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Proteomic Analysis of Bovine Longissimus Muscle Satellite Cells during Adipogenic Differentiation

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ABSTRACT : Satellite cells are skeletal muscle progenitor/stem cells that reside between the basal lamina and plasma membranes of skeletal fibers *in vivo*. These cells can give rise to both myogenic and adipogenic cells. Given the possible role for differentiation of satellite cells into adipocytes in marbling and in some pathological disorders like sarcopenia, knowledge of the proteins involved in such process remains obscure. Using two-dimensional polyacrylamide gel electrophoresis coupled with mass spectrometry, we investigated the proteins that are differentially expressed during adipogenic differentiation of satellite cells from bovine longissimus muscle. Our proteome mapping strategy to identify the differentially expressed intracellular proteins during adipogenic differentiation revealed a total of 25 different proteins. The proteins up-regulated during adipogenic differentiation of satellite cells like Cathepsin H precursor, Retinal dehydrogenase 1, Enoyl-CoA hydratase, Ubiquinol-cytochrome-c reductase, T-complex protein 1 subunit beta and ATP synthase D chain were found to be associated with lipid metabolism. The down-regulated proteins like LIM protein, annexin proteins, cofilin-1, Rho GDP-dissociation inhibitor 1 and septin-2, identified in the present study were found to be associated with myogenesis. These results clearly demonstrate that the adipogenic conversion of muscle satellite cells is associated with the up-regulated and down-regulated proteins involved in adipogenesis and myogenesis respectively. (**Key Words :** 2-DE, Satellite Cells, Adipogenesis, Myogenesis, Proteome)

INTRODUCTION

Transdifferentiation of myogenic precursor cells into adipocytes has gained much importance due to its possible role in marbling of meat animals (accumulation of intramuscular fat) and in some pathological conditions, like age related decrease in skeletal muscle, sarcopenia, due to fatty acid infiltration (Mora, 1989; Chung et al., 2006). Though both, marbling and sarcopenia involve induction of fat in the muscle, origin of such adipocytes within the muscle is unclear. Several studies have shown the role of some specialized muscle cells that make up the intramuscular fat within the muscle (Hu et al., 1995; Kokta et al., 2004). Skeletal muscle contains several kinds of stem cells of mesenchymal origin (Charge and Rudnicki, 2004; Holterman and Rudnicki, 2005) including satellite cells. Satellite cells accounts for 2-5% of muscle nuclei and reside juxtaposed to muscle fibers beneath the basal lamina (Campion, 1984; Mora, 1989). They are usually mitotically quiescent, but following mechanical stimulus or injury to muscle fibers, they are activated and proliferate to mediate postnatal growth and regeneration of muscle (Campion, 1984; Mora, 1989; Charge and Rudnicki, 2004; Holterman and Rudnicki, 2005). In addition to their myogenic property as myogenic stem cells, recent evidences indicate that some satellite cells are multipotent that can undergo adipogenic, osteogenic and chondrogenic transdifferentiation (Mauro, 1961; Asakura et al., 2001; Holterman and Rudnicki, 2005).

Numerous investigations have been focused on understanding the involvement of satellite cells in postnatal myogenesis and lipid accumulation in meat-producing animals (Mauro, 1961; Singh et al., 2007; Chung and Johnson, 2008) and in particular, studies reporting transdifferentiation of bovine satellite cells (BSC) into adipocytes are gaining importance (Burton et al., 2000; Kook et al., 2006). The adipogenic differentiation of satellite cells can be achieved by culturing them under adipogenic conditions (Kook et al., 2006) that are

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commonly used for inducing adipogenic differentiation of the adipogenic cell line, 3T3-L1 and primary stromal vascular cells (Bernlohr et al., 1985; Burton et al., 2000). Muscle and adipose differentiation involve complex processes that lead to the induction of several differentiation-linked genes, specifically either in muscle cells (Grounds et al., 1992) or in adipose cells (Kook et al., 2006). However, the factors that promote the transition from myoblast lineage to that of adipoblast are still ambiguous. But, the extrinsic factors that control muscle and adipose differentiation are clearly different. Differentiation of myoblast in cell culture is initiated by the peptide growth factor withdrawal (Florini et al., 1991; Olson, 1992), while adipose differentiation is controlled by the addition of various hormones (Ailhaud et al., 1992). Although genomic studies have revealed genes involved in adipogenic transdifferentiation (Wang et al., 2005a,b), the details of the intrinsic factors (proteins) that play an important role in such complex transdifferentiation processes is still unexplored. Proteomics is an ideal technology for detection of the changes in the protein expression as it allows comparison of two or more samples on a relatively global level with little or no prior knowledge about the possible pathways influenced by the experimental conditions. Hence, the current study was aimed to improve our understanding on the proteins that are differentially expressed during the adipogenic differentiation of satellite cells from bovine longissimus dorsi muscle using two-dimensional electrophoresis.

MATERIALS AND METHODS

Animals

Five heads of Hanwoo (Korean cattle) steers were fed and managed at feeding barn in the National Institute of Animal Science under the high quality beef production program (1997) and slaughtered at 24 months old. All experimental procedures and the care of animals were conducted in accordance with the guidelines of the Animal Care and Use Committee (IACUC) of the National Institute of Animal Science in Korea.

Cell preparation

Animal experimentation was approved by the Animal Care and Concern committee of the National Institute of Animal Science, in Suwon. Satellite cells were isolated from the bovine longissimus dorsi (LD) muscle as described by (Doumit and Merkel, 1992). Briefly, the muscles were excised, trimmed of visible connective tissue, and minced with fine sharp scissors. One gram of minced muscle was incubated for 50 min at 37°C in a solution of protease (4 mg/ml) in PBS. After enzymatic digestion, the cells were separated from the tissue fragments by repeated centrifugation at 1,200 and 300×g for 15 and 5 min respectively, followed by filtration using nylon mesh cloth. Thereafter, the filtrate was further centrifuged at 1,200 g for 15 min and the pellet was collected and reconstituted with DMEM medium supplemented with 10% Fetal Bovine Serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) and seeded in 100 mm petri-dish at a density of approximately 1×10^4 cells/cm^2. The cells were incubated at 37°C in 5% CO_2 in air.

Inducing adipogenic differentiation : For adipogenic differentiation, we plated the cell at 2×10^7/ml in 100 mm^2 dish and allowed to grow to confluence over two days. The cells were then cultured in DMEM medium supplemented with 10% FBS, 5 μM rosiglitazone, 5 μM dexamethasone, 10 μg/ml insulin, 0.2 mM ascorbic acid, 10 mM acetic acid, 0.5 mM isobutyl-1-methylxanthine and 1 mM caprylic acid. The medium was then replaced every 2 days for total 8 days. After 9th day of culture, cells on the plates were stained with Oil Red O to measure the degree of adipocyte differentiation.

Oil red O staining

Oil red O staining was performed to monitor the progression of adipocyte differentiation as described previously (Ramirez-Zacarias et al., 1992). Briefly, satellite cells of day 0, 3, 6 and 9 (after adding differentiating medium) from LD muscle were washed with PBS and then fixed using 3.7% formaldehyde for 2 min. Oil red O (0.5%) was prepared in isopropanol, mixed with water at 3:2 ratio and filtered through a 0.45 μm filter. The fixed cells were incubated with Oil red O reagent for 1 h at room temperature and then washed with water. The stained fat droplets in the cells were visualized by light microscopy and photographed.

Protein preparation for 2-DE

Proteins from satellite cells of LD on day 0 (sub-confluent bovine muscle satellite cells) and day 8 (adipogenic differentiated satellite cells) were extracted in 0.5 ml of lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 100 mM DTT, protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Following 15 min centrifugation at 15,000 rpm, the supernatant was collected and precipitated with either one or three volumes of acetone at -20°C for 2 h. The pellets collected by centrifugation were completely dried using speed-vac and re-dissolved in the 2-DE sample buffer (7 M Urea, 2 M Thiourea, 2% CHAPS, 100 mM DTT, 0.5% pH3-10NL IPG buffer) for isoelectric focusing (IEF). The concentration of the total protein in the sample was determined by Bradford's protein assay method.

2-DE and image analysis

500 μg of protein was loaded onto the immobiline dry

strips pH 3-10 NL (GE Health care). The rehydrated strips were focused on IEF system (AP Biotech, Sweden) for ~ 80 kVh at a maximum of 8,000 V in a rapid ramping mode with maximum current per strip of 50 μA. Equilibration of the immobilized pH gradient strips was performed in two steps: reduction followed by alkylation (Ahmed and Bergsten, 2005). The second dimension was run on 12.5% polyacrylamide sodium dodecyl sulphate gels (26×20 cm) with a constant voltage of 100 V for 30 min, 250 V for 6 h using the EttanDALT II system (Amersham Bioscience, Piscataway, USA). The proteins were visualized using silver or Coomassie Brilliant Blue (CBB) G-250 staining method.

The silver-stained gels were scanned using GS-800 scanner (Bio-Rad) at an optical resolution of 300 dpi. Spot detection, quantification and matching were performed using Image Master Version 7.0 (GE healthcare). A match set consisting of three images, each from one depot was created. To correct the variability due to silver staining and to reflect the quantitative variations of protein spot, the individual spot volumes were normalized by dividing their optical density (OD) values by the total OD values of all the spots present in the gel. The significance of the expression difference of proteins between undifferentiated and differentiated satellite cells from LD muscle was estimated by student's t-test, $p<0.05$ using Image Master (ver 7.0) software.

Protein identification

The CBB-stained protein spots were excised from gels using a punch and placed in 500 μl Eppendorf tubes. The proteins were digested in-gel with trypsin as described by (Hellmann, 1995). Briefly, each spot was destained with 50 μl 50% acetonitrile (ACN) in 50 mM NH_4HCO_3, incubated at 37°C for 30 min and repeated once. Then the gels were reduced and alkylated. The gel pieces were digested overnight with trypsin (20 μg/μl) in 50 mM NH_4HCO_3 containing 10% ACN. The digest was then vortexed for 30 min and dried using speed vac. The dried extracted peptides were resuspended in 1 μl solution containing pure water:ACN:trifluoracetic acid (TFA) (93:5:2).

Solution-phase nitrocellulose target preparation was used according to the method reported by (Landry et al., 2000). α-Cyano-4-hydroxycinnanic acid (CHCA) (40 mg/ml) and nitrocellulose (20 mg/ml) were prepared separately in acetone and mixed with 2-propanol at a ratio of 2:1:1. The matrix solution mixed with the sample at a ratio 1:1, 0.5-0.3 μl was spotted onto the target and dried. The immobilized samples were washed with 1% formic acid twice and dried prior to the MALDI-TOF-MS/MS analysis.

Sample peptide masses were obtained using Applied Biosystems 4700 Proteomics analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems) in the positive ion reflector mode. MS/MS analysis was performed on the 5 most abundant ions and the proteins were identified by searching the SWISS-PROT and National Center for Biotechnology Information databases using the Mascot programs (Matrix Science, London, UK). Mass accuracy was considered to be within 50 ppm for peptide mass analysis and within 100 ppm for MS/MS analysis. For protein identification, known contamination peaks such as those of keratin and autoproteolytic were removed, and molecular weight, pI and protein scores were considered.

RNA isolation and real-time RT-PCR analysis

Total RNA was isolated from the cells using TRIzol reagent (Invitrogen Co., Carlsbad) based on the manufacturer's description. The first strand cDNA was synthesized using 2 μg of total RNA as a template, oligo-dT primer and Reverse Transcriptase (Invitrogen Co.) according to the manufacturer's instructions. Measurement of the relative quantity of the cDNA of interest was carried out using SYBR Green PCR Master Mix (Applied Biosystems), 300 nM of the appropriate forward and reverse primers, and 1 ml of the cDNA mixture. The sequence of forward and reverse primers used for detection of MyoD, Myf5, Myogenin, GAPDH, PPAR-γ and FABP are given in Table 1.

RESULTS

Myogenic properties and adipogenic potential of bovine satellite cells from longissimus dorsi (LD) muscle

Bovine satellite cells (BSC) isolated from LD muscle was cultured in 10% FBS/DMEM medium and the cells

Table 1. Sequence of primers used in RT-PCR

Gene name	Forward primer	Reverse primer	Product size (bp)
MY5	ctcaggaatgccatccgctacattgaga	atccaagctggataaggagcttttatccg	216
MyoD	atcctgcgcaacgccatccgctatatcga	ctcgcgctgtagtaagtgcggtcgtagcagt	203
Myogenin	gagaagcgcagactcaagaaggtgaatga	tctgtagggtccgctgggagcagatgatc	314
PPARg	ccctggcaaagcatttgtat	ggggactgatgtgcttgaac	194
FABP	Ggggtgtggtcaccattaaa	tgacacattcagcaccatc	219
GAPDH	gggtcatcatctctgcacct	ggtcataagtccctccacga	176

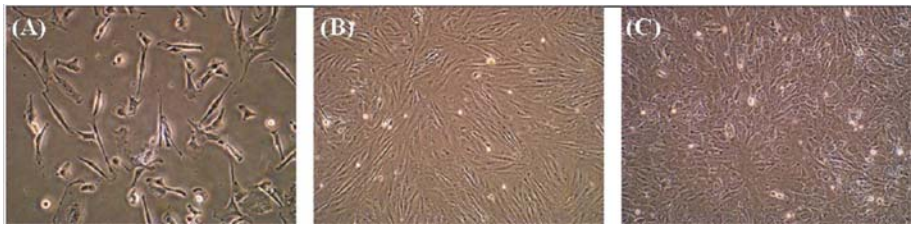

Figure 1. Morphological examination of bovine satellite cells during transdifferentiation. (A) Satellite cells appeared in the culture medium after five days of their isolation from the tissue. (B) Ten days after their culture, cells were showing a spindle-shaped fibroblastic morphology. (C) 48 h after treating with adipogenic differentiation medium, cells changed its shape from spindle to round (adipoblast morphology).

appeared in the culture medium only after day 5 (Figure 1A). After 10 days of isolation, all the cells in the culture medium attained spindle shaped fibroblastic morphology (Figure 1B). Myogenic properties of BSC were also determined by their mRNA expression levels of myogenic markers like MyoD, Myf5 and myogenin and Figure 2 shows the levels of all the three myogenic markers in BSC. Presence of mRNA of all the three genes demonstrated that, cells isolated and proliferating in the culture medium were satellite cells. Further, treatment of these cells with adipogenic induction medium for 48 h changed the fibroblastic morphology of BSC into round adipoblast (Figure 1C). Adipogenic potential of BSC was also determined by Oil Red O staining of differentiating BSC (Figure 3A-D). The cells accumulated lipid droplets after 48 h of adipogenic induction and further, fat accumulation progressively augmented with increase in period of exposure to adipogenic inducing medium. To further verify the adipogenic differentiation of BSC, the cells were analyzed for mRNA expressions of the adipogenic markers, PPAR-γ (Peroxisome proliferator-activated receptor gamma) and FABP (Fatty acid binding protein) (Figure 4). Adipogenic differentiation stimulation of BSC resulted in the prompt expression of adipogenic markers and further mRNA expression of both markers augmented on day 8. These results revealed that i) cells isolated from longissimus dorsi (LD) muscle were satellite cells and ii) when treated with adipocyte differentiation medium were committed to form adipocytes.

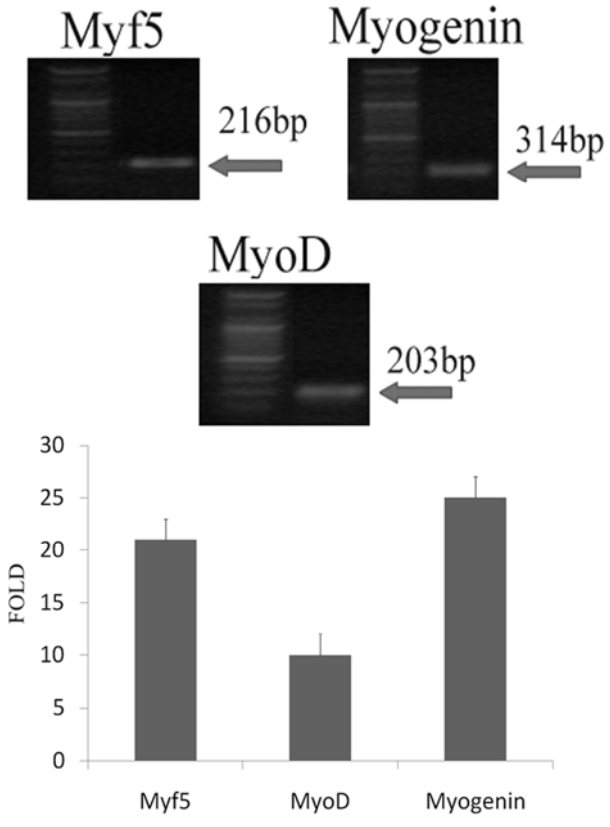

Figure 2. RT-PCR and relative mRNA levels of MyoD, Myogenin and My5 genes in total RNA isolated from proliferating bovine satellite cell cultures at day 9 from longissimus dorsi muscle. The data points in the figure are from individual assays was the average of mRNA levels obtained from triplicate culture experiments.

Differential protein expression during transdifferentiation of bovine satellite cells into adipocytes

For proteomic analysis, crude protein extracts of day 0 (sub-confluent bovine muscle satellite cells) and day 8 (adipogenic differentiating satellite cells) cells from LD muscle were separated by 2-DE. Approximately, 400 spots were detected in the silver-stained two-dimensional gels by computer-assisted image analysis. Further, to identify the differentially expressed proteins, a set of 5 gels were produced from each experiment and all the gels associated with the same collection were completely super imposable. Each spot detected by the Image Master (ver 7.0) was assigned a unique number to identify spots in a gel matching process. Twenty five spots were found to be differentially expressed between undifferentiated and adipogenic differentiating BSC (Figure 5). Among them, seven spots displayed approximately two fold or greater density and remaining eighteen spots showed lower densities (Table 2).

In particular, the expression level of spots 26, 7, 19, 9, 6, 8 and 21, which were identified as Cathepsin H precursor, Retinal dehydrogenase 1, Enoyl-CoA hydratase, Elongation

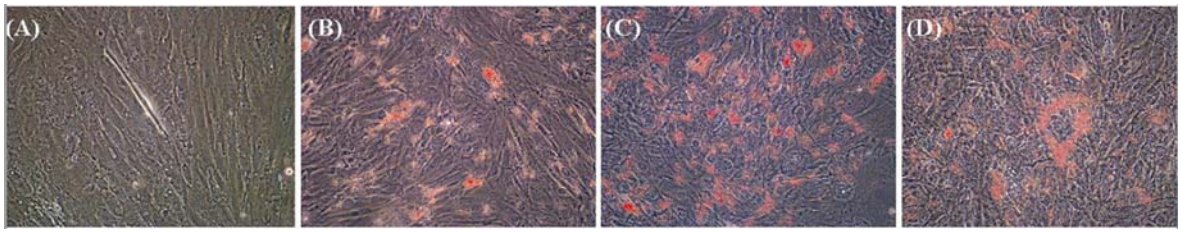

Figure 3. Oil Red O staining of transdifferentiating bovine muscle satellite cells on 0, 3, 6, 9 days. The cells were fixed and stained with Oil red O, in order to assess the degree to which lipid accumulation has taken place. (A) Undifferentiated BSC and (B-D) differentiation of satellite to adipocytes at 3, 6, 9 days.

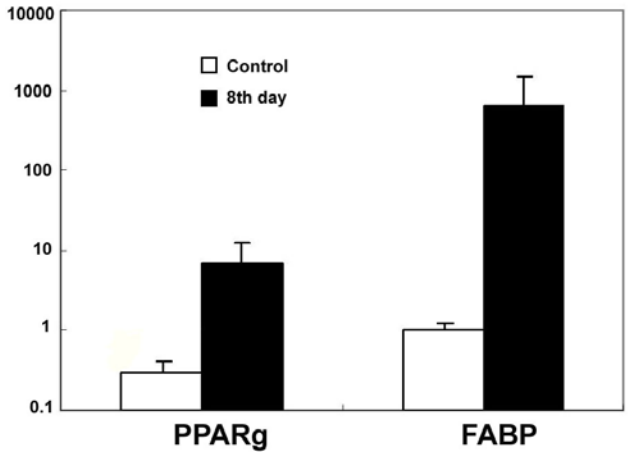

Figure 4. Relative mRNA levels of adipogenic markers, PPAR-γ and FABP in total RNA isolated from adipogenic differentiated bovine satellite cell cultures from longissimus dorsi muscle. The data points in the figure are from individual assays was the average of mRNA levels obtained from different culture experiments (SD±SE). Control represents the mRNA expression on the day 1 of the adipogenic induction.

factor Tu, Ubiquinol-cytochrome-c reductase, T-complex protein 1 subunit beta and ATP synthase D chain was 9.4, 8.7, 4.1, 3.0, 2.9, 2.9 and 2.6 fold higher in differentiating than in corresponding undifferentiated BSC. In case of spots which displayed reduced expression levels, structural proteins like Septin-2, cofilin-1 and macrophage capping protein; proteins involved in cellular processes like Annexin 1, Annexin 4, Annexin 5 and proteins involved in metabolism like phosphoglycerate mutase, triosephosphate isomerase, peptidyl-prolyl cis-trans isomerase were identified (Figure 5; Table 2). The majority of the proteins identified in our study were showing reasonably good total ion scores with more than 95% confidence interval (C.I), further supporting our identification of proteins mentioned in the Table 2.

DISCUSSION

In this study, we isolated satellite cells from bovine longissimus muscle and induced adipogenic differentiation. The isolated BSC proliferated and further showed the

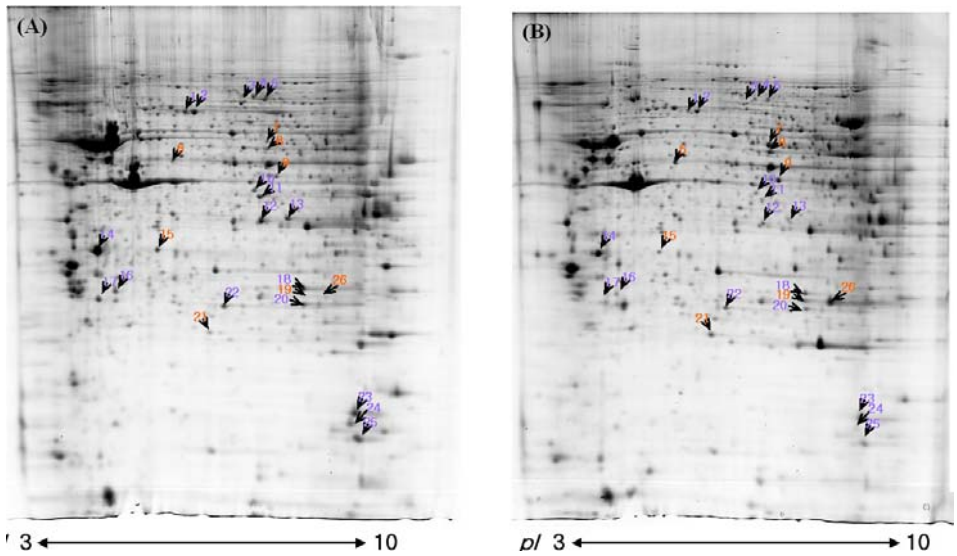

Figure 5. Representative 2-DE protein profiles of proliferating and transdifferentiating bovine muscle satellite cells. Proteins (500 μg) from subconfluent bovine muscle satellite cells (A) or adipocytes from 8th day after differentiation (B) was separated by 2-DE over the pIs range on the 3-10 NL, 12.5% acrylamide gels and stained with coomassie blue G-250. The positions of up regulated or down regulated proteins are annotated by numbered arrows.

Table 2. List of differentially expressed proteins during adipogenic transdifferentiation of satellite cells from bovine longissimus dorsi muscle

Spot No	Accession number	Protein name	Protein MW	Protein PI	Best peptide sequence	Start sequence position	End sequence position	Total ion score C.I. %	Fold change*
1	P02769	Serum albumin precursor	69248	5.82	LGEYGFQNALIVR	421	433	98.02	-0.4
					DAFLGSFLYEYSR	347	359		
					RHPEYAVSVLLR	360	371		
					KVPQVSTPTLVEVSR	437	451		
2	P02769	Serum albumin precursor	69248	5.82	RHPEYAVSVLLR	360	371	99.99	-0.5
					LGEYGFQNALIVR	421	433		
					DAFLGSFLYEYSR	347	359		
					YLYEIAR	161	167		
4	Q9CZD3	Glycyl-tRNA synthetase	81826	6.24	LLEFNQGKLPFAAAQIGNSFR	301	321	98.8	-0.4
					LGDAVEQGVINNSVLGYFIGR	382	402		
					LPFAAAQIGNSFR	309	321		
					MPCLLPSLLRATR	1	13		
					TFFSFPAVVAPFK	593	605		
5	Q9TST1	Cathepsin W precursor	41617	8.51	EVGSEEWGESVPPTCDWRK	118	136	95.6	-0.5
6	Q69BK0	Ubiquinol-cytochrome c reductase iron-sulfur subunit	29586	8.49	NAVTQFVSSMSASADVLAMAK	131	151		2.9
					EIEQEAAVELSQLR	183	196		
7	P48644	Retinal dehydrogenase 1	54668	6.23	TIPMDGNFFTYTR	143	155	100	8.7
					QAFQIGSPWR	68	77		
					GYFIQPTVFSDVTDDMR	378	394		
					YVLGNPLTPGVSQGPQIDKEQYEK	329	352		
					ELGEYGFHEYTEVK	476	489		
8	Q3ZBH0	T-complex protein 1	57308	6.19	LALVTGGEIASTFDHPELVK	322	341	99.98	2.9
		subunit beta			VQDDEVGDGTTSVTVLAAELLR	89	110		
9	P49410	Elongation factor Tu	49367	6.72	LLDAVDTYIPVPTR	239	252	100	3.0
					SLDRAEAGDNLGALVR	312	327		
					KYEEIDNAPEER	91	102		
					YEEIDNAPEER	92	102		
					GITINAAHVEYSTAAR	105	120		
					SLDRAEAGDNLGALVR	312	327		
10	Q15019	Septin-2	41461	6.15	STLINSLFLTDLYPER	51	66	96.87	-0.6
					ASIPFSVVGSNQLIEAK	233	249		
11	P40121	Macrophage capping protein	38494	5.88	YQEGGVESAFHK	116	127	100	-0.2
12	P46193	Annexin A1	38927	6.37	GVDEATIIEILTKR	59	72	100	-0.3
					GLGTDEDTLNEILASR	129	144		
					TPAQFDAEELR	114	124		
					GGPGSAVSPYPTFNPSSDVEALHK	30	53		
13	Q3B7M5	LIM protein 1	29658	6.6	LKQQSELQSQVR	74	85	100	-0.1
					GFSVVADTPELQR	97	109		
					QSFTMVADTPENLR	60	73		
					QSFTMVADTPENLR	60	73		
14	P81287	Annexin A5	35935	4.86	WGTDEEKFITIFGTR	186	200	100	-0.5
					GTVADFPGFDER	6	17		
					IDEAQVEQDAQALFQAGELK	166	185		

Table 2. List of differentially expressed proteins during adipogenic transdifferentiation of satellite cells from bovine longissimus dorsi muscle (Continued)

Spot No	Accession number	Protein name	Protein MW	Protein PI	Best peptide sequence	Start sequence position	End sequence position	Total ion score C.I. %	Fold Change*
15	P13214	Annexin A4	35735	5.55	INQTYQLQYGR	123	133	98.24	-0.5
					GLGTDEDAIINVLAYR	28	43		
					AASGFNAAEDAQTLR	9	23		
					SLEDDIRSDTSFMFQR	134	149		
16	P19803	Rho GDP-dissociation inhibitor 1	23276	5.12	VAVSADPNVPNVVVTR	58	73	99.99	-0.5
					AEEYEFLTPMEEAPK	152	166		
					AEEYEFLTPMEEAPK	152	166		
					SIQEIQELDKDDESLR	33	48		
					QSFVLKEGVEYR	99	110		
17	P13135	Calpain small subunit 1	27914	5.06	ILGGVISAISEAAAQYNPEPVPPR	56	79	100	-0.4
					SGTIGSSELPGAFEAAGFR	181	199		
					THYSNIEANESEEVR	80	94		
18	Q3SZ62	Phosphoglycerate mutase 1	28703	6.75	ALPFWNEEIVPQIK	162	175	36.68	-0.6
					VLIAAHGNSLR	180	190		
					SYDVPPPPMEPDHPFYSNISK	117	137		
					HGESTWNLENR	10	20		
19	Q58DM8	Enoyl-CoA hydratase	31223	8.82	AQFGQPEILIGTIPGAGGTQR	158	178	100	4.1
					NSNVGLIQLNRPK	44	56		
20	Q5E956	Triosephosphate isomerase	26542	6.51	VVLAYEPVWAIGTGK	160	174	100	-0.7
					HVFGESDELIGQK	100	112		
					TATPQQAQEVHEK	175	187		
21	P13620	ATP synthase D chain	18550	6.02	TIDWVAFGEIIPR	9	21	26.124	2.6
					KYPYWPHRPIETL	148	160		
					LATLPEKPPAIDWAYYK	41	57		
22	Q3T149	Heat-shock protein beta-1	22379	5.98	SATQSAEITIPVTFQAR	168	184	100	-0.7
					ALPAAAIEGPAYNR	58	71		
					LFDQAFGLPR	29	38		
					VSLDVNHFAPEELTVK	93	108		
					RVPFSLLR	2	12		
23	P23528	Cofilin-1	18360	8.26	NIILEEGKEILVGDVGQTVDDPYATFVK	45	72	99.99	-0.6
					YALYDATYETK	81	91		
					VFNDMKVR	13	20		
24	P24368	Peptidyl-prolyl cis-trans isomerase B precursor	22788	9.42	SIYGERFPDENFK	109	121	98.89	-0.7
					DTNGSQFFITTVK	138	150		
25	P22392	Nucleoside diphosphate kinase B	17287	8.52	TFIAIKPDGVQR	7	18	99.708	-0.2
					DRPFFPGLVK	57	66		
					VMLGETNPADSKPGTIR	89	105		
					VMLGETNPADSKPGTIR	89	105		
					QHYIDLKDRPFFPGLVK	50	66		
					FLRASEEHLK	40	49		
26	Q3T0I2	Cathepsin H precursor	37327	8.2	GYFLIER	311	317	100	9.4
					TPDKVNHAVLAVGYGEEK	275	292		
					GIPYWIVK	293	300		

* Negative sign indicates decreased fold change.

MyoD, Myf5 and myogenin expression, which are known to be useful markers for proliferating satellite cells (Allen and Rankin, 1990; Cooper et al., 1999). We induced the adipogenic differentiation using rosiglitazone, dexamethasone and insulin based on the previous study (Bernlohr et al., 1985). Differentiation was closely related to the activation of adipogenic transcription factor, PPAR-γ and adipogenic marker, FABP (Yada et al., 2006; Yamanouchi et al., 2006). In our study also, we found that BSC when induced with adipogenic differentiation showed PPAR-γ and FABP expression levels, further confirming that the adipogenic differentiation in BSC is via expression of a typical adipose differentiation program. Also, in the present study we found that, on adipogenic induction, the differentiating BSC began to accumulate lipid droplets. The lipid droplets, which comprise the characteristic morphology of adipocytes were shown to increase during the differentiation of BSC to adipocytes. ORO staining was used to confirm the conversion of BSC into lipid filled adipocytes during differentiation.

Numerous investigations have been focused on understanding the involvement of satellite cells for lipid accumulation within muscle in meat producing animals (Mauro, 1961; Singh et al., 2007; Chung and Johnson, 2008). The scientific knowledge on how marbling (accumulation of intramuscular fat) occurs within the muscle is limited to gene expression studies (Wang et al., 2005a,b). Genes responsible for intramuscular fatty acid formation and accumulation have been notably proposed (Bernard et al., 2007). However, no knowledge has been gained until now to reveal the proteins involved in the adipogenic differentiation of muscle satellite cells into adipocytes. Moreover, *in vitro* differentiation of muscle satellite cells into adipocytes will be an experimentally accessible system for studying the accumulation of fat within the muscle as this might be involved in marbling as well as in some pathological disorders like sarcopenia. Hence, in this study we searched and compared the differentially expressed proteins during transdifferentiation of bovine muscle satellite cells into adipocytes.

Cathepsin (Cat) H was highly expressed during adipogenic differentiation of BSC with 9.4 fold increase in its protein expression. Cathepsin proteins are the cysteine proteases that are found to control adipogenesis in humans and porcine (Yang et al., 2001). Compared with non-differentiated human preadipocytes, *in vitro* differentiated human adipocytes show elevated levels of Cat K and Cat L by 15 and 3 fold. The role of these proteins were further investigated in adipogenesis by using specific inhibitors of these proteins and inhibition of these proteins completely abolished the adipogenesis, further confirming the crucial role played by these proteins. Role of Cat proteins in adipogenesis was proposed in the remodeling of structural proteins that occur during adipose differentiation (Punturieri et al., 2000). As Cat H is highly expressed in differentiating BSC, this protein may be unique among bovine species and this can have a remodeling role during transdifferentiation of satellite cells into adipocytes. Retinaldehyde dehydrogenase (Raldh) exhibited 8.7 fold increase in its expression during differentiation. This enzyme controls the transition of retinaldehyde (Rald) to retinoic acid in vitamin A metabolism. Raldh was seen in fat cells and known to inhibit adipogenesis (Ziouzenkova et al., 2007). Higher expression of Raldh and its subsequent removal favor adipogenesis. Moreover, adipogenesis of fat cells was markedly decreased in mice lacking Raldh (Ziouzenkova et al., 2007), and increased levels of Raldh was seen in the adipocytes involved in fat accumulation (Perez-Perez et al., 2009). Taken together, these evidences suggest that Raldh protein may be positively associated with adipogenesis during transdifferentiation.

Enoyl-CoA hydratase with 4.1 fold increase in its expression during differentiation is positively correlated with adipogensis as development of intramuscular fat has been associated with fatty acid oxidation and ATP synthesis. Enoyl-CoA hydratase is an enzyme that is essential for the second step of β-oxidation in fatty acid metabolism and this process generates acetyl-CoA and ATP (Brian et al., 2002). Further, acetyl CoA is considered as one of the important molecules for *de novo* fatty acid synthesis. Lipogenesis is activated in adipocytes during differentiation, many enzymes for the lipid biosynthesis are highly up-regulated and this is considered as an energy driven process. Ubiquinol-cytochrome-c reductase (UQCRC1) was proved to be involved in the regulation of energy metabolism and balance (Kunej et al., 2007). UQCRC1 is an oligomeric enzyme that catalyzes the transfer of electrons from coenzyme QH2 to ferricytochrome c with the coupled translocation of protons across the mitochondrial inner membrane (Brandt and Trumpower, 1994). Further, it was shown that UQCRC1 gene was involved in fat deposition, energy transfer and genetic polymorphism in this gene might explain some cases of obesity in humans (Kunej et al., 2007). ATP synthase is the mitochondrial enzyme that was found to be highly expressed during differentiation with 2.6fold increase. Significant correlation between intramuscular fat content and mitochondrial enzymes has been reported (Pethick et al., 2005). Also, lipid biosynthesis requires mitochondrial enzymes as they are involved in the formation of acetyl-CoA, ATP and NADPH, that are essential for lipid biosynthesis and ATP synthase is known for oxidative respiration to generate NADPH and ATP (Kim et al., 2004). Our proteomic analysis of LD muscle BSC cells during transdifferentiation revealed a 2.9 fold higher expression of T-complex protein 1 (TCP 1). This is consistent with the previous proteomic analysis of bovine

LD muscle that showed an increase in TCP 1 protein. Further, they proposed that TCP 1 protein has a role in the remodeling of cytoskeletal protein like actin and tubulin (Kim et al., 2009). Remodeling of cytoskeletal proteins is one of the important cellular processes that occur during the adipogenesis (Renesa et al., 2005). Hence, increase in the levels of TCP-1 protein is in accordance with progression of adipogenesis, which further defines the role for TCP-1 protein during intramuscular fat accumulation.

Development of adipose tissue within skeletal muscle has been of interest to the field of domestic live stock research, since optimizing management of fat and lean growth is important to regulate carcass composition and meat quality (Asakura et al., 2001; Chung et al., 2006; Singh et al., 2007). Recent studies have shown that inhibition of myogenesis in satellite cells enabled adipogenic differentiation (Yeow et al., 2001). Also, it was reported that down regulation of muscle regulatory proteins MyoD and Myf5 that play a crucial role in myogenic differentiation enabled adipocytic differentiation (Hu et al., 1995). Some of the down regulated proteins identified in our proteomic analysis like LIM protein, annexin proteins, cofilin-1, Rho GDP-dissocaiation inhibitor 1 (Rho GDI) and septin-2 are already proved to be involved in myogeneis. LIM protein promotes myogenesis by enhancing the activity of MyoD. It was shown that over expression of LIM in C2C12 myoblasts enhanced skeletal myogenesis, whereas inhibition of LIM activity blocked terminal differentiation (Kong et al., 1996). Annexin proteins interacts with dysferlin protein and mediate the myogenesis (Lennon et al., 2003). A previous investigation has proved that cofilin is deeply involved in the regulation of actin assembly in developing skeletal muscle (Nagaoka et al., 1995). Rho GD1 protein is known to bind and alter *in vivo* expression of most of the RhoGTPase proteins (Dovas and Couchman, 2005). These RhoGTPase proteins are the crucial regulators of skeletal myogenesis (Bryan et al., 2005). Septin proteins are strongly implicated in the cytoskeletal organization during muscle cell proliferation (Kinoshita, 2006) and a proteomic study has already reported this protein to be high during myogenesis (Chaze et al., 2008). Taken together, all these evidence strongly implicates a role for these proteins in myogenesis. It has been already reported that, down regulation of proteins involved in myogenesis promotes adipogenic differentiation in myocytes (Yeow et al., 2001). Interestingly, our observation of down regulation of proteins involved in myogenesis during adipogenic differentiation of BSC does not diverge from the earlier observation.

CONCLUSION

This investigation of proteome profiling during *in vitro* adipogenic differentiation of bovine satellite cells provides the information about the proteins that may have a role in fat accumulation within the muscle. This information is important for future studies, as fat deposition within muscle is a significant process for both economical and medical point of view. To the best of our knowledge, this study is first of its kind to provide the proteomic details during transdifferentiation of bovine muscle satellite cells. Also, the data presented in the study should be useful in further understanding the role of proteins in transdifferentiaiton of satellite cells. These findings may also be relevant to the field of research concerned with human diseases, as bovine and human transdifferentiation share many commonalities.

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