X-gal staining in the intestine of transgenic chickens, whereas it was not present in the wild-type chickens. Overall, it appears that inserting the lacZ gene, which encodes beta-galactosidase, has resulted in a chicken that can utilize lactose as an energy source. This study demonstrates that transgenic technology can be used to modify nutrient utilization in domestic poultry.

MEKK1 resides within the three-tiered model of MAPK signaling at a level comparable to Raf. Overexpression of this kinase results in downstream activation of both JNK and ERK1/2. The Pennsylvania Station found that sustained MEKK1 kinase activity leads to an inhibition of myofiber formation and muscle gene expression. Interestingly, E47, the obligate dimer partner of the myogenic regulatory factors (MRFs) appears to be a target of MEKK1 signaling. MEKK1 does not alter E47 DNA binding but does severely impair the inherent transcriptional activation capabilities of E47. In addition, forced heterodimers of MyoD and E47 are DNA binding competent but fail to drive muscle-specific reporter gene activity. Thus, suppression of myogenesis by MEKK1 likely involves disruption of E47 function leading to inactivation of MRF:E protein transcriptional complexes.

The South Dakota Station continues to examine the properties of satellite cell subpopulations within single muscles that differ in their responses to growth factors. In previous work it was shown that rapidly growing satellite cell clones are more responsive to the mitogenic effects of IGF, FGF and PDGF. They are also more responsive to the proliferation and differentiation depressing effects of TGF-β. However, no differences were detected in either receptor numbers or affinities. Current work is focused on determining if the variation in growth factor responses is due to differences in the activity of the receptors. A Western Blotting technique has been developed to assay the phosphorylation state of the receptor docking protein Shc and thus estimate receptor tyrosine kinase activity. As there are no commercial antibodies produced against the turkey Shc protein, a number of
antibodies against Shc from other species have been screened. Recently one antibody has been found that seems to work very well.

Studies conducted at the Washington Station have optimized technical aspects required to culture both muscle and fat-type cells from both bovine and fish. The fat cells are unique, as they begin as mature adipocytes, de-differentiate into adipofibroblasts, proliferate like fibroblasts, then differentiate into mature fat cells. A chemically defined treatment medium (with little insulin, no dexamethasone and small amounts of MIX) has been developed that causes adipofibroblasts to fully differentiate in less than 48 hr after exposure in vitro. Initial studies have been conducted to evaluate the developmental expression of fat cell differentiation markers from both fish and beef-derived adipofibroblasts. Also collaborative studies with Canadian researchers have shown that certain forms of fatty acids promote proliferation of adipofibroblasts while others induce differentiation of the same cells.

The Washington Station has also begun a new research program on how control of somatic tissue growth and development is coordinated through the collaborative efforts of hormones, growth factors and cytokines. Although subtle species-specific differences often exist, the participating factors and their mechanisms of action are generally well conserved throughout the various vertebrate classes. Initial studies have involved the isolation and characterization of myostatin cDNA clones from several fish species. The myostatin null phenotype of domesticated animals includes extreme skeletal muscle hypertrophy compared to heterozygous and wild type animals. Although the mechanisms of action are only beginning to be determined, myostatin appears to prevent myoblast hyperplasia by inhibiting cell cycle progression past the G\(_1\) and G\(_2\) stages. These actions are mediated in part by reduced Cdk 2 levels and activity, a concomitant increase in p21 Cdk-inhibitor and consequently, the hypophosphorylation of retinoblastoma protein (Rb). The insulin-like growth factors (IGF-I & -II) similarly increase p21 activity and Rb hypophosphorylation, suggesting convergence between the signaling pathways of the IGF-I and myostatin receptors in regulating myoblast cell cycle withdrawal. However, IGF-I stimulates myoblasts to differentiate and eventually form myotubes whereas myostatin’s actions appear to be solely inhibitory. Thus, activation of the same intracellular substrate by either IGF-I or myostatin has completely opposite effects and indicates that alternative and additional pathways mediate the specific effects of each cytokine. Their differential ability to regulate skeletal muscle development, albeit via similar signaling pathways, will prove invaluable in defining the necessary biochemical and molecular mechanisms that mediate myoblast proliferation and differentiation. Previous studies have determined the developmental and stress-induced pattern of myostatin gene expression in the tilapia, Oreochromis mossambicus. The current goals are to determine the mechanisms of myostatin action at the cellular level using an alternative model organism, the rainbow trout Oncorhynkus mykiss.

**Objective 2:** Determine the nuclear mechanisms that control gene expression in skeletal muscle.

β-adrenergic agonists increase net protein accretion in skeletal muscle, and, in particular, the β-adrenergic agonist, ractopamine, stimulates skeletal α-actin expression in the pig. The Iowa Station tested the hypothesis that the full-length promoter (-1929 to +243) of the porcine skeletal α-actin-encoding gene was transcriptionally responsive to ractopamine. Porcine derived satellite cells were transiently transfected with luciferase expression constructs that contained 5’ regulatory regions of the...
porcine skeletal α-actin gene. Upon differentiation, porcine myotubes were stimulated with ractopamine (10 µM) in serum-free media and luciferase activity was evaluated. After 72 hours in treatment media, quantitative real-time RT-PCR confirmed that ractopamine treatment resulted in a fivefold increase (P < 0.05) in skeletal α-actin mRNA abundance (relative to control cultures). However, ractopamine failed to increase the transcriptional activity of the cloned skeletal α-actin promoter. Additional luciferase expression assays conducted with multiple truncated promoter constructs failed to identify a ractopamine-responsive DNA element within the proximal promoter regions. The results indicate that the accumulation of skeletal α-actin mRNA in response to ractopamine treatment may be due to posttranscriptional regulation as evidenced by the fact that known cis-acting DNA elements previously described 5’ of the porcine skeletal α-actin-encoding gene do not confer transcriptional responsiveness to ractopamine in porcine myotubes.

Ractopamine, commercially available as Paylean, promotes hypertrophy and alters myosin heavy chain (MHC) isoform content in porcine skeletal muscle, presumably through molecular mechanisms that involve the activation of β-adrenergic receptors and associated intracellular signal transduction cascades. However, sufficient mechanistic detail as to how muscle hypertrophy and fiber type plasticity is regulated by ractopamine has proven to be elusive at the molecular level. Work at the Iowa Station used the suppression subtractive hybridization (SSH) procedure to examine pre-translational gene expression after ractopamine administration in porcine skeletal muscle. Longissimus dorsi muscle samples were collected after feeding two levels of ractopamine (Paylean at 0 or 60 ppm) to maternal line pigs for 3 d and polyadenylated (poly A') mRNA was used to generate cDNA libraries for each treatment. Using the control (0 ppm) cDNA library as the driver and the ractopamine (60 ppm) cDNA library as the tester, a forward-subtracted cDNA library was generated by SSH. After amplification by PCR, the resultant amplicons were subcloned and twenty-four of 192 clones were selected for sequence analysis. Differentially expressed genes were putatively identified by sequence homology (>85%) to GenBank entries. Nine genes (calmodulin-1, phosphatase inhibitor 2, homer homolog 1, mortalin-2, DnaJ (Hsp40) homolog, Mago-nashi homolog, heterogeneous nuclear ribonucleoprotein, synthrophin, and synemin) and one expressed sequence tag (EST) were identified as being differentially expressed in longissimus dorsi muscle at the pretranslational level after ractopamine stimulation. Northern blot analysis performed on the original RNA samples confirmed that calmodulin-1 mRNA abundance increases approximately 2-fold (P < 0.05) after 3 d of ractopamine treatment. This is the first study in which the expression of a calcium-modulated protein has been implicated in the phenotypic adaptations of skeletal muscle to ractopamine in the pig. Further elucidation of the transcriptional control of calmodulin-1 by ractopamine, as well as the posttranslational role of this protein, should provide more detail as to how ractopamine alters skeletal muscle phenotype and enhances lean body composition.

Satellite cell proliferation is critical to postnatal skeletal muscle hypertrophy. Understanding the molecular mechanisms underlying hypertrophy is necessary to facilitate treatment of muscle wasting diseases and to promote increased muscle accumulation in meat animals. Previous research at the Iowa Station has suggested that JAK2 plays a role in skeletal muscle hypertrophy. Janus kinases (JAKs) are receptor-associated tyrosine kinases that are associated with cytokine receptor superfamily signaling. It was hypothesized that JAK2 was required for normal proliferation of skeletal muscle myoblasts. To elucidate the role of JAK2 in proliferation, C2C12 myoblasts were plated at a density of 1250 cells/cm². Proliferating cells were identified using BrdU incorporation. Initially, 0.2, 2 and 20 µM concentrations of Tryphostin AG490, a JAK2 specific inhibitor, were used to block JAK2 signaling. All AG490 treated cells showed a very significant (p<0.0001) decrease in proliferation. Cells treated with 20 µM
concentrations of AG490 had almost no BrdU incorporation (2.3%) vs. 0 µM (66%). Proliferating cells were identified via BrdU incorporation and counted at either 24 or 48 hours. Rescue plates were cultured with AG490 for 24 hours and then changed to normal growth media with vehicle for an additional 24 hours. Rescue cells had a significantly higher percentage of proliferation than cells continuously treated with AG490 for 24 or 48 hours (p<0.0001). These cells also had a similar percentage of proliferation as the 48-hour control cells (p>0.05). These results indicate that C2C12 myoblasts can recover from treatment with AG490 and proliferate at a similar level to control cells. Since it has been demonstrated that JAK2 plays a specific role in normal C2C12 proliferation, experiments were conducted to see if AG490 could inhibit the stretch-induced cell proliferation. Using the Flexcell 3000 stretching apparatus, cells were stretched for 12 hours in media containing AG490. Stretch significantly increased (p<0.02) proliferation of control C2C12 myoblasts. However, cells stretched in the presence of AG490 showed no evidence (p=0.79) of increased proliferation in response to stretch. The results of these experiments indicate that JAK2 is required for both normal and stretch induced proliferation in C2C12 myoblasts.

Complementary DNA macroarray and microarray experiments are being performed at the Michigan Station to identify genes that are differentially expressed in pig skeletal muscle at several fetal and postnatal ages, as well as in proliferating and differentiating pig skeletal muscle satellite cells. A cDNA library has been constructed from skeletal muscle tissue obtained from pigs at an ontogeny of ages from mid-gestation to maturity. Clones from this library are being sequenced and used for the development of cDNA microarrays. These microarrays will also be used to evaluate gene expression patterns in myogenic cell culture models. The establishment of specialized cell types during development of specific tissues involves the expression of distinct sets of cell type-specific genes. A long-term objective is to identify genes that are differentially expressed during pig skeletal muscle development. The facilities in the MSU Center for Animal Functional Genomics (CAFG) are being utilized to understand the complex mechanisms regulating pig skeletal muscle growth. A normalized porcine skeletal muscle cDNA library has been constructed (designated PoSM) using poly (A)+RNA isolated from porcine skeletal muscle tissue collected from the hind limbs of pigs at 45 and 90 days of gestation, birth, 7 weeks of age and 1 year of age. The normalized library has been plated and colonies have been robotically picked for high-throughput DNA sequence analysis. To date, sequence has been obtained and evaluated for 782 clones and cluster analysis of the data indicates that 742 unique cDNAs are present in this collection, reflecting a low redundancy rate of 5.1%. In addition, Southern blot analysis of the relative frequencies of five abundant cDNA clones in the original and normalized libraries confirmed the effectiveness of the normalization procedure. A publicly accessible database has been developed for the storage of clone data, including clone sequences, BLAST results and keywords (http://www.cafg.msu.edu).

The Michigan CAFG facilities have been used to create a cDNA microarray containing 740 randomly selected clones from the PoSM library and 28 clones derived from differential display reverse transcription polymerase chain reaction (DDRT-PCR) experiments. All clones were spotted in triplicate and arrayed in 48 8X8 patches. A portion of the bacteriophage Lambda Q gene was spotted in the top left corner of every patch as a positive hybridization control. Initial analyses utilized pools of total RNA from skeletal muscle of pigs at 60 days of gestation and 7 weeks of age that were labeled with both Cy3 and Cy5 in reciprocal experiments. Subsequent analyses utilized total RNA samples from three individual pigs at 60 days of gestation and three individual pigs at 7 weeks of age. Each 60-day sample was randomly paired with a 7-week sample for microarray screening. For two of the
arrays, the 60-day sample was labeled with Cy3 while the 7-week sample was labeled with Cy5. For the third array, the 60-day sample was labeled with Cy5 and the 7-week sample was labeled with Cy3. Normalized fluorescence intensity data was log transformed and analyzed by analysis of variance. Nine genes were identified to be differentially expressed (P<0.05). Eight genes were over expressed in the 60-day fetal samples and the remaining gene was over expressed in the 7-week postnatal samples. These results demonstrate that microarray analysis can reveal putative differentially expressed genes in developing skeletal muscle. Development of real time RT-PCR assays for validation of differential expression of these genes is underway.

A cDNA macroarray containing 327 expressed sequence tag (EST) clones was constructed at Iowa State University through a collaborative project with Dr. Chris Tuggle's laboratory (Zhao et al., 2003). This array includes ESTs from whole embryo and adult skeletal muscle, and products derived from DDRT-PCR experiments performed in the Michigan Station laboratory. This macroarray has been utilized to identify differentially expressed genes in proliferating and differentiating porcine skeletal muscle satellite cells. Clonally derived satellite cells (fourth passage) were seeded into gelatin-coated 60-mm culture dishes in MEM with 10% FBS. Fresh media was supplied at 48-h intervals, and after approximately five days, media was changed to a serum-free medium containing either 10^-6 M insulin to induce differentiation, or 10^-9 M insulin. Cultures were maintained under these conditions for three days with daily media changes, after which cells were harvested for total RNA isolation. Successful induction of differentiation was assessed by visual appraisal of the cells in culture and confirmed by evaluation of myogenin mRNA abundance using dot blot analysis. Total RNA from four clonal populations (two proliferating and two induced to differentiate) has been used to screen four copies of the macroarray. Complete analysis of the array screening results is in progress. Genes for two extracellular matrix proteins, fibronectin I (FNI) and collagen type I alpha 2 (COLIA2), identified by DDRT-PCR to be differentially expressed between fetal and postnatal muscle, appear to be up regulated when satellite cell cultures are induced to differentiate with insulin. Validation of these results by blot hybridization or real time RT-PCR analyses is in progress.

The Utah Station has initiated a project employing real-time quantitative PCR (Q-PCR) to profile gene expression in the hypertrophy-responsive gluteus medius muscle and in the hypertrophy-nonresponsive supraspinatus muscle from callipyge and normal lambs. To date, primers have been developed for several genes important to muscle growth and development using their known ovine mRNA sequences. Amongst these are genes involved with commitment to the myogenic lineage [myogenin, myf5], intracellular signal transduction [calmodulin 2], hypertrophy and protein turnover [calpains I and II, calpastatin, myostatin], muscle function [lactate dehydrogenase (LDH), myosin heavy chains (MHC) 2x, s, and 2a], autocrine/paracrine hormones [insulin like growth factors (IGF) I and II; basic fibroblast growth factor (bFGF)], and a commonly used housekeeping gene [glyceraldehyde 3 phosphate dehydrogenase (G3PDH)]. All primers were designed to achieve optimal performance in real time Q-PCR, including comparable anneal temperature and amplicon lengths, and conditions for their use with cDNA from ovine muscle are being optimized. The identity of the PCR products will be confirmed by DNA sequence analysis. Primers remain to be developed for DLK-1 (delta, drosphilia homolog-like1), an imprinted gene linked to expression of the callipyge phenotype, and for the important signal transduction molecule, calcineurin.
Objective 3: Characterize muscle proteins and their functional domains involved in myofibrillar assembly and disassembly

The Arizona Station has examined the structures of m- and µ-calpains in different calcium states using partial proteolytic cleavage. Digestion of these calpains with trypsin or chymotrypsin in the absence of calcium for periods of up to 2 hours yields a limited number of large peptide fragments, suggesting that these proteins have a very compact structure. The region between amino acids 245 and 270 was most susceptible to cleavage. Proteolytic activity reduction by proteolysis lagged behind peptide bond breakage in the 80 kDa subunit; 45% of the calpain activity remained even though there was none of the 80 kDa subunit still intact. Thus the protein could remain enzymatically active in spite of significant backbone cleavage. Addition of calcium dramatically changed the rate and extent of the tryptic or chymotryptic cleavage. Both m- and µ-calpains were degraded to fragments of less than 40 kDa in less than 5 minutes, and with m-calpain this occurred within 30 seconds. Tryptic digestion of m-calpain produced a 51 kDa fragment containing all three of the catalytic amino acids, but it retained only 2% of the original calpain activity. This suggests an important role of more distant regions of the calpains in their catalytic activity.

An area of effort of the Illinois Station involves the examination of the calpain proteolytic system in rainbow trout skeletal muscle. Components of the rainbow trout calpain proteolytic system were isolated by chromatographic techniques and their in vitro activities characterized. Two peaks of calpain activity, along with their endogenous inhibitor calpastatin, were identified. Calcium requirements for half-maximal activities for calpain I and II were 0.2 mM and 2.5 mM respectively, significantly higher than typical calcium requirements seen in mammals. Western blot analysis was used for the confirmation of calpain I and calpastatin. Incubation of trout fillets with a CaCl₂ solution was shown to reduce shear force suggesting that the calpain system may be important in postmortem proteolysis and reduction in muscle integrity in post harvest rainbow trout. Additionally, the calpain 4 (catalytic subunit) cDNA from rainbow trout has been cloned and sequenced. This represents the first reported fish and lower vertebrate full-length cDNA of Capn4. Alignments were made to other fish partial cDNA homologues and mammalian sequences. Domain V of Capn4, containing a glycine-rich consensus region in all mammals and known to play a role in membrane targeting, is absent in fish. A phylogenetic tree for the Capn4 gene super-family was constructed including members from mammals, fish and plants. The divergent Capn4 seen in fish suggests different functions and activation mechanisms of the fish calpain system.

The pH and Ca²⁺ sensitivity of myofibrillar ATPase activity plays an integral role in regulating postmortem muscle ATP utilization. The Indiana Station conducted a study to determine the influence of pH and Ca²⁺ concentration on the ATPase activity of myofibrils from red semitendinosus (RST) and white semitendinosus (WST) porcine muscles. Myofibrillar ATPase was measured at 39°C over a pH range from 5 to 7.5 and a [Ca²⁺] range from pCa 4 to 9. At maximum Ca²⁺-dependent activation (pCa 4), RST myofibrils had lower ATPase activity than WST myofibrils. This maximum activity of myofibrils from both muscle regions was not influenced from pH 7.5 to 6.5, declined between pH 6.5 and 5.75 (Hill coefficient, nH = 2.7-3.4; pH at half maximum activity, pH50 = 5.97) and was near zero at pH 5.5. At pH 7, pCa-activity relationships showed that RST required less Ca²⁺ for half-maximum activation (higher pCa50; 6.50) than WST myofibrils (pCa50 = 6.35) but had no difference in nH. At pH 7, both RST and WST myofibrils had maximum Ca²⁺-dependent, actin-activated ATPase activity at pCa≤ 6 and Ca²⁺-independent myosin ATPase activity at pCa ≥
6.75. pCa-activity relationships at different pH levels indicated that pCa₅₀ decreased with pH from pH 6.5 to 6.125 in both RST and WST myofibrils. At pH < 5.75, [Ca²⁺] did not influence ATPase activity in RST or WST myofibrils. These data show that myofibrils with predominantly fast MyHC (WST) have a higher actin-activated myosin ATPase activity than myofibrils with primarily slow MyHC isoforms (RST) at Ca²⁺ concentrations and pH values characteristic of postmortem muscle.

A study by the Indiana Station was conducted to determine the effects of postmortem pH and temperature declines on the actomyosin ATPase activity of muscle fibers expressing different MyHC isoforms. Maximum actomyosin ATPase activity was determined on individual fibers classified by MyHC expression. Samples were collected from the red (RST) and white (WST) semitendinosis muscles at 3 min and 24 h postmortem from electrically stimulated (ES) and control (NS) pork carcasses. In samples taken at 3 min postmortem, type I fibers had the lowest ATPase activity and type 2X and 2B had the highest activity, with type 2A fibers intermediate. Postmortem time and carcass treatment did not influence the ATPase activity of type I muscle fibers. ATPase activity of 2A fibers was lower in 24 h samples than in 3 min samples from ES carcasses. In 3 min and NS-24 h samples, RST type 2A fibers had lower activities than type 2A fibers from the WST. In type 2X fibers, ATPase activity decreased from 3 min to 24 h postmortem in ES carcasses. This decrease was more severe in WST 2X fibers compared to RST 2X fibers. ATPase activity in type 2B fibers did not decrease from 3 min to 24 h postmortem in NS carcasses. In ES carcasses, activity of 2B fibers decreased with time postmortem. These data suggest that fibers expressing fast MyHC isoforms have a higher ATPase activity early postmortem than slow muscle fibers but are more prone to inactivation by a rapid pH decline.

Several ongoing studies at the Iowa Station are in progress to characterize the protein components of the muscle cell cytoskeleton and the role of the cytoskeleton in muscle growth. These include: (1) Characterization of the interactions of the novel, large intermediate filament proteins synemin and paranemin with other myofibrillar and cytoskeletal proteins, particularly protein components of the costameres and Z-lines; (2) Localization of intermediate filament proteins in developing, embryonic chick skeletal muscle cell cultures at both the light and electron microscope levels, using a panel of specific antibodies; (3) Characterization of an actin binding site within the cytoskeletal, costameric protein talin; (4) Use of atomic force microscopy and analytical centrifugation to demonstrate that ADP-ribosylated desmin exists primarily as a tetramer of 53 kDa desmin polypeptides, thus indicating that ADP-ribosylation of the head domain of desmin blocks assembly of desmin by interfering with the ability of the tetramers to associate into higher oligomers in the process of assembly into 10-nm filaments; and (5) Use of RNA aptamers as probes to identify ADP-ribosylated proteins in muscle cells and to understand how ADP-ribosylation functions to inhibit terminal myogenic differentiation. Results of these studies will be presented at subsequent meetings.

Work at the Oregon Station has been conducted on calpain 5. Methods have been developed for purification of this isoform, and some characterization experiments completed. The protein autolyses in the presence of calcium, but the calcium concentration dependence is extremely high (>20 mM). The activity of the enzyme is insensitive to E64 or calpastatin. Studies are also being conducted on the role of MuRF-1 and MAFbx on protein turnover. Both these proteins have been found to be up-regulated in immobilized, denervated, and hind limb suspension muscle. Both are E3 ligases that function in preparing proteins for proteasome degradation. MuRF-1 binds to titin near the M-line of the myofibril, and is also near a site of calpain binding.
The **Wisconsin Station** has developed a new agarose gel system for electrophoretic separation of high molecular weight proteins. The electrophoretic separation of high molecular weight proteins (>500 kDa) using polyacrylamide is difficult because gels with a large enough pore size for adequate protein mobility are mechanically unstable. A 1% vertical SDS-agarose gel electrophoresis (VAGE) system has been developed that allows titin (a protein with the largest known sodium dodecyl sulfate subunit size of 3000-4000 kDa) to migrate over 10 cm in a ~13 cm resolving gel. Such migration gives clear and reproducible separation of titin isoforms. Proteins ranging in size from myosin heavy chain (~220 kDa) up to titin can be resolved on this gel system. Electroblotting of these very large proteins was nearly 100% efficient. This VAGE system has revealed two titin size variants in rabbit psoas muscle, two N2BA bands in rabbit cardiac muscle, and species differences between titins from rat and rabbit muscle. Agarose electrophoresis should be the method of choice for separation and blotting of proteins with very large subunit sizes.

An immunofluorescence microscopy method for following changes in myofibrillar-bound calpain 3 was developed at the **Wisconsin Station**. Afterward, proteolytic changes in calpain 3(p94), calpain 1, titin, and nebulin were examined in myofibrils prepared from ovine longissimus stored for 0, 1, 2, and 3 days postmortem. Western blot analysis revealed that the levels of intact calpain 3 (expressed as percentage of the level immediately postmortem) were 80%, 10% and not detectable in myofibrils prepared at 1, 2, and 3 days respectively. Western blots for calpain 1 also indicated conversion of the intact protein (80 kDa) to a 76 kDa fragment during the same time period. Thus calpains 1 and 3 appear to be activated during postmortem storage. Immunofluorescence microscopy using an IS1 region specific antibody revealed that calpain 3 staining was most intense at the sarcomere Z- and M-lines. The fluorescence intensity declined significantly during storage, paralleling changes in the proteolytic breakdown of titin and nebulin associated with these structures.

Previously, two splice variants of Cypher, a striated muscle specific PDZ-LIM domain protein, Cypher1 and Cypher2, had been reported. Four additional splice isoforms have now been characterized in collaborative work involving the **Wisconsin Station**, two of which are novel. The six isoforms can be divided into skeletal or cardiac specific classes, based on the inclusion of skeletal or cardiac specific domains. Short and long isoforms share an N-terminal PDZ domain, but the three C-terminal LIM domains are unique to long isoforms. By RNA and protein analysis, it was demonstrated that Cypher isoforms are developmentally regulated in both skeletal and cardiac muscle. It was previously shown that knockout of Cypher is neonatal lethal. To investigate the function of splice variants in vivo, rescue experiments have been performed of the Cypher null mutant by replacing the endogenous Cypher gene with cDNAs encoding either a short or long skeletal muscle isoform. In contrast to Cypher null mice, a percentage of mice that express only a short or a long skeletal muscle specific isoform can survive to at least one year of age. Although surviving mice exhibit muscle pathology, these results suggest that either isoform is sufficient to rescue the lethality associated with absence of Cypher.

**Usefulness of Findings**

The goal of this project is to increase the efficiency of lean meat production in domestic animals through the use of basic research into the biological mechanisms that regulate muscle growth and differentiation. The results summarized in this project report demonstrate that the project participants
continue to aggressively acquire information about the fundamental biological processes that regulate muscle development and growth of meat-producing animals. The committee continues to be highly productive as indicated by the number of publications appearing in the last year. Collaboration through sharing of techniques, culture models, antibodies, molecular probes, and expertise continues to be an important aspect of the project. Members have made substantial contributions toward understanding the extracellular signals, intracellular signal transduction pathways, and nuclear mechanisms that govern both myoblast/satellite cell activity and muscle protein accumulation. The committee has also made significant progress in understanding muscle protein characteristics and the regulation of myofibrillar assembly and disassembly. Committee members use and frequently improve current methods in cell and molecular biology, genomics and proteomics to address fundamental problems relevant to meat animal production. Information gained by the project continues to provide the foundation for the development of novel strategies to improve musculature, the rate and efficiency of muscle growth, and meat quality. For example, understanding how proteolytic activity of calpains is regulated may provide the opportunity to increase the rate of muscle growth with little or no increase in ingested energy, just as current understanding of calpain activation has providing novel methods to improve meat tenderization. Understanding regulation of myoblast activation, proliferation and differentiation by growth factor signaling offers potential to manipulate prenatal muscle fiber formation as well as postnatal satellite cell-mediated accumulation of myofiber nuclei. Elucidating the mechanisms that control myofiber gene expression and subsequent phenotype may provide opportunities to improve live animal efficiency. Likewise, manipulation of muscle protein isoform distribution may alter postmortem metabolism and subsequent meat quality. Developments based on fundamental research done by the committee are essential to the viability of animal agriculture.

Work Planned for Next Year

Studies will continue as described in the approved proposal. No changes in the approach or objectives are anticipated at this time.
Publications for the Year

Refereed Journal Articles

Published Full-Length Articles

Arizona


Idaho


Illinois


Indiana


Iowa

**Kansas**


**Michigan**


**Minnesota**


**North Carolina**


**Ohio**


**Oregon**


**South Dakota**


**Wisconsin**


**Washington**


**Published Abstracts**

**Arizona**


**Hawaii**


**Idaho**


**Indiana**


**Iowa**


**Kansas**


**Michigan**


**Nebraska**


**North Carolina**

Mozdziak, P.E. 2003. An undergraduate laboratory course on animal cell culture techniques *Poult. Sci.* (Suppl. 1) 82: 255.


**Wisconsin**


**Washington**


**Accepted Full-Length Articles**

**Arizona**


**Illinois**


**Indiana**


**Iowa**


**Kansas**


Michigan


Minnesota


Ohio


Pennsylvania


South Dakota


**Washington**


**Non-refereed Publications**

*Proceedings, Book Chapters, etc.*

**Arizona**


**Idaho**


**Ohio**

South Dakota


Washington


Dissertation/Thesis

Hawaii


Indiana


Nebraska


APPROVED:

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Marion Greaser
Dr. Marion Greaser, Secretary, 2003

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Elton Aberle
Dr. Elton Aberle, Administrative Advisor

12-23-03

01-07-2004

Date

Date