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Temporal profiling of the adipocyte proteome during differentiation using a 5-plex SILAC based strategy

Henrik Molina[†,‡,#], **Yi Yang**[†,‡,#], **Travis Ruch**[‡], **Jae-Woo Kim**[‡], **Peter Mortensen**[§], **Tamara Otto**[‡], **Anuradha Nalli**[Ψ], **Qi-Qun Tang**[‡], **M. Daniel Lane**[‡], **Raghothama Chaerkady**[†,‡,Ψ], and **Akhilesh Pandey**[*,†,‡,Δ]

†*McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University, Baltimore, MD 21205, USA*

‡*Department of Biological Chemistry, Johns Hopkins University, Baltimore, MD 21205, USA*

Ψ*Institute of Bioinformatics, International Technology Park, Bangalore 560066, India*

§*Center for Experimental BioInformatics (CEBI), Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark*

Δ*Departments of Pathology and Oncology, Johns Hopkins University, Baltimore, MD 21205, USA*

Abstract

The adipose tissue has important secretory and endocrine functions in humans. The regulation of adipocyte differentiation has been actively pursued using transcriptomic methods over the last several years. Quantitative proteomics has emerged as a promising approach to obtain temporal profiles of biological processes such as differentiation. Stable isotope labeling with amino acids in cell culture (SILAC) is a simple and robust method for labeling proteins *in vivo*. Here, we describe the development and application of a five-plex SILAC experiment using four different heavy stable isotopic forms of arginine to study the nuclear proteome and the secretome during the course of adipocyte differentiation. Tandem mass spectrometry analysis using a quadrupole time-of-flight instrument resulted in identification of a total 882 proteins from these two proteomes. Of these proteins, 427 were identified on the basis of one or more arginine containing peptides that allowed quantitation. In addition to previously reported molecules that are differentially expressed during the process of adipogenesis (e.g. adiponectin and lipoprotein lipase), we identified several proteins whose differential expression during adipocyte differentiation has not been documented previously. For example, THO complex 4, a context-dependent transcriptional activator in the T-cell receptor alpha enhancer complex, showed highest expression at middle stage of adipogenesis while SNF2 alpha, a chromatin remodeling protein, was downregulated upon initiation of adipogenesis and remained so during subsequent time points. This study using a 5-plex SILAC to investigate dynamics illustrates the power of this approach to identify differentially expressed proteins in a temporal fashion.

Keywords

Adipocyte; adipogenesis; proteomics; SILAC

[#]These authors contributed equally to this work.

[*]To whom correspondence should be addressed. Akhilesh Pandey M.D., Ph.D., McKusick-Nathans Institute of Genetic Medicine, 733 N. Broadway, BRB 527, Johns Hopkins University, Baltimore, MD 21205. Tel.: 410-502-6662. Fax: 410-502-7544. E-mail: pandey@jhmi.edu.

Introduction

The adipose compartment houses the largest energy reserves of the body in humans in the form of triacylglycerols and plays an essential role in maintaining the energy balance[1]. It is now well recognized that adipogenesis can occur throughout the lifetime of humans and the capacity to increase the adipocyte number is retained in adulthood[2]. Thus, identification of key players in the process of adipogenesis is important especially in regard to manipulating them for therapeutic purposes. Over the last several years, adipocytes have also been shown to possess regulatory functions in processes such as satiety and reproduction through the secretion of a variety of hormones, cytokines, growth factors and other bioactive substances, conceptualized as adipocytokines[3]. These include adiponectin, leptin, plasminogen activated inhibitor 1 (PAI-1), and other secreted factors[4]. Another important class of proteins that play a vital role in adipogenesis is transcription factors such as peroxisome proliferator activated receptor-γ[5, 6] and the CCAAT-enhancer binding protein-α[7], which have been characterized in detail during adipocyte differentiation. Thus, we sought to identify additional molecules involved in adipogenesis by analyzing nuclear and secreted compartments during the differentiation of adipocytes.

Cell lines and primary cultures are useful cell models for elucidation of molecular and developmental pathways leading to adipogenesis. 3T3-L1 preadipocytes are one of the commonly used model systems for studying the process of adipocyte differentiation. DNA microarray studies have been carried out to investigate specific cellular programs in the regulation of gene expression during differentiation of 3T3-L1 preadipocytes[8]. They found that that expression levels of 1259 transcripts changed 3-fold or more during differentiation of 3T3-L1 preadipocytes to adipocyte. In a similar study, Burton et al.[9] reported 636 transcripts to be upregulated at least 2 fold and 380 transcripts downregulated in adipocytes as compared to preadipocytes. However, since adipogenesis is a dynamic and actively regulated process, a temporal analysis of differential protein expression during adipogenesis would be crucial for understanding adipocyte biology. We have previously identified factors that are secreted by adipocytes using liquid chromatography tandem mass spectrometry (LC-MS/MS)[10]. In that study, which was not designed to be quantitative, the differential expression of a small subset of molecules was confirmed by RT-PCR and Northern blot analysis. More recently, using 1D gel based LC-MS/MS, another non-quantitative proteomic analysis was reported in which various subcellular fractions from adipocytes were characterized[11].

Thus far, there is no study describing a quantitative analysis of the dynamics of the adipocyte proteome during differentiation. Stable isotope labeling with amino acids in cell culture (SILAC) is a simple and robust *in vivo* labeling method that can be applied to studies analyzing cells at multiple time points such as during cellular differentiation. Traditionally, SILAC has been used for analyzing two or three different states. Here, we describe the development and application of a five-plex SILAC experiment using four different heavy stable isotopic forms of arginine for the analysis of nuclear and secreted proteins of adipocytes at 5 different time points during adipogenesis. We identified 882 nuclear/secreted proteins using SILAC labeling followed by 2D-LC-MS/MS. Almost half of the identified proteins from each compartment could be quantitated enabling the identification of potential regulators and markers at different states of adipocyte differentiation.

Experimental Section

Cell culture and SILAC labeling

Custom Dulbecco's modified Eagle's medium (DMEM) without arginine was purchased from Invitrogen (Carlsbad, CA). arginine-$^{13}C_4$, arginine-$^{13}C_6$, arginine-$^{13}C_6{}^{15}N_4$, and arginine-$^{13}C_6{}^{15}N_4{}^{2}H_7$ were purchased from Cambridge Isotope Laboratories (Andover, MA).

A detailed protocol for SILAC media preparation can be found at http://www.silac.org. 3T3-L1 preadipocytes were grown in normal DMEM media (light) and 4 different custom DMEM media supplemented with above 4 different isotopic forms of arginine (heavy) along with 10% calf serum. The cells from each condition were cultured through at least five passages until the complete incorporation of heavy arginine. 3T3-L1 cells were differentiated essentially as described previously[10]. Two days after the cells reached confluence (Day 0), they were induced to differentiate by changing the medium to corresponding medium supplemented with 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methyxanthine (Sigma, St. Louis, MO), 1 μM dexamethasone (Sigma), and 167 nM insulin (sigma). At the end of Day 2, the medium was replaced with DMEM supplemented with 10% fetal bovine serum and 167 nM insulin. At the end of Day 4, insulin was withdrawn, and the cells were continued to grow in corresponding media until the end of Day 7. The cells grown in light arginine, arginine-$^{13}C_4$, arginine-$^{13}C_6$, arginine-$^{13}C_6{}^{15}N_4$, and arginine-$^{13}C_6{}^{15}N_4{}^{2}H_7$ media were subjected to 18 hours serum starvation in corresponding serum free media at the end of Day 0, Day 1, Day 3, Day 5 and Day 7, respectively. After the collection of supernatant, the corresponding cells were washed 3 times in ice cold PBS and stored at −80°C until the preparation of subcellular fractions.

Preparation of protein samples

For secretome analysis, the supernatant collected from each state was combined and concentrated using a 3,000 Da cutoff Centriprep spin column (Millipore, Billerica, MA). The preparation of nuclear fraction was performed as described previously[12]. Briefly, the harvested cell pellets from each state were mixed equally according to protein concentration, which was measured using an aliquot from each sample. The cell preparations were resuspended in hypotonic lysis buffer (20mM Tris-Cl, pH 7.5; 10mM NaCl; 3 mM MgCl; 1mM DTT; 1mM sodium orthovanadate; 10mM β-glycerol phosphate) with protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany) and lysed in Dounce homogenizer with 20 strokes. The lysates were then centrifuged at 1,000g for 10 minutes at 4 °C. The nuclear extract pellet was redissolved in 6 M urea before reversed phase protein fractionation.

Oil red O staining

Oil red O staining was performed to monitor progression of adipocyte differentiation as described previously[1]. Briefly, cells on Day 0, Day 1, Day 3, Day 5 and Day 7 were washed three times with PBS and then fixed by 3.7% formaldehyde for 2 minutes. 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 one hour at room temperature and then washed with water. The stained fat droplets in the cells were visualized by light microscopy and photographed.

Reversed phase protein fractionation and tryptic digestion

Cell free protein lysates and secreted proteins were reduced and alkylated in denaturing conditions. Protein samples were adjusted to a volume of 0.3 ml followed by addition of solid urea to a final concentration of 6M. The sample was reduced with 50 μl of 50 mM dithiothreitol at 60°C for 20 minutes and alkylated by adding 50μl of 100mM iodoacetamide for 30 minutes at room temperature in the dark. The sample (500μl) was acidified using TFA (1%) and injected onto a 4.6mm × 50mm Hi Recovery Protein mRP-C_{18} column (Agilent, Santa Clara, California) using an external sample injection loop. Intact proteins was eluted using a gradient increasing from 97% buffer A (0.1% TFA) / 3% buffer B (0.08% TFA in acetonitrile) to 40% Buffer A / 60% buffer B in 36 minutes. The gradient was delivered by an Agilent 1100 series HPLC system at a flow rate of 800 μl/minute. Eluted proteins were detected by UV (280 nm) and fractions were collected every 30 seconds. Protein fractions were lyophilized completely to dryness and redissolved in 25 μl of 100mM ammonium bicarbonate containing trypsin of

concentration (40 to 110 ng) proportional to the UV signal for the given fraction (data not shown). The ratio between trypsin and substrate was kept approximately constant at 1:50. Digested fractions were lyophilized and stored at −80°C until LC-MS/MS analysis.

LC-MS/MS and Data analysis

The digested peptides were reconstituted in 5 µl of 0.1% formic acid and analyzed using reversed phase liquid chromatography coupled to QSTAR Pulsar mass spectrometer (Applied Biosystems). Liquid chromatography was set up on an Agilent 1100 series, to allow sample cleanup using trap column (75 µm × 3 cm, C18 material 5-10µm, 120Å, YMC, Japan) and peptide fractionation on an analytical column (75 µm × 10 cm, C_{18} material 5µm, 120Å, YMC, Japan). API electrospray was carried out with the help of an emitter tip 8 µM (New Objective, Woburn, MA). The peptides were eluted using a gradient of solvent A (0.1% formic acid, 3% acetonitrile) and solvent B (90% acetonitrile, 0.1% formic acid). Solvent gradient from 5 to 10 % solvent B was used for sample cleanup on trap column followed by a gradient of 10 to 40% of solvent B for peptide fractionation over analytical column. MS spectra were acquired in a survey scan (m/z range from 350 to 1200) in a data dependent mode selecting three most abundant ions for MS/MS. An exclusion time of 45 seconds were used. LCMS/MS data was acquired using Analyst QS 1.1 (MDS Sciex, Concord, Ontario, Canada) and mascot generic format (mgf) files were generated from instrument raw (*.wiff) files using the mascot.dll data import filter script in Mascot Daemon (version 2.2.2) (Matrixscience, Manchester, UK). Mascot Daemon merged data for nuclear and secretome fractions were searched in Mascot v2.2.0 (Matrixscience, Manchester, UK) search engine using mouse RefSeq database version 26 and search results were saved in Internet Explorer 6.0. Reverse database version RefSeq26r was searched to estimate the false discovery rate. The following variable modifications were used in Mascot for searching the database; arginine-$^{13}C_4$, arginine-$^{13}C_6$, arginine-$^{13}C_6{}^{15}N_2$, arginine-$^{13}C_6{}^{15}N_2{}^{2}H_7$ and oxidation of methionine. Carbamidomethylation of cysteine residues was used as a fixed modification. The mass tolerance was set 0.3 atomic mass units for precursor and 0.3 atomic mass units for fragmented ions. Relative quantitation of stable isotope labeled peptides was performed using MSQuant v1.4.3a39 downloaded from http://msquant.sourceforge.net. The “new_MSQ_quantitationModes.xml” file was modified to include base mass differences for heavy arginines and Mascot output matching string in order to MSQuant associate Mascot search results with instrument raw files. MSQuant quantitates individual peptide ratios at different time points at chromatographic peaks and calculates protein ratios with standard deviation. Mascot search results in .html format were parsed with the raw data file in MSQuant. Quantitative as well as qualitative data were manually inspected using MSQuant. Data from the MSQuant analyses were imported via a CSV format to ProteinCenter 1.2 (Proxeon A/S, Odense, Denmark) for a bioinformatics statistical analysis.

Western Blot Analysis

Western blot analysis was carried out for a subset of differentially expressed proteins. Briefly, nuclear and secreted samples from Day 0, Day 1, Day 3, Day 5 and Day 7 of adipocyte differentiation were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane (Protran, Whatman). The membranes were blocked using 3% nonfat dry milk in phosphate-buffered saline with 0.1% Tween 20 (PBS-T) for 1 hour at room temperature. After incubating the membranes with primary antibody, the bound primary antibody was detected using HRP-conjugated secondary antibody with an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ). Following antibodies with appropriate dilution were used in this study: Poly(A)-binding protein 1 (1:1000, Cell Signaling Technology, Cat No. 4992), Splicing factor, arginine/serine rich 2 (1:200, Santa Cruz, Cat No. SC-10254), Lumican (0.2µg/ml, R&D Systems, Cat No. AF2846), and Annexin V (1:500, Santa Cruz, Cat No. SC-32321).

Results and Discussion

Quantitative proteomic data

Thus far, SILAC has been most commonly used for studying 2 or 3 different states simultaneously using different isotopic forms of arginine and lysine[13]. Although it has been shown that two 3-plex SILAC experiments can be used to monitor 5 different states, it requires two different experiments carried out in parallel[14]. We have developed a 5-plex SILAC labeling strategy using five different isotopic versions of arginine to simultaneously monitor proteins derived from five different samples. This method was used to study adipocyte differentiation at five time points (Day 0, Day 1, Day 3, Day 5 and Day 7) during 7-days differentiation of pre-adipocytes to adipocytes as shown in Figure 1A. By Oil red O staining, gradual morphological changes of adipocytes during adipogenesis can be seen clearly, which were demonstrated by gradually increased fat droplets observed in the cells in this study (Figure 1B). To address the molecular alterations responsible for the adipogenic process, we took a global proteomics approach targeting two important biological compartments of the differentiating adipocytes: nuclear proteins and secreted proteins (secretome).

Our major analytical concern regarding our choice of labeled amino acids was that the deuterium labeled arginine (Arginine-$^{13}C_6{}^{15}N_4N^2H_7$), being 17 Da heavier than normal arginine, would give rise to a chromatographic shift[15]. It has also been reported that grouping deuterium next to polar groups can greatly minimize the observed shift, which is consistent with solvophobic theory[16]. The seven deuterium atoms used in the 17 Da heavier arginine are positioned in the aliphatic chain and on the secondary nitrogen and all deuterium atoms are therefore not in close proximity to the polar amine moiety. We investigated a number of peptide elution profiles to see if any chromatographic shift for the deuterium labeled peptides was observed. The elution profiles for 6 peptide sets are shown in Figure 2. The upper panel of each figure shows the measured MS spectrum for the peptide set, while the lower panels show the corresponding elution profiles for each of the five isotopic peptides indicating that these five isotopically distinct peptides indeed co-eluted in LC-MS/MS analysis. Regarding peptides labeled with ^{13}C and ^{15}N; we and others[17-19] have previously shown that the use of these isotopes does not cause any critical elution differences for peptides.

In the present study, a total of 882 proteins were identified using LC-MS/MS analysis. Of these, 581 were identified in the fraction that had been enriched for the nuclear fraction while the remaining 301 originated from the fraction defined as the secretome. From each fraction, almost half of the proteins (nucleus: 280 proteins (48%) and secretome: 147 proteins (49%)) were identified with one or more arginine containing peptides, which allowed quantitation. Fold change values of proteins from Day 1 until Day 7 were calculated using Day 0 as a reference. A Gene Ontology cellular component analysis revealed that approximately half (284) of the proteins found in the nuclear fraction were previously known nuclear proteins as shown in Figure 3A. A similar analysis of the proteins identified in the secretome revealed that 53% (149) were either membrane bound or could be classified as being secreted proteins as shown in Figure 3B.

Differentially expressed proteins in the nuclear proteome

Using a 2-fold change as a cut-off to designate up or downregulated proteins, 23 proteins showed alterations on Day 1. However, at least 150 proteins showed more than 2-fold change at each time point from Day 3 to Day 7 as shown in Table 1. These observations indicate that significant morphological and biological changes occurred during middle or late stage of adipogenesis. Among the 280 proteins quantitated from nuclear fraction, 144 proteins showed consistently increased expression at least at 3 time points.

Upregulated proteins—A heat map demonstrating the dynamics of protein expression in the nuclear fraction is shown in Figure 4. There are two major expression patterns observed in upregulated proteins: one pattern includes proteins that exhibited maximum expression on Day 3. These are represented by small nuclear ribonucleoprotein D1, PHD-finger 5A and THO complex 4. Small nuclear ribonucleoprotein D1 has been shown to provide T cell help to produce anti-dsDNA antibodies involved in lupus development[20]. PHD-finger 5A protein belongs to a novel murine multigene family that is highly conserved during evolution. The function of PHD-finger 5A is not clear but it may act as a chromatin-associated protein[21] THO complex 4, also known as ALY, mediates context-dependent transcriptional activation by facilitating the functional collaboration of multiple proteins in the T-cell receptor alpha enhancer complex[22]. Thus, these proteins may participate in transcriptional regulation in the early or middle stage of adipocyte differentiation. The other expression pattern includes proteins that showed a gradual increase in levels from Day 3 to Day 7, and are exemplified by lipoprotein lipase, isocitrate dehydrogenase 1 (NADP+) and EH-domain containing 2 proteins. Lipoprotein lipase is a key enzyme in energy metabolism, catabolizing triglycerides into free fatty acids and glycerol[23]. It is well known as an adipocyte specific gene and significantly induced in differentiated adipocytes[8, 24]. In the present study, lipoprotein lipase remained unchanged on Day 1, started to increase on Day 3 and reached peak expression on Day 7, which is consistent with previous observations. The isocitrate dehydrogenase 1 ($NADP^+$) catalyzes oxidative decarboxylation of isocitrate to α-ketoglutarate and require $NADP^+$ to produce NADPH, which plays an antioxidant role during oxidative stress[25]. Proteins that have an EH domain can carry out a variety of cellular functions ranging from regulation of the actin cytoskeleton, signal transduction and transcriptional regulation to control of the endocytic pathway. Mammalian cells express four highly homologous C-terminal EH-domain-containing paralogs (EHD1-EHD4), which are significantly similar in sequence[26]. High expression of EH-domain containing 2 impairs endocytosis of GLUT4 in cultured adipocytes and transferrin in COS cells[27]. The isocitrate dehydrogenase 1 (NADP+) and EH-domain containing 2 showed similar expression pattern with lipoprotein lipase, which indicates that both proteins might be novel potential markers of adipogenesis.

Downregulated proteins—In the nuclear faction, SNF2 alpha was downregulated at all stages (Days 1 through 7) as compared to preadipocytes (Day 0). SNF2 alpha, also designated BRM, belongs to SWI/SNF family, a chromatin remodeling complex and regulates the expression of several transcription factors in an ATP-dependent manner. It was found that its expression gradually increased during the differentiation of liver cells[28]. The process of adipocyte differentiation forced by overexpression of adipogenic regulatory proteins was inhibited upon expression of dominant-negative hBRM in several adipocytes[29]. However, we observed downregulation of SNF2 alpha from Day 1 to Day 7 during adipogenesis in the present study. One explanation for the discrepancy could be that the dominant negative does not fully abrogate the physiologic functions of SNF2 alpha in adipocytes. In this respect, the effect of SNF2 alpha deletion on adipogenesis could help explain these seemingly disparate findings. Another reason could be that we used a different inducer for driving adipocyte differentiation and that the dominant negative SNF2 alpha only inhibits when PPAR gamma activation directly drives adipocyte differentiation. In any case, our findings suggest that additional experimentation is required to understand this process in detail.

Differentially expressed proteins in the adipocyte secretome

The proteins secreted by adipocytes exert crucial roles in the energy homeostasis and other procedures in a paracrine or endocrine manner. Thus, we also examined the dynamics of expression of proteins secreted by adipocyte during adipogenesis. A total of 147 proteins were quantitated in the secretome of adipocytes from this experiment. It was found that most of the quantitated proteins showed a very low level on Day 1 (Table 2). This coincides with the

observation that the media on that particular day has a much higher viscosity, than observed on Day 0, Day 3, Day 5 and Day 7. It is believe that this greater viscosity is due to an increased secretion of Glycans. Even though the majority of proteins showed decreased expression on Day 1, there are still 6 proteins increased more than 2-fold. As shown in Table 2, abundance of more than 40 proteins changed at each time point from Day 3 to Day 7. A heat map shows the secreted proteins that were quantitated in this experiment (Figure 5).

Upregulated proteins—A subset of secreted proteins demonstrated highest expression at the Day 3 time point, which is exemplified by Biglycan, Nidogen 1 and Nidogen 2. Biglycan is one of small chondroitin sulfate proteoglycans, which comprise a subset of virtually ubiquitous extracellular proteoglycans in most connective tissue matrices[30]. Depletion of biglycan affected the differentiation of tendon stem/progenitor cells by modulating bone morphogenetic protein signaling[31]. Nieden et al. presented that Biglycan can drive embryonic stem cells to the cartilage, osteoblast or adipogenic fates depending on the supplementary co-factors[32]. In the present study, Biglycan protein existed in all stages during adipogenesis and presented a peak expression on Day 3. Considering the function of Biglycan in stem cell differentiation, our results indicate that the secreted Biglycan may assist in the differentiation of proadipocytes from Day 3 to Day 5 of maturation. Nidogen 1 and 2 are basement membrane glycoproteins and previous biochemical and functional studies indicate that they may play a crucial role in basement membrane assembly. Bader et al. found that deficiency of both nidogens in mice resulted in perinatal lethality. Nidogen 1 and 2 do not appear to be crucial in establishing tissue architecture during organ development; instead, they are essential for lung development and for maintenance and/or integrity of cardiac tissue[33]. The expression of Nidogen1 and 2 during adipogenesis showed a similar pattern: both of them had marginal expression at early and late stage of adipocyte differentiation. However, they showed the highest expression half way through the differentiation (Day 3). Thus, like Biglycan, Nidogen 1 and 2 might be involved in the middle stage of adipocyte differentiation.

Another subset of proteins showing consistently increased expression at both Day 5 and Day 7 compared with Day 0 to Day 3, such as Acrp 30. Acrp30, also called adiponectin, is encoded by adipocyte-specific apM-1 gene and specifically secreted by adipocytes. Preadipocytes with overexpressed acrp30 were observed to differentiate into adipocytes more rapidly[34]. ApM-1-mRNA and Acrp30 proteins were found to be upregulated from Day 4 to Day 9 during the adipocyte differentiation as shown by RNase protection assay and Western blot analysis[35]. Our data also showed robust expression of Acrp30 in the secretome from Day 3 to Day 7 of adipogenesis using MS analysis. Noticeably, our previous study also demonstrated that Acrp30 was highly secreted by adipocyte compared to preadipocytes[10]. Our data highlights that targeting adiponectin becomes promising strategy in the treatment of obesity related diseases.

Downregulated proteins—There are 17 proteins in secreted fraction exhibiting a consistently reduced expression during adipogenesis. Interestingly, three isoforms of tropomyosins were observed among those proteins. Tropomyosins are encoded by 4 different genes, which produce over 40 isoforms through alternative splicing[36]. Particular tropomyosin isoforms have been observed to be associated with cellular actin-based processes including the contractile ring, cortical actin, stress fibres, lamellipodial actin networks and filopodia[36]. The spatial and temporal regulation of tropomyosin isoforms are involved in the establishment of microfilaments in various tissues and cells. Thus, tropomyosin family is a predominant regulator in actin filament dynamics, which need to be tightly regulated in proliferation, apoptosis, differentiation and other cellular processes[37]. The expression of tropomyosin 1, 3 and 4 were decreased from Day 1 to Day 7 during adipocyte differentiation in our MS analysis, which indicates the tropomyosin family may be also an important player in adipogenesis. Another example for decreased proteins in secreted fraction is zyxin. Zyxin is a component of adhesion plaques that has been suggested to perform regulatory functions at those specialized

regions of the plasma membrane. Zyxin was found to be significantly upregulated in melanoma cells compared to melanocytes and its expression directly related to cell spreading and proliferation and inversely related to differentiation[38]. Here, zyxin was demonstrated to decrease during adipogenesis, which may also imply that secreted zyxin is inversely related to adipocyte differentiation.

Western blot analysis for differentially expressed proteins

In order to validate the results from SILAC-based proteomic analysis, Western blot analysis was carried out for four representative proteins. The Western blot results and corresponding mass spectrum for each protein are displayed in Figure 6. Poly(A)-binding protein 1, Splicing factor, arginine/serine-rich 2 and Lumican showed a similar expression pattern in that they were expressed at low levels on Day 0 and Day 1, but there were significantly increased on Day 3 followed by a decrease on Days 5 and 7. As shown in Figure 6, the Western blot results for these three molecules closely correlate with the SILAC data. In contrast, the Western blot analysis of Annexin V does not correlate with the quantitative mass spectrometry data. The SILAC data indicates that Annexin V shows the highest expression on Day 7 as compared to the other times. However, the Western blot shows that it has a higher expression on Days 3 and 5 than on Day 7. We think that this apparent discrepancy between MS and Western blot analysis is likely due to the non-specificity of the antibody against Annexin V. It must be noted here that a significant fraction of differentially expressed proteins that we have identified do not have any commercially available antibodies against them illustrating the power of quantitative proteomic approaches. In these cases, if additional validation is still required, AQUA type of studies (either in an MRM type of experiment or non-MRM type of experiment) can be carried out.

Conclusions

Until recently, profiling of dynamics of proteins in multiple biological conditions was limited by lack of accurate quantitative techniques. Stable isotope labeling based quantitative proteomic profiling technology has become popular mass spectrometry analysis for such analysis. Although, recent development of *in vitro* labeling methods such as iTRAQ reagents allows simultaneous proteomics comparison of up to 8 different samples, the drawbacks of these methods include biases due to labeling efficiency and limited fractionation options at protein and subcellular levels. Thus, *in vivo* labeling using the SILAC method can be used in the case of cell cultures to overcome these limitations. In this study, we were able to extend the SILAC labeling method to use up to 5 different states for a simultaneous comparison of protein abundance. We have successfully identified many differentially expressed proteins during adipogenesis, which may be of importance for this process. Further validation studies are necessary to evaluate the large number of proteins identified in differentiating adipocytes and their secretome. One of the technical disadvantages of SILAC five state experiments is limited number of amino acids, which can be made use for differential isotope labeling. Hence, use of arginine alone in this study resulted in lack of quantitation of some proteins identified in this study. Further, blocking the lysine groups and use arginine specific enzymes may increase the efficiency of this arginine based 5-state SILAC approach. Overall, this study illustrates the potential applications of this method to study spatiotemporal dynamics of a number of biological processes in cell culture-based systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Supporting Information Available: Protein name, fold-change, accession number and gene symbol for each quantitated proteins are provided as Supporting Information. This is available free of charge via the Internet at http://pubs.acs.org.

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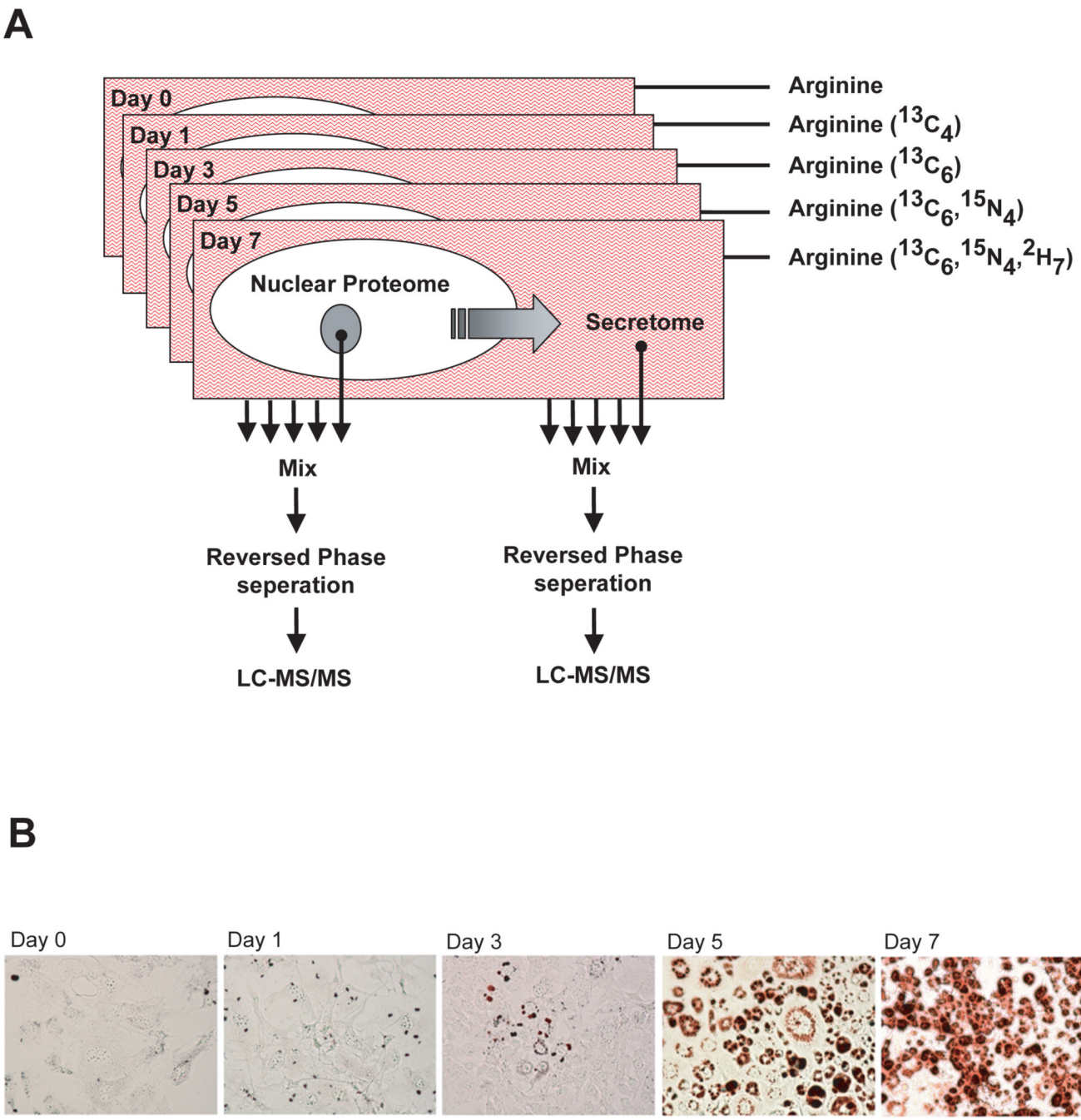

Figure 1. 5-plex SILAC strategy and Oil red O staining for adipocyte differentiation

A) Preadipocytes were grown in different cell culture media containing 5 different isotopic forms of arginine as shown until complete isotopic amino acid incorporation. After the induction of differentiation, cells were harvested on Day 0, Day 1, Day 3, Day 5 and Day 7 of the differentiation process and combined after protein normalization. In parallel, serum free media from five different cell populations were collected at the indicated time points and combined. The nuclear fraction and the secretome were processed and analyzed by LC-MS/MS. B) Differentiation of adipocytes was confirmed by Oil red O staining. Cells on Day 3, Day 5 and Day 7 show a progressive increase in red staining for fat droplets, indicating the extent of adipocyte differentiation.

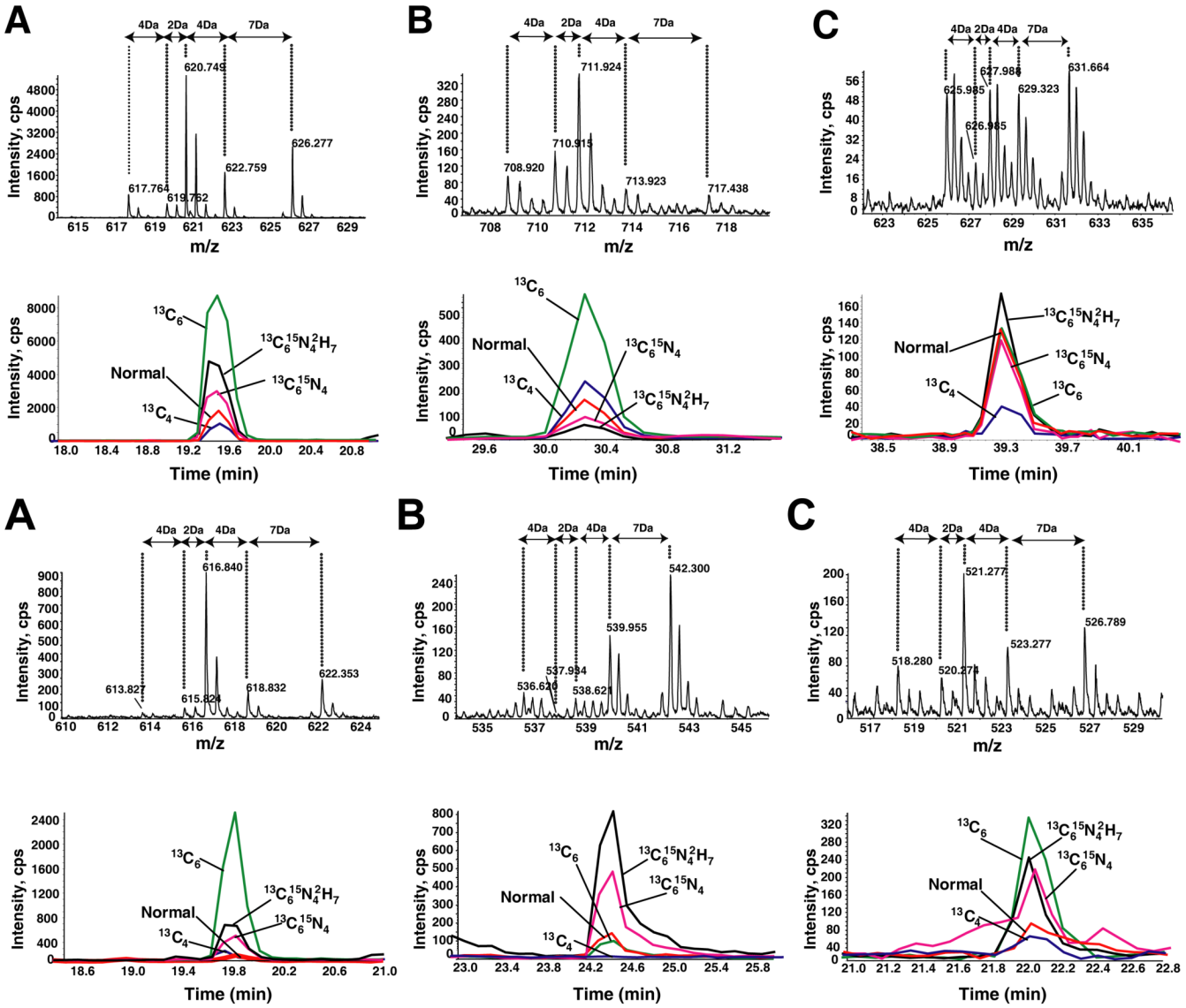

Figure 2. Mass spectra and elution profiles for SILAC labeled peptide sets

Upper panels show the mass spectra and lower panels show elution profiles. The color code of the peptide elution profiles are: red: Day 0, blue: Day 1, green: Day 3, violet: Day 5 and black: Day 7, represented by the isotopes: normal, $^{13}C_4$, $^{13}C_6$, $^{13}C_6{}^{15}N_4$, and $^{13}C_6{}^{15}N_4{}^2H_7$, respectively. The mass differences between the isotopic labeled peptides are shown in each upper panel. The sequence and charge states of the depicted peptides are as following. Figure 2 A: The peptides from secreted fraction. A: APILIATDVASR, doubly charged (DEAD box polypeptide 5), B: LGVEFDEITADDRK, triply charged (Fatty acid binding protein 2) and C: WISIMTER, doubly charged (Annexin A2). Figure 2 B: the peptides from nuclear fraction. A: IETIEVMEDR, doubly charged (similar to heterogeneous nuclear ribonucleoprotein A3 isoform 7), B: AAAITSDLLESLGR, doubly charged (osteoblast specific factor 2, fasciclin I-like), C: FGVEQDVDMVFASFIR, triply charged (pyruvate kinase 3).

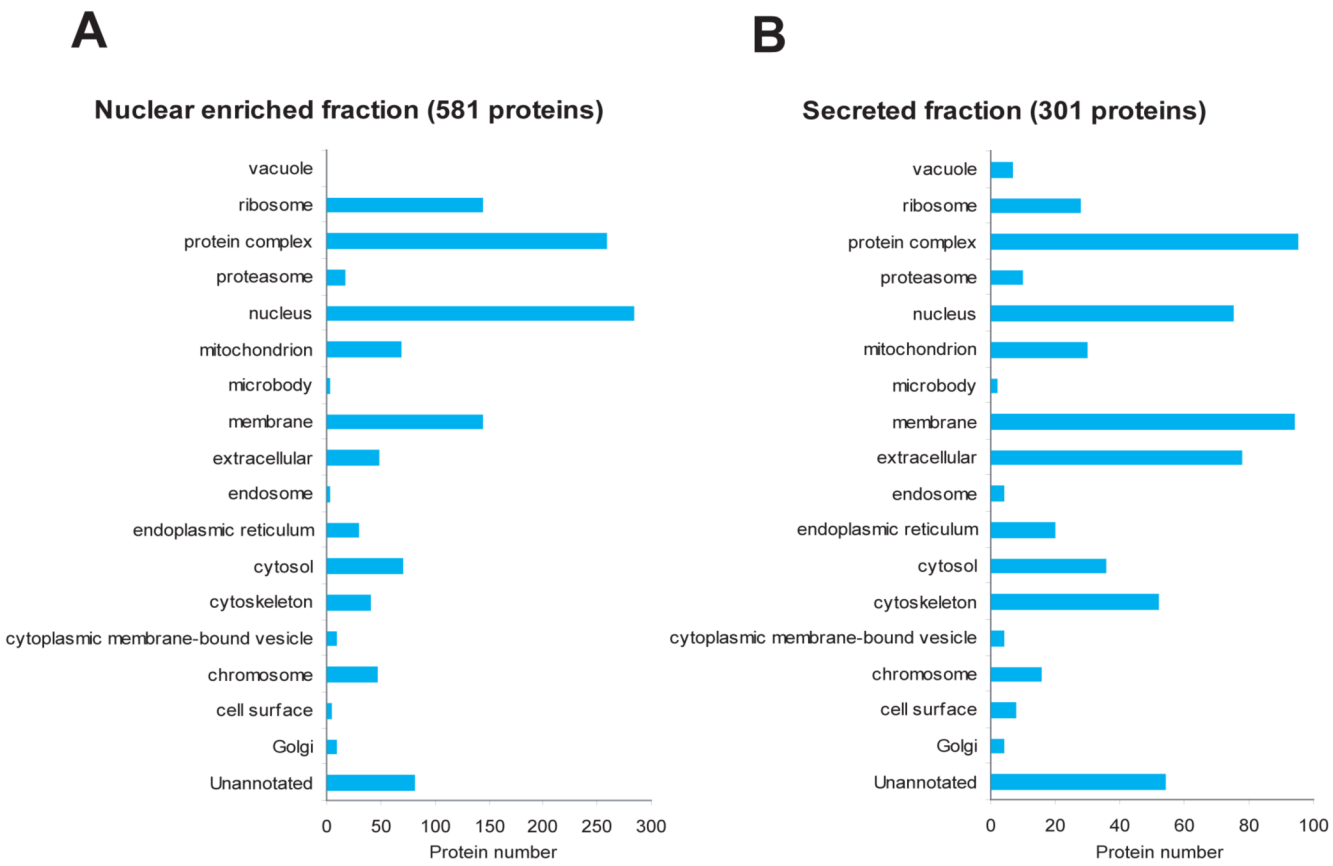

Figure 3. Gene Ontology and cellular component analysis

Gene Ontology analysis for identified nuclear and secreted proteins was performed using ProteinCenter version 1.2 (Proxeon A/S, Odense, Denmark). Panel A shows the analysis for the 581 proteins identified in the nuclear enriched fraction, while panel B shows the corresponding analysis for the proteins identified from the secretome. Approximately half (284) of the proteins found in the nuclear fraction were previously known nuclear proteins. For the secreted proteins, the analysis revealed 149 proteins were membrane or cell surface proteins.

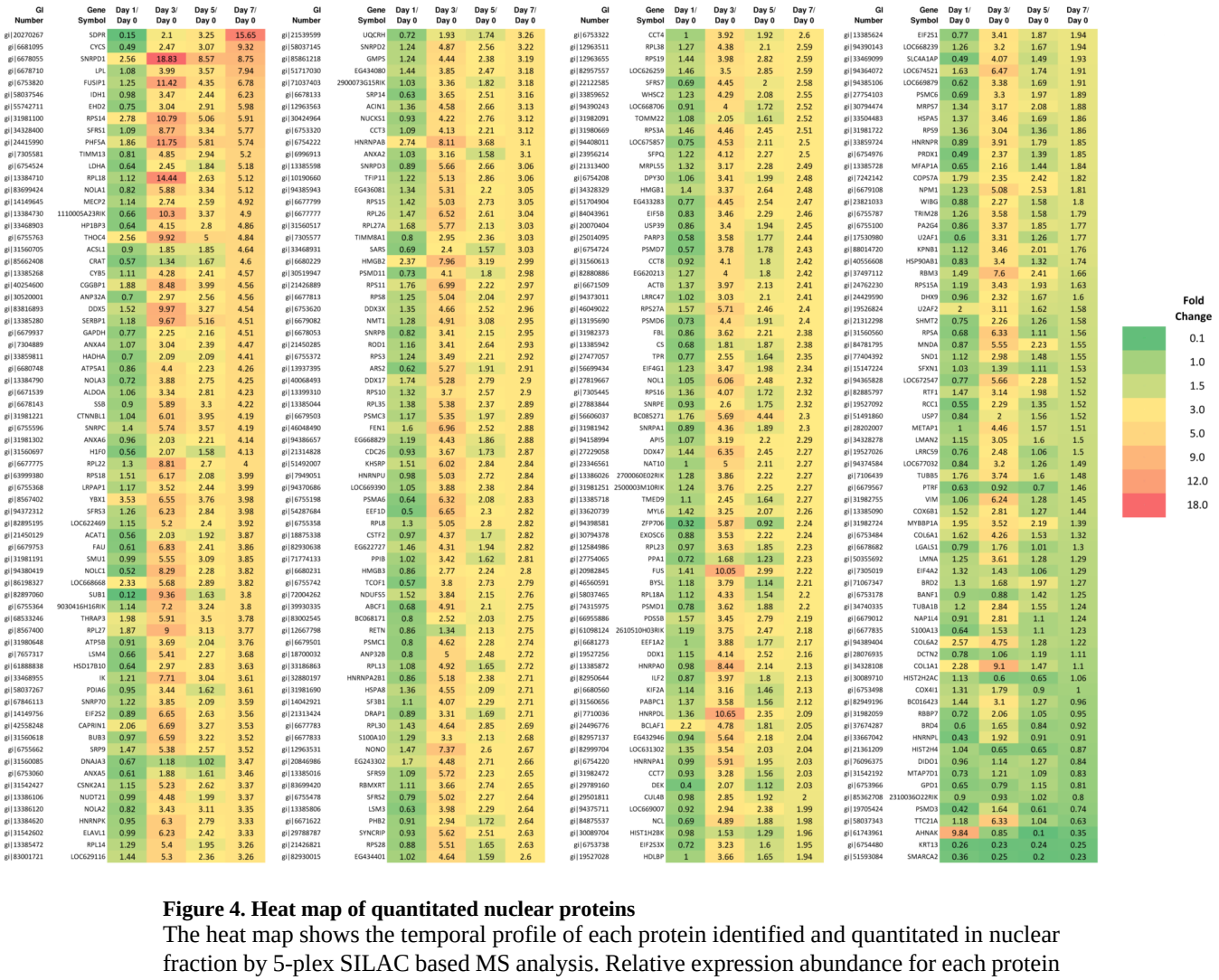

Figure 4. Heat map of quantitated nuclear proteins

The heat map shows the temporal profile of each protein identified and quantitated in nuclear fraction by 5-plex SILAC based MS analysis. Relative expression abundance for each protein is indicated in each frame using Day 0 as a reference. For reference, a color intensity scale is included in the right side.

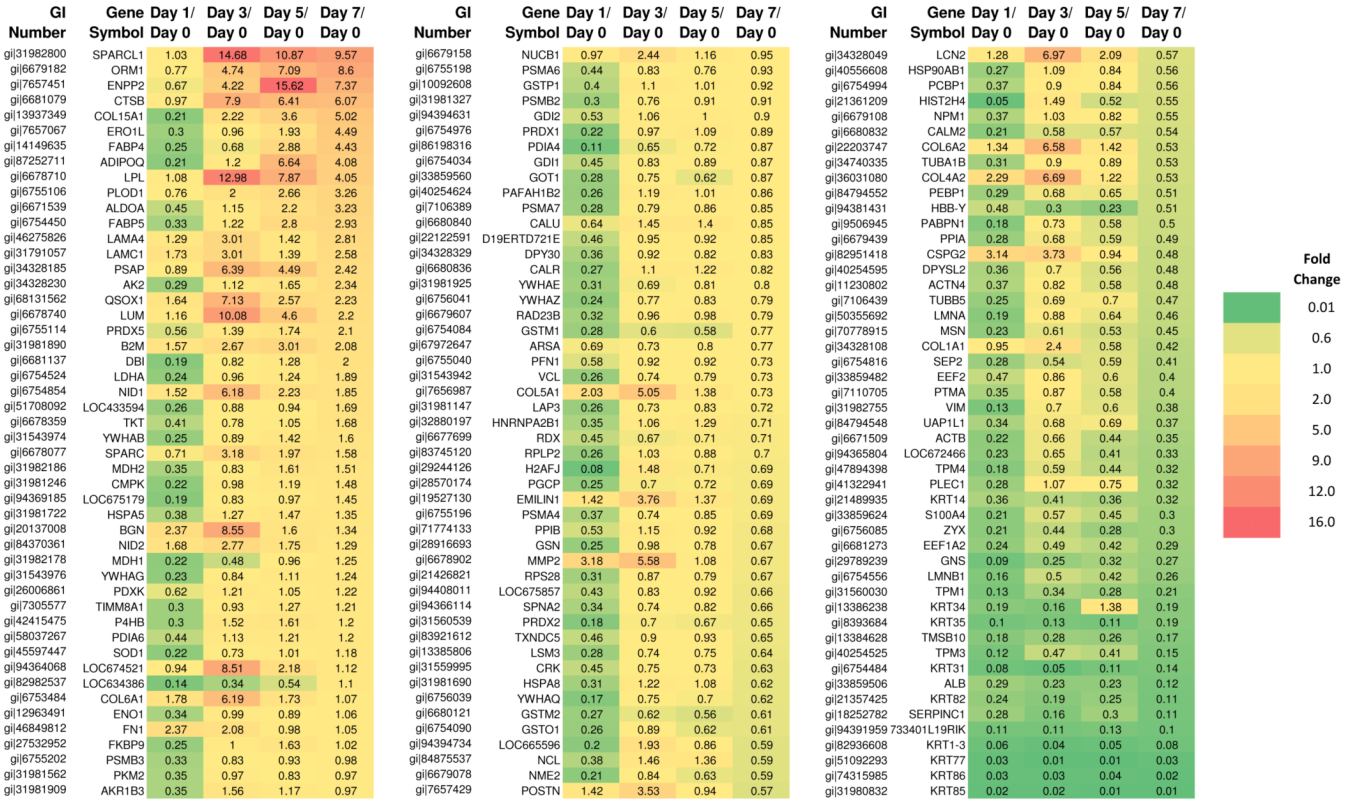

Figure 5. Heat map of quantitated secreted proteins

The heat map shows the temporal profile of each protein identified and quantitated in the secretome by 5-plex SILAC based MS analysis. Relative expression abundance for each protein is indicated in each frame using Day 0 as a reference. For reference, a color intensity scale is included in the right side.

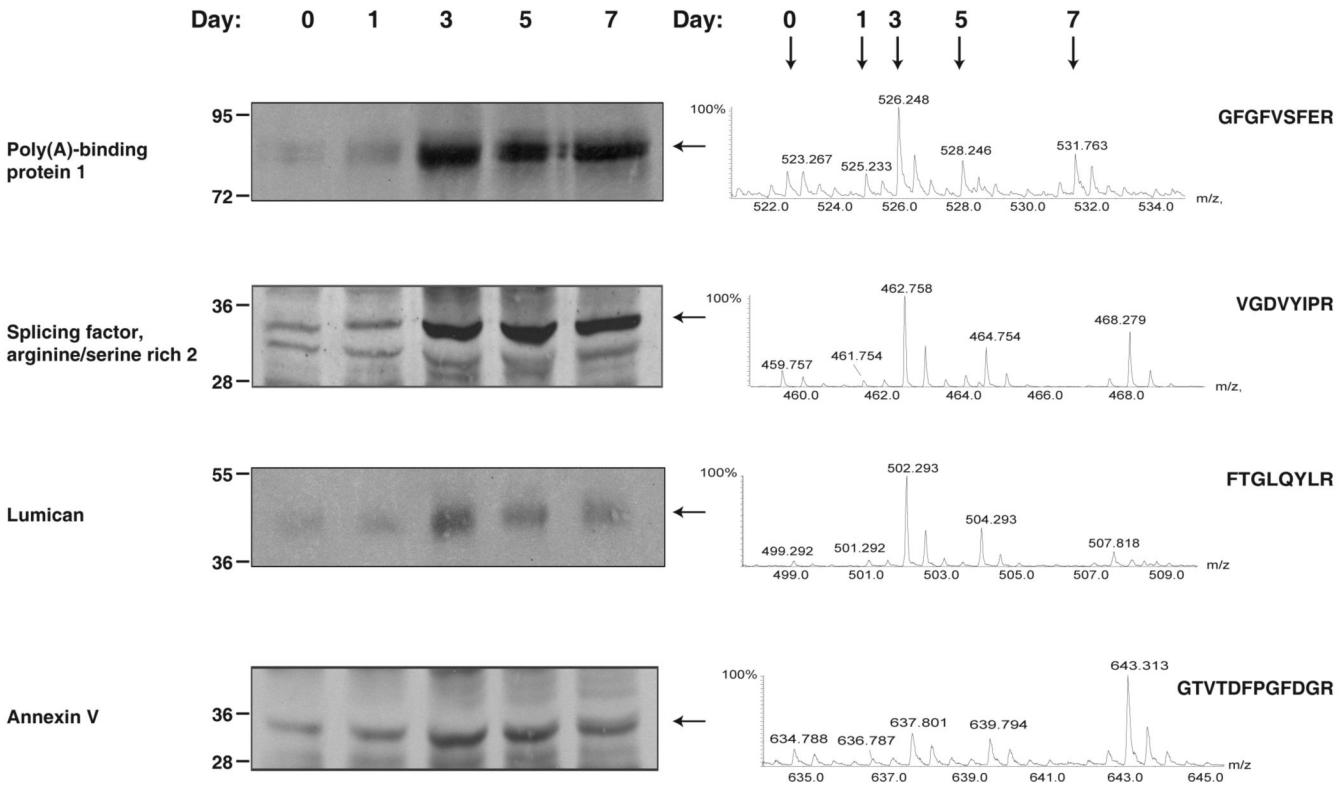

Figure 6. Western blot analysis for a subset of differentially expressed proteins

The left panel shows Western blot results with the right panel showing MS/MS spectra of a peptide from the corresponding protein.

Table 1

Distribution of nuclear proteins showing altered expression during adipogenesis

Fold-change	Day 1	Day 3	Day 5	Day 7
> 2	12	249	153	204
<0.5	11	2	2	2

Table 2

Distribution of secreted proteins showing altered expression during adipogenesis

Fold-change	Day 1	Day 3	Day 5	Day 7
> 2	6	50	41	57
<0.5	111	21	23	37