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Research Article

Profiling of the secreted proteins during 3T3-L1 adipocyte differentiation leads to the identification of novel adipokines

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Abstract. Adipose tissue is an endocrine organ capable of secreting a number of adipokines with a role in the regulation of adipose tissue and whole-body metabolism. We used two-dimensional gel electrophoresis combined with mass spectrometry to profile the secreted proteins from (pre)adipocytes. The culture medium of 3T3-L1 cells during adipocyte differentiation was screened, and 41 proteins that responded to blocking of secretion by 20 °C treatment and/or brefeldin A treatment were identified. Prohibitin, stress-70 protein, and adhesion-regulating molecule 1 are reported for the first time as secreted proteins. In addition, procollagen C-proteinase enhancer protein, galectin-1, cyclophilin A and C, and SF20/IL-25 are newly identified as adipocyte secreted factors. Secretion profiles indicated a dynamic environment including an actively remodeling extracellular matrix and several factors involved in growth regulation.

Key words. 3T3-L1 adipocyte; secretion; proteomics; extracellular matrix; growth regulation.

Introduction

Obesity is one of the most frequent nutritional disorders in western societies. It is strongly associated with type II diabetes, cardiovascular diseases and cancer [1, 2]. Obesity is characterized by excess of body fat mass, which is mostly stored in adipose tissue. In addition, adipose tissue is an endocrine organ able to secrete a number of signal molecules. Adipocyte-specific or -enriched secreted proteins, termed adipokines, have a variety of local, peripheral and central effects on metabolism, which could be links between obesity and related diseases [3, 4]. Novel secreted proteins involved in lipid and glucose metabolism have been discovered in the last 10 years, such as leptin, adiponectin, and resistin, but also proteins with alternative functions such as complement C and D (adipsin), which are also involved in triglyceride synthesis [4]. Until now, the number of systematic searches for secreted proteins from preadipocytes, adipocytes and/or adipose tissue is limited. Genes that encode adipocyte-specific or -enriched secreted or surface proteins have been screened in 3T3-L1 adipocytes and human visceral adipose tissue [5–7]. At the protein level, one-dimensional (1D)-electrophoresis and liquid chromatography-based separation combined with tandem mass spectrometry (MS/MS) have been used to identify secreted proteins during differentiation of 3T3-L1 preadipocytes to adipocytes [8].

Here we present our results for proteins secreted during adipocyte differentiation using a systematic proteomic

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approach, based on the selective blocking of secretion pathways. Proteins can be secreted via the endoplasmic reticulum (ER)/Golgi-dependent pathway to the extracellular space. In addition, some proteins are found to be secreted via ER/Golgi-independent pathways [9]. Brefeldin A (BFA) is known to inhibit protein secretion in eukaryotic cells by interfering with the function of the Golgi apparatus [10]. Treatment at 20 °C blocks both the ER/Golgi pathway as well as the ER/Golgi-independent pathways [11]. Both BFA and 20ºC treatment have been reported to totally block the secretion of leptin from 3T3-L1 adipocytes [12]. We employed mouse 3T3-L1 cells, as the model system most frequently used to study adipocytes in vitro [13]. We used 20ºC or BFA treatment to block protein secretion, allowing us to discriminate the secreted proteins from non-secreted, leaked proteins in the medium. We screened the culture medium by two-dimensional (2D) gel electrophoresis followed by MS for identification. This approach led to the discovery of novel adipokines.

Materials and methods

Chemicals were purchased from Sigma (Zwijndrecht, The Netherlands) unless stated otherwise.

Cell culture and medium protein sample preparation

Murine 3T3-L1 fibroblasts (American Type Culture Collection) were cultured and differentiated in vitro essentially as described by Bouwman et al. [14], except that preadipocytes were treated with an adipogenic cocktail for 2 days. Differentiation was monitored by the visual appearance of fat droplets in the cells. On day 0, 4, 8 and 12 of differentiation, 3T3-L1 cells cultured in 150-mm dishes were prepared for sampling by washing the cells three times with 25 ml serum-free medium (D-MEM/F-12, 1:1; Invitrogen, Breda, The Netherlands). Then, the washed cells were incubated in 12 ml phenol red-free and serum-free medium supplemented with 0.7 µM bovine insulin, 2.2 mg/l human transferrin, 10.6 nM sodium selenium and 13.1 µM ethanolamine at 37ºC or 20 °C. To block the secretion of cells with BFA, a pretreatment of adding 20 µM BFA to the medium for 1 h at 37 °C was performed before washing. After washing, the cells were incubated in the medium supplemented with 20 µM BFA at 37 °C. After 8 h, the media were harvested, and centrifuged at 4 °C, 150 g for 10 min. The supernatants were transferred to dialysis tubes (2-kDa molecular-weight-cut-off; Spectrum Laboratories, Rancho Dominguez, Calif.) and dialyzed against 20 mM NH_4HCO_3 at 4 °C for 20 h. Meanwhile, the cells were trypsinized and counted with a hemocytometer. The dialyzed medium samples were freeze-dried and dissolved in fresh rehydration buffer (8 M urea, 2% w/v CHAPS, 65 mM dithiothreitol, 0.5% v/v IPG buffer 3-10 NL (Amersham Bioscience, Roosendaal, The Netherlands)). The protein concentrations of the samples were measured by a protein assay kit (Bio-Rad, Veenendaal, The Netherlands), based on the method of Bradford. Aliquots were stored at –80ºC. Independent triplicate experiments were performed.

Two-dimensional gel electrophoresis

Medium samples derived from 2.2×10^6 cells were used for 2D gel analysis according to Bouwman et al. [14] but with 24-cm pH 3–10 NL strips. The gels were silver stained simultaneously [15]. The images of gels were obtained using a GS-800 calibrated densitometer (Bio-Rad), and processed by PDQuest 7.2 (Bio-Rad). The gel intensity was normalized with respect to the total protein amount applied on the gel. Gels from the samples with the same time point and the same treatment in three independent experiments formed one replicate group with average spot intensities (absent spots were excluded). A difference of the average value of the spot intensity between groups was considered as meaningful at a threshold ratio of 2 or more. Any spot that showed a difference between the 37 °C condition and the blocking condition, 20 °C or BFA treatment, was selected for further identification. To simplify MS-dependent identification of spots, parallel gels were made with higher sample loading, and stained with Coomassie Brilliant Blue. The differentially expressed spots were excised using an automated spot cutter (Bio-Rad).

MS and protein identification

Spots were in-gel digested and peptide-mass fingerprints were obtained by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) (Waters, Almere, The Netherlands) as described by Bouwman et al. [14]. Some spots that could not be identified by MALDI-TOF analysis were further processed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) as described by Dumont et al. [16], allowing a maximum of one missed cleavage. The MALDI-TOF data were screened against the Swiss-Prot database using the ProteinLynx GlobalServer (Waters), and against the National Center for Biotechnology Information non-redundant mouse database using Mascot (http://www.matrixscience.com/search_form_select.html) and Profound (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe). The MS/MS data were screened against the GenBank non-redundant mouse database using Mascot and Sequest (ThermoFinnigan, San Jose, Calif.).

1D and 2D Western blotting

To confirm the identity of adiponectin, adipocyte medium samples were analyzed by 1D or 2D Western blot. For preparing the 1D blot, proteins were separated

on a denaturing 12.5% gel. For the 2D blot, samples were separated on immobilized 11-cm (Bio-Rad) pH 3–10 non-linear strips followed by second-dimensional separation on 12.5% Criterion gels (Bio-Rad), according to the manufacturer's instruction. The separated proteins were transferred to Hybond ECL nitrocellulose membrane (Amersham Bioscience). An additional 2D gel was run in parallel followed by silver staining for reference. After blocking, the blots were probed with an anti-mouse adiponectin monoclonal antibody (Chemicon, Chandlers Ford, UK), washed, and incubated with a horseradish peroxidase-conjugated secondary antibody (DAKO, Glostrup, Denmark). The signals were detected by Super-Signal West Pico Chemiluminescent reagent and CL-XPose clear blue X-ray film (Perbio Science, Etten-leur, The Netherlands).

Amino acid sequence search for features of secreted proteins

For the identified secreted protein candidates without a signal peptide as indicated by Swiss-Prot, the amino acid sequence was analyzed [17] with SignalP V2.0.b2 and TMAP. SignalP [18] predicts the presence and location of signal peptide cleavage sites in the N-terminal part (first 70 residues). TMAP [19] predicts the number of transmembrane domains.

Results

Identification of secreted proteins from 3T3-L1 cells during adipocyte differentiation

On day 12 of differentiation, more than half of the cells showed adipogenic conversion characterized by visible fat droplets inside the cells. The secreted proteins in cell culture medium were harvested at day 0, 4, 8, and 12, and profiled using 2D gel electrophoresis (fig. 1A). From a total of 1077 spots, 193 responded to blocking conditions; 158 spots were blocked by 20 °C as well as by BFA treatment, 32 spots were blocked only by the 20 °C treatment. One of these spots, which was later identified as SF20/IL-25, was blocked at day 4 and 8, but not at day 12. Another three spots were only blocked by the BFA treatment. In total, from 161 spots, the proteins could be identified by MALDI-TOF MS and LC-MS/MS, and are marked on the master gel (fig. 1B). These spots represent 41 different proteins (table 1).

Secretion features of candidate proteins

Of the 41 candidate proteins, 26 were reported in the literature as secreted proteins. With the exception of cyclophilin A, these proteins are located in the extracellular space as annotated in the Swiss-Prot or the SOURCE (http://source.stanford.edu) database. However, 5 of these known extracellular proteins are without a secretion signal peptide as annotated in the database: collagen V alpha 1, galectin-1, macrophage migration inhibitory factor (MIF), stromal cell-derived growth factor SF20/IL-25, and cyclophilin C. To screen for the potential secretion features, these 5 proteins and cyclophilin A were analyzed together with the remaining 15 proteins by the SignalP and TMAP programs. In three proteins, collagen V alpha 1, SF20/IL-25, and cyclophilin C, a secretion signal peptide sequence could be identified. Furthermore, prohibitin, stress-70 protein, and adhesion-regulating molecule 1 were found to harbor a predicted secretion signal sequence indicating that these are genuine secreted proteins. Peroxiredoxin 1 might be anchored to the membrane because it has a predicted N-terminal transmembrane helix. Transitional endoplasmic reticulum ATPase and alpha enolase have a predicted transmembrane helix in the middle of the sequence, which is a feature of a transmembrane protein. The remaining 12 proteins, including the known secreted proteins galectin-1 and MIF, lack both the secretion signal sequence and the membrane anchor sequences (table 2).

Strongly up-regulated secretion of adipocyte markers during differentiation

Complement C3 that was detected at a low level in the medium of preadipocytes clearly increased during differentiation. Adipsin was also detected, but only in the medium of day 8 and 12, as two groups of spots within a pI range of 3.5–4.5 at ± 41 and ± 48 kDa, respectively (fig. 1B, spot ID 15). This is in line with the already reported glycosylation [20], but also suggests additional forms of post-translational modification. Adiponectin was identified as well (fig. 2A, B) and followed the same secretion pattern as adipsin. Although different forms of adiponectin have been reported, only one form was detected by us which was shown to be glycosylated by the presence of a mass peak of 1679 in the spectrum [21]. The secretion of adiponectin was only blocked by BFA. Treatment of 20 °C instead seemed to increase the secretion somehow (fig. 2C), as was confirmed by 1D Western blotting (fig. 2D).

Different changes of extracellular matrix components and their modifying proteins during adipocyte differentiation

Collagens made an important contribution to the secretion profile of 3T3-L1 cells, since they represented 106 out of 193 analyzed spots. These spots belong to eight different proteins related to five types of collagen. All types of collagen were secreted at any time point during differentiation but with a different profile. Collagens I and III were found as major types in both preadipocyte and adipocyte medium. The summed intensities of spots of these types of collagen generally showed first a decrease and then an increase during differentiation. At day 12, the

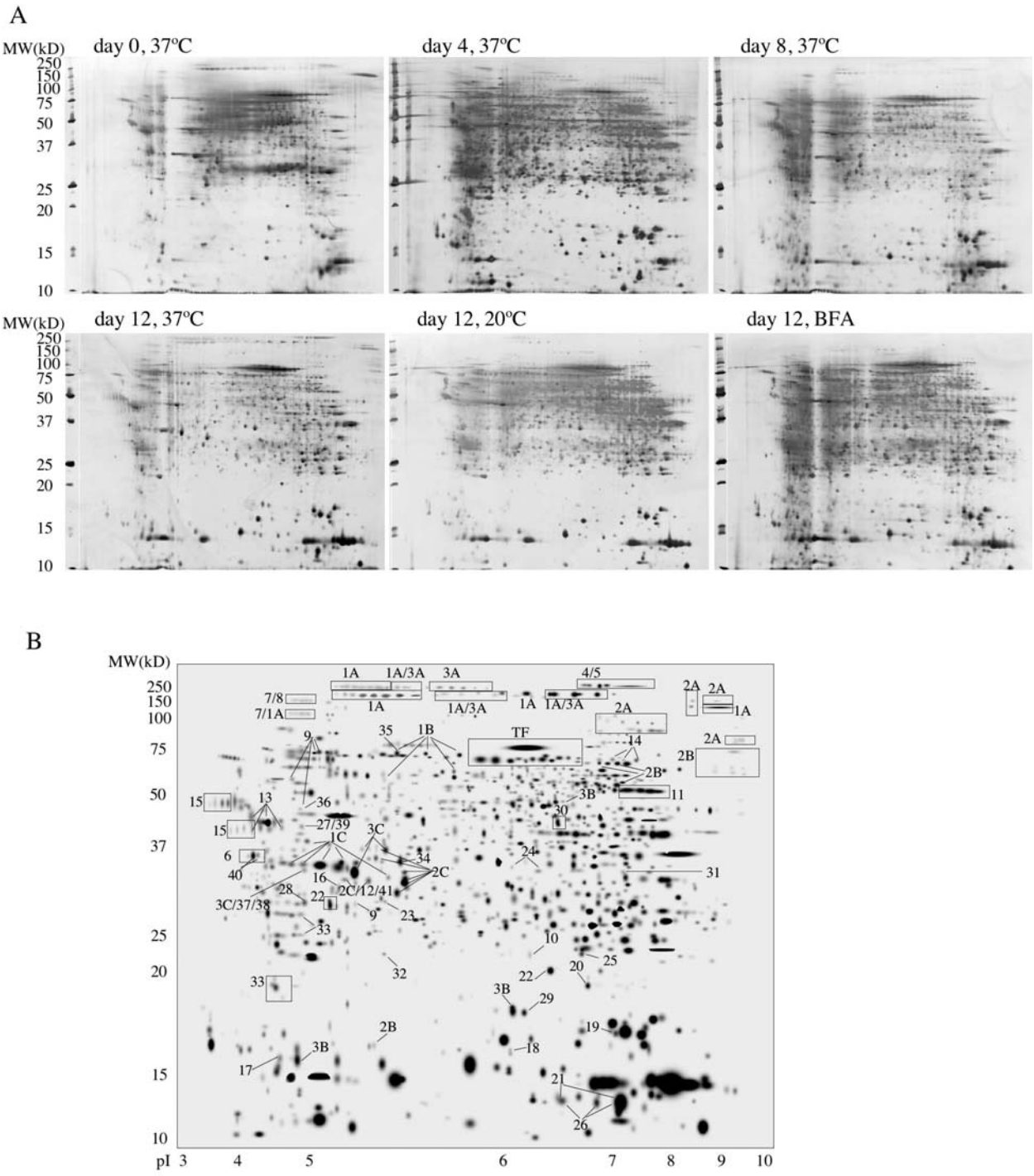

Figure 1. Medium proteins from 3T3-L1 cells during differentiation separated on 2D gels. (*A*) Representative gels of medium proteins, derived from 2.2×10^6 3T3-L1 cells on day 0, 4, 8, and 12, and on day 12 treated with 20 °C or 20 µM BFA. (*B*) The master gel with identified (mixed) spots. Spot numbers refer to the ID of the protein in table 1 (TF, human transferrin).

level of secretion per cell of these two types of collagen was again that of day 0 (fig. 3). The level of secretion of collagens IV, V and VI showed an increase during differentiation (fig. 3). We also identified four proteins that are involved in the process of collagen modification and degradation: matrix metalloproteinase-2, metalloproteinase inhibitor 2, procollagen C-proteinase enhancer protein, and protein-lysine 6-oxidase. For those proteins also, the secretion level was found to change during differentiation (fig. 3).

Another extracellular matrix component SPARC (osteonectin), a modulator of cell matrix interaction and col-

Table 1. Identification of candidate secreted proteins from 3T3-L1 cells during differentiation.

ID	Accession number	Protein description	Number of spots	blocked by		Change during differentiation[a]	Function	Secretion[b]
				20 °C	BFA			
Extracellular matrix and related proteins								
1A	P11087	(pro)collagen type I alpha 1	35	yes	yes	±, v	extracellular matrix component	I
1B		collagen type I alpha 1 fragment	5	yes[c]	yes	+		I
1C		collagen type I alpha 1 C-terminal propeptide	6	yes[c]	yes[d]	±, v	collagen catabolism by-product	I
2A	Q01149	(pro)collagen type I alpha 2	15	yes	yes	±, v	extracellular matrix component	I
2B		collagen type I alpha 2 fragment	10	yes	yes[d]	+		I
2C		collagen type I alpha 2 C-terminal propeptide	8	yes	yes	±, v	collagen catabolism by-product	I
3A	P08121	(pro)collagen type III alpha 1	19	yes	yes	±, v	extracellular matrix component	I
3B		collagen type III alpha 1 fragment	3	yes	yes	+		I
3C		collagen type III alpha 1 C-terminal propeptide	4	yes	yes	±, v	collagen catabolism by-product	I
4	P02463	(pro)collagen type IV alpha 1	5	yes	yes	+	extracellular matrix component	I
5	P08122	(pro)collagen type IV alpha 2	5	yes	yes	+	extracellular matrix component	I
6	O88207	collagen type V alpha 1 fragment	3	yes	yes	+	extracellular matrix component	I
7	Q04857	(pro)collagen type VI alpha 1	8	yes	yes	+, ^	extracellular matrix component	I
8	Q02788	(pro)collagen type VI alpha 2	1	yes	yes	+, ^	extracellular matrix component	I
9	P33434	matrix metalloproteinase-2 and fragment	6	yes	yes	±	collagen catabolism	I
10	P25785	metalloproteinase inhibitor 2	1	yes	yes	+	inhibitor of metalloproteinases	I
11	Q61398	procollagen C-proteinase enhancer protein	6	yes	yes	±	collagen catabolism	I
12	P28301	protein-lysine 6-oxidase	2	yes	yes	±, v (m)	modification for fibrous collagen	I
13	P07214	SPARC, osteonectin	4	yes	yes	++	modulator of cell-matrix interaction	I
Lipid metabolism								
14	P01027	complement C3 beta chain	3	yes	yes	++	stimulates triglyceride synthesis	I
15	P03953	adipsin	8	yes	yes	++	stimulates triglyceride synthesis	I
16	Q60994	adiponectin	1	no	yes	++	control of fat metabolism and insulin sensitivity	I
Growth regulation								
17	P16045	galectin-1	1	yes	no	++	growth regulator	I
18	Q9CPT4	stromal cell-derived growth factor SF20/IL-25	1	no	no	++	stimulates lymphocyte proliferation	I
19	P17742	cyclophilin A	1	yes	no	+, ^	protein folding, immune regulation	I
20	P30412	cyclophilin C	1	yes	no	+	protein folding, immune regulation	I
21	P34884	macrophage migration inhibitory factor	2	yes	yes	± (m)	proinflammatory cytokines	I
22	P97298	pigment epithelium-derived factor (fragment)	3	yes	yes	±, v	neurotrophic protein	I
23	P24142	prohibitin	1	yes	no	–	inhibits DNA synthesis	II
Others								
24	O09164	extracellular SOD	2	yes	yes	++	antioxidant	I
25	P35700	peroxiredoxin 1	1	yes	no	++	antioxidant	II
26	P01887	beta-2 microglobulin	3	yes	yes	± (m)	cell defense	I
27	Q61646	haptoglobin	1	yes	no	++ (m)	acute-phase response, proteolysis	I
28	P02571	gamma-actin fragment	1	yes	yes	+	cytoskeleton	II
29	P18760	cofilin, non-muscle isoform	1	yes	no	++	control of actin polymerization	II
30	P13020	gelsolin fragment	2	yes	yes	+, ^	actin-depolymerizing factor	I
31	O88569	heterogeneous nuclear ribonucleoprotein A2/B1	1	yes	yes	++, ^	pre-mRNA processing	II

Table 1 (continued)

ID	Accession number	Protein description	Number of spots	blocked by		Change during differentiation[a]	Function	Secretion[b]
				20 °C	BFA			
High expression on day 4 and only blocked by 20 °C treatment								
32	Q9DCX2	ATP synthase D chain, mitochondrial	1	yes	no	±, ^	proton transport	II
33	P20152	vimentin fragment	5	yes	no	±, ^	intermediate filament	II
34	P02570	beta-actin fragment	1	yes	no	++, ^	cytoskeleton	II
From mixed spots								
35	P38647	stress-70 protein, mitochondrial	1	no	yes	++ (m)	chaperone	II
36	Q9JKV1	adhesion-regulating molecule 1	1	yes	yes	– (m)	cell adhesion	II
37	P48036	annexin A5	1	yes	yes	± (m)	phospholipid binding	II
38	Q9JM65	non-clathrin coat protein epsilon-COP	1	yes	yes	± (m)	protein transport	II
39	Q01853	transitional endoplasmic reticulum ATPase fragment	1	yes	no	++ (m)	protein transport	II
40	Q60817	NASCENT polypeptide-asso-ciated complex alpha polypeptide	2	yes	yes	+ (m)	protein transport, transcription coactivator	II
41	P17182	alpha enolase fragment	2	yes	yes	±, v (m)	glycolysis	II

[a] Comparing the secretion level between day 0 and 12, a difference ≥ twofold was considered as meaningful, ≥ eightfold as strong. +, increased; ++, strongly increased; –, decreased; ±, no change; ^, highest, and v, lowest at day 4; m, mixed spots.
[b] Proteins with (I) and without (II) secretion evidence, respectively, in the Swiss-Prot, SOURCE, or PubMed database.
[c] One spot was not blocked by 20 °C, only by BFA.
[d] One spot was only blocked by 20 °C, not by BFA.

Table 2. Predicted secretion features of the candidate secreted proteins from 3T3-L1 cells during differentiation.

ID	Protein	Annotated location[a]	Number of transmembrane helices[b]	Presence of signal peptide[c]
6	collagen type V alpha 1	Exs	1	yes
18	SF20/IL-25	Exs	1	yes
20	cyclophilin C	Cyt, Exs	1	yes
17	galectin-1	Exs	0	no
21	macrophage migration inhibitory factor	Exs	0	no
19	cyclophilin A	Cyt	0	no
36	adhesion-regulating molecule 1	Mem	0	yes
23	prohibitin	Cyt	1 (N)	yes
35	stress-70 protein, mitochondrial	Mit	1 (N)	yes
25	peroxiredoxin 1	Cyt	1 (N)	no
39	transitional endoplasmic reticulum ATPase (fragment)	Gol, ER, Nuc	1 (middle)	no
41	alpha enolase (fragment)	Cyt	1 (middle)	no
28	gamma-actin (fragment)	Cyt	0	no
29	cofilin, non-muscle isoform	Nuc, Cyt	0	no
31	heterogeneous nuclear ribonucleoprotein A2/B1	Nuc	0	no
32	ATP synthase D chain, mitochondrial	Mit	0	no
33	vimentin (fragment)	Cyt	0	no
34	beta-actin (fragment)	Cyt	0	no
37	annexin A5	Mem	0	no
38	non-clathrin coat protein epsilon-COP	Mem, Gol	0	no
40	NASCENT polypeptide-associated complex alpha polypeptide	Nuc, Cyt	0	no

[a] From Swiss-Prot database. Exs, extracelluar space; Cyt, cytoplasm; Mem, membrane; Gol, Golgi apparatus; ER, endoplasmic reticulum; Mit, mitochondrion; Nuc, nucleus.
[b] Predicted by TMAP program; N, N-terminal.
[c] Predicted by SignalP program.

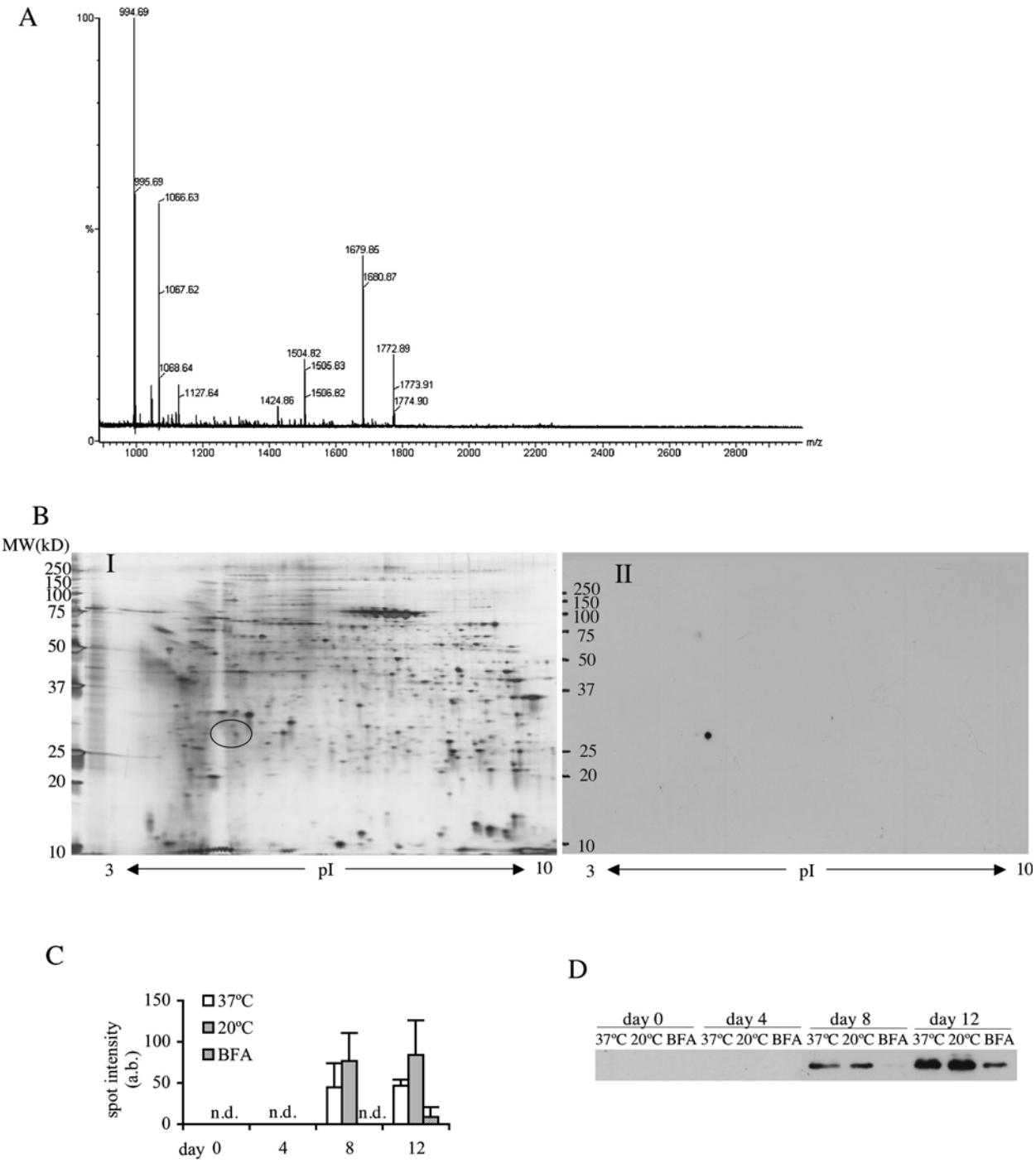

Figure 2. Identification of adiponectin and its secretion during 3T3-L1 adipocyte differentiation under unblocked and blocked conditions. (*A*) MALDI-TOF mass spectrum of tryptic peptide mixture derived from adiponectin. (*B*) Spot detection on 2D Western blot. One gel loaded with 15 µg proteins derived from 5.7×10^5 day 8 adipocytes was silver-stained (I). Another gel loaded with 7.5 µg proteins was blotted and probed with an anti-adiponectin antibody (II). The area of the adiponectin spot is circled. (*C*) Secretion levels of adiponectin detected by 2D gel on day 0, 4, 8, 12 of differentiation, at 37 °C, 20 °C, and with 20 µM BFA, respectively. The values were expressed as the mean ± SD of gel spot intensity in arbitrary units (a.b.) from three independent experiments; n.d., not detectable. (*D*) A representative 1D blot showing the secretion levels of adiponectin for the same conditions. Every lane was loaded with medium proteins derived from 5×10^4 cells.

lagen synthesis [22], was strongly up-regulated during differentiation, in line with previous reports [5, 8].

Secretion patterns of growth-modulating factors during differentiation

The growth-modulating factors galectin-1, cyclophilin A and C, stromal cell-derived growth factor SF20/IL-25, MIF, pigment epithelium derived factor (PEDF) and prohibitin were identified in our experiments (table 1, fig. 4). The secretion of the first four of these proteins was clearly up-regulated during differentiation, whereas the secretion of prohibitin, a known negative regulator of growth, decreased at day 12 to about half of its level compared with that at day 0. For MIF, the pattern could not be determined by 2D gel analysis because it was mixed with beta-2 microglobulin as detected by LC-MS/MS. The fragments of PEDF corresponding to the C- and N-terminal half of the protein showed a similar pattern as collagens I and III. For the complete protein, the pattern could not be determined by 2D gel analysis because it was in an area of high abundant spots, but Western blotting revealed the same pattern as for the fragments (data not shown).

Other proteins secreted by 3T3-L1 cells during differentiation

The acute-phase reactant haptoglobin and the self-defense protein beta-2 microglobulin were identified in the medium at day 12, but they appeared on the gel as mixed spots with a fragment of transitional endoplasmic reticulum ATPase and with MIF, respectively. Of these, haptoglobin was not yet present at day 0.

We identified two proteins, extracellular superoxide dismutase and peroxiredoxin 1, presenting as antioxidants in the medium of adipocytes. Both were hardly detectable at day 0, but showed strong up-regulation toward day 12. According to our criteria, a component of the cytoskeleton, a fragment of gamma-actin, and two actin filament modulators, cofilin and gelsolin (table 1), were identified as secreted factors. While these three proteins showed an increase in secretion level between day 0 and 12, gelsolin reached its highest level at day 4.

Finally, a ribonucleosome component, heterogeneous nuclear ribonucleoprotein A2/B1, was found to be present in the medium and strongly up-regulated upon differentiation (table 1).

Cellular proteins in the medium responding to 20 °C or BFA treatment

We clustered the spots that showed the highest expression at day 4, when the cells were easy to detach from the culture dish during incubation with the serum-free medium, and that were only blocked by the 20 °C treatment. Three cellular proteins met these criteria (table 1), whose presence in the medium was possibly due to cell leakage, as discussed below.

We observed an additional group of cellular proteins among the potentially secreted factors, which are involved in protein synthesis and transport or which are membrane proteins. However, these were all identified from mixed spots (table 1). Only stress-70 protein and adhesion-regulating molecule 1 have a potential signal peptide, and therefore might be true secreted proteins.

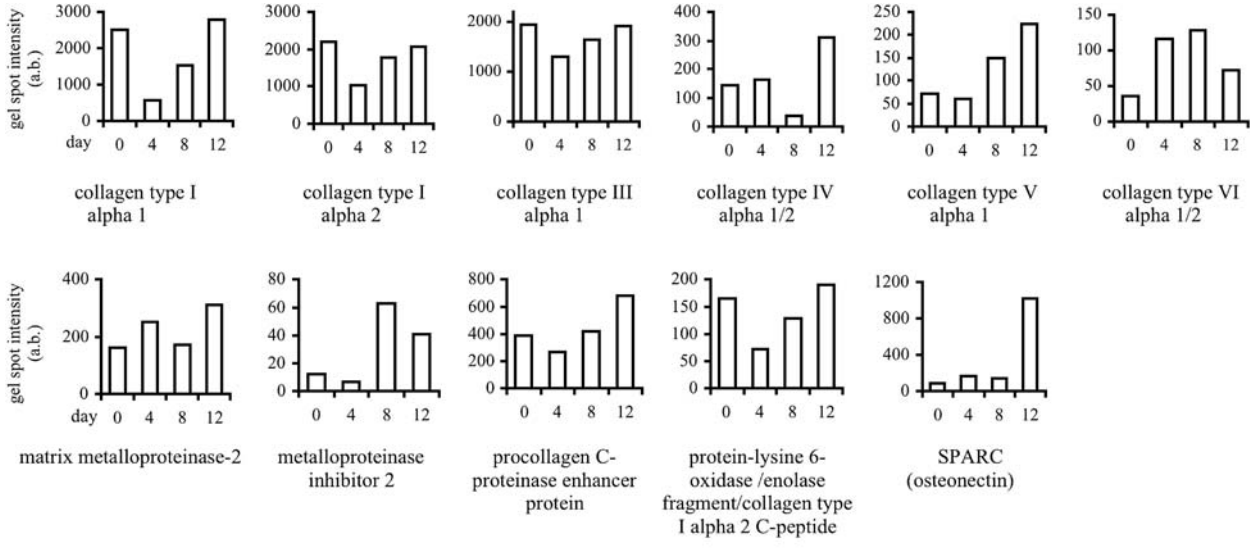

Figure 3. Secretion levels of extracellular matrix components and related proteins from 3T3-L1 cells during differentiation. Medium was sampled at 37 °C without BFA on day 0, 4, 8, and 12 during differentiation. The gel intensity of one protein in arbitrary units (a.b.) was expressed as the sum of the gel intensities of all spots containing this protein. The gel intensity of each spot is the mean from three independent experiments.

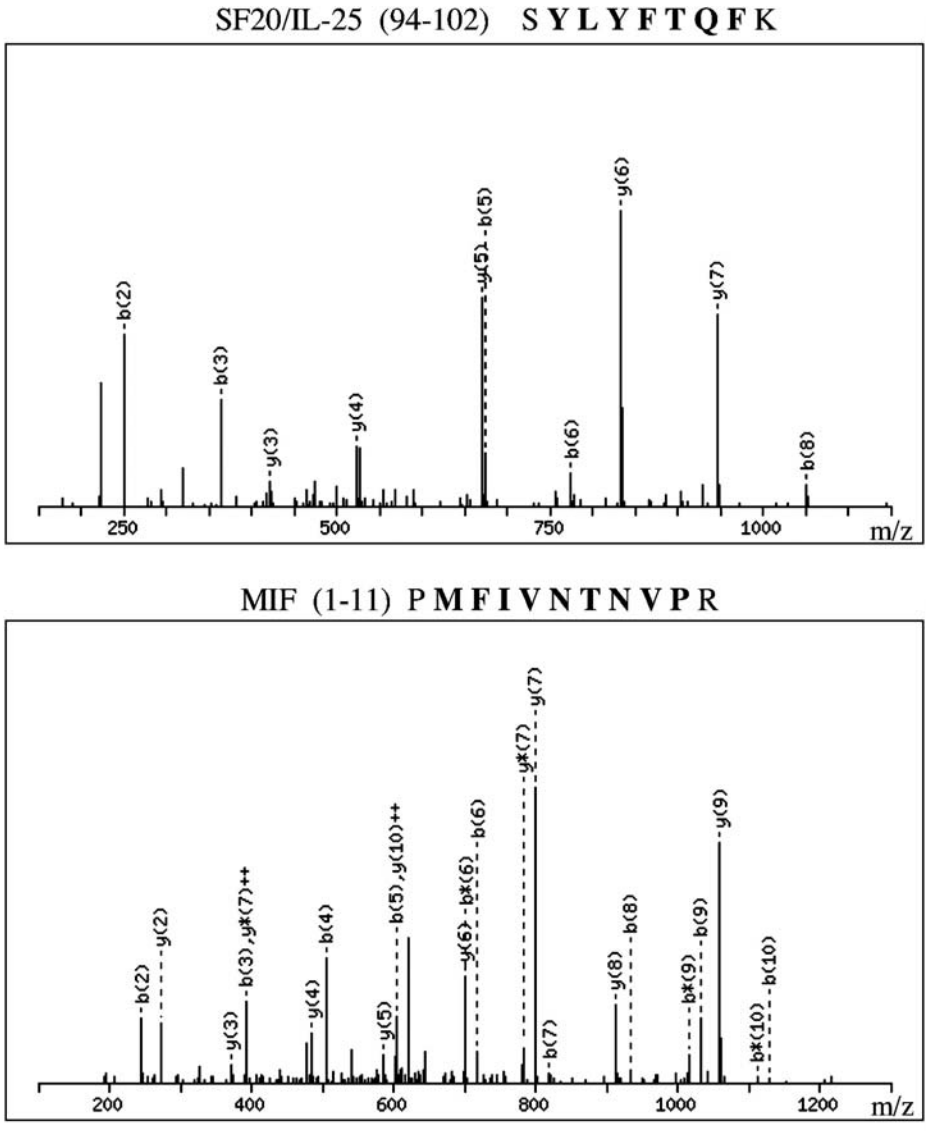

Figure 4. LC-MS/MS identification of SF20/IL-25 and MIF. Spectra were obtained as indicated in Materials and methods.

Discussion

We used a systematic proteomic approach with secretion blocking and identified novel secreted proteins during 3T3-L1 adipocyte differentiation. Previously, Kratchmarova et al. [8] identified a number of factors secreted by 3T3-L1 adipocytes, some of which were also found here. In contrast to our findings, they reported a down-regulation of PEDF and identified haptoglobin as a major secreted protein. The HCNP precursor protein was also detected by us, but with a less than twofold change during differentiation. The differences between the studies may be due to the experimental approach or to the analysis of different subtypes of these proteins.

Efficacy and limitations of the approach

The validity of our procedure was demonstrated by the detection of several known adipocyte-specific secreted proteins: complement C3, adiponectin and adipsin. The efficacy of the blocking method was confirmed by Western blot analysis of adiponectin (fig. 2) and PEDF (not shown). Nevertheless, some spots contained more than one protein, obscuring the secretion pattern of individual proteins. At day 4 under serum-free conditions, we observed a considerable number of floating cells at 37 °C, but not at 20 °C. In addition, the protein concentration of the harvested medium was highest at 37 °C at day 4, indicating that the detachment of cells might be associated with increased leakage of cellular proteins. Using the 20 °C treatment as a single selection criterion, cellular proteins with the highest expression in the medium at day

4 might be aberrantly categorized as being secreted. For some cellular proteins, fragments were observed to be blocked by both 20°C and BFA. This may be due to the differential expression of proteinases, which by themselves are blocked by these treatments.

Some well-known adipokines, including leptin, resistin, plasminogen activator inhibitor-1, vascular endothelial growth factor, and collagen type VI alpha 3, were not identified. This can be explained in several ways. Like leptin, they may have a low abundance in the medium of 3T3-L1 adipocytes and occur below the detection level of our system. 3T3-L1 cells may behave differently from in vivo (pre)adipocytes, but there is recent evidence that some adipokines and cytokines like interleukin-6 (Il-6) and tumor necrosis factor-α are mainly expressed by other cells in the stromal-vascular part of adipose tissue and are not released by adipocytes [23, 24]. Furthermore, by 2D gel electrophoresis, only part of the secretory proteome is visualized. In addition, adipokines may occur as spots mixed with other prominent unblocked proteins. Finally, some proteins are possibly secreted in a way not affected by temperature or BFA.

New features of extracellular proteins discovered by this blocking strategy

A number of new aspects of protein secretion during adipocyte differentiation were revealed. One of these is the different response of secreted proteins to the blocking conditions, which may reflect their different secretion pathways. The proteins whose secretion could not be blocked by BFA, such as galectin-1 [9], cyclophilin A and C, SF20/IL-25, and cofilin, are secreted through an ER/Golgi-independent pathway. Furthermore, the inconsistent blocking behavior of SF20/IL-25 suggests that mature 3T3-L1 adipocytes possess an additional secretion pathway, which is only present in adipocytes. In addition, our approach revealed unique features of adiponectin secretion by its positive response to 20°C treatment. The slightly up-regulated secretion of adiponectin at 20°C might relate to observations showing increased adiponectin mRNA and serum concentrations in mice exposed to cold [25].

There are two obvious routes by which proteins can end up in the extracellular space. One is through active secretion pathways, which may involve an N-terminal secretion signal peptide [9]. The other is through passive shedding of proteins with a transmembrane domain into the extracellular space by proteolytic enzymatic mechanisms [26]. We found that the structural analysis of a protein does not provide conclusive information about its intra- or extracellular location, and that a functional assay like our blocking procedure has an important added value. For example, the structure of cyclophilin A does not give any indication about its secretion, but it has been reported as a secreted growth factor [27]. As described above, fragments of some cellular proteins might be shedded. One of those is alpha enolase, a well-known key enzyme in glycolysis with a single transmembrane helix as revealed by TMAP analysis. Indeed, alpha enolase has been reported as a cell surface protein [28, 29]. Our findings indicate that it is also present on 3T3-L1 adipocytes. There is evidence that the plasminogen cascade is required for adipocyte differentiation [30], in which alpha enolase can serve as a receptor of plasminogen.

Remodeling of the extracellular matrix in adipose tissue development

During adipocyte differentiation, the cell morphology converts from a fibroblast-like to a spherical shape. This requires a dramatic remodeling of the extracellular matrix [13, 31]. The most obvious change was the dramatic reduction in fibrillar collagens I and III at the early stage of differentiation, which corresponds to the increased likelihood of cells to detach from the culture dish at day 4 in serum-free medium. The decrease in fibrillar collagens probably provides a more permissive environment for adipocyte expansion [32]. At later stages of differentiation, we observed a clear increase in fibrillar collagens. This regulated decrease and increase may explain some of the contradictory reports at the mRNA and protein levels [33–36]. In accordance with the in vivo basement membrane [37], we observed that the main component collagen IV is up-regulated during differentiation. Interestingly, the basement membrane may not only offer structural support to cells and tissues, but can also be an important regulator of vascularization [38] which is a fundamental aspect of adipose tissue development [39].

Growth regulation as a new aspect of adipokines revealed by novel adipocyte-secreted proteins

Except for well-known adipocyte secreted proteins, such as complement C3, adiponectin, adipsin, osteonectin, several collagens, matrix metalloproteinase-2, metalloproteinase inhibitor 2, haptoglobin, and a housekeeping protein beta-2 microglobulin [40], our identification method confirmed five other secreted proteins that have been reported in only a very limited number of studies, such as MIF [41], protein-lysine 6-oxidase [42], extracellular superoxide dismutase [43], gelsolin, and PEDF [8]. Furthermore, five proteins were newly identified as adipocyte secreted factors that have previously been reported as being secreted by other cell systems: procollagen C-proteinase enhancer protein [44], galectin-1, cyclophilin A and C, and SF20/IL-25. Moreover, three proteins (with a signal peptide sequence) are reported here for the first time as secreted factors: prohibitin, stress-70 protein, and adhesion-regulating molecule 1. Seven out of these 13 confirmed and newly identified secreted proteins have a function in growth regulation.

MIF as an inhibitor of the random migration of macrophages might play a role in the significant infiltration of macrophages into white adipose tissue as recently discovered [23]. MIF is associated not only with the immune response but also with cell growth and differentiation during wound healing and carcinogenesis [45].

PEDF is a member of the serine protease inhibitor superfamily [46]. It is highly expressed in human adipose tissue [47] and may function in anti-angiogenesis and innervation [48, 49]. We observed that 3T3-L1 cells secrete about the same amount of PEDF per cell at day 0 and day 12, but much less at day 4. This pattern is different from the previous report [8] but in line with that of collagens I and III, to which PEDF specifically binds in vitro [50].

Galectin-1 is expressed in a wide variety of cells and tissues including adipose tissue. Under different circumstances, it acts as a mitogen or as an inhibitor of cell proliferation, differentiation, and apoptosis [51]. The increasing secretion of galectin-1 during 3T3-L1 differentiation suggests an active involvement in adipose tissue development.

Cyclophilin A and C are intracellular immunophilin family proteins known to be distributed ubiquitously. They have peptidyl-prolyl cis-trans isomerase activity and act as molecular chaperones [52, 53]. Cyclophilin A does not have the structural characteristics for secretion, but was reported to be secreted from vascular smooth muscle cells as an autocrine growth factor [27] and from macrophages [54]. We observed first an up-, then a down-regulation of cyclophilin A during adipocyte differentiation. However, these quantifications may have been affected by some leakage of the cellular protein pool.

SF20/IL-25, like cyclophilin C, was also originally isolated from a mouse bone marrow stromal cell line [55]. In vitro findings indicated that this growth factor supports proliferation of lymphoid cells. However, these results could not be reproduced [56]. We have found that it is strongly up-regulated between days 0 and 4 of adipocyte differentiation and maintained at a high level throughout the differentiation process, indicating that this growth factor could be involved in adipogenesis and adipose tissue development.

Prohibitin is a fundamentally important gene ubiquitously expressed from yeast to humans and originally identified as an inhibitor of DNA synthesis [57]. It was detected in the mitochondria inner cell membrane and the nucleus. Recently, it has been established as a vascular (membrane) maker of adipose tissue in mouse and humans [58]. Our result shows that prohibitin is secreted into the medium of both preadipocytes and adipocytes, and that its concentration levels off at day 12. The presence of a possible signal peptide sequence is in line with our finding, but its function in the extracellular space, like its function on the cell surface, remains unknown.

Adhesion-regulating molecule 1 was discovered as a novel cell adhesion-promoting factor that was up-regulated in breast cancer cells [59]. Our 2D gel experiment showed its presence in 3T3-L1 adipocyte medium, but did not provide more information about its blocking behavior and regulation, because it was in a mixed spot.

From the above descriptions, we can conclude that differentiating adipocytes secrete proteins that are able to stimulate or inhibit the growth of immune cells, vascular endothelium cells, neuronal cells or tumor cells. From those factors for which the secretory profile was determined, the positive factors, galectin-1, cyclophilin A and C, and SF20/IL-25, were up-regulated, and the negative factor prohibitin was down-regulated. In vivo, therefore, these factors are likely involved in regulating defined processes associated with adipose tissue development like vascularization and innervation.

Most of these growth factors have been shown to play different roles in tumor development, including proliferation (SF20/IL-25) [55, 56], invasion (galectin-1) [60], and angiogenesis (MIF) [61]. The addition of 3T3-L1 adipocyte-derived secreted factors to breast cancer cells in vitro induced transcriptional programs of tumorigenesis including increased cell proliferation, invasive potential, survival, and angiogenesis, and type VI collagen was identified as a critical factor in this effect [62]. We therefore propose that the above-mentioned factors may also contribute to the tumorigenic potential of 3T3-L1 adipocytes. One should note that (some of) the tumor-related activities might merely be due to the immortalized nature of 3T3-L1 cells. However, the fact that the secretion profile of these factors changes during adipocyte differentiation implies that their secretion is a true characteristic of adipogenesis.

Conclusion

In conclusion, we have performed protein profiling with 2D gel electrophoresis and MS to study the secretion profiles of 3T3-L1 adipocytes during differentiation, using BFA and temperature blocking to distinguish between active secretion and leakage. Secretion profiles were determined, indicating a dynamic environment including an actively remodeling ECM and several factors involved in growth regulation. In addition, several new secreted proteins were discovered by this strategy, whose biological functions can now be further explored.

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