Project No. and Title:
NC1184: Molecular Mechanisms Regulating Skeletal Muscle Growth and Differentiation
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Annual Meeting Dates: 27-Oct-2012 to 28-Oct-2012

Participants:
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Min Du, Washington State University
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Michael Zeece, University of Nebraska
Steven Jones, University of Nebraska
Deb Hamernik – Administrative Advisor
Mark Mirando - NIFA Representative

Brief Summary of Minutes of Annual Meeting:
Members not attending: Donald McFarland, South Dakota State University; Sandra Velleman, Ohio State University; Yong Soo Kim, University of Hawaii; Gale Strasburg, Michigan State University; Rod Hill, Idaho; J.E. Minton, Kansas; Ronald Allen, University of Arizona; Catherine Ernst, Michigan State University; Rajkumar Prabhu, Mississippi State University; Shihuan Kuang, Purdue University; Shijun Li, University of Wisconsin; Michael Zeece, University of Nebraska; Steven Jones, University of Nebraska.

The annual meeting of the NC-1184 technical committee meeting was held at Virginia Polytechnic Institute and State University, on October 27-28, 2012, and was hosted by Dr. Sally Johnson from the Department of Animal and Poultry Science. On October 27th, the group was welcomed by Dr. Alan Grant, Dean of the College of Agricultural Sciences. NIFA Representative, Dr. Mark Mirando, made brief remarks via teleconference to the group regarding changes at NIFA, the USDA funding outlook, and status of funding RFA revisions. The business meeting was chaired by the Administrative Advisor, Dr. Deb Hamernik. Next year's annual meeting of the NC-1184 committee will be held at North Carolina State University. The group decided that the 2013 (2014 location is Iowa State University) meeting will be held at North Carolina State University, Paul Modziak, chair. The remainder of the day was filled by oral station reports summarizing each station's contributions to the objectives of the NC-1184 project. The meeting adjourned, and the group met for dinner at the Gerrard residence. The

meeting reconvened on October 28 and the remaining stations reported on their activities. Following completion of the oral reports, the meeting adjourned for the year.

Accomplishments

Objective 1. Characterize the signal transduction pathways that regulate skeletal muscle growth and metabolism including the influence of endogenous growth factors and various production practices.

The **Illinois Station** reported the following: In swine, IGF2 is of particular interest as a quantitative trait loci (QTL) controlling muscle growth and fat deposition. This effect was first reported in crosses of Large White pigs with either wild European boars or pigs of the Peitran breed and explained 15-30% of variation in muscle mass and 10-20% of variation in back fat. The mutation was later identified as a single base pair substitution in intron 3 of IGF2 (g3072A). The substitution altered a conserved CpG island that is hypomethylated in skeletal muscle and is thought to be a binding site for a repressor or other regulatory element. The mutation resulted in an increase in IGF2 expression in postnatal muscle though it did not alter IGF2 expression in fetal muscle. Increased postnatal IGF2 expression may be responsible for increased differentiation in satellite cells resulting in increased muscle hypertrophy. However, the phenotype of IGF2 intron 3 g3072A animals has not been fully characterized; therefore, the mechanism by which this mutation alters muscle growth remains unclear. Interestingly, IGF2 is one of many imprinted genes in that only one allele of IGF2 is expressed. In the instance of IGF2 it is the paternal allele which is expressed.

The **Illinois Station** sought to more fully characterize the phenotype of the IGF2 mutation and determine the mechanism of increased muscle growth in pigs carrying a paternal A allele, IGF2 mutant and wild-type animals will be produced with a common genetic background. First, a complete growth curve of body weight and length will be established from fetal development through market weight. Feed intake and efficiency will be measured in finishing pigs and related to common carcass measurements. Muscle hyperplasia, hypertrophy and fiber type will be investigated. Finally, expression and protein levels of IGF2 and other relevant genes, throughout the life span of market animals will be established.
At this time, offspring have been collected at days 60 and 90 of gestation and length and weight measured. At d60, female offspring carrying paternal A alleles were heavier than female offspring carrying paternal G alleles, but male offspring of different genotype did not differ in weight. Length was also similar between genotypes in both males and females at d60 gestation. Similarly, body weights, weights of longissimus dorsi and semitendinosus muscles and length were similar between genotypes at d90 of gestation.

The **Indiana Station** reports that skeletal muscles in the limb and body trunk are composed of heterogeneous myofibers expressing different isoforms of myosin heavy chain (Myh), including type I (slow, Myh7), IIA (intermediate, Myh2), IIX (fast, Myh1), and IIB (very fast, Myh4). We report here that myostatin (Mstn) positively regulates slow but negatively regulates fast Myh isoforms. Mstn was expressed at higher levels in the fast muscle myoblasts and myofibers than in the slow muscle counterparts. Interestingly, Mstn knockout led to a shift of Myh towards faster isoforms,

suggesting an inhibitory role of Mstn in fast Myh expression. Consistently, when induced to differentiate, Mstn null myoblasts formed myotubes preferentially expressing fast Myh. Conversely, treatment of myoblasts with a recombinant Mstn protein upregulated Myh7 but downregulated Myh4 gene expression in newly formed myotubes. Importantly, both Mstn antibody and soluble activin type 2B receptor inhibited slow Myh7 and promoted fast Myh4 expression, indicating that myostatin acts through canonical activin receptor to regulate the expression of Myh genes. These results demonstrate a role of myostatin in the specification of myofiber types during myogenic differentiation.

Dr. M.V. Dodson of the **Washington Station** has been focused on establishing more effective methods to isolate mature, lipid-filled, adipocytes from intramuscular adipose depots of beef cattle. Isolating a more representative population of (purified) adipocytes possessing the ability to dedifferentiate and form proliferative-competent progeny (stem?) cells is one end-point of this research. Rather than using flasks as ceiling culture supports for mature adipocytes, flat cell culture ware of various brands and sizes were used to culture the adipocytes, with the idea that flat-bottomed cell culture plates could be inverted, protected from contamination by inserting these plates into progressively larger ones and used for cell propagation, cloning or single cell analyses. Methods for such cell culture have been summarized in the journal-*Methods*. Impact of such research is to allow more scientists to use the culture procedures, perform more research with mature adipocytes and help define the mechanisms of dedifferentiation of these important [but still relatively unknown] cells.

Dr. Min Du, also of the **Washington Station**, continued to define the mechanisms regulating myogenic and adipogenic commitment of mesenchymal stem cells. Their studies demonstrated that AMP-activated protein kinase promotes myogenic differentiation through enhancing the expression of myogenin, as well as b-catenin. AMPK inhibits the activity of histone deacetylase 5 and promotes the histone acetylation of myogenic gene promoters, a process mediated by myocyte enhancer factor 2C (MEF2C). His group also show that Zfp423, a newly identified transcription factor, initiates adipogenic commitment of bovine progenitor cells. These studies will enhance understanding of mechanisms regulating mesenchymal stem cell differentiation, which provides molecular targets for enhancing lean/fat ratio and efficiency of livestock production.

The objective of the **South Dakota Station** study was to determine the effects of 17β-estradiol (estradiol) on avian myogenic satellite cell proliferation, differentiation, and the gene expression of proteins important in regulating skeletal muscle growth and development. Increasing levels of estradiol were administered in basal medium containing additional nutrients. Female-derived pectoralis major (PM) satellite cell proliferation was stimulated at a level of 10-9 M following 4 days of treatment. Male PM satellite cell proliferation was increased at 10-12M estradiol. Likewise, male biceps femoris (BF) satellite cells increased proliferation with 10-12M estradiol. Turkey embryonic myoblast proliferation, however, was not responsive to estradiol following 3 days under these conditions. Estradiol had no effect on the differentiation of any of the 4 groups of cells. Likewise, glypican-1 expression was unaffected by estradiol treatment. MyoD expression decreased in male PM but not BF cells. Female PM cells and embryonic myoblasts were also unaffected by estradiol

administration. Estradiol decreased myogenin expression in male satellite cells, but had no effect on female cells. There was a slight decrease in myogenin expression in embryonic myoblasts. The results demonstrate a direct effect of estradiol on avian satellite cell proliferation independent of glypican-1, and decreased expression of MyoD and myogenin in some myogenic cells, coinciding with increased cellular proliferation.

The objective of the additional **South Dakota Station** study was to determine the effects of fatty acids on the proliferation, differentiation, and expression of syndecan-4 and glypican-1 in avian myogenic satellite cells (SC). SC derived from the pectoralis major (PM) and biceps femoris (BF) muscles of the turkey and chicken were individually administered 8 different fatty acids in defined medium during proliferation. A parallel set of turkey SC were induced to differentiate. Highest levels of proliferation of turkey PM and BF SC occurred in cultures containing oleic acid. Linoleic acid and oleic acid were equipotent in supporting proliferation of chicken SC. Microscopic examination revealed that inclusion of docosahexaenoic acid or eicosapentaenoic acid was toxic towards both PM and BF SC from both species. Linolenic acid and arachidonic acid diminished levels of differentiation. Expression of glypican-1 varied between treatments to a greater extent with turkey BF than with PM SC. Expression in chicken PM and BF SC demonstrated a similar pattern in response to treatments. Turkey PM syndecan-4 expression varied between treatments, whereas expression in turkey BF SC was similar between treatments. Expression in chicken SC varied little between treatments. The results demonstrate species and muscle-specific differences in the parameters examined. It is proposed that changes in lipid raft receptor interactions may contribute to these observed differences.

The objective of the **Minnesota Station** study was to assess the role of estrogen receptor -α (ESR1) and the Type 1 insulin-like growth factor receptor (IGFR1) in E2-stimulated proliferation of cultured BSC. Although the exact mechanism(s) by which Estradiol (E2) enhances muscle growth in a number of species including humans and cattle, is not known, Estradiol treatment has been shown to stimulate proliferation of cultured bovine satellite cells (BSC). This is particularly significant because satellite cells are the source of nuclei needed to support postnatal muscle fiber hypertrophy and are thus crucial in determining the rate and extent of muscle growth.
To accomplish this, we have used small interfering RNA (siRNA) to silence expression of *ESR1* or *IGFR1* and assessed the effects on E2-stimulated proliferation in BSC cultures. In BSC treated with nonspecific siRNA, Estradiol significantly ($P < 0.05$) stimulates proliferation under conditions in which neither IGF1 nor IGF2 expression is increased; however, treatment of *ESR1*- or *IGFR1*-silenced cells with E2 does not significantly stimulate proliferation. These results indicate that both ESR1 and IGFR1 are required in order for E2 to stimulate proliferation in BSC cultures. The fact that treatment with ESR1 siRNA completely suppresses the ability of E2 to stimulate proliferation suggests that ESR2 (estrogen receptor-β) is not involved in E2 stimulated BSC proliferation. Additionally, the fact that this occurs under culture conditions in which neither IGF1 nor IGF2 mRNA expression is increased strongly suggests that E2 activates IGFR1 via a mechanism that does not involve increased IGF1 or IGF2 binding to the receptor.

Systemic and local factors accompany normal functions, such as exercise, or dysfunctional conditions such as aging, obesity and disease to alter the maintenance, function and regeneration of skeletal muscle. The **Virginia Station** recently discovered the existence of a family of proteins, known as the regenerating gene (Reg) family, previously uncharacterized in skeletal muscle. Although the precise functional roles for this protein family are unclear, various family members affect several cellular processes, such as proliferation, apoptosis and adhesion. To elucidate a role for this protein family in skeletal muscle, we are characterizing the existence and function of family members during skeletal muscle inflammation, degeneration and regeneration following a well-established muscle damage protocol. Further studies are currently underway to definitively identify the role(s) of the Reg family of proteins during periods of differential skeletal muscle mass and function (growth and muscular dystrophy).

Though much is known about satellite cells, information is limited regarding how populations of SCs differ with muscle fiber type, especially in pigs. The objective of this study was to isolate and culture SCs from red (RST) and white (WST) portions of the semitendinosus muscle of neonatal and adult pigs and determine their capacity to proliferate, differentiate and express various myosin heavy chain (MyHC) isoforms *in vitro*. Muscle from neonatal pigs yielded nearly ten times more ($P < 0.001$) presumptive satellite cells as those from adult pigs, with fusion percentages close to 60% for the former. RST yielded more ($P < 0.001$) SCs per gram muscle compared to WST, 8.1 ± 0.2 ×104 cells versus 6.7 ± 0.1 ×104 cells/gram muscle in young pigs, and 9.7 ± 0.4 x103 cells versus 5.5 ± 0.4x103 cells/gram muscle in adult pigs, respectively. Likewise, satellite cells from RST proliferated faster ($P < 0.001$) than those from WST across both ages, as indicated by a shorter cell doubling time, 18.6 ± 0.8 h versus 21.3 ± 0.9 h in young pigs, and 23.2 ± 0.7 h versus 26.7 ± 0.9 h in adult pigs, respectively. As a result of shorter times to confluence, satellite cells from RST also formed myotubes earlier than those SCs originating from WST. Once differentiated, however, SC from WST differentiated and fused faster ($P < 0.05$) as evidenced by fusion percentage within the first 24 h, 41.6% versus 34.3%, respectively; but reached similar ultimate fusion percentages similar to WST by 48 h. Over 90% of MyHC expressed in maximally fused SC cultures from both RST and WST was restricted to the embryonic isoform. Type IIX MyHC mRNA was not detected in any culture. Myotube cultures from RST expressed more ($P < 0.01$) type I MyHC isoform mRNA than those from WST, whereas those cultures from WST expressed more ($P<0.05$) type II (including type IIA and type IIB) MyHC transcripts. These data show SC cultures from porcine fast and slow muscles express MyHC profiles largely reflective of their muscle of origin and argue satellite cells are partially restricted to a particular muscle phenotype in which they are juxta-positioned. Understanding the molecular nature of these intrinsic control mechanisms may lead to improved strategies for augmenting meat animal growth or muscle regeneration.

The **North Carolina Station** contributed the following:
Lentiviral vectors are an effective method of introducing transgenes into the genome of early stage embryos because they transduce both dividing and non-dividing cells. Lentiviral pseudoparticles containing the coding sequence for the fluorescent protein DsRed were injected into freshly laid leopard gecko eggs. Tissue samples were collected from hatchlings, and the samples were tested for presence of the transgene. Of the injected gecko population, a greater than 89% of efficiency of

transgenesis was confirmed using PCR screening. Histological evaluations revealed the presence of DsRed in injected gecko organs; with protein production concentrated in the muscle, kidney, heart, and brain. Therefore, lentiviral vectors appear to be viable technology to create transgenic geckos. Once the geckos are bred through germline transmission; they are another model organism that can be used to study muscle development and cell lineage analysis.

Skeletal muscle is composed of metabolically heterogeneous myofibres that exhibit high plasticity at both the morphological and transcriptional levels. The objective of this study was to employ microarray analysis to elucidate the differential gene expression between the tonic-red anterior latissimus dorsi (ALD) muscle, the phasic-white posterior latissimus dorsi (PLD) and mixed-phenotype biceps femoris (BF) in 1-week-and 19-week-old male turkeys. A total of 170 differentially expressed genes were identified in the muscle samples analysed ($P < 0.05$). Gene GO analysis software was utilized to identify top gene networks and metabolic pathways involving differentially expressed genes. Quantitative real-time PCR for selected genes (BAT2D, CLU, EGFR and LEPROT) was utilized to validate the microarray data. The largest differences were observed between ALD and PLD muscles, in which 32 genes were over-expressed and 82 genes were under-expressed in ALD1-PLD1 comparison, and 70 genes were over-expressed and 70 under-expressed in ALD19-PLD19 comparison. The largest number of genes over-expressed in ALD muscles, as compared to other muscles, code for extracellular matrix proteins such as dystroglycan and collagen. The gene analysis revealed that phenotypically red BF muscle has high expression of glycolytic genes usually associated with the white muscle phenotype. Muscle-specific differences were observed in expression levels of genes coding for proteins involved in mRNA processing and translation regulation, proteosomal degradation, apoptosis and insulin resistance. The current findings may have large implications in muscle-type-related disorders and improvement of muscle quality in agricultural species.

Hens were placed on feed formulations targeting 0, 100 ppm/kg body weight (100), 200 ppm/kg body weight (200), and 400 ppm/kg Body weight (400) in January of 2010 CP31398. A final necropsy was accomplished on November 15, 2011. A flock of 876 hens at 104 weeks of age was obtained for the chemopreventative trial. Three hundred birds were killed at 104 weeks of age, and. one hen was found with oviductal cancer, and two were classified as having metastasized reproductive and gastrointestinal cancers. The remaining flock of 576 birds was divided into the four treatment groups, each containing 144 birds. Each treatment group was administered the layer hen maintenance diet containing varying levels of CP-31398 (CP). The control treatment group received 0 ppm CP-31398, the low dose treatment group received 100 ppm CP-31398, the medium dose treatment group received 200 ppm CP-31398, and the high dose treatment group received 400 ppm CP-31398 (>99% purity; Indofine Chemical Company, Inc. Hillsborough, NJ). Therefore, the targeted feed levels were 15, 30, and 60 ng/mg CP-31398. At week 12 of the trial, it became evident that the hens in the high dose treatment group (400ppm CP-31398) were experiencing liver toxicity likely from the higher concentration of CP-31398. Similar toxicity was experienced in rats that were ingesting high levels of the chemopreventive compound (Rao et al., 2009). The birds receiving 400ppm of CP-313198 were immediately removed from the chemopreventive treatment

for 30 days, and were then reintroduced at 300ppm CP-31398 to avoid liver hemorrhage for the remaining 76 weeks of the chemopreventive study. Following the decrease in the amount of CP-31398 administered to the high dosage treatment group, there were few incidences of liver toxicity and hemorrhage. Mortalities and euthanized hens were necropsied weekly to determine the cause of death, all tumors and perceived adenocarcinomas were removed, and excised tissue was fixed in 10% neutral formalin buffer for 24 hours. The samples were then moved to 70% ethanol and stored at approximately 4°C until a histological analysis could be completed. At the end of the 22-month chemoprevention trial, the remaining 401 birds of approximately 192 weeks of age were killed by cervical dislocation and tumor incidence was recorded based upon gross pathology. Tissue samples collected during the necropsy were fixed in 10% neutral formalin buffer, transferred to 70% ethanol, and stored at approximately 4°C for histological analysis of any purported tumors. CP-31398 above 200 ppm appears to have reduced ovarian cancer tumor incidence rates.

The **Iowa Station** is currently studying Duchenne muscular dystrophy (**DMD**), which is caused by a mutation in the dystrophin gene resulting in a dystrophin protein deficiency. Of interest, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (**PGC-1α**) has been shown to increase mitochondrial biogenesis, decrease free radical injury, and increase expression of utrophin.

The **Iowa Station** found that AAV-mediated PGC-1α gene transfer to neonatal mdx mice, a mouse model of DMD, decreased muscle injury and improved muscle function at six week of age. Next, we established that this strategy was also effective in a rescue paradigm where gene transfer occurs following disease onset. Associated with decreased muscle injury was increased utrophin protein expression and increased gene expression of dystrophin-glycoprotein complex members, oxidative metabolism, and satellite cell activation compared to control limbs. In a parallel experiment neonatal mice were injected with the virus driving PGC-1α expression and subjected to a downhill injury protocol at six weeks of age. We again found that PGC-1α was protective to dystrophic muscle. Together, these data support a role for PGC-1α pathway activation as a therapeutic approach for DMD.

To increase the clinical relevance of this work we began exploring the possibility of using pharmaceuticals to activate this pathway. We selected quercetin, a nutraceutical, as our agent of choice as there is a substantial rationale to suspect it will activate the PGC-1α pathway, is orally available, and has a well-established safety record. Dystrophin-deficient mice were fed quercetin (0.2% or 0% dietary quercetin) from 3-9 months of age when diaphragms were removed for histological and biochemical evaluation. We found that quercetin-treated animals had a 20% increase in the number of muscle cells, indicating interruption of the disease process and preservation of muscle fibers. Further, central nucleation and infiltrating cells were decreased by approximately 35% indicating that quercetin-treated mice had less muscle injury. Supporting the notion of less injury and inflammation was that TNFα expression was decreased by more than 50% in treated mice compared to control. Further, expression of mitochondrial transcription factor A, cytochrome C ($p<0.07$), and cytochrome C oxidase 2 were increased 1.5-2-fold in diaphragms from

treated mice compared to control. Together with data collected during PGC-1α gene transfer, our results implicate quercetin as a therapeutic for dystrophin-deficiency.

The **New Jersey Station** was specifically focused on regulation of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway by amino acids and phytoecdysteroids in skeletal muscle. Phytoecdysteroids are a class of chemicals synthesized by plants. The most common phytoecdysteroid, 20-hydroxyecdysone (20HE) is found in many plants such as spinach and quinoa. Anabolic effects of phytoecdysteroids are reported in a variety of mammalian animals, including rodents, sheep, pigs, and Japanese quail. Part of the anabolic effect of phytoecdysteroids is through increasing protein synthesis, but the mechanistic details are largely unknown. Recent data suggest that activation of protein kinase B (PKB/Akt) is similar to growth factors such as IGF-I. Based on previous reports in cultured myocytes, we hypothesize phytoecdysteroids will activate mTORC1 signaling in skeletal muscle. Moreover, we hypothesize that the effect of phytoecdysteroids on mTORC1 is distinct from that of amino acids. Herein we propose to elucidate the molecular processes that regulate muscle growth by phytoecdysteroids, and to determine if these anabolic effects can be amplified in combination with protein nutrition. After completion of these studies we expect to show to what extent consumption of phytoecdysteroids activate mTORC1 signaling in skeletal muscle and to determine if the anabolic effect of these chemicals complements that of amino acids. These results can potentially be used to develop natural products that can improve muscle growth of domestic animals and thus benefit the meat industry.

Objective 2. Characterize the cellular and molecular basis of myogenesis.

The **Texas Station** utilized a unique cattle population to investigate differential gene expression and signal transduction in skeletal muscle resulting from inheritance of *Bos taurus* or *Bos indicus* alleles of genes associated with skeletal muscle growth, development and proteolysis.

Considerable emphasis was placed on identifying regions of the genome that harbor genes for traits that directly impact the consumer, such as marbling and tenderness. For mapping genes associated with production efficiency and nutrient utilization, a resource population of multiple F2 families of Nellore-Angus, Brahman-Angus, and Brahman-Hereford was generated at the Research Station in McGregor, TX. DNA was collected for all animals, and phenotype scored for multiple characteristics including disposition, feed intake, age at puberty, and carcass and meat traits of steers.

Outputs: Single-channel, whole genome microarray data were generated from 48 longissimus samples from steers from the McGregor herd. For each steer, additional collected data include Warner-Bratzler shear force from electrically-stimulated and non-stimulated carcass halves, carcass traits, and SNP50 genome data. Network gene expression analyses combined with genotype analyses resulted in the identification of a new family of genes that affects shear force in beef.

The Illinois Station using qPCR, expression of 7 genes (*Ezh2, Gpc3, Mdk, Mest, Mycn, Peg3,* and *Plagl1*) was evaluated in skeletal muscle, heart and liver samples from male and female wild-type

mice at 0, 1, 3, 5 and 7 weeks of age. All expression was normalized to 18s ribosomal RNA, standardized to a common sample used on each plate and expressed as a fold-change compared to newborn males of each tissue. Orthogonal contrasts were used to differentiate between linear, quadratic, and cubic patterns of expression among tissues.
In general, expression declined from 1 or 7 days of age when compared to 49 days of age for all the genes in all the tissues except for expression of Ezh2 and Mycn, which was not altered by age in liver. Some differences in expression were observed between sexes with the majority of the differences occurring at 49 days. Expression was usually increased in females compared with males at this time point. Occasionally, males had greater expression than females; this was observed in liver at 1 day of age for Gpc3, Mest, and Plagl1 and muscle at 35 days of age for Mest. There were some similarities in the patterns of gene expression between muscle and heart or liver, but, there was not a single gene that had the same pattern of expression in all 3 tissues. Overall, these data suggest that the downregulation of these growth regulating genes with age might play a role in the coordinated cessation of muscle growth similar to organ growth with limited differences between sexes.

At the **Ohio Station**, current research efforts are focused on understanding the mechanism of how the heparan sulfate family of proteoglycans may be involved in the regulation of muscle growth properties. There are 6 glypican family members with only glypican-1 found in skeletal muscle. The report this year progresses research completed on syndecan-4.

Syndecan-4 core protein is composed of extracellular, transmembrane, and cytoplasmic domains. The cytoplasmic domain functions in transmitting signals into the cell through the protein kinase C alpha (PKCα) pathway. The glycosaminoglycan (GAG) and N-linked glycosylated (N-glycosylated) chains attached to the extracellular domain influence cell proliferation. The current study investigated the function of syndecan-4 cytoplasmic domain in combination with GAG and N-glycosylated chains in turkey muscle cell proliferation, differentiation, fibroblast growth factor 2 (FGF2) responsiveness, and PKCα membrane localization. Syndecan-4 construct or syndecan-4 construct without the cytoplasmic domain and with or without the GAG and N-glycosylated chains were transfected or co-transfected with a small interfering RNA targeting syndecan-4 cytoplasmic domain into turkey muscle satellite cells. The overexpression of syndecan-4 mutants increased cell proliferation but did not change cell differentiation compared to syndecan-4. Syndecan-4 cytoplasmic domain and GAG and N-glycosylated chain mutants had increased cellular responsiveness to FGF2 during proliferation. Syndecan-4 increased PKCα cell membrane localization, whereas the syndecan-4 mutants had decreased PKCα cell membrane localization compared to syndecan-4. However, compared to the cells without transfection, syndecan-4 mutants increased cell membrane localization of PKCα. These data indicated that the syndecan -4 cytoplasmic domain and the GAG and N-glycosylated chains are critical in syndecan-4 regulating satellite cell proliferation, responsiveness to FGF2, and PKCα cell membrane localization.

Syndecan-4 oligomerization through the cytoplasmic domain has been shown to be necessary for PKCα activity in cell types other than skeletal muscle whereas monomeric syndecan-4 is unable to activate PKCα. We have demonstrated that skeletal muscle syndecan-4 forms oligomers and associates with α-actinin. The deletion of the syndecan-4 cytoplasmic domain, however, did not prevent oligomer formation, but cell membrane localization of syndecan-4 with

the deletion of the cytoplasmic domain was significantly inhibited as well as interaction with α-actinin. These data suggest that the syndecan-4 cytoplasmic domain may play a primary role in the cell membrane localization of syndecan-4 rather than oligomer formation.

Using Cre/LoxP-based cell lineage tracing in mice, the **Indiana Station** has identified a population of aP2-expressing progenitors in the stromal vascular fraction (SVF) of both white and brown adipose tissues. The aP2-lineage progenitors reside in the adipose stem cell niche and express adipocyte progenitor markers, including CD34, Sca1, Dlk1, and PDGFRα. When isolated and grown in culture, the aP2-expressing SVF cells proliferate and differentiate into adipocytes upon induction. Conversely, ablation of the aP2 lineage greatly reduces the adipogenic potential of SVF cells. When grafted into wild-type mice, the aP2-lineage progenitors give rise to adipose depots in recipient mice. Therefore, the expression of aP2 is not limited to mature adipocytes, but also marks a pool of undifferentiated progenitors associated with the vasculature of adipose tissues. Our finding adds to the repertoire of adipose progenitor markers and points to a new regulator of adipose plasticity.

The **Indiana Station** also demonstrated that hypoxic culture conditions favor the quiescence of satellite cell-derived primary myoblasts by upregulating Pax7, a key regulator of satellite cell self-renewal, and downregulating MyoD and myogenin. During myoblast division, hypoxia promotes asymmetric self-renewal divisions and inhibits asymmetric differentiation divisions without affecting the overall rate of proliferation. Mechanistic studies reveal that hypoxia activates the Notch signaling pathway, which subsequently represses the expression of miR-1 and miR-206 through canonical Hes/Hey proteins, leading to increased levels of Pax7. More importantly, hypoxia conditioning enhances the efficiency of myoblast transplantation and the self-renewal of implanted cells. Given the robust effects of hypoxia on maintaining the quiescence and promoting the self-renewal of cultured myoblasts, we predict that oxygen levels in the satellite cell niche play a central role in precisely balancing quiescence versus activation, and self-renewal versus differentiation, in muscle stem cells in vivo.

At the **Virginia Station,** a series of studies have been conducted aimed at understanding how hyperthermia influences the set points of several metabolic pathways within skeletal muscle. It appears that during heat stress skeletal muscle experiences mitochondrial dysfunction leading to impaired cellular energy status. This may have broad implications for the reduced growth and heat intolerance seen during heat stress especially if skeletal muscle is not able to make necessary contributions to whole-body energy homeostasis. Taken together, heat stress alters skeletal muscle gene expression in a manner that affects skeletal muscle metabolism and function, which may be important for whole-body metabolism and overall physiological adaptation to hyperthermia.

AMP-activated protein kinase (AMPK) is activated by upstream kinases and negatively regulated by protein phosphatases. Intracellular calcium mediates protein phosphatase 2A (PP2A), which is in a heterotrimeric complex with the PR72 subunit. The PR72 subunit contains two calcium binding sites formed by EF hands. Our previous studies have shown that chronic calcium exposure decreases AMPK activity. To define the specific molecular mechanism whereby calcium can deactivate AMPK, activities of AMPK and PP2A were analyzed in C2C12 muscle cell cultures and

skeletal muscle tissues from mutant pigs possessing the AMPKγ3-mutation or the ryanodine receptor (RyR1) calcium gating mutation, or both. C2C12 myotubes treated with calcium releasing agent (caffeine) for 10 h decreased ($P < 0.05$) AICAR-induced AMPK activity to control levels and this negative effect was eliminated by ryanodine receptor stabilizer, dantrolene. Interestingly, muscle from pigs with the RyR1 mutation and C2C12 cells administered with 10 h caffeine showed higher ($P < 0.05$) PP2A activity compared to controls. More importantly, the inhibitory effect of caffeine on AMPK activity was attenuated by the PP2A inhibitor, calyculin A or siRNA induced knockdown of PP2A. These data show the inhibitory effect of chronic calcium on AMPK activity is exerted through the activation of PP2A.

The **Wisconsin Station** previously identified a rat strain deficient in titin splicing. Using genetic mapping, we found a loss-of-function mutation in RBM20 as the underlying cause for the pathological titin isoform expression. Mutations in human RBM20 have previously been shown to cause dilated cardiomyopathy. We showed that the phenotype of Rbm20 deficient rats resembles the human pathology. Deep sequencing of the human and rat cardiac transcriptome revealed an RBM20 dependent regulation of alternative splicing. Additionally to titin we identified a set of 30 genes with conserved regulation between human and rat. This network is enriched for genes previously linked to cardiomyopathy, ion-homeostasis, and sarcomere biology.

Mechanism of RBM20

Titin, a sarcomeric protein expressed primarily in striated muscles, is responsible for maintaining the structure and biomechanical properties of muscle cells. Titin undergoes developmental size reduction from 3.7 MDa in neonates to primarily 2.97 MDa in the adult. This size reduction results from the gradually increased exon skipping between exons 50-219 of titin mRNA. Our previous study reported that *Rbm20* is the splicing factor responsible for this process. In this work we investigated its molecular mechanism. We demonstrate that *Rbm20* inhibits titin splicing and mediates exon skipping by binding to titin pre-mRNA to mask the splice sites. The exons in the Rbm20-occupied regions are ultimately skipped. The two *Rbm20* speckles found in nuclei from muscle tissues were identified as aggregates of *Rbm20* protein on the partially processed titin pre-mRNAs using combined antibody immunofluorescence and in situ hybridization. Cooperative repression and alternative 3' splice site selection were found to be employed by *Rbm20* to skip different subsets of titin exons, and the splicing pathway selected depends on *Rbm20* expression levels that vary with tissue type and developmental age.

Objective 3. Characterize mechanisms of protein assembly and degradation in skeletal muscle

At the **Nebraska Station**, bovine skeletal muscle (stearnomandibularis) is being used to investigate the fraction of myofibrils called easily releasable myofilaments (ERM). The method for isolating ERM was essentially based on the procedure described by Neti et al (1) with a few modifications. ERM isolated were characterized by electron microscopy and 2-dimentional electrophoresis. Two dimensional separations of ERM filaments showed considerable difference in the pattern of major myofibrillar proteins when compared to myofibrils. Specifically, major protein spots corresponding to actin, tropomyosin, and troponin were much great number than in the myofibril fraction. Mass

spectroscopy was used to confirm the identity of these addition spots. Most recently MS analysis has shown that the additional spots represent post translational modifications of myofibrillar proteins. For example, eight spots identified as post translationally alpha actin from Bos taurus were found to contain varying degrees of phosphorylation, glycosylation, biotinylation, and Lys-linked polyglutamate. Examination of these polypeptides has revealed similar post-translational modifications in the isoforms of tropomyosin, troponin, and other myofibrillar proteins. Specifically, the MS data show evidence of ubiquitin tagged myofibrillar proteins that likely resulted from ubiqutiylation by muscle-specific ligase (MuRF1 & TIM 32). This latter finding provides significant evidence for the proteasome pathway for degradation of myofibrillar protein.

The **Wisconsin Station** examined length dependent activation in skeletal muscle. The magnitude of length dependent activation in striated muscle has been shown to vary with titin isoform. Recently, a rat that harbors a homozygous autosomal mutation (HM) causing preferential expression of a longer, giant titin isoform was discovered (Greaser et al. J. Mol. Cell. Cardiol. 44:983, 2008). Here, we investigated the impact of titin isoform on myofilament force development and cross-bridge cycling kinetics as function of sarcomere length (SL) in tibialis anterior skeletal muscle isolated from wild type (WT) and HM. Skeletal muscle bundles from HM rats exhibited reductions in passive tension, maximal force development, myofilament calcium sensitivity, maximal ATP consumption, and tension cost at both short and long sarcomere length (SL=2.8 μm and SL= 3.2 μm, respectively). Moreover, the SL-dependent changes in these parameters were attenuated in HM muscles. Additionally, myofilament Ca2+ activation–relaxation properties were assessed in single isolated myofibrils. Both the rate of tension generation upon Ca2+ activation (kACT) as well as the rate of tension re-
development following a length perturbation (kTR) were reduced in HM myofibrils compared to WT, while relaxation kinetics were not affected. We conclude that presence of a long isoform of titin in the striated muscle sarcomere is associated with reduced myofilament force development and cross-bridge cycling kinetics, and a blunting of myofilament length dependent activation.

Thyroid hormone levels play an important role in cardiac regulation, and a state of hypothyroidism leads to dilated forms of cardiomypathy. Inappropriate thyroid levels can also impact the sarcomeric protein titin, which is responsible for maintaining passive tension and structural integrity. The effects of hypothyroidism were studied after administration of propylthiouracil (PTU) to wild type (WT) and homozygous mutant (Hm) rats (the latter of which express a giant titin isoform). Enzyme-linked immunosorbent assay results demonstrated that PTU lowered thyroxine levels in the treated rats. Electrophoretic cardiac myosin heavy chain analysis indicated that hypothyroidism induced a transition to predominantly beta myosin heavy chain expression, while Wt control samples continued to express an age appropriate range of alpha and beta isoforms. PTU treated Wt rats had a larger N2BA to N2B titin ratio, and the slower migrating N2BA was in higher proportion than the faster N2BA. PTU did not affect titin isoform expression in Hm. Because it is believed that thyroid hormone regulates titin isoform expression through the PI3K/Akt pathway, we investigated several participants by immunoblotting. The Akt PH domain was not altered by PTU administration, although expression was higher in Hm rats. Akt phospho-Ser473 did not vary between treatments, but mTOR phospho-Ser2448 demonstrated a higher

phosphorylation state in Wt. Echocardiography data showed significantly decreased fractional shortening in Hm control, Hm PTU, and Wt PTU relative to Wt control. Wt control also exhibited a higher ejection fraction and a lower isovolumic relaxation time compared to the other treatments. It can be concluded that hypothyroidism has significant effects on titin isoform expression and other muscle functional characteristics.

Impact Statements

- An expression network and gene variants were identified that are associated with shear force variability that remains in beef after electrical stimulation of carcasses. These findings can be applied to improve genetic selection strategies for the beef cattle industry.
- Coached a high school student participating in an Agricultural Biotechnology class to a top 10 finish in a state biotechnology expo competition.
- Information about project was presented to animal science students from the Universidad Autonoma Chapinga, Mexico. July 30. Texas A&M University, College Station, TX.
- Research efforts from this project are included in public Genomics Exhibition at the George Bush Presidential Library & Museum Aug 27, 2012-July 5, 2013.
- Data from this project used to train Brazilian graduate students in a 40-hr Functional Genomics course at UNESP-Jaboticabal, Sao Paulo, Brazil, May 21-25.
- The results presented by the **Ohio Station** for Objective 2 are the first demonstration of a biological function of the proteoglycan N-glycosylated chains for a heparan sulfate proteoglycan.
- The N-glycosylated and glycosaminoglycan chains affect satellite cell properties critical for muscle growth which will likely impact muscle mass accumulation affecting meat yield and potentially meat quality.
- The cytoplasmic domain of syndecan-4 through its effect on cytoskeletal organization may play a significant role in muscle cell migration and the formation of muscle fibers.
- Myostatin facilitates slow and inhibits fast myosin heavy chain expression during myogenic differentiation.
- Fatty acid binding protein 4 expression marks a population of adipocyte progenitors in white and brown adipose tissues.
- Two-dimensional electrophoretic separation of ERM filament proteins revealed additional isoforms of myofibrillar proteins that were identified as being derived from post-translational events. The fact that they were only observed in the ERM fraction (not in myofibrils) suggests that these modifications are part of the myofibril disassembly/degradation process.
- Finding ubiquitin associated with these myofibrillar proteins further supports the hypothesis that post-translational modifications found principally the ERF fraction are part of the mechanism of myofibrillar protein degradation.
- The major outcome of this research is new knowledge that will be disseminated by publication in peer reviewed journals.
- We anticipate that the characterization of alternative splicing in skeletal muscles will provide new clues about the efficiency of protein synthesis and its control.

- Training of graduate students and post-doctoral fellows is being accomplished through this project.

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Adams, A. L. Acclimation of Holstein calves to transit stress: the integration of endocrine, immune, and behavior systems. Doctor of Philosophy Dissertation, Texas A&M University, August, 2012.

Carter, M. J. Inflammatory gene expression in goats in response to transport. Master of Science Thesis, Texas A&M University, August, 2012.

Hollinger, K., Gardan-Salmon, D., Santana, C., Rice, D., Snella, E., and Selsby, J.T. Rescue of dystrophic skeletal muscle by PGC-1α involves restored expression of dystrophin associated protein complex components and satellite cell signaling. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. *In Review.*

Won, Samantha, “Acute and chronic heat stress alters the metabolic profile of skeletal muscle in growing swine” (M.S. degree conferred August 2012).

Invited presentations and abstracts

Adams, A. L., T. H. Friend, G. A. Holub, L. R. Berghman, P. K. Riggs, S. M. Garey, C. L. Terrill, and M. J. Carter. 2012. Stress affects plasma serotonin, but not tryptophan, in Holstein steer calves. J. Anim. Sci. Vol. 90, Suppl. 3/J. Dairy Sci. Vol. 95, Suppl. 2, Abstract T1.

Adams, K. K., L. R. Chenault, J.Valenta, R. N. Vaughn, A. K. Torres, K. J. Kochan, T. H. Welsh Jr., R. D. Randel, F. M. Rouquette Jr., A. D. Herring, and P. K. Riggs. 2012. Expression profiling of testicular sense and antisense RNA transcripts of Brahman bulls. J. Anim. Sci. Vol. 90, Suppl. 3/J. Dairy Sci. Vol. 95, Suppl. 2, Abstract M34.

Angel, J. M., E.L. Abel, P. K. Riggs, and J. DiGiovanni. 2012. The skin promotion susceptibility locus Psl1.2 is a complex locus consisting of at least 5 subloci. 11th Annual Meeting of the Complex Trait Community, June 12-15, Paris, France.

Baer, A.A, J.C. Jones, K.A. Jones, and A.C. Dilger (2012) Synergistic effects of β-adrenergic agonists and zinc supplementation. ASAS Midwest Section Meeting, Abstract 243P.

Baumgard L. H. and R. P. Rhoads. 2012. Heat stress and post-absorptive metabolic perturbations. J. Anim. Sci. Vol. 90, E-Suppl. 3: 5.

Boddicker, R. L., N. J. Boddicker, J. N. Rhoades, S. Pearce, J. Johnson, M. C. Lucy, T. J. Safranski, N. K. Gabler, J. T. Selsby, S. Lonergan, J. Patience, R. P. Rhoads, J. C. M. Dekkers, L. H. Baumgard, and J. W. Ross. 2012. Heat stress experienced in utero alters postnatal body composition parameters in growing pigs. J. Anim. Sci. Vol. 90, E-Suppl. 1: W401.

Boddicker, R.L. Boddicker, N.J., Rhoades, J.N., Pearce, S., Johnson, J., Lucy, M.C., Safranski, T.J., Gabler, N.K., Selsby, J.T., Patience, J., Rhoads, R.P., Baumgard, L.H., Ross, J.W. Heat stress experienced in utero alters postnatal body composition parameters in growing pigs. American Society of Animal Science Annual Meeting. Phoenix, AZ, July 15-19, 2012.

Brown, Waggy, Peterson, M. Du, S. Nair, J. Li, P. Thomas. 2012. Gender disparity in cardiac AMPK activation in response to exhaustive exercise. American College of Sports Medicine 59th Annual Meeting, San Francisco, CA, May 29 to June 2, 2012.

Cruzen SM, Harris AJ, Hollinger K, Selsby JT, Gabler NK, Lonergan SM, Huff-Lonergan E. Gilts selected for low residual feed intake have potential for decreased protein degradation. International Congress of Mean Science and Technology. Montreal, Canada, August 12-17, 2012.

Fu, X., M. J. Zhu, and M. Du. 2012. Eph and ephrin expression during C2C12 myogenesis. *Experimental Biology Meeting*, San Diego, CA, April 21 to 25.

Griner, J. M. J. Zhu, and M. Du. 2012. Lysyl oxidase pre-peptide promotes adipogenesis in 3T3-L1 cells. *Experimental Biology Meeting*, San Diego, CA, April 21 to 25.

Guo, W., S. Li, S. Schafer, H. Schultz, T.A. Hacker, K.W. Supe, N. Hubner, M. Gotthardt, and M.L. Greaser. 2012. RBM20 regulates titin splicing, and defects result in cardiomyopathy. Myofilament Proteins as Structural Scaffolds and Mediators of Function meetings, Madison, WI.

Hollinger K, Snella L, Shanely RA, and Selsby JT. Dietary quercetin supplementation alleviates disease related muscle injury in dystrophic muscle. FASEB, San Diego, CA, April, 2012.

Hollinger K, Snella E, Shanely RA, and Selsby JT. A quercetin enriched diet slows disease progression in dystrophic skeletal muscle. Advances in Skeletal Muscle Biology in Health and Disease. Gainesville, FL, February 22-24, 2012.

Hollinger K, Rice* D, Snella E, and Selsby JT. PCG-1α over-expression rescues dystrophic muscle by modifying gene expression. FASEB, San Diego, CA, April, 2012.
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Huang, Y., A. K. Das, M. J. Zhu, and M. Du. 2012. Zfp423 promotes adipogenic differentiation of bovine stromal vascular cells. *The ADSA-AMPA-ASAS-CSAS-WSASAS Joint Annual Meeting*, Phoenix, AZ, July 15 to 19.

Hulsman, L. L., M. R. S. Fortes, A. Reverter, R. Vaughn, P. K. Riggs, R.K. Miller, J.O. Sanders, A. D. Herring, C. A. Gill, and D. G. Riley. 2012. Characterization of enrichment terms essential to beef sensory characteristics in a Nellore-Angus population utilizing Bayesian Inference. ASAS Southern Section, Feb 4-7, Birmingham, Alabama

Ing, N. H., D. W. Forrest, P. K. Riggs, T. Bryan, A. Lee, S. Loux, C. C. Love, S. P. Brinsko, D. D. Varner, T. H. Welsh, Jr. 2012. Dexamethasone Decreases Expression of Genes Involved in Cholesterol Biosynthesis and Steroidogenesis in Stallion Testes. Endocrine Society Annual Meeting, Jun 23-26, Houston, TX.

Johnson, J. S., R. L. Boddicker, S. Pearce, M. V. Sanz-Fernandez, M. C. Lucy, T. J. Safranski, N. K. Gabler, R. P. Rhoads, J. W. Ross, J. Patience, S. Lonergan, L. H. Baumgard, and J. Selsby. 2012. Gestational thermal environment alters postnatal response to heat stress. FASEB J. 26:1079.4.

Jones, J. C. (2012) *Reductions in expression of growth regulating genes are observed in skeletal muscle and organs with age*. M. S. Thesis, University of Illinois, Urbana-Champaign.

Jones, J. C. and A. C. Dilger (2012) Similar reductions in expression of proliferative genes are observed in skeletal muscle and organs with age. The FASEB Journal, 26:1086.6.

Kamanga-Sollo, E., M. E. White, M. R. Hathaway, and W. R. Dayton. 2012. Role of estrogen receptor-α (ER-α) and insulin-like growth factor receptor-1 (IGFR-1) in estradiol-stimulated proliferation of cultured bovine satellite cells. *J. Anim. Sci.* 90, Supplement 3: 547 (Abstract).

Lucy M. C., T. J. Safranski, J. N. Rhoades, J. W. Ross, N. K. Gabler, R. P. Rhoads, and L. H. Baumgard. 2012. Litter characteristics and thermoregulatory behavior of first parity sows exposed to a controlled heat stress (HS) during gestation. J. Anim. Sci. Vol. 90, E-Suppl. 3: 843.

McFarland, Douglas C., Sandra G. Velleman, Jane E. Pesall, Cynthia S. Coy. 2012. Effects of estradiol on avian myogenic satellite cell proliferation and expression of heparan sulfate proteoglycans, MyoD and myogenin. FASEB J. 26:1075.20

McFarland, D.C., S. G. Velleman, J. E. Pesall, and C. S. Coy. 2011. Effect of fatty acids on avian satellite cell proliferation, differentiation and heparan sulfate proteoglycan expression. FASEB J. 25:1051.43.

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Pleitner, J., W. Guo, B. Schmitt, J. Patel, T. Hacker, and M. Greaser. 2012. Hypothyroidism effects on wild type and mutant rats with altered cardiac titin expression. Biophys. J. Supplement.

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Rhoads R. P. and L. H. Baumgard. 2012. The physiology of heat stress: A shift in metabolic priorities at the systemic and cellular levels. J. Anim. Sci. Vol. 90, E-Suppl. 3: 840.

Sanz- Fernandez M. V., S. C. Pearce, L. R. Long, N. K. Gabler, J. F. Patience, M. E. Wilson, M. T. Socha, R. P. Rhoads, and L. H. Baumgard. 2012. Effects of supplemental zinc amino acid complex on physiology and performance in heat-stressed growing pigs. J. Anim. Sci. Vol. 90, E-Suppl. 3: 166.

Selsby JT. Early characterization of a novel porcine model of Becker muscular dystrophy. Muscular Dystrophy Association Fall Education Seminar. Des Moines, IA, October 13, 2012.

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Thorson J., S. Sharpton, L. Prezotto, R. Cardoso, P. Riggs, J. Edwards, M. Amstalden, G. Williams. 2012. Expression of hypothalamic RF-amide related peptide 3 (RFRP3) and adenohypophyseal G protein-coupled receptor 147 (GPR147) during the breeding and non-breeding seasons in the mare. Society for the Study of Reproduction Meeting, Aug. 12-16, State College, Pennsylvania, Abstract 482.

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Book chapters

Dayton, W. R. and M. E. White. 2012. Mechanisms of anabolic steroid action in bovine skeletal muscle. In: G.P. Cobb, editor, Veterinary Pharmaceuticals. American Chemical Society Books (in press).

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Greaser, M.L. and W. Guo. 2012. “Post-mortem Muscle Chemistry” In: “Handbook of Meat and Meat Processing, Second Edition”, ed. YH Hui, CRC Press, Boca Raton, FL, pp 70-78.

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Dodson, M.V. and S. Wei. 2012. What are we doing right? *NACTA Journal* 56(3):

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