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Proteomic Functional Characterization of Bovine Stromal Vascular Cells from Omental, Subcutaneous and Intramuscular Adipose Depots

Ramanna Valmiki Rajesh, Seong-Kon Kim, Mi-Rim Park, Jin-Seon Nam, Nam-Kuk Kim, Seulemina Kwon, Duhak Yoon, Tae-Hun Kim and Hyun-Jeong Lee*

Division of Animal Genomics and Bioinformatics, National Institute of Animal Science, Rural Development Administration, #564 Omockchun-dong, Suwon, Korea

ABSTRACT : Anatomically separate fat depots differ in size, function, and contribution to pathological states such as the metabolic syndrome. We isolated pre-adipocytes from different adipose depots, omental, subcutaneous and intramuscular, of beef cattle, and cultured *in vitro* to determine the basis for the variations and attribute these variations to the inherent properties of adipocyte progenitors. The proliferating cells from all depots before the confluence were harvested and the proteome was analyzed by a functional proteomic approach, involving 2-DE and MALDI-TOF/TOF. More than 252 protein spots were identified, selected and analyzed by Image Master (ver 7.0) and MALDI-TOF/TOF. Further, our analysis showed that there were specific differences in proteome expression patterns among proliferating precursor cells from the three depots. Sixteen proteins were found to be differentially expressed and these were identified as proteins involved in cellular processes, heat shock/chaperones, redox proteins, cytoskeletal proteins and metabolic enzymes. The results also enabled us to understand the basic roles of these proteins in different inherent properties exhibited by adipose tissue depots. (**Key Words :** 2-DE, Stromal Vascular Cells, Adipose Depot, Proteome)

INTRODUCTION

Adipose tissue is recognized not only as the main site of energy storage, but also as a complex, essential, and highly active metabolic and endocrine organ. For several decades, it has been appreciated that adipose tissue is regionally heterogeneous with respect to metabolic function (Arner, 1997; Kirkland et al., 1984, 1997). Adipocytes isolated from different depots differ in size, lipoprotein lipase release, lipid synthetic capacity, fatty acid incorporation and other characteristics (Hartman, 1985; Edens et al., 1993; Fried et al., 1993; Hube et al., 1996; Caserta et al., 2001). The interdepot variations observed solely a result of influences extrinsic to adipose cells (including their hormonal and paracrine micro environment, local nutrient availability, innervations, and anatomic constraints), or intrinsic differences in the innate characteristics of an adipocyte also contribute? Regional variations have been observed in replicative potential, fatty acid transfer and adipogenic differentiation of preadipocytes originating from various depots from the same individuals cultured under identical conditions (Hauner and Entenmann, 1991; Djian et al., 1993; Kirkland et al., 1996; Adams et al., 1997; Niesler et al., 1998). So, interdepot variations may not be solely a result of extrinsic influences, also inherent differences among the adipose cells likely to contribute.

The cellular development associated with adipose tissue growth involves both cellular hypertrophy (increase in size) and hyperplasia (increase in number). Hypertrophy is the result of excess triglyceride accumulation in existing adipocytes due to a positive energy balance (more intake and less energy expenditure). Hyperplasia (or "hypergenesis") is a general term referring to the proliferation of cells within an organ or tissue. Both these processes together are termed as adipogenesis that involves proliferation and differentiation of preadipocytes (adipose precursor cells). Preadipocytes are believed to be present throughout life (Prins and O'Rahilly, 1997) and are typically studied *in vitro* using both preadipocyte primary cultures (derived from the stromal vascular fraction of adipose tissue from various species including humans) (Loffler and Hauner, 1987; Novakofski and Hu, 1987; Gregoire et al., 1990; Litthauer and Serrero, 1992; Ramsay

* Corresponding Author: Hyun-Jeong Lee. Tel: +82-31-290-1594, Fax: +82-31-290-1594, E-mail: hyunj68@korea.kr
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et al., 1993) and established cell lines, of murine origin, committed to the adipocyte lineage (Cornelius et al., 1994). Evidence from cell line studies suggests that the proliferation of preadipocytes occurs prior to differentiation (Smyth et al., 1993; Cornelius et al., 1994). Cell proliferation studies involving either by directly i.e., incorporation of 3H-thymidine or 5-bromo-2-dexoyuridine (BrdU) or indirectly (increase in the fat cell number) revealed the proliferation of adipose precursor cells rather than mature fat cells per se (Hausman et al., 2001). Also studies of de novo labeling of replicating preadipocytes *in vivo* showed that age and anatomical site or depot greatly influence the timing and rate of hyperplastic growth (Kirkland and Harris, 1980). There are many factors to consider in evaluating or defining preadipocyte proliferation, and they have been extensively studied (Cryer et al., 1992; Ramsay et al., 1993). In the present study, we have made an attempt to evaluate the different inherent factors of proliferating preadipocytes from different depots by the functional proteomic approach.

MATERIALS AND METHODS

Animals

Five heads of Hanwoo (Korean cattle) steers were fed and managed in the feeding barn at Livestock Research Institute under the high quality beef production program (1997) and slaughtered at 24 months old. All experimental procedures and 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

Immediately, after stunning and exsanguination, the muscle and fat portions between the 6th to 7th rib were removed, and the subcutaneous and intramuscular fat depots were sampled from this rib section aseptically. The omental adipose tissue was taken within the lesser curvature of the abomasum. All these tissue samples were kept in sterile saline (0.154 M NaCl, 37°C) for recovery of stromal vascular cells (Cianzio et al., 1982). The stromal vascular fraction of adipose tissue was prepared as described by Cryer et al. (1987). Tissue was sliced and cells were released by collagenase digestion in the Krebs Ringer Bicarbonate (KRB) buffer (1.22 mM CaCl2) for 1 h. The digested tissue was filtered through a nylon mesh to separate cells from undigested tissue fragments and debris. The filtrate was centrifuged at 2,500 rpm for 5 min at room temperature. The pellet was washed twice by centrifugation (2,500 rpm, 5 min) with Hank`s Balanced Salt Solution (HBSS) and resuspended in a medium containing M199 supplemented with 10% Fetal Bovine Serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) and seeded in 10 mM petri-dish at a density of approximately 2,500 cells/cm^2. The cells were incubated at 37°C in 5% CO_2 in an air, with a change in the culture medium on every second day.

Cell counting

Cell number was determined at day 2, 4, 6, 8 and 10 post plating. Cell cultures were washed three times with saline, then trypsinized with calcium and magnesium free Hank's solution containing 0.2% trypsin and finally counted in a "Countess Automated Cell Counter" from Invitrogen Ltd, USA. Cell viability was assessed by trypan blue exclusion. Regardless of anatomical origin, 95% of cells excluded trypan blue.

Protein preparation for 2-DE

Proteins from stromal vascular cells were extracted in 0.5 ml of lysis buffer (7 M Urea, 2M Thiourea, 4% CHAPS, 100 mM DTT, protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Following 15min 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 were collected by centrifugation and then completely dried using speed-vac. Dried samples were 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 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 a Coomassie Brilliant Blue (CBB) G-250 staining method. The CBB-stained gels were scanned using a 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 for variability due to CBB staining and 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 protein between three depots was estimated by Student's t-test, $p<0.05$ and was done 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 et al. (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 a 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 was 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 samples were then dried for a second time prior to the MALDI-TOF-MS/MS analysis.

Sample peptide masses were obtained using the 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 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 auto proteolytic were removed, and molecular weight, pI and protein scores were considered.

Western blot analysis

Western blot analysis was carried out for a subset of four differentially expressed proteins. For western blotting, 40 µg of sample proteins were separated on SDS-PAGE and gels were transferred to PVDF membranes (Millipore) in the ice-cold transfer buffer (25 mM Tris-Cl, pH 8.3, 1.4% glycine, 20% methanol) at 250 mA for 90 min. Membranes were 1 treated with blocking buffer containing 5% non-fat milk (Becton, Dickinson and Company, MD, USA) in TBS/T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) and incubated overnight at 4°C. Primary goat mouse-HSPB1 (sc-1049), goat-anti-CFL (sc-32158) and goat-anti-ACTB (sc-47778) antibodies, all from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), at 1:400 dilutions and goat-anti-PRDX 1 (NBPI-006064; Novus Biologicals), at 1:5,000 were used in TBS/T with 5% non-fat milk. Following 2 h of incubation with primary antibodies, membranes were washed three times for 10 min each with 10 ml of TBS/T. Horseradish peroxidase-labeled (HRP) anti-goat and anti-mouse secondary antibody (sc-2020; sc-2005) was diluted 1:5,000 in TBS/T with 5% non-fat milk and incubated with the membranes for 1 h. After three 10-min washes, membranes were visualized using a chemiluminescent HRP substrate (Millipore) and a VersaDoc imaging system (Bio-Rad). All experiments were repeated in triplicate.

RESULTS

Depot origin affects the proliferation rate

The stromal vascular cells of adipose tissue were prepared from three depots, omental, subcutaneous and intramuscular (Figure 1A, B and C). Cells were proliferated *in vitro* in M199 supplemented 10% FBS with antibiotics (Figure 1D, E and F). During the initial days of cultivation, proliferation rate and pattern of preadipocytes from all the three depots were similar. As the cells reached confluence (day 10), preadipocytes from the intramuscular depot were showing higher proliferative rates in comparison to other two depots (Figure 1G). Although preadipocytes from all the three depots were showing different proliferation rate, the pattern of proliferation was found to be similar.

Mapping proteins expressed in preadipocytes from different depots

Proteome of the proliferating preadipocytes was analyzed by 2D gel coupled with MALDI TOF/TOF. Gels with separated proteins from omental (Figure 2A), subcutaneous (Figure 2B) and intramuscular (Figure 2C) depots are displayed in Figure 2. 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. Methodical replicates of silver stained gels of the same sample showed high reproducibility (95%) by comparison using Image Master ver 7.0 software. Spots were isolated and identified from CBB-stained gels in order to generate a 2-DE-proteome map. Using the 2-DE technology, 252 spots were mapped and 138 spots out of them were identified (Table 1). To classify identified proteins, 16 functional categories were established based on

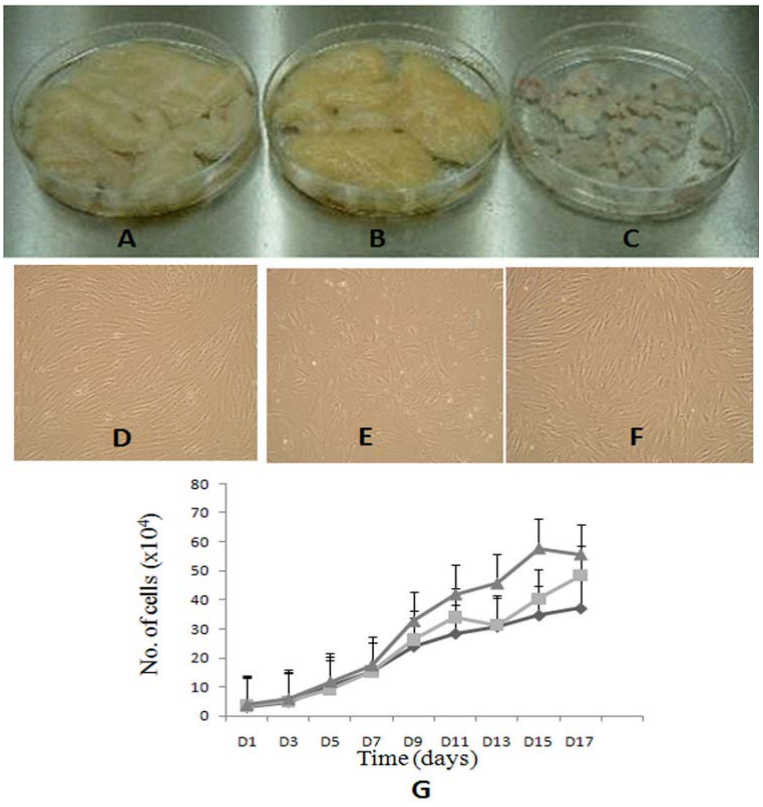

Figure 1. Adipose tissue from omental (A), subcutaneous (B), intramuscular (C). Proliferating stromal vascular cells from omental (D), subcutaneous (E), intramuscular adipose (F) tissue at end of the 9th day; (G) comparison of the proliferative rate between preadipocytes from three depots and results expressed here are the mean±SEM of triplicate experiments.

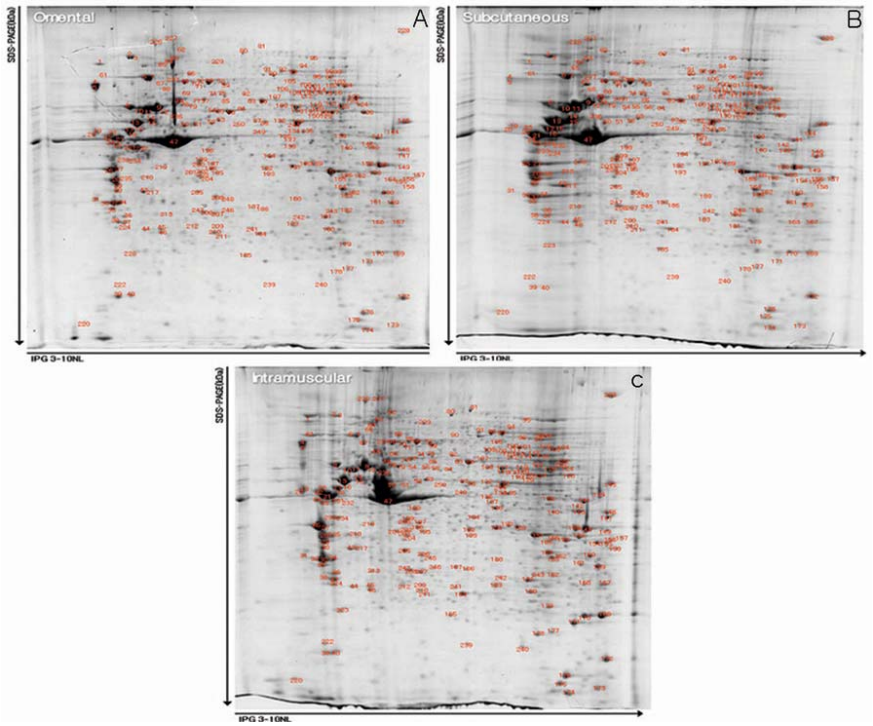

Figure 2. Two-dimensional polyacrylamide gel electrophoresis. Two-dimensional PAGE was performed with protein lysates prepared from proliferating preadipocytes from omental, subcutaneous and intramuscular depot. The gels were silver stained and was used as reference gels for our analysis.

Table 1. i) Common proteins identified from the proliferating preadipocytes from omental, subcutaneous and intramuscular depots*

Spot No.	Protein name	Protein score	Protein score (C.I %)	Swiss Prot Number	MW	pI
6	78 kDa glucose-regulated protein precursor (GRP 78)	228	100	P11021	72,332.96	5.07
7	Protein disulfide-isomerase precursor (EC 5.3.4.1) (PDI)	111	100	P09103	57,143.6	4.19
8	Vimentin	93	100	P48616	53,727.8	5.06
10	Tubulin beta-2 chain (Beta-tubulin class-II)	165	100	P32882	49,953.06	4.78
14	ATP synthase subunit beta, mitochondrial precursor (EC 3.6.3.14)	192	100	P00829	56,283.53	5.15
15	Protein disulfide-isomerase A6 precursor (EC 5.3.4.1)	87	100	Q922R8	48,100.39	5
22	Cis-2,3-dihydrobiphenyl-2,3-diol dehydrogenase (EC 1.3.1.56)	58	64	P72220	28,906.96	5.44
26	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (EC 1.17.4.3)	62	85	Q886Z0	39,763.85	6.36
30	HERV-K_1p13.3 provirus ancestral Env polyprotein	62	84	P61568	21,461.53	6.29
32	Hippocalcin-like protein 1 (Visinin-like protein 3) (VILIP-3)	59	67	P62749	22,338.24	5.32
33	Non-muscle caldesmon	62	91	Q27976	60,547.75	6.34
43	Annexin A5 (Annexin V) (Lipocortin V) (Endonexin II)	139	100	P81287	36,088.86	4.86
44	Calpain small subunit 1 (CSS1)	103	100	P13135	27,931.39	5.06
48	Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	81	100	P19378	70,804.92	5.24
49	60 kDa heat shock protein, mitochondrial precursor (Hsp60)	141	100	P18687	60,988.56	5.84
57	Prolyl 4-hydroxylase alpha-2 subunit precursor (EC 1.14.11.2)	67	95	Q60716	61,002.13	5.55
58	Stress-70 protein, mitochondrial precursor	128	100	P38646	73,680.50	5.81
60	Endoplasmin precursor (Heat shock protein 90 kDa beta member 1)	72	98	Q95M18	92,429.70	4.76
64	Actin, alpha sarcomeric/skeletal (Actin alpha 3)	89	100	P04752	41,983.85	5.22
70	Vacuolar ATP synthase catalytic subunit A, ubiquitous isoform	89	100	P31404	68,344.11	5.41
73	Annexin A6 (Annexin VI) (Lipocortin VI) (P68) (P70)	72	98	P08133	75,873.27	5.42
79	Tryptophanyl-tRNA synthetase (Tryptophan--tRNA ligase) (TrpRS)	106	100	P17248	53,812.05	5.49
80	Gelsolin	89	99	Q3SX14	80,645.69	5.58
81	Vinculin (Metavinculin)	190	100	Q64727	116,714.36	5.77
82	T-complex protein 1 subunit alpha (TCP-1-alpha) (CCT-alpha)	80	100	P28480	60,359.65	5.86
86	Vacuolar ATP synthase subunit B, brain isoform (EC 3.6.3.14)	84	100	P21281	56,500.73	5.57
89	Protein disulfide-isomerase A3 precursor (EC 5.3.4.1) (ERP60)	84	100	P38657	56,929.56	5.86
100	WD repeat protein 1 (Actin-interacting protein 1) (AIP1) (NORI-1)	120	100	O75083	66,193.52	6.17
101	WD repeat protein 1 (Actin-interacting protein 1) (AIP1)	123	100	O88342	66,406.70	6.11
102	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial precursor	76	99	Q8BH04	70,527.90	6.92
103	Transketolase (EC 2.2.1.1) (TK)	101	100	Q6B855	67,905.81	7.56
104	4-aminobutyrate aminotransferase (EC 2.6.1.19)	63	88	P14010	55,489.33	8.92
105	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	67	95	Q73Q55	33,417.44	8.66
106	Stress-induced-phosphoprotein 1 (STI1)	103	100	Q60864	62,582.11	6.4
107	Glutaminase kidney isoform, mitochondrial precursor (GLS)	65	92	O94925	73,461.10	7.85
108	Retinal dehydrogenase 1 (EC 1.2.1.36) (RalDH1) (RALDH 1)	115	100	P48644	54,805.76	6.24
109	Lamin-A/C (70 kDa lamin) (NY-REN-32 antigen)	114	100	P02545	74,139.49	6.59

Table 1. ii) Common proteins identified from the proliferating preadipocytes from omental, subcutaneous and intramuscular depots* (Continued)

Spot No.	Protein name	Protein score	Protein score (C.I %)	Swiss Prot Number	MW	pI
112	T-complex protein 1 subunit zeta (TCP-1-zeta) (CCT-zeta) (CCT-zeta-1)	66	95	Q3MHL7	57,956.17	6.32
113	Putative HTH-type transcriptional regulator protein MJ0300	61	80	Q57748	33,111.20	6.98
114	Lamin-A/C	130	100	P48678	30,776.86	8.98
118	T-complex protein 1 subunit beta (TCP-1-beta) (CCT-beta)	162	100	P80314	57,477.24	5.94
121	T-complex protein 1 subunit eta (TCP-1-eta) (CCT-eta)	122	100	Q2NKZ1	59,442.79	6.78
122	Pyruvate kinase isozyme M2 (EC 2.7.1.40)	88	100	P52480	57,844.89	7.17
126	ATP synthase subunit alpha heart isoform, mitochondrial precursor	192	100	P19483	59,719.64	9.21
127	Glutamate dehydrogenase 1, mitochondrial precursor (EC 1.4.1.3)	129	100	P00366	61,511.97	7.25
128	Serine hydroxymethyltransferase, mitochondrial precursor (EC 2.1.2.1)	135	100	Q3SZ20	55,605.50	7.67
129	Septin-11	68	96	Q5R8U3	49,033.00	6.89
131	Transcriptional regulator IE63 homolog	60	75	P28939	51,320.88	9.65
132	Alpha-enolase (EC 4.2.1.11) (NNE)	97	100	Q9XSJ4	47,326.13	6.37
134	Alpha-centractin (Centractin) (Actin-RPV) (ARP1)	114	100	P61163	42,613.74	6.19
135	Elongation factor Tu, mitochondrial precursor (EF-Tu)	171	100	P49410	49,398.26	6.72
136	Isocitrate dehydrogenase [NADP] cytoplasmic (EC 1.1.1.42)	151	100	Q9XSG3	46,785.42	6.13
137	Septin-2	83	100	Q5RA66	41,503.47	6.15
139	Citrate synthase, mitochondrial precursor (EC 2.3.3.1)	97	100	Q29RK1	51,772.54	8.16
140	Synaptotagmin-15 (Synaptotagmin XV) (SytXV)	67	95	Q8C6N3	47,270.32	8.18
141	Phosphoglycerate kinase 1 (EC 2.7.2.3)	129	100	P00559	44,602.68	8.68
143	Fructose-bisphosphate aldolase A (EC 4.1.2.13) (Muscle-type aldolase)	101	100	P00883	39,342.89	8.31
145	Elongation factor 1-alpha 1 (EF-1-alpha-1) (Elongation factor 1 A-1)	87	100	P62629	50,113.84	9.1
146	Annexin A2 (Annexin II) (Lipocortin II) (Calpactin I heavy chain)	91	100	P07355	38,604.04	7.57
147	Aspartate aminotransferase, mitochondrial precursor (EC 2.6.1.1)	79	100	P12344	47,513.53	9.19
148	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (GAPDH)	90	100	P00355	35,836.05	8.51
149	Heterogeneous nuclear ribonucleoproteins A2/B1	152	100	P22626	37,429.70	8.97
152	L-lactate dehydrogenase A chain (EC 1.1.1.27) (LDH-A)	124	100	P19858	36,597.64	8.12
153	Malate dehydrogenase, mitochondrial precursor (EC 1.1.1.37)	87	100	P00346	35,595.63	8.93
154	Heterogeneous nuclear ribonucleoproteins A2/B1	179	100	O88569	37,402.67	8.97
155	Calponin-1 (Calponin H1, smooth muscle) (Basic calponin)	87	100	Q08091	33,355.59	9.05
158	Heterogeneous nuclear ribonucleoprotein A1 (Helix-destabilizing protein)	197	100	P04256	34,212.26	9.2
160	Voltage-dependent anion-selective channel protein 1 (VDAC-1)	139	100	P21796	30,772.60	8.62
161	Non-structural RNA-binding protein 53	65	93	P30212	57,645.33	8.10
162	Iron sulfur cluster assembly protein 1, mitochondrial precursor	63	89	Q6FJY3	22,900.85	9.51
163	Potassium voltage-agted channel subfamily D member 1	61	82	Q03719	71,697.79	8.56
165	Calponin (Calponin H2, smooth muscle)	75	99	Q5RFN6	33,697.05	6.94
168	Proteasome subunit alpha type 7	75	99	O14818	27,886.85	8.60
169	Transgelin (smooth muscle protein 22-alpha)	166	100	Q9TS87	22,598.85	8.87

Table 1. iii) Common proteins identified from the proliferating preadipocytes from omental, subcutaneous and intramuscular depots* (Continued)

Spot No.	Protein name	Protein score	Protein score (C.I %)	Swiss Prot Number	MW	pI
170	Peroxiredoxin 1	114	95	Q5E947	22,195.39	8.59
171	Transgelin 2	69	97	Q5E9F5	22,426.49	8.40
172	Peptidyl-propyl cis-trans isomerase B precursor (EC 5.2.1.8)	104	100	P80311	23,746.52	9.33
174	Peptidyl-propyl cis-trans isomerase A precursor (EC 5.2.1.8)	64	90	P62935	17,869.35	8.34
175	UPF0343 protein MK0485	63	90	Q8TY21	37,111.78	6.00
179	GLuthathione S-transferase P (EC 2.5.1.18)	79	99	P28801	23,613.19	6.89
180	Triosephosphate isomerase (EC 5.3.1.1)	93	99	Q5E956	26,689.51	6.45
181	Phosphoglycerate mutase 1 (EC 5.4.2.1)	69	97	Q3SZ62	28,851.99	6.67
183	Peroxiredoxin 6	125	100	O77834	24,920.01	6.02
184	Heat shock protein beta-1 (HSP 27)	105	100	Q3T149	22,379.33	5.98
185	DNA-directed RNA polymerase beta chain	62	86	Q4L3K4	135,006.84	6.14
187	Purine nucleoside phosphorylase	105	100	P55859	32,036.54	5.92
186	Cofilin (non-muscle isoform)	87	99	Q5E9F7	18,587.70	8.26
188	Proteasome subunit alpha type 1 (EC 3.4.25.1)	77	99	P25786	29,555.59	6.15
189	Heterogeneous nuclear ribonucleoprotein M	67	96	P52272	77,515.53	8.84
191	Annexin A1 (Lipocortin I)	139	100	P46193	38,951.68	6.38
192	Glutamyl-tRNA (GLn) amidotransferase subunit A	65	93	O06491	52,663.74	5.39
193	26S proteasome non-ATPase regulatory subunit 14	91	99	O35593	34,577.04	6.06
194	Isocitrate dehydrogenase (NAD) subunit, mitochondrial precursor	110	100	P41563	39,667.84	6.76
195	Cell division control protein 2 homolog (p34 protein kinase)	64	92	Q9DGA5	34,605.13	8.60
197	60S acidic ribosomal protein P0	92	99	Q95140	34,370.62	5.72
198	26S Proteasome non-ATPase regulatory subunit 13	92	99	Q5E964	42,866.34	5.44
199	Actin alpha sarcomeric/cardiac	62	85	P20399	42,033.00	5.23
200	Actin, cytoplasmic 2	79	99	P63256	41,877.95	5.39
201	Tubulin beta 2 chain	123	100	P83130	49,955.05	4.77
202	Peroxiredoxin 4	112	100	Q9BG12	30,721.98	6.01
205	Annexin A4	182	100	P13214	35,735.06	5.55
206	F-actin capping protein subunit beta isoforms 1 and 2	64	90	P14315	30,610.64	5.69
207	Chloride intracellular channel protein 4	75	99	Q9XSA7	28,727.07	5.60
208	Prohibitin	207	99	P35232	29,804.10	5.57
211	Proteasome subunit beta type 4 (EC 3.4.25.1)	60	95	Q3T108	29,031.08	5.52
212	Actin, cytoplasmic 1	58	94	O18840	41,736.73	5.29
213	Proteasome subunit beta type 3 (EC 3.4.25.1)	92	99	Q58DU5	28,405.17	5.19
228	Collagen alpha-2 (I) chain precursor	79	99	P02465	129,063.58	9.23
236	Actin, cytoplasmic I	122	100	Q71FK5	41,736.73	5.29
237	Ena/VASP-like protein	55	87	O08719	42,094.88	8.74
238	Rho GDP-disassociation inhibitor 1 (Rho-GD1 alpha)	97	100	P19803	23,421.40	5.12
242	Phosphoglycerate mutase I (EC 5.4.2.1)	58	93	Q9DBJ1	28,831.99	6.67
243	Annexin A2 (Lipocortin II)	61	97	P04272	38,612.07	6.92
250	26S protease regulatory subunit 7 (protein MSSI)	124	100	P46471	48,647.88	5.72
251	Prolyl4-hydoxylyase alpha-1 subunit precursor	102	100	Q5RAG8	61,049.30	5.70
252	Elongation factor 2 (EF-2)	81	99	P13639	95,338.14	6.41

* Proteins (isoforms) that appeared in three or more spots are Septin-11 and Retinal dehydrogenase (3 spots); Lamin A/C (6 spots); Vimentin (10 spots).

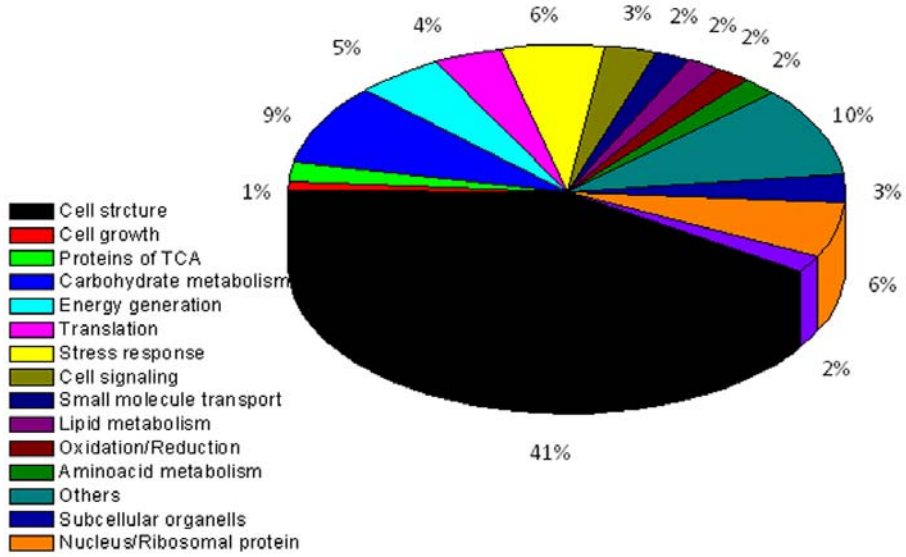

Figure 3. Functionality of common identified proteins in proliferating preadipocytes from three depots. The 16 functional protein categories found were based on the n = 138 individual proteins and are largely assigned according to the gene ontology database with additional help from Swissprot. The largest group comprising 41% was related to cell structure and next being proteins of carbohydrate metabolism (9%). The proteome of the proliferating preadipocytes was categorized based on the information contained in Swiss-Prot. The "Other" function category (10%) includes cytokine, ion channel, iron-binding protein, signal transduction, and transcription.

information from Gene Ontology database and additional information from ExPasy (http://www.expasy.org/sprot/). About 41% of the proteins were related to cell structure, 9% to carbohydrate metabolism, 6% stress response, 5% of proteins in energy generation, followed by the groups of proteins involved in translation, lipid metabolism, cell signaling and others comprise of 10% of total identified proteins (Figure 3).

Differential protein expression of preadipocytes from three depots

To evaluate the differential protein expression in proliferating preadipocytes from omental, subcutaneous and intramuscular depots, we used a 2 fold cutoff ($p<0.05$) from our image analysis studies to designate up and down regulated proteins. Figure 4A, B and C represent the differential protein expression of omental versus subcutaneous, subcutaneous versus intramuscular and omental versus intramuscular depots respectively. This comparative analysis revealed the presence of 16 different proteins, and they were categorized into 6 functional groups based on the gene ontology function: heat shock proteins/chaperones (HSPB 1, HSPA 9 and HSPA 5); redox (PRDX 6, 4 and 1); cytoskeleton (ACTB, CFL1, TAGLN and GSN); cell processes (ANXA 6, 5 and 4); metabolism (LDHB and ALDH1A1) (Table 2).

Our image analysis along with in-depth spot analysis (lower panel of Figure 4A, B and C) revealed omental depot with higher expressions of ANXA 6, HSPB1, TAGLN, ALDH1A1 and CALD 1, whereas in the subcutaneous depot, expressions of PRDX 6 and GSN were found to be high. Differential protein expression analysis between omental and intramuscular depots showed higher expressions of HSPA 5, HSPA 9, CALD 1, PRDX 4, ALDH1A1 and TAGLN in omental, while ANAX 4 and ACTB in intramuscular depots. Results from subcutaneous versus intramuscular depots revealed higher expression of ANAX 5, PRDX 1 and LDHB in intramuscular, whereas CFL 1 was found to be high in the subcutaneous depot.

Western blot analyses

For western blot analyses, immunoblots prepared with the protein extracts from proliferating preadipocytes from all the three depots used in the proteomic analysis. These immunoblots were further probed with antibodies for detection of a subset of four proteins (CFL 1, ACTB, HSPB 1 and PRDX 1) that are differentially expressed in our proteomic analyses (Figure 5). Our immunoblot results were found be corroborating with our proteomic analyses, further confirming the differential expression of proteins during preadipocyte proliferation.

DISCUSSION

The basis for depot specific differences in proliferating preadipocytes has not been studied. Most of the studies until now are concentrated on the regulation of adipocyte differentiation, as it is considered to be the most important event during adipogenesis. In the present study, proliferation rate of preadipocytes although was same in the

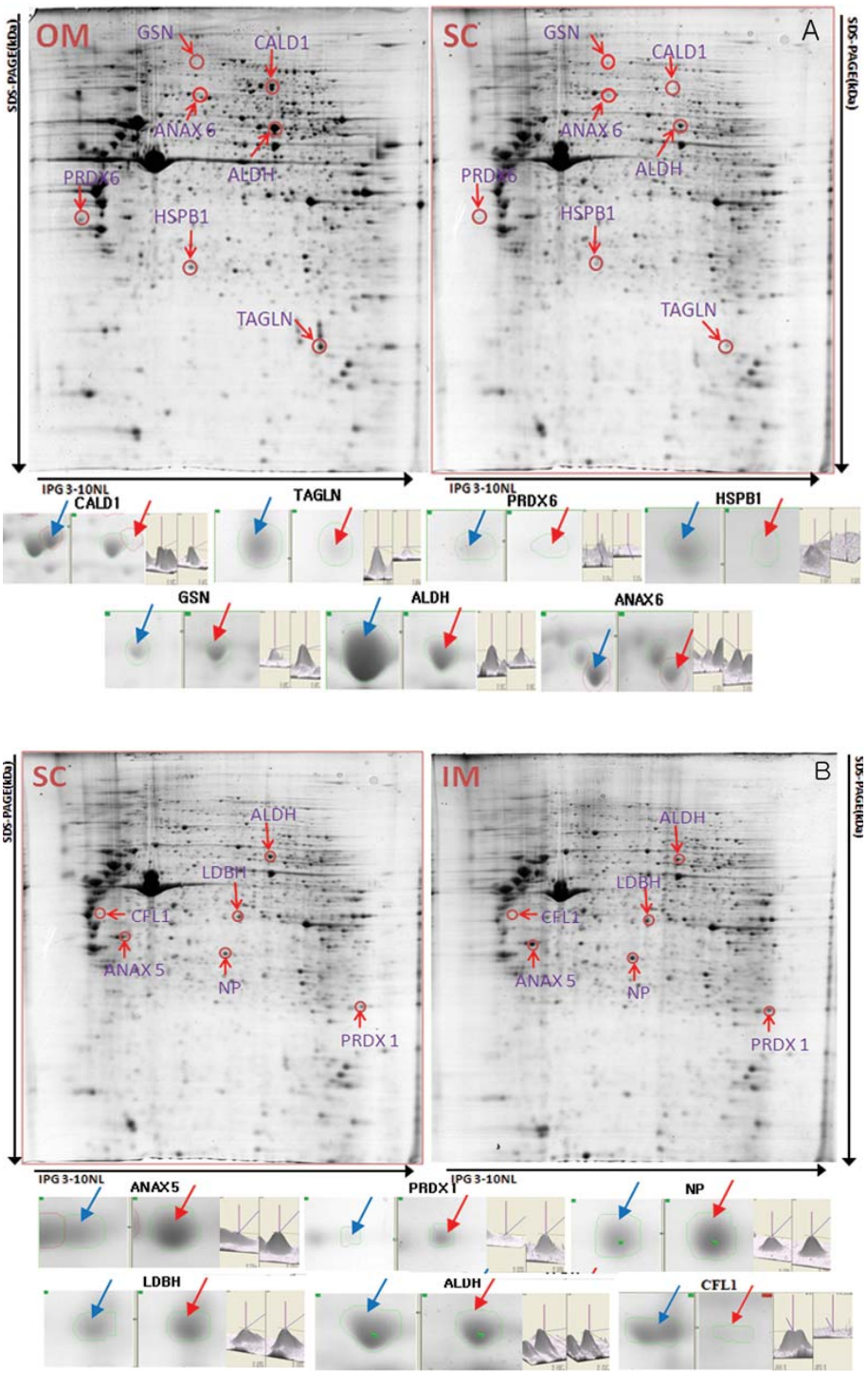

Figure 4. i) 2-DE map of proteins from proliferating preadipocytes from different depots. Protein of spots of coomassive stained 2-DE gels (non linear pH 3-10) of proliferating preadipocytes from different depots were isolated and identified via MALDI-MS/MS: (A) differentially expressed proteins between omental (OM) and subcutaneous (SC) depots. (B) subcutaneous and depots and intramuscular (IM) (C) omental and intramuscular depots. The lower panel in all the three figures reveals in-depth analysis of the protein spots and their representation in 3D view. Proteins that are identified as differentially regulated are summarized in Table 2. The proteins discussed in these figures specify at least a 2 fold change in their protein expression compared to each other.

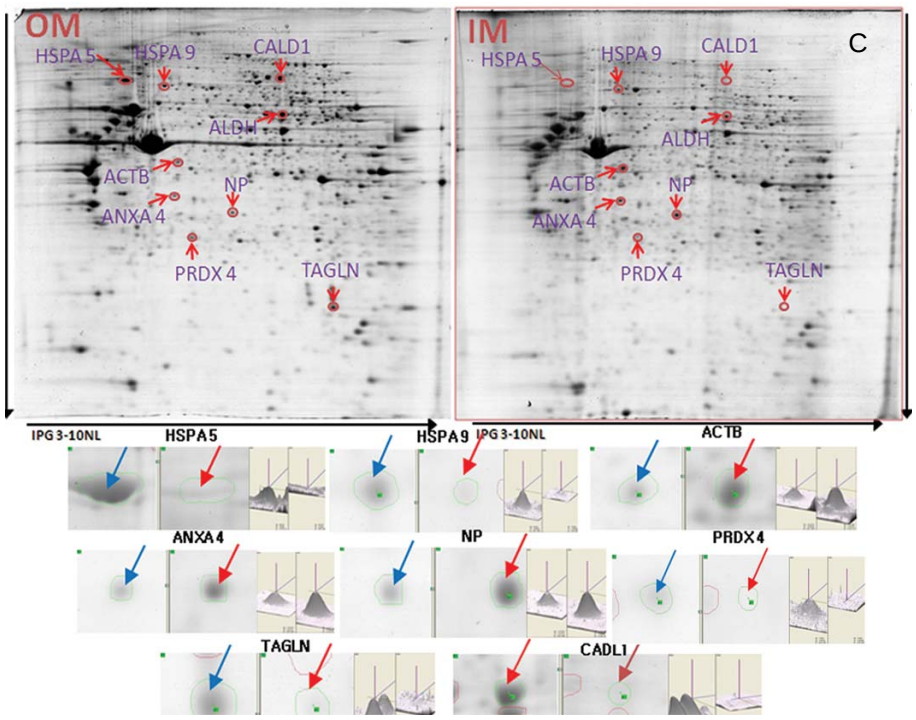

Figure 4. ii) 2-DE map of proteins from proliferating preadipocytes from different depots. Protein of spots of coomassive stained 2-DE gels (non linear pH 3-10) of proliferating preadipocytes from different depots were isolated and identified via MALDI-MS/MS: (A) differentially expressed proteins between omental (OM) and subcutaneous (SC) depots. (B) subcutaneous and depots and intramuscular (IM) (C) omental and intramuscular depots. The lower panel in all the three figures reveals in-depth analysis of the protein spots and their representation in 3D view. Proteins that are identified as differentially regulated are summarized in Table 2. The proteins discussed in these figures specify at least a 2 fold change in their protein expression compared to each other.

Table 2. Comparison of differentially expressed proteins from proliferating preadipocytes of omental, subcutaneous and intramuscular depots

Function	Name protein	OM vs. SC	OM vs. IM	SC vs. IM
Cell processes	Annexin A6 (ANXA 6)	Low in SC/High in OM	-	-
	Annexin A5 (ANXA 5)	-	-	Low in SC/High in IM
	Annexin A4 (ANXA 4)	-	Low in OM/High in IM	-
Heat shock /chaperone	Heat shock 27 kDa protein 1 (HSPB 1)	Low in SC /High in OM	-	-
	Heat shock 70 kDa protein 9 (HSPA 9)	-	Low in IM/High in OM	-
	Heat shock 70 kDa protein 5 (HSPA 5)	-	ND in IM	-
Redox	Peroxiredoxin 6 (PRDX 6)	Low in OM/High in SC	-	-
	Peroxiredoxin 4 (PRDX 4)	-	Low in IM/High in OM	-
	Peroxiredoxin 1 (PRDX 1)	-	-	Low in SC/High in IM
Cytoskeleton	Actin, beta (ACTB)	-	Low in OM/High in IM	-
	Cofilin 1 [non-muscle] (CFL 1)	-	-	High in SC/Low in IM
	Transgelin (TAGLN)	Low in SC/High in OM	Low in IM/High in OM	-
	Caldesmon 1 (CALD 1)	ND in SC	ND in IM	-
	Gelsolin (GSN)	Low in OM/High in SC	-	-
Metabolism	Lactate dehydrogenase B (LDHB)	-	-	Low in SC/High in IM
	Aldehyde dehydrogenase 1 (ALDH1A 1)	Low in SC/High in OM	Low in IM/High in OM	-

OM = Omental; SC = Subcutaneous; IM = Intramuscular; ND = Not detected.
The differential expression was determined from the in-depth protein spot analysis along with 3D analysis of the spots (please refer bottom panel of Figure 4A, 4B, 4C for OM vs. SC, SC vs. IM and OM vs. IM respectively).

initial stages of growth, as cells reached confluence, the preadipocytes from the intramuscular depot were found to have higher proliferation rates. It has been demonstrated that preadipocytes from different depots in rats (Kirkland et al., 1992; Lacasa et al., 1997) porcine (Samulin et al., 2008) and humans (Tchkonia et al., 2002; Van Harmelen et al., 2004) vary in proliferation rate. The depot-specific differences in proliferation rate observed in our study can be attributed to the inherent changes in the protein expression that control the proliferation. This can be considered as a valid reason as the preadipocytes were grown in similar *in vitro* conditions. Also, the reason for us to select preadipocytes for this study is, despite exposure to hormonal manipulations *in vivo*, such as estrogen treatment, hypophysectomy, or castration, preadipocytes cultured from various depots retain distinct cell-dynamic and biochemical responses relative to other depots (Kirkland et al., 1992; Lacasa et al., 1997).

Until now, proteomic comparative analyses of proliferating preadipocytes from different adipose depots of bovine are lacking. But, proteomic analysis of the adipocyte was performed using cell lines such as 3T3-L1 preadipocytes. Many studies involving the effect of hormones (Fasshauera et al., 2002), apopototic factors (Renes et al., 2005) and oligosaccharides (Rahman et al., 2008) on differentiating 3T3-L1 preadipocytes are in literature. Our proteomic analyses displayed about 16 differently expressed proteins, and they belong to similar categories observed in proteomic analysis of primary cultures of human adipose-derived stem cells (James et al., 2005). Further, our immunoblot results for a sub set of four different proteins were found to be corroborating with our proteomic analyses, further confirming the differential expression of proteins during proliferation. *In vivo* studies demonstrated that preadipocyte proliferation, differentiation and their interrelations are very complex and reported to be defined by a number of factors including age, hormones, species and depot (Hartman, 1985). Most of the proteins that were differentially expressed during proliferation in this study were also reported during differentiation of 3T3-L1 cells (Adachi et al., 2007; Barcelo-Batllori and Gomis, 2009). Although studies from Petrak et al. (2008) and Wang et al. (2009) have shown that, many of our differentially expressed proteins as common cellular stress proteins, but most of them were already reported to have a role in cell proliferation and lipid metabolism. Hence, we intend to discuss the role of differently expressed proteins in our subsequent sections.

Differential expression of proteins during preadipocyte proliferation from different depots

Heat shock proteins/chaperones-Preadipocyte proliferation has resulted in the induction of three different heat shock proteins/chaperones (HSPB1, HSPA9 and HSPA5). Apart from proteomic analyses, higher expression of HSPB1 was confirmed in our immunoblot analyses (Figure 5). HSPB1 was already reported to be high in omental preadipocytes (Perez-Perez et al., 2009), and it has been shown to be involved in the modulation of adipocyte differentiation and metabolism. It was reported that HSPB1 interacts with insulin-like growth factor receptor 1 and its signal transducer, the serine/theronine kinase Akt, which together modulate adipocyte metabolism (Rane et al., 2003; Shan et al., 2003).

HSPA9 protein is highly expressed in proliferating omental preadipocytes and this protein is very well shown to be involved in adipogenesis. In fact, omental fat was already reported to have over expression of heat shock proteins (Perez-Perez et al., 2009). Moreover, there is a growing body of literature linking chaperone-like molecules to adipogenesis, obesity, and diabetes (Cherian and Abraham, 1995; Kurucz et al., 2002; Kumar et al., 2004; Ozcan et al., 2004), For example, adipogenesis in 3T3-L1

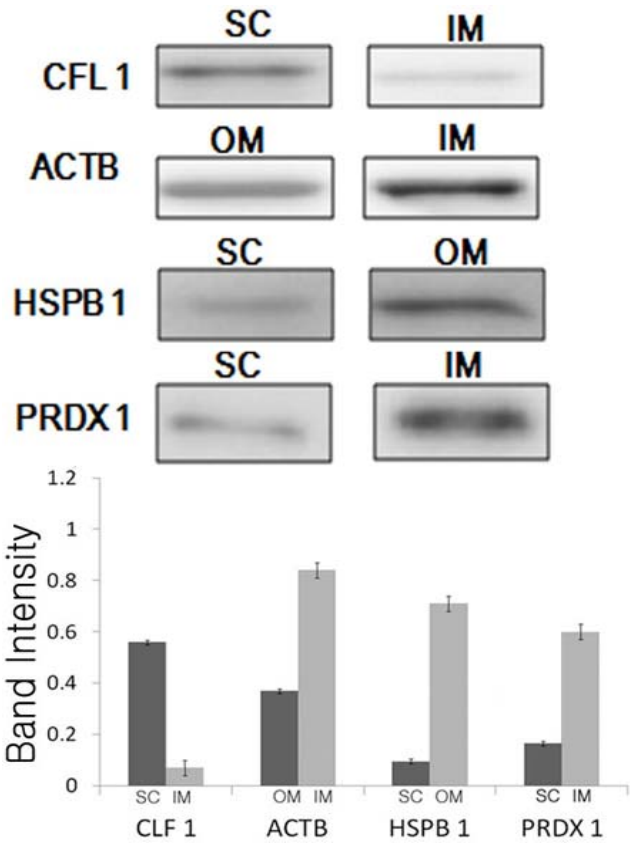

Figure 5. For immunoblot detection, protein lysates from proliferating preadipocytes of omental (OM), subcutaneous (SC) and intramuscular (IM) depots were examined on immunoblots using antibodies detecting CLF 1, ACTB, HSPB 1 and PRDX 1. As a loading control and for normalization, the blot was immunostanied with antibody against protein GAPDH, which was found to have similar protein expression among three depots (p value is 0.1612; $p>0.05$). Data represent mean±SE (n = 3).

cells is accompanied by increased expression of the chaperone-related immunophilin, FK-binding protein 51 (Yeh et al., 1995). Moreover, the nuclear hormone receptors that control adipogenic transcription, the glucocorticoid receptor, and the peroxisome proliferation-activated receptor, are sequestered in the cytosol as a complex with HSPA 9 prior to ligand activation (Hache et al., 1995; Young et al., 2005). It is interesting to note that clinical studies have linked polymorphisms in HSPA 9 to an increased risk for obesity and type 2 diabetes (Chouchane et al., 2001; Zouari Bouassida et al., 2004). It has been also reported that preadipocytes from the omental depot are more susceptible to apoptosis (Niesler et al., 1998). Presence of HSPA 9 in omental preadipocytes may protect them from apoptosis, because HSPA9 also called as mortalin and is known to have antiapoptotic role. Mortalin was shown to inhibit apoptosis by inactivating p53 (Wadhwa et al., 1998; Kaul et al., 2001).

HSPA 5 also known as glucose-regulated protein 78 kDa (GRP 78) was found to be absent in intramuscular depot. GRP 78, is well known to positively modulate the adipogenesis. This protein is critical to the regulation of a transcription factor, X-box binding protein 1 (XBP1) (Shi et al., 2009) and this XBP 1 was shown to play a key role in adipocyte differentiation by acting as a critical regulator of the morphological and functional transformations during adipogenesis (Sha et al., 2009). Hence, the absence of this protein expression in the intramuscular depot may be one of the factors that contribute to the low level of fat seen in this depot from adult animal.

Redox : Three anti-oxidative proteins peroxiredoxin1, 4 and 6 (PRDX 1, 4, 6) are found to be differently expressed in our proteomic analysis. PRDX 1, 4 and, 6 proteins were highly expressed in proliferating preadipocytes from intramuscular, omental and, subcutaneous depots respectively. It has been reported that proliferating cells are always at the risk of the oxidative damage (Villani et al., 2000). So, all the proliferating preadipocytes from three depots are been guarded by one or the other anti-oxidant protein. More specifically, PRDX 1 was reported to be over expressed to the high proliferative signals (Prosperi et al., 1998) and the presence of this protein in high levels from the intramuscular depot also supports our observation of high proliferative rate among the preadipocytes from this depot. High levels of PRDX 4 in omental preadipocytes are related to the anti-apoptotic function and as already mentioned that preadipocytes from the omental depot are more sensitive to apoptotic stimuli. Anti-apoptotic nature of PRDX 4 is by activating NF-kB (Jin et al., 1997). PRDX 6 plays a role in protecting cells from cell death by its antioxidant property (Hoehn et al., 2003).

Cytoskeleton : Actin (ACTB), cofilin 1 (CFL 1) and transgelin (TAGLN) are the three proteins that have a role in cytoskeleton development are found to be differently expressed across the depot specific proliferating preadipocytes. ACTB was shown to play a critical role in cell architecture/mobility and is essential for various life cyclic processes (Weil et al., 2009). In fact, mechanical tension, acting through the actin filament complex, can control the differentiation status of adult stromal stem cells (Takenouchi et al., 2004). Furthermore, ACTB was gradually shown to increase during adipose conversion of bovine intramuscular preadipocyte cells (McBeath et al., 2004), suggesting that a rearrangement of cytoskeletal proteins has a role in the intracellular accumulation of lipid droplets (Luegmayr et al., 1996). Cofilins are a family of actin-binding proteins and are expressed in all eukaryotic cells. Presence of CFL 1 in subcutaneous adipose tissue is already reported (Choi et al., 2003). TAGLN, also called as SM22α although is a structural protein, we here implicate this protein to involve in adipogenic differentiation. Transcriptional up regulation of SM22α down regulates protein kinase C (PKC) (Mayr et al., 2004). This down regulation of PCK has a direct effect on 3T3-F442A cell adipogenic differentiation (Fleming et al., 1999). In the case of gelsolin (GSN), which is absent in the omental depot may have a role as an anti-apoptotic factor. GSN inhibits apoptosis by inhibiting the TNF-α, which is one of the proteins that actively participate in apoptosis (Sezen et al., 2009).

Cellular processes : Annexins are the family of calcium-dependent phospholipid binding proteins. In our study, we found three annexins (VI, V and IV) differently expressed among the depots. The preadipocytes from the intramuscular depot were found to express more ANXA 5, and adipocytes from this depot was shown to have less amount of fat than omental. Studies have shown that ANXA 5 inhibits two important proteins that have the direct regulation on fat synthesis. Protein kinase C (PKC) is inhibited by ANXA 5 via a mechanism of phospholipids (PS) sequestration, as annexin proteins are known to have domains that are involved in phospholipid binding. This sequestration of phospholipids by ANXA 5 may lead to a reduction in the amounts of PS that is required for PKC activation (Dubios et al., 1998). In fact, PKC promotes adipogenic commitment and is essential for the terminal differentiation of 3T3-F442A preadipocytes (Webb et al., 2003). PKC is also involved in the phosphorylation of CCAAT-enhancer-binding protein (C/EBP) family and these proteins are transcription factors known to have direct interaction with adipocyte differentiation genes (Darlington et al., 1998). ANXA 5 also inhibits the A_2 (PLA_2) activity by inhibiting PKC, which is required for activating PLA_2 through phosphorylation (Mira et al., 1997). A study from Gao and coworkers has shown that PLA_2 is a differentiation specific enzyme activity in adipogenic cell line and

adipocyte precursors in primary culture (Gao and Serrero, 1990). Hence, inhibition of both proteins by ANXA 5 in the intramuscular depot will definitely play a major role in adipogenic differentiation and lipogenic pathways.

Metabolism : Adipogenesis in preadipocytes is accompanied by the induction of proteins associated with glycolysis and other enzymes that participate in the metabolism of the cell. In this category, we found two metabolically active enzymes that are differentially expressed in the preadipocytes. Aldehyde dehydrogenase 1 (ALDH1A1) was found to be in its higher expression in omental preadipocytes in comparison to the other two depots. There is also a study reporting the increased levels of ALDH1A1 in omental fat (Perez-Perez et al., 2009). Moreover, mice lacking ALDH1A1were preserved from developing diet-induced obesity and insulin resistance (Ziouzenkova et al., 2007), and it was also shown that increased levels of ALDH1A1 in the obese omental fat might involve in fat accumulation (Perez-Perez et al., 2009).

Lactate dehydrogenase B (LDHB) is another important enzyme highly expressed in the preadipocytes of intramuscular depot. LDHB is an important enzyme in the glycolytic pathway as it converts pyruvate, the final product of glycolysis to lactic acid. It also performs the reverse reaction during the cori cycle in the liver generating ATP. LDHB is seen to be more expressed in cells that are involved in fast proliferation (Pan et al., 1991). This justifies our observation of fast proliferation of intramuscular preadipocytes and high expression of LDHB.

Most of the proteins that are differentially expressed in proliferating preadipocytes like, a number of annexins, cytoskeletal and heat shock proteins have already been reported in the proteome analysis of differentiating preadipocytes (Aboulaich et al., 2004; James et al., 2005). Hence, the proteins discussed in the current study will have their profound effect either on differentiation or metabolism of adult adipocytes. In addition, further studies like protein specific gene regulation for these proteins will throw light on their hidden pathways in adipocyte development.

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