Published September 8, 2015

Coordinated gene expression between skeletal muscle and intramuscular adipose tissue in growing beef cattle[1]

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ABSTRACT: Previous research indicates that metabolism and fiber type of skeletal muscle is related to intramuscular lipid content. It is hypothesized that changes in skeletal muscle gene expression influence adipose tissue development. The objective of this study was to determine differences in the metabolism and intercellular signaling of skeletal muscle fibers within the same muscle group that could be responsible for the initiation of intramuscular adipose tissue development and differentiation. Longissimus dorsi muscle samples were collected from steers (n = 12; 385 d of age; 378 kg BW) grazing wheat pasture. Longissimus muscle samples were dissected under magnification and sorted into 3 categories based on visual stage of adipose tissue development: immature intramuscular adipose tissue (MM), intermediate intramuscular adipose tissue (ME), and mature intramuscular adipose tissue (MA). Additionally, muscle fibers lying adjacent to each intramuscular adipose tissue (IM) category and those not associated with IM tissue were collected and stored separately. Quantitative real-time PCR was used to determine relative fold change in genes involved in metabolism, angiogenesis, formation of extracellular matrix, and intercellular signaling pathways in both LM and IM samples. Gene expression data were analyzed using a GLM that included the fixed effect of tissue. Pearson correlation coefficients were also computed between gene expression in LM and IM tissue samples that were at the same stage of development. *Fatty acid binding protein 4* and *peroxisome proliferator-activated receptor* γ mRNA expression were 3.56- and 1.97-fold greater ($P < 0.05$) in ME and MA IM compared with MM IM whereas *delta-like 1 homolog* mRNA expression was 1.43-fold less ($P < 0.01$) in MA IM compared with MM IM, indicating successful separation into different development categories. Genes associated with metabolism and angiogenesis in LM tissue showed no differences among stages of development. Myostatin expression did not change in LM tissue; however, expression of *myostatin receptor* and *follistatin* mRNA decreased ($P < 0.01$) as IM matured. *Collagen type I* and *collagen type VI* mRNA expression were 2.5- and 1.32-fold greater in LM associated with MM IM than in LM associated with ME IM. Angiogenic growth factors in MM IM tissue had a strong positive correlation ($r \geq 0.69$) with angiogenic growth factors in LM associated with MM IM; however, no correlation was observed in ME or MA IM. These data indicate a coordinated effort between LM and IM in early stages of IM development.

Key words: gene expression, intercellular signaling, intramuscular fat

 J. Anim. Sci. 2015.93:4302–4311
doi:10.2527/jas2015-8886

INTRODUCTION

Narrow profit margins in animal agriculture have made improving the efficiency of animal growth an important focus. When cattle are marketed on a carcass basis rather than on a live basis, producers receive premiums for carcasses with above-average intramuscular fat (**IM**) and minimal subcutaneous fat. Therefore, understanding the mechanisms regulating intramuscular adipose tissue development is economically important.

[1]This project was supported by the Beef Checkoff, the Agriculture and Food Research Initiative Special Grants no. 2009-34198-19830 and 2010-34198-20812, and the Agriculture and Food Research Initiative Grant no. 2010-65206-20605 from the USDA National Institute of Food and Agriculture

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Received January 7, 2015.
Accepted June 19, 2015.

Some studies have shown that the type of growing program affects body composition and feedlot performance of beef cattle (Carstens et al., 1991; Drouillard et al., 1991; Hersom et al., 2004). Data collected from the Vetlife Benchmark Performance database reported a slight decline in USDA Choice carcasses since 1999, despite an increase in USDA yield grades of 4 or 5 (Anderson and Gleghorn, 2007). These data indicate that carcasses are reaching a fatter end point without an increase in quality grade.

Meeting the challenge of increasing meat quality will require a comprehensive knowledge of gene expression changes that occur between muscle and IM. Development of intramuscular adipose tissue occurs within the perimysium of muscle bundles. Because intramuscular adipose tissue develops within close proximity to muscle fibers, it is believed that signaling between myogenic cells and adipocytes may influence IM development (Kokta et al., 2004). Intramuscular adipose tissue develops in close association with capillary networks (Harper and Pethick, 2004). Hausman and Richardson (2004) reported that angiogenesis is an important aspect of adipose tissue development and that differentiation of adipocytes may be regulated by factors that stimulate the formation of blood vessels.

The hypothesis for the current study was that LM in close proximity to IM will have elevated expression of growth and angiogenic factors when compared with LM not in close proximity to IM that corresponds with increased development of IM in that specific location within the muscle. The objective was to determine the expression of genes involved in intercellular signaling pathways and angiogenesis in skeletal muscle and intramuscular adipose tissue at different stages of development.

MATERIALS AND METHODS

Animals and Treatments

Before the initiation of these studies, all procedures for animal care, handling, and sampling were approved by the Oklahoma State University Institutional Animal Care and Use Committee (protocols AG-50-372 and AG-09-15). Twelve Angus steers from the Range Cow Research Center, South Range Unit, near Stillwater, OK, were used for this study. Details regarding cattle and pasture management and treatments were reported by Sharman et al. (2013). Tissue samples used in this experiment were harvested from steers grazing winter wheat pasture at a high stocking rate (3.21 steers/ha) to achieve a low rate of BW gain and steers grazing wheat pasture at a low stocking rate (0.99 steers/ha) to achieve a high rate of BW gain. Samples were collected from steers harvested at the first intermediate harvest in Exp. 1 (n = 3 per treatment) and the intermediate harvest in Exp. 2 (n = 3 per treatment). Experiments 1 and 2 were conducted in 2 consecutive years and steers were harvested at 2 different endpoints. Steers from Exp 1. were harvested at a common age, and steers from Exp. 2 were harvested at similar BW.

Tissue Collection

Longissimus muscle samples were collected from steers following hide removal as described by Lancaster et al. (2014) and stored at –80°C in RNAlater (Invitrogen, Life Technologies, Grand Island, NY). Dissection of LM tissue samples occurred after being frozen in RNAlater. During the duration of dissection, thawed tissue samples were stored on ice and in RNAlater. Small pieces (approximately 1 by 1 by 1 cm) of LM samples were evaluated under magnification (0.9–4x; model SZ30 binocular dissecting microscope; Olympus Corp., New York, NY) and dissected according to the maturity of the intramuscular adipose tissue within steer. Intramuscular adipose tissue was sorted based on visual assessment (Fig. 1) into 1 of 3 categories: immature intramuscular adipose tissue (**MM**), intermediate intramuscular adipose tissue (**ME**), and mature intramuscular adipose tissue (**MA**). The following criteria were used to define IM categories: immature IM consisted of primarily loose connective tissue with

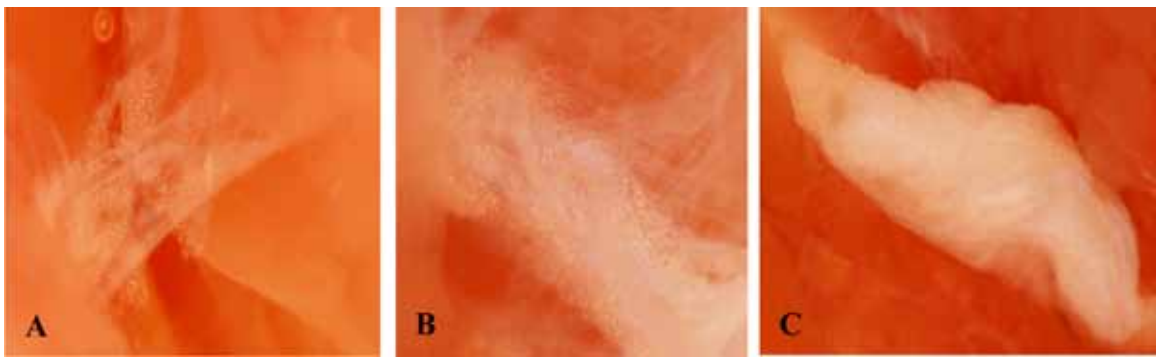

Figure 1. Photographs representing the different stages of intramuscular adipose tissue development using a dissecting microscope and camera: A) immature stage, B) intermediate stage, and C) mature stage of development of intramuscular tissue between muscle bundles.

a few visible fat adipocytes, intermediate IM consisted of clumps of fat adipocytes without visible vasculature, and mature IM consisted of densely packed adipocytes with highly visible vasculature. Intramuscular adipose tissue was removed from muscle tissue and stored separately according to development category. Muscle fibers lying immediately adjacent to intramuscular deposits were collected and stored separately according to the development category of the IM with which they were associated. The remaining muscle tissue of the small piece was discarded. Muscle tissue from small pieces devoid of any IM was also collected and stored separately as muscle fibers not associated with any intramuscular adipose tissue.

Ribonucleic Acid Extraction

Total RNA was isolated from muscle and adipose tissues using TRIzol reagent (Ambion, Life Technologies, Grand Island, NY) following the manufacturer's procedures. Following RNA isolation, a cleanup procedure was used to remove additional fat from the sample and removal of any carryover guanidine isothiocyanate from the TRIzol procedure by adding chloroform (1:1 ratio) to isolated RNA. Mixture was centrifuged at 4°C for 5 min at 20,000 × *g*. The upper aqueous phase was removed and transferred to a fresh tube and then phenol:chloroform:isoamyl alcohol (25:24:1) was added in a 1:1:1 ratio. The mixture was centrifuged at 4°C for 5 min at 20,000 × *g*. The upper aqueous phase was transferred to a fresh tube and 100% ethyl alcohol was added (2.5:1 ratio) along with 3 *M* sodium acetate (pH 5.2; 0.1:1 ratio) and incubated for 60 min at –80°C. Following incubation, RNA was centrifuged at 4°C for 30 min at 20,000 × *g* and the supernatant was discarded. The RNA pellet was washed with 75% ethyl alcohol and allowed to air dry before resuspension in diethylpyrocarbonate-treated water and stored at –80°C. The quantity of RNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA integrity was determined using gel electrophoresis.

Total RNA (1.0 μg) was used to synthesize cDNA using a reverse transcription kit containing genomic DNA wipeout buffer (QuantiTect; Qiagen Inc., Valencia, CA). Following reverse transcription and amplification, samples were quantified using a NanoDrop ND-1000 spectrophotometer. Complementary DNA samples were stored at –20°C until gene expression analysis was performed.

Quantitative Real-Time PCR Protocol

Quantitative real-time PCR (**qRT-PCR**) was used to determine the mRNA expression level of specific genes of interest. Gene-specific primers were designed using exonic sequences obtained from the National Center for Biotechnology Information using a Primer3 software package (Rozen and Skaletsky, 1999). When possible, primers were designed to be intron spanning to prevent amplification of contaminating genomic DNA. Specificity of each primer set was evaluated by comparing primer sequences to the GenBank database using the blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Complementary forward and reverse primer sequences for each primer pair were evaluated with the OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA). Primers used in muscle tissue are listed in Table 1 and primers used in intramuscular adipose tissue are listed in Table 2. A Bio-Rad CFX96 real-time detection system (Bio-Rad Laboratories, Hercules, CA) was used to quantify mRNA abundance. For both tissue types, qRT-PCR reactions contained 7.5 μL of RT^2 Fast SYBR Green Supermix for iQ (SABiosciences, Frederick, MD), 0.25 μL of 25 μ*M* forward primer (400 n*M*), 0.25 μL of 25 μ*M* reverse primer (400 n*M*), and 100 ng of cDNA and were performed using a MyiQ Real Time PCR detection system (Bio-Rad Laboratories). Thermal cycling parameters were 95°C for 5 min followed by 40 cycles of 95°C for 10 s, optimum annealing temperature for 30 s, and then 72°C for 30 s. A melt curve analysis was performed after amplification to verify the specificity of each gene. For each gene, all reactions displayed a single peak melt temperature (±0.5°C), indicating a unique product was produced.

Gene Expression

A normalization factor was calculated using geNorm software (Biogazelle, Zwijnaarde, Belgium) by calculating the geometric mean of 4 reference genes. The genes used as reference genes for muscle tissue were *tyrosine 3/tryptophan 5 monooxygenase activation protein zeta* (*YWHAZ*), *succinate dehydrogenase subunit A flavoprotein* (*SDHA*), *ribosomal protein S9* (*RPS9*), and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). For the adipose tissue normalization factor, the reference genes *peptidyprolyl iomerase A* (*PPIA*), *actin,* β (*ACTB*), *RPS9*, and *YWHAZ* were used. Relative expression for each gene was computed using the $2^{\Delta Ct}$ method. The threshold cycle (**Ct**) values for each gene were multiplied by the ratio of the natural log of 2 to the natural log of the qRT-PCR reaction efficiency factor for each individual gene. Multiplying the Ct value with the ratio of the qRT-PCR reaction efficiency adjusts the Ct value for differences in qRT-PCR efficiency. The relative expression values of the target genes in both muscle and adipose tissue were calculated as 2(normalization factor – adjusted target Ct) × 10^3

Table 1. Primers used to quantify mRNA expression of genes in longissimus muscle tissue using quantitative real-time PCR

Gene name[1]	Accession	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size, bases	Reaction efficientcy, %
ND2	NC_006853	AATTCCACCACCACTACCCTGTCA	GAGAGTGGCAAGAATTAGGACGGT	81	96.0
COX3	NC_006853	ACGTCATCATTGGGTCCACCTTCT	GCTTCAAAGCCGAAGTGGTGGTTA	91	91.9
PAX7	ENSBTAT00000019924	AGAAAGCCAAGCACAGCATC	TCGGGTTCTGACTCCACATC	85	106.2
MYOG	ENSBTAT00000007923	CTCCCATCGCGCCTCCTG	GCAGATTGTGGGCGTCTG	162	94.1
MYF5	ENSBTAT00000019924	CAGCGTCTACTGTCCTGATGT	CTGGAGTTGCAGGTTGAGAA	182	99.2
MYH1	NM_174117	CCCACTTCTCCCTGATCCACTAC	TTGAGCGGGTCTTTGTTTTTCT	246	96.5
MYH2	NM_001166227	GCTGCGTCTTCTCACTTGGT	CCACCTTCTCTGCTCTGGAT	219	94.7
MYH7	NM_174727	GGGCAAGAAGAGGAGTGAGG	CGGTAATCAGGATGGACTGG	104	97.0
VEGFA	NM_174216	ACTTCTGCGCTGTTCTCGTTC	CTCTTCCTTCTCTTCTTCCTCCTC	139	99.1
ANGPT1	NM_001076797	GGTCAGAAGAAAGGAGCGAGT	GAATAGCAGCGAGGAAAGCA	98	91.7
ANGPT2	NM_001098855	CTGAGCGGGTGGTTTATTAC	CCGTGCTGAACCTGATACTG	154	89.9
COL1A1	NM_001034039	TGGCAAGAACGGAGATGATG	CCATCCAAACCACTGAAACC	147	101.2
COL1A2	NM_174520	GGCCCAAGTGGAGATAGAGG	AGCAAAGTTCCCGCCAAG	138	97.6
COL6A2	NM_001075126	TCCACGAGAAGCACGAGAG	CCAGGTCGGAGAAGAGTGTC	90	90.1
MMP2	NM_174745	GTCTTCGACGGCATCTCTC	TTCTCCTCCTGTGGGTCTTC	173	82.2
TIMP4	NM_001045871	CCAAATCACCACCTGCTATG	TACCCGTAGAGCTTCCGTTC	96	87.1
MSTN	NM_001001525	GTTTGGCTTGGCGTTACTCA	TTCCTTCTGCTCGCTGTTCT	178	99.1
IGF1	NM_001077828	ATCACATCCTCCTCGCATCT	CTGTCTCCGCACACGAACT	131	98.6
LEPR	NM_001012285	TGGCTTAGAATCCCTTCCTC	TCGGTTTCCCTACTCCTTCC	115	98.8
ADIPOR1	NM_001034055	AAGCACCGGCAGACAAGAG	ATCGTGAAGTGCATGGTAGG	77	98.9
ADIPOR2	NM_001040499	AAGGTCTGGGAAGGTCGATG	ATGTTGCCTGTCTCGGTGTG	158	96.1
YWHAZ	NM_174814	CGCTACTTGGCTGAGGTTG	GCTTCTTGGTATGCTTGCTGTG	77	93.8
SDHA	NM_174178	TGGAAGGTCTCTGCGCTATG	GGATGGACCCGTTCTTCTATG	120	96.2
RPS9	NM_001101152	TGGATGAGGGCAAGATGAAG	ACGATGAAGGACGGGATG	187	96.2
GAPDH	NM_00103404	AGCGACACTCACTCTTCTACCTTC	ACTCTTCCTCTCGTGCTCCTG	191	90.7

[1]*ND2 = NADH dehydrogenase subunit 2*; *COX3 = cytochrome c oxidase subunit III*; *PAX7 = paired box 7*; *MYOG = myogenin*; *MYF5 = myogenic factor 5*; *MYH1 = myosin, heavy chain 1*; *MYH2 = myosin, heavy chain 2*; *MYH7 = myosin, heavy chain 7*; *VEGFA = vascular endothelial growth factor A*; *ANGPT1 = angiopoietin-1*; *ANGPT2 = angiopoietin-2*; *COL1A1 = collagen type I alpha 1*; *COL1A2 = collagen type I alpha 2*; *COL6A2 = collagen type VI alpha 2*; *MMP2 = matrix metallopeptidase 2*; *TIMP4 = tissue metallopeptidase inhibitor 4*; *MSTN = myostatin*; *IGF1 = insulin-like growth factor 1*; *LEPR = leptin receptor*; *ADIPOR1 = adiponectin receptor 1*; *ADIPOR2 = adiponectin receptor 2*; *YWHAZ = tyrosine3/tryptophan 5 monooxygenase activation protein zeta*; *SDHA = succinate dehydrogenase subunit A flavoprotein*; *RPS9 = ribosomal protein S9*; *GAPDH = glyceraldehyde-3-phosphate dehydrogenase*.

(Chung and Johnson, 2009). This procedure allowed statistical analysis of linear mRNA expression values after being adjusted for difference in qRT-PCR reaction efficiencies.

Statistical Analysis

Messenger RNA relative expression data were analyzed using a GLM (PROC GLM; SAS Inst. Inc., Cary, NC) with experiment, development category, treatment, year × development category, year × treatment, and development category × treatment interactions as fixed effects in the model. Treatment differences were declared significant when $P \leq 0.05$ for overall *F*-test. Least squares means were separated using Fisher protected LSD with $\alpha = 0.10$. Pearson correlation coefficients were also computed between gene expression in muscle and its related intramuscular adipose tissue for each stage of development using PROC CORR of SAS.

RESULTS AND DISCUSSION

Intramuscular Adipose Tissue Gene Expression

Messenger RNA expression of genes evaluated in intramuscular adipose tissue is reported in Table 3. Year and treatment had no effect on gene expression, so only the main effect of development category is reported. *Delta-like 1 homolog* (*DLK1*) mRNA expression was 1.43-fold less ($P = 0.002$) whereas *peroxisome proliferator-activated receptor* γ (*PPAR*γ; $P = 0.02$) and *fatty acid binding protein 4* (*FABP4*; $P < 0.0001$) increased 1.97- and 3.56-fold, respectively, as IM developed. *DLK1* is responsible for maintenance of preadipocytes by inhibiting their differentiation into adipocytes. The expression pattern of *DLK1* in this study is similar to reports by Smas and Sul (1993), who reported high *DLK1* expression in preadipocytes but absence in adipocytes. *Peroxisome proliferator-activated receptor* γ mRNA ex-

Table 2. Primers used to quantify mRNA expression of genes in intramuscular adipose tissue using quantitative real-time PCR

Gene name[1]	Accession	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size, bases	Reaction efficiency, %
DLK1	NM_174037	CGACATGACCACCTTCACC	CAGACCGCACAGAGAGACAG	113	80.5
PPARγ	NM_181024	TTCTCCAGCATTTCCACTCC	GACGCTTTATCCCCACAGAC	233	103.9
FABP4	NM_174314	AGCTGCACTTCTTTCTCACC	TGACACATTCCAGCACCATC	404	96.0
G3PDH	NM_001035354	ATCAATGGAGACAGGCAGAAG	TTTGGAGAGGGACTAGGCAAC	199	90.3
FASN	NM_001012669	AAGCAGGCACACAATATGGAC	TGAAGTCAAAGAAGAAGGAGAGG	244	87.7
VEGFA	NM_174216	ACTTCTGCGCTGTTCTCGTTC	CTCTTCCTTCTCTTCTTCCTCCTC	139	104.1
ANGPT1	NM_001076797	GGTCAGAAGAAAGGAGCGAGT	GAATAGCAGCGAGGAAAGCA	98	108.8
ANGPT2	NM_001098855	CTGAGCGGGTGGTTTATTAC	CCGTGCTGAACCTGATACTG	154	91.3
IGF1R	XM_002696504	ATCCAGGCCACCTCTCTCTC	CCAAGCCTCCCACTATCAAC	142	87.0
IGFBP1	NM_174554	TGCCAGCGAGAACTCTACAA	AGATCCTCTTCCCACTCCAA	193	77.9
IGFBP2	NM_174555	GGACGGGAACGTGAACTTG	GTGCTGCTCCGTGACCTTCT	109	96.5
IGFBP3	NM_174556	TTTCCCACTCAGCCATTC	CAACAAGCCACTCGTCTTCC	152	89.0
IGFBP4	NM_174557	GGAAGGGAAGAGGTCAGAGG	ACAAACGGAGGAGGAAGGAG	164	93.6
IGFBP5	NM_001105327	GAGCAAGCCAAGATCGAAAG	TCTCAGCTCCTCCCACGAAC	190	94.4
IGFBP6	NM_001040495	GCGTACAAGACACTGAGATGG	GGTCACAATTAGGCACGTAGAG	114	90.4
ACVR2B	NM_174495	AACGGCACTACCTCGGACT	ACTCGTGTCCTGGGCTTAGA	99	99.5
FST	NM_175801	GAGCTGTGCCCTGAGAGTAA	TCCTCGTCTTCGGTGTCTTC	167	96.0
ADIPOQ	NM_174742	CCATCGCCTCCTACTTCCAC	GGGATCTTCCATGTTGTCCTC	138	93.0
LEP	NM_173928	ACTAGACCGGAGCTGGGATT	GAGGGAATCTTGCTTGATGG	122	99.7
WNT5B	NM_001205628	AGGAGCACATGGCCTACATC	TGCAGGACTCTCCCAAAGAC	127	94.4
PPIA	NM_178320	GGTACTGGTGGCAAGTCCAT	GCCATCCAACCACTCAGTCT	159	93.5
ACTB	NM_173979	CTGGACTTCGAGCAGGAGAT	GGATGTCGACGTCACACTTC	208	93.9
RPS9	NM_001101152	TGGATGAGGGCAAGATGAAG	ACGATGAAGGACGGGATG	187	95.2
YWHAZ	NM_174814	CGCTACTTGGCTGAGGTTG	GCTTCTTGGTATGCTTGCTGTG	77	99.0

[1]*DLK1 = delta-like 1 homolog*; *PPARγ = peroxisome proliferator-activated receptor gamma*; *FABP4 = adipocyte fatty acid binding protein*; *G3PDH = glycerol-3-phosphate dehydrogenase*; *FASN = fatty acid synthase*; *VEGFA = vascular endothelial growth factor*; *ANGPT1 = angiopoietin-1*; *ANGPT2 = angiopoietin-2*; *IGF1R = insulin-like growth factor 1 receptor*; *IGFBP1 = insulin-like growth factor binding protein 1*; *IGFBP2 = insulin-like growth factor binding protein 2*; *IGFBP3 = insulin-like growth factor binding protein 3*; *IGFBP4 = insulin-like growth factor binding protein 4*; *IGFBP5 = insulin-like growth factor binding protein 5*; *IGFBP6 = insulin-like growth factor binding protein 6*; *ACVR2B = activin A receptor IIB*; *FST = follistatin*; *ADIPOQ = adiponectin*; *LEP = leptin*; *WNT5B = wingless type MMTV integration site family, member 5*; *PPIA = peptidylprolyl isomerase A*; *ACTβ = actin, beta*; *RPS9 = ribosomal protein S9*; *YWHAZ = tryrosine 3/tryptophan 5 monooxygenase activation protein, zeta*.

pression was expected to increase as number of differentiated adipocytes became larger due to the role of *PPARγ* in promoting differentiation of adipocytes (Rosen and MacDougald, 2006). *Fatty acid synthase* (*FASN*) mRNA expression was similar and *glycerol-3-phosphate dehydrogenase* (*G3PDH*; $P = 0.004$) mRNA expression was 1.80-fold lower in MA IM compared with MM IM. The lack of change in *FASN* mRNA expression and lower mRNA expression of *G3PDH* in MA IM are somewhat surprising. It may indicate maximum development of these IM deposits such that lipid deposition has slowed, but the lack of change in *FASN* does not fit this scenario. The reason for this is unknown at this time. It has been well documented that differentiation of adipocytes is controlled by programmed changes in gene expression (Kokta et al., 2004). Veerkamp et al. (1991) indicated that *FABP4* is responsible for the transport of fatty acids into adipocytes and *FABP4* mRNA expression in intramuscular adipose tissue was shown to steadily increase as intramuscular adipose tissue developed (Pickworth et al., 2011). These results agree with the current study in which *FABP4* was greater in more developed intramuscular deposits compared with MM adipose tissue. *FASN*, which is responsible for synthesis of fatty acids, has been shown to have a positive correlation with increasing IM content (Jeong et al., 2012); however, in this study, no difference in *FASN* was detected across development levels. The results of this study demonstrate an increase in the number of differentiated adipocytes, indicating that we were successful in separating intramuscular adipose tissue into different stages of development based on visual assessment under the microscope. Taken together, these results indicate that within a single animal, not all intramuscular deposits are at the same stage of development, supporting the idea that IM development occurs throughout the lifetime of an animal.

Messenger RNA expression of *vascular endothelial growth factor A* (*VEGFA*), *angiopoietin-1*

Table 3. Relative mRNA expression ($2^{\Delta Ct} \times 10^3$) of genes evaluated in intramuscular adipose tissue of different stages of development

Gene name[1]	MM[2]	ME[2]	MA[2]	SEM	*P*-value
Adipogenesis					
DLK1	164.92[a]	176.69[a]	119.57[b]	10.84	0.002
PPARγ	5.10[b]	10.02[a]	10.07[a]	1.35	0.02
FABP4	973.1[c]	4,031.9[a]	2,902.1[b]	428.9	<0.0001
G3PDH	3,828.9[a]	3,711.8[a]	2,123.7[b]	371.4	0.004
FASN	311.31	284.95	356.75	93.71	0.86
Angiogenesis					
VEGFA	9.88	7.94	8.55	0.88	0.30
ANGPT1	0.47	0.34	0.30	0.11	0.52
ANGPT2	40.84	29.23	32.46	3.76	0.09
Intercellular signaling					
IGF1R	76.14[a]	49.06[b]	72.06[a]	7.39	0.03
IGFBP1	66.89	92.02	60.42	11.42	0.14
IGFBP2	0.97[b]	0.87[b]	2.16[a]	0.15	<0.0001
IGFBP3	320.82[b]	515.65[a]	514.45[a]	32.39	<0.0001
IGFBP4	570.89	623.46	592.14	29.01	0.44
IGFBP5	710.99	508.03	615.94	59.24	0.07
IGFBP6	4,622.0[a]	2,376.6[b]	1,988.8[b]	231.7	<0.0001
ACVR2B	7.17[a]	5.49[b]	4.27[b]	0.52	0.002
FST	172.76[a]	130.44[b]	101.82[b]	15.27	0.01
ADIPOQ	197.33	309.87	319.52	50.65	0.18
LEP	4.58[b]	10.64[a]	11.58[a]	2.09	0.05

[a–c]Least squares means without a common superscript differ ($P < 0.05$).

[1]*DLK1 = delta-like 1 homolog*; *PPARγ = peroxisome proliferator-activated receptor gamma*; *FABP4 = adipocyte fatty acid binding protein*; *G3PDH = glycerol-3-phosphate dehydrogenase*; *FASN = fatty acid synthase*; *VEGFA = vascular endothelial growth factor*; *ANGPT1 = angiopoietin-1*; *ANGPT2 = angiopoietin-2*; *IGF1R = insulin-like growth factor 1 receptor*; *IGFBP1 = insulin-like growth factor binding protein 1*; *IGFBP2 = insulin-like growth factor binding protein 2*; *IGFBP3 = insulin-like growth factor binding protein 3*; *IGFBP4 = insulin-like growth factor binding protein 4*; *IGFBP5 = insulin-like growth factor binding protein 5*; *IGFBP6 = insulin-like growth factor binding protein 6*; *ACVR2B = activin A receptor IIB*; *FST = follistatin*; *ADIPOQ = adiponectin*; *LEP = leptin*; *WNT5B = wingless type MMTV integration site family, member 5B*.

[2]MM = immature intramuscular adipose tissue; ME = intermediate intramuscular adipose tissue; MA = mature intramuscular adipose tissue.

Table 4. Relative mRNA expression ($2^{\Delta Ct} \times 10^3$) of genes evaluated in longissimus dorsi muscle tissue associated with different intramuscular adipose tissue development

Gene name[1]	NF[2]	MM[2]	ME[2]	MA[2]	SEM	*P*-value
Metabolism						
ND2	3,596.4	2,911.6	2,769.1	2,951.5	309.2	0.25
COX3	34,858.6	37,546.3	37,878.4	37,462.8	3,527.3	0.92
Satellite cell activity						
PAX7	0.19	0.27	0.18	0.19	0.04	0.27
MYOG	32.27	34.22	30.54	33.10	4.21	0.93
MYF5	2.83	3.25	3.26	3.41	0.27	0.49
Fiber type						
MYH1	4,462.7	5,386.1	3,359.9	3,578.0	821.3	0.26
MYH2	14,568.9	15,925.4	9,013.9	10,962.2	2,609.6	0.20
MYH7	4,559.9	5,673.7	3,754.0	3,613.4	785.6	0.21
Angiogenesis						
VEGFA	27.95	41.53	23.58	26.33	7.16	0.27
ANGPT1	11.55	14.37	7.97	10.11	3.27	0.54
ANGPT2	11.85	18.08	10.73	10.70	3.62	0.39
Extracellular matrix						
COL1A1	18.64[b]	35.29[a]	12.42[b]	11.74[b]	5.68	0.01
COL1A2	13.85[b]	23.78[a]	11.13[b]	10.62[b]	2.81	0.004
COL6A2	102.27[b]	140.68[a]	106.55[b]	113.77[b]	10.25	0.04
MMP2	61.06	69.85	52.25	60.34	5.78	0.18
TIMP4	78.51	76.30	71.36	77.99	5.06	0.72
Intercellular signaling						
IGF1	1.71	1.57	1.49	1.57	0.17	0.83
MSTN	8.22	9.08	8.44	8.03	1.14	0.92
ADIPOR1	30.72	33.03	29.84	30.76	1.70	0.55
ADIPOR2	29.31[a]	91.56[b]	122.74[c]	142.06[d]	8.01	<0.0001
LEPR	0.33	0.41	0.41	0.39	0.60	0.76

[a–d]Least squares means without a common superscript differ ($P < 0.05$).

[1]*ND2 = NADH dehydrogenase subunit 2*; *COX3 = cytochrome c oxidase subunit III*; *PAX7 = paired box 7*; *MYOG = myogenin*; *MYF5 = myogenic factor 5*; *MYH1 = myosin, heavy chain 1*; *MYH2 = myosin, heavy chain 2*; *MYH7 = myosin, heavy chain 7*; *VEGFA = vascular endothelial growth factor A*; *ANGPT1 = angiopoietin-1*; *ANGPT2 = angiopoietin-2*; *COL1A1 = collagen type I alpha 1*; *COL1A2 = collagen type I alpha 2*; *COL6A2 = collagen type VI alpha 2*; *MMP2 = matrix metallopeptidase 2*; *TIMP4 = tissue metallopeptidase inhibitor 4*; *MSTN = myostatin*; *IGF1 = insulin-like growth factor 1*; *LEPR = leptin receptor*; *ADIPOR1 = adiponectin receptor 1*; *ADIPOR2 = adiponectin receptor 2*.

[2]NF = longissimus muscle not associated with intramuscular adipose tissue; MM = longissimus tissue associated with immature intramuscular adipose tissue; ME = longissimus tissue associated with intermediate intramuscular adipose tissue; MA = longissimus tissue associated with mature intramuscular adipose tissue.

(*ANGPT1*), and *angiopoietin-2* (*ANGPT2*) genes involved in angiogenesis remained constant as adipose tissue developed. *Wingless type MMTV integration site family, member 5B* (*WNT5B*) mRNA expression was 2.38-fold greater ($P < 0.0001$) in MA IM than in MM and ME IM. Evaluation of *wingless type MMTV integration site family, member 10B* (*WNT10B*) was attempted, but mRNA expression was undetectable in intramuscular adipose tissue. The Wnt signaling pathway influences adipogenesis by intersecting with the transcriptional cascade of adipocyte differentiation (Rosen and MacDougald, 2006). *WNT5B* is responsible for promotion of differentiation of adipocytes (Kanazawa et al., 2005) and increased as IM development progressed. However, *WNT10B*, which has inhibitory effects on adipocyte differentiation, had undetectable levels of mRNA expression in this study. Tan et al. (2006) reported that when adipogenesis was induced in bovine bone marrow stromal cells, *WTN10B* expression was decreased and undetectable by Day 3 after induction of adipogenesis.

Muscle Gene Expression

Messenger RNA expression of genes evaluated in LM is reported in Table 4. Year and treatment had no effect on gene expression, so only the main effect of development category is reported. Messenger RNA expression of genes associated with metabolism, *cytochrome C oxidase subunit III* (*COX3*) and *NADH dehydrogenase subunit 2* (*ND2*), were similar among LM associated with different adipose tissue maturity categories. There were no changes in mRNA expression of genes indicative of muscle fiber type, *myosin heavy chain 1* (*MYH1*), *myosin heavy chain 2* (*MYH2*), and *myosin heavy chain 7* (*MYH7*), among LM associated with the differing development categories of adipose tissue. Previously, IM content has been positively correlated (r = 0.46 to 0.49) with fiber area of oxidative muscle fiber types (Melton et al., 1974; May et al., 1977) and number of capillaries per muscle fiber (r = 0.63; Melton et al., 1975). In addition, Kim et al. (2009) reported that gene expression of *NADH dehydrogenase subunit 2* and *cytochrome c oxidase subunit III* explained 84 and 97%, respectively, of the variation in IM content of loin muscle in Korean cattle. However, the lack of differences in *ND2*, *COX3*, and myosin heavy chain isoforms among LM associated with different stage of IM maturity indicate little direct influence on development of intramuscular adipose tissue.

Satellite cell activity was evaluated in muscle tissue by measuring the mRNA expression of *paired box 7* (*PAX7*), *myogenin* (*MYOG*), and *myogenic factor 5* (*MYF5*). There was no difference in mRNA expression in muscle associated with different adipose deposits, indicating that satellite cell activity does not appear to affect IM development. Messenger RNA expression of *VEGFA*, *ANGPT1*, and *ANGPT2*, all genes involved in angiogenesis, was not different in LM associated with different adipose tissue development.

Messenger RNA expression of genes associated with extracellular matrix (**ECM**) was affected in LM associated with increasing IM development. Expression of *collagen type I α 1* (*COL1A1*; P = 0.01), *collagen type I α 2* (*COL1A2*; P = 0.004), and *collagen type VI α 2* (*COL6A2*; P = 0.04) mRNA were 2.47-, 2.02-, and 1.31-fold greater, respectively, in muscle associated with immature intramuscular adipose compared with the other development categories and LM not associated with IM. However, mRNA expression of genes responsible for the remodeling of ECM, *matrix metallopeptidase 2* (*MMP2*) and *tissue metallopeptidase inhibitor 4* (*TIMP4*), remained constant in LM associated with different stages of IM development. Messenger RNA expression of *matrix metallopeptidase 9* (*MMP9*) was not detectable in skeletal muscle tissue (data not shown). Messenger RNA expression changes in components of the ECM were observed in muscle tissue associated with increasing development of intramuscular adipose tissue. Extracellular matrix is essential for intramuscular adipose development because adipocytes are embedded within connective tissue (Moody and Cassens, 1968; Du and Carlin, 2012). The current study showed that both *collagen type I* and *collagen type VI* exhibited the same mRNA expression pattern, with muscle associated with immature intramuscular adipose development having the highest expression. On the other hand, a study using murine 3T3-L1 cells reported that the expression levels of collagens were different as adipocytes differentiated, with *collagen type I* expression being decreased early and increasing late in development whereas *collagen type VI* expression was the reverse, with expression being highest early in development and decreasing late (Aratani and Kitagawa, 1988). Tan et al. (2006) reported that in adipogenesis of bovine bone marrow stromal cells, an increase in mRNA expression of *COL1A1* and *COL1A2* occurred. A microarray study evaluating nutritional restriction effects on gene expression in muscle tissue reported a downregulation of *COL1A1* and *COL1A2* in nutrient-restricted cattle (Byrne et al., 2005). Animals that have a genetic predisposition for high levels of IM development have been shown to have increased levels of *collagen type I* and *collagen type VI* (Nakajima et al., 1998; Wang et al., 2009). These results indicate that muscle fibers have a role in constructing the ECM for development of intramuscular adipose tissue during the early stage of adipose development.

Intercellular Signaling Pathway Gene Expression

Insulin-like growth factor I mRNA expression remained constant in muscle tissue regardless of adipose tissue development category. Changes in *IGF1 receptor* (*IGF1R*) mRNA expression in adipose tissue were detected with 1.51-fold lower expression (P = 0.03) in ME than in MM and MA categories. Messenger RNA expression of IGFBP in IM were different among adipose tissue development categories, but changes were not consistent between IGFBP. Messenger RNA expression of *IGFBP-2* (P < 0.0001) and *IGFBP-3* (P < 0.0001) increased 2.35- and 1.61-fold with increasing development; conversely, *IGFBP-6* mRNA expression decreased 2.32-fold (P < 0.0001) with increasing development. Messenger RNA expression of *IGFBP-1*, *IGFBP-4*, and *IGFBP-5* was similar among development categories. The *IGFBP* are known to have different effects on IGF activity and, therefore, would be expected to have different patterns of mRNA expression. *IGFBP-2* is known to have solely inhibitory effects on IGF-mediated actions (Collett-Solberg and Cohen, 2000). The increase in *IGFBP-2* in more devel-

oped adipose tissue suggests that it may play a role in reducing the proliferative effects of *IGF-I* on adipocytes allowing them to transition toward lipid accumulation. Both *IGFPB-3* and *IGFBP-5* have inhibitor and stimulator effects on *IGF-I*, making it difficult to establish the implication of these changes, especially because the mRNA expression pattern of *IGFBP-3* increased whereas *IGFBP-5* showed no change. An in vitro study conducted using bovine bone marrow stromal cells reported an increase in *IGFBP-3*, *IGFBP-4*, and *IGFBP-5*, with *IGFBP-5* having the greatest increase (Tan et al., 2006). The results are contradictory to the results shown in our in vivo study, indicating that other extracellular signals may affect IGFBP activity. These changes in IGFBP mRNA expression throughout the development of intramuscular adipose tissue implies that they may be responsible for the response of intramuscular adipose tissue to local or systemic IGF-I. *IGFBP-6* mRNA expression was highest in immature intramuscular adipose tissue and decreased as depots became more developed. *IGFBP-6* is the only IGFBP that has a higher affinity for IGF-II and preferentially binds with it (Collett-Solberg and Cohen, 2000), suggesting that IGF-II levels may be elevated in less developed intramuscular adipose tissue. Elevated levels of IGF-II were observed in stromal-vascular cells from 75-d-old pigs, but when adipogenesis was induced in these cells, IGF-II levels remained steady (Hausman et al., 2002). However, our study is inconclusive about the expression pattern of IGF-II because its expression was not measured.

Messenger RNA expression of myostatin's receptor, *activin A receptor IIB*, and *follistatin* measured in intramuscular adipose tissue did differ with development category. *Myostatin* mRNA expression in muscle tissue remained constant as intramuscular adipose tissue developed; however, the expression of myostatin receptor (*ACVR2B*; $P = 0.002$) decreased 1.68-fold as intramuscular adipose tissue became more developed. Additionally, *follistatin* (*FST*; $P = 0.01$), a myostatin inhibitor, decreased 1.70-fold with increasing development of intramuscular adipose tissue. Collectively, these data suggest that as intramuscular adipose tissue develops, it becomes desensitized to the inhibitory effects of *myostatin* on the differentiation of adipocytes (McPherron et al., 1997; Hirai et al., 2007a,b).

Adipokines and their receptors present in muscle were also evaluated. *Adiponectin* (*ADIPOQ*) mRNA expression remained constant as adipose developed, which is contrary to what was expected. However, the large variation in *ADIPOQ* mRNA expression may have prevented us from detecting increased expression in more developed IM. Expression level of the adiponectin receptors had differing expression patterns in LM. *Adiponectin receptor 1* (*ADIPOR1*) mRNA expression in LM did not change with increasing IM development, but *adiponectin receptor 2* (*ADIPOR2*) mRNA expression increased 4.84-fold ($P < 0.0001$) as muscle became associated with more developed intramuscular adipose tissue. Interestingly, Ahima (2006) reported that *ADIPOR2* is mainly expressed in liver. Whether the increase in *ADIPOR2* expression is prompted due to intramuscular development or inherent to muscle fibers, which results in development of intramuscular adipose tissue at that location, is not clear from these data. As expected, *leptin* (*LEP*) mRNA expression in IM increased 2.43-fold ($P = 0.05$) as IM development increased, although *leptin receptor* (*LEPR*) mRNA expression in LM remained constant among development categories. *Leptin* mRNA expression in the current study followed the expected pattern that as the number of differentiated adipocytes increased, so did the mRNA expression of *leptin*. However, the mRNA expression of *leptin receptor* measured in muscle tissue remained constant in muscle regardless of the development of the associated adipose tissue. An attempt was made to evaluate the resistin signaling pathway; however, *resistin* mRNA in intramuscular tissue and *resistin receptor* mRNA in muscle were not detectable (data not shown). Previous research (Argilés et al., 2005) indicates that adipokine production is proportional to the size of intramuscular adipose tissue mass and would increase as the number of differentiated adipocytes increases. Actions of both *leptin* and *adiponectin* have the same result on skeletal muscle, which is an increase in fatty acid oxidation and glucose uptake by muscle tissue (Muoio et al., 1997; Kokta et al., 2004), indicating that muscle closely associated with intramuscular adipose tissue development would have improved insulin sensitivity and utilize fatty acids for energy more than skeletal muscle not associated with intramuscular adipose tissue development. This may synergistically increase IM development in these microenvironments within skeletal muscle.

Pearson Correlations between Muscle and Intramuscular Adipose Tissue

Correlations were calculated between expression of genes in intramuscular adipose tissue and the muscle tissue that corresponded with each IM development category (Table 5). In immature IM and its associated LM, mRNA expression of *VEGFA*, *ANGPT1*, and *ANGPT2* in LM had a strong positive correlation with mRNA expression of *ANGPT1* and *ANGPT2* ($r = 0.69$ to 0.94), *FASN* and *PPARγ* ($r = 0.89$ to 0.96), and *ADIPOQ* and *LEP* ($r = 0.56$ to 0.94) in intramuscular adipose tissue. Interestingly, similar correlations were not observed between LM and IM of the other adipose

tissue development categories. These data suggest that there is a highly coordinated set of changes that occur between skeletal muscle and intramuscular adipose tissue during the early development of intramuscular adipose tissue. The correlations observed is supported by Rupnick et al. (2002), who administered antiangiogenic agents into obese mice and observed a reduction in fat content over the control mice, concluding that there is a correlation between adipogenesis and angiogenesis. Collectively, these data demonstrate that the development of vasculature and adipose tissue are interrelated, especially during early development.

In conclusion, development of intramuscular adipose tissue is a complex process that is influenced by a variety of signals. The close proximity of intramuscular adipose tissue to muscle tissue during development suggests that intercellular signaling between these 2 tissues is crucial for development of intramuscular adipose tissue. Early in the development of intramuscular adipose tissue, changes in muscle that effect remodeling of the ECM along with angiogenesis are critical for development of intramuscular adipose tissue. The strong correlation of angiogenic growth factors in LM with angiogenic growth factors and markers of adipocyte differentiation in immature intramuscular adipose tissue suggest that there is a highly coordinated change that occurs between skeletal muscle and IM during the early stage of adipose development. However, the mechanisms of intramuscular adipose tissue development are still not fully clear and more in vitro and in vivo studies are needed to further elucidate the pathways and mechanisms involved in intramuscular adipose tissue development. Further understanding the interactions between skeletal muscle and adipose tissue during intramuscular development could allow for development of management strategies that optimize carcass quality.

Table 5. Correlations of angiogenic gene expression in longissimus muscle (LM) with gene expression in corresponding immature, intermediate, and mature intramuscular adipose tissue (intramuscular fat [IM])

Item	*VEGFA*[1] (LM)	*ANGPT1*[1] (LM)	*ANGPT2*[1] (LM)
Immature IM[2]			
FASN	0.96*	0.91*	0.91*
PPARγ	0.89*	0.91*	0.92*
ANGPT1	0.91*	0.94*	0.94*
ANGPT2	0.79*	0.69*	0.69*
ADIPOQ	0.93*	0.95*	0.96*
LEP	0.72*	0.56*	0.56
Intermediate IM			
FASN	0.01	–0.35	–0.75*
PPARγ	–0.18	–0.20	–0.58*
ANGPT1	–0.19	–0.18	–0.53
ANGPT2	–0.15	–0.12	–0.35
ADIPOQ	–0.19	–0.43	–0.32
LEP	0.54	–0.18	–0.61*
Mature IM			
FASN	0.37	–0.06	–0.38
PPARγ	0.37	0.18	–0.12
ANGPT1	0.42	0.26	–0.13
ANGPT2	–0.14	0.29	0.52
ADIPOQ	0.19	–0.22	–0.07
LEP	0.79*	–0.11	–0.43

[1]*VEGFA = vascular endothelial growth factor A*; *ANGPT1 = angiopoietin-1*; *ANGPT2 = angiopoietin-2*.

[2]*FASN = fatty acid synthase*; *PPARγ = peroxisome proliferator activated receptor gamma*; *ADIPOQ = adiponectin*; *LEP = leptin*.

*Correlations are significantly different from 0 ($P < 0.05$).

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