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"Luiz de Queiroz" College of Agriculture

Transcriptome changes associated with muscle and intramuscular connective tissue growth in cull cows under different recovery gain rates

Daiane Aparecida Fausto

Thesis presented to obtain the degree of Doctor in Science. Area: Animal Science and Pastures

Piracicaba
2016

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my parents Remi Fausto and Marilena Stedille Fausto, for all support and love!

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"Never sure of anything, because wisdom begins with doubt." (Freud)

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RESUMO

Mudanças no transcriptoma associadas com o crescimento muscular e do tecido conjuntivo intramuscular em vacas de descarte submetidas a diferentes taxas de recuperação do ganho

A taxa de renovação do estroma e proteínas miofibrilares define o crescimento muscular, e pode afetar a qualidade da carne, afetando turnover de colágeno e taxa proteolítica. Há uma falta de informação sobre as mudanças no processo de remodelação de proteína muscular em resposta à taxa de recuperação do ganho de peso, observada durante a “realimentação” depois de subnutrição, o que pode ser alterado em animais mais velhos. Alterações no tecido muscular durante o período de recuperação pode ser indicada pelo perfil de expressão diferencial de genes, pela técnica de sequenciamento de RNA. Os objetivos deste trabalho foram avaliar as alterações do transcriptoma na musculatura de vacas de descarte Nelore submetidas a: 1) a recuperação do peso em condições de pastejo; e 2) a recuperação da desnutrição em diferentes taxas de ganho de pesos. No primeiro experimento, os animais foram divididos em: grupo de manutenção (manutenção de peso e de alta escore corporal); Recuperação do ganho (recuperação de baixo escore corporal com o ganho de peso corporal moderado em 0,6 kg / dia sob pastejo). No segundo experimento, os animais foram divididos em três grupos em confinamento: Controle (abatidos com baixo escore de condição corporal), ganho moderado (0,6 kg de ganho de peso vivo por dia) durante a estação seca e alta recuperação de ganho (1,2 kg de ganho de peso vivo por dia) durante a estação seca. Nos dois estudos, amostras do *Longissimus dorsi* foram coletadas após o abate e imediatamente congeladas até à análise de sequenciamento ser realizada. No primeiro experimento, os genes encontrados no tratamento de recuperação do ganho foram relacionados com a resposta inflamatória como: 4A semaphorin (*SEMA4A*), solute carrier family 11 member 1 (*SLC11A1*), Ficolin 2 (*FCN2*) e placental growth factor (*PGF*). No segundo experimento, o osteonectina (*SPARC*), colágeno tipo IV subunidades 1 (*COL4A1*) foram alguns dos genes mais expressos para ambas as taxas de ganho de recuperação de remodelação do tecido conjuntivo. Para ganho moderado, foram identificadas proteínas miofibrilares estruturais como: Myosin IE (*MYO1E*), Myosin, Heavy Chain 11 (*MYH11*), (*MYOG*) and actinin, alpha 4 (ACTN4). No tratamento de alta recuperação de ganho, genes como CLL de célula B / 9 linfoma (BCL9), peroxisome proliferator-activated receptor alpha (*PPARA*), Diacylglycerol O-Acyltransferase 2 (*DGAT2*) and Phosphatidylinositol 4-Kinase, Catalytic, Beta (*PI4KB*) indicam maior deposição de tecido adiposo. Em resumo, observou-se que a deposição muscular durante a recuperação do ganho de peso envolve a regulação da expressão de vários genes relacionados com a matriz extracelular (ECM), corroborando com modelos de inflamação e similar a fibrose observada em animais maduros. Além disso, no grupo HG, genes relacionados com a síntese de colágeno e a deposição de gordura, também foram encontrados, indicando contribuição do tecido conjuntivo durante o crescimento muscular. Estes resultados são importantes para a compreensão do desenvolvimento do tecido como um todo, e auxiliam no progresso do conhecimento científico sobre a remodelação muscular durante a recuperação do ganho de peso e sua influência sobre estruturas de proteínas e vias intracelulares.

Palavras-chave: Bovino; Matriz extracelular; Colágeno; Crescimento muscular

ABSTRACT

Transcriptome changes associated with muscle and intramuscular connective tissue growth in cull cows under different recovery gain rates

The renewal rate of stromal and myofibrillar proteins defines muscle growth, and can affect the quality of meat, by affecting collagen turnover and proteolytic rate. There is a lack of information on changes in the muscle protein remodeling process in response to the recovery weight gain rate observed during "realimentation" after undernutrition, which may be altered in older animals. Changes in muscle tissue during the recovery period may be indicated by the differential expression profile of genes after RNA sequencing. The objectives of this study were to evaluate transcriptome changes in the muscle of Nellore cull cows subjected to: 1) recovery weight gain under grazing conditions; and 2) recovery from undernutrition at different weight gain rates. In the first experiment, the animals were divided into two groups and subjected to one of two nutritional managements under grazing conditions: maintenance (maintenance of weight and high body condition score under grazing conditions) and recovery gain (recovery from low body condition score with moderate body weight gain of 0.6 kg/day under grazing conditions). In the second experiment, the animals were divided into three groups and subjected to one of three nutritional managements under feedlot conditions: control (slaughtered at low body condition score), moderate recovery gain (MG; 0.6 kg of daily live weight gain) during the dry season, and high recovery gain (HG; 1.2 kg of daily live weight gain) during the dry season. In both experiments, samples of longissimus dorsi muscle were collected after slaughter and immediately frozen until sequencing analysis could be performed. In the first experiment, genes related to inflammatory response, such as semaphorin 4A (*SEMA4A*), solute carrier family 11 member 1 (*SLC11A1*), ficolin-2 (*FCN2*), and placental growth factor (*PGF*), were expressed at higher levels during recovery gain. In the second experiment, osteonectin (*SPARC*) and collagen type IV subunits 1 (*COL4A1*) were expressed at higher levels in both recovery gain and connective tissue remodeling. For MG, structural myofibrillar proteins such as myosin IE (*MYO1E*), myosin, heavy chain 11 (*MYH11*), myogenin (*MYOG*), and actinin, alpha 4 (*ACTN4*) were identified. In the HG treatment, the B-cell CLL/lymphoma 9 (BCL9), peroxisome proliferator-activated receptor alpha (*PPARA*), diacylglycerol O-Acyltransferase 2 (*DGAT2*), and phosphatidylinositol 4-Kinase, catalytic, and beta (*PI4KB*) genes indicated more deposition of adipose tissue. In summary, we observed that muscular deposition during recovery weight gain involved the regulation of expression of several genes related to the extracellular matrix (ECM), corroborating the inflammatory and -like models observed in mature animals. Moreover, in the HG group, genes related to collagen synthesis and fat deposition were also found, indicating the important contribution of connective tissue during muscle growth. These results are important for understanding tissue development as a whole, and will assist in the progress of scientific knowledge on muscle remodeling during recovery weight gain and its influence on protein structures and intracellular routes.

Keywords: Bovine; Extracellular matrix; Collagen; Muscle growth

1 INTRODUCTION

It has already been established that growth alters rates of DNA, RNA, and protein accretion, ultimately affecting skeletal muscle (BEERMANN, 2004) and myofibrillar protein turnover (KOOHMARAIE et al., 2002), which is involved with certain cell routes related to meat tenderness. This quality attribute has been a problem for the meat industry (MORGAN et al., 1991; SAVELL; SHACKELFORD, 1992), since consumers identify it as a primary sensory trait in purchasing decisions (DELGADO et al., 2006; MENNECKE et al., 2007). In addition, tenderness involves complex interdependent biochemical mechanisms (RILEY et al., 2007) that involve protein degradation (LONERGAN, 2001; HUFF-LONERGAN et al., 1996; HO et al., 1997; GEESINK; KOOHMARAIE, 1999), lipid content (WOOD et al., 2003), connective tissue maturity and content (PURSLOW, 2005; MCCORMICK et al., 2009), and heat shock proteins (BERNARD et al., 2007), which have a strong genetic component. These mechanisms comprising the different deposition patterns of tissues interact in response to nutritional management strategies (BOLEMAN et al., 1996) and affect tenderness, which makes it difficult to predict this particular quality trait (OUALI, 1990; KOOHMARAIE, 1996).

Several studies have evaluated changes in the transcriptome of genes associated with muscle remodeling in response to nutritional challenges (LEE; HOSSNER, 2002; LEE; ENGLE; HOSSNER, 2002; BYRNE et al., 2005). These changes indicate the possibility of modifying proteins that are part of the muscle structure involved in meat tenderness, such as components of the ECM and cytoskeleton. However, no information exists on muscle remodeling in mature cows, even though they contribute significantly to meat production, especially in Brazil, where a significant portion of slaughters fall into this category. In 2015, cull cows represented close to 34% of the Brazilian bovine herd slaughtered (IBGE, 2015). Despite advances in our understanding of the impact of growth rate on myofibrillar metabolism and tissue deposition in muscle, the basics of these processes are still not fully understood.

Nutrient-genome interaction with other signaling networks allows the integration of cellular control of dietary intake and internal regulatory mechanisms (DAUNCEY; KATSUMATA; WHITE, 2004). In addition, studies on transcriptomes are

relevant to meat scientists, providing potential information on the biological characteristics of muscle (HOCQUETTE et al., 2007; BALDWIN et al., 2012).

Moreover, a major contribution to the process involving accelerated proteolysis via the proteasome-ubiquitin pathway has been recognized as the principal cause of muscle atrophy under a number of catabolic conditions (JAGOE; GOLDBERG, 2001). Genes involved in connective tissue remodeling, such as SPARC, have also been identified; some in relation to tissue injury or high turnover rates with an important role within the ECM (BRADSHAW; SAGE, 2001), and others as serine protease inhibitors (SENTANDREU; COULIS; OUALI, 2002).

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2 LITERATURE REVIEW

2.1 Influence of growth rate on muscle remodeling

During animal development and growth prior to maturity, body tissues grow in specific waves, starting with nervous tissue and followed by bone, muscle, and adipose tissue, which accumulates at a faster rate with higher weight gain in mature animals (OWENS et al., 1993). This differential tissue accretion is also modified by nutritional restriction, which alters muscle fiber type profiles by increasing the contribution of oxidative myofibers. These are spared during restrictive conditions (BYRNE et al., 2005). In addition, collagen renewal processes are directly related to the rate of muscle growth (FISHELL et al., 1985).

When muscle is subjected to regeneration after atrophy, the first phase is characterized by the release of growth factors and cytokines to signal the proliferation of cells and the infiltration of inflammatory cells (TIDBALL; VILLALTA, 2010). In the first stage, recovery is characterized by an inflammatory process, where macrophages are recruited (MCLENNAN, 1996) to promote muscle regeneration as mediators of tissue remodeling. The support of myogenesis and myofiber growth is described as a second phase of muscle recovery from atrophy (CHAZAUD et al., 2009).

The inflammatory reaction is followed by the activation of satellite cells, maturation of newly formed myofibers (CICILIOT; SCHIAFFINO, 2010), and remodeling. Simultaneously, angiogenesis and the proliferation of fibroblasts occur, the latter enabling the muscle to synthesize ECM components that are degraded during the formation of new myofibers (SERRANO; MUÑOZ-CÁNOVES, 2010). However, if stem cells (satellite cells) lose their repair capacity during skeletal muscle remodeling, excessive ECM accumulation takes place, leading to fibrosis, which is characterized by increased amounts of collagen types I and III, fibronectin, elastin, proteoglycans, and laminin (UEZUMI et al., 2011). It can also provoke the infiltration of adipocytes (NATARAJAN; LEMOS; ROSSI, 2010), and may be accentuated by age (ALEXAKIS; PARTRIDGE; BOU-GHARIOS, 2007). In addition, some researchers have found that transforming growth factor-beta1 (TGF-β1) may be associated with the induction of myofibroblastic differentiation in myogenic cells in injured skeletal muscle (LI et al., 2004).

2.2 Components of the extracellular matrix

The main constituent of connective tissue is the ECM, which is primarily composed of connective fibers made up of collagen (95%), elastin, and an amorphous substance composed of proteoglycans and glycoproteins (JUNQUEIRA; CARNEIRO, 2004). The collagen molecules are composed of a combination of triple alpha-helix subtypes that define the collagen types. The primary types involved in skeletal muscle development are I, III, IV, V, VI, XI, XII, XIV, XV, and XVIII (NISHIMURA et al., 1997).

During intramuscular connective tissue remodeling, the enzymes responsible for degrading almost all ECM components are the metalloproteinases, or MMPs (NAGASE; VISSE; MURPHY, 2006). This enzyme system is formed primarily of zinc-dependent endopeptidases that degrade collagen, elastin, fibronectin, laminin, and proteoglycans and can regulate the turnover of ECM macromolecules that are involved in the regulation of preadipocyte growth, thus contributing to adipose tissue development in skeletal muscle (BAILEY; LIGHT, 1989). Among these is MMP-2, which plays an important role in regulating the integrity and composition of the ECM (CARMELI et al., 2004). The MMP system is regulated by specific inhibitors, known as TIMPs (tissue inhibitors of matrix metalloproteinases). Most notably, TIMP-1 and TIMP-2 are capable of inhibiting the activity of any MMP, with a preference for the inhibition of MMP-2 and MMP-9, respectively (KJAER, 2004). The occurrence of co-expression between the various major components of the MMP system in bovine muscle, particularly MMP-2, TIMP-1, and TIMP-2 (BALCERZAK et al., 2001), may indicate the presence of an important regulatory mechanism in the alteration of the ECM. The types of collagen expressed during this process have also been studied.

2.2.1 Proteoglycans

The proteoglycans are considered modulators of growth factor activities (KRESSE; SCHÖNHERR, 2001). They contain one or more sulfated polysaccharide chains (glycosaminoglycans) that are covalently linked to a central protein (KJELLEN; LINDAHL, 1991; ESKO, 1991) and play an important role in the proliferation (RUOSLAHTI, 1989), growth (GALLAGHER, 1989), cell adhesion (RUOSLAHTI, 1989) and determination of mechanical properties of tissues

(ROUGHLEY; LEE 1994; PINS et al., 1997). There are reports of the degradation of proteoglycans in the ECM, which would be a weakening factor in the intramuscular connective tissue of bovine muscle (NISHIMURA et al., 1996).

Proteoglycan expression changes as development in embryonic chicken muscle occurs, changing a matrix rich in chondroitin sulfate to a complex array containing a lower level of chondroitin sulfate, heparan, and dermatan (YOUNG et al., 1989; FERNANDEZ et al., 1991). This process has also been reported in other species (VELLEMAN et al., 1999; YOUNG et al., 1990). Different types of proteoglycans expressed in skeletal muscle have special functions during the process of differentiation, but the expression pattern appears to be conserved among them.

2.2.1.2 Decorin

Decorin, the main proteoglycan in skeletal muscles, is a macromolecule belonging to the family of leucine-rich proteoglycans, which is divided into subfamilies according to amino acid sequences and genes (IOZZO, 1997). It participates in cell growth by modulating some growth factors (LI; MCFARLAND; VELLEMAN, 2008), and regulates the formation and stability of collagen fibrils and fibers (NISHIMURA, 2015).

The decorin subfamily contains an N-terminal domain substituted with chondroitin sulfate/dermatan chains. It is the main proteoglycan in skeletal muscles, and interacts with collagen (primarily types I and II) during fibrogenesis (VOGEL et al., 1984; HEDBOM; HEINEGARD, 1989; BROWN; VOGEL, 1989). This interaction appears to be mediated by the central protein and dermatan sulfate chains (FONT et al., 1993). In previous studies, beef muscles with divergent tenderness differed in their content of dermatan sulfate (PEDERSEN et al., 1999), a glycosaminoglycan present in decorin. In the muscles of adult bovine animals, decorin is found in the endomysium and perimysium (EGGEN et al., 1994). The architecture of collagen fibrils can be modified by decorin in bovines (NISHIMURA et al., 2003) and mice (DANIELSON et al., 1997). Other *in vitro* studies on extracellular components found interactions between decorin and collagen that modulate the formation of collagen fibers (SCOTT, 1988; WEBER et al., 1996).

2.2.2 Adhesive glycoproteins

Adhesive glycoproteins are so named because they participate in adhesion between cells, fibers, and ECM macromolecules. These molecules contain a predominant protein that is associated with carbohydrates (JUNQUEIRA; CARNEIRO, 2004). Fibronectin is one of the main adhesive glycoproteins in the intramuscular connective tissue matrix. It binds to integrins, which act as transmembrane receptors of information between cells and the matrix. Such communication or signaling between cells and the matrix results in modulation of the growth and function of tissues, including migration, cell survival or death, and responsiveness to growth factors. Different tissues have different matrices, which change as the animal ages (VELLEMAN, 2003).

2.3 Studies related to the extracellular matrix and its impact on meat

Skeletal muscle is mainly composed of muscle fibers and surrounding intramuscular connective tissues of three types: endomysium, epimysium, and perimysium (NISHIMURA, 2015). In theory, rapid growth increases the synthesis of new soluble collagen that dilutes the mature existing collagen (ABERLE et al., 1981; FISHELL et al., 1985). However, the relationship between nutrition management and changes in connective tissue is difficult to determine (HALL; HUNT, 1982). MMP-2 activity has also been associated with collagen degradation and tenderness of meat in sheep with a high growth rate (SYLVESTRE et al., 2002). However, this effect may be related to turnover from the collagen fraction, which would influence meat tenderness independent of the action of MMPs during the *postmortem.* On the other hand, collagen degradation (TORNBERG, 1996) and changes in the structure of connective tissue (TAYLOR; KOOHMARAIE, 1998) were not observed in muscles of cattle and sheep, respectively. During aging, a decrease in the turnover of connective tissue occurs, along with the formation of mature crosslinks (HAUS et al., 2007) that directly affect meat tenderness (MCCORMICK, 1994). The amount of mature collagen is a result of the degree of intramuscular renewal, as defined by the rates of synthesis, crosslinking formation, and degradation conducted by MMP activity (BAILEY; LIGHT, 1989). It plays an important role during fibrosis, because

connective tissue accumulates when its rate of synthesis is greater than its rate of degradation.

In mice, age has been associated with fibrosis (BRACK et al., 2007) and with accumulation of ECM (ALEXAKIS, PARTRIDGE; BOU-GHARIOS, 1981). Moreover, the synthesis of glycosaminoglycan is high in young tissues but decreases with growth and maturation (SCHOFIELD; WEIGHTMAN, 1978); changes in proteoglycans may also change with age. However, during severe fibrosis, the level of decorin is reduced (ZANOTTI et al., 2005). Decorin has been related to anti-fibrosis agents in skeletal muscle (SATO et al., 2003). It can inhibit myostatin (MSTN) and TGF-β1, which probably act together to amplify the fibrotic process (ZHU et al., 2007).

2.4 Hypotheses

1. A moderate recovery weight gain under grazing conditions during body condition recovery is sufficient to elicit changes in proteins and protease gene expression that are associated with the renewal of major ECM components in cull cows.

2. The greater gain rate achieved under feedlot conditions is necessary to increase connective tissue growth and turnover of ECM major proteins by altering the transcription of associated proteins and protease genes.

2.5 Objective

To evaluate transcriptome profile changes related to protein turnover in the muscle of cull cows under different recovery gain rates.

2.6 Specific objectives

1. To evaluate transcriptome changes in muscle of Nellore cull cows subjected to recovery weight gain under grazing conditions.
2. To evaluate recovery from undernutrition changes muscle gene expression in mature Nellore cull cows differentially depending on the rate of weight.

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3 TRANSCRIPTOME CHANGES IN MUSCLE OF NELLORE CULL COWS SUBJECTED TO RECOVERY WEIGHT GAIN UNDER GRAZING CONDITIONS

Abstract

The rate of tissue structural remodeling is related to muscle growth, and may affect some routes involved in meat quality attributes. However, it is uncertain which main routes are changed in mature animal muscle, as in *Bos taurus indicus* cull cows under grazing conditions. In grazing production systems, there is a period of undernutrition followed by realimentation, which affects muscle protein turnover and the remodeling of the connective and muscle tissues. Our hypothesis was that a moderate growth rate during the body condition recovery period would elicit changes in the expression of genes involved in ECM renewal. The aim of this study was to evaluate transcriptome changes in the muscle tissue of *Bos taurus indicus* cull cows subjected to recovery weight gain under grazing conditions. Thirty-eight Nellore cull cows were divided randomly into two different management groups: 1) Maintenance (MA) of cows with high body condition score (BCS = 8.0); and 2) Recovery gain (RG) from weight loss by moderate growth under high forage availability. After slaughter, RNA analysis was performed on the *longissimus dorsi* muscle. A total of 13,145 genes were identified in the transcriptome after quality filtering, alignment, and normalization. Only 17 genes were differentially expressed ($P_{adj} < 0.2$), and these were grouped by biological process, indicating tissue remodeling. In the MG group, a greater abundance of myostatin (*MSTN*), insulin-like growth factor binding protein 5 (*IGFBP5*), and methyltransferase-like 7A (*METTL7A*) transcripts was observed. Semaphorin 4A (*SEMA4A*), solute carrier family 11 member 1 (*SLC11A1*), and ficolin-2 (*FCN2*) were expressed at higher levels in the RG, which may indicate an inflammatory response during tissue regrowth. Signaling factors related to the negative control of satellite cell proliferation in adults and muscle protein synthesis were less abundant in the RG group. The only gene related to anabolic processes that was more abundant in the MA group was related to fat deposition. On the other hand, transcriptome analysis did not reveal any differences in expression of proteases involved in muscle protein turnover and related to collagen in *Bos taurus indicus* cull cows experiencing recovery gain from low body condition scores. The few genes that were differentially expressed in the experiment showed muscle repair-related changes during RG based on the greater expression of genes involved in inflammatory responses and the lower expression of negative regulators of muscle cell proliferation and hypertrophy.

Keywords: Connective tissue; Growth; Protein metabolism; Proteases; Extracellular matrix; Inflammatory process

3.1 Introduction

There is growing interest in studying gene expression associated with muscle structural remodeling due to nutritional challenges (BYRNE et al., 2005; LEE; HOSSNER, 2002; LEE; ENGLE; HOSSNER, 2002) to improve our understanding of

the impact of these genes on the phenotypic characteristics of animals. The changes reported in the literature point to the possibility of modifying structures that are part of the muscle physiological pathways. These are ultimately involved in meat tenderization, regulating genes from the intermediary metabolism as well as genes involved in connective tissue turnover, which may be compromised by caloric restriction (BYRNE et al., 2005).

Compensatory growth alters the response of cells based on endocrine status and nutrient availability. These integrated signals are reflected in one of the key hormones related to growth rate, insulin-like growth factor I (IGF-I), which plays a role in protein synthesis (ELLENBERGER et al., 1989) and satellite cell proliferation and differentiation (BARTON-DAVIS; SHOTURMA; SWEENEY, 1999). The IGF-I action mode involves its binding proteins (IGFBP), which transport it in the blood and are also altered during feed restriction (LEE et al., 2005). The IGFBPs protect IGFs against proteolysis and potentiate or inhibit its biological actions (CLEMMONS, 1998) or function through IGF-independent mechanisms (XI et al., 2006).

In theory, diets that promote rapid growth can lead to increased rates of protein turnover, including turnover of collagen molecules (FISHELL et al., 1985; ABERLE et al., 1981; ARCHILE-CONTRERAS, MANDELL; PURSLOW, 2010). This increase in collagen turnover has been observed during recovery gain after a period of body weight loss (ALLINGHAM; HARPER; HUNTER, 1998). Our hypothesis was that a moderate growth rate during body condition recovery is sufficient to elicit changes in gene expression that are related to muscle ECM renewal. The aim of this study was to evaluate transcriptome changes in muscle of Nellore cull cows subjected to recovery weight gain under grazing conditions.

3.2 Material and Methods

3.2.1 Treatments

The experiment took place at the Brazilian National Beef Cattle Research Center/EMBRAPA with 38 Nellore cull cows aged 4 to 12 years. The weight and average body condition of the cows at the beginning of the trial period was 447.9 ± 37.3 Kg and 7.23 ± 0.97, respectively. All experimental procedures were approved by

the environmental (CEAP; protocol #66) and animal (CEUA) ethical committees of the Luiz de Queiroz College of Agriculture, University of São Paulo.

The animals were randomly divided into two groups and subjected to one of two nutritional managements: 1) Maintenance group (MA): high forage (*Brachiaria decumbens*) availability to promote weight maintenance of cows with high body condition scores (BCS = 8.0); and 2) Recovery gain group (RG): low forage availability causing weight loss, followed by high forage (*Brachiaria decumbens*) availability and supplementation to induce compensatory gain. The supplementation in the dry season (0.6 kg/animal/day) consisted of 20.6% corn grain, 20% soybean meal, 25% soybean hull, 12% urea, 0.4% monensin sodium, and mineral salt. Calculations of nutritional supplementation value were based on the recommendations of the National Research Council (1996). Animals were serially slaughtered after 1, 41, 103 and 137 days (considering the presented increment in body condition score within the slaughter intervals) at the Meat Laboratory of the Beef Cattle Research Center/EMBRAPA in Campo Grande, MS, Brazil. Immediately after slaughter, samples of *longissimus dorsi* muscle were collected and frozen in liquid nitrogen (-80°C). They were kept frozen at -80°C until sequencing analysis could be performed. The differences between groups used the gene expression data of all the animals from each treatment, not considering the slaughter time. Our approach was to identify genes were consistently changed in the growth curve during the recovery gain.

3.2.2 RNA Extraction, library, and sequencing

Extraction of RNA from *longissimus dorsi* muscle samples (100 mg) from each animal (n = 38) was performed using 1 mL of Trizol reagent (Life Technologies, Carlsbad, CA, USA). The extracted RNA was quantified using a spectrophotometer (NanoDrop 200, Thermo Scientific, Wilmington, Delaware, USA). The integrity of the material was verified using 1% agarose gel and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) (RIN: RNA Integrity Number). Only samples with RINs greater than 7 were used for the next steps. After this stage, 2 µg of total RNA from each sample was purified and fragmented using a TruSeq RNA Sample Prep Kit v2. RNA messenger molecules (2 to 4% of the total RNA) were split from the remaining RNA by connecting them to poly-T tails adhered to magnetic beads. After

being purified and fragmented, the first cDNA tape was synthesized using random primers (hexamers) and reverse transcriptase enzyme; once synthesized, it was passed to the messenger RNA removal step for synthesis of the second cDNA tapes, which were purified using magnetic beads (Agencourt® Ampure XP, Beckman Coulter).

The double cDNA tapes were repaired to stand “blunt end,” followed by adenylation of the 3' extremities and the correct connection of the adapters, which are necessary for correct hybridization in the flow cell and allow molecule sequencing. Next, the DNA fragments were enriched via polymerase chain reaction (PCR); only the fragments with adapters were selected and amplified using specific primers that connected themselves to the end of the adapters. This library was then purified and validated in a Bioanalyzer, which measured the quantity and size of the fragments present in the sample. All samples were diluted to 10 nM, since in this stage, it is possible to multiplex the samples through specific index usage (7 bp oligonucleotides). After the preparation phase, the samples were clustered in flow cell cBot (Illumina, San Diego, USA). For this step, we used an SR TruSeq Kit, a Cluster Kit v2-CBOT-SH, and a set of capillaries that transferred the samples and reagents into the flow cell (HiSeq cBot manifold). After clustering, the flow cell with 38 libraries was sequenced in HiScanSQ (Illumina) at the Genomics Center, University of Sao Paulo/ESALQ, Piracicaba, São Paulo, Brazil. A read was defined as a 100 bp cDNA fragment sequenced from a paired end.

3.2.3 Mapping and Counting Reads

The sequencing data quality was evaluated with FastQC [http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/]. Seqclean software [https://bitbucket.org/izhbannikov/seqyclean/downloads] was used with 24 Phred quality parameters for maximum average error. Vector and adaptor sequences from the UniVec database [https://www.ncbi.nlm.nih.gov/tools/vecscreen/univec/] were used as a guide to remove possible contaminants from the quality filter. The reads were mapped using TopHat 2.0.10 (TRAPNELL; PACHTER; SALZBERG, 2009) and Bowtie2 v2.1.0 (LANGMEAD et al., 2009) against the UMD3.1 *Bos taurus taurus* masked genome available at Ensembl [http://www.ensembl.org/Bos_taurus/Info/Index/], with a maximum of one mismatch

allowed. To quantify the read counts, the HTSeq v0.5.4p2 program (ANDERS; HUBER, 2010) was used with the model nonempty intersection; reads that aligned on more than one gene were considered ambiguous and were not counted. Normalization of the expression analysis data was performed by the R differential gene expression analysis package (DESeq2) (LOVE; HUBER; ANDERS, 2014), considering growth rates (maintenance and recovery gain) and age of animal in the model, followed by the Benjamini-Hochberg (1995) correction for multiple tests on the obtained *P* values, which was used to control the false discovery rate (FDR).

3.2.4 Enrichment analysis

The Functional Annotation Clustering function of the Database for Annotation, Visualization and Integrated Discovery (DAVID) v. 6.7 (HUANG; SHERMAN; LEMPICKI, 2009) used the GOTERM, Sp_Pir_KEYWORDS, and KEGG_PATHWAY analyses to create clusters that showed decreasing values of enrichment scores for the genes. The Benjamini and Hochberg correction applied to the DAVID enrichments was the *P*-value adjusted to ≤ 0.09. Only genes that were differentially expressed (*P*-value ≤ 0.001 and *P*-value adjusted ≤ 0.2) were submitted to enrichment analysis.

3.2.5 Uncharacterized proteins, microRNA-targets, and Ingenuity Pathway Analysis (IPA)

The uncharacterized, differentially expressed proteins were annotated by comparison with orthologous genes at BioMart Ensembl [http:www.ensemble.org/biomart]. Analysis of the microRNA targets was performed by the TargetScanHuman database (http://www.targetscan.org/) and miRBase (http://www.mirbase.org/). In addition, enrichment analysis was performed using QIAGEN's Ingenuity Pathway Analysis (IPA, Qiagen Redwood City, www.qiagen.com/ingenuity). We used IPA to identify upstream genes in the dataset that could potentially explain the observed genes expressed in our data (target molecules).

3.3 Results and Discussion

Identification of 13,145 genes in the muscle transcriptome was possible following quality filtering, alignment, and normalization procedures. Seventeen genes that were differentially expressed ($P_{adj} < 0.2$) were grouped by biological function through functional annotation clustering analysis (Table 1). The distribution of fold-change data of the genes expressed in both treatments was homogeneous (Figure 1).

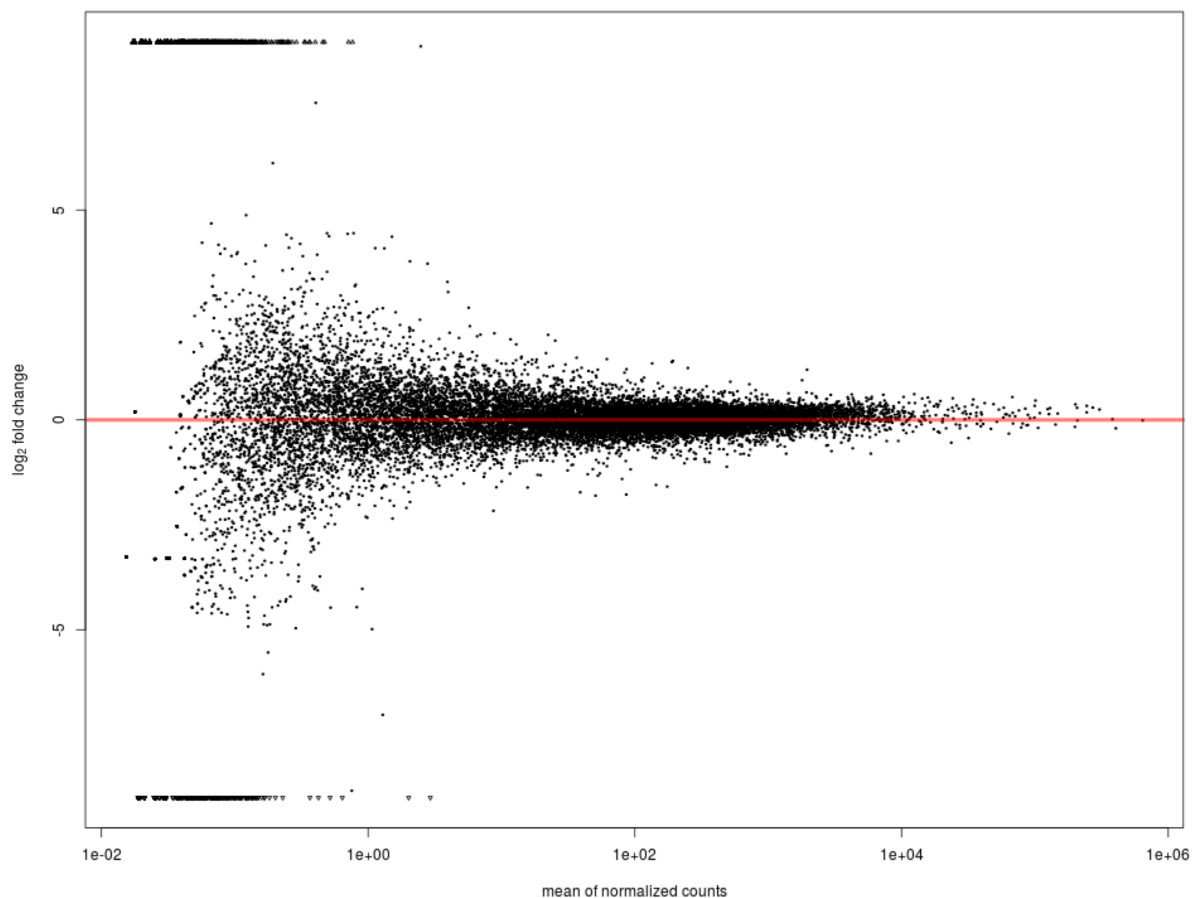

Figure 1. MA plot of *longissimus dorsi* muscle of mature *Bos taurus indicus* cull cows subjected to recovery weight gain under grazing conditions, showing gene distribution according to log2foldchange

In the group of differentially expressed genes annotated by Biomart Ensembl (Table 1), the *METTL7A* gene that was expressed in two transcripts in the MA group is related to lipid metabolism (BRASAEMLE; WOLINS, 2012); specifically to lipid

droplet formation (BOUCHOUX et al., 2011). This is to be expected since these animals presented high body score and weight. A greater abundance of *MSTN* transcripts (Table 1) was also observed in the MA group. This protein is a member of the transforming growth factor-β superfamily, which has been considered a novel and unique negative regulator of muscle mass (RODGERS; GARIKIPATI, 2008; MCCROSKERY et al., 2003). *MSTN* inhibits the expression of myogenic regulatory factors (RIOS et al., 2002) and satellite cell proliferation (FRY et al., 2014). The latter authors showed that proliferation is important in regulating muscle adaptations to hypertrophic growth in adults. Therefore, it seems plausible that cows under recovery weight gain, which are experiencing muscle hypertrophy, would present lower amounts of this negative growth regulator.

Transcripts of another protein related to growth factors, *IGFBP5*, were also more abundant in the muscle of cows from the MA group. In these animals, we expected to find signs of cessation of muscle growth. *IGFBP5* has been shown to be an important regulator of *IGF-I* local action (JACKMAN; KANDARIAN, 2004) by sequestering this growth factor, leading to decreases in protein synthesis (BAXTER, 2000). However, *IGFBP-5* may have effects that are unrelated to its *IGF*-regulatory role (TRIPATHI et al., 2009; MIYAKOSHI et al. 2001; TANNO et al., 2005). IPA analysis showed that *IGF-I* was the upstream gene regulating the greater expression of *IGFBP5* and *MSTN* (P = 0.023) in the MG group. This reinforces the idea that *IGF-I*, *IGFBP5*, and *MSTN* regulated muscle gain in this group of cows. Betacellulin (*BTC*) has been reported in fibroblastic cells (WATANABE et al., 1994) with mitogenic action.

SEMA4A, Slc11A1, PGF, and *FCN2* were more strongly expressed in the RG group (Table 1). *SEMA4A* is part of a large family of extracellular proteins (ROTH et al., 2009), and is involved in the regulation of cell migration and muscle angiogenesis (MEDA et al., 2012). It has also been linked to immune response (KUMANOGOH et al., 2002; KUMANOGOH et al., 2005), an important pathway related to renewal. *Slc11A1* is related to immune response and defense as well (DING et al., 2014), and *PGF* is related to the inflammatory process (CLAUS et al., 1996), pointing to macrophage secretory and physiological function in the RG group. *FCN2* is related to soluble collagen-like proteins that are involved in immune defense (GARRED et al., 2009). It is involved in connective tissue remodeling in mature animals via the

apoptosis of myofibroblasts, endothelial cells, and macrophages (JENSEN et al., 2007), which can also indicate immune responses during tissue regrowth.

In summary, inflammatory responses seem to increase during regeneration in recovery growth as a response to remodeling. Tissue remodeling of the ECM has been shown to involve an initial inflammatory process (FIELDING et al., 1993) followed by interactions between fibroblasts and satellite cells that normally lead to fibrogenic processes (MURPHY et al., 2011).

Table 1 – Differentially genes expressed[#] in muscle from mature *Bos taurus indicus* cull cows between maintenance of high body score and recovery gain conditions under grazing

	Gene name	Gene ID	Base Mean	log2(FC)*	P-Value	Padj#
1	IGFBP5	ENSBTAG00000007062	144.26	-1.5	<0.0001	0.1
2	SEMA4A	ENSBTAG00000012228	10.00	1.3	<0.0001	0.1
3	FCN2	ENSBTAG00000048155	8.84	2.1	<0.0001	0.1
4	SLC11A1	ENSBTAG00000015520	14.13	1.5	<0.0001	0.1
5	NAP1L3	ENSBTAG00000019407	7.39	-1.4	<0.0001	0.2
6	UNC80	ENSBTAG00000015415	8.71	-2.2	0.0001	0.2
7	NEPN	ENSBTAG00000039574	38.94	-1.7	0.0002	0.2
8	CIB2	ENSBTAG00000010981	52.88	-1.3	0.0001	0.2
9	*U. PROTEIN	ENSBTAG00000032899	44.35	-1.3	0.0002	0.2
10	METTL7A	ENSBTAG00000025005	337.68	-1.0	0.0001	0.2
11	PGF	ENSBTAG00000013688	112.45	1.0	0.0001	0.2
12	RAB15	ENSBTAG00000003474	37.00	1.2	0.0002	0.2
13	KIF3C	ENSBTAG00000019138	33.57	-1.1	0.0002	0.2
14	BTC	ENSBTAG00000004237	877.91	-0.6	0.0002	0.2
15	INTS7	ENSBTAG00000018548	172.50	0.5	0.0002	0.2
16	TMLHE	ENSBTAG00000011648	25.50	-0.8	0.0003	0.2
17	MSTN	ENSBTAG00000011808	86.34	-1.8	0.0003	0.2

[#]annotated by BioMart Ensembl; *log2 (FC): log2(foldchange); Negative values: upregulation in maintenance; Positive values: upregulation in recovery gain; #$P_{adj:}$ adjusted P value for multiple testing with the Benjamini-Hochberg procedure (FDR); [&]U. protein – uncharacterized protein

DAVID functional annotation enrichment analysis showed significant groupings of genes in the extracellular spaces and disulfide bond functional clusters (Table 2). In the RG group, *FCN2* genes were clustered in the extracellular space and disulfide

bonds. *PGF* and *SMA4A* were also included in the disulfide bonds. From the enrichment data, it appears that *NEPN* was related to extracellular space ($P < 0.1$) and *MSTN* to disulfide bonds ($P < 0.1$) in the MG group. In addition, *BTC* and *IGFBP5* were found in both pathways.

Table 2 – Functional Annotation Clustering[&] of genes from mature *Bos taurus indicus* cull cows during maintenance of high body score or recovery gain under grazing conditions

Annotation Cluster*	Representative Annotation Terms	Count	P-Value	P_{adj}[#]
GOTERM_CC_ALL	extracellular space	4	0.003	0.09
SP_PIR_KEYWORDS	disulfide bond	6	0.004	0.09
SP_PIR_KEYWORDS	Signal	6	0.009	0.10
SP_PIR_KEYWORDS	Glycoprotein	6	0.013	0.11
GOTERM_CC_ALL	extracellular region	6	0.002	0.12
SP_PIR_KEYWORDS	Secreted	5	0.008	0.12
SP_PIR_KEYWORDS	growth factor	3	0.004	0.16
GOTERM_CC_ALL	extracellular region part	4	0.011	0.20
GOTERM_MF_ALL	growth factor activity	3	0.004	0.25
GOTERM_MF_ALL	receptor binding	4	0.009	0.28
GOTERM_MF_ALL	protein binding	9	0.032	0.53
GOTERM_BP_ALL	positive regulation of biological process	4	0.054	1.00
GOTERM_BP_ALL	biological regulation	8	0.055	1.00
GOTERM_BP_ALL	regulation of cellular process	7	0.093	1.00
GOTERM_BP_ALL	regulation of biological process	7	0.120	1.00
GOTERM_BP_ALL	positive regulation of cellular process	3	0.180	1.00

[&]analyzed by DAVID tool (annotation clusters had a group of enrichment scores of 1.82); *Annotation Cluster; SP_PIR: single protein of protein information resource; MF: molecular function; BP: biological process; CC: cellular component; [#]P_{adj}: adjusted *P* value for multiple testing with the Benjamini-Hochberg procedure (FDR)

A group of cytokines, oncostatin M (*OSM*), chemokine ligand 2 (*CCL2*), and interleukin 6 (*IL6*), were identified as upstream regulators of *SEMA4A*, *PGF*, and *SLC11A1* (Appendixe A) in the RG group ($P < 0.0076$, Table 3). The cytokines regulate the gene expression in ECM connective tissue by binding to specific receptors on the surface of fibroblasts (CROMBRUGGHE; VUORIO; KARSENTY, 1990). Moreover, cytokines can influence anabolic and catabolic processes as well as programmed cell death (ZOICO; ROUBENOFF, 2002; CHANG; YANG, 2000). Some cytokines act as potent inducers of MMP activity, which in turn stimulates IGF-binding protein proteolysis, downregulating *IGF-I* activity and favoring ECM

degradation (KJAER, 2004) and increased collagen transcription in response to remodeling of the connective tissue. This produces a large number of advanced glycation end products, reducing the impact on the synthesis of other types of collagen (DEGROOT et al., 2001).

Table 3 – Upstream regulators[&] of genes from mature *Bos taurus indicus* cull cows during recovery gain under grazing conditions

Upstream regulator	Molecule Type	*P* value	Target molecules
RBPJ	transcription regulator	0.0028	PGF
SHH	Peptidase	0.0045	PGF
EPAS1	transcription regulator	0.0054	PGF
MTF1	transcription regulator	0.0061	PGF
DLX3	transcription regulator	0.0104	PGF
FOXD1	transcription regulator	0.0067	PGF
HIF1A	transcription regulator	0.0192	PGF
mir-182	microRNA	0.0242	PGF
miR-182-5p	mature microRNA	0.0218	PGF
AGT (SerpinA8)	growth factor	0.0279	PGF
OSM	Cytokine	0.0393	PGF
HRAS	Enzyme	0.0439	PGF
SMARCA4	transcription regulator	0.0150	SLC11A1
Tnf (family)	Group	0.0048	SLC11A1
PIAS2	transcription regulator	0.0061	SLC11A1
ATF3	transcription regulator	0.0391	SLC11A1
CCL2	Cytokine	0.0432	SLC11A1
IL6	Cytokine	0.0076	SEMA4A

[&]Analysis by Ingenuity Pathway Analysis (IPA)

We also found miR-182-5p (Table 3), a miRNA upstream regulator of the *PGF* gene. This molecule has 345 transcripts with a total of 354 conserved sites and 72 poorly conserved sites. It is involved in membrane protein ectodomain proteolysis, membrane protein proteolysis as well as in the cell cycle and apoptosis (KRISHNAN et. al., 2013). However, interaction of the proteins and post-translational modifications may play a major role in remodeling. The other microRNA found (miR-182) had 100 transcripts with sites, comprising 93 conserved sites and 112 poorly conserved sites. It also regulates the activity of the MMP-2 and MMP-9 extracellular proteases (SACHDEVA et al., 2014). Although, there were no major changes in proteins that played a major role in the strength of the matrix (Table 4).

Table 4. Major extracellular matrix-related genes identified as unaltered in mature *Bos taurus indicus* cull cows between maintenance of high body score and recovery gain conditions

	Gene name	Gene ID	Base Mean	log2(FC)*	*P* Value	P_{adj} #
1	ADAMTS2	ENSBTAG00000014665	86.474	0.1	0.718	0.9
2	COL1A2	ENSBTAG00000013472	1507.5	-0.1	0.993	0.9
3	COL3A1	ENSBTAG00000021466	2934.1	-0.1	0.966	0.9
4	COL4A1	ENSBTAG00000012849	5880.2	0.1	0.476	0.9
5	COL4A2	ENSBTAG00000025210	3351.4	0.1	0.503	0.9
6	DCN	ENSBTAG00000003505	2546.5	0.4	0.214	0.9
7	ELASTIN	ENSBTAG00000019517	433.35	0.1	0.762	0.9
8	HSPG2	ENSBTAG00000017122	2694.9	-0.1	0.963	0.9
9	SPINT2	ENSBTAG00000000182	336.72	0.4	0.008	0.5
10	SERPINH1[&]	ENSBTAG00000001027	1304.2	0.1	0.735	0.9
11	MMP2	ENSBTAG00000019267	604.3	0.5	0.048	0.8
12	MMP14	ENSBTAG00000014824	42.949	0.2	0.517	0.9
13	TIMP2	ENSBTAG00000010899	488.1	0.1	0.560	09

*log2 (FC): log2(foldchange); Negative values: upregulation in maintenance; Positive values: upregulation in recovery gain; [#]P_{adj}: adjusted *P* value for multiple testing with the Benjamini-Hochberg procedure (FDR); [&]HSP47

3.4 Conclusion

Recovery gain provoked changes in genes associated with growth factors that are involved in the control of muscle cell proliferation and protein synthesis during recovery gain.

However, the transcriptome for proteases possibly involved in muscle protein turnover and related to collagen and other major ECM components was not affected in mature females experiencing recovery gain.

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4 RECOVERY FROM UNDERNUTRITION CHANGES MUSCLE GENE EXPRESSION IN MATURE NELLORE CULL COWS DIFFERENTIALLY DEPENDING ON THE RATE OF WEIGHT GAIN

Abstract

Differential growth rates during recovery from undernutrition in mature animals may change the remodeling process in the stromal and myofibrillar proteins as well as in the muscle proteolytic system. Gene expression profile screening based on the number of readings obtained from cDNA libraries may provide important information regarding tissues changes during weight recovery. The objective of this study was to assess changes in muscle gene expression profiles in *Bos taurus indicus* cull cows subjected to moderate and high gain rates during recovery from undernutrition, with a specific focus on connective tissue growth. Thirty-two cows with low body condition scores were randomly assigned to base (BS; n = 4; low body condition), high recovery gain (HG; n = 11; 1.2 kg of daily live weight gain) and moderate recovery gain (MG; n = 17; 0.6 kg of daily live weight gain) groups during the dry season. The animals were slaughtered serially following 0 (BS), 51, 74, and 104 days on feedlot. Comparison was made between the cows in BS and each of the recovery gain rate groups. Analysis was performed on *longissimus dorsi* samples collected immediately after slaughter. After extraction of total RNA, the samples were loaded to a sequencer (HiScanSQ, Illumina). Data normalization and analysis of differential expression were done using the R software DESeq procedure, followed by correction by Benjamini-Hochberg multiple tests for the *P* values obtained. There were 24,616 genes with quantitatively measured expression; 11,779 and 9175 resulted from comparisons between BS and MG or HG, respectively. A total of 416 (BS x MG) and 355 (BS x HG) genes were differentially expressed during recovery gain ($P < 0.05$). These genes could be grouped by their biological functions of protein turnover, extracellular matrix (ECM), and tissue remodeling. Both recovery gain rates showed regulation of genes involved in connective tissue remodeling, including osteonectin (*SPARC*), collagen type IV subunits 1 (*COL4A1*), serpin peptidase inhibitor, clade A, member 8 (*SERPINA8*) and bone morphogenetic protein 2 (*BMP2*). The MG group showed differential gene expression for collagen, Type IV, Alpha 2 (*COL4A2*), heparan sulfate proteoglycan 2 (*HSPG2*), serpin peptidase inhibitor, clade H member 1 (heat shock protein 47; *SERPINH1/HSP47*), lysyl oxidase-like 4 (*LOXL4*) and sparc/osteonectin (*SPOCK2*). In the HG group, we observed genes related to collagen, type I, alpha 2 (*COL1A2*) and three MicroRNAs (bta-mir-2302, bta-mir-1842, bta-mir-2382), as well as two genes related to adiposity: peroxisome proliferator-activated receptor alpha (*PPARA*) and diacylglycerol O-acyltransferase 2 (*DGAT2*). The gene expressions of intramuscular connective tissue and muscle tissue change during recovery from undernutrition in a way that is consistent with the remodeling of the stromal proteins in the ECM. However, in the HG group, genes related to fat deposition were also found. Those changes are similar to fibrosis-like growth model common to muscle regeneration in mature animals.

Keywords: Connective tissue; Growth; Adiposity; Old animals

4.1 Introduction

Protein synthesis and degradation rates increase in cattle due to compensatory growth (JONES et al., 1990), and studies have shown that collagen synthesis in connective tissue is regulated by nutritional restriction (LAURENT, 1987; SPANHEIMER et al., 1991). These changes point to the possibility of modifying structures that are part of the muscle scaffold and are potentially related to meat tenderness, such as cytoskeletal and ECM structures (MCCORMICK, 2009), proteinase systems, and calcium signaling (KOOHMARAIE; GEESINK, 2006), which may also involve some of the regulated genes. This study provides insights into the changes that are taking place and may aid in understanding how recovery affects muscle growth and characteristics in mature animals.

Animal diets that provide fast growth increase the renewal of muscle protein relative to diets that provide normal weight gain (THERKILDSEN, 2005) by changing the crosslinks of connective tissue (MCCORMICK, 1994), and this can improve the sensory traits and collagen characteristics of the muscle (ABERLE et al., 1981; FISHELL et al., 1985). Moreover, it is important to highlight that before muscle tissue renewal starts, an inflammatory response is stimulated (ZACKS; SHEFF, 1982), with invasion of macrophages followed by the formation of new myofibers (CICILIOT; SCHIAFFINO, 2010) and then remodeling (SCHULTZ et al., 1985). If these events are not coordinated, an accumulation of ECM occurs, causing fibrosis (ALEXAKIS; PARTRIDGE; BOU-GHARIOS, 2007); this is more significant in adult animals. Moreover, there have been advances in understanding 1) the role of nutrition and growth rate in myofiber metabolism and other tissue deposition into the muscle, 2) how the increase in growth changes biological mechanisms, and 3) how the outcomes of these relate to ECM and myofiber metabolism.

Our hypothesis was that the greater rate of weight gain achieved under feedlot conditions during recovery from undernutrition would be necessary to increase ECM protein turnover (i.e., collagen) by altering the transcription of proteins and protease genes to elicit changes in tenderness. The aim of this study was to assess recovery from undernutrition changes muscle gene expression in mature Nellore cull cows differentially depending on the rate of weight.

4.2 Material and Methods

4.2.1 Treatments

The experiments were performed at the Brazilian National Beef Cattle Research Center/EMBRAPA on 32 Nellore cull cows aged 6 to 17 years. The experimental procedures were approved by the environmental (CEAP, protocol #66) and animal (CEUA) ethics committees from the “Luiz de Queiroz” College of Agriculture, University of São Paulo.

The animals were randomly divided into three groups and were subjected to one of three nutritional managements: 1) Base (BS, N=4 slaughter at the beginning of the experiment, very low body condition; score 4); moderate recovery gain (MG; N=17, 0.6 kg of live weight gain) during the dry season; and high recovery gain (HG; N=11, 1.2 kg of live weight gain) during the dry season. The diet for the MG group was 100% sorghum silage “ad libitum” and for the HG group was composed of sorghum silage (35%), ground corn (52%), soybeans meal 45% (10%), limestone (1.64%), urea (0.56%), ammonium sulfate (0.05). It was balanced based on nutritional values from National Research Council (NRC; 1996), for this category. All animals received mineral supplementation. The animals were slaughtered serially after 0 (BS), 51, 74, and 104 days on feedlot at the Meat Laboratory of the Brazilian Agricultural Research Corporation in Campo Grande, MS, Brazil, following the procedures recommended by the Inspection of Industrial Sanitation for Products of Animal Origin (RIISPOA), and inspected by the Municipal Inspection Service (SIM). Immediately after slaughter, samples from the *longissimus dorsi* muscle were collected and frozen in liquid nitrogen (-80°C). They were kept frozen at -80°C until sequencing analysis could be performed. The differences between groups used the gene expression data of all the animals from each treatment, not considering the slaughter time. Our approach was to identify genes were consistently changed in the growth curve during the recovery gain.

4.2.2 RNA Extraction, library, and sequencing

RNA extraction from the *longissimus dorsi* muscle samples (100 mg) from each animal (n = 32) was performed using 1 mL of Trizol reagent (Life Technologies,

Carlsbad, CA, USA). The extracted RNA was quantified using a spectrophotometer (NanoDrop 200, Thermo Scientific, Wilmington, Delaware, USA). The integrity of the material was verified using 1% agarose gel and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) (RIN: RNA Integrity Number). Only samples with RINs greater than 8 were used for the next steps. After this stage, 2 μg of total RNA from each sample was purified and fragmented using a TruSeq RNA Sample Prep Kit v2 to split RNA messenger molecules (2 to 4% of the total RNA) from the remaining RNA by connecting these molecules to poly-T tails adhered to magnetic beads. After purification and fragmentation, the first cDNA tape was synthesized using random primers (hexamers) and reverse transcriptase enzyme. Next, the messenger RNA was removed and the second cDNA tapes were synthesized and then purified using magnetic beads (Agencourt® Ampure XP, Beckman Coulter).

The double cDNA tapes were repaired to stand "blunt end," followed by the adenylation of the 3' extremities and the correct connection of the adapters, which are necessary for correct hybridization in the flow cell and allow molecule sequencing. Next, DNA fragments were enriched via polymerase chain reaction (PCR), whereby only fragments with adapters are selected and amplified using specific primers that connect themselves to the end of the adapters. This library was then purified and validated in Bioanalyzer, which measured the quantity and size of the fragments present in the sample. All samples were diluted to 10 nM, since in this stage it is possible to multiplex the samples through specific index usage (7 bp oligonucleotides). After the preparation phase, the samples were clustered in flow cell cBot (Illumina, San Diego, USA). For this step, we used an SR TruSeq Kit, a Cluster Kit v2-CBOT-SH, and a set of capillaries that transferred the samples and reagents into the flow cell (HiSeq cBot manifold). After clustering, the flow cell with 38 libraries was sequenced in HiScanSQ (Illumina) at the Genomics Center, University of Sao Paulo/ESALQ, Piracicaba, São Paulo, Brazil. A read was defined as a 100 bp cDNA fragment sequenced from a paired end.

4.2.3 Mapping and Counting Reads

The sequencing data quality was evaluated with FastQC [http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/]. Seqclean software [https://bitbucket.org/izhbannikov/seqyclean/downloads] was used with 24 Phred

quality parameters for the maximum average error. Vector and adaptor sequences from the UniVec database [https://www.ncbi.nlm.nih.gov/tools/vecscreen/univec/] were used as guides to remove possible contaminants for quality filtering. The reads were mapped using TopHat 2.0.10 (TRAPNELL; PACHTER; SALZBERG, 2009) and Bowtie2 v2.1.0 (LANGMEAD et al., 2009) against the UMD3.1 *Bos taurus taurus* masked genome available at Ensembl [http://www.ensembl.org/Bos_taurus/Info/Index/], with a maximum of one mismatch allowed. To quantify the read counts, HTSeq v0.5.4p2 (ANDERS; HUBER, 2014) was used with the model nonempty intersection, in which reads that aligned on more than one gene were considered ambiguous and were not counted. Normalization of the data expression analysis was performed by the R differential gene expression analysis package (DESeq2) (LOVE; ANDERS, 2014), considering growth rate treatments (BS x MG), (BS x HG) and (MG x HG) and age of animal in the model, followed by the Benjamini-Hochberg (1995) correction for multiple tests on the obtained *P* values to control the false discovery rate (FDR).

4.2.4 Enrichment analysis

For the data set that include all genes differentially expressed, the Functional Annotation Clustering DAVID tools (HUANG; SHERMAN; LEMPICKI, 2009) used GOTERM, SP_PIR_KEYWORDS and KEGG_PATHWAY analyses to create clusters that show decreasing values of gene enrichment scores. The Benjamini-Hochberg correction applied to the DAVID enrichment was Padj <0.05 for genes that were considered differentially expressed with a P-value <0.05.

Furthermore, a small portion of the significantly upregulated genes in each treatment (MG and HG), which could also be identified in the most enriched groups by DAVID tools, were reported in order discuss changes in gene expression pattern. Even though the enrichment groups were not significant in this set of data, it was still used as a guide to select candidate genes.

4.2.5 Novel transcripts, Uncharacterized proteins, microRNA-targets, and Ingenuity Pathway Analysis (IPA)

For novel transcripts, genome2seq was used to determine the FASTA sequence, which was annotate based on sequence homology by the Basic Local

Alignment Search Tool (BLAST, http://www.blast.ncbi.nlm.nih.gov/Blast.gov). The differentially expressed uncharacterized proteins were annotated by searching for orthologous genes at BioMart Ensembl [http:www.ensemble.org/biomart]. Analysis of microRNA-targets was performed using the TargetScanHuman database (http://www.targetscan.org/). In addition, enrichment analysis was performed using QIAGEN's Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, www.qiagen.com/ingenuity). We used IPA to identify upstream genes in the dataset that could potentially explain the observed genes expressed in our data (target molecules).

4.3 Results

4.3.1 Differential expression

After the quality filtering, alignment, and normalization procedures, 11,779 genes were identified in the transcriptome for BS x MG comparison and 9175 genes were identified for BS x HG. Among those, 416 (BS x MG) and 355 genes (BS x MG) were differentially expressed ($P < 0.05$). In the comparison between MG and HG just the cell division cycle 34 gene (CDC34; ENSBTAG00000002098) was upregulated for HG (P value = 0.001). It appear to be part of the family of ubiquitin conjugating enzymes.

The distribution of fold-change data of the genes expressed ($P < 0.05$) in both treatments is presented in Figures 1 and 2 for MG and HG, respectively. From the upregulated genes, fifty-four common genes were identified in both growth treatments (Table 2), related mostly to extracellular region, regulation of biosynthetic process, protein binding. Besides those common genes, it was chosen fifteen candidate genes per group (Table 3 and 4) based on their appearance in the most enriched groups by DAVID tools, in order to discuss changes in gene expression pattern between growth groups.

Figure 1. Volcano plot from *longissimus dorsi* muscle of mature *Bos taurus indicus* cull cows subjected to medium recovery gain (MG; 0.6 kg of live weight gain) during the dry season, showing gene distribution (BS x MRG) according to log2foldchange

Figure 2. Volcano plot from longissimus dorsi muscle of mature Bos taurus indicus cull cows subjected to high recovery gain (HG; 1.2 kg of live weight gain) during the dry season, showing gene distribution (BSx MRG) according to log2foldchange

4.3.2 Upregulated genes common to both recovery gain rate groups

The fifty-four different genes were differentially expressed in the same way in both recovery gain rate groups (Table 2). These included interesting genes involved in connective tissue remodeling as an inflammatory response, such as interferon regulatory factor 2 binding protein 1 (*IRF2BP1*), immunoglobulin superfamily, member 3 (*IGSF3*), and parathymosin (*PTMS*). These may present a concerted response that depends largely on macrophage secretion (CHAZAUD et al., 2009), which is the first stage of tissue remodeling (MCLENNAN, 1996). In addition, an increase in *COL4A1* and *SPARC* (Table 2), which are related to tissue injury or high turnover rates and play important roles within the ECM (BRADSHAW; SAGE, 2001),

suggests that connective tissue remodeling and growth were related to increased ECM component transcripts. Also presented was heat shock protein 70 (*HSP 70,* Table 1), which is related to the prevention of atrophy (SENF et al. 2008) and associated with cellular repair (MCARDLE et al., 2004).

The increase in collagen type IV in the connective tissue (Table 2) and mature collagen molecules of cows can be expected, since it has been observed in the basement membrane during aging in other animal models. Muscle growth during recovery also presented upregulated genes of insulin-like growth factor binding protein 6 (*IGFBP6*). Bernard et al. (2009) found this gene to be downregulated during high muscle growth. Moreover, *IGFBP-6* may be relevant for aging because it can avoid senescence-associated growth arrest (MICUTKOVA et al., 2011). Several ribosomal genes (Table 1) were more strongly expressed in the muscle of cows slaughtered at the beginning of the experiment (control group), including tRNA methyltransferase (*TRMT5*; ENSBTAG00000011796), aspartyl-tRNA synthetase (*DARS*; ENSBTAG00000009949), general transcription factor IIA, 2 (*GTF2A2*; ENSBTAG00000010298), ribosomal protein L10a (*RPL10A*; ENSBTAG00000019494), and similar to ribosomal protein S28 (*RPS28*; ENSBTAG00000002468). These genes, which are related to protein synthesis, may be an indication that the synthetic apparatus in the control animals was not downregulated, and that muscle waste was accompanied by greater protein degradation. In addition, with the stabilization of weight, muscle (very poor body condition) could maintain or increase expression of proteins associated with the remodeling response (BATT et al., 2006).

Table 1 - Differentially expressed genes[&] related to the RNA metabolic process in muscle from mature *Bos taurus indicus* culls in the control group

	Medium recovery gain				High recovery gain			
ID	baseMean	log2FD[#]	P-value	P_{adj}*	baseMean	log2FD[#]	P-value	P_{adj}*
DARS	554.02	0.6126	<0.0001	0.0021	525.10	-0.4457	0.0019	0.0489
RPS28	1126.27	-0.8321	<0.0001	0.0037	1028.363	-0.6789	0.0001	0.0086
GTF2A2	170.04	-0.5413	<0.0001	0.0041	145.67	-0.7614	0.0008	0.0324
RPL10A	607.44	-0.7708	0.0002	0.0113	553.05	-1.1077	0.0008	0.0321
TRMT5	87.375	-0.6999	0.0005	0.0208	80.58	-0.6745	0.0018	0.0475

[&#]annotated by BioMart Ensembl; [#]log2(FC): log2(foldchange); *[#]P_{adj}: adjusted *P* value for multiple testing with the Benjamini-Hochberg procedure (FDR)

Table 2 – Differentially genes expressed[#] in muscle from mature *Bos taurus indicus* cull cows presented in moderate and high recovery gain under feedlot conditions during the body condition

			Medium recovery gain				High recovery gain			
	Gene name	ID	Base Mean	log2FD[*]	Pvalue	Padj	Base Mean	log2FD[*]	Pvalue	Padj[&]
1	IRF2BP1	ENSBTAG00000000154	234.24	0.7649	0.00001	0.0022	213.16	1.1985	<0.0001	<0.0001
2	COL11A2	ENSBTAG00000000601	41.48	1.6654	0.00084	0.0291	32.98	2.4761	<0.0001	<0.0001
3	NDRG2	ENSBTAG00000000843	2395.06	0.6558	0.00004	0.0045	2322.84	0.9293	0.0004	0.0226
4	TLE2	ENSBTAG00000001153	69.48	0.9714	0.00077	0.0283	74.14	1.4787	<0.0001	0.0025
5	NDST1	ENSBTAG00000001692	190.70	0.4564	0.0008	0.0287	181.40	0.6502	0.0001	0.0121
6	DLGAP4	ENSBTAG00000001741	624.29	0.9061	<0.0001	<0.0001	552.07	0.7959	0.0017	0.0468
7	HAGHL	ENSBTAG00000002481	71.519	1.1096	<0.0001	<0.0001	68.36	0.9463	0.0015	0.0443
8	ZC3H7B	ENSBTAG00000002782	192.34	0.6186	0.0021	0.0487	186.69	0.8072	<0.0001	0.0013
9	PCSK7	ENSBTAG00000003052	167.39	0.6126	<0.0001	0.0037	165.28	0.6924	0.0001	0.0074
10	CCDC6	ENSBTAG00000003189	437.08	1.0129	0.00012	0.0090	418.87	1.0925	0.0018	0.0468
11	ATF5	ENSBTAG00000003457	64.90	1.9213	<0.0001	0.0024	51.27	1.7354	<0.0001	0.0025
12	IGSF3	ENSBTAG00000004849	31.49	1.5141	0.00085	0.0293	34.73	1.4975	0.0002	0.0136
13	GOSR1	ENSBTAG00000004961	36.51	1.2186	0.00001	0.0018	31.24	1.1193	0.0016	0.0453
14	BMP2	ENSBTAG00000005111	43.33	1.1180	0.0019	0.0451	43.82	1.3420	0.0004	0.0226
15	WNK1	ENSBTAG00000005221	1214.73	0.5630	0.0020	0.0462	1146.41	0.8040	<0.0001	0.0018
16	RREB1	ENSBTAG00000005980	378.89	0.9929	0.0002	0.0135	358.19	1.3797	<0.0001	<0.0001
17	IKBKG	ENSBTAG00000006268	129.18	0.4539	0.0001	0.0060	123.79	0.8339	<0.0001	0.0005
18	CNPY4	ENSBTAG00000007016	66.14	0.8605	<0.0001	0.0034	59.99	0.8163	0.0017	0.0468
19	Hsp70s	ENSBTAG00000007807	134.39	0.7562	0.0016	0.0400	99.91	0.8711	0.0017	0.0468
20	SPRY4	ENSBTAG00000008250	76.86	1.1595	<0.0001	<0.0001	79.55	1.3634	<0.0001	0.0005
21	RNF19B	ENSBTAG00000008330	244.99	0.6885	0.00002	0.0028	249.14	0.8419	0.0001	0.0109
22	ZNF335	ENSBTAG00000010130	172.13	0.6960	0.00027	0.0147	172.07	0.8581	<0.0001	0.0025
23	ANKRD52	ENSBTAG00000011762	204.61	1.2252	<0.0001	0.0076	197.71	1.4004	<0.0001	0.0003
24	HCFC1	ENSBTAG00000011904	517.89	0.8892	0.0004	0.0184	463.31	0.7865	0.0003	0.0182
25	BCAR1	ENSBTAG00000012002	193.46	0.7499	<0.0001	0.0028	183.24	0.9149	0.0006	0.0274
26	AGT/ SerpinA8	ENSBTAG00000012393	39.66	1.5243	0.0005	0.0208	33.46	1.8283	0.0002	0.0136

27	COL4A1	ENSBTAG00000012849	3878.34	1.1081	<0.0001	0.0031	4201.49	1.1596	0.0005	0.0241
28	CALM2	ENSBTAG00000014583	5948.55	0.9281	0.0013	0.0368	6563.94	1.1312	0.0003	0.0210
29	SPARC	ENSBTAG00000014835	5040.54	1.2803	0.0007	0.0272	5480.43	1.2674	0.0002	0.0141
30	STK40	ENSBTAG00000015969	409.49	0.6567	0.0003	0.0157	426.84	1.0523	<0.0001	0.0056
31	PHLDB1	ENSBTAG00000016197	692.72	0.6399	<0.0001	0.0036	674.72	0.8292	0.0001	0.0075
32	EEF2K	ENSBTAG00000017662	1197.85	0.6830	0.0004	0.0198	1103.45	0.8386	0.0013	0.0406
33	PTPRS	ENSBTAG00000018052	244.97	0.8360	<0.0001	<0.0001	232.07	0.8332	0.0001	0.0074
34	PTMS	ENSBTAG00000018451	401.18	1.1720	<0.0001	<0.0001	404.72	1.3615	<0.0001	0.0001
35	CELSR2	ENSBTAG00000018804	57.38	1.1369	<0.0001	0.0090	48.27	1.2484	0.0019	0.0482
36	IPO13	ENSBTAG00000018887	2035.80	0.4534	0.0015	0.0393	2005.55	0.7321	<0.0001	0.0032
37	INPPL1	ENSBTAG00000019167	1045.44	0.3790	0.0019	0.0451	1003.14	0.7132	<0.0001	0.0010
38	RHBDF	ENSBTAG00000019805	145.22	0.6672	0.00016	0.0108	136.86	0.6965	0.0005	0.0249
39	HIRA	ENSBTAG00000019888	27.98	1.9177	0.00204	0.0470	13.74	1.4991	0.0017	0.0467
40	A2VDS5	ENSBTAG00000020061	343.82	1.0474	0.00003	0.0037	283.41	0.9958	0.0010	0.0354
41	OSBP	ENSBTAG00000020332	354.43	0.4063	0.0001	0.0095	332.04	0.6418	<0.0001	0.0054
42	PKD1	ENSBTAG00000020619	467.06	0.6965	0.00048	0.0214	444.83	0.6482	0.0011	0.0378
43	C7orf26	ENSBTAG00000021248	144.68	0.5477	0.0002	0.0126	132.57	0.5230	0.0011	0.0355
44	IGFBP6	ENSBTAG00000021467	419.34	0.8601	0.00055	0.0234	378.35	0.6843	0.0001	0.0114
45	ZBTB7B	ENSBTAG00000021512	342.79	0.5990	0.0001	0.0090	364.67	0.8443	0.0004	0.0214
46	VCL	ENSBTAG00000021879	2726.57	0.8945	<0.0001	<0.0001	2465.89	0.8657	0.0004	0.0214
47	TRIL	ENSBTAG00000024340	16.61	1.6317	0.00056	0.0237	17.84	1.7021	0.0006	0.0274
48	FAM53C	ENSBTAG00000030529	707.91	0.5054	<0.0001	0.0006	653.49	0.5043	0.0005	0.0249
49	PNPLA6	ENSBTAG00000032137	133.73	0.6388	<0.0001	0.0027	126.22	0.7983	0.0001	0.0098
50	SCN4B	ENSBTAG00000039340	886.06	1.4914	0.00020	0.0126	1329.33	2.1197	0.0000	0.0010
51	CDR2L	ENSBTAG00000039466	14.93	1.4218	0.00021	0.0128	12.35	1.4303	0.0017	0.0468
52	MED16	ENSBTAG00000047217	101.73	0.7553	0.00047	0.0211	99.41	0.7657	0.0002	0.0138
53	INSIG1	ENSBTAG00000001592	57.63	1.1748	0.0018	0.0436	60.05	1.2048	0.0001	0.0084
54	FAM189B	ENSBTAG00000047731	81.82	0.8119	<0.0001	0.0008	83.60	0.8828	0.0008	0.0316

[#]annotated by BioMart Ensembl; *log2(FC) – log2(foldchange); [#]P_{adj}: adjusted P value for multiple testing with the Benjamini-Hochberg procedure (FDR)

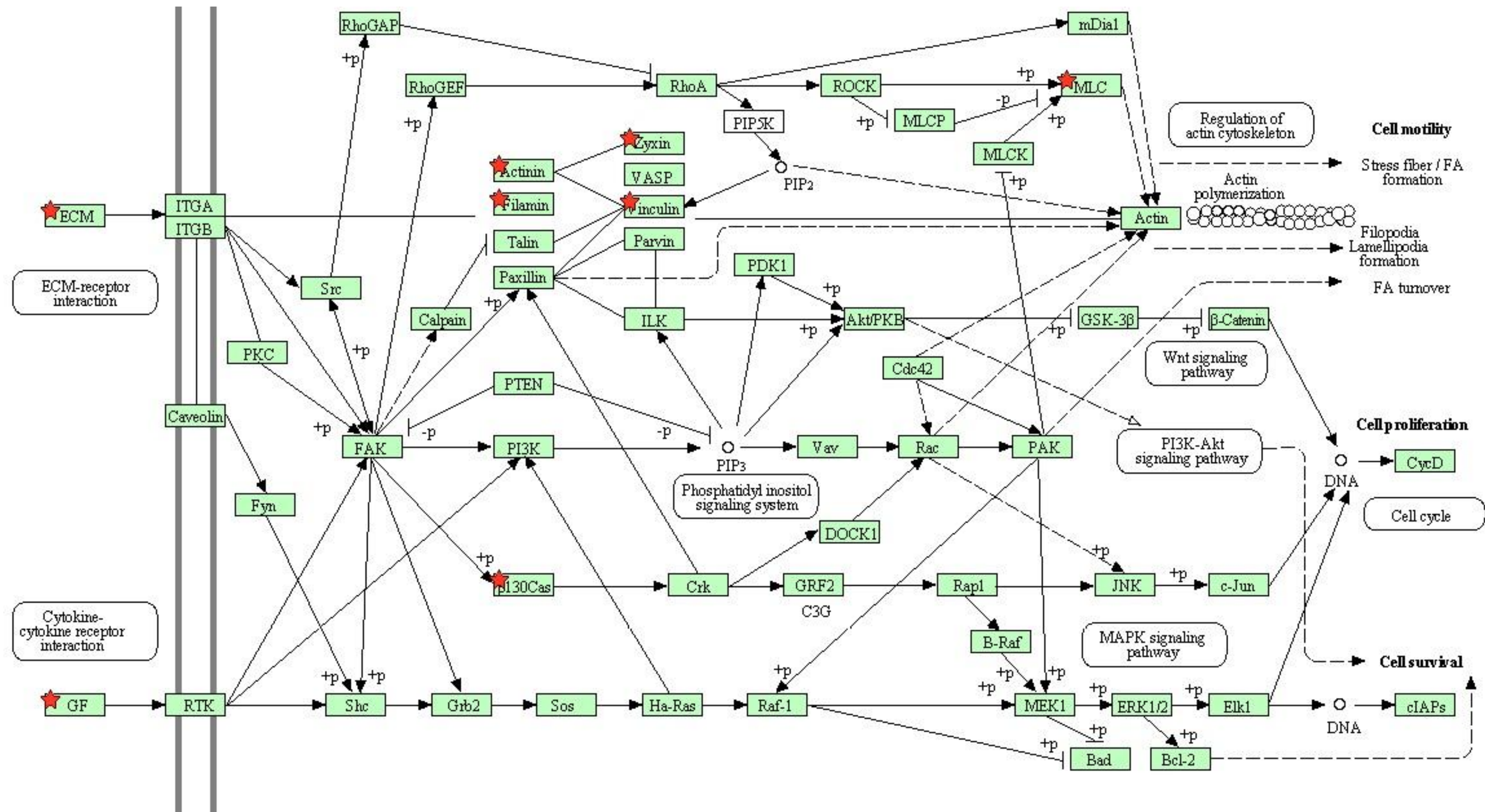

Figure 3. Focal adhesion pathway present in the nutritional managements moderate recovery gain - MG (0.6 kg of daily live weight) gain)

4.3.3 Upstream regulator gene analysis by IPA

Despite recent advances in understanding the regulation of collagen gene expression using QIAGEN's Ingenuity Pathway Analysis (Table 3), the mechanism controlling the group of genes that is upregulated in both recovery gain groups suggested that one or more genes are also involved in the regulation of collagen gene expression (JIMENEZ et al., 2001). Associated with the upstream regulator gene analysis by the IPA program in both recovery gain treatments were two microRNAs: mir-182 and miR-29b-3p (Table 2). Mir-182, which targets *COL11A2* (Table 4), is involved with the cytoskeleton, macromolecule metabolic processes, and apoptosis (CEKAITE et al., 2012). Target genes present in these roles are B-cell CLL/lymphoma 10 (*BCL10*), BCL2/adenovirus E1B 19kDa interacting protein 3 (*BNIP3*), and tumor necrosis factor, alpha-induced protein 8 (*TNFAIP8*). This microRNA has 100 transcripts with sites, including 93 conserved sites and 112 poorly conserved sites. MiR-29b-3p was previously reported to target *SPARC* in a study on rats (BOT; DĘBSKI; LUKASIUK, 2013).

One important group of upstream regulators was clustered at the cell death annotation after enrichment in DAVID, with a score of 2.18 ($P < 0.0001$). The genes presented in this group were DNA-damage-inducible transcript 3 (*DDIT3*), angiotensinogen (serpin peptidase inhibitor, clade A, member 8; *AGT/SERPINA8*), collagen type IV alpha 3 (*COL4A3*), fibroblast growth factor 2 (*FGF2*), fibroblast growth factor receptor 3 (*FGFR3*), interleukin 1 (*IL1B*), interleukin 3 (*IL3*), and phosphoinositide-3-kinase catalytic alpha polypeptide (*PIK3CA*). Most of these regulators are cytokines and growth factors that mainly affect connective tissue, as would be expected in a growth model that privileges fibrogenic development of the muscle.

The other interesting result was the regulation of *BMP2* by MMP2 (Table 3). The MMP family of zinc-containing proteolytic enzymes breaks down ECM proteins (NAGASE; WOESSNER Jr., 1999). Changes in tissue inhibitors of matrix metalloproteinases (TIMPs) were not detected. *BMP-2* belongs to the *TGF-β* superfamily and has been shown to play a role in chondrocyte differentiation and matrix maturation (LI et al., 2003).

We further demonstrated that changes occurred in the ECM, but these may be limited to minor components related to connective tissue scaffolding. Collagen type

IV transcripts represent this limited connective tissue remodeling in mature animals, where a large number of advanced glycation end products exert a feedback signal to reduce other types of collagen synthesis as well as MMP-initiated collagen degradation. Before remodeling, events related to growth factor and cytokines occur (CROMBRUGGHE; VUORIO; KARSENTY, 1990). The cytokines promote the migration, proliferation, and survival of various cell types, whereas macrophages are responsible for the inflammation process (CICILIOT; SCHIAFFINO, 2010); this continues until muscle remodeling and growth occur.

Table 3 - Upstream regulators genes& in muscle from mature Bos taurus indicus cull cows presented in moderate and high recovery gain under feedlot conditions during the body conditions

	Upstream Regulator	Molecule Type	p-value	Target molecules
1	Proteasome	chemical protease inhibitor	0.0232	BMP2
2	Collagen type II	Complex	0.0109	BMP2
3	WNT3A	Cytokine	<0.0001	BMP2, COL11A2, SPARC
4	SCGB1A1	Cytokine	0.0124	COL4A1
5	IL1B	Cytokine	0.0257	ATF5,BMP2, SPARC
6	IL3	Cytokine	0.0268	ATF5,BMP2
7	Ubiquitin	Group	0.0209	BMP2
8	IL1	Group	0.0270	BMP2, SPARC
9	FGF2	growth factor	0.0288	SPARC, SPRY4
10	TGFB1	growth factor	0.0336	BMP2, CELSR2, COL4A1, SPARC
11	LEP	growth factor	0.0417	COL4A1,SPARC
12	AGT	growth factor	0.0441	COL11A2,COL4A1
13	MAP3K3	Kinase	0.0178	BMP2
14	FGFR3	Kinase	0.0224	SPARC
15	PIK3CA	Kinase	0.0330	SPARC
16	MAP3K1	Kinase	0.0413	COL4A1
17	miR-29b-3p	mature microrna	0.0102	COL4A1,SPARC
18	mir-182	Microrna	0.0308	COL11A2
19	COL4A3	Other	0.0186	COL4A1
20	CTSS	Peptidase	0.0163	COL4A1
21	MMP2	Peptidase	0.0277	BMP2
22	ZNF219	transcription regulator	0.0023	COL11A2
23	DNAJB6 (Hsp40)	transcription regulator	0.0070	SPARC
24	CTBP2	transcription regulator	0.0124	SPRY4
25	MYCN	transcription regulator	0.0150	COL4A1,SPARC
26	CREBBP	transcription regulator	0.0345	SCN4B,TLE2
27	CSHL1	transcription regulator	0.0436	BMP2
28	SNAI1	transcription regulator	0.0451	SPARC
29	ITGB4	transmembrane receptor	0.0077	SPARC
30	IL10RA	transmembrane receptor	0.0235	BMP2, SPARC

[&]Analysis by Ingenuity Pathway Analysis (IPA)

4.4 Base x MG

The fifteen genes differentially expressed in the MG (Table 4) were found in the following enrichments groups: extracellular matrix organization, extracellular matrix, extracellular region. In addition, in the MG treatment, the focal adhesion pathway (Figure 3) for enriched KEGG was significant ($P < 0.02$). The genes present

in this pathway were actinin alpha 4 (*ACTN4*), breast cancer anti-estrogen resistance 1 (*BCAR1*), *COL4A1*, *COL4A2*, filamin A, alpha (*FLNA*; actin binding protein 280), myosin, light chain 9, regulatory (*MYL9*), placental growth factor (*PGF*), vinculin (*VCL*), and zyxin (*ZYX*). This pathway is important because the cytoskeleton (Figure 3) of a cell connects to the ECM (RIVELINE et al., 2001). It is organized to connect the ECM with actin cytoskeleton (HILDEBRAND; SCHALLER; PARSONS, 1993), and may signal cell growth (SCHLAEPFER, HAUCK; SIEG, 1999).

LOXL4, *PGF*, and *SerpinH1/HSP47* are other connective tissue-related genes that showed significant changes in expression (Table 1). The former is important for the formation of mature collagen triple-helices. The heparan sulfate proteoglycans (*HSPG*) are also present in the extracellular compartment and interact with adhesion molecules and growth factors (BRANDAN; LARRAÍN, 1998). Moreover, *SPOCK2*, from the *SPARC* glycoprotein family, is expressed during tissue remodeling (BORNSTEIN, 1995). We also identified several structural myofibrillar proteins, Myosin IE (*MYO1E*), Myosin Heavy Chain 11 (*MYH11*), and myogenin (*MYOG*), that responded positively to the recovery growth. Previous reports have shown an upregulation of the myosin regulatory chain after compensatory growth in cattle (LEHNERT et al., 2006).

Table 4 - Differentially genes expressed[&] annotated by BioMart Ensembl from *Bos taurus indicus* cull cows submitted to moderate recovery weight gain rate under feedlot condition

	Gene name	Gene ID	Base Mean	log2(FC)*	P-Value	Padj[#]
1	GFOD2	ENSBTAG00000018908	45.6309	0.6797	0.00064	0.0255
2	ZYX	ENSBTAG00000017970	734.4485	0.6869	0.00031	0.0164
3	MYO1E	ENSBTAG00000021538	158.8939	0.6919	0.00145	0.0387
4	ACTN4	ENSBTAG00000011215	926.2848	0.7488	0.00111	0.0350
5	HSPG2	ENSBTAG00000017122	2510.337	0.7731	0.00190	0.0453
6	FLNA	ENSBTAG00000011190	1989.381	0.8912	0.00023	0.0135
7	COL4A2	ENSBTAG00000025210	2388.819	0.9041	0.00024	0.0136
8	MYL9	ENSBTAG00000011473	479.5974	1.0359	0.00022	0.0133
9	MYOG	ENSBTAG00000006030	102.4932	1.0399	0.00045	0.0208
10	SERPINH1 / HSP47	ENSBTAG00000001027	594.4890	1.1857	0.00035	0.0175
11	MYH11	ENSBTAG00000015988	624.1959	1.2080	0.00146	0.0387
12	LOXL4	ENSBTAG00000020895	63.65500	1.3388	0.00193	0.0455
13	PGF	ENSBTAG00000013688	52.8659	1.3563	0.00118	0.0360
14	POSTN	ENSBTAG00000012409	27.6964	1.9888	0.00050	0.0220
15	SPOCK2	ENSBTAG00000008827	1073.1340	2.2876	<0.001	0.0001

[#]log2(FC) – log2(foldchange); [##]P_{adj}: adjusted *P* value for multiple testing with the Benjamini-Hochberg procedure (FDR)

4.5 Base x HG

The fifteen genes differentially expressed in the MG (Table 4) were found in the following enrichments groups: apoptosis, macromolecule localization, ECM, oxidoreductase, and biosynthetic processing, including three microRNAs: bta-mir-1842, bta-mir-2382, and bta-mir-2302 (Table 5). According to the TargetScanHuman database (http://www.targetscan.org/), bta-mir-1842 has 100 transcripts with conserved sites, comprising 171 conserved sites. It can target *MYL9* and fatty acid binding protein 3 (*FABP3*), and can be involved in macromolecular complex subunit organization and ATP binding. Bta-miR-2382 has 125 transcripts with conserved sites, comprising 133 conserved sites and 113 poorly conserved sites. It is involved in developmental processes, biological regulation, phosphoprotein, and positive regulation of transcription. The other microRNA was bta-mir-2302, which targets genes related to the ECM such as serpin peptidase inhibitor, clade F, member 2 (*SERPINF2*), superoxide dismutase 3, extracellular (*SOD3*), (2',3'-cyclic nucleotide 3' phosphodiesterase (*CNP*), *LOXL4*, and syndecan 4 (*SDC4*). Other important genes such as ubiquitin specific peptidase 36 (*USP36*), calcium channel flower domain

containing 1 (*CACFD1*), and ubiquitin-conjugating enzyme E2M (*UBE2M*) are also targeted by this microRNA.

The *BCL9* gene was more strongly expressed in the HG group, which may indicate signaling that facilitates the conversion of satellite cells from myogenic to fibrogenic lineages and the associated tissue fibrosis in mature animals. The expression of *BCL9* seems to be necessary to mediate *WNT* signaling for myogenic progression and differentiation (BRACK et al., 2009), and this signaling is associated with conversion of the cell lineages in muscle of older animals (BRACK et al., 2007).

Genes related to the ECM and skeletal muscle were also identified in the HG group. *COL1A2* is a structural component of the ECM, and its contribution is expected in older animals (MAYS; BISHOP; LAURENT, 1988) along with collagen, type XIII, alpha 1 (*COL13A1*). Other genes, such as *PPARA*, *DGAT2*, and *PI4KB*, which are related to fat acid synthesis in adipose tissue, were also differentially expressed in the HG group. These results may suggest a pronounced ECM renewal in the HG group. Calpain 6 (*CAPN6*), which has a non-proteolytic function, is related to the suppression of skeletal muscle development and regeneration in *CAPN6*-deficient mice (TONAMI et al., 2013). These authors classified *CAPN6* as a suppressor of skeletal muscle differentiation. In this respect, the expression of CAPN6, also points to fibrosis-like growth in the physiology of cows submitted to high recovery gain

Table 5 - Differentially expressed genes annotated by BioMart Ensembl from *Bos indicus* cull cows submitted to high rates under feedlot conditions during the body condition

	Gene name	Gene ID	Base Mean	log2(FC)*	P-Value	Padj#
1	PI4KB	ENSBTAG00000007320	346.5510	0.4392	0.0011	0.0375
2	PSKH1	ENSBTAG00000018037	191.9507	0.5348	0.0017	0.0468
3	NFIX	ENSBTAG00000018229	1007.8530	0.8040	0.0012	0.0393
4	CAPN6	ENSBTAG00000000828	268.2329	0.8056	0.0016	0.0462
5	PPARA	ENSBTAG00000008063	182.0755	0.8448	0.0018	0.0479
6	BCL9	ENSBTAG00000002422	47.5846	0.9087	0.0007	0.0285
7	bta-mir-1842	ENSBTAG00000037500	75.5286	1.0893	0.0017	0.0468
8	bta-mir-2382	ENSBTAG00000044606	99.0436	1.0950	0.0007	0.0286
9	COL1A2	ENSBTAG00000013472	1998.8622	1.1402	0.0010	0.0351
10	DGAT2	ENSBTAG00000001154	417.5709	1.3255	0.0004	0.0214
11	bta-mir-2302	ENSBTAG00000045250	9.9179	1.8459	0.0010	0.0351
12	USP35	ENSBTAG00000012944	10.9748	1.8948	0.0007	0.0292
13	COL13A1	ENSBTAG00000011741	7.8424	1.9125	0.0020	0.0500
14	TPSB1	ENSBTAG00000007325	23.0818	2.0395	0.0010	0.0351
15	PCK1	ENSBTAG00000001936	30.4277	2.0618	<0.0001	0.0012

#log2(FC) – log2(foldchange); ## P_{adj}: adjusted *P* value for multiple testing with the Benjamini-Hochberg procedure (FDR)

4.6 Conclusion

Muscle gene expression is changed during recovery gain from undernutrition in mature animals, consistent with relevant fibrogenic and ECM growth. In addition, the HG group showed genes related to fat deposition that also point to a fibrosis-like growth pattern.

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5 GENERAL CONCLUSIONS

Muscle growth rate is regulated by the proliferation and incorporation of satellite cells into existing muscle fiber, protein synthesis, and degradation as well as connective tissue growth. These processes change with age and nutritional management. For this reason, a major interest for meat scientists is the verification of gene expression to identify potential candidates that can be further characterized for use in determining how cattle management and nutrition influence beef tenderness.

In the transcriptome studies developed in this thesis for proteases that are potentially involved in muscle protein turnover and related to collagen, we did not show gene differential expression in mature females experiencing recovery weight gain under grazing. However, provoked changes to genes related to the inflammatory response during regrowth point to the renewal of tissue. In the feedlot experiment, changes in muscle tissue were observed during the protein turnover provided by recovery gain management, indicating remodeling through genes associated with ECM renewal due to the increase in recovery weight gain. Although two stages were observed, in the MG group, we identified genes related to structural proteins in the process of renewal. This may be related to the maturation of newly formed myofibers by satellite cells. In the HG group, it appears that high deposition of ECM materials resulted in a fibrosis pattern.

In summary, nutritional management can modify gene expression in Nellore cull cows subjected to different weight gain rates; however, this is achieved mostly by genes related to the ECM in a fibrosis-like pattern, which may be detrimental to meat texture.

APPENDIXE

Appendixe A

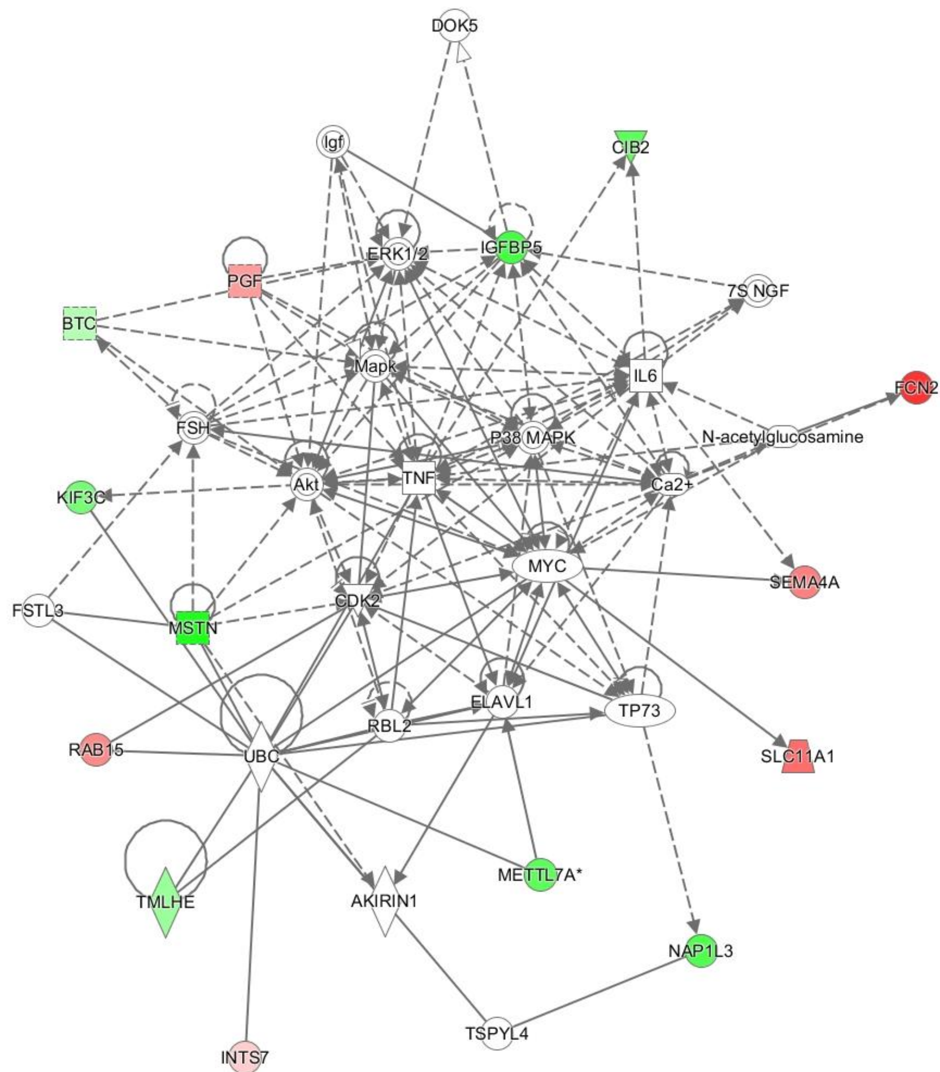

Figure 1. Network of the genes from *Bos indicus* cull cows submitted to high growth rate under grazing conditions during the body condition recovery, calculate by Ingenuity Pathway Analysis of. Genes in uncolored nodes were not identified as differentially expressed in this experiment. Node color indicates expression level of gene: red = up-regulated in recovery gain group, green = up-regulated in maintenance