Animal (2019), **13:2**, pp 333–340 © The Animal Consortium 2018
doi:10.1017/S1751731118001490

animal

Transcriptome changes in muscle of Nellore cows submitted to recovery weight gain under grazing condition

D. A. Fausto[1], A. L. J. Ferraz[2], E. F. Delgado[1†], S. C. S. Andrade[1a], L. L. Coutinho[1] and G. L. D. Feijó[3]

[1]*Animal Science Department, 'Luiz de Queiroz' College of Agricultural/University of Sao Paulo, Brazil (11 Padua Dias Avenue, 13418 900 Piracicaba, Sao Paulo), Brazil;* [2]*Animal Science Department, Mato Grosso do Sul State University, Brazil (University City of Aquidauna, 79200-000), Brazil;* [3]*Beef Cattle Research Center/ Embrapa, Campo Grande, MS, Brazil (830 Rádio Maia, 79106-550, Campo Grande, Mato Grosso do Sul), Brazil*

(Received 27 June 2017; Accepted 22 May 2018; First published online 9 July 2018)

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. In all, 38 Nellore cull cows were divided randomly into two different management groups: (1) Maintenance (MA) and (2) Recovery gain (RG) from weight loss by moderate growth under high forage availability. After slaughter, RNA analysis was performed on the Longissimus thoracis muscle. Semaphorin 4A, solute carrier family 11 member 1, and Ficolin-2 were expressed in the RG, which may indicate an inflammatory response during tissue regrowth. Signaling factors, such as Myostatin, related to fibroblast activation, 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 were more abundant in the MA group was related to fat deposition. The 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: extracellular matrix, growth, inflammatory process, protein metabolism, proteases

Implications

There is a lack of information about the concerted changes related with muscle remodeling during recovery weight gain observed in 'realimentation' after undernutrition, which may be limited in old animals such as cull cows under grazing. The results showed changes during recovery gain with impact on few important genes involved in regulatory pathways of muscle growth, without affecting the major genes related to connective tissue when compared with fat animals. The latter presented upregulation of genes related to adipose tissue deposition. This is a contribution to scientific knowledge about muscle remodeling during recovery gain in mature animals. However, the transcriptome for proteases and related to collagen and other major extracellular matrix components was not affected during the recovery growth.

Introduction

There is growing interest in studying gene expression associated with muscle structural remodeling due to nutritional challenges (Byrne *et al*., 2005; Lee et al., 2002) to improve our understanding of the impact of these genes on the phenotypic characteristics of animals. Although animals from different breeds, including early- and late-maturing biological types, differ in tissue growth, carcass composition and marbling (Cuvelier *et al*., 2006), the changes in gene expression 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 those 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, IGF-I, which plays a role in protein synthesis (Ellenberger *et al*., 1989) and satellite cell proliferation and differentiation (Barton-Davis *et al*., 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

[a] Present address: Genetic and Evolutionary Biology Department, University de Sao Paulo (Matao street, 14, 05508-090, Sao Paulo, Sao Paulo), Brazil.
[†] E-mail: efdelgad@usp.br

IGF-independent mechanisms (Xi *et al*., 2006). Moreover, before muscle tissue renewal starts, an inflammatory response is stimulated, with invasion of macrophages followed by the formation of new myofibers and then remodeling (Ciciliot and Schiaffino, 2010). If these events are not coordinated, an accumulation of extracellular matrix occurs, causing fibrosis (Mann *et al*., 2011); this is more significant in adult animals. The remodeling is likely muscle-dependent as muscles respond differently to compensatory growth, with changes in collagen properties according to muscle responses to restricted feeding levels (Cassar-Malek *et al*., 2004).

In theory, diets that promote rapid growth can lead to increased rates of protein turnover, including turnover of collagen molecules (Archile-Contreras *et al*., 2010). This increase in collagen turnover has been observed during recovery gain after a period of BW loss. 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 extracellular matrix (ECM) renewal. The aim of this study was to evaluate transcriptome changes in the muscle of Nellore cull cows subjected to recovery weight gain under grazing conditions.

Material and methods

Treatments

All experimental procedures were approved by the environmental (CEAP; protocol no. 66) and animal (CEUA) ethical committees of the Luiz de Queiroz College of Agriculture, University of São Paulo. The experiment took place at the Brazilian Beef Cattle Research Center with 38 Nellore cull cows, aged 4 to 12 years, that were kept under grazing condition on pasture of *Brachiaria decumbens* grass, randomly divided into two groups based on body condition score (BCS): (1) Maintenance group (MA) – cows (age = 6.68 years ± 0.42; initial live weight = 490 kg ± 7.80) maintained at high BCS (initial = 8.42 ± 0.05) under grazing with high forage availability during the entire experimental period; and (2) Recovery gain group (RG) – cows (age = 7.28 ± 0.48; lLW = 411.42 ± 4.22) with low BCS (initial 5.05 ± 0.14) due to weight loss at overgrazed pasture during 129 days (pre-experimental period) followed by weight gain and BCS recovery under high forage availability. All the animals were given mineral supplementation.

The animals were serially slaughtered at the Meat Laboratory of the Beef Cattle Research Center/EMBRAPA in Campo Grande/MS – Brazil, starting after 36 days ($n = 10$; MA = 5 *v.* RG = 5) days into experimental conditions (grazing on pasture with high forage availability), followed by slaughters at 77 ($n = 10$; MA = 5 *v.* RG = 5), 139 ($n = 10$; MA = 5 *v.* RG = 5) and 173 ($n = 8$; MA = 4 *v.* RG = 4) days of high forage availability. The slaughter intervals were set to ~35 days, but also considered noticeable increment in BCS in the RG group (Figure 1). Immediately after slaughter, samples of *Longissimus thoracis* 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 that consistently changed in the growth curve during the recovery gain.

RNA extraction, library and sequencing

Extraction of RNA from *L. thoracis* 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, DE, 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 > 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 complementary DNA (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; Brea, California, United States).

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 PCR; only the fragments with adapters were selected and amplified using

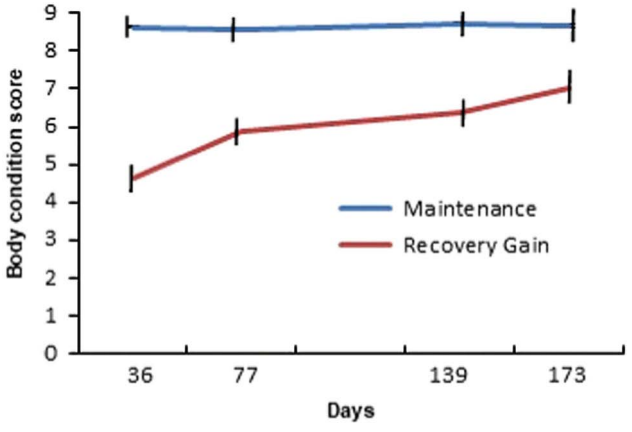

Figure 1 Body condition score (BCS) from mature Nellore cull cows under maintenance of high body score *v.* recovery gain at grazing conditions. Maintenance group: cows maintained at high body condition score (BCS > 8; 1 – being extremely thin, 4 – bone structure no longer noticeable and 9 – very fat) under grazing with high forage (*Brachiaria decumbens*) availability. Recovery gain group: cows with low body condition score (BCS ~ 5) due to weight loss under overgrazed pasture, followed by BCS recovery under high forage (*B. decumbens*) availability. The slaughters started after 36 days into experimental conditions (grazing on pasture with high forage availability). Bars indicate standard error.

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 as 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, CA, USA). 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.

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 *et al*., 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 and 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 *et al*., 2014).

Statistics

Differentially expressed genes between treatments (MA × RG) were identified using the DESeq2 package from R/Bioconductor (Love *et al*., 2014). The -in function 'estimate Size Factors' was used to obtain the normalized counts, that is, baseMean values, which are the number of reads divided by the size factor or normalization constant. Transcripts with baseMean < 5 considering all samples were removed to avoid artifacts due to low coverage. The method used to test for differential expression was the negative binomial distribution, followed by false discovery rate (Benjamini and Hochberg, 1995). A multifactor design was performed where the growth rate was the variable of interest, and the age was accounted as the controlled variable that might contribute to the changes on the treatment (the type variable in a design $=$ ~type + condition).

Enrichment analysis

The Functional Annotation Clustering function of the Database for Annotation, Visualization and Integrated Discovery (DAVID) v. 6.7 (Huang *et al*., 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 $\leq$ 0.09. Only genes that were differentially expressed (*P*-value ≤ 0.001 and *P*-value adjusted ≤ 0.2) were submitted to enrichment analysis.

Uncharacterized proteins, microRNA targets, and Ingenuity Pathway Analysis

The uncharacterized differentially expressed genes 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, CA, USA; 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).

Results

Identification of 13 145 genes in the muscle transcriptome was possible following quality filtering, alignment and normalization procedures. There were no changes in the expression of genes that have been reported playing major roles in the strength of the matrix, such as: *ADAM Metallopeptidase with Thrombospondin Type 1 Motif 2, Collagen type I α 2, Collagen type III α 1, Collagen type IV α 1, Collagen type IV α 2, Decorin, Elastin, Heparan sulfate proteoglycan core protein, Serine Peptidase Inhibitor, Kunitz Type 2, Serpin Family H Member 1, Matrix Metallopeptidase 2, Matrix Metalloproteinase-14, TIMP Metallopeptidase inhibitor 2* (Table 1). Those results indicate that components related to connective tissue scaffolding maybe were not modified.

Insulin-like growth factor binding protein 5 (IGFBP5), *Semaphorin 4A* (SEMA4A), *Ficolin-2* (FCN2), *Solute carrier family 11 member 1* (SLC11A1), *Nucleosome assembly protein 1 like 3, Unc-80 homolog, Nephrocan* (NEPN), *Calcium and integrin binding family member 2, Uncharacterized protein, Methyltransferase-like 7A* (METTL7A), *Placenta growth factor* (PGF), *Member RAS oncogene family, Kinesin family member 3C, Betacellulin, Integrator complex subunit 7, Trimethyllysine hydroxylase, epsilon* and *Myostatin* (MSTN), totaling seventeen genes, were differentially expressed (*P* adjusted < 0.2)(Table 2) and were grouped by biological function through functional annotation clustering analysis. The distribution of fold-change data of the genes expressed in both treatments was homogeneous. The uncharacterized protein was annotated by comparison with orthologous genes at BioMart Ensembl and identified as transcripts METTL7A.

In the group of differentially expressed genes annotated by Biomart Ensembl, the METTL7A gene was expressed in two

Table 1 *Major extracellular matrix-related candidate genes in* Longissimus thoracis *muscle from mature Nellore cull cows under maintenance*[1] *of high body condition score (BCS)* v. *recovery*[2] *gain at grazing conditions*

Genes	ID	Name	Base Mean	log2(FC)[3]	*P*-value	P_{adj} [4]
ADAMTS2	ENSBTAG00000014665	*ADAM Metallopeptidase With Thrombospondin Type 1 Motif 2*	86.474	0.1	0.718	0.9
COL1A2	ENSBTAG00000013472	*Collagen type I α 2*	1507.5	−0.1	0.993	0.9
COL3A1	ENSBTAG00000021466	*Collagen type III α 1*	2934.1	−0.1	0.966	0.9
COL4A1	ENSBTAG00000012849	*Collagen type IV α 1*	5880.2	0.1	0.476	0.9
COL4A2	ENSBTAG00000025210	*Collagen type IV α 2*	3351.4	0.1	0.503	0.9
DCN	ENSBTAG00000003505	*Decorin*	2546.5	0.4	0.214	0.9
ELASTIN	ENSBTAG00000019517	*Elastin*	433.35	0.1	0.762	0.9
HSPG2	ENSBTAG00000017122	*Heparan sulfate proteoglycan core protein*	2694.9	−0.1	0.963	0.9
SPINT2	ENSBTAG00000000182	*Serine Peptidase Inhibitor, Kunitz Type 2*	336.72	0.4	0.008	0.5
SERPINH1[5]	ENSBTAG00000001027	*Serpin Family H Member 1*	1304.2	0.1	0.735	0.9
MMP2	ENSBTAG00000019267	*Matrix Metallopeptidase 2*	604.3	0.5	0.048	0.8
MMP14	ENSBTAG00000014824	*Matrix metalloproteinase-14*	42.949	0.2	0.517	0.9
TIMP2	ENSBTAG00000010899	*TIMP metallopeptidase inhibitor 2*	488.1	0.1	0.560	0.9

[1]Maintenance group: cows maintained at high BCS (BCS > 8; 1 – being extremely thin, 4 – bone structure no longer noticeable and 9 – very fat) under grazing with high forage (*Brachiaria decumbens*) availability.
[2]Recovery gain group: cows with low BCS (BCS ~ 5) due to weight loss under overgrazed pasture, followed by BCS recovery under high forage (*B. decumbens*) availability.
[3]log2 (FC): log2(fold change); negative values: upregulation in maintenance; positive values: upregulation in recovery gain.
[4]P_{adj}: adjusted *P*-value for multiple testing with the Benjamini–Hochberg procedure (false discovery rate).
[5]HSP47.

Table 2 *Differentially genes expressed*[1] *in* Longissimus thoracis *muscle from mature Nellore cull cows under maintenance*[2] *of high body condition score (BCS)* v. *recovery gain*[3] *at grazing conditions*

Genes	ID	Name	BaseMean	log2(FC)[4]	*P*-value	P_{adj} [5]
IGFBP5	ENSBTAG00000007062	*IGF binding protein 5*	144.26	−1.5	< 0.0001	0.1
SEMA4A	ENSBTAG00000012228	*Semaphorin 4A*	10.00	1.3	< 0.0001	0.1
FCN2	ENSBTAG00000048155	*Ficolin-2*	8.84	2.1	< 0.0001	0.1
SLC11A1	ENSBTAG00000015520	*Solute carrier family 11 member 1*	14.13	1.5	< 0.0001	0.1
NAP1L3	ENSBTAG00000019407	*Nucleosome assembly protein 1 like 3*	7.39	−1.4	< 0.0001	0.2
UNC80	ENSBTAG00000015415	*Unc-80 homolog*	8.71	−2.2	0.0001	0.2
NEPN	ENSBTAG00000039574	*Nephrocan*	38.94	−1.7	0.0002	0.2
CIB2	ENSBTAG00000010981	*Calcium and integrin binding family member 2*	52.88	−1.3	0.0001	0.2
[6]U. PROTEIN	ENSBTAG00000032899	*Uncharacterized protein*	44.35	−1.3	0.0002	0.2
METTL7A	ENSBTAG00000025005	*Methyltransferase-like 7A*	337.68	−1.0	0.0001	0.2
PGF	ENSBTAG00000013688	*Placenta growth factor*	112.45	1.0	0.0001	0.2
RAB15	ENSBTAG00000003474	*Member RAS oncogene family*	37.00	1.2	0.0002	0.2
KIF3C	ENSBTAG00000019138	*Kinesin family member 3C*	33.57	−1.1	0.0002	0.2
BTC	ENSBTAG00000004237	*Betacellulin*	877.91	−0.6	0.0002	0.2
INTS7	ENSBTAG00000018548	*Integrator complex subunit 7*	172.50	0.5	0.0002	0.2
TMLHE	ENSBTAG00000011648	*Trimethyllysine hydroxylase, epsilon*	25.50	−0.8	0.0003	0.2
MSTN	ENSBTAG00000011808	*Myostatin*	86.34	−1.8	0.0003	0.2

[1]Annotated by BioMart Ensembl.
[2]Maintenance group: cows maintained at high BCS (BCS > 8; 1 – being extremely thin, 4 – bone structure no longer noticeable and 9 – very fat) under grazing with high forage (*Brachiaria decumbens*) availability.
[3]Recovery gain group: cows with low BCS (BCS ~ 5) due to weight loss under overgrazed pasture, followed by BCS recovery under high forage (*B. decumbens*) availability.
[4]log2 (FC): log2(fold change); negative values: upregulation in maintenance group; positive values: upregulation in recovery gain group.
[5]P_{adj}: adjusted *P*-value for multiple testing with the Benjamini–Hochberg procedure (false discovery rate).
[6]U. protein – uncharacterized protein.

transcripts in the MA group. A greater abundance of MSTN transcripts, as well as IGFBP5 (Table 2), another protein related to growth factors, were also observed in the MA group. Transcripts for SEMA4A, Slc11A1, PGF and FCN2 were more expressed in the RG group (Table 3).

The enrichment analysis from DAVID Functional Annotation showed group functionally related to extracellular space (GOTERM_cellular component_ALL) and disulfide bond functional (Single protein of protein information resource_ KEYWORDS) (Table 3). Others cluster that were not significant

Table 3 *Functional Annotation Clustering*[1] *of genes with main cellular, molecular and biological processes identified in* Longissimus thoracis *muscle from mature Nellore cull cows under maintenance*[2] *of high body condition score (BCS)* v. *recovery gain*[3] *at grazing conditions*

Annotation clusters	Representative annotation terms	Count	*P*-value	P_{adj}[4]
GOTERM_CC_ALL	Extracellular space	4	0.003	0.09
GOTERM_CC_ALL	Extracellular region part	4	0.011	0.20
GOTERM_CC_ALL	Extracellular region	6	0.002	0.12
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
SP_PIR_KEYWORDS	Secreted	5	0.008	0.12
SP_PIR_KEYWORDS	Growth factor	3	0.004	0.16
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

CC = cellular component; SP_PIR = single protein of protein information resource; MF = molecular function; BP = biological process.
[1]Analyzed by DAVID tool (annotation clusters had a group of enrichment scores of 1.82).
[2]Maintenance group: cows maintained at high BCS (BCS > 8; 1 – being extremely thin, 4 – bone structure no longer noticeable and 9 – very fat) under grazing with high forage (*Brachiaria decumbens*) availability.
[3]Recovery gain group: cows with low BCS (BCS ~ 5) due to weight loss under overgrazed pasture, followed by BCS recovery under high forage (*B. decumbens*) availability.
[4]P_{adj}: adjusted *P*-value for multiple testing with the Benjamini–Hochberg procedure (false discovery rate).

also appears as Signal (Single protein of protein information resource_KEYWORDS), Glycoprotein (Single protein of protein information resource_KEYWORDS), Extracellular region (GOTERM_cellular component_ALL), Secreted (Single protein of protein information resource_KEYWORDS), Growth factor (Single protein of protein information resource_KEYWORDS), Extracellular region part (GOTERM_ cellular component_ALL), Growth factor activity (GOTERM_molecular function_ALL), Receptor binding (GOTERM_molecular function_ALL), Protein binding (GOTERM_molecular function_ALL), Positive regulation of biological process (GOTERM_biological process_ALL), Biological regulation (GOTERM_biological process_ALL), Regulation of cellular process (GOTERM_biological process_ALL), Regulation of biological process (GOTERM_biological process_ALL), Positive regulation of cellular process (GOTERM_BP_ALL) (Table 3). In the RG group, FCN2 genes were clustered in the extracellular space and the disulfide bond cluster. 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.

Recent advances allowed the understanding of the gene expression regulation using QIAGEN's Ingenuity Pathway Analysis, which resulted in the identification of upstream regulators genes controlling upregulated expression in RG group as follow: *Recombination signal binding protein for immunoglobulin kappa J region*, *Sonic hedgehog*, *Endothelial PAS domain protein 1*, *Metal-responsive transcription factor-1*, *Distal-Less Homeobox 3*, *Forkhead Box D1*, *Hypoxia inducible factor-1α*, miR-182, miR-182-5p, *Angiotensinogen* (AGT), *Oncostatin M* (OSM), *c-Harvey-ras*, *SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4* (SMARCA4), *Tumor necrosis factor* (TNF (family)), *Protein inhibitor of activated STAT-2*, *Activating transcription factor 3*, *Chemokine ligand 2* (CCL2), *Interleukin* (IL6) (Table 4). Those genes were grouped in molecule type as a Transcription regulator, Peptidase, microRNA, Mature microRNA, Growth factor, Cytokine and Enzyme. One group of cytokines, represented by OSM, CCL2 and IL6 (Table 4), were identified as upstream regulators of SEMA4A, PGF and SLC11A1 in the RG group and the IGF-I was the upstream gene regulating the greater expression of IGFBP5 and MSTN ($P = 0.023$) in the MA cows. This may indicate the idea that IGF-I, IGFBP5 and MSTN regulated muscle gain in those animals.

We observed two MicroRNAs (miR-182-5p and miR-182, Table 4), that are small noncoding RNA, as upstream regulator genes. The miR-182-5p (Table 4), was found as an upstream regulator of the PGF gene. This molecule has 345 predicted target transcripts with a total of 354 conserved sites and 72 poorly conserved sites. The other microRNA found (miR-182, Table 4) had 100 predicted target transcripts with sites, comprising 93 conserved sites and 112 poorly conserved sites.

Discussion

The METTL7A gene that was more expressed in the MA group is related to lipid metabolism (Brasaemle and Wolins, 2012), specifically to lipid droplet formation (Bouchoux *et al.*, 2011). This is to be expected as those animals presented high

Table 4 *Upstream regulators[1] of upregulated genes expressed in* Longissimus thoracis *muscle from mature Nellore cull cows under maintenance[2] of high body condition score (BCS)* v. *recovery gain[3] at grazing conditions*

Upstream regulators	Gene name	Molecule type	*P*-value	Target molecules
RBPJ	*Recombination signal binding protein for immunoglobulin kappa J region*	Transcription regulator	0.0028	PGF
SHH	*Sonic hedgehog*	Peptidase	0.0045	PGF
EPAS1	*Endothelial PAS domain protein 1*	Transcription regulator	0.0054	PGF
MTF1	*Metal-responsive transcription factor-1*	Transcription regulator	0.0061	PGF
DLX3	*Distal-Less Homeobox 3*	Transcription regulator	0.0104	PGF
FOXD1	*Forkhead Box D1*	Transcription regulator	0.0067	PGF
HIF1A	*Hypoxia-inducible factor-1α*	Transcription regulator	0.0192	PGF
miR-182	miR-182	microRNA	0.0242	PGF
miR-182-5p	miR-182-5p	Mature microRNA	0.0218	PGF
AGT (SerpinA8)	*Angiotensinogen*	growth factor	0.0279	PGF
HRAS	*c-Harvey-ras*	Enzyme	0.0439	PGF
OSM	*Oncostatin M*	Cytokine	0.0393	PGF
IL6	*Interleukin*	Cytokine	0.0076	SEMA4A
CCL2	*Chemokine ligand 2*	Cytokine	0.0432	SLC11A1
SMARCA4	*SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4*	Transcription regulator	0.0150	SLC11A1
PIAS2	*Protein inhibitor of activated STAT-2*	Transcription regulator	0.0061	SLC11A1
ATF3	*Activating transcription factor 3*	Transcription regulator	0.0391	SLC11A1
TNF (family)	*Tumor necrosis factor*	Group	0.0048	SLC11A1

[1]Genes regulators identified by Analysis by Ingenuity Pathway Analysis involved in upregulation of genes identified in the recovery gain treatment.
[2]Maintenance group: cows maintained at high BCS (BCS >8; 1 – being extremely thin, 4 – bone structure no longer noticeable and 9 – very fat) under grazing with high forage (*Brachiaria decumbens*) availability.
[3]Recovery gain group: cows with low BCS (BCS ~ 5) due to weight loss under overgrazed pasture, followed by BCS recovery under high forage (*B. decumbens*) availability.

BCS associated with slight increase in LW during the experiment, which points to increased adiposity in the muscle with cessation of muscle cell growth. The latter process may be regulated by greater IGFBP5 expression in the MA cows, which would link to a decline in protein synthesis, considering that IGFBP5 is an important regulator of IGF-I local action (Jackman and Kandarian, 2004) by sequestering this growth factor and affecting protein accretion (Baxter, 2000). Nonetheless, IGFBP-5 may have effects that are unrelated to its IGF-regulatory role (Tripathi *et al*., 2009).

Moreover, MSTN transcripts (Table 2) presenting greater abundance in the MA group and is considered unique negative regulator of muscle mass (Rodgers and Garikipati 2008). 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 enabling muscle adaptation 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.

The extracellular tissue remodeling in the RG cows involved genes (SEMA4A and SLC11A1) related to immune response and defense. 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*., 2005), an important pathway related to renewal. SLC11A1 is related to immune response and defense (Ding *et al*., 2014). Furthermore, greater PGF expression may indicate macrophage secretory and physiological function as well as stimulation of angiogenesis in the RG group. This gene was related to the inflammatory process (Claus *et al*., 1996) and angiogenesis in muscle (Viita *et al*., 2008). In summary, those results together points to an increase in inflammatory responses 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). These may present a concerted response that depends largely on macrophage secretion, which is the first stage of tissue remodeling (Ciciliot and Schiaffino, 2010). For tissue renewal to occur, the migration and proliferation of myofibroblasts are important to produce current ECM (Mann *et al*., 2011).

The identified increase in FCN2 expression would be part of connective tissue remodeling program in the RG cows as this protein is related to soluble collagen-like proteins (Garred *et al*., 2009). Those molecules are identified in apoptosis of myofibroblasts, endothelial cells, and macrophages (Jensen *et al*., 2007), which are part of renewal process in extracellular muscle tissue.

Although we did not observe changes in the major components of connective tissue, there are indications that renewal is marked by inflammatory response while the other repair states were not present during the whole recovery period and remodeling of the regenerated muscle tissue in RG cows. Probably alterations in the extracellular

components that would elicit, for instance, changes in collagen properties would be related to high extracellular matrix turnover only observed in greater growth rates at younger age as was reported in negative correlation between average daily gain and total collagen or heat soluble collagen in *Longissimus* muscle from pasture fed beef cattle (Archile-Contreras *et al*., 2010). Moreover, lower MSTN expression in RG cows may be related with regulation of fibroblast, as myostatin is involved in fibroblast activation and fibrosis (i.e. progressive deposition of collagen and other extracellular matrix proteins such as fibronectin and vimentin) both *in vivo* and *in vitro* (Li *et al*., 2008). Although myostatin was able to stimulate procollagen (Type I and Type III) mRNA and fibronectin protein expressions in mice skeletal muscle, MSTN expression is reduced during muscle repair from injury (Zhu *et al*., 2007). Moreover, those authors showed that myostatin knockout mice had fewer fibrotic connective tissue deposits with the smaller area between regenerating myofiber in injured muscle. This could be the action of cytokines regulating gene expression in ECM connective tissue by binding to specific receptors on the surface of fibroblasts (Crombrugghe *et al*., 1990).

The genes related to immune and inflammatory responses allowed the identification of cytokines (Table 4) that are upstream regulators of those genes, indicating that these cytokines may have acted at some point during recovery growth. It could be the case of observed upregulated PGF in RG group as a response to Oncostatin. This cytokine was responsible for increased levels of PGF mRNA and protein in *rheumatoid arthritis* synovial fibroblast (Tu *et al*., 2013). The upstream regulator gene *Oncostatin* is involved with the proliferation of fibroblasts (Ihn and Tamaki, 2000) and production of collagen and glycosaminoglycan Another identified upstream regulator gene, IL-6 contributes to overall inflammation and subsequently to fibrosis (Atamas and White, 2003). Tumor necrosis factor activated macrophages that appear to be key players in pathologic processes that are associated with fibrosis (Song *et al*., 2000) because they are activated by profibrotic factors. It demonstrates that the renewal may had favored some fibrosis, but it was not constant throughout the experiment to elicit any changes in ECM gene expression.

Although identified as upstream regulatory elements, as the cytokines discussed before, it is worth mentioning that miR-182-5p (Table 4) is involved in processes related to tissue remodeling, such as membrane protein ectodomain proteolysis, membrane protein proteolysis as well as in the cell cycle and apoptosis (Krishnan *et al*., 2013). The other microRNA identified (miR-182) as an upstream regulator of PGF, also regulates the activity of the MMP-2 and MMP-9 extracellular proteases (Sachdeva *et al*., 2014).

Conclusion

We concluded that recovery gain resulted in changes in genes associated with growth factors that are involved in the control of muscle and fibroblast cell proliferation and protein synthesis. However, the transcriptome for proteases possibly involved in muscle protein turnover and related to collagen and other major extracellular matrix components were not affected during long-term moderate recovery gain in mature bovine females under grazing condition.

Acknowledgments

This work would have not been possible without the partnership of the Genomics Center at the University of Sao Paulo/ESALQ, Piracicaba, São Paulo, Brazil and the National Beef Cattle Research Center/EMBRAPA, and funding from FAPESP 13-11772-4. LLC is a recipient of CNPq research productivity scholarship.

Declaration of interest

The authors declare that they have no conflicts of interest.

Ethics statement

The environmental and animal ethics committees approved the protocols used in this work.

Software and data repository resources

The data presented here are not deposited in any official repository.

References

Anders S and Huber W 2010. Differential expression analysis for sequences count data. Genome Biology 11, R106.1–R.106.

Archile-Contreras A, Mandell IB and PURSLOW PP 2010. Disparity of dietary effects on collagen characteristics and toughness between two beef muscles. Meat Science 86, 491–497.

Atamas SP and White B 2003. Cytokine regulation of pulmonary fibrosis in scleroderma. Cytokine & Growth Factor Reviews 14, 537–550.

Baxter RC 2000. Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. American Journal of Physiology: Endocrinology and Metabolism 278, E967–E976.

Barton-Davis ER, Shoturma DI and Sweeney HL 1999. Contribution of satellite cells to IGF-I induced hypertrophy of skeletal muscle. Acta Physiologica 167, 301–305.

Benjamini Y and Hochberg Y 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B 57, 289–300.

Bouchoux J, Beilstein F, Pauquai T, Guerrera C, Chateau D, Ly N, Alqub M, Klein C, Chambaz J, Rousset M, Lacorte J, Morel E and Demignot S 2011. The proteome of cytosolic lipid droplets isolated from differentiated Caco-2/TC7 enterocytes reveals cell-specific characteristics. Biology of the Cell 103, 499–517.

Brasaemle DL and Wolins NE 2012. Packaging of fat: an evolving model of lipid droplet assembly and expansion. Journal of Biological Chemistry 287, 2273–2279.

Byrne KA, Wang YH, Lehnert SA, Harper GS, Mcwilliam SM, Bruce HL and Reverter A 2005. Gene expression profiling of muscle tissue in Brahman steers during nutritional restriction. Journal of Animal Science 83, 1–12.

Cassar-Malek I, Hocquette JF, Jurie C, Listrat A, Jailler R, Bauchart D, Briand Y and Picard B 2004. Muscle-specific metabolic, histochemical and biochemical responses to a nutritionally induced discontinuous growth path. Animal Science 79, 49–59.

Ciciliot S and Schiaffino S 2010. Regeneration of mammalian skeletal muscle. Basic mechanisms and clinical implications. Current Pharmaceutical Design 16, 906–914.

Claus M, Weich H, Breier G, Knies U, Rockl W, Waltenberger J and Risau W 1996. The vascular endothelial growth factor receptor flt-1 mediates biological activities. The Journal of Biological Chemistry 271, 17629–17634.

Clemmons DR 1998. Role of insulin like growth factor binding proteins in controlling IGF action. Molecular and Cellular Endocrinology 140, 19–24.

Crombrugghe B, Vuorio T and Karsenty G 1990. Control of type I collagen genes in scleroderma and normal fibroblasts. Rheumatic Diseases Clinics of North America 16, 109–123.

Cuvelier C, Cabaraux JF, Dufrasne I, Clinquart A, Hocquette JF, Istasse L and Hornick JL 2006. Performance, slaughter characteristics and meat quality of young bulls from Belgian Blue, Limousin and Aberdeen Angus breeds fattened with a sugar-beet pulp or a cereal-based diet. Animal Science 82, 125–132.

Ding X, Zhang X, Yang Y, Ding Y, Xue W, Meng Y, Zhu W and Yin Z 2014. Polymorphism, expression of natural resistance-associated macrophage protein 1 encoding gene (NRAMP1) and its association with immune traits in pigs. Asian-Australasian Journal of Animal Sciences 27, 1189–1195.

Ellenberger MA, Johnson DE, Carstens GE, Hossner KL, Holland MD, Nett TM and Nockels CF 1989. Endocrine and metabolic changes during altered growth rates in beef cattle. Journal of Animal Science 67, 1446–1454.

Fielding RA, Manfredi TJ, Ding W, Fiatarone MA, Evans WJ and Cannon JG 1993. Acute phase response in exercise. III. Neutrophil and IL-1 beta accumulation in keletal muscle. The American Journal of Physiology 265, R166–R172.

Fry CS, Lee JD, Jackson JR, Kirby TJ, Stasko SA, Liu H, Dupont-Versteegden EE, Mccarthy JJ and Peterson CA 2014. Regulation of the muscle fiber microenvironment by activated satellite cells during hypertrophy. The FASEB Journal 28, 1654–1665.

Garred P, Honoré C, Ma YJ, Munthe-fog L and Hummelshoj T 2009. MBL2, FCN1, FCN2 and FCN3: the genes behind the initiation of the lectin pathway of complement. Molecular Immunology 46, 2737–2744.

Huang DW, Sherman BT and Lempicki RA 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocols 4, 44–57.

Ihn H and Tamaki K 2000. Oncostatin M stimulates the growth of dermal fibroblasts via a mitogen-activated protein kinase-dependent pathway. J Immunol 165, 2149–2155.

Jackman RW and Kandarian SC 2004. The molecular basis of skeletal muscle atrophy. American Journal of Physiology – Cell Physiology 287, C834–C843.

Jensen ML, Honore C, Hummelshoj T, Hansen BE, Madsen HO and Garred P 2007. Ficolin-2 recognizes DNA and participates in the clearance of dying host cells. Molecular Immunology 44, 856–865.

Krishnan K, Steptoe AL, Martin HC, Wani S, Nones K, Waddell N, Mariasegaram M, Simpson PT, Lakhani SR, Gabrielli B, Vlassov A, Cloonan N and Grimmond SM 2013. MicroRNA-182-5p targets a network of genes involved in DNA repair. RNA 19, 230–242.

Kumanogoh A, Shikina T, Suzuki K, Uematsu S, Yukawa K, Kashiwamura S, Tsutsui H, Yamamoto M, Takamatsu H, Komitamura EP, Takegahara N, Marukawa S, Ishida I, Morishita H, Prasad DV, Tamura M, Mizui M, Toyofuku T, Akira S, Takeda K, Okabe M and Kikutani H 2005. Non redundant roles of Sema4A in the immune system: defective T cell priming and Th1/Th2 regulation in Sema4A-deficient mice. Immunity 22, 305–316.

Langmead B, Trapnell C, Pop M and Salzberg Sl 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biology 10, 1–25.

Lee SH, Engle TE and Hossner KL 2002. Effects of dietary copper on the expression of lipogenic genes and metabolic hormones in steers. Journal of Animal Science 80, 1999–2005.

Lee HG, Hidari H, Kang SK, Hong ZS, Xu CX, Kim SH, Seo KS, Yoon DH and Choi YJ 2005. The relationships between plasma insulin-like growth factor (IGF)-1 and IGF-binding proteins (IGFBPs) to growth pattern, and characteristics of plasma IGFBPs in steers. Asian Australasian Journal of Animal Sciences, Gwanak-gu 18, 1575–1581.

Li ZB, Kollias HD and Wagner KR 2008. Myostatin directly regulates skeletal muscle fibrosis. Journal of Biological Chemistry 283, 19371–19378.

Love MI, Huber W and Anders S 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15, 550–571.

Mann CJ, Perdiguero E, Kharraz Y, Aguilar S, Pessina P, Serrano AL and Muñoz-Cánoves P 2011. Aberrant repair and fibrosis development in skeletal muscle. Skeletal Muscle 1, 21.

Meda C, Molla F, Pizzol M, Regano D, Maione F, Capano S, Locati M, Mantovani A, Latini R, Bussolino F and Giraudo E 2012. Semaphorin 4A exerts a proangiogenic effect by enhancing vascular endothelial growth factor – a expression in macrophages. The Journal of Immunology 188, 4081–4092.

Murphy MM, Lawson JA, Mathew SJ, Hutcheson DA and Kardon G 2011. Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. Development 138, 3625–3637.

Rios R, Carneiro I, Arce VM and Devesa J 2002. Myostatin is an inhibitor of myogenic differentiation. American Journal of Physiology – Cell Physiology 282, C993–C999.

Rodgers BD and Garikipati DK 2008. Clinical, agricultural, and evolutionary biology of myostatin: a comparative review. Endocrine Reviews 29, 513–534.

Roth L, Koncina E, Satkauskas S, Crémel G, Aunis D and Bagnard D 2009. The many faces of semaphorins: from development to pathology. Cellular and Molecular Life Sciences 66, 649–666.

Sachdeva M, Jeffrey KM, Lee CL, Zhang M, Li Z, Dodd RD, Cason D, Luo L, Ma Y, Mater DY, Gladdy R, Lev DC, Cardona DM and Kirsch DG 2014. MicroRNA-182 drives metastasis of primary sarcomas by targeting multiple genes. The Journal of Clinical Investigation 124, 4305–4319.

Song E, Ouyang N, Horbelt M, Antus B, Wang M and Exton MS 2000. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. Cellular Immunology 204, 19–28.

Trapnell C, Pachter L and Salzberg SL 2009. TopHat: discovering splice junctions with RNA-seq. Bioinformatics 25, 1105–1111.

Tripathi G, Salih DAM, Drozd AC, Cosgrove RA, Cobb LJ and Pell JM 2009. IGF-independent effects of insulin-like growth factor binding protein-5 (Igfbp5) in vivo. The FASEB Journal 23, 2616–2626.

Tu HJ, Lin TH, Chiu YC, Tang CH, Yang RS and Fu WM 2013. Enhancement of placenta growth factor expression by Oncostatin m in human rheumatoid arthritis synovial fibroblasts. Journal of Cellular Physiology 228, 983–990.

Viita H, Markkanen J, Eriksson E, Nurminen M, Kinnunen K, Babu M, Heikura T, Turpeinen S, Laidinen S, Takalo T and Ylä-Herttuala S 2008. 5-lipoxygenase-1 prevents vascular endothelial growth factor A- and placental growth factor-induced angiogenic effects in rabbit skeletal muscles via reduction in growth factor mRNA levels, NO bioactivity, and downregulation of VEGF receptor 2 expression. Circulation Research 102, 177–184.

Xi G, Kamanga-Sollo E, Hathaway MR, Dayton WR and White ME 2006. Effect of constitutive expression of porcine IGFBP-3 on proliferation and differentiation of L6 myogenic cells. Domestic Animal Endocrinology 31, 35–51.

Zhu J, Li Y, Shen W, Qiao C, Ambrosio F, Lavasani M, Nozaki M, Branca MF and Huard J 2007. Relationships between Transforming Growth Factor-β 1, Myostatin, and Decorin: implications for skeletal muscle fibrosis. The Journal of Biological Chemistry 282, 25852–25863.