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Differential Proteome Expression of *in vitro* Proliferating Hanwoo Stromal Vascular Cells from Omental, Subcutaneous and Intramuscular Depots in Response to Hormone Deprivation and IGF-1, Estradiol-17β Addition

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ABSTRACT

The aim of this study was to analyze the proteome expressions of proliferating stromal vascular cells from Hanwoo omental, subcutaneous and intramuscular depots subjected to hormone deprivation and IGF-1, Estradiol-17β addition. For hormone deprivation or addition studies, the cells were either grown in 10% charcoal-dextran stripped fetal bovine serum (CD-FBS) or in 10% FBS supplemented medium. Further, to analyze the effect of insulin like growth factor (IGF-1) and 17β-Estradiol (E2), cells were grown in 10% CD-FBS containing IGF-1 (10 ng/ml) or E2 (10 nM). The results showed that hormone deprivation had a negative impact on proliferation among the cells from all depots without any growth difference. On comparison of proliferation levels, higher levels were observed in cells that were grown in 10% FBS than in 10% CD-FBS alone or with IGF-1/E2. Proteome expression from preadipocytes grown in hormone deprivation conditions were compared by 2D-DIGE and MALDI-ToF/ToF. A total of twelve different proteins were found to be differentially expressed under hormone deprivation conditions. Further, our proteomic analysis with DIGE under IGF-1 and E2 addition revealed four proteins with differential expression levels. Moreover, the results highlighted in this study offer a role for each differentially expressed protein with respect to their effect in positive or negative regulation on proliferation.

(**Key words :** Stromal vascular cells, Preadipocytes, Proliferation, IGF-1, 17β-Estradiol)

INTRODUCTION

In order to improve the beef quality, it is very important to understand the factors that can regulate preadipocyte proliferation and differentiation in cattle. The mass of adipose tissue in cattle is controlled by the preadipocyte proliferation (hyperplasia) and differentiation (hypertrophy).

Also, increase in preadipocyte number was shown to be involved in adipose tissue deposition and expansion observed in adipose tissue development (Brook et al., 1972). Hyperplasia is related to the generation of new adipocytes from precursor cells, a process that we call adipogenesis. Adipogenesis is a particular system, which involves two major events: preadipocyte proliferation, and adipocyte differentiation. Both processes are tightly regulated and the cross-talk that exists between them determines the final adipocyte phenotype of the cell (Fajas, 2003).

The pattern of distribution of fat has an important implication in meat industry, marbling (deposition of intramuscular fat) improves the palatability and acceptability of beef by affecting the taste and tenderness of the meat (Nishimura et al., 1999). The expansion of adipose tissue depends not only on the ability of mature adipocytes to alter their lipid storage capacity, but also on the rate of proliferation of preadipocytes and the subsequent differentiation into mature adipocytes (Dieudonne et al., 1982; Krotkiewski et al., 1983; Rebuffe-Scrive et al., 1985). Preadipocytes are believed to be present throughout life and are typically studied *in vitro* using preadipocyte primary cultures (Prins and O'Rahilly, 1997). Furthermore, the culture system used in culturing bovine preadipocytes was also used for rat, porcine and human cells (Rong et al., 2007). Evidence from cell line studies suggests that the proliferation of preadipocytes occur prior to differentiation (Smyth et al., 1993; Cornelius et al.,

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1994). Cell proliferation studies involving either by directly i.e., incorporation of 3H-thymidine or 5-bromo-2-deoxyuridine (BrdU) or indirectly (increase in 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).

The adipocyte number and size are regulated in a coordinated manner; there is strong evidence that an interaction of a number of hormones may be responsible for the regulation of fat mass. Previous studies indicate that growth factors, insulin, glucocorticoids and sex steroids hormones enhance preadipocytes proliferation (Deslex et al., 1987; Hauner et al., 1987; Roncari and Van, 1978). Also, it was shown that preadipocytes of adipose depots were subjected to apoptosis during serum deprivation (Niesler et al., 1998; Papineau et al., 2003). Hence, in back ground of such studies, the aim of this work was to look for changes in the proteome expression in relation to growth rate under hormone deprivation and IGF-1, E2 addition. So, this study details the comparison of growth rate and DIGE (Fluorescence Difference Gel Electrophoresis) dependent proteomic characterization during hormone deprivation and IGF-1, E2 addition on proliferating preadipocytes from bovine omental, subcutaneous and intramuscular depots.

MATERIALS AND METHODS

1. Animals

Five heads of Hanwoo (Korean cattle) steers were fed and managed in the feeding barn at National Institute of Animal Science under 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.

2. Cell preparation

Immediately after stunning and exsanguination, the muscle and fat portions between the 6^{th} to 7^{th} ribs 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, 37ºC) 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 Krebs Ringer Bicarbonate (KRB) buffer (1.22 mM $CaCl_2$) for 1 h. The digested tissue was filtered through a 250 µm nylon mesh screen 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 medium containing M199 supplemented with 10% Fetal Bovine Serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were incubated at 37ºC in 5% CO_2 in air. After the cells reach confluence, subsequently the cells were seeded in 10 cm petri-dish at a density of approximately 2×10^4 cells/cm^2 and were also cultured in medium containing 10% charcoal dextran stripped fetal bovine serum (CD-FBS; Gibco-BRL), together with IGF-1 (10 ng/ml; Sigma, I3769) and E2 (10nM); Sigma, E1132). The medium was changed every second day allowing the cells to proliferate until confluence (about 10 days).

(1) Cell Counting

Cell number was determined at day 3, 6, 9 and 12 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. Whatever their anatomical origin, 95% of cells excluded trypan blue.

3. 2D-DIGE (2-dimensional gel electrophoresis/Fluorescence Difference Gel Electrophoresis)

(1) Protein preparation and 2D-DIGE

Proteins from proliferating stromal vascular cells (12^{th} day) were extracted on ice for at least 30 min with 30 µl lysis buffer (pH 8.5) containing 7 M urea, 2 M thiourea, 30 mM Tris and 4% CHAPS (Rabilloud, 1998). Following the centrifugation at 20000×g for 20 min at 4ºC, the supernatant

was removed and the pellets were collected and then completely dried using speed-vac. Dried samples were redissolved in 2-DE sample buffer (7M Urea, 2M Thiourea, 2% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 100 mM Dithiothreitol (DTT), 0.5% pH 3-10NL Immobilized pH gradient (IPG) buffer. Then the concentration of total protein in the sample was determined by 2D-Quant Kit (GE healthcare), using BSA as a standard. 50 μg of protein per sample were labeled with 400 pmol of Cy3 or Cy5 or Cy2 (internal standard) (CyDyes, GE Healthcare) for 30 min on ice in the dark. After the labeling reaction with 1 μl lysine (10 mM) for 10 min, the differently labeled samples were pooled with 50 μg of unlabeled, equal amount of each sample mixture and then mixed with a double volume of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% (v/v) IPG buffer pH 3-10 and 2% (w/v) DTT). The mixed samples were applied onto 24 cm IPG non-linear pH 3-10 gel strips, which had been rehydrated at 20ºC for at least 10 h in 450 μl DeStreak solution (GE Healthcare) with 0.5% (v/v) IPG buffer pH 3-10 NL. Isoelectric focusing (IEF) was carried out for a total of 88 kVh at 20ºC in five steps with a gradual increase of voltage (300 V for 4 h, 600 V for 4 h, gradient from 600 V to 1000 V for 4 h, gradient from 1000 V to 5000 V for 4 h and to total 8000 V) using the IPGphor 3 system (GE Healthcare). For the second dimension, the IPG strips were first reduced for 15 min in SDS-equilibrium buffer (6 M urea, 50 mM Tris- HCl, pH 8.8, 30% glycerol, 2% SDS, trace of bromophenol blue and 0.5% (w/v) DTT) and then carbamido-methylated for 15 min in the same buffer containing 4.5% (w/v) iodoacetamide instead of DTT. The second dimension was performed on 12% polyacrylamide gels (20×24 cm) using the Ettan Dalt six system (GE Healthcare) at 10 mA per gel for 1 h, and then with an increased current of 15 mA per gel until the bromophenol blue band reached the end of the gel cassette. Electrophoresis was carried out under continuous cooling at 15ºC.

(2) Gel image analysis and spotting

The in-gel fluorescence was scanned on a Typhoon 9400 (GE Healthcare) at appropriate wavelengths to detect Cy3 and Cy5 specific emission corresponding to the protein concentration in every single spot (Tonge et al., 2001). The intra-gel spot detection of the multiplexed gel images was performed using differential in-gel analysis (DIA) included in the DeCyder 6.5 software (GE Healthcare). The images for each gel were merged; spot boundaries were detected followed by a normalization of the spot volumes revealing differential spots. One-way analysis of variance, ANOVA, was adopted to determine significant protein expression differences among proliferating preadipocytes from three depots. Subsequently, significantly under or over-expressed proteins were identified by multiple comparisons using the Student's t-test. For protein identification the gels were stained with Flamingo Pink (Bio Rad) according to the manufacturer's instructions. Spots of interest were selected on the gel by comparing with the pattern of the 2D-DIGE protein pattern. The selected spots were excised from gels using a punch and placed in 500 μl Eppendorf tubes. In-gel tryptic digestion and mass spectrometry were followed.

4. Protein identification

The 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 37ºC for 30 min and repeated once. Then the gels were reduced and alkylated. The gel pieces were digested overnight with trypsin (20μg/μl) in 50 mM NH_4HCO_3 containing 10% ACN. The digest were 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:trifluoroacetic acid (TFA) (93:5:2).

Solution-phase nitrocellulose target preparation was used according to the method reported by Landry et al. (2000). The 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 the 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 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 auto-proteolytic products were removed, and molecular weight, isoelectric point (pI) and protein scores were considered.

RESULTS

1. Effect of hormone deprivation and IGF-1, E2 addition on proliferation of preadipocytes from bovine omental, subcutaneous and intramuscular adipose depots

Figs. 1 A, B represents the proliferation curves of pre-adipocytes from omental, subcutaneous and intramuscular adipose depots grown in hormone deprived medium (10% CD-FBS) and 10% FBS from day 1 to 12 after seeding. All the preadipocytes grown in both above mentioned conditions were showing similar growth rate up to day 3, from the day 6 the preadipocytes grown in 10% CD-FBS were found to have their growth rate in declined phase. In contrast, the growth rate of preadipocytes in 10% FBS from all the depots was increased by a factor of at least four ($p<0.05$). Thus the presence or absence of hormones (CD-FBS were shown to have less concentrations of important hormones such as testosterone, estrogen, cortisone and progesterone) influence the growth of preadipocytes. However, while comparing the growth of preadipocytes in 10% FBS among the depots, the growth rate was higher in omental compared to intramuscular and subcutaneous preadipocytes on 12th day.

The above experiment does not establish the effect of any particular hormone on the growth rate of preadipocytes. This prompted us to compare the preadipocyte proliferation rates with effect to the addition of well known cell proliferators IGF-1 or E2 to the medium containing 10% CD-FBS. Figs. 2A, B displays the proliferation curves of preadipocytes grown in 10% CD-FBS with addition of either one of the hormones, respectively. From the figure it is clear that, the preadipocytes from all the depots were showing lesser growth rates than that was observed in preadipocytes grown in 10% FBS. As the preadipocytes from three depots, grown either in 10% CD-FBS or with IGF-1/E2 were showing initial growth rates similar to the preadipocytes from 10% FBS medium and later the growth rate noticeably decreased, we here consider that more than one hormone is necessary to support proliferation. Further, to investigate the effect of

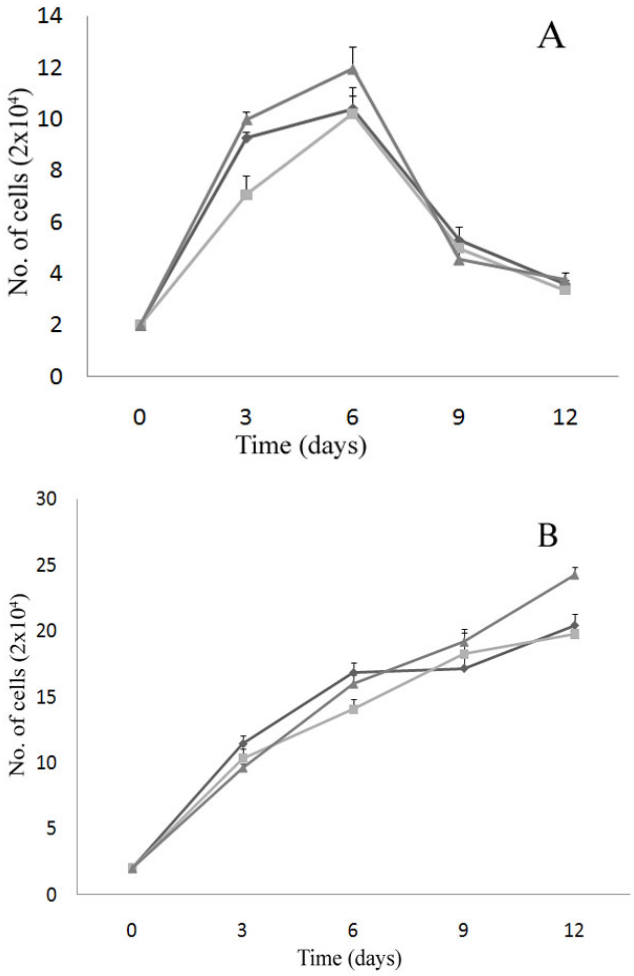

Fig. 1. Influence of hormone deprivation on the growth rate of preadipocytes from omental, subcutaneous and intramuscular depots. Preadipocytes from three depots were prepared and plated in medium containing 10% CD-FBS (A) or 10% FBS (B). On the indicated days, cells were collected and counted as mentioned in methods. Comparison of the growth rate between preadipocytes from omental (-◆-), subcutaneous (-■-) and intramuscular (-▲-) depots expressed here are the mean ± SEM of triplicate experiments. The growth rate of preadipocytes in 10% FBS from all the depots was increased by a factor of at least four ($p<0.05$).

hormone deprivation and IGF-1 or E2 addition on proteome expression of preadipocytes, we analyzed the proteome of the proliferating preadipocytes from all the depots at all treated conditions using 2D-DIGE coupled with MS for the identification of differentially expressed proteins, which may play a crucial role in determining the proliferation of preadipocytes.

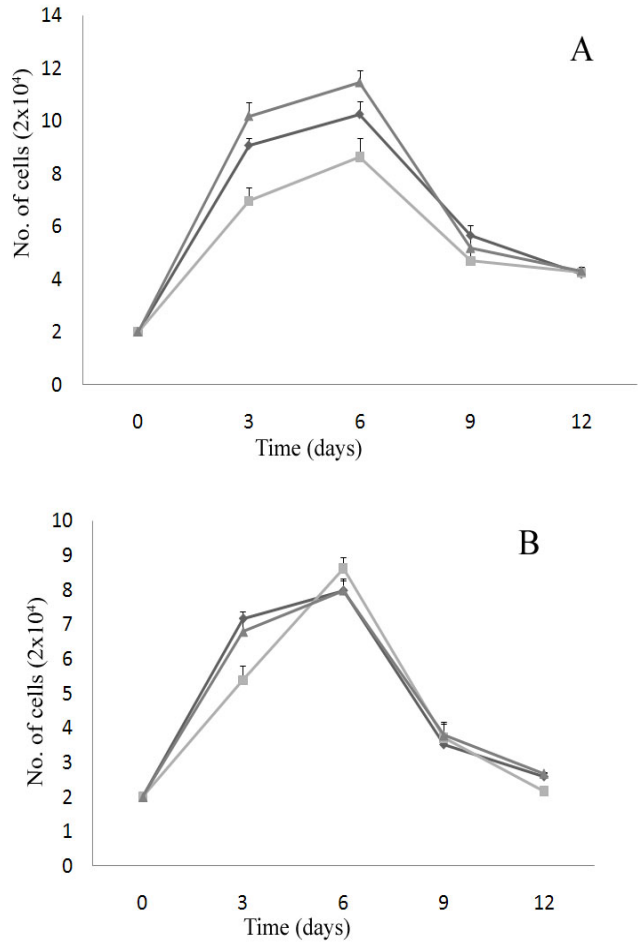

Fig. 2. Influence of IGF-1 and E2 addition on the growth rate of preadipocytes from omental, subcutaneous and intramuscular depots. Preadipocytes from three depots were prepared and plated in medium containing 10% CD-FBS+ IGF-1 [10 ng/ml] (2A) or E2 [10nM] (2B). On the indicated days, cells were collected and counted as mentioned in methods. Comparison of the growth rate between preadipocytes from omental (-◆-), subcutaneous (-■-) and intramuscular (-▲-) depots expressed here are the mean ± SEM of triplicate experiments.

2. Differential protein expression of preadipocytes from omental, subcutaneous and intramuscular adipose depots grown in hormone deprivation and addition conditions

To investigate the differences in protein expression of preadipocytes grown in hormone deprivation and IGF-1, E2 addition conditions, whole protein extracts of preadipocytes from three depots grown in 10% FBS or 10% CD-FBS with IGF-1/E2 were separated by 2D-DIGE. For our proteomic analysis, we choose the preadipocytes from day 12, as maximum difference in growth rate was observed. According to the DeCyder program used for analyzing the spots, differentially expressed proteins were identified. Spots which exhibited significant differential expression during proliferation at various conditions (t-test, $p<0.05$), were isolated from the gel stained with Flamingo Pink for more detailed characterization. Comparison of protein samples from preadipocytes grown in 10% FBS and CD-FBS, showed twelve different proteins to have differential expression. Across three depots, Transgelin-2, WD repeat protein 55, Lamin-A/C, Apolipoprotein A-IV, Caspase-precursor, LASP-1 and Vimentin were found to be highly expressed, while Hemoglobin subunit beta-C, Calponin-1, BID, Ras association domain-containing family protein 5, Cytochrome C oxidase polypeptide are expressed low (Fig. 3; Table 1).

In case of IGF-1/E2 addition, not many differences were found and only four proteins, Vimentin, Purine nucleoside phosphorylase, Protein yippee-like 2 and Annexin 2 were found to be highly expressed in E2 (Fig 4; Table 2). Further, we consider that the proteins that are comparatively highly expressed during 10% FBS treatment (high growth rate) are involved directly or indirectly in proliferation of preadipocytes, where as proteins that are showing their high expression in 10% CD-FBS or with IGF-1/E2 (low growth rate) treatment may have their role in cell death.

DISCUSSION

Hormones are the major regulatory factors that are critical for adipocyte development and function. An extensive array of hormones and growth factors modulate adipocyte development and activity, including growth hormone, thyroid hormone, glucocorticoids, catecholamines, glucagon, insulin, and insulin-like growth factor. Among them, steroid hormones play a crucial role in the adipose tissue accumulation and distribution (Rodriguez-Cuenca et al., 2005). In the present study, we found that the preadipocytes grown in 10% CD-FBS have low growth rates compared to the preadipocytes grown in 10% FBS. CD-FBS is stripped off from important hormones such as testosterone, estrogen, cortisone and progesterone (as per manufacturer's protocol) and all these belongs to steroid hormone group. Also, it has been demonstrated that charcoal-stripping of serum not only

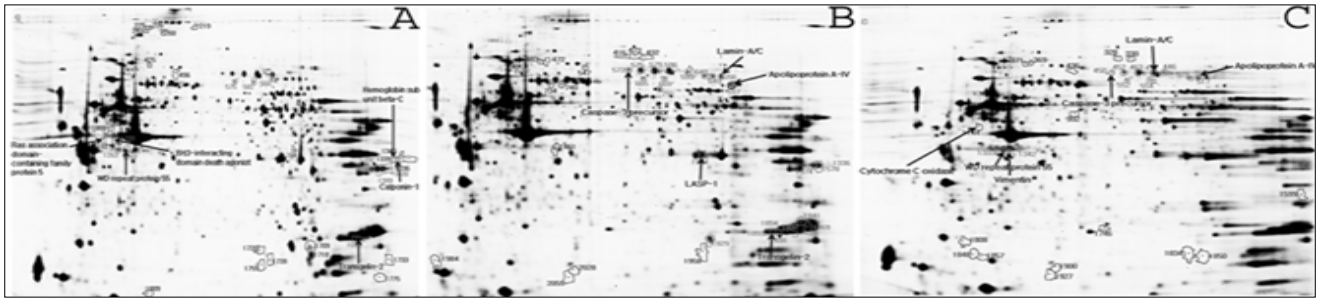

Fig. 3. 2D-DIGE of preadipocyte proteins. Protein extracts from omental, subcutaneous and intramuscular preadipocytes grown in 10% CD-FBS or 10% FBS supplemented medium (day 12) were labeled with CyDyes Cy3, Cy5 and Cy2, respectively, and were mixed and separated on a 2-DE gel using 24 cm pH 3-10 NL strips in the first dimension and 12% SDS-PAGE gels in the second dimension. Gels were scanned to obtain single images, and the figures represented here are the overlay of the two dyes (Cy3, Cy5) of omental (A), subcutaneous (B) and intramuscular (C) preadipocytes proteins. The same gel, after fluorescence imaging, was stained with Flamingo pink and scanned. Differentially expressed protein spots as indicated in Table 1 are represented in the corresponding gels with their legends.

Table 1. Differentially expressed proteins of preadipocytes grown in FBS and CD-FBS supplemented medium from adipose depots

Protein name	Omental		Subcutaneous		Intramuscular	
	CD-FBS	FBS	CD-FBS	FBS	CD-FBS	FBS
Transgelin-2	↓	↑	↓	↑	NC	NC
WD repeat protein 55	↓	↑	NC	NC	↓	↑
Lamin-A/C	NC	NC	↓	↑	↓	↑
Apolipoprotein A-IV	NC	NC	↓	↑	↓	↑
Caspase-3-precursor	NC	NC	↓	↑	↓	↑
Hemoglobin subunit beta-C	↑	↓	NC	NC	NC	NC
Calponin-1	↑	↓	NC	NC	NC	NC
BH3-interacting domain death agonist (BID)	↑	↓	NC	NC	NC	NC
Ras association domain-containing family protein 5	↑	↓	NC	NC	NC	NC
LIM and SH3 domain protein 1 (LASP-1)	NC	NC	↓	↑	NC	NC
Vimentin	NC	NC	NC	NC	↓	↑
Cytochrome C oxidase polypeptide	NC	NC	NC	NC	↑	↓

'↑' – Upregulated; '↓' – Downreguated; 'NC' – No change
Differential expression of proteins indicated here is based on our results from DeCyder 6.5 software. For differential expression, we considered the area of the spot and also the peak of the spot and determined the maximum spot volume. Spots, whose difference in fluorescence intensity was ≥1.2 fold was considered as differentially expressed proteins.

eliminates a variety of steroids, but also agents such as fatty acids and to a lesser extent, growth factors (Lindquist and de Alarcon, 1987). Hence, absence of these hormones will have a profound effect on the proliferation of preadipocytes from all the depots. *In vitro*, it is well established that glucocorticoids, or the glucocorticoid analogue dexamethasone, enhance preadipocytes recruitment in presence of serum in nearly every adipocyte line and all SV cells studied (Hausman et al., 1993; 1998; Kras et al., 1999). The mechanism by which these hormones control the adipose tissue growth is not clear. One possible mechanism would be the regulation of key proteins in adipose tissues at the genomic level by transcriptional means. But, this would require steroid receptors to be present in the adipose tissues and convincing evidence now exists that adipose tissues install many such receptors (Pedersen et al., 1996). Another possible mechanism would be the regulation of secondary messengers at the membrane and this would require the presence of steroid hormones receptors on the plasma membrane, and there is now evidence of such receptors (Pietras et al., 2001). But the quantitative effect of these hormones on the adipose tissue development depends on the

Fig. 4. 2D-DIGE of preadipocyte proteins. Protein extracts from omental, subcutaneous and intramuscular preadipocytes grown in 10% CD-FBS with IGF-1 or E2 supplemented medium (day 12) were labeled with CyDyes Cy3, Cy5 and Cy2, respectively, and were mixed and separated on a 2-DE gel using 24 cm pH 3-10 NL strips in the first dimension and 12% SDS-PAGE gels in the second dimension. Gels were scanned to obtain single images, and the figures represented here are the overlay of the two dyes (Cy3, Cy5) of omental proteins (A), subcutaneous proteins (B) and intramuscular (C) preadipocytes. The same gel, after fluorescence imaging, was stained with Flamingo pink and scanned. Differentially expressed protein spots as indicated in Table 1 are represented in the corresponding gels with their legends.

Table 2. Proteins expressed differentially in preadipocytes from adipose depots grown in medium supplemented with IGF-1 and E2

Protein name	Omental		Subcutaneous		Intramuscular	
	IGF 1	E2	IGF 1	E2	IGF 1	E2
Vimentin	↓	↑	↓	↑	NC	NC
Purine nucleoside phosphorylase	NC	NC	↓	↑	↓	↑
Protein yippee-like 2	NC	NC	↓	↑	NC	NC
Annexin 2	NC	NC	NC	NC	↓	↑

'↑' – Upregulated; '↓' – Downreguated ; 'NC' – No change
Differential expression of proteins indicated here is based on our results from DeCyder 6.5 software. For differential expression, we considered the area of the spot and also the peak of the spot and determined the maximum spot volume. Spots, whose difference in fluorescence intensity was ≥1.1 fold was considered as differentially expressed proteins.

number of receptors expressed on adipose tissue. Thus, the presence of hormones and their receptors play a critical role in the adipocyte and tissue development.

Growth of preadipocytes in 10% FBS was high compared to 10% CD-FBS, and comparing the growth rate among the depots, high growth rate was seen in omental preadipocytes on day 3 and 6, while intramuscular preadipocytes on 9th and 12th day. Two reasons can be attributed for such depot specific differential growth rate, concentration of the hormonal receptors and the depot specific inherent hormone availability. Previous studies have shown the variations in the mRNAs of steroid hormone receptors that are depot specific and these variations could be a key in the understanding the adipose tissue steroid responsiveness (Dieudonne et al., 2000). To date, several studies demonstrate a role of adipose tissue as steroid reservoir and in addition to serving as a steroidal reservoir, adipose tissue is one of the most important extragonadal source of steroids, due to the specific expression of steroidogenic enzymes, such as aromatase (Belanger et al., 2002), suggesting a potential impact on the local adipose tissue metabolism that would be independent of plasma hormone milieu (Simpson et al., 2000). However, the differential role of the adipose depots in steroid metabolism remains unclear.

Comparing the growth of preadipocytes in the medium containing IGF-1 and E2, in both conditions, the growth rate was more or less similar. But, the growth rate was more for preadipocytes in the medium with IGF-1 compared to E2. However, comparing with preadipocytes in 10% FBS, both hormones were showing low growth rate. This shows that both hormones could not substitute the role of the hormones that are present in 10% FBS. Previous reports have mentioned the proliferative role of IGF-1 and E2 on preadipocytes (Finaly et al., 2004; Park et al., 2006). The contrasting roles of these hormones in proliferation found in our study may be explained as; the earlier studies have been conducted in

examining the effect of IGF-1 on proliferation was in presence of serum factors or conditioned medium that has other hormones except IGF-1 (Wright et al., 1995).

The growth rate for preadipocytes growing in IGF-1 supplemented medium was similar to the preadipocytes in 10% FBS in initial stages. But, in later stages the growth rate came down dramatically. The same pattern was seen with preadipocytes in E2 supplemented medium. This decrease in growth may be attributed to the cell death and it may be due to apoptosis. This conclusion may be correct as the previous studies have shown that, IGF-1 can acts as pro-apoptotic factor depending on the conditions. IGF-1 has been reported to increase Fas-induced apoptosis in human osteoblasts (Kawakami et al, 1998), also IGF-1 has been found to increase apoptosis in serum-starved glioma and hepatoma cells (Yang et al., 1996; Xu et al., 1997). Even in 3T3-L1 cells, the IGF-1 increased apoptosis induced by TNF-α in serum free medium. Further, it was also proved that, presence of serum factors can over ride the apoptotic function of IGF-1 (Niesler et al., 2000).

E2 induces cell proliferation in human uterus stroma as well as in the luminal and glandular epithelium by signaling through its transcription factor, ERα, mediated by IGF-1 receptor (Tong et al., 2002). Jae et al. (2006) demonstrated that E2 stimulates proliferation of mouse embryonic stem cells with the involvement of MAPKs and CDKs. However, Dubey et al. (2000) suggested that estradiol inhibits smooth muscle cell growth by activating the cAMP synthesis and the conversion of cAMP to adenosine mediates the inhibitory effect on vascular smooth muscle cell. Also, it was proposed that E2 may have a negative effect on the proliferation rate, as previous studies have shown that the downstream metabolite of E2, 2-methoxyestradiol, can inhibit proliferation (Pico et al., 1988). In our study, there seemed to be stimulatory and inhibitory effects of IGF-1 and E2 on the preadipocyte proliferation, respectively. Further investigation of the dose effect of IGF-1 and E2 on each depot specific preadipocytes will throw more light on the differences and mechanism underlying in such differences.

1. Differential protein expression

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) has been the workhorse of proteomics allowing the resolution of several thousand proteins in a single sample. The limitations of this technique has been low sensitivity, reduced dynamic range and gel to gel variability. Difference gel electrophoresis (DIGE) circumvents these issues associated with traditional 2D PAGE and allows more accurate and sensitive quantitative proteomics studies. Hence, in the present study we used 2D DIGE for differential protein expression analysis.

(1) Proteins highly expressed in preadipocytes grown in FBS

Transgelin-2, WD repeat protein 55, Lamin-A/C, Apolipoprotein A-IV, Caspase-3-precursor, LASP-1 and Vimentin were found to be highly expressed in preadipocytes grown in FBS. We have demonstrated that preadipocytes from three depots showed higher growth rate in FBS compared to CD-FBS. So, proteins that are highly expressed in FBS may have a role in proliferation of preadipocytes. Transgelin-2 and Vimentin are two cytoskeletal proteins that play a role in organization of the cytoskeleton and shown to express by serum or growth factors (Sommers et al., 1994; Van de Klundert et al., 1995; Liao et al., 2007). Transgelin-2 has been revealed to be involved in proliferation through Ras activation (Hoffrogge et al., 2006). WD-repeat proteins are one of the fast-expanding conservative protein families playing critical roles in many essential biological functions like signal transduction, transcription regulation, cell cycle progression, and cell proliferation (Li and Roberts, 2001) and it was shown that, these functions are due to its involvement in the removal of incompletely processed rRNA intermediates (Iwanami et al., 2008).

Lamin A/C is a nuclear structural protein and the proliferative function of this protein may be due to its role in nuclear assembly. This protein is known to interact with a number of proteins that serve key regulatory roles, including transcription factors (Dreuillet et al., 2002; Lloyd et al., 2002). Similarly, LASP-1, also called as LIM and SH3 protein 1 recently identified as a special focal adhesion protein involved in cell proliferation. Small interfering RNA (si-RNA) studies targeted against the LASP-1 expression arrested the cell growth in G_2/M phase of the cell cycle and proliferation of the si-RNA transfected cells was suppressed by 90% (Grunewald et al., 2007). In the case of Caspase-3-precursor and Apolipoprotein A-IV, these two proteins could act antagonistically on proliferation. While, caspase-3-precursor is considered to be one of the key apoptotic proteins involved in the programmed cell death (Mancini et

al., 1998); Apolipoprotein A-IV may play an antagonistic role due to its function in attenuating the apoptosis (Spaulding et al., 2006).

(2) Proteins highly expressed in preadipocytes grown in CD-FBS

The growth rate of preadipocytes from CD-FBS was similar to FBS in initial stages, but during the later stages (day 12), the growth rate was decreased. This shows that the proteins that are expressed in later stages from preadipocytes in CD-FBS medium may have an important role in cell death. Hemoglobin subunit beta belongs to hemorphins family i.e. endogenous fragments of β-globin, known to play a role in cell death by inducing cytotoxicity (Blishchenko et al., 2002). Hemoglobin subunit beta is known as LVV-hemorphin-7 and it participates in the cell death by the induction of cytolysis (Kampa et al., 1997). Calponin-1 is a 34-kd member of the calponin family and was found to inhibit cell proliferation independently due to its effect on cell contractility (Takahashi et al., 1993; Takahashi et al., 1995). Also, it was shown that, calponin-1may also depress the rate of cell proliferation by inhibiting actomyosin-dependent processes (Horiuchi et al., 1999). BH3-interacting domain (BID) is a member of the Bcl-2 family of proteins and was shown to participate in apoptosis as it is a substrate for caspase-8. This caspase-8 cleaves BID at the C-terminal to generate tBID. This tBID then translocates to the mitochondria and induces cytochrome c release either in a Bax-dependent or independent manner (Wei et al., 2000; Eskes et al., 2000) and the end result is cell death by apoptosis. Ras association domain-containing family protein 5 (Nore 1), activated forms of Ras associated proteins can exert profound negative effects on cellular growth and survival (Ghosh et al., 1996). The ability of activated Ras associated proteins to induce senescence, necrosis, cell cycle arrest, differentiation, or apoptosis is well documented (Sewing et al., 1997; Zhu et al., 1998; Chi et al., 1999).

2. Differential protein expression of preadipocytes from IGF-1 and E2 supplemented medium

Vimentin, Purine nucleoside phosphorylase (PNP), Annexin A2 and Protein yippee-like are four proteins that were found to be highly expressed from preadipocytes in E2. The low growth rate of preadipocytes in E2 tempted us to propose and explain the cell death role displayed by these highly expressed proteins. Vimentin is a type III intermediate filament cytoskeletal protein, and is shown to have both proliferative and anti-proliferative effects (Penuelas et al., 2005). The proteolytic cleavage of vimentin by caspase 3, 6 and 7 generate a proapoptotic amino-terminal product that interacts with filament assembly (Byun et al., 2001).

Purine nucleoside phosphorylase (PNP) is called as suicide gene. Suicide genes induce cell death by apoptosis or necrosis (Lal et al., 2000). PNP converts certain adenosine analogs into highly toxic metabolites that readily diffuse across cell membranes (Sorscher et al., 1994; Hughes et al., 1995; 1998) and interfere with DNA, RNA, and protein synthesis, killing dividing, as well as non-dividing cells (Parker et al., 1998). Annexin A2 is a Ca^{2+}-dependent phospholipid-binding protein which has been implicated in a number of membrane related events (Rescher and Gerke, 2004). It is proposed to be involved in p53-mediated apoptosis (Hung et al., 2008). Protein yippee-like 2 is also identified as small unstable apoptotic protein [SUAP], this protein was shown to be upregulated in cells that are undergoing apoptosis due to IL-3 deprivation (Baker, 2003).

CONCLUSION

In summary, our study demonstrates the effect of removal of steroid hormones on the growth rate of preadipocytes from adipose depots. Also, we found that neither IGF-1 nor E2 can promote the growth rate of preadipocytes independently and were not able to substitute the roles played by steroid hormones present in FBS. Our present proteomic approach on adipose depots under hormonal deprivation and IGF-1, E2 addition has revealed proteins that are differently expressed. Although we were not able to find the depot specific differences in protein expression, the results obtained in our study could be key in understanding the roles played by these proteins in cell proliferation or cell death during hormone deprivation and IGF-1, E2 addition conditions. This approach will be a stepping stone to unravel the hidden mechanism in such hormone specific studies carried out in adipose depot development.

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